Development of Inhibitors in the IL-6/GP130/JAK/STAT Pathway as Therapeutic Agents

DISSERTATION

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By

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Abstract

STAT3 is hyperactive in a wide variety of cancers, including leukemias, lymphomas and numerous solid tumors. JAK2 is a critical upstream activator of STAT3, and has itself been implicated in cancer, as well as a number of myeloproliferative disorders. There are four JAKs and seven STATs that exist in mammals. The JAK/STAT signaling pathway is activated in response to numerous hormones and cytokines. IL-6 is one of these cytokines, as it is a primary activator of STAT3 signaling.

IL-6 signals through the transmembrane protein, GP130, with its receptor α subunit. IL-6 first binds to its receptor α subunit, and they together bind GP130. Two complete units then dimerize to form the signaling-competent hexameric receptor complex. After activation of the complex, JAKs, which are constitutively associated with GP130, become phosphorylated and activate several signaling cascades, including the STAT pathway.

Once STATs are phosphorylated by JAKs, they dimerize and translocate to the nucleus, where they regulate the transcription of target genes. STAT3 is anti-apoptotic in nature, and its target genes include those corresponding to the proteins Bcl-2 and Bcl-xL, which mediate cell survival, among others. However, other STATs, such as STAT1 and STAT2, are pro-apoptotic in nature. This makes the selective targeting of STAT3 desirable.
Unfortunately, there are not currently any selective JAK2 or STAT3 inhibitors on the market, as the structural similarities between JAKs and STATs have made the development of inhibitors for a specific JAK or STAT difficult. There are several JAK2-specific inhibitors that are currently in clinical trials, which is promising. However, there has only been one phase 0 clinical trial involving the direct targeting of STAT3.

In this document, the development of small molecule inhibitors of JAK2 and STAT3 is reported. Some of the compounds that have been synthesized and evaluated were based on the structures of WP1066 and AG490, which are known to inhibit JAK2. However, the majority of the compounds were directed at STAT3.

There are several current strategies for the direct inhibition of STAT3. These involve targeting the N-terminal, DNA binding, or SH2 domains. The STAT3 inhibitors reported herein target the SH2 domain. Computational modeling has suggested that the occupation of three sites of the SH2 domain is ideal for binding, and analogues have been synthesized accordingly. These compounds are able to block STAT3 phosphorylation, dimerization and subsequent STAT3-mediated gene expression.

The majority of these compounds are derivatives of the natural product, curcumin. Curcumin has previously been shown to act as an inhibitor of STAT3. Computational modeling has suggested that it is able to bind to JAK2 as well, making it a dual inhibitor. However, it has limited potency and bioavailability, and also has numerous biological targets. These features have limited its therapeutic usefulness. In this document, the development of curcumin analogues with drastically improved potency and selectivity for STAT3 is reported.
The development of 1,4-naphthoquinones and improved analogues of LLL12 is also reported in this document. LLL12 is a potent STAT3 inhibitor that was developed in Dr. Li’s laboratory. Its structure was derived from the first ever reported STAT3 SH2 domain inhibitor, STA-21, which was identified through virtual screening. The structural similarities to LLL12 of known 1,4-naphthoquinones, such as plumbagin and juglone, led to the development of additional 1,4-naphthoquinones as well, which were evaluated for their abilities to inhibit STAT3.
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<thead>
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<th>Description</th>
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<tbody>
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<td>ABC-like</td>
<td>Activated-B-cell-like</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>AGC</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>AMKL</td>
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<td>AML</td>
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<td>B-ALL</td>
<td>B-Cell acute lymphoblastic leukemia</td>
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<td>CaM-KK</td>
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</tr>
<tr>
<td>CBM</td>
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</tr>
<tr>
<td>cIAP2</td>
<td>Cellular inhibitor of apoptosis 2</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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</tr>
<tr>
<td>CIS</td>
<td>Cytokine-inducible SH2 protein</td>
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<td>CLF</td>
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<td>DLBCL</td>
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<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<td>2D NMR</td>
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<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
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<tr>
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<td>Epidermal growth factor</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shifts assay</td>
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<tr>
<td>Epo</td>
<td>Erythropoietin</td>
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<tr>
<td>ERK</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cells</td>
</tr>
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<td>ET</td>
<td>Essential thrombocytemia</td>
</tr>
<tr>
<td>F</td>
<td>Fibroblasts</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>Band 4.1, ezrin, radixin and moesin</td>
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<td>Fibroblast growth factor</td>
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<td>FP</td>
<td>Fluorescence polarization</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<tr>
<td>Gab</td>
<td>Grb2-associated binding protein</td>
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<td>GTPase-activating protein</td>
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<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GP130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<tr>
<td>Grb2</td>
<td>Growth factor receptor binding protein 2</td>
</tr>
<tr>
<td>h</td>
<td>Human</td>
</tr>
<tr>
<td>HCC</td>
<td>Human hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epithelial growth factor receptor 2</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HHV8</td>
<td>Human herpes virus-8</td>
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<tr>
<td>hIL-6</td>
<td>Human interleukin-6</td>
</tr>
<tr>
<td>hIL6-Rα</td>
<td>Human interleukin-6 receptor alpha subunit</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
</tr>
<tr>
<td>HM</td>
<td>Hydrophobic motif</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HP1α</td>
<td>Heterochromatin protein 1 alpha</td>
</tr>
<tr>
<td>HR</td>
<td>Homology region</td>
</tr>
<tr>
<td>HTFM</td>
<td>High-throughput fluorescence microscopy</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>Human T cell lymphotropic virus 1</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>hyper-IL-6</td>
<td>Fusion protein of IL-6/sIL-6Rα</td>
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xxxvi
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<thead>
<tr>
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<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>Interleukin-6 receptor alpha subunit</td>
</tr>
<tr>
<td>IL-11Rα</td>
<td>Interleukin-11 receptor alpha subunit</td>
</tr>
<tr>
<td>IRF1</td>
<td>Interferon-regulatory factor-1</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>Janus kinase homology</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile idiopathic arthritis</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>KH97</td>
<td>A granulocyte/macrophage colony-stimulating factor receptor-associated molecule</td>
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<tr>
<td>KLD</td>
<td>Kinase-like domain</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma associated herpesvirus</td>
</tr>
<tr>
<td>LDL-R</td>
<td>Low-density lipoprotein receptor</td>
</tr>
<tr>
<td>LGL</td>
<td>Large granular lymphocyte leukemia</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LIFR</td>
<td>Leukemia inhibitory factor receptor</td>
</tr>
<tr>
<td>Lk</td>
<td>Linker</td>
</tr>
<tr>
<td>LMW-DSP2</td>
<td>Low molecular weight-dual specificity phosphatase 2</td>
</tr>
<tr>
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<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>5-LOX</td>
<td>5-Lipoxygenase</td>
</tr>
<tr>
<td>LPMC</td>
<td>Lamina propria mononuclear cells</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>Murine</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mb</td>
<td>Membrane-bound</td>
</tr>
<tr>
<td>MC</td>
<td>Melanoma cells</td>
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<tr>
<td>MDL-A</td>
<td>(+)-Madindoline A</td>
</tr>
<tr>
<td>MDL-B</td>
<td>(+)-Madindoline B</td>
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<tr>
<td>MEK</td>
<td>MAP/ERK kinase</td>
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<td>mIL-6</td>
<td>Murine IL-6</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>mP</td>
<td>Millipolarization units</td>
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<tr>
<td>MPDs</td>
<td>Myeloproliferative disorders</td>
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<tr>
<td>mTor</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>ND</td>
<td>Not determined</td>
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<tr>
<td>NGFR</td>
<td>Nerve-growth-factor receptor</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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NP-40
Nonidet P-40

N-terminus
Amino terminus

O
Osteoblasts

ODN
Oligonucleotide

OSM
Oncostatin M

OSMR
Oncostatin M receptor

p
Phospho

PCM1
Pericentriolar material 1

PDGF
Platelet-derived growth factor

PDK1
3’Phosphoinositide-dependent kinase 1

PGE
Prostaglandin

PH
Pleckstrin homology

PHLPP
PH domain leucine-rich repeat protein phosphatase

PIAS
Protein inhibitor of activated STAT

PI3K
Phosphatidylinositol-3-kinase

PIP2
Phosphatidylinositol-4,5-bisphosphate

PIP3
Phosphatidylinositol-3,4,5-trisphosphate

PIPLC
Phosphatidylinositol-specific phospholipase C

PKA
Protein kinase A

PKB
Protein kinase B (Akt)

PKC
Protein kinase C

PKR
Protein kinase double-stranded RNA-dependent

xxxix
<table>
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<tr>
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<tr>
<td>PMBL</td>
<td>Primary mediastinal B-cell lymphoma</td>
</tr>
<tr>
<td>PMF</td>
<td>Primary myelofibrosis</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor-gamma</td>
</tr>
<tr>
<td>PrlR</td>
<td>Prolactin receptor</td>
</tr>
<tr>
<td>PRMT1</td>
<td>Protein arginine methyl-transferase 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homology deleted on chromosome ten</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
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<td>Protein tyrosine phosphatase</td>
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<td>PTP1B</td>
<td>Protein-tyrosine phosphatase 1B</td>
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<td>PTP-BL</td>
<td>Protein tyrosine phosphatase-basophil like</td>
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<td>PTPRT</td>
<td>Protein tyrosine phosphatase receptor T</td>
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<td>Phosphotyrosine</td>
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<td>Polycythemia vera</td>
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<td>r</td>
<td>Recombinant</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
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<td>Rheumatoid arthritis</td>
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<td>rhIL-6</td>
<td>Recombinant human IL-6</td>
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<td>RPN1</td>
<td>Ribophorin 1</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>rtPCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
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</table>
sGP  Soluble glycoprotein
sGP130Fc  Fusion protein of the extracellular portion of GP130 and the Fc region of IgG1
SH2  Src homology 2
Shc  SH2 and collagen homology domain containing protein
SHIP  SH2 domain-containing inositol polyphosphate 5-phosphatase
SHP2  Src homology 2 domain-containing tyrosine phosphatase 2
sIL6-Rα  Soluble interleukin-6 receptor alpha subunit
S6K1  p70 Ribosomal S6 protein kinase 1 (p70^{6k})
SM  Smooth muscle cells
SOCS  Suppressor of cytokine signaling
Sos  Son of sevenless
SPR  Surface plasmon resonance
SR  Serine-rich
STAT  Signal transducer and activator of transcription
SUMO  Small ubiquitin-related modifier
TAD  Transactivation domain
TAL1  T-cell acute lymphocytic leukemia protein 1
TC45  The nuclear form of T-cell protein tyrosine phosphatase
TCPTP  T-cell protein tyrosine phosphatase
TCZ  Tocilizumab
TF  Transcription factor
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<th>Term</th>
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<td>T Helper type I</td>
</tr>
<tr>
<td>Th2</td>
<td>T Helper type II</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TpoR</td>
<td>Thrombopoietin receptor</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine kinase 2 (a member of the JAK family)</td>
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<tr>
<td>uSTAT3</td>
<td>Unphosphorylated STAT3</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>vIL-6</td>
<td>Viral IL-6</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter 1: IL-6 and its Signaling Complex

1.1 IL-6-Type Cytokines

1.1.1 Introduction

The interleukin (IL)-6-type cytokines consist of long type I cytokines that are phylogenetically related.\(^1\)\(^2\) Its members include IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC).\(^2\)\(^3\) Members of this class act in a highly pleiotropic manner, and are also quite redundant in their abilities to transduce biological activities.\(^2\) Ultimately, their signaling cascades result in the activation of target genes that are involved in cell death, cell survival, growth and differentiation.\(^3\) They are involved in hematopoiesis, and have pro- and anti-inflammatory outcomes.\(^3\)

1.1.2 Receptor α-Subunits

All members of the IL-6 family of cytokines transduce their signals via one or more of the functionally and structurally similar type I cytokine transmembrane β-subunits: glycoprotein 130 (GP130), leukemia inhibitory factor receptor (LIFR), or oncostatin M receptor (OSMR).\(^2\)\(^3\) However, the signaling mechanisms of the different family members have some variability.\(^2\) IL-6, IL-11, and CNTF must first bind their specific α-subunits before the cellular signal can be transduced through the β-subunits.\(^3\) Still, some unique variations in signaling exist.\(^3\) The CNTF receptor alpha subunit
(CNTFRα) is unique in that it is often anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) linkage,\textsuperscript{4,5} while the membrane-bound (mb) IL-6 receptor alpha subunit (IL-6Rα) and IL-11 receptor alpha subunit (IL-11Rα) often contain transmembrane components.\textsuperscript{2,3,6} Interestingly, membrane association of any of the α-subunits is not a requirement for signaling to occur, and as a result, the presence of soluble versions of the α-subunits are also sufficient to induce cellular responses.\textsuperscript{2,7} Also, LIF and OSM are unique in that they are both able to directly bind to β-subunits and transduce their signals without an α-subunit.\textsuperscript{4,6,8}

CNTF is unique in that human CNTF has been found to be capable of utilizing either mbIL-6Rα or the soluble IL-6Rα (sIL-6Rα) as a substitute for CNTFRα;\textsuperscript{4} these findings have also been confirmed in mice.\textsuperscript{9} CLC, on the other hand, utilizes CNTFRα as opposed to an α-subunit that is specific to itself.\textsuperscript{3} It is the most recently identified member of the IL-6 family,\textsuperscript{10} and it appears to be involved in unique mechanisms of both secretion and receptor recruitment.\textsuperscript{3,11,12} For efficient CLC secretion to occur, cytokine-like factor 1 (CLF)\textsuperscript{12} or CNTFRα\textsuperscript{11} expression is required. Interestingly, the stable secreted complex of CLC/CLF has been shown to be a ligand for CNTFRα in both mouse and human cells.\textsuperscript{9,12,13} It has been postulated that CLC/CLF may recruit a receptor besides CNTFRα as well, but such a receptor has yet to be identified.\textsuperscript{9} A secreted CLC/CNTFRα composite can also induce signaling.\textsuperscript{3,11}

Lastly, it is also suspected that an α-subunit for CT-1 exists.\textsuperscript{3,6,14,15} In 1996, Pennica and colleagues reported that phosphatidylinositol-specific phospholipase C (PIPLC) was able to inhibit the effects that CT-1 had on motoneurons.\textsuperscript{15} They also did
not observe any binding between CT-1 and the GPI-linked CNTFRα subunit.\textsuperscript{15} These data led them to suggest that there may be a novel GPI-linked α-subunit to which CT-1 binds.\textsuperscript{15} However, to date, the presence of this subunit has not been verified.\textsuperscript{3,6}

1.1.3 Receptor β-Subunits and the Receptor Complexes

When IL-6 or IL-11 bind to their respective α-subunits they interact with GP130, a β-subunit, to induce homodimerization as a hexameric complex.\textsuperscript{2,6,16,17,18} CNTF, LIF, CLC and CT-1 signaling results in the heterodimerization of GP130 and LIFR.\textsuperscript{2,3,5,6,19,20,21} Human OSM has the ability to signal via both GP130-LIFR and GP130-OSMR heterodimers.\textsuperscript{3,6,22,23} The general components of the complete receptor complexes for several of the cytokines discussed are portrayed below (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{IL-6-Type cytokine receptor complexes (adapted and reprinted by permission from Macmillan Publishers Ltd: Nature Reviews. Neuroscience,\textsuperscript{21} © 2007. This figure was originally adapted from The Biochemical Journal,\textsuperscript{3} © the Biochemical Society).}
\end{figure}
1.1.4 Differences in Signaling Depending on the Receptor Complex

There has been some evidence that signaling resulting from GP130 homodimers, GP130-LIFR heterodimers, or GP130-OSMR heterodimers may differ slightly.\(^2\,6\,24\,25\,26\) In studying growth factor induced macrophage differentiation in M1 leukemia cells, Tanigawa and colleagues enforced T-cell acute lymphocytic leukemia protein 1 (TAL1) expression, using a TAL1 retrovirus.\(^24\) They found that while LIF- or OSM-induced macrophage differentiation was disturbed, IL-6-induced differentiation was not.\(^24\) These findings led them to suggest that LIFR-GP130 heterodimers might utilize a pathway that is able to be inhibited by TAL1, while GP130 homodimers utilize an alternate pathway.\(^24\)

Kuropatwinski and colleagues found that in the mouse hepatoma cell line, Hepa1, STAT5 recruitment and activation was much more prominent through signaling via LIFR or OSMR than IL-6Rα.\(^25\) They also found that, when comparing the cytoplasmic receptor domains responsible for signaling, OSMR requires its distal box 3 sequence motif contained in the 36 residue carboxyl-terminal region for signaling, while LIFR did not have the same requirements.\(^25\) From their studies, they also suggested that there were differences in signaling between the three cytokine receptors, and that it is the composition of the cytoplasmic domains of the receptors that determines signaling specificity.\(^25\)

Lastly, Starr and coworkers developed hypothetical receptors that contained the extracellular domain of the granulocyte colony-stimulating factor (G-CSF) receptor linked to the cytoplasmic and transmembrane portions of LIFR or GP130.\(^26\) They expressed them in M1 leukemia cells, embryonic stem cells, and Ba/F3 cells in order to
analyze the resulting effects on the induction of differentiation, the inhibition of differentiation, and cell proliferation, respectively. They found that their model GP130 homodimer was able to produce a G-CSF-induced signal in all three types of cells, while their model LIFR homodimer was only able to do so in embryonic stem cells. They also suggested that there may by differences in the abilities of the cytoplasmic portions of GP130 and LIFR to propagate signals.

1.1.5 Signaling Similarities of IL-6-Type Cytokines

Despite this evidence for differences in signaling between the dimers formed, the fact remains that all IL-6-type cytokines recruit at least one GP130 molecule to their active receptor complex. As a result, all IL-6-type cytokines result in cellular signaling via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt(PKB)/mTor) pathway, and the mitogen-activated protein kinase (MAPK) pathway (Figure 2).

![Figure 2. Pathways activated by GP130.](image-url)
Due to this redundancy, and the fact that GP130, JAKs and STATs are ubiquitously expressed, it is not surprising that all IL-6-type cytokines can elicit often overlapping biological responses in vivo.2,3,6 The in vivo and in vitro functional redundancies that have been observed for several of the IL-6-type cytokines are shown in Table 1.39

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>LIF</th>
<th>OSM</th>
<th>IL-11</th>
<th>CNTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of lipoprotein lipase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acute phase protein synthesis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Hematopoietic stem cell growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>M1 leukemic differentiation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Megakaryocyte differentiation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Myeloma growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ig production</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Bone resorption</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Embryonic stem cell growth</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Neuronal differentiation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Induction of the junB gene</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: The activity indicated by (+) was exhibited by the complex of cytokine and its soluble receptor α-chain

Table 1. Functional redundancies of IL-6-type cytokines (adapted and reprinted with permission from © John Wiley & Sons, Inc.)39

However, there are mechanisms for specific biological responses to be obtained from the various IL-6-type cytokines. Although many of the proteins involved in IL-6-type cytokine signaling are ubiquitously expressed, the α-subunits, as well as the OSMR and LIFR β-subunits, are not.2,6 Since these subunits are required for signaling, differences in expression of these subunits can determine how a certain cell type will
respond, or not respond, to a specific cytokine.\textsuperscript{2,3,6} Also, the synthesis and secretion of cytokines are under temporal and spatial control.\textsuperscript{6} As a result, it should not be surprising that each of the IL-6 type cytokines have unique characteristics.\textsuperscript{6} The prominent tissues responsible for synthesizing the various IL-6-type cytokines and the effects of the cytokines on the tissues are shown (Table 2).\textsuperscript{6}

1.1.7 Effects of Gene Knockouts

Due to the critical role that the GP130/JAK/STAT pathway plays in IL-6-type cytokine signaling, and the often redundant ability of IL-6-type cytokines to initiate signaling, it is no surprise that gene knockouts (KOs) of GP130, JAK or STAT3 in mice have been shown to lead to lethal phenotypes, while mice that are deficient in only one of the cytokines tend to survive, although not without consequences.\textsuperscript{2,6} When Yoshida and colleagues targeted the disruption of GP130 in mice, they found that mouse embryos containing the homozygous null mutation died prior to birth, while heterozygous mice did not display any abnormalities.\textsuperscript{40} Rodig and coworkers found that mice that were homozygous null for JAK1 were born undersized, failed to nurse, and died shortly after birth.\textsuperscript{41} Both Parganas and colleagues as well as Neubauer and coworkers reported that JAK2-deficient mice died prenatally.\textsuperscript{42,43} Both groups cited the absence of erythropoiesis as a cause.\textsuperscript{42,43} Takeda and colleagues targeted STAT3 and were not able to obtain any viable homozygous null mice from a heterozygote cross.\textsuperscript{44} As a result of these findings, they concluded that STAT3 is essential for early embryonic development in mice.\textsuperscript{44}
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sites of synthesis</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Many tissues including blood, cartilage, bone marrow, skin, lung and central nervous system</td>
<td>Haematopoietic tissues, lung, gastrointestinal tract, bone, central nervous system, thymus, connective tissues, skin, uterus and testis</td>
</tr>
<tr>
<td>IL-11</td>
<td>Many tissues, including heart, liver, endometrium, pituitary, central nervous system, gut, kidney, lung and thymus</td>
<td>Blastocyst implantation</td>
</tr>
<tr>
<td>LIF</td>
<td>Many tissues, including heart, liver, endometrium, pituitary, central nervous system, gut, kidney, lung and thymus</td>
<td>Blastocest implantation</td>
</tr>
</tbody>
</table>

**Table 2. Sites of synthesis and effects of IL-6-type cytokines (this table originally appeared in The Biochemical Journal, © the Biochemical Society).**

On the other hand, KO mice deficient in a specific cytokine have been shown to survive. Two groups studied CNTF-deficient mice in the mid 1990’s, and both of them found that CNTF-deficient mice were viable, although in adulthood they did exhibit a loss of motor neurons and a corresponding reduction of muscle strength. In contrast, no mice that were homozygous null for CNTFRα survived longer than 24 hours after birth, possibly due to the fact that CNTFRα is utilized by both CNTF and CLC. While in the aforementioned study CNTFRα was shown to be critical for survival, IL-11Rα has been shown to not be required for survival, although females deficient in IL-11Rα were infertile. Lastly, when Kopf and colleagues disrupted the IL-6 gene in mice, they found that they developed normally. However, the mice were not able to efficiently control infection with vaccinia virus or Listeria monocytogenes, and they exhibited an impaired inflammatory acute phase response (APR) following infection or tissue damage.

1.2 IL-6 Trans-Signaling

1.2.1 Introduction

One crucial mechanism in IL-6-type cytokine signaling is accommodated by the fact that, as previously mentioned, α-subunits do not have to be associated with the membrane for signaling to occur. In fact, they lack any intrinsic signaling capabilities of their own, whether they are membrane-associated or not. This mechanism by which cells that do not express the α-subunits for, and are normally unresponsive to, IL-6, IL-11 or CNTF, become responsive due to the presence of extracellular α-subunits, was termed
“trans-signaling” by Rose-John and Heinrich in 1994.\textsuperscript{50,51} This mechanism of generating "cytokine diversity" was almost simultaneously proposed by Hirano and colleagues.\textsuperscript{39} The generation of soluble α-subunits is a normally functioning mechanism, but when present in excess has been associated with a number of diseases.\textsuperscript{7}

For example, IL-6Rα is primarily expressed by hepatocytes and leukocytes.\textsuperscript{7,52,53,54} However, when sIL-6Rα is produced, other cell types expressing mbGP130 can become responsive to IL-6.\textsuperscript{2} This is an important mechanism in acute inflammation, as the production of sIL-6Rα by neutrophils enhances lymphocyte recruitment to inflamed tissues.\textsuperscript{55,56} SIL-6Rα has also been implicated in liver regeneration, as well as the differentiation and survival responses of neurons.\textsuperscript{7,57}

\subsection*{1.2.2 Trans-Signaling and Disease}

On the other hand, elevated levels of sIL-6Rα have also been observed in a number of disorders.\textsuperscript{2,6,7} It was found that, in Crohn’s disease, trans-signaling involving sIL-6Rα was responsible for preventing mucosal T-cell apoptosis, and is ultimately partly responsible for the continuation of chronic intestinal inflammation.\textsuperscript{58} When Keul and colleagues evaluated the serum of patients suffering from juvenile chronic arthritis, they found that there were elevated sIL-6Rα concentrations, while soluble GP130 (sGP130) and IL-6 autoantibody concentrations were not significantly different from controls.\textsuperscript{59} When the synovial fluid and serum concentrations of rheumatoid arthritis (RA) patients was evaluated, it was found that they exhibited elevated levels of IL-6 and sIL-6Rα.\textsuperscript{60,61} Interestingly, the concentration of both IL-6 and sIL-6Rα was also found to be
significantly higher in the synovial fluids of RA patients when compared to osteoarthritis patients. Elevated sIL-6Rα levels have also been reported in patients suffering from human immunodeficiency virus (HIV) and multiple myeloma (MM).

It should be noted that the majority of the time soluble receptors have antagonistic activity, while the soluble versions of IL-6-type cytokine α-subunits display agonistic activity. However, sGP130 is also present in high levels in human serum, and it does act as a natural inhibitor of the IL-6/sIL-6Rα complex. Human serum levels of sGP130 are usually around 400 ng/mL, while sIL-6Rα levels are significantly lower, having been reported at about 35-75 ng/mL. Interestingly, homeostatic serum levels of IL-6 are significantly lower still, and in healthy individuals were found to have a range of about 1-17 pg/mL. IL-6 levels are elevated in certain disorders as part of the immune response, but are almost always still lower than sIL-6Rα levels.

Figure 3. Antagonistic activity of sGP130 on IL-6 signaling (this figure originally appeared in Clinical Science, © the Biochemical Society).
Interestingly, when Jostock and colleagues created recombinant sGP130 (rsGP130), they found that it inhibited the IL-6/sIL-6Rα induced proliferation of Ba/F3 cells, but that it did not have an antagonistic effect on the IL-6 induced proliferation of Ba/F3 cells expressing mbGP130 and mbIL-6Rα. This suggests that sGP130 does not inhibit IL-6 alone, and that its primary role is to inhibit trans-signaling. Müller-Newen and colleagues evaluated the effects of IL-6, IL-6/sIL-6Rα, and sGP130 on four different cell types. They found that cells not expressing IL-6Rα were much more vulnerable to the antagonistic effect of sGP130 when the cells were stimulated with both IL-6/sIL-6Rα and sGP130 than when cells expressing IL-6Rα were stimulated with both IL-6 and sGP130. This goes along with the previously conceived notion that the high levels of sIL-6Rα and sGP130 act together as an extracellular buffer in order to modulate the systemic effects of IL-6.

However, it has recently been shown that under certain conditions sGP130 can have a significant inhibitory effect on classical signaling as well. This is of critical importance, since derivatives of sGP130 are being developed as potential treatments for inflammatory diseases. The effects that sIL-6Rα and sGP130 will have on specific cells are complicated, and appear to be related to the local concentrations of IL-6, sIL-6Rα and sGP130, as well as the equilibrium between IL-6, IL-6/sIL-6Rα, and IL-6/sIL-6Rα/sGP130.

There have been numerous studies reporting on the binding affinities of IL-6 to IL-6R, and sGP130 to IL-6Rα and the IL-6/IL-6Rα complex. Narazaki and colleagues showed that a complex of sIL-6Rα and sGP130 was not able to be formed in the absence
of IL-6. In contrast, Gaillard and coworkers reported that sIL-6Rα and sGP130 were able to interact in the absence of IL-6, although with a lower affinity when compared to the interactions of sGP130 with the IL-6/sIL-6Rα complex. However, there is agreement on the point that IL-6 does not interact with sGP-130 in the absence of IL-6Rα.

Taga and colleagues were the first to describe the receptor-binding of IL-6. They examined several cell lines expressing IL-6Rα and found the dissociation constants (Kd’s) to be within the range of 130 to 640 pM. They did note that the presence of other, lower-affinity binding sites could not be excluded due to the concentrations of IL-6 used in their experiments. This is in accordance with Rose-John and colleagues, who found that 125I-labeled human recombinant IL-6 bound to NIH/3T3 cells expressing IL-6Rα with a Kd of 440 pM.

Hibi and coworkers were the first to describe the low-affinity binding of IL-6 to IL-6Rα and the high affinity binding of GP130 to the IL-6/IL-6Rα complex. Stoyan and colleagues reported that sIL-6Rα bound IL-6 with a Kd of 1.5 nM. Zohlnhöfer and coworkers were the first to quantize both values, and found that IL-6Rα bound IL-6 with low affinity (Kd ≈ 500 pM) while the high affinity binding (Kd ≈ 60 pM) probably resulted from the binding of GP130 to the IL-6/IL-6Rα complex. Weiergräber and colleagues later confirmed the low affinity binding of IL-6 to IL-6Rα, finding that sIL-6Rα bound 125I-labeled IL-6 with a Kd of 500 pM.

Since then, binding studies have been used to guide the design of administrable treatments. For example, a designer fusion protein of IL-6/sIL-6Rα (hyper-IL-6) was
found to bind a fusion protein containing the extracellular region of GP130 and the Fc portion of immunoglobulin (Ig) G1 (sGP130Fc) with a $K_d$ of 4.2 nM. Jostock and colleagues tested sGP130Fc on lamina propria mononuclear cells (LPMC) from patients with Crohn’s disease. They found that sGP130Fc was able to inhibit the anti-apoptotic effects of sIL-6Rα on LPMC cells, and suggested sGP130Fc’s potential for therapeutic use. Tenhumberg and colleagues found the $K_d$ to be 597 pM, and also discovered an improved protein with a $K_d$ of 122 pM, which signified its therapeutic relevance. When interpreting the comprehensive binding data it is important to consider, as Jostock and colleagues noted, that the specific techniques used can have a significant impact on the calculated binding constants. The important points are that IL-6 binds IL-6Rα with low affinity, GP130 binds the IL-6/sIL-6Rα complex with high affinity, sGP130 and mbGP130 have similar affinities to the IL-6/IL-6Rα complex, and sIL-6Rα and mbIL-6Rα have similar affinities to IL-6.

The binding affinities and the concentrations of the IL-6 signaling members that have been discussed are important because they can have a drastic effect on the signaling that occurs in vivo. Although sIL-6Rα acts as an agonist in the absence of sGP130, when sGP130 is present it can instead potentiate the ability of sGP130 to act as an antagonist. Müller-Newen and colleagues found that when IL-6Rα-expressing cells were stimulated with only IL-6, the antagonistic activity of sGP130 was weak, but when sIL-6 was added it increased the antagonistic abilities of sGP130, since formation of IL-6/sIL-6Rα complexes allowed for the high affinity binding of sGP130 to the complexes, thereby rendering IL-6 inactive. They suggested that, theoretically, assuming a $K_d$ of
500 pM for the formation of the IL-6/sIL-6Rα complex, a roughly 50% reduction of bioactive IL-6 should result due to the formation of the ternary IL-6/sIL-6Rα/sGP130 complex, unless the concentration of IL-6 is approximately equal to, or greater than, the concentration of sIL-6Rα. Assuming a 10-fold higher affinity for ternary complex formation predicts that at observed physiological concentrations the bioavailability of IL-6 is significantly decreased.

Recently, Garbers and colleagues have suggested similar effects. From their calculations, if a five-fold molar excess of sIL-6Rα is present relative to IL-6, then roughly 50% of IL-6 should be trapped in ternary complexes. They demonstrated that classical signaling in Ba/F3 cells can be inhibited if the concentration of sIL-6Rα exceeds the concentration of IL-6. They explained this by the rationalization that when an IL-6/sIL-6Rα/sGP130 complex is formed, an IL-6/sIL-6Rα complex is removed from the equilibrium between free IL-6, free sIL-6Rα, and the IL-6/sIL-6Rα complex. This favors the formation of additional IL-6/sIL-6Rα complexes, which can then also be trapped in ternary complexes. As this process continues, free IL-6 is removed and classical signaling can be inhibited. However, in their experiments, they did find that about 300 times more sGP130Fc was needed to inhibit the classical signaling in cells expressing IL-6Rα than the trans-signaling in cells lacking mbIL-6Rα. They concluded that only high concentrations of sGP130Fc were sufficient to significantly shift the equilibrium between free IL-6, free sIL-6Rα, and the IL-6/sIL-6Rα complex, and that classical signaling will only be affected when high concentrations of sGP130Fc are present and the concentration of sIL-6Rα exceeds the concentration of IL-6 (Figure 4).
There are a couple of important points to consider. MbGP130 can be present in high local concentrations, and when it is, it will be able to compete with sGP130 in IL-6 binding. Also, as a result of cellular activation or a number of pathological conditions, the local concentrations of sIL-6Rα and sGP130 might be significantly different than their concentrations in plasma. This could alter their inhibitory capabilities. Lastly, although higher serum concentrations of sIL-6Rα than IL-6 have been reported by many groups, their concentrations at a specific location might be quite different. Indeed, at 4
and 12 hours after administering the irritant, carrageenan, to mice, Rabe and colleagues found there to be significantly higher amounts of IL-6 than sIL-6Rα at the site of inflammation.77

The importance lies with the fact that trans-signaling has been implicated in a number of diseases, including asthma, RA, chronic inflammatory bowel disease, peritonitis and colon cancer, while classical signaling plays a role in many desirable processes.65,78 In a recent review, it was concluded that the anti-inflammatory or regenerative activities of IL-6 are a result of classical signaling, while the pro-inflammatory activities of IL-6 are a result of trans-signaling.79 For many pathologies inhibiting trans-signaling is desirable, while blocking classical signaling can be detrimental.65,77,87

In a murine polymicrobial sepsis model, the administration of a low dose of sGP130Fc increased survival from 45% to 100%, administration of a high dose of sGP130Fc increased survival to only 80%, and administration of an anti-IL-6 monoclonal antibody (mAb) did not improve survival.80 The higher rate of survival at the lower dose of sGP130Fc could be a result of the higher dose inhibiting both classical signaling and trans-signaling. This could also explain why the non-specific mAb, which inhibits both types of signaling, failed to improve the survival rate.65,80 Further elucidation of the involvement of classical signaling and trans-signaling in specific diseases will be critical, since in many instances sGP130Fc, which specifically targets trans-signaling at low doses, may prove to be superior to treatments such as the mAb tocilizumab (TCZ), which nonspecifically blocks both types of signaling.65,79
1.2.3 Generation of Soluble Receptors

There are normally two mechanisms that are involved in the generation of soluble receptors: differential mRNA splicing and proteolytic shedding (Figure 5). Müllberg and colleagues have shown that both sIL-6Rα and sGP130 can be generated through proteolytic shedding, and that shedding of sIL-6Rα is regulated by protein kinase C (PKC). MbIL-6Rα and mbGP130 are proteins of 80 and 130 kDa, respectively. However, the smaller soluble forms identified by Müllberg and colleagues were 55 and 100 kDa. SIL-6Rα has also been found to be generated through an alternative splicing mechanism. Lastly, Diamant and coworkers discovered that sGP130 can be generated through alternative splicing as well, and that the resulting sGP130 lacks the transmembrane region of mbGP130. The alternatively spliced sIL-6Rα also lacks the transmembrane portion, and it contains a novel sequence at the carboxyl terminus (C-terminus), GSRRRGSCGL, which does not alter its properties.

Recently, Ahmed and colleagues evaluated the effects of epigallocatechin-3-gallate (EGCG) in an induced arthritis rat model. EGCG (Figure 6) is the major catechin found in green tea, and it exhibits anti-inflammatory, chemopreventive and chemotherapeutic properties. Interestingly, EPCG appears to act by up-regulating the alternative splicing mechanism that produces sGP130. It is also interesting that with an increase in sGP130 production a corresponding decrease in mbGP130 expression was observed. EPCG will need to be further evaluated for its therapeutic potential, and if its promise holds true, it could be the first of a class of compounds that act by up-regulating the production of sGP130.
Figure 5. Mechanisms of generating soluble receptors (reprinted with permission from © Elsevier).^7

Figure 6. (–)-Epigallocatechin-3-O-gallate.

Two enzymes belonging to the ADAM (a disintegrin and metalloproteinase) family have been found to be involved in the generation of sIL-6Rα through proteolytic shedding.^56,79 When ADAM17 is activated, the result is rapid IL-6Rα proteolysis, while ADAM10 appears to be involved in the slower constitutive shedding of IL-6Rα.^[79,89] After
cleavage by ADAM10 or ADAM17, another enzyme, γ-secretase, is responsible for cleaving the C-terminal fragment of IL-6Rα, thereby removing the remaining membrane stub. This could potentially result in an IL-6Rα intracellular domain fragment, which is thought to be rapidly degraded, as opposed to being involved in nuclear signaling.

Under apoptotic stimulation, ADAM17-mediated IL-6Rα shedding is induced, and this shedding was found to be caspase-dependent, but PKC-independent. This mechanism is thought to be critical in the resolution of inflammation, since the shedding of IL-6Rα by neutrophils undergoing apoptosis results in the recruitment of monocytic cells, which play a role in clearing the apoptotic neutrophils. It has also been found that when cells are depleted of cholesterol, PKC-independent IL-6Rα shedding is induced. While high levels of cholesterol have been considered detrimental for years, this finding suggests that constant low levels of cholesterol could favor inflammatory processes, play a role in the pathogenesis of a number of diseases, and at least partly explain why low levels of cholesterol are correlated with increased mortality.

1.3 Endocytosis: Down-Regulation of the Receptor Complex

1.3.1 Introduction

After IL-6 stimulates GP130 signaling, it is quickly internalized via endocytosis, along with its receptor complex, which results in the down-regulation of its surface receptor. In a rat model, after injecting recombinant human IL-6 (rhIL-6), the cytokine was cleared with biphasic kinetics; it was initially rapidly cleared with a half-life of 3 minutes, and the remaining rhIL-6 was cleared with a slower half-life of roughly 55
minutes.\textsuperscript{92} The majority of rhIL-6 removed from circulation was found in the liver, which is not surprising, since hepatocytes are one of the main cell types that express IL-6R\textalpha.\textsuperscript{6,92} Interestingly, over time the amount of rhIL-6 in the liver decreased, and a corresponding increase in rhIL-6 was observed in skin fibroblasts.\textsuperscript{93} It was found that the degradation of rhIL-6, in fact, occurs in these cells.\textsuperscript{93} However, it is important to note that hepatocytes have been found to degrade IL-6 as well, so the liver is involved in both classical IL-6 signaling as well as the degradation of IL-6.\textsuperscript{6,94}

Receptor-bound IL-6 was observed to have a half-life of 15 minutes in rat hepatocytes.\textsuperscript{94} Internalized IL-6 reached its maximum at 30 minutes, where afterwards, the radiolabel appeared in a degraded state.\textsuperscript{94} It was also found that as IL-6 was internalized, its surface-receptors were lost.\textsuperscript{73} The soluble receptor, sIL-6R\textalpha, is also able to be internalized, and it has been shown that once sIL-6R\textalpha is internalized, it undergoes lysosome-mediated degradation.\textsuperscript{95} IL-6R\textalpha is not recycled after internalization, and this is thought to be an important mechanism for temporarily preventing additional stimulation.\textsuperscript{73}

1.3.2 Dependence on a Di-Leucine Internalization Motif

It has been found that internalization of IL-6 with the entire receptor complex is dependent on an intracellular domain of GP130 containing the amino acid sequence TQPLLDSEER.\textsuperscript{96} It was found that the di-leucine internalization motif was critical for endocytosis and lysosomal degradation to occur, and that substituting an upstream serine residue with alanine drastically decreased the internalization rate.\textsuperscript{97} This suggests that
serine-phosphorylation may be critical for sufficient internalization to occur.\textsuperscript{97} It was later found that phosphorylation of S782, located close to the amino terminus (N-terminus) of the di-leucine pair (L786 and L787), is a key regulator of the membrane expression of the receptor.\textsuperscript{98,99} S780 was found to be required for the phosphorylation of S782 to occur.\textsuperscript{98} However, S780 does not get phosphorylated in this process.\textsuperscript{98} In contrast, the cytoplasmic portion of mbIL-6R\textalpha{} is not critical for endocytosis.\textsuperscript{96}

1.3.3 Independence from the JAK/STAT Pathway

It was found that once primary rat hepatocytes were exposed to IL-6, JAK2 reached its most active state within 2 minutes.\textsuperscript{100} GP130 phosphorylation follows JAK2 phosphorylation, reaches its maximum within another 2 minutes, and then declines to its baseline level within the next hour.\textsuperscript{100} At least two more hours were required for additional phosphorylation to occur after adding more IL-6, which suggests that GP130 is internalized after activation.\textsuperscript{100} For further activation to occur, \textit{de novo} GP130 synthesis was necessary.\textsuperscript{100} This is not surprising, considering that high levels of GP130 mRNA are expressed in many cell types.\textsuperscript{101,102}

Interestingly, although GP130 has been shown to be a substrate of JAK2,\textsuperscript{100} it has been shown using a chimeric receptor system that the endocytosis of GP130 is independent of the activation of the JAK/STAT pathway.\textsuperscript{103} However, after receptor-stimulation via IL-6, STAT reaches its maximum activation at 15 to 30 minutes, and yet it is still deactivated within 60 to 90 minutes.\textsuperscript{104} When comparing endocytosis-competent and -incompetent receptor chimeras in the melanoma cell line A375, it was found that
there was no significant difference in cell proliferation, which showed that STAT activity is not down-regulated by the internalization of the receptor complex.\textsuperscript{104} Instead, \textit{de novo} synthesis of inhibitors resulting in negative feedback are responsible for this.\textsuperscript{104}

1.4 The IL-6/IL-6R\textalpha/GP130 Receptor Complex

1.4.1 IL-6

The structure of IL-6 was solved in 1997.\textsuperscript{105,106} It is a 26 kDa, 185 amino acid peptide, which contains five \(\alpha\)-helices, with four of them making up a classical four-helix bundle with an ‘up-up-down-down’ conformation.\textsuperscript{105,106,107,108} Bazan was the first to describe this conformation, and contributed a great deal to the initial understanding of cytokine-receptor binding.\textsuperscript{109,110,111,112,113,114}

To determine the regions of human IL-6 (hIL-6) that are important for binding, chimeras were created that contained portions of both murine IL-6 (mIL-6) and hIL-6.\textsuperscript{115} Ultimately, three sites were found to be involved in binding.\textsuperscript{116} The site I epitope is responsible for binding to IL-6R\textalpha.\textsuperscript{116} It is located at the C-terminus of helix D, as well as part of the C-terminal portion of the A-B loop and the N-terminal portion of helix B.\textsuperscript{116} Sites II and III are involved in binding to GP130, and are dependent on the interaction of IL-6 with IL-6R\textalpha.\textsuperscript{116,117} Site II is located on residues of helixes A and C, while site III is made up of residues located on the C-D loop and the N-terminus of helix D. The binding sites are portrayed in the mIL-6 model (Figure 7).\textsuperscript{116}
1.4.2 The IL-6/IL-6Rα Binary Complex

The cDNA for human IL-6Rα (hIL-6Rα) encodes a protein that is 468 amino acids long, which consists of a signal peptide that is composed of 19 amino acids and extracellular, transmembrane and cytoplasmic regions as well. The predicted molecular weight of IL-6Rα would be 50 kDa, but in its mature glycosylated form it weighs 80 kDa. The extracellular portion is composed of 339 amino acids, and consists of three domains that are each approximately 100 amino acids in length. The transmembrane and cytoplasmic domains are composed of 28 and 82 amino acids, respectively. As it might be inferred from the previous discussion of sIL-6Rα, both the cytoplasmic and transmembrane domains of IL-6Rα do not play a role in signal
transduction. As a result, it is not surprising that unlike other growth factor receptors, the cytoplasmic domain of IL-6Rα does not contain a tyrosine/kinase domain.

Of the three extracellular domains, the N-terminal domain I is Ig-like, while domains II and III are fibronectin type III (FnIII) modules. The two FnIII modules make up the cytokine binding domain (CBD), which is also known as the cytokine binding module (CBM). Only the CBD is responsible for the binding of IL-6 and signaling via GP130. Like other members of this receptor family, IL-6Rα contains four conserved cysteine residues in domain II, a hinge region containing conserved proline residues, and a WSxWS box (where “x” is a non-conserved residue) in the C-terminal domain III (Figures 8 and 9). The WSxWS box helps form the shape that is needed for ligand binding. It has been found that domain III alone is capable of binding IL-6, but that domain II is also required for association with GP130 to occur.

Figure 8. The tertiary folding of β-strands in receptor domains (this figure was originally published in Proceedings of the National Academy of Sciences of the United States of America, © National Academy of Sciences). Circles: residues characteristic of class 1 receptors, diamonds: residues characteristic of class 2 receptors, square boxes: globally conserved amino acids.
Figure 9. The domains (D₁, D₂ and D₃) of sIL-6Rα (this figure was originally published in Proceedings of the National Academy of Sciences of the United States of America, © National Academy of Sciences). L: loop.

Through use of a model, it has been suggested that hydrophobic and aromatic contacts make up a hydrophobic core and account for the majority of the binding free energy, and that the core is surrounded by hydrophilic residues that are involved in recognition. Domain III of IL-6Rα has been found to be greatly involved with the binding of IL-6 at the site I binding epitope, and it contains 18 residues that directly contact IL-6. IL-6Rα contains two phenylalanine residues (Phe²²⁹ and Phe²⁷⁹) that together contribute 48% of the total buried surface area. Phe²²⁹ is predicted to be at the center of the binding site, and has been identified as a “hotspot.” IL-6 contributes charge interactions (such as with Arg¹⁷⁹ and Lys¹⁷¹) that surround the hydrophobic interactions.
1.4.3 The IL-6/IL-6R/GP130 Receptor Complex

GP130 is a 130 kDa protein, while sGP130 has been detected as a 100 kDa protein, which corresponds to the mass of GP130 when the cytoplasmic and transmembrane domains are absent.\textsuperscript{67} The ectodomain of GP130 is expected to be 65 kDa, but the additional 35 kDa observed for sGP130 is a result of glycosylation.\textsuperscript{67} From cloning and characterizing cDNA encoding for GP130, it was found that the cDNA encodes a 22 amino acid signal peptide, an extracellular region consisting of 597 amino acids, a short transmembrane domain (TM) consisting of 22 amino acids, and a cytoplasmic domain of 277 amino acids.\textsuperscript{17} Mature GP130 consists of 896 amino acids, which corresponds to a molecular mass of 101 kDa.\textsuperscript{17,116,121} However, it contains 14 potential sites for N-linked glycosylation, which results in it having a higher mass in its fully mature form.\textsuperscript{17,116,121}

The extracellular portion of GP130 consists of an N-terminal Ig-like domain (domain I), two FnIII domains that make up the CBM (domains II and III), and three additional membrane-proximal FnIII domains.\textsuperscript{3,116} These three addition domains are not needed for ligand binding.\textsuperscript{116,125} However, domain V has been implicated in the disulfide-linked homodimerization of GP130 and activation of the receptor.\textsuperscript{126,127} The Ig-like domain is also not required for ligand binding, but it is needed for signaling to occur.\textsuperscript{126,128} The CBM contains a WSxWS motif, characteristic cysteine residues and a hinge region containing proline residues, while the Ig-like domain is thought to conform to a seven-stranded Ig-like conformation.\textsuperscript{111,126,129,130} All six extracellular domains, as well as the transmembrane (TM) and cytoplasmic portions, are shown in Figure 10.
Figure 10. A schematic representation of the domains of GP130 (adapted from The Biochemical Journal, © the Biochemical Society).3

The IL-6/IL-6Rα complex forms the composite site II epitope that interacts with domains II and III of GP130.16,131 This epitope contains two different interactions. One interaction forms at the hinge region of GP130, between domains II and III, and the A and C helical faces of IL-6.16 The second interaction occurs between domain III of IL-6Rα and domain III of GP130.16

Phe169 of GP130 is critical for the first interaction, and mutational studies have revealed that it is required for all cytokine interactions.16,126,129 IL-6 contributes numerous polar residues (Arg24, Lys27, Arg30 and Asp34) that are situated in a way that their polar head groups are projecting toward the solvent; they lie flat in the interface.16 In contrast, the methylene groups contribute to the hydrophobic interactions.16 Concerning the second interaction, the overall shapes of domain III of IL-6Rα and domain III of GP130 are complementary; IL-6Rα provides a hump that fits into a cavity of GP130.16 Analysis of this second interaction demonstrates why IL-6 must first bind IL-6Rα before it can bind GP130, as this interaction significantly enhances the binding affinity.16
The site III epitope is unique to IL-6 type cytokines. Like the site II epitope, it also consists of two interactions.\textsuperscript{16} Contributing to the first interaction are 20 residues of IL-6 and 21 residues of GP-130.\textsuperscript{16} This interaction mainly involves the N-terminal region of helix D and the A-B loop of IL-6, and the bottom β sheet of domain I of GP130.\textsuperscript{16} A major contributor to the interaction is the A-B and C-D interhelical loops of IL-6, which form a groove that the N-terminus of GP-130 (Leu\textsuperscript{2} to Cys\textsuperscript{6}) fits into.\textsuperscript{16} Trp\textsuperscript{157} of IL-6 is critical for the first interaction.\textsuperscript{16} Through evaluating mutants of mIL-11 to analyze the binding to GP130, it was found that Trp\textsuperscript{147} of mIL-11 (analogous to Trp\textsuperscript{157} of IL-6) was critical to the site III epitope and for binding to occur.\textsuperscript{132} Paonessa and colleagues found that substituting Trp\textsuperscript{157} and Asp\textsuperscript{160} with Arg resulted in a lack of efficient homodimerization.\textsuperscript{117} Similar results were obtained when substituting Thr\textsuperscript{162} with Asp.\textsuperscript{117}

Concerning the second interaction, the top of domain I of GP130 interacts with domain II of IL-6Rα.\textsuperscript{16} The second interaction was not initially expected, but is able to form due to the curvature of the B and D helical axes of IL-6 that contribute the site I epitope.\textsuperscript{16} The site I epitope of IL-6 is found at one end of the arched surface, which results in IL-6Rα sticking out at an angle, allowing it to interact with the top of domain I of GP130.\textsuperscript{16} As a result, this interaction is dependent on the site I interaction.\textsuperscript{16} All 10 interactions of the complete hexameric IL-6/IL-6Rα/GP130 complex are shown (Figure 11).\textsuperscript{16}

In summary, IL-6 first binds IL-6Rα via the site I epitope to obtain the ability to bind GP130.\textsuperscript{126,131,133} This IL-6/IL-6Rα complex forms a composite site II epitope, which interacts with domains II and III of GP130, forming a trimolecular recognition complex.
with 1:1:1 stoichiometry, that by itself in not capable of signaling. An interaction between the site III epitope of IL-6 and IL-6Rα with the Ig-like domain I of GP130, which is unique to IL-6 type cytokines, allows for the transition to the higher-order and activated hexameric complex, which is signaling competent. The functional receptor complex is composed of IL-6, IL-6Rα, and GP130 with a stoichiometry of 2:2:2.

Figure 11. The extracellular domains of the hexameric IL-6/IL-6Rα/GP130 complex (reprinted with permission from © American Association for the Advancement of Science).
1.4.4 The Viral IL-6/GP130 Complex

Interestingly, viral IL-6 (vIL-6), which is encoded by Kaposi’s sarcoma associated herpesvirus (KSHV, also known as human herpes virus-8 (HHV8)), and is expressed in Castleman’s disease, Body-cavity based lymphoma, and Kaposi’s sarcoma, is able to directly activate GP130 without using an α-subunit.\textsuperscript{131,136} It interacts with GP130 through primarily hydrophobic contacts.\textsuperscript{131} The activated complex in this case is a tetramer (as opposed to a hexamer), consisting of two complexes that are cross-linked via interactions between the site III epitope of vIL-6 and the Ig-like domain of GP130.\textsuperscript{131}

1.5 Targeting IL-6 and GP130

1.5.1 IL-6 Signaling and Disease

As it has been discussed throughout this chapter, IL-6 has been implicated in the pathogenesis of a number of disorders. These include Crohn’s disease,\textsuperscript{58,66} juvenile chronic arthritis,\textsuperscript{59} RA,\textsuperscript{60,61} HIV,\textsuperscript{53} MM,\textsuperscript{6,62} asthma\textsuperscript{65,78,137,138} Kaposi’s sarcoma,\textsuperscript{136,139} body cavity-based lymphomas,\textsuperscript{136,140} Castleman’s disease,\textsuperscript{136,141,142} peritonitis\textsuperscript{65,78,143} and colon cancer.\textsuperscript{65,78,144,145} A number of other types of cancer also involve IL-6 signaling, primarily through the JAK/STAT pathway.\textsuperscript{146} They will be discussed in the next chapter.

1.5.2 Inhibitors of IL-6/GP130 Signaling

Studies involving TCZ and sGP130Fc have been mentioned. TCZ, sGP130Fc, therapies that act through similar mechanisms, and the small molecule inhibitor of GP130, madindoline, will be discussed here.
TCZ

TCZ was the first IL-6 blocker to be developed. It binds to both sIL-6Rα and mbIL-6Rα, inhibiting both classical and trans-signaling. It has been shown to bind to IL-6Rα with a $K_d$ of 2.53 ± 0.12 nM. It has been approved for the treatment of Castleman’s disease, RA, systemic juvenile idiopathic arthritis (JIA), and polyarticular JIA. It is currently in clinical trials for a number of other disorders as well (Table 3).

Other Protein-Based IL-6 Inhibitors Under Development

The current compounds that are being investigated as IL-6 blockers are all proteins, and more specifically, almost all of them are antibodies. They all fall under one of three classes (Figure 12, Table 4). The first class, which TCZ belongs to, targets IL-6Rα, and inhibits both classical and trans-signaling. The second class targets IL-6, also inhibiting both classical and trans-signaling. The third class, which currently consists of one sGP130Fc fusion protein, targets trans-signaling specifically. However, as was discussed previously, it can also inhibit classical signaling under certain conditions.
<table>
<thead>
<tr>
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<tr>
<td>Relapsing polychondritis</td>
<td>NCT01041424</td>
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<td>Type II diabetes, obesity</td>
<td>NCT01073826</td>
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<td>Ankylosing spondylitis</td>
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<td>Graves’ ophthalmopathy</td>
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<td>Cardiovascular disease in rheumatoid arthritis</td>
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<td>Polymyalgia rheumatica</td>
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<td>Giant cell arteritis</td>
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<td>Acute graft-versus-host disease</td>
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<td>Non-ST elevation myocardial infarction</td>
<td>NCT01491074</td>
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<td>Systemic sclerosis</td>
<td>NCT01532869</td>
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<td>Transplant rates in highly sensitized patients awaiting kidney transplantation</td>
<td>NCT01594424</td>
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<td><strong>European Union Clinical Trials Registry</strong></td>
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<td>Ankylosing spondylitis</td>
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<td>Systemic sclerosis</td>
<td>2011-001460-22</td>
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<td><strong>UMIN-CTR clinical trials (Japan)</strong></td>
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<tr>
<td>Anti-neutrophil cytoplasmic antibody-associated vasculitis</td>
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<td>Takayasu arteritis</td>
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Table 3. The evaluation of TCZ in clinical trials in the USA, EU and Japan (adapted and reprinted with permission from © Ivyspring International Publisher).\textsuperscript{141}
Figure 12. The three modes of IL-6 inhibition that are currently under investigation (reprinted with permission from © the American Society for Clinical Investigation).
<table>
<thead>
<tr>
<th>Targeting strategy/Compound</th>
<th>Company</th>
<th>Specificity</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Targeting IL-6Ra</td>
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<td></td>
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<tr>
<td>REGN88 (SAR153191)</td>
<td>Regeneron/Sanofi-Aventis</td>
<td>Fully human IL-6Ra-specific mAb</td>
<td>RA, Ankylosing spondylitis</td>
</tr>
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<td>ALX-0061</td>
<td>Ablynx</td>
<td>IL-6Ra-specific nanobody</td>
<td>RA</td>
</tr>
<tr>
<td>2) Targeting IL-6</td>
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<td></td>
<td></td>
</tr>
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<td>UCB Inc.</td>
<td>IL-6-specific mAb</td>
<td>RA</td>
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<tr>
<td>CNTO136 (Sirukumab)</td>
<td>Centocor Inc.</td>
<td>Fully human IL-6-specific mAb</td>
<td>RA</td>
</tr>
<tr>
<td>CNTO328 (eCLB, Siltuximab)</td>
<td>Centocor Inc.</td>
<td>Chimeric IL-6-specific mAb</td>
<td>Castleman's disease, MM, Prostate cancer, Solid tumors, Metastatic renal cell carcinoma, Metastatic kidney cancer</td>
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<tr>
<td>ALD518 (BMS-945429)</td>
<td>Alder Biopharmaceuticals/Bristol-Myers Squibb</td>
<td>Humanized IL-6-specific mAb, aglycosylated</td>
<td>RA, Non–small cell lung cancer–related fatigue and cachexia</td>
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<td>Avidia</td>
<td>Anti-IL-6/anti-Fc avimer protein</td>
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<td>3) Targeting IL-6/sIL-6Ra</td>
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<tr>
<td>FE301</td>
<td>Conaris/Ferring</td>
<td>sGP130Fc fusion protein</td>
<td>Crohn's disease</td>
</tr>
</tbody>
</table>

Table 4. Therapeutic proteins that are currently under investigation (adapted with permission from © the American Society for Clinical Investigation).
Madindoline

(+)-Madindoline A (MDL-A) and (+)-madindoline B (MDL-B) were isolated from *Streptomyces nitrosporeus*, and their structures were elucidated in the mid 1990’s ([Figure 13](#)). They were found to be potent IL-6 inhibitors. Importantly, they were found to be selective, as both of them inhibited the growth of IL-6 dependent MH60 cells, but not IL-6-independent MH60 cells. Their IC$_{50}$ values for the IL-6 dependent MH60 cells were 8 and 30 µM, respectively.

![Figure 13. (+)-Madindoline A and (+)-madindoline B.](#)

In an effort to confirm the structural assignments, and to assign the relative and absolute configurations, Omura and colleagues performed and reported the first total synthesis of MDL-A and (-)-madindoline B, the enantiomer of the naturally occurring MDL-B, in 2000. Their total synthesis was also necessary since they were no longer being produced in the original bacteria. Since then, a number of total syntheses for the madindolines have been reported.

MDL-A is noncytotoxic and is highly selective for IL-6 and IL-11, making it a lead compound as an IL-6 inhibitor. It has been shown to inhibit both IL-11- and IL-6-
induced osteoclastogenesis \textit{in vitro}.\textsuperscript{160} \textit{In vivo}, it has also been shown to inhibit IL-6-stimulated serum amyloid A production.\textsuperscript{160} In contrast, MDL-A did not inhibit the growth of IL-2-dependent CTLL-2 cells, the growth of IL-3-dependent Ba/F3 cells, TNF\textalpha activity in mouse fibrosarcoma L929 cells, or the LIF-induced differentiation of M1 cells to macrophage-like cells.\textsuperscript{160} Interestingly, MDL-A is shown to act through binding to GP130, but it does not inhibit the formation of the IL-6/IL-6R\alpha/GP130 complex.\textsuperscript{160} This suggests that its primary mode of action is to inhibit the dimerization of the trimeric complexes.\textsuperscript{160}

Through surface plasmon resonance (SPR)-based biosensor analysis, it was found that MDL-A binds GP130 with a \( K_\text{d} \) of 288 \( \mu \text{M} \), which is of relatively low affinity.\textsuperscript{161} Under standard denaturing conditions, GP130 was able to be released from MDL-A, which suggests that the interaction is non-covalent.\textsuperscript{161} Interestingly, the 3a-tetrahydrofuro[2,3-\textit{b}]indole ring alone was shown to not bind to GP130, suggesting that the diketo cyclopentene moiety is required for binding.\textsuperscript{161} However, whether or not the two halves function synergistically, or if the diketo cyclopentene moiety alone was sufficient for binding, was not determined.\textsuperscript{161}

Since MDL-A has been shown to bind to GP130, to not inhibit the formation of the initial IL-6/IL-6R\alpha/GP130 complex, and to not inhibit LIF, MDL-A is thought to interact with domain I of GP130.\textsuperscript{160,161} This would be a rational conclusion in light of the fact that LIF does not require domain I of GP130 for signaling, while in IL-6 signaling domain I of GP130 is not required for the formation of the IL-6/IL-6R\alpha/GP130 complex, but is required in the transition to the signaling-competent hexameric complex.\textsuperscript{16,160,161}
While the properties of MDL-A make it a promising lead compound, its relatively low affinity to GP130 would suggest that higher affinity analogues would need to be developed in order for there to be therapeutic relevance.\textsuperscript{161}

In recent years, analogues have been developed which have helped provide insight into the structure-activity relationships (SARs) of MDL-A-derived compounds.\textsuperscript{153} The length of the longer of the alkyl chains of the diketo cyclopentene moiety seems to be highly important, as the addition of five methylene groups to the chain increased the activity by a factor of 3.9.\textsuperscript{153} Acyl analogues of the 3a-hydroxy moiety also generally increased activity, as did bromination and iodination of the C5 position of the hydroxyfuroindoline aromatic ring.\textsuperscript{153}

However, additional analogues need to be developed in order to attain GP130 inhibitor(s) with therapeutic relevance. This is one of the current and ongoing projects in Dr. Fuchs’ laboratory. Still, downstream of GP130 are JAK2 and STAT3. Their roles in diseases and the inhibitors that have been developed to target them will be discussed in the next chapter.
Chapter 2: The JAK/STAT Pathway as a Molecular Target

2.1 GP130 Signaling

2.1.1 Introduction

GP130 undergoes disulfide-linked homodimerization upon the binding of IL-6/IL-6Rα, which leads to activation of GP130 signaling.\textsuperscript{106,162} As previously stated, the main signaling pathways activated by IL-6 are the PI3K/Akt/mTor, MAPK, and JAK/STAT pathways.\textsuperscript{27}

2.1.2 Activated JAK

There are six tyrosine residues in GP130’s cytoplasmic region.\textsuperscript{17,163} Members of the JAK protein family are constitutively associated with GP130, and become activated following receptor dimerization.\textsuperscript{164} Following activation, JAKs phosphorylate tyrosine residues on the cytoplasmic tail of GP130.\textsuperscript{6} Phosphorylation at the membrane-proximal Y759 of GP130 (or Y757 for mGP130) allows for the recruitment of the Src homology 2 (SH2) domain-containing tyrosine phosphatase 2 (SHP2), its phosphorylation, and activation of the PI3K/Akt/mTor and MAPK cascades.\textsuperscript{2,6,165} The consensus binding site for SHP2 (as well as the competing suppressor of cytokine signaling (SOCS)-3, which will be discussed later) is YxxV, where Val is located at the +3 position.\textsuperscript{2,165} In contrast, phosphorylation at the membrane-distal tyrosine residues located near the C-terminus of GP130 (human: Y767, Y814, Y905 and Y915; murine: Y765, Y812, Y904 and Y914)
results in the recruitment of the SH2 domain-containing STAT proteins (primarily STAT1 and STAT3). The consensus binding site for STAT is YxxQ, where Gln is located at the +3 position. The STATs are then tyrosine-phosphorylated, undergo homo- and/or hetero-dimerization via the reciprocal interactions of the phosphotyrosine (pTyr) residues and SH2 domains, translocate to the nucleus, and activate the transcription of target genes. The signaling complex for the murine model and the activation of the signaling pathways is shown (Figure 14).

Figure 14. Activation of the JAK/STAT, MEK/ERK (MAPK), and PI3K/Akt pathways as a result of the tyrosine phosphorylation of GP130 (reprinted with permission from © John Wiley & Sons, Inc.).
The Y759 binding site of GP130 is crucial for the GP130-mediated activation of the MAPK cascade.\textsuperscript{3,165} After this site is phosphorylated, SHP2 is able to bind, and then is itself tyrosine-phosphorylated by JAK1.\textsuperscript{168} Following its phosphorylation, SHP2 is able to interact with growth factor receptor binding protein 2 (Grb2).\textsuperscript{2,3,169} Grb2 is constitutively associated with son of sevenless (Sos), a GDP/GTP Ras exchange factor.\textsuperscript{2,3,170} Tyr\textsuperscript{542} and Tyr\textsuperscript{580}, located near the C-terminus of SHP2, are thought to be involved in the interaction with the Grb2/Sos complex.\textsuperscript{3,169} Ras becomes activated, and GTP-bound Ras results in extracellular-regulated kinase (ERK) activation, which results in activation of the Ras-Raf-ERK1/2 MAPK cascade.\textsuperscript{2,3,165,170} It should be noted that OSMR is able to activate the MAPK cascade as well. However, in contrast to LIFR and GP130, it recruits the adapter protein Shc (SH2 and collagen homology domain containing protein) to Tyr\textsuperscript{861}, which recruits Grb2 to its phosphorylated Tyr\textsuperscript{317}, allowing for the activation of the MAPK cascade.\textsuperscript{3,171}

Tyrosine-phosphorylated SHP2 can also form complexes with Grb2-associated binding proteins (Gabs) 1 and 2 and the p85 subunit of PI3K, leading to the activation of the Akt pathway.\textsuperscript{2,36} Gab1 is tyrosine phosphorylated in response to numerous cytokines, including IL-3, IL-6, IFNα and IFNγ.\textsuperscript{36} Gab1 contains a pleckstrin homology (PH) domain, as well as tyrosine residues, which make for potential binding sites for numerous adapter molecules containing SH2 domains following phosphorylation.\textsuperscript{36} Interestingly, Gab1 expression enhances GP130-mediated ERK2 activation, which signifies that Gab1 is also involved in activating the Ras-Raf-MAPK cascade.\textsuperscript{3,36} Mutation of the SHP2 binding site of GP130, Tyr\textsuperscript{759}, abolished the interactions of Gab1 with PI3K and SHP2, as
well as ERK2 activation. The presence of dominant negative p85 PI3K or Ras also inhibited the activation of ERK2. These findings suggest that PI3K, SHP2 and Ras are all involved in Gab1-mediated ERK activation.

2.1.3 The MAPK Cascade

The Ras-Raf-MEK-ERK signaling cascade, also known as the MAPK cascade, is involved in a number of cellular processes, including cell adhesion, cell migration, differentiation, cell cycle progression, cell survival, proliferation, metabolism and transcription. Ras mutations are found in a number of cancers. Mutations to Raf also contribute to cancer. For example, gain of function mutations in the genes encoding K-Ras and B-Raf are present in approximately 50% of colorectal cancers. Importantly, even in the absence of oncogenic mutations, the MAPK pathway is up-regulated in many cancers.

Ras is inactive with GDP bound, but becomes active when GTP is bound. The guanine nucleotide exchange factor (GFR) Sos promotes the activation of Ras, while GTPase-activating proteins (GAPs) increase the rate at which Ras-mediated GTP hydrolysis occurs. It should be noted that Ras activation can be facilitated by a number of receptors, and that Ras is localized to the plasma membrane as a result of farnesylation.

Ras-GTP then interacts with Raf family members at the Ras-binding domain. Upon homo- or hetero-dimerization of the protein serine/threonine Raf kinases, they become active and catalyze the phosphorylation of the MAP/ERK kinases (MEKs) 1 and
MEK1 and MEK2 are dual-specificity kinases that phosphorylate ERK1 and ERK2 (MAPKs) on tyrosine and then threonine residues. ERK1 and ERK2 are the only known substrates of MEK1 and MEK2. It should be noted that Raf kinases have narrow substrate specificity as well.

In contrast, the ERKs have been documented to have around 160 substrates. Many of these substrates are transcription factors, some are cytosolic and others are located in cellular organelles. Given the involvement ERK1/2 have with transcription factors, it is not surprising that they can translocate to the nucleus following activation. Due to the number of substrates of ERK1/2, one could imagine that determining their overall effects is complicated. It should be noted that Ras is also capable of interacting with several families of effector proteins, and as a result, can affect other pathways. However, that in-depth topic is outside of the scope of this discussion. Activation of the SHP2-mediated MAPK cascade is shown below (Figure 15).

Figure 15. The MAPK cascade.
2.1.4 The PI3K/Akt/mTor Pathway

The members of the Akt family are signaling proteins that respond to a large number of signals.\textsuperscript{30} They are involved in metabolism, motility, cell-cycle progression, cell survival and transcription.\textsuperscript{30} They belong to the PKA, PKG and PKC related kinase (AGC) family.\textsuperscript{182} There are three members of the human Akt family, which all contain a N-terminal PH domain, a kinase domain, and a C-terminal regulatory domain that contains a hydrophobic motif (HM).\textsuperscript{182} Increased activity of the PI3K/Akt pathway has been implicated in many cancers.\textsuperscript{183} Constitutive activation of Akt can occur through numerous mechanisms. However, this by itself is not believed to be sufficient for tumorigenesis.\textsuperscript{30} Instead, it is thought to aid in cancer progression through the promotion of cell survival and proliferation, and by increasing the metabolic capacity of cells.\textsuperscript{30} It has also implicated in the pathogenesis of diabetes.\textsuperscript{184} GP130-mediated Akt activation has been implicated in KSHV pathogenesis as well.\textsuperscript{185}

PI3K can be activated through numerous routes, which involve the activation of receptor tyrosine kinases (RTKs) due to the binding of growth factors, G-protein coupled receptors, or oncogenes like Ras.\textsuperscript{29} PI3K is a heterodimeric protein that contains a p85 regulatory subunit and a p110 catalytic subunit.\textsuperscript{177,186} PI3K can be activated by direct binding to the SH2 domain of its regulatory subunit by certain receptors, as is the case with EpoR.\textsuperscript{183,187,188} Ras can recruit and activate PI3K by directly binding its catalytic subunit.\textsuperscript{183} PI3K can also be activated by a complex formation between the SH2 domain of its regulatory subunit and adapter proteins. This results in conformational changes in the regulatory subunit and brings the catalytic subunit to the cell membrane, where it is in
close proximity to its phospholipid substrates.\textsuperscript{183,187} This is the case in Gab-mediated activation.\textsuperscript{189}

Activated PI3K phosphorylates membrane phosphoinositides at the D-3 position of the inositol ring, and is involved in the production of the biologically active phosphatidylinositol-3,4-bisphosphate from phosphatidylinositol-4-phosphate and phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2).\textsuperscript{29,177,183} PTEN (tumor suppressor phosphatase and tensin homology deleted on chromosome ten) and SHIP (SH2 domain-containing inositol polyphosphate 5-phosphatase) oppose the actions of PI3K by removing a phosphate at either the 3 or 5 position of PIP3.\textsuperscript{30} Wortmannin and LY294002 are two known inhibitors of PI3K.\textsuperscript{30}

PIP3 binds to the PH domain of Akt, recruiting it to the cell membrane.\textsuperscript{29,190,191} PIP3 is also involved in the translocation and activation of 3’phosphoinositide-dependent kinase 1 (PDK1), through binding its PH domain and recruiting it to the cell membrane.\textsuperscript{29,183} Akt is activated by being phosphorylated first at Thr\textsuperscript{308} within the activation loop, and then at Ser\textsuperscript{473}, which is located at the C-terminal HM.\textsuperscript{29} PDK1 is responsible for phosphorylating Akt at Thr\textsuperscript{308}.\textsuperscript{192} Ca\textsuperscript{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) has also been shown to phosphorylate Akt at Thr\textsuperscript{308}.\textsuperscript{193,194}

In contrast, a number of kinases have been implicated in the phosphorylation of Akt at Ser\textsuperscript{473}.\textsuperscript{29} One of these is integrin-linked kinase (ILK), which is thought to act as an adapter, by either recruiting another kinase or inhibiting a specific Ser\textsuperscript{473} phosphatase.\textsuperscript{194,195} DNA-dependent protein kinase (DNA-PK) has been shown to directly phosphorylate Ser\textsuperscript{473} of Akt.\textsuperscript{196,197} Interestingly, PDK1 is thought to gain Ser\textsuperscript{473} kinase
activity by interacting with other protein(s).\textsuperscript{198} Even more interestingly, it has been suggested that Akt regulates itself via autophosphorylation at Ser\textsuperscript{473}.\textsuperscript{199} Lastly, mTor, a downstream effector of PI3K/Akt, has also been shown to phosphorylate Akt.\textsuperscript{200,201} In contrast, both protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP) are able to directly inactivate Akt through dephosphorylation.\textsuperscript{30,202,203} The activation of PI3K, PIP3, PDK1 and Akt (PKB) in response to an activated RTK is shown below (Figure 16).\textsuperscript{182}

Akt has numerous cellular substrates, and once active, it can translocate to the nucleus and exert its effects on a number of transcription factors.\textsuperscript{29,177} It regulates a

\textbf{Figure 16. Activation of the Akt pathway (reprinted with permission from © Elsevier).}\textsuperscript{182}
number of proteins which control survival, cell proliferation and growth, among other processes.\textsuperscript{183} More than 100 nonredundant substrates for Akt have been reported.\textsuperscript{204,205}

The large kinase, mTor, exists in two complexes, mTorC1 and mTorC2.\textsuperscript{29,184} Akt activates mTorC1 through both a direct and indirect mechanism, while mTorC2 is responsible for activating Akt via phosphorylation of Ser\textsuperscript{473}.\textsuperscript{29,184} Along with Akt, abnormal mTor activation is also implicated in many cancers.\textsuperscript{200} Interestingly, it has been found recently that mTorC2 activity is critical for a number of roles in a variety of cancers, while it is less essential for normal cells.\textsuperscript{201} As a result, mTorC2 has recently become a promising target.\textsuperscript{201}

\textit{2.1.5 Termination of GP130/JAK/STAT Signaling}

The structures, interactions, implications in disease, and current treatments of the JAKs and STATs will be discussed in the following sections. First, the mechanisms of signal termination will be described.

**Protein Inhibitor of Activated STAT (PIAS) Proteins**

The PIAS (protein inhibitors of activated STAT) proteins bind to tyrosine-phosphorylated STATs, and as a result, prevent their dimerization, translocation to the nucleus, and gene activation.\textsuperscript{2,206,207} Four PIAS family members have been shown to inhibit STAT activity: PIAS1, PIAS3, PIASX, PIASY.\textsuperscript{208,209} It should be noted that different splice variants of PIAS family members also exist.\textsuperscript{208}
PIAS1 was initially identified as Gu/RNA helicase II binding protein (GBP), through use of a yeast two-hybrid system. It was later discovered that this protein, PIAS1, inhibits STAT1 activity. It was found through co-immunoprecipitation analysis that PIAS1 could associate with STAT1, but not with STAT2 or STAT3. Furthermore, the \textit{in vivo} interaction between STAT1 and PIAS1 was found to be dependent on the phosphorylation of STAT1 at Tyr\textsuperscript{701}. Also, PIAS1 has been shown to interact with dimeric STAT1, but not monomers of STAT1. Interestingly, the methylation of STAT1 at Arg\textsuperscript{31} by protein arginine methyl-transferase 1 (PRMT1) is thought to inhibit the interaction between STAT1 and PIAS1, thereby increasing the DNA binding activity of STAT1.

PIAS3 was the first PIAS family member that was shown to inhibit STAT activity. PIAS3 directly binds to STAT3, but was only shown to do so in cells that contained active STAT3 as a result of ligand stimulation. PIAS3 was able to inhibit the DNA-binding activity of STAT3 and the corresponding gene activation. PIAS3 was also shown to not interact with STAT1.

PIASX and PIASY are the two more recently identified PIAS family members. It should be noted that PIAS family members can act through distinct mechanisms. Whereas PIAS1 and PIAS3 inhibit DNA binding, PIASX and PIASY do not block STAT DNA binding, but still inhibit STAT-mediated gene activation. PIASX and PIASY are thought to act by recruiting histone deacetylases (HDACs), and possibly other co-repressor molecules, to their complexes. PIASX and PIASY negatively regulate STAT4 and STAT1 activity, respectively.
Small ubiquitin-related modifier (SUMO) has been shown to be added to target proteins by SUMO E3 ligases in both yeast and mammals.\textsuperscript{215,216,217,218} In contrast to the ubiquitin system, which often leads to degradation, the sumoylation of target proteins usually modulates their functions by affecting their localization and/or activation, and protecting them from degradation by the ubiquitin system.\textsuperscript{216} E3 SUMO ligase activity, which involves many targets, has been shown for all members of the PIAS family.\textsuperscript{218,219,220} STATs are among the targets of PIASs for sumoylation.\textsuperscript{219} For example, STAT1 is sumoylated at Lys\textsuperscript{703}, close to a critical residue, Tyr\textsuperscript{701}.\textsuperscript{221,222} Presumably, the sumoylation of STAT family members, particularly STAT1 and STAT3, has great significance, and it has been suggested that it represses STAT activity.\textsuperscript{208,219,222,223,224} However, the exact effects of the sumoylation of STAT family members is controversial, as they remain to be clarified.\textsuperscript{208,219,223,224}

There are several mechanisms by which PIAS proteins may inhibit transcription factors (TFs) (\textbf{Figure 17}).\textsuperscript{223} First, as stated above, in some cases, they can block the DNA binding activity of TFs.\textsuperscript{223} In contrast, they may instead recruit co-repressors, such as HDACs, to the complex.\textsuperscript{223} Sumoylation may positively or negatively affect transcription depending on the context.\textsuperscript{223} Or, lastly, PIAS proteins may sequester TFs to subnuclear structures.\textsuperscript{223}
Suppressor of Cytokine Signaling (SOCS) Proteins

Eight members of the SOCS protein family have been identified (SOCS1-7 and cytokine-inducible SH2 protein (CIS)). SOCS family members consist of a central SH2 domain, a variable-length N-terminal domain, and a C-terminal SOCS box. The SOCS SH2 domain is involved in binding to specific pTyr motifs, while the SOCS box consists of three α-helices that are bound to an E3 ubiquitin ligase complex. It has been implicated in the ubiquitination and subsequent proteasomal degradation of target proteins.

SOCS1 and SOCS3 play the most critical role in IL-6/JAK/STAT signaling. SOCS family members act through a classical negative feedback mechanism, where they are up-regulated in response to certain cytokines, and then act to attenuate cytokine signaling. However, the different SOCS family members display distinct mechanisms of inhibition (Figure 18).
SOCS1 binds directly to tyrosine-phosphorylated JAKs, directly inhibiting JAK activity.\cite{208, 227, 228, 229} For example, SOCS1 has been shown to bind to Tyr^{1007} of JAK2, which is located within the activation loop.\cite{226, 230} In contrast, SOCS3 has been shown to display high-affinity binding to the SHP2 docking site of GP130, Tyr^{759}.\cite{231, 232} SOCS3 has also been shown to bind to Tyr^{1007} of JAK2, although with lower affinity.\cite{226, 233, 234} For SOCS family members, multi-step interaction models have been suggested, where SOCS may first associate with the receptor, and then associate with JAK.\cite{3, 226} In fact, a partial crystal structure of the ternary complex was recently solved, and it showed that SOCS3 uses the two opposing surfaces of its SH2 domain to bind to GP130 and JAK2.\cite{235}
Tyrosine phosphorylation of GP130 was required for SOCS3 binding, while binding between SOCS3 and JAK2 was independent of JAK2 phosphorylation.\textsuperscript{235} Other SOCS family members, such as SOCS2 and CIS, appear to inhibit STAT signaling by directly competing for the STAT pTyr binding sites of several receptors.\textsuperscript{208,226} However, GP130 has not been implicated as one of these receptors.\textsuperscript{3,208,226} As a result, SOCS2 and CIS are not thought to be significantly involved in GP130 signaling.\textsuperscript{3,208,226} SOCS4-7 have also not been implicated in IL-6/GP130/JAK/STAT signaling.\textsuperscript{226} It should be noted that nearly all SOCS family members have been shown to have multiple binding partners.\textsuperscript{226}

In unstimulated cells, the SOCS proteins are usually expressed at low levels, but become rapidly induced following cytokine stimulation.\textsuperscript{208,236} Still, the mRNA expression profiles of the SOCS family members are not identical.\textsuperscript{236} Following stimulation with IL-6 or GH, SOCS1 and SOCS3 are rapidly induced and their mRNA levels peak at around one hour, but return to baseline by about hour four.\textsuperscript{228,236,237} In contrast, SOCS-2 has lower initial levels, but increases over a longer period of time.\textsuperscript{228,236,237}

The up-regulation of SOCS1 and SOCS3 are largely induced by the STATs.\textsuperscript{238} Naka and colleagues found that SOCS1 mRNA expression was inhibited by transfecting M1 cells with a dominant negative STAT3 mutant, which indicates that SOCS1 is a STAT3 target gene.\textsuperscript{227} This is not surprising considering that the promoter of the SOCS1 gene contains STAT3 and STAT6 binding sequences.\textsuperscript{227} Interestingly, Saito and colleagues found that in IFNγ-induced expression of SOCS1, STAT1 acted indirectly by inducing the expression of the transcription factor IFN regulatory factor 1 (IRF-1), which in turn was responsible for inducing SOCS1 expression.\textsuperscript{238,239}
The transcription of SOCS3 is activated by STAT3. For the gene encoding mSTAT3, a STAT1/STAT3 binding element is located at -72 to -64 upstream of the transcription start site. It was found to be essential for LIF-induced SOCS3 promoter activity. It was also shown that SOCS3 expression was inhibited in AtT-20 cells that were transfected with dominant negative STAT3. However, it should be noted that the up-regulation of SOCS3 can occur in a STAT3-independent manner as well, which involves the activation of protein kinase A (PKA) through increased cAMP concentrations.

Interestingly, SOCS3 has also been found to be tyrosine-phosphorylated at Tyr and Tyr, located within the SOCS box, in response to IL-2, epidermal growth factor (EGF), Epo and platelet-derived growth factor (PDGF). In one study, it was found that this phosphorylation occurred when JAK1 or JAK2 was co-expressed, but only weakly when JAK3 was expressed. In another study, phosphorylated SOCS3 was found to inhibit the activation of STAT5, but to also maintain the activation of the MAPK cascade by binding to the Ras inhibitor, RasGAP. Interestingly, JAK-mediated phosphorylation of SOCS3 has been shown to decrease the SOCS3 half-life and enhance its proteasome-mediated degradation. In this sense, the phosphorylation of SOCS3 could be a mechanism to regulate the amount of negative feedback provided by SOCS3.

The SOCS box was found to bind to elongins B and C, which signifies that SOCS may be involved in targeting proteins for proteasomal degradation. The heterodimeric elongin BC complex has been shown to be able to link to a Cullin-5/Rbx1
complex to form an E3 ubiquitin ligase complex.\textsuperscript{219,249} Indeed, the SOCS box was found to be essential for the proteasomal degradation of TEL-JAK2, a fusion protein that results from specific translocations that are associated with human leukemia.\textsuperscript{219,250} It should be noted that TEL transcription factor-JAK2 fusion proteins are present in human leukemias, result from chromosomal translocations, and produce constitutively activated JAK2.\textsuperscript{251,252}

Ubiquitination occurs through a multi-step process (\textbf{Figure 19}).\textsuperscript{219} Ubiquitin is first linked to the ubiquitin-activating enzyme, E1, which is powered by ATP hydrolysis.\textsuperscript{219} Ubiquitin is adenylated by E1 and then moved to a conjugating enzyme, E2.\textsuperscript{219} E2 then associates with the E3 complex and ubiquitin is transferred.\textsuperscript{219}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure19.png}
\caption{The SOCS1-mediated ubiquitination of JAK2 (this figure originally appeared in The Journal of Biological Chemistry. Samuel Wormald and Douglas J. Hilton. \textit{The Journal of Biological Chemistry}. 2004; 279:821-4. © the American Society for Biochemistry and Molecular Biology.).\textsuperscript{219}}
\end{figure}
SOCS1 has been shown to mediate wild-type (WT) JAK2 ubiquitination and subsequent proteasomal degradation.\textsuperscript{253} While JAK2 was monoubiquitinated in unstimulated cells, stimulation with IL-3 or interferon-\(\gamma\) (IFN\(\gamma\)) resulted in the polyubiquitination and subsequent proteasomal degradation of tyrosine-phosphorylated JAK2.\textsuperscript{253} Phosphorylation at Tyr\textsuperscript{1007} was found to be essential for proteasomal degradation.\textsuperscript{253}

SOCS proteins have been shown to be involved in the ubiquitination of many targets, but the targets are usually not receptors.\textsuperscript{226} The exact contributions of the SOCS box for SOCS family members is still being investigated, but for both SOCS1 and SOCS3, it has been demonstrated that lacking the SOCS box motif results in prolonged or increased responses to cytokines.\textsuperscript{226,254,255} It should be noted that STAT3 has also been found to be ubiquitinated and to undergo proteasomal degradation, although the involvement of SOCS was not implicated.\textsuperscript{256}

SOCS proteins serve a critical role in signaling, and reduced expression or inactivation of them can lead to sustained signaling.\textsuperscript{224} The SOCS3 is gene has been found to be hypermethylated at CpG islands of the promoter in lung cancer, breast cancer, mesothelioma, and human hepatocellular carcinoma (HCC) cell lines, as well as primary HCC and lung cancer tissue samples.\textsuperscript{257,258} This methylation silences SOCS3 and significantly contributes to constitutive STAT3 activity.\textsuperscript{257,258} Restoration of SOCS3 leads to a down-regulation of STAT3, suppression of growth, and induction of apoptosis.\textsuperscript{257,258} Hypermethylation of both the SOCS3 and SOCS1 promoters have also been reported in head and neck squamous cell carcinoma (HNSCC) and Barrett’s
adenocarcinoma, with the SOCS3 promoter being hypermethylated in a greater percentage of samples in both cases.\textsuperscript{259,260} Overexpression of WT SOCS3 in carcinoma cells exhibiting silenced SOCS3 led to a down-regulation of STAT3, Bcl-2 and Bcl-xL, as well as the suppression of growth and induction of apoptosis.\textsuperscript{259} This silencing of SOCS3 can clearly play a major role in the constitutive activation of STAT3 and the associated disorders that result.\textsuperscript{257,260,261}

**Protein Tyrosine Phosphatase (PTP) Activity**

IL-6 signal transduction involves the phosphorylation of a number of components. Protein tyrosine phosphatases (PTPs) are able to regulate signaling by targeting the phosphorylated residues.\textsuperscript{3} The discussion of PTPs is quite complex, and their actions are an on-going focus of research. The main PTPs that play a role in JAK/STAT signaling, and particularly STAT3 signaling, will be discussed here.

**STAT3**

It was recently shown that TC45, the nuclear form of T-cell PTP (TCPTP), as well as SHP1 and SHP2, are involved in the dephosphorylation of activated STAT3.\textsuperscript{262} Interestingly, in mouse keratinocytes, TC45 was localized in the cytoplasm, but following UVB radiation, it accumulated in the nucleus, where a corresponding rapid dephosphorylation of STAT3 occurred.\textsuperscript{262} As STAT3 was dephosphorylated, the expression of two of STAT3’s target genes, cyclin D1 and c-Myc, were also down-regulated.\textsuperscript{262} The nuclear form of TCPTP had also previously been shown to form
complexes with STAT3 in the nuclei of M1 myeloid leukemia cells.\textsuperscript{263} Furthermore, it has been shown in mouse embryonic fibroblasts that PKR (protein kinase double-stranded RNA-dependent) negatively regulates both STAT1 and STAT3 tyrosine phosphorylation, but not serine phosphorylation, by activating TCPTP, which dephosphorylates them in the nucleus.\textsuperscript{264}

SHP1 and SHP2 have also been implicated in STAT3 signaling elsewhere.\textsuperscript{265,266,267} Both butein (3,4,2',4'-tetrahydroxychalcone) and acetyl-11-keto-β-boswellic acid have been shown to down-regulate STAT3 signaling by inducing the expression of SHP1.\textsuperscript{265,266} SHP2 has been shown to regulate the STAT3 pathway in neural stem cells.\textsuperscript{267} Gain-of-function mutations in PTPN11, the gene encoding for SHP2, results in diminished STAT3 signaling, and has been implicated in the pathogenesis of Noonan syndrome and juvenile myelomonocytic leukemia.\textsuperscript{268} However, it remains unclear as to whether SHP1 and SHP2 are capable of directly dephosphorylating STAT3, or whether they down-regulate STAT3 activity by dephosphorylating other signaling components.

PTP receptor T (PTPRT), a membrane localized PTP and the most often mutated PTP in human cancers, has also been shown to directly dephosphorylate STAT3.\textsuperscript{269} Through the use of a modified substrate-trapping assay, it was shown that STAT3 is a direct substrate of PTPRT.\textsuperscript{269} This was shown in the colorectal cancer cell lines, SW480 and HT29, as well as the embryonic kidney cell line, HEK293T.\textsuperscript{269} In contrast, STAT5 was not bound by PTPRT, and its level of phosphorylation did not change with PTPRT levels.\textsuperscript{269} It was also shown that in the colorectal cancer cell line, HCT116, the levels of
protein expression of two of STAT3’s target genes, Bcl-xL and SOCS3, were also down-regulated in response to PTPRT.\textsuperscript{269} Expression of PTPRT also resulted in the presence of significantly less STAT3 in the nucleus when compared to control.\textsuperscript{269} Moreover, it was shown that PTPRT specifically dephosphorylated STAT3 at Tyr\textsuperscript{705}, where phosphorylation at this site has been found to be critical for STAT3 activity.\textsuperscript{269}

It was also found that low molecular weight-dual specificity phosphatase 2 (LMW-DSP2), which is specifically and abundantly expressed in murine testes, down-regulates STAT3-mediated signaling.\textsuperscript{270} Phosphorylation of STAT3 at Tyr\textsuperscript{705} and Ser\textsuperscript{727} were significantly reduced when LMW-DSP2 was expressed.\textsuperscript{270} IL-6-induced SOCS3 expression was reduced in HeLa cells that were transfected with LMW-DSP2 as well.\textsuperscript{270} Furthermore, it was shown that STAT3 immunoprecipitated with WT STAT3, but not STAT3 which contained Phe at residue 705 in HEK293T cells, which suggests that LMW-DSP2 directly interacts with STAT3 in a phosphotyrosine-dependent manner.\textsuperscript{270} To confirm the existence of the interaction \textit{in vitro}, GC-1 testicular cells were used to demonstrate that anti-LMW-DSP2 immunoprecipitates contain STAT3, which suggests that LMW-DSP2 interacts with STAT3 under normal conditions.\textsuperscript{270} Interestingly, when LMW-DSP2 was over-expressed, it also dephosphorylated JAK2 at tyrosine in HEK293T cells.\textsuperscript{270}

\textbf{JAKs}

CD45, the prototypic transmembrane PTP, has been found to be capable of directly dephosphorylating JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) \textit{in
In both in vitro and in vivo, CD45 has been shown to down-regulate IL-3-mediated cellular proliferation, as well as Epo-dependent hematopoiesis and antiviral responses. TYK2 and JAK2, but not JAK1, have also been shown to be substrates for protein-tyrosine phosphatase 1B (PTP1B). Lastly, both JAK1 and JAK3 have been identified as substrates of TCPTP.

STATs 1, 4, 5 and 6

TCPTP has been shown to have other STAT substrates besides STAT3. TC45 has been shown to dephosphorylate nuclear STAT1. TCPTP has also been shown to directly dephosphorylate both STAT5a and STAT5b. Interestingly, it appears as though part of the interaction between STAT5 and TCPTP is pTyr-independent, since a measurable level of STAT5/TCPTP complexes could be immunoprecipitated even when mutant TCPTP and vanadate, a potent PTP inhibitor, were present. Interactions between TCPTP and STAT6 have also been demonstrated, and it is suggested that STAT6 may be a nuclear substrate for PTP under physiological conditions. TCPTP overexpression abrogated IL-4-induced STAT6 phosphorylation and gene expression in activated-B-cell-like (ABC-like) diffuse large B-cell lymphomas (DLBCLs).

Nakahira and colleagues identified PTP-basophil like (PTP-BL) as a phosphatase that interacts with members of the STAT family. It was identified in a yeast two-hybrid screen in the search for STAT4-interacting molecules. They showed that PTP-BL directly interacts with, and dephosphorylates, STAT4, and that a PTP-BL deficiency in CD4+ T cells results in prolonged STAT4 and STAT6 activation, which leads to elevated
T helper 1 and 2 cell differentiation. Furthermore, they demonstrated that PTP-BL was not only involved in the dephosphorylation of STAT4 and STAT6, but of STAT1, STAT3 and STAT5 as well.

**SHP1**

SHP1 has been shown to be involved in the termination of both JAK1 and JAK2 signaling. SHP1 is able to reversibly associate with IFNα receptor complexes, which mediate JAK/STAT signaling. It was found in murine macrophages lacking SHP1 that JAK1 and STAT1α displayed significantly increased levels of tyrosine phosphorylation, while the levels of TYK2 and STAT2 activation were not significantly changed. SHP1 is also able to associate with tyrosine-phosphorylated EpoR, which mediates JAK2 signaling. It was found that mutant cells lacking Tyr at 429 of EpoR were hypersensitive to Epo stimulation, and that JAK2 displayed prolonged autophosphorylation. Jiao and colleagues also evaluated EpoR/JAK2 signaling. In Cos-7 cells, they showed that SHP1 was able to directly associate with, and dephosphorylate, JAK2. Interestingly, they found that the association of SHP1’s SH2 domain with pTyr-EpoR was not required for SHP1 phosphatase activity, which suggests that the direct interaction between JAK2 and SHP1 was the more critical one.

It should be noted that, in some circumstances, SHP1 can help to activate JAK/STAT signaling. You and colleagues overexpressed WT and catalytically inactive SHP1 in HeLa cells. After stimulating the cells with EGF or IFNγ, STAT activity was analyzed. They found that the catalytically inactive SHP1 reduced STAT activity in
both cases, while native SHP1 either had no effect or resulted in increased activity.\textsuperscript{283} Likewise, EGF-induced activation of the MAPK cascade was inhibited by catalytically inactive SHP1, but was increased by native SHP1.\textsuperscript{283} It should be noted that in these experiments the widely distributed form of SHP1 was used, not the form found primarily in hemopoietic cells.\textsuperscript{283} The two forms differ at their N-terminus, which could have a significant effect on their activities.\textsuperscript{283} None the less, these data demonstrate that SHP1 can have an activating or inhibiting effect on STAT signaling, depending on the cell type and the specific JAKs/STATs involved.\textsuperscript{283}

**SHP2**

It has been shown that JAK1 and JAK2 can interact with SHP2 and phosphorylate SHP2 at Tyr\textsuperscript{304} and Tyr\textsuperscript{327}.\textsuperscript{284} The JAK/SHP2 interactions were shown to not be dependent on the phosphatase activity, or SH2 region, of SHP2.\textsuperscript{284} Interestingly, in response to IFN stimulation, it was found that JAK1, but not JAK2 phosphorylation levels, were significantly increased in SHP2\textsuperscript{−/−} fibroblasts.\textsuperscript{285} This suggests that SHP2 is a negative regulator of JAK1 signaling, possibly via direct inactivation of JAK1.\textsuperscript{285} However, this was not specifically shown.\textsuperscript{285}

Like SHP1, SHP2 has also been show to both positively and negatively regulate JAK/STAT signaling.\textsuperscript{282,286} In particular, SHP2 has been shown to have a positive role in IL-3 signal transduction in hematopoietic cells.\textsuperscript{287} In SHP2\textsuperscript{−/−} cells, JAK2 activation via tyrosine phosphorylation was significantly reduced, as was the activation of a downstream effector, STAT5.\textsuperscript{287} In this case, JAK activation was impaired.\textsuperscript{287}
However, when WT SHP2 was over-expressed in hematopoietic cells, the results were not as suspected; JAK2 activation was increased, but the tyrosine-phosphorylation of STAT5 was reduced. This led to the finding that STAT5 is a direct and physiological substrate of SHP2, and that SHP2 directly dephosphorylates STAT5.

To make matters more complicated, STAT5 activity has also been shown to be suppressed when SHP2 is deleted in murine mammary glands. It is thought that SHP2 increases STAT5 activity through the JAK2/prolactin receptor (PrlR) complex. Interestingly, STAT3 activity was slightly up-regulated when SHP2 was deleted, which suggests that the regulation of STAT family members by PTPs is quite complex.

It has been found that the initial IL-6-mediated activation of STAT3 is not significantly affected by the presence or absence of SHP2. However, the basal level of STAT3 phosphorylation that occurs in the absence of IL-6 is inversely related to SHP2 concentrations. As a result, it has been proposed that SHP2 might not be significantly involved in regulating signaling shortly after ligand-binding, but could instead be responsible for repressing a basal level of kinase activity, at least in IL-6/JAK1/STAT3 signaling.

Lastly, it has also been shown that STAT1 is a substrate of SHP2, and that SHP2 is able to directly dephosphorylate STAT1 at both Tyr and Ser. Following stimulation with EGF or IFNγ, they both localized to the nucleus, where they associated. The C-terminal region of SHP2, which is responsible for PTP activity, was shown to interact with the C-terminal region of STAT1, which contains the SH2 transcriptional activation domain.
Summary of PTPs

The role of PTPs in JAK/STAT signaling, particularly SHP1 and SHP2, is quite complex. However, the direct substrates of the various PTPs provide valuable insight into what signaling components are directly inactivated by PTPs. They are summarized below (Table 5). That fact that SHP1 and SHP2 can regulate JAK/STAT signaling in both a positive and regular manner is intriguing, and further studies will hopefully reveal their specific roles in cell signaling. In evaluating their effects, the cell type, receptor system, and specific signaling components involved must be taken into consideration.

<table>
<thead>
<tr>
<th>PTP</th>
<th>Confirmed Target(s)</th>
<th>STAT(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>JAK1, JAK2, JAK3, TYK2</td>
<td></td>
</tr>
<tr>
<td>LMW-DSP2</td>
<td>JAK2</td>
<td>STAT3</td>
</tr>
<tr>
<td>PTP1B</td>
<td>JAK2, TYK2</td>
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<tr>
<td>PTP-BL</td>
<td></td>
<td>STAT1, STAT3, STAT4, STAT5, STAT6</td>
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<tr>
<td>PTPRT</td>
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<td>STAT3</td>
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<tr>
<td>SHP1</td>
<td>JAK1</td>
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<td>STAT1, STAT5</td>
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<tr>
<td>TCPTP</td>
<td>JAK1, JAK3</td>
<td>STAT1, STAT3, STAT5A, STAT5B, STAT6</td>
</tr>
</tbody>
</table>

Table 5. The confirmed targets of JAK/STAT PTPs.

2.1.6 GP130 Requirements for JAK Binding

As previously stated, the cytoplasmic region of GP130 contains 277 amino acids. GP130 contains box 1 and box 2 motifs in the cytoplasmic region, which are conserved among cytokine receptors. Murakami and colleagues were the first to identify the two boxes. The homology region (HR), which contains the two boxes, is located in the membrane-proximal portion of GP130, located from 10 to 61 residues.
away from the TM. With the exception of IL-7R, they found that the box 1 sequence, Pro-Xaa-Pro, was present in all of the receptors that they evaluated: hGP130, hG-CSFR, hIL-2Rβ, human erythropoietin receptor (hEpoR), a granulocyte/macrophage colony-stimulating factor receptor-associated molecule (KH97), mIL-3R, hIL-7R, and hIL-4R. The sequence for IL-7R was Pro-Xaa-His. They also found that box 2 was conserved in hGP130, hG-CSFR, hIL-2Rβ, hEpoR, and KH97. Box 1 is eight amino acids in length and proline-rich, while box 2 consists of a cluster of hydrophobic amino acids followed by one or two positively charged residues.

The cytoplasmic portion of GP130 also contains a serine-rich (SR) region and four GTP-binding motif-like sequences: NKRD, EVSA, DAFG and GBGTEGQV. One of these sequences is located between the HR and TM, while the other three are located near the C-terminus. Figure 20 depicts Murakami’s portrayal of the cytoplasmic region of GP130, where the GTP-binding motif-like sequences are represented by *’s, EC represents the extracellular region, and the I Cs represent the various GP130 constructs that were used in the experiments. It was found that neither the SR region nor the three membrane-distal GTP-binding motif-like sequences were required for generation of a growth signal. In contrast, substituting the two highly conserved box 1 proline residues with serine resulted in the cells becoming unresponsive to IL-6 and a lack of the tyrosine-phosphorylation of GP130. Disturbing the three conserved box 1 hydrophobic amino acids produced a nonfunctional GP130 mutant as well, and it was also found that the seven-residue amino acid sequence in box 2, IEANDKK, was required for IL-6-mediated signal transduction to occur. There is also
a 32-residue interbox region that contains six positively charged amino acids in the N-terminal half of the region and three negatively charged amino acids in the remainder of the region.\(^{293}\) Interestingly, it was found that substituting five of the six positively charged residues abolished the IL-6 response, while substituting two of the three negatively charged residues did not.\(^{293}\)

![Figure 20. The cytoplasmic region of GP130 (this figure was originally published in Proceedings of the National Academy of Sciences of the United States of America, © National Academy of Sciences).\(^{293}\)](image)

Narazaki and colleagues found that JAK2 was rapidly tyrosine-phosphorylated, as was JAK1 to a lesser extent, following GP130 homodimerization in mouse embryonic stem cells, myeloid leukemic M1 cells, and pro-B cells.\(^{295}\) Stimuli that are known to result in GP130 homodimerization were found to up-regulate the kinase activity of JAK2.\(^{295}\) However, similar to the results obtained by Murakami and coworkers, they found that GP130 mutants with deletions in box 2 or proline-to-serine substitutions in box 1 were unable to propagate the expected signal.\(^{295}\) They also found that in BAF.B03 cells expressing either WT or mutant GP130 (which still contained the box 1 and box 2 motifs), JAK2 tyrosine phosphorylation was induced by the IL-6/IL-6R\(\alpha\) complex, although to a lesser extent in the mutants.\(^{295}\) Furthermore, they demonstrated that
disrupting the tyrosine phosphorylation of JAK2 by introducing mutant GP130 co-relates with a lack of biological responsiveness to IL-6/IL-6Rα.\textsuperscript{295}

Tanner and colleagues found that the box 1 motif was essential for the association of JAKs with cytokine receptors.\textsuperscript{296} Interestingly, they determined that while box 2 was not required for the association of JAKs, it still was required for the activation of JAKs, since no JAK autokinase activity could be detected when box 2 was absent.\textsuperscript{296} They also found that sequences within a given receptor are critical for specificity, since JAK2 was able to associate with the GP130, EpoR and GHR chimeras, while JAK1 was only able to associate with the GP130 chimera.\textsuperscript{296} Their findings also suggest that multiple domains of JAK are responsible for receptor-binding.\textsuperscript{296} However, the tyrosine-kinase domain is not likely to be one of these.\textsuperscript{296}

The interbox region has also shown to be important for the interaction between JAK1 and GP130. Haan and colleagues showed that a single amino acid substitution (a replacement of Trp\textsuperscript{666} with Ala) in the interbox region not only abrogated STAT activation, but precluded JAK1’s association with GP130 as well.\textsuperscript{294} Furthermore, they found that when one WT GP130 dimerized with a mutant GP130 that was incapable of associating with JAK1, there was still no activation of STAT.\textsuperscript{294} As a result, it is thought that JAK must associate with both units of GP130 for their mutual activation to occur.\textsuperscript{294}

Haan and colleagues later found that while the absence of box 2 did not significantly affect the binding of over-expressed JAK1, it did drastically reduce the binding of JAK1 at physiological levels.\textsuperscript{297} Also, substituting the conserved Trp\textsuperscript{652} residue with Ala in box 1 still resulted in JAK1 associating with GP130, but
phosphorylation of JAK1 did not occur and signaling was completely terminated.\textsuperscript{293,297} Substituting this conserved Trp residue resulted in a total lack of signal transduction, even when only one of the GP130 units contained the mutant, which signified that the mutation is dominant.\textsuperscript{297} Substituting Pro\textsuperscript{671} and Pro\textsuperscript{672} with Ala or Phe\textsuperscript{676} with Ala in the interbox region resulted in less activation of STAT despite normal JAK1 association.\textsuperscript{297} In contrast, substituting Tyr\textsuperscript{683} with Phe in the interbox region did not affect JAK1 association or STAT activation.\textsuperscript{297}

Lastly, Greiser and colleagues demonstrated that shifting the HR of GP130 to a C-terminal location abrogated the binding of JAK1 to GP130, which suggests that JAK1 may require membrane proximity.\textsuperscript{298} Inserting one to four alanine residues in the membrane-proximal region, which slightly shifts the HR away from the plasma membrane, did not significantly affect JAK1 binding, but did decrease JAK1 tyrosine phosphorylation.\textsuperscript{298} Surprisingly, the level of STAT activation was not co-related with the level of JAK1 phosphorylation in the mutants.\textsuperscript{298} A reasonable explanation would be that adding additional Ala residues results in a twisting of GP130.\textsuperscript{298} As a result, a high level of JAK1 phosphorylation might still occur, but STAT might not be in a proper orientation to become activated.\textsuperscript{298}

For all of these studies, it must be taken into consideration that certain alterations to GP130 might have not just had the attended effects. Instead, the overall structure of the protein might have also been altered, which would make it difficult to interpret the results. An alternate depiction of the domains of GP130, including the cytoplasmic tyrosine residues, as well as boxes 1 and 2, is shown below (Figure 21).
2.2 JAK: Not Just Another Kinase

Members of a certain protein tyrosine kinase (PTK) family were named the Janus kinases (JAKs) after the Roman God with two heads, Janus, because both JAK1 and JAK2 contained a C-terminal PTK domain and as a second phosphotransferase-related domain.\textsuperscript{299,300} The name also had another meaning, ‘just another kinase,’ due to the fact that their functions remained elusive despite their ubiquitous expression.\textsuperscript{300,301} They were discovered in the early 1990’s through the use of low-stringency hybridization or PCR-based strategies.\textsuperscript{251,302,303,304,305,306,307} To say that they have turned out to be ‘not just another family of kinases’ would be an understatement.
2.2.1 Members of the JAK family

Four members of the JAK family exist in mammals: JAK1, JAK2, JAK3 and TYK2.\(^{251}\) Their genes are located on chromosomes 1p31.3, 9p24, 19p13.1 and 19p13.2, respectively.\(^{251}\) The JAKs are large proteins, with molecular weights between 120 to 140 kDa.\(^{251}\) They are also composed of over 1,100 amino acids.\(^{251}\) They are nonreceptor tyrosine kinases that are constitutively associated with their receptors.\(^{251}\) Since nematode worms and slime molds do not express STATs, but do express JAKs, it is thought that the JAKs came later in evolution than the STATs.\(^{251}\) The JAKs contain roughly 20 exons.\(^{251}\) Alternately spliced forms have been shown to exist, but the functional significance of these forms has not been elucidated.\(^{251}\)

The JAKs are ubiquitously expressed, with the exception of JAK3, which is expressed primarily in hematopoietic cells.\(^{251}\) JAK3 is constitutively expressed in natural killer (NK) cells and thymocytes, while its expression can also be induced in B cells, T cells and myeloid cells.\(^{307,308,309,310,311,312}\) They are required for many immunological processes, as well as hematopoiesis.\(^{313,314,315}\)

The critical roles that the JAKs play is not surprising considering that they are involved in the signaling of many different cytokines and receptors (Figure 22).\(^{313}\) It should be noted that cytokine receptors must contain the common gamma chain (γc) subunit in order to activate JAK3.\(^{313}\) It is also noteworthy that the cytokine receptors sharing the common beta chain (βc) subunit, as well as the homodimeric cytokine receptors, which include EpoR, GHR, PrlR, the thrombopoietin receptor (TpoR) and G-CSFR, are almost exclusively dependent on JAK2 for signaling.\(^{313,315}\)
The JAKs are often involved in regulating the surface expression of cytokine receptors, although the mechanisms of regulation are variable. For example, the N-terminal domain of JAK2 has been shown to bind EpoR in the endoplasmic reticulum and up-regulate its expression. In contrast, TYK2 promotes the surface expression of IFNαR1 chain by slowing down its degradation. Also, JAK1 has been implicated in the promotion of OSMR surface expression.
2.2.2 JAK Structure

The JAKs consist of seven domains that are highly conserved; they are called the JAK homology (JH) domains.\textsuperscript{301,305,313} Located at the C-terminus are the kinase (JH1) and pseudokinase (JH2) domains.\textsuperscript{313} The JH1 domain contains the typical characteristic features of a catalytic tyrosine kinase domain.\textsuperscript{313} This includes tyrosine residues within the activation loop region, a conserved Asp residue that plays a role in the phosphotransfer reaction in the catalytic loop, and a GxGxxG motif that is located in the nucleotide-binding loop.\textsuperscript{308,313}

The JH2 domain has many similarities to the JH1 domain.\textsuperscript{299,313} However, it does not contain characteristic residues of an active tyrosine kinase, and has been long-thought to be catalytically inactive.\textsuperscript{299,313} Interestingly, it was recently shown that the JH2 domain of JAK2 is a dual-specificity kinase that phosphorylates Ser\textsuperscript{523} and Tyr\textsuperscript{570} of JAK2, both of which are negative regulatory sites.\textsuperscript{319} As a result, it is not surprising that the basal level of JAK2 activity increased when the catalytic activity of JH2 was inactivated.\textsuperscript{319} It was previously thought that the JH2 domain functions as a negative regulator of the JH1 domain, but this recent finding sheds light upon its mechanism of inhibition.\textsuperscript{319,320}

The primary structures of the combined JH3 and JH4 domains have resemblance to an SH2 domain.\textsuperscript{313,321} However, it has been found that the JAK SH2 domain is not responsible for fulfilling a classical SH2 function in JAK/STAT signaling.\textsuperscript{321} SH2 domains contain a conserved Arg residue, which is responsible for contacting the pTyr residue of the SH2 domain-recognized motif.\textsuperscript{321} Substituting this conserved Arg with Lys has been repeatedly shown to make SH2 domains non-functional.\textsuperscript{322,323,324,325} However,
replacement of Arg\textsuperscript{466} with Lys in JAK1 did not affect the subcellular distribution of JAK1, the ability of JAK1 to bind to GP130 and OSMR, or the signaling capacity of JAK1.\textsuperscript{321} Still, the SH2 domain of JAK1 does play structural roles in up-regulating the surface expression of OSMR and receptor-binding, and is thought to contribute to scaffolding.\textsuperscript{321}

At the N-terminus are domains JH5-JH7, which make up the band 4.1, ezrin, radixin and moesin (FERM) motif.\textsuperscript{313,326} The FERM domain is believed to be responsible for receptor binding.\textsuperscript{327} For example, a region in the FERM domain of JAK1 has been found to be required for adequate binding of GP130 to occur.\textsuperscript{328}

The FERM domain is also thought to regulate catalytic activity.\textsuperscript{329} Tyr\textsuperscript{119}, located within the FERM domain of JAK2, has been shown to be a phosphorylation site.\textsuperscript{330} Following Epo stimulation, phosphorylation at this site has been shown to down-regulate JAK2 activity by causing JAK2 to dissociate from the receptor.\textsuperscript{330} A mutant containing Phe at this site, which is incapable of being phosphorylated, is able to more stably associate with the receptor complex.\textsuperscript{330} This has been shown for Tpo and GH as well.\textsuperscript{330}

Thus, for at least the three mentioned receptors, Tyr\textsuperscript{119} is a site of negative regulation.\textsuperscript{330}

Results from a recent study of a Y613E JAK2 mutant also suggest that the JAK2 FERM domain interacts with EpoR to regulate kinase activity.\textsuperscript{331} The presence of the Y613E mutant resulted in constitutive JAK2 activation, but only when EpoR was present.\textsuperscript{331} It should be noted that the substitution of Tyr with Glu acts as a phosphorylation mimic.\textsuperscript{330} As a result, it is suggested that unassociated JAK2 is locked into an inactive state, but that receptor steric constraints are relieved upon FERM
binding, which allows activation to occur. However, even though receptor binding through the FERM domain is necessary for JAK2 activation, the FERM domain regulates JAK2 activity in an inhibitory fashion in general. The FERM domain of JAK3 has also been implicated in receptor binding and regulation of kinase activity. The domains of the JAK family are shown below (Figure 23).

Figure 23. The general structure of JAK proteins (reprinted with permission from © Elsevier). KLD: Kinase-like domain.
2.2.3 JAK2 Phosphorylation Sites

A number of activity-regulating phosphorylation sites have been identified. Tyr\textsuperscript{119} of the FERM domain of JAK2 has already been mentioned.\textsuperscript{330} Tyr\textsuperscript{913} of the JH1 domain of JAK2 has been identified as an autophosphorylation site that negatively regulates JAK2.\textsuperscript{334} Tyr\textsuperscript{221} within the FERM domain is a phosphorylation site that has been suggested to be a positive regulator of JAK2 activity, although its effect on activity is disputed.\textsuperscript{335,336} Tyr\textsuperscript{813} has been shown to be a site of autophosphorylation.\textsuperscript{337,338} It acts as a binding site for the adaptor protein, SH2-B\beta, which enhances JAK2 activity, and was identified in GH signaling.\textsuperscript{337,338} As previously mentioned, Tyr\textsuperscript{570} within the JH2 domain is a phosphorylation site that is involved in the negative regulation of JAK2 activity.\textsuperscript{335} Autophosphorylation of Ser\textsuperscript{523}, located in the linker region between the SH2-like and JH2 domains, has already been discussed as well.\textsuperscript{319} It negatively regulates JAK2 activity, and is the only residue of JAK2 that is constitutively phosphorylated.\textsuperscript{319,339} It should be noted that Ser\textsuperscript{523} could be \textit{cis}-phosphorylated, while other sites, such as Tyr\textsuperscript{570}, are thought to be \textit{trans}-phosphorylated by the JH2 domain of another JAK2 molecule.\textsuperscript{319}

Critically, JAK2 contains a YY motif (Tyr\textsuperscript{1007} and Tyr\textsuperscript{1008}) in the activation loop of the JH1 domain.\textsuperscript{340} They are both sites of auto- or transphosphorylation.\textsuperscript{340} Mutation of Tyr to Phe at 1008 did not significantly affect kinase activity, while phosphorylation at Tyr\textsuperscript{1007} was found to be essential for JAK2 activity.\textsuperscript{340} This is not surprising, since most kinases require phosphorylation in their activation loop in order to be fully active.\textsuperscript{313} This is the critical event that occurs in JAK activation and signal transduction.\textsuperscript{313,340,341,342}
Some of the common sites of JAK phosphorylation, including the YY motif of JH1, are shown below (Figure 24).\textsuperscript{251}

![Common phosphorylation sites of JAK](image)

**Figure 24.** Common phosphorylation sites of JAK (this figure originally appeared in Genome Biology. Yamaoka, K., Saharenin, P., Pesu, M. Hlot III, V. E. T., Silvennoinen, O. and O’Shea, J. J. *Genome Biology.* 2004; 5:253. © BioMed Central Limited.).\textsuperscript{251}

### 2.2.4 JAKs and Disease: The Quest for Selectivity

All four members of the JAK family have been implicated in disease in some way or another. Patent applications that have been submitted recently indicate that TYK2-specific inhibitors are being investigated for the treatment of multiple sclerosis.\textsuperscript{343} This is rational in light of the fact that TYK2 has been implicated in autoimmune diseases, and that mice deficient in TYK2 show resistance to experimental autoimmune encephalomyelitis, which is a model for multiple sclerosis.\textsuperscript{344,345,346}

JAK3 inhibitors were originally investigated as immunosuppressive agents that could be used to prevent solid organ transplant rejection.\textsuperscript{344,347,348} More recently, they have been investigated for their potential to treat autoimmune diseases like RA and psoriasis.\textsuperscript{349} Notably, JAK3 is thought to be an ideal target, due to the fact it only signals through $\gamma_c$-containing cytokine receptors.\textsuperscript{350} Tofacitinib (CP690,550; Pfizer) is a JAK3-
specific inhibitor that was recently approved by the US FDA for the treatment of RA (Figure 25).\textsuperscript{314,327,351} It is the only JAK-selective drug that is currently approved in the US.

![Figure 25. Tofacitinib (CP690,550; Pfizer).](image)

While JAK1, JAK2 and JAK3 have all been implicated in cancer to some extent, JAK2 is by far the most implicated.\textsuperscript{327} Therefore, JAK2-specific inhibitors are highly desirable.\textsuperscript{327} For example, JAK1 plays a role in the signaling of IL-6-type cytokines, $\gamma_c$-associated receptors and type II receptors.\textsuperscript{313,327} As a result, undesired inhibition of JAK1 could have drastic consequences on many different cytokine-mediated processes.\textsuperscript{327}

However, the high degree of structural similarities between the JAKs has made the development of JAK2-specific inhibitors difficult.\textsuperscript{327} Ruxolitinib (INCB18424; Incyte/Novartis) is the only other JAK2 inhibitor that is approved by the US FDA (Figure 26).\textsuperscript{314,352} It is authorized for the treatment of primary myelofibrosis (PMF), postpolycythemia vera myelofibrosis, and postessential thrombocythemia myelofibrosis.\textsuperscript{352} However, it is a pan-JAK inhibitor that inhibits both JAK1 and JAK2.\textsuperscript{314,352} It should be noted that a number of other JAK inhibitors, some of which are specific for JAK2, are in clinical trials.\textsuperscript{314}
2.2.5 JAK2 and Disease

JAK2 has been implicated in numerous myeloproliferative disorders (MPDs). These include atypical chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and PMF. Specifically, JAK2 V617F was identified in over 95% of PV patients and 50% or more of ET and PMF patients. JAK2 has been implicated in a number of types of acute leukemia as well. These include B-cell precursor acute lymphoblastic leukemia (BCP-ALL), B-cell acute lymphoblastic leukemia (B-ALL), acute myelogenous leukemia (AML), and a subtype of AML, acute megakaryoblastic leukemia (AMKL). Furthermore, constant activation of STAT3, and STAT5 to some extent, have been shown in numerous solid tumors, including prostate, pancreatic, breast, ovarian and hepatic carcinomas. While STAT3 can be activated by several kinases, in 15 out of 16 human solid tumor cell lines, it was found that JAK2 was the most critical upstream activator of STAT3. Of the cell lines tested, the only exception was the gastric cell line, MKN45.
JAK2 can be hyper-activated by a couple of direct mechanisms. First, JAK2 has been shown to be involved in genetic recombination, which can result in a fused oncogenic allele and the subsequent expression of JAK2 fusion proteins, which are often hyperactive. The genes that JAK2 are recombined with are usually transcription factors, and include pericentriolar material 1 (PCM1), TEL, BCR, and ribophorin 1 (RPN1). Interestingly, translocations involving the other JAK family members have not been observed.

Mutations of JAK2 have been shown to produce hyper-active JAK2 as well. For example, different JAK2 mutations found in myeloproliferative neoplasms were found to reduce JH2 activity. The V617F JAK2 mutation is an acquired somatic mutation that is present in the hematopoietic cells of a number of patients suffering from MPDs. It results from a point mutation (exon 14) which produces a guanine to thymidine substitution. Among other observed traits, the cells of MPD patients carrying the autonomous V617F mutation showed reduced phosphorylation of Tyr. Val of the JAK2 JH2 domain is thought to play an important role in the inhibitory effects of JH2 on JH1. The substitution at this site leads to constitutive JAK2 activity. The JH2 domain appears to normally negatively regulate JAK2 by increasing its K_m for both ATP and other substrates. The V617F mutation has been shown to not affect the K_m of JAK2 for ATP, and to not greatly alter the V_max of JAK2. Instead, it appears that it results in hyperactivation, at least in part, by significantly decreasing the K_m of JAK2 for peptide substrates. Interestingly, studies have indicated that while the FERM domain plays an inhibitory role in WT JAK2, it
plays a role in hyper-activating JAK2 when the V617F mutation is present. There are other mutations observed in MPNs, such as K539L (exon 12) and R683S (exon 16), which are characterized by hyperactive JAK2 as well. This all suggests that abrogated JH2 activity contributes to the pathogenesis of MPNs. The R683G or R683S mutations are detected in 15% of B-ALL patients, and are thought of as biomarkers for B-ALL.

Lastly, it should be noted that JAK2 has recently been implicated in the STAT-independent regulation of gene expression. Activated JAK2 regulates a number of genes that lack STAT binding sites in their regulatory portions, which suggests that a STAT-independent pathway is involved in their regulation. The presence of JAK2 in the nuclei of hematopoietic cells has been demonstrated. In the nucleus, JAK2 directly phosphorylates histone H3 at Tyr. This inhibits the binding of heterochromatin protein 1 alpha (HP1α). This increased displacement of HP1α by the abnormally activated JAK2 seen in malignancies is thought to reduce the potential tumor-suppressor functions of HP1α. In support of this suggestion, JAK2 and JMJD2C, a lysine-specific histone H3 demethylase, were found to cooperate to epigenetically modify the genome, and to play a role in both primary mediastinal B-cell lymphoma (PMBL) and Hodgkin lymphoma (HL). This again reiterates the need for the development of JAK2-specific inhibitors.
2.2.6 JAK2 Inhibitors

There are a number of selective and non-selective JAK2 inhibitors that are currently in clinical trials for the treatment of MPDs or cancer (Figure 27). They include AZD1480 (AstraZeneca), CYT387 (YM Biosciences), TG101348 (TargeGen), SB1518 (ONX0803; Onyx, S*Bio), SB1578 (S*Bio), SB1317 (S*Bio), LY2784544 (Eli Lilly and Company) and BMS-911543 (Bristol-Myers Squibb). In addition, INCB28050 (LY3009104, Baricitinib; Incyte/Lilly) is being evaluated for its therapeutic effectiveness in treating RA, XL-019 (Exelixis) is being evaluated in MPDs, and both AC-430 (Ambit Biosciences) and AEG41174 (AEgera) are being evaluated in cancer. SB1518, AC-430, AEG41174 and XL-019 are selective for JAK2. Still, a selective JAK2 inhibitor is yet to reach the market.
Figure 27. JAK2 inhibitors that are currently in clinical trials.

Other JAK inhibitors have shown promise, and they include pyridone 6 (2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one), TG101209, AG490 and the related compounds LS104 (formerly known as CR4) and WP1066 (Figure 28). The SAR studies of AG490/WP1066 analogues will be discussed in the proceeding chapter. In addition, a number of the STAT3 inhibitors that will be discussed throughout this document are also predicted to bind JAK2.
Figure 28. Promising lead compounds for JAK inhibition.

2.3 The STATs

The STATs were first identified in the early 1990’s by the pioneering work of Darnell and co-workers. Since the proteins identified were found to be involved in both signal transduction and the activation of transcription, they were termed STATs. STAT3 was identified by two independent groups in 1994. It was identified by Akira and colleagues as a protein that is tyrosine-phosphorylated, and then translocates to the nucleus in response to numerous cytokines that share GP130 as part of their receptor complexes. It was also described as a transcription factor that binds to IL-6-responsive elements in the promoters of numerous APR genes. It was simultaneously identified by Zhong and co-workers as a DNA-binding protein that becomes activated via phosphorylation in response to EGF and IL-6, but not IFNγ. However, although STAT3 is not tyrosine-phosphorylated in response to IFNγ, IFNγ has been shown to up-regulate the expression of STAT3.
2.3.1 Members of the STAT family

Seven mammalian STAT genes have been identified, which encode STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Alternate splice forms of STAT1, STAT3 and STAT4 have also been identified. In all cases, the alpha (α) isoform is the longer of the two, while the beta (β) isoform lacks amino acids at the C-terminus. STAT1β and STAT4β lack 38 and 44 residues at the C-terminus, respectively. STAT3β lacks 55 residues, but also has seven residues at its C-terminus that are not found in STAT3α.

STATs 1, 3, 4, 5a and 5b are between 750 and 795 amino acids in length; STATs 2 and 6 are approximately 850 amino acids in length. The STATs received their name because they are responsible for transducing signals in the cytoplasm and also act as nuclear transcription factors. They are differentially activated by JAKs in response to a number of cytokines, and are involved in a variety of processes (Table 6).

STAT1

STAT1 is the primary mediator of transcription in IFN signaling. It plays an important role in adaptive and innate immune responses. Given its role, it is not surprising that disruption of the STAT1 gene in mice led to increased susceptibility to viral diseases and infections by microbial pathogens. Human patients expressing STAT1 mutants were also found to be susceptible to mycobacterial infections. STAT1 is pro-apoptotic, promotes inflammation, and is an antagonist of proliferation.
STAT1 can signal as a homodimer, STAT1-STAT2 heterodimer, or STAT1-STAT3 heterodimer.\textsuperscript{414}

<table>
<thead>
<tr>
<th>Cytokine</th>
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<tr>
<td>CNTF</td>
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<td>Tpo</td>
<td>JAK2, TYK2</td>
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Table 6. The major JAKs and STATs that are activated by cytokine receptors (adapted and reprinted by permission from Macmillan Publishers Ltd: Oncogene, \textsuperscript{315} © 2007).
STAT2

STAT2 is involved in type I IFN signaling (IFNα/β).\textsuperscript{415,416} STAT1 homodimers and STAT1-STAT2 heterodimers are produced in response to type 1 IFN signaling.\textsuperscript{417} STAT2 is the largest STAT in mammals, consisting of 850 amino acids.\textsuperscript{413} Interestingly, evidence for STAT2 homodimerization is lacking.\textsuperscript{413} Like STAT1, STAT2 is immunostimulatory and pro-apoptotic in nature.\textsuperscript{363,413,416}

STAT4

STAT4 is essential for optimal development of the human T helper type I (Th1) lineage, and a deficiency in STAT4 leads to the impaired development of these cells.\textsuperscript{418} It is normally expressed in lymphocytes.\textsuperscript{419} STAT4 is activated via IL-12 signaling,\textsuperscript{418} and it plays a role in IL-27 signaling as well.\textsuperscript{413,420} STAT4 signals as a homodimer,\textsuperscript{414} and is heavily involved in the production of the proinflammatory cytokine, IFNγ.\textsuperscript{363,418,421} STAT4-deficient mice are susceptible to infection from intracellular organisms, but are also resistant to autoimmune disorders that involve a Th1 response.\textsuperscript{421}

STAT5

STAT5a and STAT5b have extremely similar sequences, with about 96% of the identities of their amino acids being the same.\textsuperscript{413} As a result, they are quite functionally redundant.\textsuperscript{413} However, one can be favored over the other in certain situations.\textsuperscript{413} STAT5 has been found to be critical in lymphoid development and differentiation.\textsuperscript{422} STAT5 is largely implicated in cancer, although not as much so as STAT3.\textsuperscript{362} STAT5 signals as a
homodimer.\textsuperscript{414} It is activated in response to a large number of cytokines.\textsuperscript{363} It is anti-apoptotic and pro-proliferative in nature, and promotes differentiation.\textsuperscript{363}

**STAT6**

STAT6 is primarily activated in response to IL-4 and IL-13.\textsuperscript{423} STAT6 signals as a homodimer.\textsuperscript{414} Like STAT2, STAT6 exhibits a relatively large transactivation domain.\textsuperscript{413} It is largely involved with T helper cell type II (Th2) function.\textsuperscript{421,424} Like STATs 3 and 5, STAT6 is also involved in anti-apoptotic activities.\textsuperscript{363}

2.3.2 *STAT Structure and Function*

The STAT proteins are characterized by an N-terminal domain, a coiled-coil domain, a DNA-binding domain (DBD), a linker (Lk) domain, an SH2 domain, and a C-terminal transactivation domain (TAD) (Figure 29).\textsuperscript{362,413} The N-terminal domain is approximately 125 residues in length.\textsuperscript{413} It is capable of mediating interactions between two STAT dimers in the process of tetramer formation.\textsuperscript{362} Although it is not a requirement for STAT function, this interaction can act as a stabilizer for the translocation of two STAT dimers to nearby DNA sites.\textsuperscript{362} This region is conserved, and deletions in this region may abrogate STAT phosphorylation.\textsuperscript{329} This domain has also been shown to be involved in the STAT1-STAT1 interactions that allow for dephosphorylation of activated STAT1 to occur.\textsuperscript{425} Furthermore, it is thought to be involved in nuclear transport and cooperative DNA binding to tandem $\gamma$-activated sequence (GAS) elements.\textsuperscript{329,413,426,427}
The coiled-coil domain roughly consists of residues 135-315. It contains a four-α-helix bundle and a broad hydrophilic surface. It is involved in protein-protein interactions and is responsible for binding regulatory proteins and other transcription factors. The coiled-coil domain has also been implicated in receptor binding, nuclear export and tyrosine phosphorylation.

![Figure 29. The general structure of STAT proteins](image)

The DBD approximately consists of residues 320 to 480. It consists of a β-barrel Ig fold, which is responsible for directing binding to GAS elements. It directly contacts the STAT-binding sites of gene promoters. With the exception of STAT2, STAT homodimers differentially bind greater than 10 GAS elements that have a TTNNNNNAA consensus sequence. Generally speaking, dimeric STATs generally bind DNA targets sites with nanomolar binding constants.

The Lk domain generally consists of residues 480 to 580 (465-585 for STAT3). It connects the DBD to the SH2 domain. Its role is to facilitate the correct conformations between the two domains. It also interacts with both domains.
and is suggested to play a role in DNA-binding capability and transcriptional regulation.\textsuperscript{419}

The SH2 domain consists of residues 575 to 680, in general.\textsuperscript{413} It is the most conserved of the STAT domains.\textsuperscript{419} It is critically important in signaling, and is responsible for the recruitment of STATs to specific pTyr motifs of receptors, association with and activation by JAK, and the binding of the pTyr residue to its dimerization partner.\textsuperscript{419} The SH2 domain is made up of an anti-parallel β-sheet that is bordered by two α-helices, which forms a pocket.\textsuperscript{419} A conserved Arg (Arg\textsuperscript{609} for STAT3) is found at the bottom of the pocket, and is critical for the pTyr interaction.\textsuperscript{419}

STAT dimerization depends on reciprocal SH2-pTyr interactions.\textsuperscript{419} All STATs contain a tyrosine residue around position 700, which when phosphorylated, is responsible for this interaction.\textsuperscript{413} This residue lies just adjacent to the SH2 domain, and its location keeps an intramolecular interaction between the SH2 domain and the pTyr residue from taking place.\textsuperscript{413}

The remaining residues at the C-terminus make up the TAD.\textsuperscript{413} The TADs of the STATs vary considerably.\textsuperscript{419} This variability is thought to partly explain how different STATs are able to associate with different transcriptional regulators.\textsuperscript{413} The TAD plays a role in transcriptional activation via interacting with other proteins, like histone acetyltransferases.\textsuperscript{362} All STATs, with the exceptions of STAT2 and STAT6, contain a Ser in the TAD, which can result in enhanced transcriptional activities when phosphorylated (Figure 30).\textsuperscript{329,362}
The nuclear import and export of STATs appears to be variable. Some STATs, such as STATs 1 and 2, are only found present in the nucleus in large amounts following their phosphorylation. In contrast, STAT3 is found in both the nucleus and the cytoplasm under basal conditions. Importins α5, α7 and β have been found to play a role in the nuclear passage of phospho (p)-STAT3. In contrast, importin α3 has been implicated in the pTyr-independent nuclear import of STAT3. Following the dephosphorylation of STAT3 in the nucleus, exportin 1 facilitates its translocation back to the cytoplasm. Interestingly, it was recently reported that STAT3 is imported into and exported from the nucleus independent of tyrosine phosphorylation.

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**Figure 30. Structures of the STATs** (reprinted by permission from Macmillan Publishers Ltd: Nature Reviews. Cancer, © 2004).
STATs can also be acetylated, sumoylated, ubiquitylated and methylated; these changes affect their nuclear localization, dimerization, stability, transcriptional activity, and association with histone deacetylases and histone acetyltransferases.\textsuperscript{150} Several STATs have been found to form tetramers, and it suggested that they might from higher-ordered complexes as well.\textsuperscript{414}

STATs do not always function through the conical pathway, and further functions are still being investigated. Some STATs, such as STAT3, are thought to have cytoplasmic functions as well.\textsuperscript{433} PSTAT3 has been found to act as an adaptor protein for PI3K.\textsuperscript{437} Monomeric STAT1 has been found to play a role in apoptosis and the expression of several genes.\textsuperscript{438} Interestingly, a crystal structure of unphosphorylated STAT3 (uSTAT3) bound to DNA was recently reported, which suggests that uSTAT3 can also have transcriptional control over certain genes.\textsuperscript{439} It was previously known that uSTAT3 is able to bind to unphosphorylated NFκB, accumulate in the nucleus, and activate certain κB-dependent genes.\textsuperscript{440,441} STAT3 phosphorylated at Ser\textsuperscript{727} has been observed in the mitochondria in electron transport complexes, and is thought to be required for the optimal function of the electron transport chain.\textsuperscript{442} It has also been found to support Ras-dependent malignant transformation in the mitochondria, even when the STAT3 mutant at hand was not capable of being tyrosine-phosphorylated or binding DNA.\textsuperscript{443} All of this shows that STAT3 plays other important roles in the cell besides acting as a transcription factor.\textsuperscript{443}
2.4 STAT3

2.4.1 Characteristics of STAT3

The STAT3-encoding gene is found on chromosome 17q21. Like other STATs, STAT3 is activated by the JAKs via phosphorylation at Tyr (Tyr\(^{705}\)). Phosphorylation at Ser\(^{727}\) by a number of kinases (ERK1, ERK2, JNK, H-7-sensitive kinase, p38) also enhances the transcriptional activity of STAT3. STAT3 signals as a homodimer or a STAT1-STAT3 heterodimer.

STAT3 is critical for the APR. It is also essential for development, which is evidenced by the fact that targeted disruption of the STAT3 gene in mice led to embryonic lethality. STAT3 is needed for the GP130-mediated G\(_1\) to S phase cell cycle transition. It acts in a pleiotropic manner, and it modulates the transcription of genes that are involved in proliferation, cell differentiation, angiogenesis, apoptosis, metastasis and immune responses. For example, STAT3’s target genes include the anti-apoptotic proteins Bcl-2, Bcl-xL, survivin, Mcl-1, and cellular inhibitor of apoptosis 2 (cIAP2).

2.4.2 STAT3 Isoforms

As previously mentioned, two isoforms of STAT3, STAT3\(\alpha\) and STAT3\(\beta\), have been identified. STAT3\(\alpha\) is 770 amino acids in length and has a molecular weight of roughly 92 kDa, while the truncated STAT3\(\beta\) is has a molecular weight of approximately 83 kDa. Both STAT3\(\alpha\) and STAT3\(\beta\) are activated by
phosphorylation at Tyr$^{705}$. However, there are some notable differences, many of which are still being analyzed.$^{444,454}$

It has been suggested that the two splice forms are differentially activated by certain cytokines.$^{405}$ In addition, in transfected COS cells, STAT3β was found to be constitutively activated and to have greater DNA-binding activity.$^{454}$ However, it was STAT3α that was found to have greater transcriptional activity relative to the DNA-binding activity.$^{454}$ This is not surprising, since STAT3β lacks the Ser$^{727}$ phosphorylation site, and phosphorylation of this site is implicated in increased transcriptional activity (Figure 31).$^{444}$

![Figure 31. Structures of STAT3α and STAT3β (reprinted from Biochemical Journal, © the Biochemical Society).$^{444}$](image)

STAT3β has been primarily reported as a negative regulator of STAT3α.$^{451}$ However, a recent report has shown that expression of STAT3β can lead to prolonged and enhanced phosphorylation of STAT3α, as well as its nuclear retention.$^{444}$ Both isoforms have been expressed in all cell types and tissues that have been analyzed, and
STAT3α is usually the major isoform present. Both isoforms have specific functions, which is evidenced by the differences in their transcriptional activities and target genes.

Two additional isoforms, STAT3γ and STAT3δ, have also been identified. STAT3γ has a molecular weight of 72 kDa, and is a C-terminal truncated form that is generated from STAT3α following translation via limited proteolysis. It is suggested that elastase may be the main enzyme responsible for producing STAT3γ. STAT3δ is predicted to be 64 kDa. However, the physiological roles of STAT3γ and STAT3δ remain unclear.

2.4.3 STAT3β DNA-Binding

A crystal structure of a STAT3β homodimer bound to dsDNA provided some insight into its mechanism of binding (Figure 32). The sugar-phosphate backbones of the two DNA strands are contacted by four loops of each STAT monomer. Three of these loops extend from the DBD, while the fourth is part of the Lk domain. Some other polar residues in the Lk and SH2 domain (Lys, Lys and Gln) project toward the DNA, but are not in close enough proximity to the DNA for direct interactions to occur. For one strand of DNA, His, Lys and Gln contribute polar contacts to the phosphate groups, while Met and Val interact with the backbone sugars in a hydrophobic fashion. For the other DNA strand, Arg, the amide backbone of Val, Ser and Gln contribute polar interactions, while Ile and Val interact with the
ribooses of the DNA. The bound DNA is somewhat underwound, and adopts a B-form DNA-like conformation.


The SH2 domain-pTyr interactions were also described. Direct polar interactions with pTyr\textsuperscript{705} are contributed by Lys\textsuperscript{591}, Arg\textsuperscript{609}, Ser\textsuperscript{611} and Ser\textsuperscript{613}. Strands C and D have residues that form a hydrophobic pocket, which Leu\textsuperscript{706} packs against.
PTyr$^{705}$ and the close-by residues 702-709 bind in trans to the other monomer, while residues 708-712 display intramolecular interactions with the SH2 domain in cis.$^{432}$ This clamps the monomers together, and allows the dimer to “grip” the DNA.$^{432}$ Thr$^{708}$, Phe$^{710}$ and Cys$^{712}$ also contribute side chains that intercalate with the other monomer of the complex.$^{432}$

2.4.4 STAT3 Activation

STAT3 can be activated in response to a number of growth factors, cytokines and hormones.$^{150,261,364,455}$ These include IL-6 and IL-10 family members,$^{150,455}$ leptin,$^{459}$ TNFα,$^{460}$ IFNγ,$^{400}$ EGF,$^{399}$ growth hormone (GH),$^{461}$ PDGF$^{462}$ and hepatocyte growth factor (HGF),$^{463}$ among others. Besides the JAKs, the Src family kinases are also significant contributors to the phosphorylation of STAT3.$^{464,465,466,467}$ However, as stated, JAK2 is thought to be the most critical upstream activator of STAT3,$^{314,364}$ and out of the four JAKs and seven STATs that have been identified, STAT3 activation due to IL-6 signaling is a primary pathway that has been implicated in the promotion of tumorigenesis.$^{150}$

2.4.5 STAT3 and Cancer

As it has been mentioned, STAT3 is constitutively active in a large variety of cancers. STAT3 has been implicated in a number of blood tumors.$^{362}$ These include MM,$^{33}$ the leukemias: human T cell lymphotropic virus 1 (HTLV-1)-dependent leukemia,$^{468}$ AML,$^{469}$ and large granular lymphocyte leukemia (LGL);$^{470}$ and the
lymphomas: Epstein-Barr virus (EBV)-related lymphoma, Burkitt’s lymphoma, mycosis fungoides/cutaneous T-cell lymphoma, classical HL, non-HL, and anaplastic large cell lymphoma (ALCL).

STAT3 is also activated in a number of solid tumors. These include breast cancer, melanoma, ovarian cancer, non-small cell lung carcinoma, prostate cancer, pancreatic cancer, renal cell carcinoma, and head and neck cancer. Furthermore, elevated levels of STAT3 are often linked to a poor prognosis, and this is the case with colorectal cancer. While STAT1, STAT3 and STAT5 are activated in a number of different cancers, STAT3 is the primary activated STAT in lymphomas, melanoma, ovarian cancer, lung cancer, pancreatic cancer and prostate cancer.

There are a number of mechanisms that facilitate constitutive STAT3 activation. Hyperactive IL-6 stimulation via autocrine or paracrine loops can lead to constitutive STAT3 activation. The methylation of the promoters of SOCS1 and SOCS3 is often observed in primary tumors and cancer-derived cell lines, which results in their decreased expression and the up-regulation in activity of the JAKs and STATs. Lowered expression or inactivation of the PIAS and PTP proteins can also lead to sustained STAT3 activity. The activating JAK mutations have already been discussed. It should be noted that while activating somatic mutations in JAK2 are often found in hematologic malignancies, their occurrence is significantly lower in solid tumors.

Interestingly, in contrast to the mutations found in JAK2, activating mutations in STAT3 have not been historically implicated. This suggests that in the majority of
cases STAT3 must become constitutively active through the other mechanisms that have been mentioned. However, a recent report showed that activating STAT3 somatic mutations were found in a high percentage of patients that suffered from LGL. All of the mutations were found in the SH2 domain-containing region, and the Y640F and D661V mutations were found to increase STAT3 transcriptional activity.

Regardless of how STAT3 becomes constitutively activated, it is an ideal drug target for a number of reasons. While directly inhibiting STAT3 has been challenging, and the targeting of the upstream kinases is a viable alternative, the problem with targeting kinases remains: tyrosine-kinase inhibitors can have multiple potential targets. Transcription factors, such as STAT3, are the final players that are responsible for directly inducing gene-expression patterns that lead to malignancy. Successful inhibition of a single transcription factor can stop the effects of numerous abnormal upstream signaling components that cause its constant activation. Furthermore, inhibiting a single transcription factor could successfully inhibit abnormal and detrimental signaling without affecting a number of other signaling cascades that may not be problematic.

STAT3 is the most frequently activated STAT in human cancers, while STAT1 and STAT5 are also commonly activated. However, STAT1 is not likely to play a negative role in cancer. In contrast, it is thought to play pro-apoptotic and antiproliferative roles, acting as a tumor suppressor. As a result, targeting STAT1 in cancer should be avoided. STAT5 is implicated in the pathogenesis of cancer, although not as much so as STAT3. However, the interplay between the STATs can be
complicated, as it was recently reported that breast cancer cell lines exhibiting STAT3 and STAT5 activation were more susceptible to paclitaxel and vinorelbine when compared to cells that only exhibited STAT3 activation. Studies have shown that STAT6 is also implicated in the pathogenesis of some lymphomas and leukemias. Both STAT5 and STAT6 are also therapeutic targets of interest.

Importantly, while inhibiting STAT3 or STAT5 can induce apoptosis in cancer cells, there is evidence that STAT3 signaling might not be as critical to normal cell function. It has been noted that STAT3 is critical for development, as disrupting the STAT3 gene in mice results in embryonic death. However, tissue-specific knockouts of STAT3 in non-cancerous cells, including T cells, macrophages, neutrophils, keratinocytes and mammary gland cells, have shown that many tissues are able to proliferate and survive in the absence of STAT3, although not without consequences. Inhibiting STAT3 in normal cells with dominant-negative STAT3 or peptidomimetics also does generally not lead to apoptosis, suggesting that cancer cells might rely on high levels of activated STAT3 for growth and survival, while normal cells can tolerate decreased levels of STAT3 activity.

It should be noted that long-term inhibition of STAT3 signaling has negative consequences in vivo. Dominant-negative mutations in the STAT3 gene are implicated in hyper-IgE syndrome, an immunodeficiency disorder. It has also been found that eliminating STAT3 signaling can eventually result in Th1-associated autoimmunity, but there is a period of time before that occurs. A therapeutic window does exist for
targeting STAT3 in the treatment of cancer,\textsuperscript{511} and there are several approaches to inhibiting STAT3 that have been employed.

\subsection*{2.4.6 Targeting STAT3}

Targeting STAT3 is a favored therapeutic approach.\textsuperscript{363} Over the past 10 or so years there have been numerous attempts to design therapeutic STAT3 inhibitors. Most indirect inhibitors of STAT3 have already been discussed (IL-6 Abs, sGP130Fc, MDL, JAK inhibitors). However, there is one other important class of indirect STAT3 inhibitors that should be mentioned. Leptomycin B and ratjadone A are two anti-fungal metabolites produced by \textit{Streptomyces} and \textit{Sorangium cellulosom}, respectively (\textbf{Figure 33}).\textsuperscript{512,513} They have both been shown to be inhibitors of exportin 1,\textsuperscript{512,513} and to result in the nuclear accumulation of STAT3.\textsuperscript{434,514,515} Retention of STAT3 in the nucleus leads to its dephosphorylation, and since it is unable to be transported back to the cytoplasm for further activation, STAT3 signaling is inhibited.\textsuperscript{434} Promisingly, after ratjadone A treatment, transformed fibroblasts exhibiting constitutive STAT3 activity were induced to undergo apoptosis, while the effect was only minor in normal NIH/3T3 fibroblasts.\textsuperscript{434} This suggests that nuclear export inhibitors could be therapeutically relevant.\textsuperscript{434}

However, such inhibitors might have detrimental consequences due to their lack of specificity; other proteins depend on exportin 1 for nuclear export.\textsuperscript{224} In general, all indirect mechanisms of STAT3 inhibition have two major problems. First, if an upstream target is inhibited then other pathways which might be critical to regular function might also be affected. Secondly, inhibiting an upstream target still allows for the potential of
STAT3 activation through an alternate pathway to occur. This could be especially problematic if there are multiple events that have led to STAT3 activation (excess cytokine stimulation, activating JAK mutation, decreased SOCS or PIAS expression, etc.).

![Leptomycin B](image1.png)

**Leptomycin B**

![Ratjadone A](image2.png)

**Ratjadone A**

**Figure 33. Structures of leptomycin B and ratjadone A.**

As a result, directly targeting STAT3 is a favored therapeutic approach.\(^{363}\) Over the past 10 plus years multiple strategies have been employed in attempts to design therapeutic STAT3 inhibitors. Different inhibitors have been developed that are directed at the N-terminal domain, DBD, and SH2 domain of STAT3.
N-Terminal Domain Inhibitors

The N-terminal domain of STAT3 is involved in the formation of a tetramer from two STAT3 dimers, as well as other protein-protein interactions.\textsuperscript{431} The second of the eight helices is highly implicated in the domain function, and has been targeted.\textsuperscript{516} A library of helix 2 analogues were synthesized and tested for their abilities to suppress STAT3 signaling.\textsuperscript{516} A palmitoylated 13-residue peptide (KYLKHLQELYRTD) was found to have a GI\textsubscript{50} of $1.8 \pm 0.2 \, \mu M$ in MCF7 breast cancer cells.\textsuperscript{516} The peptide analogues were able to induce apoptosis in breast cancer cells, but not in normal breast cells or fibroblasts deficient in STAT3.\textsuperscript{516} The peptide, ST3-H2A2, has been found to inhibit growth, and to induce apoptosis in DU145 and PC3 prostate cancer cells as well.\textsuperscript{517} No effective non-peptide inhibitors directed toward the N-terminal domain have been reported to date, but these data do confirm that the N-terminal domain is a viable drug target.\textsuperscript{516}

DBD Inhibitors

Several platinum (IV) compounds (CPA-1, CPA-3, CPA-7 and IS3 295) have been reported to interfere with STAT3 DNA-binding.\textsuperscript{518,519} CPA-1, CPA-7 and IS3 295 have been shown to be two to four times more selective for STAT3 dimers than STAT1 dimers, and their IC\textsubscript{50} values for disrupting STAT3 DNA-binding are 5.0, 1.5 and 1.4 \mu M, respectively.\textsuperscript{518,519} IS3 295 was shown to interact with STAT3 with noncompetitive inhibition kinetics.\textsuperscript{519} The exact site of interaction with STAT3 is not known, but cysteine residues were implicated in the interaction.\textsuperscript{519}
Galiellalactone (Figure 34) is a fungal metabolite that was found to inhibit IL-6 signaling by binding activated STAT3 dimers in their DBDs.\textsuperscript{520} It was proposed that galiellalactone might inhibit STAT3 binding by forming a covalent bond via a Michael addition of the sulfur atom of Cys\textsuperscript{468} into the $\alpha,\beta$-unsaturated ester of galiellalactone.\textsuperscript{520} Galiellalactone has been shown to induce apoptosis in PC3 and DU145 prostate cancer cells, and inhibit STAT3-mediated luciferase activity with an IC\textsubscript{50} of approximately 5 $\mu$M.\textsuperscript{521} However, galiellalactone does exert some inhibitory effects on other pathways as well.\textsuperscript{520}

![Figure 34. Structure of galiellalactone.](image)

There have been numerous reports on the development of STAT3 decoy oligonucleotides (ODNs), which competitively inhibit the DBD of STAT3 by competing with the specific cis elements that exist in the promoter regions of target genes.\textsuperscript{515,522,523,524,525,526,527,528,529,530,531} To date, the only clinical trial involving the targeting of STAT3 involved a STAT3 decoy ODN.\textsuperscript{527} Patients with head and neck squamous cell carcinoma received intratumoral injections of the decoy.\textsuperscript{527} Following treatment there was evidence of a decrease in the expression of STAT3 target genes in
the patients’ tumors.\textsuperscript{527} Furthermore, toxicities were not reported and a maximum tolerated dose was never reached.\textsuperscript{527}

However, the decoy failed to reduce tumor growth in mice harboring cancer xenografts.\textsuperscript{527} One explanation for this is that decoy ODNs are often subject to degradation and thermal denaturation \textit{in vivo}.\textsuperscript{527} Another limitation to STAT3 decoy ODNs is that STAT1 and STAT3 prefer similar DNA targets; obtaining specificity for STAT3 is difficult.\textsuperscript{515,532} An improved cyclic decoy was designed that had a longer serum half-life and inhibited tumor growth in a mouse model.\textsuperscript{527} The decoy may prove to be an effective therapeutic agent, but further studies are required.\textsuperscript{527} To date, only one other decoy ODN has been evaluated in a clinical setting, and it was not being investigated in relation to STAT3 or cancer.\textsuperscript{533}

\textbf{SH2 Domain Inhibitors}

There have been a number of small molecule and peptide-like inhibitors that have been designed to target the STAT3 SH2 domain. There have also been G-rich, quartet forming oligodeoxynucleotides that have been shown to inhibit STAT3 activity by interacting with the SH2 domain.\textsuperscript{534,535,536,537} The oligodeoxynucleotide, T40214, is a promising candidate, as it was able to inhibit IL-6-mediated STAT3 activation in PC3 prostate cells with an IC\textsubscript{50} of 5 \textmu M.\textsuperscript{535} However, optimization of the physiochemical properties of the G-quartet oligodeoxynucleotide may need to occur in order to facilitate any type of clinical development.\textsuperscript{538} Here, the peptidomimetic and notable small molecule SH2 domain inhibitors will be discussed.
In the early 2000’s McMurray and colleagues identified the high-affinity hexapeptide, Ac-pYLPQTV-NH₂, by assaying pTyr hexapeptides that were derived from the known STAT3 docking sites of G-CSFR, LIFR, GP130, EGFR and IL-10R.\textsuperscript{539} Using electrophoretic mobility shifts assays (EMSAs), they measured the abilities of peptides to inhibit STAT3 dimerization and DNA binding.\textsuperscript{539} Ac-pYLPQTV-NH₂ was the highest-affinity peptide of those evaluated, achieving an IC\textsubscript{50} of 150 nM.\textsuperscript{539} Since then, McMurray and colleagues have used this lead peptide to identify extremely potent inhibitors of the STAT3 SH2 domain.\textsuperscript{540,541,542,543,544,545,546,547}

They have identified compounds with sub-100 nM K\textsubscript{i} values in the STAT3 fluorescence polarization (FP) assay.\textsuperscript{545} PM-73G (Figure 35), a phosphopeptide mimic prodrug with increased cell permeability and stability against phosphastase activity, has been shown to inhibit STAT3 phosphorylation and the growth of breast tumor xenografts in a mouse model \textit{in vivo}.\textsuperscript{547} However, its IC\textsubscript{50} value in MDA-MB-468 cells was roughly 30 \(\mu\)M, despite the fact that it completely abrogated STAT3 phosphorylation at Tyr\textsuperscript{705} at a concentration of 0.5 \(\mu\)M.\textsuperscript{545} Even at 25 \(\mu\)M, PM-73G inhibited the phosphorylation of off-targets focal adhesion kinase (FAK), Akt and STAT5.\textsuperscript{545} So at the level that it is cytotoxic it is not specific for STAT3, and yet it is able to abolish phosphorylation at 705 at much lower concentrations.\textsuperscript{545} As a result, it is suggested that inhibition of the phosphorylation at 705 alone is not sufficient to block downstream gene expression or induce apoptosis.\textsuperscript{545,547}
Others have designed peptidomimetic compounds targeting the SH2 domain as well. Wang and co-workers have succeeded in designing compounds that bind with high affinity and have notable IC$_{50}$ values in cancer cells.$^{548,549}$ The most active compound in inducing apoptosis in cancer cells, CJ-1383 (Figure 36), was found to have a K$_i$ of 0.95 µM in the STAT3 FP assay.$^{549}$ Although they have reported other compounds that bind with higher affinity, CJ-1383 displayed the most cellular activity.$^{549}$ It achieved IC$_{50}$ values of 11.2 and 3.6 µM in MDA-MB-231 and MDA-MB-468 breast cancer cells, respectively.$^{549}$

Figure 36. Structure of CJ-1383.
Turkson and colleagues have also designed a number of peptidomimetic compounds targeting the SH2 domain. ISS 610 disrupted STAT3 DNA binding with an IC$_{50}$ of 42 ± 23 µM.$^{550}$ More recently, they identified a lead peptidomimetic compound, 14aa.$^{551}$ 14aa was observed to have a $K_i$ value of 5 ± 1 µM in the STAT3 FP assay, bind STAT3 with a $K_d$ of 900 nM, and disrupt STAT3 DNA binding with an IC$_{50}$ of 73.1 ± 6 µM.$^{551}$ However, they observed little tumor cytotoxicity (IC$_{50}$ values of roughly 50 µM in several cell lines), which they credited to poor metabolic stability and cell permeability.$^{551}$

Low cell permeability and stability lead to low biological activities.$^{538}$ These are two key traits that peptide-like inhibitors exhibit, which often leads to the need of very high concentrations in order to have the desired effects in vivo.$^{538}$ Additionally, any peptide-like compound containing a pTyr moiety would presumably have significantly more problems with membrane permeability.$^{538}$ As a result, non-peptidic small molecule inhibitors, which could be more cell permeable, are desired.$^{538}$

The notable small molecule inhibitors of the SH2 domain are shown (Figure 37). STA-21 was the first non-peptidic small molecule that was reported to bind to the SH2 domain of STAT3.$^{552}$ It was identified through virtual screening.$^{552}$ It was shown to inhibit STAT3-dependent luciferase reporter activity in MDA-MB-435s (breast) and Caov-3 (ovarian) cells, both of which express constitutively active STAT3.$^{552}$ At tested concentrations of 20 and 30 µM, it significantly induced apoptosis in MDA-MB-435s and MDA-MB-231 breast cancer cell lines.$^{552}$
Stattic was also identified through virtual screening.\textsuperscript{553} Stattic was found to have an IC\textsubscript{50} of 5.1 ± 0.8 µM in the STAT3 FP assay.\textsuperscript{553} Stattic is thought to act through an irreversible mechanism.\textsuperscript{553} It was shown to nearly abolish STAT3 phosphorylation in HepG2 cells at a concentration of 20 µM.\textsuperscript{553} However, at a concentration of 10 µM and an incubation time of 24 hours, Stattic induced less than 20 and 10% apoptosis in MDA-MB-435s and MDA-MB-231 cells, respectively.\textsuperscript{553}

Cpd 188 was identified through virtual screening as well.\textsuperscript{554} In an SPR assay, it inhibited the binding of a pTyr-containing peptide ligand to STAT3 with an IC\textsubscript{50} of 20 µM.\textsuperscript{554} It also inhibited IL-6-mediated STAT3 phosphorylation and nuclear translocation with IC\textsubscript{50} values of 73 and 39 µM, respectively.\textsuperscript{554} These were determined in an immunoblot assay with HepG2 cells and in a high-throughput fluorescence microscopy (HTFM) assay with murine embryonic fibroblasts, respectively.\textsuperscript{554} Cpd 188 induced apoptosis in MDA-MB-468, MDA-MB-231 and MDA-MB-435 cell lines with EC\textsubscript{50} values of 0.73, 3.96 and 7.01 µM, respectively.\textsuperscript{554} In Contrast, it induced apoptosis in cell lines without constitutively active STAT3, MDA-MB-453 and MCF7, with EC\textsubscript{50} values of 15.5 and 17.19 µM, respectively.\textsuperscript{554} The compounds identified were selective for STAT3 over STAT1, which the authors attributed to targeting the SH2 domain at three sites, one of which is a hydrophobic pocket that acts as a selectivity filter.\textsuperscript{554}

The oxazole based inhibitor, S3I-M2001, was found to disrupt STAT3-STAT3 DNA binding with an IC\textsubscript{50} of 79 ± 9 µM, as determined by EMSA analysis.\textsuperscript{555} However, it had similar activity against STAT3-STAT1 dimers (IC\textsubscript{50} of 92 ± 11 µM), and was only about twice more active against STAT3-STAT3 dimers than STAT1-STAT1 dimers.\textsuperscript{555}
Furthermore, in cell assays, it only achieved IC₅₀ values in the 50 to 100 µM range in several cell lines exhibiting constitutively active STAT3.⁵⁵⁵

STX-0119 was identified through a virtual screen.⁵⁵⁶ In HeLa cells, it inhibited STAT3 transcription with an IC₅₀ of 74 µM in a STAT3-dependent luciferase reporter gene assay.⁵⁵⁶ It also disrupted STAT3 dimerization with an IC₅₀ of 9 µM in a fluorescence resonance energy transfer (FRET)-based STAT3 dimerization assay.⁵⁵⁶ It was able to induce apoptosis in MDA-MB-468 cells.⁵⁵⁶ It also showed decent selectivity for STAT3 over STAT1, and even more selectivity for STAT3 over STAT5.⁵⁵⁶

S3I-201 was also initially identified through virtual screening.⁵⁵⁷ S3I-201 was shown to inhibit STAT3 DNA-binding with an IC₅₀ of 86 ± 33 µM in vitro through EMSA analysis.⁵⁵⁷ It was roughly two times more selective for STAT3-STAT3 DNA binding than STAT3-STAT1 or STAT5-STAT5.⁵⁵⁷ It was about four times more selective for STAT3-STAT3 than STAT1-STAT1 DNA binding.⁵⁵⁷

Since then improvements have been made. S3I-201.1066 was able to inhibit STAT3 DNA-binding with an IC₅₀ of 35 ± 9 µM.⁵⁵⁸ It also inhibited binding in the STAT3 FP assay with an IC₅₀ of 20 ± 7.3 µM.⁵⁵⁸ Through SPR analysis, it was shown to bind to STAT3 with a Kₐ of 2.74 µM.⁵⁵⁸

More recently, S3I-1757 displayed an improved IC₅₀ of 13.5 ± 0.5 µM in the STAT3 FP assay.⁵⁵⁹ In cell studies, it was also shown that S3I-1757 inhibits STAT3 dimerization.⁵⁵⁹ However, it has apparent IC₅₀ values of >50 µM in MDA-MB-231 and MDA-MB-468 breast cancer cells, as determined by the MTT assay.⁵⁵⁹
BP-1-102 has the highest binding affinity of any S3I-201 analogue that has been reported on, to date. It inhibited peptide binding in the STAT3 FP assay with an IC$_{50}$ of 4.1 µM. It also showed selectivity, only inhibiting STAT1 and STAT5 binding in their corresponding FP assays with IC$_{50}$ values in the 25 to 30 µM range. Its IC$_{50}$ for inhibiting STAT3 DNA-binding in vitro was 6.8 ± 0.8 µM. Through SPR analysis, BP-1-102 was also found to bind STAT3 with a K$_d$ of 504 nM. IC$_{50}$ values for its cytotoxic activities in cancer-derived cell lines were not reported, although it appears to achieve IC$_{50}$ values around 10 µM in DU145 cells, and below 10 µM in MDA-MB-231 cells.

Computational modeling was used in the discoveries of LLL3 and LLL12, which were based on the structure of STA-21 and synthesized in Dr. Li’s lab. LLL3 was shown to inhibit STAT3-specific DNA binding activity in U373 glioblastoma and MDA-MB-231 breast cancer cells. In MDA-MB-231 cells, it was also shown to inhibit STAT3-dependent transcriptional activity. At a concentration of 10 µM, LLL3 decreased cell viability to 60% or below in several glioblastoma cell lines.

LLL12 was shown to inhibit STAT3 DNA binding, but not STAT1 DNA binding. It also inhibited IL-6-mediated STAT3 phosphorylation and STAT3-dependent transcriptional activities. Moreover, in five out of six cancer cell lines, LLL12 achieved IC$_{50}$ values of 0.16 to 0.97 µM. In the other cell line, SK-BR-3 (breast), it still achieved an IC$_{50}$ of 3.09 µM. LLL12 is the most potent STAT3 inhibitor to date.

Nonetheless, there are still currently no small molecule STAT3 inhibitors in clinical trials. More potent and selective STAT3 inhibitors with improved
pharmacokinetic properties are desired, and the discovery and development of such compounds has been a major focus. Finding compounds that are bioavailable, cell permeable, and display limited toxicity to non-cancerous cells are some issues that have needed to be overcome.

Finding inhibitors that are significantly more selective for STAT3 than STAT1 is another critical issue. It is true that STAT3 and STAT5 have SH2 domains with quite similar 3\° structures. However, they have different amino acid sequences in the peptide binding regions, which allow for differences in the binding affinities of inhibitors to be achieved. On the other hand, STAT3 and STAT1 have SH2 domains that have similar sequences and 3\° structures, which makes obtaining selectivity difficult. Regardless, JAK inhibitors cannot achieve selectivity for STAT3 over STAT5 or STAT1, so the discovery of more potent STAT3-specific inhibitors is required.
Figure 37. Notable small molecule inhibitors of the STAT3 SH2 domain.
Chapter 3: Development of AG490 and WP1066 Analogues

3.1 Introduction

The role of JAK2 in disease has already been thoroughly discussed in chapter 2. In an attempt to design more potent JAK2 inhibitors, the structures of AG490 and WP1066 were looked to as a starting point. Structurally, they have similarities with the natural product, curcumin,\textsuperscript{564} which is the focus of the majority of the research presented in this document (Figure 38). It is important to note that, as it will be discussed in chapter 6, computational modeling predicts that curcumin is able to bind to both JAK2 and STAT3.

![Structures of AG490, WP1066 and curcumin.](image_url)

Figure 38. Structures of AG490, WP1066 and curcumin.

Structurally, all three compounds contain aromatic rings on both sides and at least one enone. All three compounds are also expected to adopt fairly planar structures due to
the aromaticity of the compounds, their high degrees of conjugation, and the possibility for resonance stabilization when they exist in planar conformations. The catechol moiety that AG490 contains could be problematic concerning its likeliness in being a suitable drug candidate; catechols can often react with membranes, proteins and DNA, leading to non-repairable damage.\textsuperscript{565} AG490 is able to inhibit JAK2/STAT3 signaling \textit{in vitro}, but only with IC\textsubscript{50} concentrations in the range of 25 to 100 \textmu M, which leads to poor activity \textit{in vivo}.\textsuperscript{384,388,566,567,568} WP1066 is an analogue of AG490, and it has been shown to disrupt JAK2 activity and induce apoptosis in AML cells.\textsuperscript{569} It has also been shown to be active against V617F JAK2.\textsuperscript{388} WP1066 contains a methyl substituent that AG490 lacks, and contains a bromopyridine moiety instead of a catechol.

3.2 Computational Modeling

Computational modeling was carried out in order to get a picture of how AG490 and WP1066 interact with JAK2 (Figure 39). They were predicted to bind to the ATP binding site of JAK2, within the JH1 domain, with similar conformations. A crystal structure of the PTK domain of JAK2 with the inhibitor, pyridone 6, bound within the binding site has previously been obtained (please refer to section 2.2.6 for the structure of pyridone 6).\textsuperscript{570} It is able to offer some insight into its 3D structure, and how inhibitors will be able to bind.\textsuperscript{327}

The catechol and bromopyridine rings of AG490 and WP1066 both appear to bind to the ATP binding site, which is located at the interface of the hinge region. Another major interaction is provided by the nitrile groups, which orient themselves into the
oxyanion hole. The extra methyl substituent of WP1066 is thought to provide hydrophobic interactions as well, although the exact nature of the interaction was not determined. As can be seen, the benzyl groups of both compounds do not extend all of the way towards the substrate pocket of JAK2. This suggests that they do not play a major role in the binding affinity, leaving room for improvements to be made.

![Computational docking of AG490 and WP1066 into the ATP binding site of JAK2. (This work was performed by the lab of Dr. Chenglong Li.)](image)

3.3 Synthetic Procedures

One of the attractive features of AG490 analogues is that they can be readily synthesized. Analogues were synthesized in a two-step reaction sequence, as shown (Scheme 1). Briefly, the substituted benzylamines were added into the cyanoester under neat conditions. Subsequent elimination yielded the corresponding benzylamides. The benzylamides were then condensed with substituted aldehydes to yield the desired AG490 analogues.
3.4 Development of Analogues and SAR Studies

When analyzing the structure of AG490, one can see three moieties that can be altered. They are the catechol, enone and benzylamine portions. The nitrile group was left unaltered, since computational modeling suggested that it was important for binding. The length of the chain connecting the aromatic portions was also left unchanged.

![Scheme 1. General procedure for the synthesis of AG490 analogues. (Analogues were synthesized by Dr. Dalia Abdelhamid and Michael Corcoran.)](image)

3.4.1 Importance of the Methyl and Bromopyridine Moieties

The first goal was to investigate the importance of the benzylic methyl substituent and bromopyridine moiety. Four sets of analogues were synthesized to gain an understanding of how much the methyl substituent contributes to binding (Figure 40). In the case of WP1066 (FLLL101), the methyl substituent does not appear too critical, as the inhibition of cell growth in DU145 and MDA-MB-468 cells was similar at the indicated concentrations of FLLLS 101 and 104 (Figures 41 and 42). The IC$_{50}$ values obtained in MDA-MB-468 cells also support this conclusion (Table 7).
FLLLs 102 and 103 were inactive in all cell lines in which they were evaluated (Table 7). As a result, the importance of the methyl substituent for these analogues was not able to be determined. However, for FLLLs 113 and 114, a significant reduction in activity in DU145 cells at concentrations of 10 and 50 µM resulted from the removal of the methyl substituent. A similar effect was also seen in MDA-MB-468 cells. For FLLLs 115 and 116, a general reduction in activity at a 10 µM concentration in DU145 and MDA-MB-468 cells was also observed with the removal of the methyl substituent. The IC$_{50}$ values achieved by the compounds in MDA-MB-468 cells, in general, demonstrate that the presence of the methyl substituent results in increased activity.
Figure 40. Structures of FLLLs 101-104 and FLLLs 113-116.
Figure 41. Activities of AG490 analogues with or without a methyl substituent in DU145 cells. Percent inhibition of cell growth is shown.

Figure 42. Activities of AG490 analogues with or without a methyl substituent in MDA-MB-468 cells. Percent inhibition of cell growth is shown.
It can also be seen from these initial studies that the styrol moiety is very critical. While the presence or absence of the benzylic methyl substituent clearly had an effect, substituting the bromopyridine ring with 4-hydroxy-3-methoxybenzene resulted in a near complete lack of activity. Removal of either the bromine or aromatic nitrogen also resulted in decreased activity, although it was only minor. This signified that changing the substitution pattern of the styrol might be able to improve activity, and that extending the benzylic alkyl substituent might also be able to improve potency.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DU145</td>
</tr>
<tr>
<td>FLLL101 (WP1066)</td>
<td>7.5 ± 1.3</td>
</tr>
<tr>
<td>FLLL102</td>
<td>&gt;50</td>
</tr>
<tr>
<td>FLLL103</td>
<td>&gt;50</td>
</tr>
<tr>
<td>FLLL104</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL113</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL114</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL115</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL116</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL118</td>
<td>7.65 ± 0.61</td>
</tr>
<tr>
<td>FLLL119</td>
<td>3.66 ± 0.36</td>
</tr>
<tr>
<td>FLLL120</td>
<td>&gt;150</td>
</tr>
<tr>
<td>FLLL121</td>
<td>33.4 ± 2.7</td>
</tr>
<tr>
<td>FLLL122</td>
<td>&gt;150</td>
</tr>
<tr>
<td>FLLL123</td>
<td>17.0 ± 1.6</td>
</tr>
<tr>
<td>FLLL126</td>
<td>7.74 ± 0.54</td>
</tr>
<tr>
<td>FLLL132</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL138</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL139</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL144</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 7. Antiproliferative activities of AG490 analogues. ND: not determined. (Values for FLLLs 102 and 103 were obtained by Michael Corcoran, as were the values for FLLL101, with the exception of the value for the MDA-MB-468 cells.)
3.4.2 Effects of a Benzylic Ethyl Substituent

Due to the observed importance of the methyl substituent, and the fact that computational modeling signified that it might contribute hydrophobic interactions, the effects that a methyl to ethyl substitution would have on the activity of the AG490 analogues was explored. In an attempt to gain an increase in binding due to the substituted styrol, a number of analogues were also synthesized and evaluated that contained various substitution patterns (Figure 43).

![AG490 analogues featuring an ethyl substituent.](image)

The compound containing the 3,5-dimethoxybenzene substituent, FLLL118, was less active than FLLL101 in both cell lines (Figures 44 and 45). As was the case with FLLLs 102 and 103, the 4-hydroxy-3-methoxybenzene-containing FLLL120 also showed
a lack of activity. FLLLs 124 and 125 also exhibited low activity, and FLLL123 showed somewhat reduced activity when compared to FLLL101 as well.

Figure 44. Activities of AG490 analogues featuring a methyl to ethyl substitution in DU145 cells. Percent inhibition of cell growth is shown.

Figure 45. Activities of AG490 analogues featuring a methyl to ethyl substitution in MDA-MB-468 cells. Percent inhibition of cell growth is shown.
However, FLLL119 exhibited similar activity to FLLL101 at concentrations of 10 and 50 µM in both DU145 and MDA-MB-468 cell lines. Furthermore, from comparing IC₅₀ values, it can be seen that FLLL119 is more than twice as potent as FLLL101 in all three cell lines in which they were examined (DU145, MDA-MB-468 and MDA-MB-231). FLLL119 was the most potent in MDA-MB-231 cells, where it achieved an IC₅₀ value of less than 2 µM.

A couple of important points can be inferred from these data. The first is that an ethyl group leads to greater activity than a methyl substituent at the benzylic position. Since computational modeling predicts that the methyl substituent contributes hydrophobic interactions, it could be reasoned that the ethyl substituent is able to contribute more of these interactions, leading to increased binding.

The second important point is a reiteration of what was demonstrated by the SAR studies of the first series: the substitution pattern of the styrol is absolutely critical for binding. FLLLs 101, 104 and 119, which all contain a bromopyridine moiety, exhibit relatively similar activities. In contrast, FLLLs 102, 103 and 120, which all contain a 4-hydroxy-3-methoxybenzene moiety, all demonstrate a lack of activity. So while the substitution at the benzylic carbon is important, it appears that the substitution pattern of the styrol by far has the greater effect. The data suggests that the same substitution patterns which appear to make curcumin active fail to increase the activity of WP1066 and AG490 analogues.
3.4.3 Methylation of the Benzylamine Nitrogen

To determine the contributions of the benzylamine to the activity of the AG490 analogues, a series of derivatives were synthesized, where all of the compounds were methylated at the amine nitrogen (Figure 46). It should be noted that the methylation of various positions of biologically active small molecules can be used to determine the important sites of interaction between small molecules and their biological targets.\textsuperscript{571} By methylating the amine nitrogen, a hydrogen bond donating group is being removed and a hydrophobic methyl group is being added. The effects of the methylation and the identity of the styrol were able to be studied by leaving the benzyl substituent unaltered in all of the derivatives.

Figure 46. N-Methylated AG490 analogues.
Consistent with the previous results, FLLL122, which contains the 4-hydroxy-3-methoxybenzene moiety, exhibited low activity in both DU145 and MDA-MB-468 cell lines (Figures 47 and 48). FLLL121, which contains the 3,5-dimethoxybenzene moiety, displayed decent activity at a 50 µM concentration, achieving an IC\textsubscript{50} of 33.4 ± 2.7 µM in DU145 cells. It also achieved an IC\textsubscript{50} of 17.5 ± 0.8 µM in MDA-MB-231 cells. However, it is significantly less active than FLLL118, which contains the 3,5-dimethoxybenzene moiety, has an ethyl substituent at the benzylic carbon, and is not N-methylated.

Figure 47. Activities of N-methylated AG490 analogues in DU145 cells. Percent inhibition of cell growth is shown.

FLLL128 only achieves noteworthy inhibition at a concentration of 50 µM in DU145 cells, which is still significantly better than FLLLs 127 and 129, which show no
activity in DU145 cells at the tested concentrations. Even at 50 µM, they resulted in a less than 20% inhibition of growth in MDA-MB-468 cells. In contrast, FLLL126, which contains the bromopyridine moiety, showed only slightly decreased activity in both cell lines when compared to FLLL101. In general, these data suggest that N-methylated derivatives exhibit slightly lower, but still similar activity to the derivatives that are not N-methylated. It also reiterates the importance of the substituted styrol.

![Graph](image)

**Figure 48. Activities of N-methylated AG490 analogues in MDA-MB-468 cells.** Percent inhibition of cell growth is shown.

3.4.4 Methylation of the Benzylamine Aromatic Ring

Computational studies indicated that the benzyl moiety does not extend all the way toward the substrate pocket or greatly contribute to the binding of AG490 and
WP1066 to JAK2. As a result, it could be inferred that improvements could be made in order to increase binding. Three series of analogues were synthesized that contained benzyl groups that were methylated at either the ortho, meta or para positions (Figure 49). An additional analogue, FLLL149, was also synthesized in order to further study the importance of the benzyl group and analyze what contributions an additional carbonyl group could make.

![AG490 analogues featuring a methylated benzyl group](image-url)

Figure 49. AG490 analogues featuring a methylated benzyl group.
In DU145 cells, the only compounds that were able to achieve similar activity to FLLL101 were FLLls 144 and 149 (Figure 50). Consistent with previous data, the findings for the bromopyridine-containing FLLL144 again reiterate the importance of the bromopyridine moiety. Since the rest of the compound displayed limited activity, it was difficult to determine which position of methylation contributed the most to activity. The activity of FLLL149 suggests that the benzyl group might not be at all critical for activity, and that an additional carbonyl group might be beneficial for activity.

Figure 50. Activities of AG490 analogues featuring a methylated benzyl moiety in DU145 cells. Percent inhibition of cell growth is shown.
Interestingly, while only one of the bromopyridine-containing analogues showed significant activity in DU145 cells, in MDA-MB-468 cells, all of the bromopyridine-containing derivatives, FLLLs 132, 138 and 144, were significantly active at concentrations of both 10 and 50 µM (Figure 51). The achieved IC$_{50}$ values of the compounds indicate that the o-methylated compound, FLLL132, is more active than the p-methylated compound, FLLL144.

![Figure 51. Activities of AG490 analogues featuring a methylated benzyl moiety in MDA-MB-468 cells. Percent inhibition of cell growth is shown.](image)

The o-methylated analogue, FLLL132, achieved an improved IC$_{50}$ value in MDA-MB-468 cells relative to FLLL101, while the m-methylated compound, FLLL138,
exhibited similar activities to FLLL101. However, due to the still relatively similarities in IC$_{50}$ values in MDA-MB-468 cells, and the fact that FLLL101 still achieved the highest inhibition at 10 µM in DU145 cells, it is hard to determine whether or not the methylation of the benzyl group significantly contributed to activity. Still, at the very least, it appears to not hinder the activity of the derivatives.

The compounds featuring the 3-hydroxy-5-methoxybenzene substituent, FLLLs 133, 139 and 145, were all able to inhibit the cell growth of MDA-MB-468 cells by greater than 50% at a concentration of 50 µM. FLLL139 achieved an IC$_{50}$ of 22.6 ± 3.9 µM in these cells, demonstrating modest activity. Computational data suggests that the presence of both a hydrogen bond donor and acceptor in the binding site would aide in binding. The methoxy and hydroxyl substituents of FLLL139 provide this, and might be the reason for its observed activity.

3.5 Summary of SAR Studies of AG490 Analogues

Some valuable pieces of information were able to be learned from the SAR studies of AG490 analogues. The first is that the identity of the substituted styrol is absolutely critical to activity. Several analogues that were only altered at this location showed a complete lack of activity when compared to WP1066. The bromopyridine-containing compounds were the most active in every series. It is possible that another moiety at this location might result in increased activity. However and unfortunately, none of the aromatic moieties that were tested showed improved activity when compared to the bromopyridine moiety. However, several of the 3,5-substituted analogues, such as
FLLLs 118, 121, 123 and 139, at least showed modest activity. They were, in general, more active than the 3,4-substituted analogues.

It is also clear that the presence of a benzylic alkyl substituent does contribute to binding. In general, compounds featuring a methyl substituend had more activity than compounds lacking an alkyl group at this position, and compounds featuring an ethyl substituent displayed the most activity. FLLL119, which contains an ethyl substituent at this position, was the most active out of all of the compounds that were evaluated. It was more potent than WP1066 in all three cell lines in which IC\textsubscript{50} values were obtained. While these results were promising, an analogue that exhibited vastly improved activity was not discovered.

Lastly, it was found that N-methylated derivatives exhibited slightly decreased activity, while analogues that had been methylated on the benzyl ring exhibited similar to increased activity. While the N-methylated FLLL126 had similar potency to WP1066, it was still slightly less active. The o- and m-methylated analogues, FLLL132 and 138, both showed similar activities to WP1066.

Additional SAR studies will need to be performed in order to achieve a more potent JAK2 inhibitor. One objective will be to discover a moiety that is more active than the bromopyridine substituent. The position of the bromine on the pyridine, and its effects on activity, should also be studied. Also, since the compounds featuring a benzylic ethyl substituent exhibit the most activity, the alkyl chain could be continued to be extended until a decrease in activity is observed. Through this, the optimal length of the chain could be determined. Lastly, since methylating the benzyl ring appears to result in
an increase in activity, additional alterations on that side of the scaffold should be made in an attempt to further extend the molecule. Theoretically, this should result in observed increases in binding and activity.
Chapter 4: Initial SAR Studies of Curcumin Analogues

4.1 Introduction

1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, otherwise known as curcumin, is a natural product that is found in turmeric (*Curcuma longa*) (Figure 52). It is found in the rhizomes, and is commonly used as a food preservative, flavoring agent, coloring agent and spice. It is also used for decoration. Besides these uses, there is also documentation which shows that curcumin has been used for medicinal purposes in Ayurveda, which is the traditional Indian system of medicine, for over 6,000 years. It has been used to treat many diseases, including liver disease, RA, infectious diseases, atherosclerosis, diabetes and different types of cancer. Curcumin makes up 77% of commercial curcumin, while the related curcuminoids, demethoxycurcumin and bisdemethoxycurcumin, are present at 17 and 3%, respectively (Figure 53).

Importantly, curcumin has both chemotherapeutic and chemopreventive properties in relation to a number of cancer types. Advantageously, curcumin is quite non-toxic; even when it was administered at doses of up to 10 grams per day, no dose-limiting toxicity was reached. The molecular scaffold of curcumin is also quite simple; it consists of two aromatic rings that are joined to the central 1,3-diketone by olefin bonds. At the same time, it still contains a number of functional groups which
can be optimized. Together, these properties make curcumin an ideal starting point for SAR studies and lead optimization.\textsuperscript{574}

![Diagram of tautomeric forms of curcumin]

**Figure 52. The tautomeric forms of curcumin.**

![Diagram of curcuminoids]

**Figure 53. The two other curcuminoids present in commercial curcumin.**

As shown in Figure 52, curcumin can undergo tautomerization, and naturally exists as a mixture of the diketone and keto-enol forms.\textsuperscript{574} Computational modeling predicts that the keto-enol form is lower in energy than the diketone form by 6.7 and 6.9 kcal/mol in gas and solution phases, respectively.\textsuperscript{577} This is thought to be due to the
acidity of the protons that are attached to the central methylene carbon, the stabilizing intramolecular hydrogen bonding that can occur in the keto-enol form, and the fact that the keto-enol form is fully conjugated. The keto-enol form, which can form an intramolecular hydrogen bond, prefers the syn conformation. In contrast, the lone pair repulsion of the carbonyls of the diketone form repel each other, and as a result, the diketone form prefers the anti conformation. In support of this prediction, X-ray crystallography has confirmed the preference of the keto-enol form. The solution structure of curcumin has also been analyzed by nuclear magnetic resonance (NMR) spectroscopy in a number of solvents and from a pH range of 3 to 9. In all cases, curcumin was found to exist in the keto-enol form, further supporting the computational predictions.

The number of therapeutic effects that curcumin has can be attributed to the fact that is shows activity on a plethora of molecular targets. Several of these targets are highly related with cancer cell proliferation, and include STAT3, NFκB, peroxisome proliferator-activated receptor-gamma (PPARγ), and activating protein-1 (AP-1). Some of its other targets include cytosolic phospholipase A2 (cPLA2), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), protein kinases such as human epithelial growth factor receptor 2 (HER2), EGFR, Akt, and MAPK, and proteins which regulate the cell cycle and apoptosis.

Unfortunately, this poor selectivity of curcumin is one of the characteristics that limits its therapeutic usefulness. Another characteristic of curcumin that limits its usefulness is that it exhibits limited bioavailability. In fact, its limited bioavailability
might contribute to it being tolerated at such high doses; if significant amounts of curcumin are not being absorbed, then its supposed lack of toxicity might not be entirely accurate. It is also physically and metabolically unstable, which further hinders its usefulness.\textsuperscript{574}

Curcumin has been found to decompose rapidly in neutral and basic buffers.\textsuperscript{595} For example, approximately 90\% of curcumin has been shown to decompose in 0.1 M phosphate buffer at 37\(^{\circ}\)C and pH 7.2 within 30 minutes.\textsuperscript{595} However, it should be noted that curcumin is significantly more physically stable in human blood.\textsuperscript{595} Still, curcumin is subject to extensive phase I and phase II metabolism through sulfation, glucuronidation, oxidation and reduction, both \textit{in vitro} and \textit{in vivo}.\textsuperscript{596,597,598,599,600,601}

To overcome these limitations and improve the selectivity, stability and bioavailability of curcumin-related compounds, a number of analogues have been synthesized and evaluated.\textsuperscript{573,574,581,582,583,584,585,586,587,588,589,590} Varying the substitution patterns of the aromatic rings has been a primary aim in many prior studies.\textsuperscript{574} The olefin bonds have not been greatly modified since they are largely viewed as linkers.\textsuperscript{574} However, they still are considered to be important for activity.\textsuperscript{574}

In this chapter, the synthesis and evaluation of 24 curcumin analogues compounds will be reported. It should be noted that this work, as well as much of the discussion up this point, was initially published in Bioorganic & Medicinal Chemistry Letters. Fuchs, J. R.; Pandit, B.; Bhasin, D.; Etter, J. P.; Regan, N.; Abdelhamid, D.; Li, C.; Lin, J.; Li, P.-K. \textit{Bioorganic & Medicinal Chemistry Letters}. 2009; 19:2065-9. © Elsevier Ltd.\textsuperscript{574}
4.2 Rationale and Synthesis of Initial Curcumin Analogues

The initial curcumin analogues were synthesized and evaluated in order to gain an understanding of what structural features were required for anticancer activity in prostate and breast cancers. The development of STAT3 inhibitors was always the goal in sight. However, it was first necessary to evaluate what type of curcumin-related compounds could lead to high levels of antiproliferative activity and show selectivity for cancer cells over non-cancerous cells.

As it has been discussed, curcumin and related structures can have effects on numerous molecular targets. As a result, the activity of any given compound could be due to the modulation of a single target or multiple targets. Comparing the effectiveness of the large number of analogues that have been synthesized is also challenging, since one often has to compare between cell lines. Cell lines can exhibit drastic differences between one another in cell signaling; a pathway that is up-regulated in one cancer cell line might not be up-regulated in another. This often makes comparing the activities of compounds between cell lines unreliable, which makes it hard to determine the SARs of analogues.

For consistency, all synthesized analogues were tested in four cancer cell lines. Two of the cell lines were prostate cancer cell lines. They include the androgen-dependent cell line, LNCaP, and the androgen-independent cell line, PC3. The other two cell lines were derived from breast cancers and include the estrogen-dependent cell line, MCF7, and the estrogen-independent cell line, MDA-MB-231. To determine selectivity, compounds were also evaluated in a spontaneously immortalized, but non-malignant
mammary epithelial cell line, MCF10A. Cells were treated with compounds for three days, as it is described in the experimental section.

Two series of compounds were initially synthesized and evaluated. The heptadienedione series consists of curcumin and FLLLs 1-4, 7-10, 17, 17S, 18, 18S and 21, while the pentadienone series consists of FLLLs 11-14, 11S, 12S, 16, 16S, 20, 22 and 23 (Tables 8 and 9). FLLLs 3, 4, 10, 17, 18 and 21 were synthesized via aldol condensation between 2,4-pentanodione and specified aldehydes, using the procedure established by Venkateswarlu and colleagues (Scheme 2).\textsuperscript{573} The treatment of curcumin with hydrazine or N-methylhydrazine in acetic acid yielded FLLLs 7 and 8, respectively (Scheme 3).\textsuperscript{605,606,611}

FLLLs 11-14, 16, 20, 22 and 23 were synthesized by condensing acetone with the specified benzaldehydes under standard protic conditions (Scheme 4).\textsuperscript{612} FLLLs 1, 2, 11S, 12S, 16S, 17S and 18S all contain a sulfamate. They were prepared by reacting chlorosulfonamide (ClSO\textsubscript{2}NH\textsubscript{2}) with the specified sulfamate-precursors in dimethylacetamide (DMA) at room temperature for 24 hours, according to the procedure of Okada and colleagues (Scheme 5).\textsuperscript{613}
Table 8. The heptadienedione series of curcumin analogues. (Analogues were synthesized by Jonathan Etter and Drs. James Fuchs, Deepak Bhasin, Nicholas Regan and Dalia Abdelhamid.)
Table 9. The pentadienone series of curcumin analogues. (Analogues were synthesized by Drs. James Fuchs, Deepak Bhasin, Nicholas Regan and Dalia Abdelhamid.)

Scheme 2. Syntheses of curcumin and FLLLs 3, 4, 10, 17, 18 and 21.
Scheme 3. Syntheses of FLLLs 7 and 8.

Scheme 4. Syntheses of FLLLs 11-14, 16, 20, 22 and 23.
Scheme 5. Syntheses of FLLLs 1, 2, 11S, 12S, 16S, 17S and 18S. The synthesis of FLLL1 from curcumin is shown as an example.

4.3 Antiproliferative Activities of Initial Curcumin Analogues

The majority of the modifications for the heptadienedione series focused on the substitution patterns of the aromatic rings. The results are summarized in Table 10. The first observation to be made is that the removal of the 4-hydroxyl groups of curcumin resulted in a decrease in activity in all cell lines that were evaluated (FLLL4). Incorporating additional methoxy groups into curcumin’s structure yielded similar results (FLLL17). In contrast, removing the 3-methoxy groups from curcumin had little effect (FLLL3). Exchanging the methoxy and hydroxyl substituents in curcumin’s structure resulted in a complete loss of antiproliferative activity (FLLL18).

FLLLs 7 and 8 were created to mimic the keto-enol form of curcumin, which as it has been discussed, is the predominant naturally occurring form. FLLL7 exhibited similar activities in all four cancer cell lines, and was three- to four-fold better than curcumin.
Ishida and colleagues observed slightly improved, but similar activities in MCF7, PC3 and LNCaP cells. FLLL7 has also been reported to be an androgen receptor antagonist, and to have antioxidant, anti-inflammatory, anti-angiogenic and COX-inhibitory activities. Whether or not there is any correlation between the anti-angiogenic, anti-androgenic and antiproliferative activities of FLLL7 is uncertain. It is important to note that the N-methylated version, FLLL8, exhibited an approximate three-fold reduction in activity when compared to FLLL7. It only showed slightly more activity than curcumin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PC3</th>
<th>LNCaP</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>19.8 ± 2.1</td>
<td>19.6 ± 3.7</td>
<td>21.5 ± 4.7</td>
<td>28.4 ± 4.3</td>
</tr>
<tr>
<td>FLLL1</td>
<td>7.5 ± 1.8</td>
<td>5.9 ± 1.7</td>
<td>5.5 ± 1.2</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>FLLL2</td>
<td>19.3 ± 5.4</td>
<td>20.0 ± 1.0</td>
<td>15.1 ± 3.5</td>
<td>14.3 ± 1.1</td>
</tr>
<tr>
<td>FLLL3</td>
<td>27.3 ± 6.6</td>
<td>19.7 ± 2.9</td>
<td>24.3 ± 1.9</td>
<td>21.9 ± 2.0</td>
</tr>
<tr>
<td>FLLL4</td>
<td>40.0 ± 4.9</td>
<td>34.7 ± 6.6</td>
<td>25.9 ± 9.5</td>
<td>31.9 ± 11.1</td>
</tr>
<tr>
<td>FLLL7</td>
<td>5.6 ± 2.0</td>
<td>3.4 ± 0.9</td>
<td>5.9 ± 0.6</td>
<td>6.6 ± 1.9</td>
</tr>
<tr>
<td>FLLL8</td>
<td>16.2 ± 1.4</td>
<td>12.1 ± 1.3</td>
<td>15.8 ± 1.2</td>
<td>20.4 ± 3.0</td>
</tr>
<tr>
<td>FLLL9</td>
<td>12.9 ± 2.3</td>
<td>20.2 ± 1.7</td>
<td>17.8 ± 5.9</td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>FLLL10</td>
<td>5.9 ± 1.3</td>
<td>3.9 ± 0.6</td>
<td>5.4 ± 0.8</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>FLLL17</td>
<td>37.2 ± 4.1</td>
<td>21.1 ± 4.3</td>
<td>37.6 ± 6.1</td>
<td>41.7 ± 1.2</td>
</tr>
<tr>
<td>FLLL17S</td>
<td>13.1 ± 2.1</td>
<td>10.4 ± 1.6</td>
<td>4.7 ± 0.7</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>FLLL18</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FLLL18S</td>
<td>7.4 ± 1.6</td>
<td>7.7 ± 1.5</td>
<td>5.5 ± 0.4</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>FLLL21</td>
<td>20.9 ± 4.8</td>
<td>6.8 ± 0.6</td>
<td>15.4 ± 1.5</td>
<td>7.2 ± 1.1</td>
</tr>
</tbody>
</table>

Table 10. Antiproliferative activities of the heptadienedione series of curcumin analogues. (The majority of these values were obtained by Dr. Bulbul Pandit.)
The limiting properties of curcumin, such as its metabolic instability, have already been discussed. Two of the metabolic modifications to curcumin, glucoronidation and sulfation, occur at the 4-OH positions.\textsuperscript{597,598,601} It has been previously shown that the protection of the alcohol groups via methylation improves the stability of the compounds.\textsuperscript{600} Also, sulfamate derivatives of numerous steroids, including estradiol, have been characterized by improved absorption, which has led to increased activity.\textsuperscript{614,615}

FLLLs 1, 9, 10 and 17S were synthesized with this in mind. FLLLs 1 and 17S are sulfamate derivatives, FLLL10 is a methoxy derivative, and FLLL9 is an acetate derivative. All of these compounds exhibited significantly greater activity than curcumin. FLLL18S, which is also a sulfamate derivative but lacks a free hydroxyl group, also exhibited two- to four-fold more activity than curcumin. FLLLs 21 and 2 are the mono-protected versions of FLLLs 1 and 10. In general, they exhibited activities between those of curcumin and the fully protected analogues. Also, the methoxy and sulfamate derivatives exhibit similar activity, but display increased activity when compared to the acetate derivative, FLLL9.

The activities of the pentadienone analogues are shown in Table 11. All of the analogues exhibited potent antiproliferative activities, achieving IC\textsubscript{50} values of less than 10 µM in all cancer cell lines. Notably, FLLLs 12S and 22 achieved sub-micromolar IC\textsubscript{50} values in certain cell lines. All of the analogues were 2 to 50 times more potent than curcumin.
FLLL11 is the monocarbonyl version of curcumin. It is roughly five to eight times more potent than curcumin, and offers a good comparison between the two series of compounds. However, unlike the heptadienedione series, when the methoxy and hydroxyl substituents are exchanged in the pentadienone series there is no significant change in activity (FLLL20). The addition or elimination of a methoxy group was also tolerated (FLLLs 12 and 16).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL11</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>FLLL11S</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>FLLL12</td>
<td>3.6 ± 1.3</td>
</tr>
<tr>
<td>FLLL12S</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>FLLL13</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>FLLL14</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>FLLL16</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td>FLLL16S</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>FLLL20</td>
<td>5.9 ± 0.9</td>
</tr>
<tr>
<td>FLLL22</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>FLLL23</td>
<td>4.6 ± 0.2</td>
</tr>
</tbody>
</table>

Table 11. Antiproliferative activities of the pentadienone series of curcumin analogues. (The majority of these values were obtained by Dr. Bulbul Pandit.)

It is also interesting that while protecting the hydroxyl groups in the heptadienedione series had a significant impact on activities, doing so in the pentadienone series did not greatly alter the activities (FLLLs 11S, 12S, 14 and 16S). All of this suggests that the two series of compounds might be acting through different mechanisms or undergoing different metabolic pathways.
As previously mentioned, compounds were also tested in MCF10A cells in order to determine their selectivities. As shown in Table 12, the synthesized curcumin analogues were significantly more selective than curcumin. Curcumin exhibited only a slightly higher IC$_{50}$ in MCF10A cells than in cancer cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF10A</th>
<th>PC3</th>
<th>LNCaP</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>30.1 ± 3.7</td>
<td>19.8 ± 2.1</td>
<td>19.6 ± 3.7</td>
<td>21.5 ± 4.7</td>
<td>28.4 ± 4.3</td>
</tr>
<tr>
<td>FLLL7</td>
<td>&gt;50</td>
<td>5.6 ± 2.0</td>
<td>3.4 ± 0.9</td>
<td>5.9 ± 0.6</td>
<td>6.6 ± 1.9</td>
</tr>
<tr>
<td>FLLL10</td>
<td>31.5 ± 7.8</td>
<td>5.9 ± 1.3</td>
<td>3.9 ± 0.6</td>
<td>5.4 ± 0.8</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>FLLL13</td>
<td>&gt;50</td>
<td>2.5 ± 0.5</td>
<td>2.1 ± 0.9</td>
<td>2.7 ± 0.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>FLLL22</td>
<td>&gt;50</td>
<td>2.1 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>FLLL23</td>
<td>&gt;50</td>
<td>4.6 ± 0.2</td>
<td>1.7 ± 0.6</td>
<td>2.4 ± 1.0</td>
<td>2.4 ± 0.4</td>
</tr>
</tbody>
</table>

Table 12. Antiproliferative activities of selected analogues in cancer-derived and non-cancer-derived cell lines. (The majority of the values were obtained by Dr. Bulbul Pandit.)

Selectivity ratios were calculated by dividing the IC$_{50}$ values in MCF10A cells by the lowest IC$_{50}$ values that were observed in the cancer cell lines. FLLLS 7, 10, 13, 22 and 23 exhibited selectivity ratios of at least five-fold. On top of being the most potent analogue, FLLL22 was also found to be the most selective analogue that was evaluated.

4.4 Summary of Initial SAR Studies

In summary, two series of compounds consisting of a total of 24 analogues were synthesized and evaluated in cancerous and non-cancerous cells. Evaluated compounds
were found to be potent and selective. FLLL22 was the most promising analogue, as it was the most potent and most selective of the compounds evaluated.

In these studies the monocarbonyl analogues were generally more active than the diketone analogues. However, it cannot be assumed that compounds from the two series are operating through the same mechanism. In support of this are the findings that eliminating the 4-hydroxyl groups, exchanging the hydroxyl and methoxy substituents, inserting an additional methoxy group, or protecting the alcohols had a significant impact on the resulting activities of the diketone analogues, while for the monocarbonyl series these alterations had little impact.

The results from the SAR studies of the heptadienedione series offer some interesting insight. While elimination of the 3-methoxy substituents was tolerated, the elimination of the 4-hydroxyl substituents resulted in decreased activity. A decrease in activity was also observed upon the addition of a third aromatic substituent. Also, exchanging the methoxy and hydroxyl substituents resulted in the elimination of activity. In contrast, protecting the hydroxyl substituents, especially as methoxy or sulfamate analogues, drastically increased the activity of the compounds.

The fact that these studies confirmed that curcumin analogues could be both potent in and selective for cancer cells was promising. These data encouraged the development of additional analogues that were aimed at a specific target, STAT3. The findings from the SAR studies provided general rules to follow in order to develop potent compounds.
Chapter 5: The STAT3 Fluorescence Polarization Assay

5.1 Introduction

Extensive work has been performed with the STAT3 FP assay in the search for STAT3 inhibitors. The FP assay will be discussed here, since the majority of the remaining chapters will include FP data. The FP assay is critical for the validation of STAT3 SH2 domain inhibitors. While Western blots, DNA-binding assays and others can demonstrate the down-regulation of the STAT3 pathway, they are unable to directly confirm the mechanism of action (MOA) of STAT3 inhibition. In contrast, the FP assay is able to demonstrate direct SH2 domain binding.

5.1.1 Background

In 1999, Haan and colleagues reported the binding of the isolated STAT3 SH2 domain to various phosphopeptides using an enzyme-linked immunosorbent assay (ELISA). The phosphopeptide sequences used were based on the GP130 and LIFR tyrosine motifs, as well as the sequence surrounding STAT3 at Tyr. At a pH of 7.5, complex formation was not observed. The suggested reasoning for this is that at that pH, STAT3 dimerization was occurring prior to the binding of the phosphopeptides to the SH2 domain. At a pH of 5.5, binding was observed. However, the determination of binding constants at pH 5.5 might be unreliable due to the acidic
conditions, and the possible effects that they might have on the conformation of the protein.\textsuperscript{539,616}

In 2001, Turkson and colleagues reported on measuring the ability of part of the sequence of the STAT3 pTyr motif that binds to the STAT3 SH2 domain in dimer formation, PpYLKTK, to inhibit STAT3 dimerization via EMSA analysis.\textsuperscript{507} This sequence is a shorter version of one of the sequences analyzed by Haan and coworkers.\textsuperscript{507,616} Turkson and colleagues were able to confirm that PpYLKTK was able to disrupt STAT3 dimer formation, while the unphosphorylated peptide, PYLKTK, had no effect.\textsuperscript{507} They also found that acetylating the N-terminus of the phosphopeptide slightly improved binding, and they suggested that this was due to the ability of the acetyl group to mask the positive charge of the N-terminal amino group.\textsuperscript{507} Importantly, they also found that while the Y-1 position was essential, it was not necessary for the identity of this residue to be Pro.\textsuperscript{507} It should be noted that acetylating the N-terminus was found to be critical in peptides where Tyr was N-terminal, but much less critical if an additional residue was present at the Y-1 position.\textsuperscript{507}

As was briefly touched upon in the section on targeting STAT3, in 2003, McMurray and colleagues reported on the abilities of pTyr-containing hexapeptides to inhibit STAT3 dimerization and DNA binding.\textsuperscript{539} Their hexapeptide sequences were derived from the known STAT3 docking sites of EGFR, LIFR, G-CSFR, IL-10R and GP130.\textsuperscript{539} They also analyzed the STAT3-derived hexapeptide, pYLKTKF.\textsuperscript{539} All of the peptides that they analyzed had been acetylated at the N-terminus and were C-terminal amides.\textsuperscript{539} Their analysis was also performed using an EMSA.\textsuperscript{539}
Their hexapeptides achieved IC$_{50}$ values of 0.15 to 150 µM. An EGFR-based sequence exhibited the weakest binding, while both an LIFR- and GP130-based sequence exhibited sub-micromolar IC$_{50}$ values. The sequence surrounding Tyr$^{904}$ of GP130, pYLPQTV, was superior to all other hexapeptides analyzed, achieving an IC$_{50}$ of 0.15 µM. It exhibited activity that was more than 100 times superior to that of the STAT3-based peptide, pYLKTKF, which only achieved an IC$_{50}$ of 20 µM.

In 2004, Schust and Berg reported the development of a high-throughput FP assay for STAT3. Gly had previously been successfully utilized as a spacer for the N-terminal labeling of SH2 domain-binding peptides in STAT1/STAT6 and Src FP assays. This inspired them to design the phosphoheptapeptide, 5-carboxyfluorescein (5-FAM)-GpYLPQTV-NH$_2$, which they used as a probe. 5-FAM-GpYLPQTV-NH$_2$ bound to STAT3 with a K$_d$ of 150 ± 10 nM at a 50 mM NaCl concentration when 10 nM of probe was used.

They found that increasing the NaCl concentration resulted in a decrease in binding affinity; at concentrations of 100, 150 and 200 mM NaCl the K$_d$ values increased to 190 ± 20, 310 ± 30 and 430 ± 50 nM, respectively. They also found that the addition of DMSO resulted in a slight decrease in the K$_d$ values when the salt concentration was fixed at 50 mM. The presence of 5, 10 and 15% DMSO resulted in the K$_d$ values decreasing by 1, 11 and 19%, respectively.

Similar to the results of McMurray and colleagues, Schust and Berg found that the native STAT3 Tyr$^{705}$ version of the peptide, 5-FAM-PpYLKTKFI, only bound weakly to STAT3, and that the shorter Pro to Gly substituted peptide, 5-FAM-GpYLKTKF, did not
significantly bind to STAT3 either.\textsuperscript{617} Obviously, since STAT3 does dimerize, it would be illogical to conclude that the native STAT3 Tyr\textsuperscript{705} motif is unable to bind to the SH2 domain. The more plausible explanation is that the GP130-based heptapeptide contains the residues that are necessary to bind to STAT3, while the STAT3-based heptapeptide lacks certain residues that are critical for binding. In support of this conclusion, when Haan and colleagues used much longer sequences that contained additional N-terminal residues, they found that the STAT3 SH2 domain bound similarly to their probes that were GP130 pY767-, pY814-, pY905-, pY915-, or STAT3 pY705-based, while the GP130 pY759-based peptide, which is known to bind to the SH2 domain of SHP2, showed only weak binding at a pH of 5.5.\textsuperscript{616} When a mutation was introduced at position 770 of the GP130 pY767-based peptide, the binding was greatly reduced,\textsuperscript{616} which further supports this conclusion.

Regardless, Schust and Berg created a useful tool for analyzing the ability of inhibitors to bind to the STAT3 SH2 domain. They successfully demonstrated that when 150 nM STAT3 and 10 nM 5-FAM-GpYLPQTV-NH\textsubscript{2} were used, competitor peptides, such as Ac-pYLPQTV-NH\textsubscript{2} and pYLPQTV-NH\textsubscript{2}, could inhibit the FP signal in a dose-dependent manner.\textsuperscript{617} Importantly, they also demonstrated that the unphosphorylated version, Ac-YLPQTV-NH\textsubscript{2}, was unable to do so.\textsuperscript{617}

\textit{5.1.2 Variations to the STAT3 FP Assay}

Since its development, a number of minor variations to the STAT3 FP assay have been utilized. As previously stated, Schust and Berg used constant concentrations of 150
nM STAT3 and 10 nM probe in determining the IC$_{50}$ values of SH2 domain inhibitors.$^{617}$ These concentrations have been used in a number of other studies.$^{551,553,558,621}$ General incubation times of STAT3 with probe range from 15 to 45 minutes,$^{551,553,558,617,621,622}$ although test compounds have also been incubated with STAT3 for 60 minutes prior to the addition of probe.$^{553,551,558}$ Schust and Berg also showed that the K$_d$ only changes slightly over a 24 hour period, which removes time constraints.$^{617}$ Incubations have regularly been performed at room temperature,$^{617,621,622,623}$ but they have also been performed at multiple temperatures (22, 30 and 37°C).$^{553}$ In some instances, test compounds were incubated with STAT3 at 30°C prior to the addition of probe at room temperature.$^{551,558}$ The K$_i$ values have been calculated according to the equation, K$_i$ = IC$_{50}$/(1 + [STAT3]/K$_d$).$^{551,621}$

The general FP buffer conditions are pH 7.4-7.6, 50 mM NaCl, 10 mM HEPES, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 0.1% Nonidet P-40 (NP-40).$^{617,622}$ Variations to this, such as substituting EDTA with Na$_4$EDTA,$^{623}$ using 1% NP-40,$^{623}$ adding 5% DMSO,$^{621}$ adding 10% DMSO,$^{553}$ and removing DTT,$^{553,558}$ have also been reported. DTT was removed from the buffer because it was found to react with Stattic in a nucleophilic fashion, which led to an observed reduction in Stattic’s activity.$^{553}$ Removing DTT did not have a significant effect on the performance of the FP assay.$^{553}$

Different probes have also been used in the FP assay, although the probe of Schust and Berg, 5-FAM-GpYLPQTV-NH$_2$, has been most commonly used.$^{551,553,558,617,621}$ McMurray and colleagues have used the probe, 5-FAM-ApYLPQTV-NH$_2$, at a 10 nM final concentration, and STAT3 at a final concentration of
80 nM in their FP assay.\textsuperscript{623} Wang and colleagues have used 5-FAM-β(Ala)-β(Ala)-GpYLPQTV as a probe.\textsuperscript{548} Lastly, Hao and colleagues have used 5-FAM-SpYLPQTV as a probe, and at a concentration of 3 nM.\textsuperscript{622}

5.2 Initial Utilization of the STAT3 FP Assay

Before proceeding, is should be mentioned that the majority of the data presented in this section was originally published in PLoS ONE. Bill, M. A.; Nicholas, C.; Mace, T. A.; Etter, J. P.; Li, C.; Schwartz, E. B.; Fuchs, J. R.; Young, G. S.; Lin, L.; Lin, J.; He, L.; Phelps, M.; Li, P.-K.; Lesinski, G. B. PLoS ONE. 2012; 7:e40724.\textsuperscript{624}

5.2.1 Initial Optimization of Fluorescent Probe Concentrations

In order to determine the optimal concentration of fluorescent probe, the labeled peptide, 5-FAM-SpYLPQTV, was diluted to concentrations of 0.5 to 15 nM in FP buffer. Fluorescence and fluorescence polarization readings were taken to find the optimal concentration of probe to be used in binding assays (\textit{Figures 54 and 55}).\textsuperscript{625} The fluorescence readings indicated a 10-fold increase in intensity (tracer/background) at a concentration of 4 nM 5-FAM-SpYLPQTV. This indicated that the probe’s signal was 10 times greater than the background at this concentration. Fluorescence polarization readings (in millipolarization units (mP)) also indicated that at this concentration the signal of the probe was constant. Furthermore, Hao and colleagues had previously used a concentration of 3 nM when utilizing the same fluorescent peptide.\textsuperscript{622} As a result, 4 nM 5-FAM-SpYLPQTV was used for binding experiments.
Figure 54. Fold increase in fluorescence intensity based on fluorescence measurements at various concentrations of 5-FAM-SpYLPQTV.

Figure 55. Fluorescence polarization readings at various concentrations of 5-FAM-SpYLPQTV.
5.2.2 Initial Validation of the STAT3 FP Assay

Two standard saturation curves were run in order to verify that the assay was functional. The FP values were recorded 45 to 90 minutes after increasing concentrations of STAT3 (final concentrations of 0 to 1000 nM) were incubated with 5-FAM-SpYLPQTV (final concentration of 4 nM) when 8 nM of the competitor peptide, SpYLPQTV, was or was not present (Figure 56). The specific binding is shown, and was defined as the contribution to the signal from the labeled bound ligand.\(^{625}\)

![saturation curves](image)

**Figure 56.** Saturation curves demonstrating the differences in mP when STAT3 and 5-FAM-SpYLPQTV were incubated in the presence or absence of SpYLPQTV.

As can be seen, a pronounced right-handed shift was observed when 8 nM of the competitor peptide, SpYLPQTV, was present. Scatchard plot analysis was utilized to determine the \(K_d\) values for the two curves (Figure 57). In the absence of SpYLPQTV,
the $K_d$ was determined to be 163.7 nM, which is similar to the value of $150 \pm 10$ nM that Schust and Berg observed for their fluorescent probe, GpYLPQTV-NH$_2$.\textsuperscript{617} When 8 nM SpYLPQTV was present in the mixture, the $K_d$ shifted to 823.0 nM. SpYLPQTV is able to directly compete for binding to the STAT3 SH2 domain. Such a large shift is expected, since the unlabeled phosphorylated peptide is expected to have a similar binding affinity to STAT3 as that of the labeled peptide. This shift demonstrated that the assay was functional.

![Scatchard plot](image)

**Figure 57. Scatchard plot of the data obtained from the saturation curves.**

Scatchard plot analysis was also used to obtain the $B_{max}$ values, which were determined to be 191.0 and 194.8 mP in the absence and presence of 8 nM SpYLPQTV, respectively. Similar values are expected, since SpYLPQTV is expected to inhibit 5-FAM-SpYLPQTV in a competitive manner. Hill plots were also constructed, and the
resulting slopes were found to be 0.9985 and 1.001 (Figure 58). Non-cooperative binding is identified by a Hill slope of 1. The obtained data was consistent with this, demonstrating that the binding of the probe was non-cooperative, as expected.

![Hill plot of the data obtained from the saturation curves.](image)

**Figure 58.** Hill plot of the data obtained from the saturation curves.

### 5.3 Altered Utilization of the STAT3 FP Assay

#### 5.3.1 Rationale for Altering the STAT3 FP Assay Conditions

In these initial experiments, after validating the functionality of the STAT3 FP assay, the competitive efficiencies of FLLL32 and FLLL62 at 50 µM were evaluated by running saturation curves in the absence or presence of either FLLL32 or FLLL62, and observing the shift in the $K_d$. The methods for these experiments are described later in the experimental section, and the data will be presented in the next chapter on FLLLs 31,
and 62. However, the discussion of the advantages to this method, and why it was necessary to alter the conditions of the assay and the way in which compounds were evaluated, will be discussed here.

There are several advantages to performing experiments in the manner described above. As it will be discussed in more detail later, using a fixed concentration of inhibitor is a practical way to deal with solubility issues. Compounds, such as FLLL32, are not soluble in aqueous solutions at concentrations that would be needed in order to observe a greater than 50% inhibition of the FP signal. As a result, IC$_{50}$ and K$_i$ values are not able to be determined for these compounds in this assay. Using a fixed concentration of inhibitor and varying concentrations of STAT3 allows one to gather multiple data points for compounds, determine the shift in K$_d$ values, and make direct comparisons between inhibitors at a given concentration. This method also allows for other values, such as B$_{max}$ and the Hill slope, to be compared as well.

However, there are numerous disadvantages to this method. The first is that this method for performing the FP assay is not commonly employed, which makes it difficult to compare results with others that have been published. The STAT3 protein is also costly (currently $574 for 50 µg), and using varying concentrations of STAT3, including concentrations of up to or over 1000 nM, becomes extremely expensive in a short amount of time.

Ideally, IC$_{50}$ and K$_i$ values should be derived, as these values are the most practical ones to compare with other reported data. These values are derived by first determining the K$_d$ for fluorescent ligand/protein binding, and then holding the
concentrations of both fluorescent ligand and protein fixed, while varying the concentration of inhibitor. This method is also advantageous from a cost point of view, since after initially obtaining the $K_d$, the concentration of protein can be held at concentrations that are much lower than the maximal concentrations used to obtain saturation curves. Even if, due to solubility issues, IC$_{50}$ and $K_i$ values are not able to be determined and derived, the percent inhibition of the FP signal can be calculated and comparisons can be made.

5.3.2 Validation of the STAT3 FP Assay Using Altered Conditions

As it has been mentioned, 10 nM of the fluorescent probe, 5-FAM-GpYLPQTV-NH$_2$, has been most commonly utilized in STAT3 FP assays.$^{551,553,558,617,621}$ This probe is close to identical in structure to the probe used in the FP assays reported in this document, 5-FAM-SpYLPQTV. As a result, this concentration of probe was used for all additional experiments, in the hopes of obtaining data that was able to be compared to other reports.

Schust and Berg had used up to 15% DMSO in their FP buffer in their initial report,$^{617}$ and 10% DMSO was used in evaluating Stattic.$^{553}$ In an attempt to increase the solubility of test compounds in FP buffer, 10% DMSO was used as a component of the buffer in all remaining FP experiments.

As it has been mentioned, DTT has also been removed from the FP buffer,$^{553,558}$ since it was found to react with Stattic.$^{553}$ Importantly, the removal of DTT did not affect the functionality of the FP assay.$^{553}$ Since the presence or absence of DTT was shown to
not impact the FP assay, and since other compounds could also potentially react with DTT in a similar fashion, DTT was also removed from the buffer.

Before proceeding, it should be mentioned that the majority of the data presented in the remainder of this section was initially published in Bioorganic & Medicinal Chemistry. Bhasin, D.; Chettiar, S. N.; Etter, J. P.; Mok, M.; Li, P.-K. Bioorganic & Medicinal Chemistry. 2013; 21:4662-9. © Elsevier Ltd.

Saturation curves were obtained by incubating increasing concentrations of STAT3 (0 to 1200 nM) in the presence of 10 nM 5-FAM-SpYLPQTV (Figure 59), which is similar to the methods of Turkson and coworkers. The $K_d$ was derived to be 136 nM. This value is similar to the value of 150 nM, which was reported by Schust and Berg when using 5-FAM-GpYLPQTV-NH$_2$ as a probe. The higher-affinity binding of 5-FAM-SpYLPQTV that was observed was expected, as an 11% decrease in $K_d$ has been reported in the presence of 10% DMSO.

At fixed concentrations of 150 nM STAT3 and 10 nM 5-FAM-SpYLPQTV, the competitor peptide, SpYLPQTV, showed greater than 90% inhibition of the fluorescence polarization signal at concentrations as low as 2 µM (Figure 60). The IC$_{50}$ was derived to be 329 nM. This is in line with a similar competitor peptide, GpYLPQTV-NH$_2$, which has been found to have an IC$_{50}$ of 300 nM.

In contrast, the unlabeled and non-phosphorylated counterpart, SYLPQTV, only inhibited the FP signal by $8.73 \pm 4.36\%$ at a concentration of 100 µM. This small amount of inhibition resulting from the presence of SYLPQTV was presumably due to non-specific binding. Together, these data demonstrated that the phosphorylated competitor
peptide was able to inhibit the binding of the fluorescent probe to the STAT3 SH2 domain, while the un-phosphorylated counterpart was not. These data also demonstrated that the STAT3 FP assay was functional.

Figure 59. Specific binding when increasing concentrations of STAT3 were incubated with 10 nM 5-FAM-5pYLpQTV.
5.4 Inhibitory Activities of P1-P17 and STAT3 Decoys

Numerous compounds have been evaluated in the STAT3 FP assay. The majority of this data will be presented in the remaining chapters of this document, where the FP data will be presented along with the additional biological data. However, for some compounds evaluated in the STAT3 FP assay the other data, actual compound identification numbers and structures will not be shown out of respect for a collaborative and ongoing research project (P1-P17). The FP data for the STAT3 decoys will also be discussed in this section, since it will not be discussed elsewhere in this document.
5.4.1 Inhibitory Activities of P1-P12

Initially, 12 compounds were evaluated in the FP assay. Compounds were initially evaluated at a single concentration of 500 µM to identify compounds that had the ability to bind to the STAT3 SH2 domain (Figure 61). As can be seen, three compounds, P1, P6 and P8, inhibited the FP signal by greater than 50% at this concentration. P1 showed the highest inhibitory activity, achieving greater than 90% inhibition of the FP signal. P3 also exhibited decent potency, achieving 41.0 ± 1.2% inhibition.

![Figure 61. Inhibitory activities of P1-P12 (500 µM) and SpYLPQTV (100 µM) in the STAT3 FP assay.](chart.png)
Since P1, P3, P6 and P8 all demonstrated activity, they were further evaluated over a range of 500 to 1.95 µM. Unfortunately, the inhibitory activities of P3, P6 and P8 dropped rapidly. At a concentration of 250 µM, none of them inhibited the FP signal by greater than 25%. In contrast, P1 maintained activity at lower concentrations (Figure 62). P1 was found to have an IC$_{50}$ of 73.9 ± 7.1 µM, and a K$_i$ of 34.9 ± 3.5 µM.

Figure 62. Inhibitory activities of P1 in the STAT3 FP assay.

Two issues were encountered in the evaluation of these compounds: low solubility in the FP buffer and high FP readings resulting from the presence of only compound and probe. P2 was not entirely soluble at 500 µM. P7 also had significant solubility issues, as did P4, P5, P6 and P12, although to a lesser extent. The presence of P6 at high concentrations along with the probe also resulted in anomalous and high FP readings, even in the absence of STAT3. This was an issue for P1 and P10 as well,
although to a lesser extent. As it will be reiterated throughout the discussion of FP data, these are several key issues that, if problematic enough for the given compound(s), can make data hard to interpret. A lack of solubility in the FP buffer can result in false negatives, and it is not possible to obtain precise and accurate results for compounds that result in high FP readings in the absence of STAT3, since the resulting signal significantly interferes with the FP assay.

5.4.2 Inhibitory Activities of P13-P17

Five additional compounds were later evaluated in the FP assay. They were designated as P13-P17. To provide an idea of how high FP readings resulting from test compounds can be problematic, a chart is shown (Figure 63). As it can be seen on the far left, when only 10 nM 5-FAM-SPYLPQTV was present (in FP buffer), a tracer signal of about 100 mP was observed. When 150 nM STAT3 was also present in the mixture, a signal of close to 250 mP was observed; this represents the maximal reading that will be observed with the specified concentrations of fluorescent probe and STAT3.

When 100 µM of the competitor peptide was also present in the mixture, the readings were relatively the same regardless of whether STAT3 was present or not; this is because the excess of SpYLPQTV was binding to STAT3 and nearly completely inhibiting the binding of the fluorescent probe. In contrast, the presence of the negative control, SYLPQTV, had little effect on the FP readings, since it was not able to significantly bind to STAT3.
The rest of the bars represent the FP readings when only 10 nM 5-FAM-SpYLPQTV and the indicated compounds at the indicated concentrations were present in the mixture. Compounds such as P15 and P16 are fairly ideal for the FP assay. When they were incubated at numerous concentrations with only 5-FAM-SpYLPQTV, the readings were similar to the readings when only 5-FAM-SpYLPQTV was present in the mixture.

![Figure 63. Fluorescence polarization readings resulting from various mixtures of peptides or compounds in buffer.](image)

However, P14 to some extent, P13 to more of an extent, and P17 to the largest extent, interfered with the FP signal at higher concentrations. It should be noted that some
of the compounds evaluated also had trouble going into solution. P13 and P17 were not able to go entirely into solution at a concentration of 500 µM. P14 was less soluble in the FP buffer. At 125 µM or higher, the solutions containing P14 were slightly foggy. P15 and P16 were soluble at all tested concentrations.

This brings up several key issues that are drawbacks to the FP assay. However, it should first be made clear that the obtained FP values are not related to the concentration of fluorophore, but are instead related to the rotational mobility of the fluorophore. The less rotational mobility the fluorophore has, the larger the FP readings will be. This is why a dramatic increase in FP readings is seen as more of the low molecular weight probe is bound to the high molecular weight protein; the rotational mobility of the fluorophore is decreased.

The high FP readings that were observed at higher concentrations of P13 and P17 could possibly be a result of the test compounds forming aggregates in solution, which the fluorescent probe could non-specifically bind. If aggregates of test compounds of high enough (overall) molecular weights are formed, and the probe is able to bind to them, high FP measurements are observed. This is a cited problem, and is particularly a problem at over 100 µM concentrations of certain test compounds. Several compounds have even been observed to form aggregates at concentrations below 100 µM. It should be noted that this does not occur with the majority of test compounds, but this could be an issue for compounds with hydrophobic moieties that prefer to interact with each other in aqueous conditions.
Another problem that can occur in the FP assay is light scattering, which can result from the insolubility of test compounds. In the best case scenario for insoluble compounds, if light scattering is not an issue, the data is not reliable since the compound is not able to go into solution at the indicated concentrations. If light scattering is an issue, abnormally high FP values are often observed, since scattered light can be highly polarized. If the solution is turbid enough, decreased values could also be observed, since the excitation and emission light could be diverted from the normal path of the instrument. Light scattering resulting from insolubility could have also contributed to the readings observed when P13, P14 and P17 were present at concentrations at which they were not entirely soluble.

Lastly, the autofluorescence of test compounds can also interfere with the FP assay. Compounds are generally screened at μM concentrations, while probes are used at nM concentrations. As a result, compounds that are fluorescent, even if they are much less fluorescent than the probe, can often interfere with the assay. Fluorescent compounds can mask the signal resulting from the probe and lead to decreased FP readings. However, if the fluorescent compounds tend to aggregate in solution, then they can also lead to abnormally high FP readings, due to their effectively large sizes and high molecular weights. Still, if light is absorbed in the region where fluorescein is excited by colored compounds, then there will not be too significant of interference, as long as the optical density is equal to or less than 1 OD. The autofluorescence of test compounds can result in extreme complications, as the fluorescent probe, the fluorescent probe bound to protein, the fluorescent compound, and
the fluorescent compound bound to protein can all contribute to the observed FP measurements.

The major drawback, which compounds the problem, is that high concentrations of test compounds are often necessary for FP assays. So if test compounds are not active enough at lower concentrations and interfere with the FP readings at higher concentrations, the FP assay cannot be used to obtain thorough data for them. This problem can be seen in the evaluation of P13 and P17.

P13 and P17 were initially evaluated in the FP assay at 250 and 125 µM respectively, due to the high FP readings that resulted from their presence at the standard initial screening concentration. Since P15 and P16 did not appear to be problematic for the assay, they were screened at 500 µM. P14 was initially screened at 500 µM as well, since it did not appear to be overly-problematic in the assay (Figure 64).
Figure 64. Initial evaluation of P13-P17 in the STAT3 FP assay.

As can be seen, at concentrations that were suitable for the assay, neither P13 nor P17 were able to achieve greater than 50% inhibition of the FP signal. As a result, their IC$_{50}$ values were not able to be determined. Still, their inhibitory activities were respectable relative to a number of other compounds that have been evaluated. At a 500 µM concentration, P15 and P16 achieved greater than 50 and 40% inhibition of the FP signal, respectively, and were evaluated at additional concentrations (Figure 65).
Figure 65. Further evaluation of P15 and P16 in the STAT3 FP assay.

Surprisingly, P16 achieved greater than 50% inhibition in the FP assay at a concentration of 250 µM, when it had failed to do so at a concentration of 500 µM. However, in light of the relatively large amount of error that existed in the initial readings, it is likely that the inhibitory activity of P16 at 500 µM is in actuality equal to or greater than its inhibitory activity at 250 µM. Regardless, it maintained inhibitory activity at lower concentrations, showing the highest level of activity in the FP assay of the five compounds evaluated. In contrast, the inhibitory activity of P15 dropped off dramatically as the concentration was reduced to 250 µM and below.
5.4.3 STAT3 Decoys

The effectiveness of STAT3 ODNs has been discussed in the section on targeting STAT3. The first, and to this point the only human clinical trial targeting STAT3 has recently been concluded, and the results have been published: Sen, M.; Thomas, S. M.; Kim, S.; Yeh, J. I.; Ferris, R. L.; Johnson, J. T.; Duvvuri, U.; Lee, J.; Sahu, N.; Joyce, S.; Freilino, M. L.; Shi, H.; Li, C.; Ly, D.; Rapireddy, S.; Etter, J. P.; Li, P.-K.; Wang, L.; Chiosea, S.; Seethala, R. R.; Gooding, W. E.; Chen, X.; Kaminski, N.; Pandit, K.; Johnson, D. E.; Grandis, J. R. Cancer Discovery. 2012; 2:694-705. © American Association for Cancer Research.527 When the paper was initially under review, a reviewer had asked if the STAT3 decoy ODNs were able to bind to uSTAT3 in addition to pSTAT3. Since the STAT3 FP assay utilizes uSTAT3, it was used to evaluate the ODNs. There was reason to be skeptical about the use of the STAT3 FP assay in evaluating whether or not the decoys could bind STAT3, since the decoys target the DBD, while the STAT3 FP assay is used to determine SH2 domain inhibition. However, the results were intriguing.

The STAT3 decoy and cyclic decoy have similar sequences and structures, with the difference being that the cyclic decoy is cyclized. The cyclic decoy was designed to improve the serum half-life via increased stability.527 The STAT3 decoy mutants differ at one critical nucleotide, and have been shown to not bind to STAT3.522,527 The parental and STAT3 decoys were evaluated in the FP assay (Figure 66).
Intriguingly, the STAT3 decoys were able to significantly inhibit the binding of the probe to the SH2 domain. At a concentration of 500 µM, both the parental STAT3 decoy and mutant were able to significantly inhibit the FP signal, by over 60 and 45%, respectively. When all four ODNs were evaluated at 10 µM, the parental STAT3 decoy still inhibited the FP signal by greater than 40%, the cyclic STAT3 decoy achieved just under 20% inhibition, and the mutants achieved little or no inhibition of the FP signal.

In another assay, phosphorylated STAT3 has been shown to bind the STAT3 decoy and cyclic STAT3 decoy with K_d values of 1.22 x 10^{-7} and 2.52 x 10^{-7} M, respectively. This signifies that the parental decoy has roughly two-fold higher affinity.
to \( p \text{STAT3} \) when compared to the cyclic decoy. Interestingly, the results from the FP assay also indicated that the parental decoy is able to more efficiently inhibit the binding of the fluorescent probe when compared to the cyclic decoy. The inhibitory activity of the mutant at 500 \( \mu \text{M} \) signifies that the single nucleotide substitution may not be enough to completely abrogate binding to \( u \text{STAT3} \). However, at a concentration of 10 \( \mu \text{M} \), the \( \text{STAT3} \) mutant did not inhibit the FP signal, while the parental decoy achieved a substantial degree of inhibition.

These findings are very interesting in light of the recent report that \( u \text{STAT3} \) is able to dimerize and directly bind to M67 dsDNA, which has an identical sequence to the STAT3 decoys at the critical positions.\(^{439} \) It was found that the association between two \( p \text{STAT3} \) molecules is roughly twice as favorable when compared to \( u \text{STAT3} \), but that after association, the abilities of \( p \text{STAT3} \) and \( u \text{STAT3} \) to associate with M67 dsDNA are nearly identical.\(^{439} \)

The results from the FP assay were peculiar at the time, but are fairly logical in light of these recent findings. Previously, \( u \text{STAT3} \) was not thought to be able to bind to dsDNA. However, if the STAT3 used in the FP assay is able to dimerize to some extent, subsequent binding of the decoys could favor the dimerized structure over free STAT3. Then the fluorescent probe might not be able to access the SH2 domain, which could lead to inhibition in the FP assay. More studies will have to be carried out to confirm all of the MOAs of the decoy ODNs, but these findings are intriguing to say the least.
5.5 Future Directions

There are numerous advantages to the FP format. FP assays can be performed quickly, with a degree of simplicity, and for a relatively low cost.\textsuperscript{627} The use of radioisotopes, separation steps or filtration steps are not required for FP assays.\textsuperscript{627} Taking FP readings does not destroy samples, so multiple readings can be taken.\textsuperscript{627} Other methods that can detect molecular interactions without using radioisotopes include two-dimensional NMR (2D NMR) spectroscopy, frontal chromatography, SPR and calorimetry.\textsuperscript{629} However, FP assays are ideal for high-throughput screening (HTS), as they are homogenous add, mix and read assays.\textsuperscript{632}

The major issues that are present in FP assays, and the STAT3 FP assay in particular, have been discussed. In one study, it was found that roughly 2\% of library compounds evaluated were fluorescent at 480 nm excitation/530 nm emission, which are the excitation and emission wavelengths for fluorescein.\textsuperscript{618} In another study, which evaluated a library of 1,280 compounds at 10 \(\mu\)M concentrations, it was found that 19 of the compounds had fluorescent intensities of at least 50\% relative to 1 nM fluorescein.\textsuperscript{632}

Shifting to longer excitation/emission wavelengths decreased the number of fluorescent compounds in both cases.\textsuperscript{618,632} Using longer wavelength (red-shifted) probes such as BODIPY® TMR or the cyanine-based Cy5 could be advantageous in this respect.\textsuperscript{627,630,631,632} The intensity of scattered light also decreases with wavelength, and using red-shifted probes would eliminate some of the interference resulting from light scattering.\textsuperscript{627,630,631,632} However, the drawbacks to red-shifted probes, such as Cy5, are their high costs and generally smaller shifts in polarization, which lead to smaller assay
Regardless, the peptide used as the STAT3 probe could be labeled with a red-shifted tag, which could eliminate some of the interference in the FP assay.

Since many of the compounds tested are not entirely soluble in the FP buffer, it would also be preferable to change the composition of the buffer in order to make test compounds more soluble. If compounds were soluble in the FP buffer, even at high concentrations, then the true abilities of the compounds to bind to the STAT3 SH2 domain could be more accurately determined. Albumin has been utilized as a drug carrier, and has been shown to increase the solubility of curcuminoids. Curcuminoids have also been shown to have increased solubility in serum. These findings are of particular interest, since many of the compounds that have been evaluated in the assay are curcumin-related. Finding the appropriate concentrations of such ingredients that could be used in the FP assay to increase solubility of test compounds without interfering with the assay would be ideal.

STAT1 and STAT5 FP assays have also been utilized, and it would be beneficial to evaluate all test compounds for inhibitory activities against all three STAT proteins (STATs 1, 3 and 5) in order to determine selectivity. Also, the fact that the GP130-based peptides, pYLPQTV and 5-FAM-GpYLPQTV-NH₂, have been shown to bind with higher affinity to STAT3 than the STAT3 Tyr⁻⁷⁰⁵-based peptides, pYLKTKF and 5-FAM-PpYLKTKFI, has already been discussed. However, as mentioned, the longer versions of the peptides were found to bind similarly to STAT3 at a pH of 5.5. Since one of the goals in developing STAT3 SH2 domain binders is to identify inhibitors that block STAT3 dimerization, it would be logical to perform binding assays with the
probe that is able to most closely mimic STAT3 dimerization. Perhaps a longer version of the STAT3 Tyr\textsuperscript{705}-based peptide could have a similar or improved binding affinity when compared to the GP130-based peptides. While the probes containing the sequence pYLPQTV undoubtedly bind the STAT3 SH2 domain, using a STAT3 Tyr\textsuperscript{705}-based probe in a secondary assay could further confirm that the STAT3 SH2 domain inhibitors are able to block dimerization.

While all of these ideas are intriguing and might lead to substantial improvements to the assay, STAT3 will first need to be cloned, expressed in cells and purified, in-house, so that an ample of amount of STAT3 protein is available, and its cost becomes irrelevant. This has been previously achieved.\textsuperscript{558,617} Unfortunately, in the initial attempt to do so, the resulting STAT3 protein that was obtained was not able to bind to the probe. Presumably, too short of a sequence was expressed and purified, and it did not fold in the same manner as native STAT3, which led to a lack of binding. Once this hurdle is jumped over, all of the other ideas for improving the assay can be attempted. However, until that time comes, the STAT3 protein is simply too costly to use in attempting to improve the assay in ways that may not end up being successful.
Chapter 6: The Story of FLLLs 31, 32 and 62

6.1 Introduction

Aggarwal and colleagues were the first to show that curcumin was able to inhibit the IL-6-mediated phosphorylation of STAT3.\textsuperscript{564} However, as it has been discussed, curcumin has activity against a number of targets. Discovering curcumin analogues that specifically and potently inhibit the JAK2/STAT3 pathway, with a particular emphasis on STAT3, has been a primary goal.

There have been well over 100 curcumin-derived compounds that have been synthesized in the laboratories of Dr. Tom Li and Dr. James Fuchs. Out of the many compounds that have been synthesized, there have been several that have exhibited vast improvements in one regard or another, and they have directed the future directions that were taken in order to continuously improve the potency and pharmacokinetic profiles of the curcumin-derived STAT3 inhibitors. Three of those compounds (FLLL31, FLLL32 and FLLL62) will be discussed in this chapter, and the story of their development will be described. It should be mentioned that much of the data presented in this chapter concerning FLLLs 32 and 62 was originally published in PLoS ONE. Bill, M. A.; Nicholas, C.; Mace, T. A.; Etter, J. P.; Li, C.; Schwartz, E. B.; Fuchs, J. R.; Young, G. S.; Lin, L.; Lin, J.; He, L.; Phelps, M.; Li, P.-K.; Lesinski, G. B. \textit{PLoS ONE}. 2012; 7:e40724.\textsuperscript{624}
6.2 Alkylating the Central Carbon of Curcumin (FLLLs 31 and 32)

As it was mentioned in chapter 4, protecting the hydroxyl substituents was found to greatly improve the potency of the curcumin analogues.\(^{574}\) That finding helped to guide future synthetic efforts, and the majority of the potent curcumin analogues that have been developed do not contain a free hydroxyl substituent, although a number of compounds containing a free hydroxyl group have been synthesized in order to determine SARs. As a result, it will be seen that the three potent compounds presented in this chapter all contain methoxy substituents at both the 3 and 4 positions. Protecting the hydroxyl substituents as methoxy groups is logical in light of the fact that dimethoxycurcumin, which contains the 3,4-dimethoxy substituents, has been shown to be superior to curcumin in regards to achieved plasma concentrations, stability, and potency in cancer cells.\(^{574,612,636}\)

6.2.1 Computational Modeling of Curcumin and Alkylated Analogues

Computational modeling guided the next significant improvement that was to be made. The two forms of curcumin and analogues which feature substitutions at the central carbon of curcumin were docked into the ATP binding site of JAK2 and the SH2 domain of STAT3. The predicted binding energies were calculated (Table 13).

For JAK2, both the diketone and keto-enol forms of curcumin are predicted to bind to the ATP binding site, although with different orientations. A carbonyl of the diketone form of curcumin is predicted to interact with the oxyanion hole, while the keto-enol form extends further into the pocket due to its longer conformation. The alkylated versions of curcumin are restricted to the diketone form, since the central carbon no
longer contains protons and tautomerization can no longer occur. As can be seen, with the exception of the dibutyl analogue, all alkylated curcumin analogues are predicted to bind with equal or improved energetics relative to curcumin.

Table 13. Predicted binding energies of curcumin and alkylated derivatives to JAK2 and STAT3. (This work was performed by Drs. Katryna Cisek and Chenglong Li.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Predicted Binding Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin (keto-)</td>
<td>JAK2: -9.4  STAT3: -8.1</td>
</tr>
<tr>
<td>Curcumin (enol-)</td>
<td>JAK2: -9.5  STAT3: No Binding</td>
</tr>
<tr>
<td>R,R, (dimethyl)</td>
<td>JAK2: -9.6  STAT3: -8.1</td>
</tr>
<tr>
<td>R,R, (dibutyl)</td>
<td>JAK2: No Binding  STAT3: No Binding</td>
</tr>
<tr>
<td>R,R, (cyclopropyl)</td>
<td>JAK2: -9.5  STAT3: -7.6</td>
</tr>
<tr>
<td>R,R, (cyclopentyl)</td>
<td>JAK2: -10.3 STAT3: -8.0</td>
</tr>
<tr>
<td>R,R, (cyclohexyl)</td>
<td>JAK2: -10.3 STAT3: -8.5</td>
</tr>
</tbody>
</table>

Concerning binding to the STAT3 SH2 domain, it is clear that the diketone forms of curcumin and derivatives are favored over the keto-enol forms. The keto-enol form of curcumin is not even predicted to bind to the SH2 domain. This is because the planar structure of curcumin does not allow it to obtain the “bent” conformation that is necessary for binding to the Tyr\(^{705}\) site and hydrophobic side pocket. This is unfortunate in relation to its ability to bind STAT3 in actuality, since as it has been discussed, the keto-enol form is the predominant one.\(^{574,577,578,579}\)
As can be seen, the dimethyl- and cyclohexyl-containing derivatives are predicted to bind to the SH2 domain with equal or higher affinity when compared to the diketone form of curcumin. The cyclohexyl-containing derivative is predicted to display the most improved binding. This is because the cyclohexyl moiety is predicted to interact with the Leu\textsuperscript{706} site in addition to the Tyr\textsuperscript{705} site and hydrophobic side pocket of the SH2 domain. Curcumin is unable to do so.

The docking of the diketone form of curcumin and the cyclohexyl-containing derivative, FLLL32, into the ATP binding site of JAK2 (left) and the SH2 domain of STAT3 (right) is shown (Figure 67). The binding modes to both JAK2 and STAT3 are different for curcumin and FLLL32. For STAT3, the two aromatic groups of curcumin prefer to interact with the Tyr\textsuperscript{705} site and the hydrophobic pocket. In contrast, the two aromatic groups of FLLL32 are predicted to interact with the Tyr\textsuperscript{705} and Leu\textsuperscript{706} sites, while the cyclohexyl moiety is predicted to interact with the hydrophobic pocket.

6.2.2 Synthesis of Alkylated Analogues

Unalkylated curcumin analogues were first synthesized via aldol condensation between 2,4-pentanedione and 3,4-dimethoxybenzaldehyde, using the procedure established by Venkateswarlu and colleagues\textsuperscript{573} as it was discussed in chapter 4. The alkylation was then performed by first deprotonating the central carbon with K\textsubscript{2}CO\textsubscript{3}. Following deprotonation, the specified monoiodoalkanes or diiodoalkanes were added to the reaction mixture and allowed to react overnight at room temperature, in an S\textsubscript{N}2 reaction (Scheme 6).
Figure 67. Binding modes of curcumin and FLLL32 to JAK2 (left) and STAT3 (right). (This work was performed by Drs. Katryna Cisek and Chenglong Li.)

Scheme 6. General procedure for the alkylation of curcumin analogues. (FLLLS 31, 32 and 62 were synthesized by Eric Schwartz in the lab of Dr. James Fuchs.)
6.2.3 Biological Evaluation of FLLL31 and FLLL32

From the computational data, it was decided that the methoxy-protected dimethyl- and cyclohexyl-containing derivatives should first be synthesized and evaluated (FLLLS 31 and 32, **Figure 68**). They were evaluated in several cancer cell lines (**Table 14**). As it can be seen, both FLLL31 and FLLL32 are vastly more potent than curcumin in all cell lines. In DU145, MDA-MB-468 and MDA-MB-231 cells, they were found to be 10- to 20-fold more potent than curcumin. FLLL32 was more active than FLLL31 in DU145 cells, while their activities were similar in MDA-MB-468 and MDA-MB-231 cells.

**Figure 68.** Structures of curcumin, FLLL31 and FLLL32.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DU145</td>
</tr>
<tr>
<td>Curcumin</td>
<td>30.8 ± 1.9</td>
</tr>
<tr>
<td>FLLL31</td>
<td>2.70 ± 0.82</td>
</tr>
<tr>
<td>FLLL32</td>
<td>1.41 ± 0.06</td>
</tr>
</tbody>
</table>

**Table 14.** Activities of curcumin, FLLL31 and FLLL32 in cancer cell lines.
Since FLLLs 31 and 32 were designed to inhibit STAT3, their abilities to inhibit STAT3 DNA binding were evaluated (Figure 69). MDA-MB-231 cells were treated with 10 µM of FLLL31 or FLLL32 for 24 hours and the levels of STAT3 DNA binding were measured. Both FLLL31 and FLLL32 were able to inhibit binding. Roughly 20% binding occurred in treated cells when compared to the DMSO control. It should be noted that curcumin was also evaluated at a concentration of 10 µM, and it also resulted in the reduction of STAT3 DNA binding. However, only a 30% decrease in DNA binding was observed after treatment with curcumin. This data demonstrates that, relative to curcumin, FLLLs 31 and 32 have drastically improved activities against STAT3.

Figure 69. Amount of STAT3 DNA binding in MDA-MB-231 cells following a 24 hour treatment with 10 µM FLLL31 or FLLL32. (This data was obtained in the lab of Dr. Jiayuh Lin.)
In order to determine specificity, STAT1 DNA binding was also determined in MDA-MB-231 cells following a 24 hour treatment with FLLL31, FLLL32 or curcumin (Figure 70). None of the three compounds significantly reduced the DNA binding activity of STAT1. This data demonstrates, as was hoped, that the curcumin analogues FLLL31 and FLLL32 are specific for STAT3 in this regard.

![Figure 70. Amount of STAT1 DNA binding in MDA-MB-231 cells following a 24 hour treatment with 10 μM curcumin, FLLL31 or FLLL32. (This data was obtained in the lab of Dr. Jiayuh Lin.)](image)

To further evaluate the activities of FLLL32, it was tested in a luciferase assay, which evaluated that STAT3-dependent transcriptional activity that occurred in the cells (Figure 71). MDA-MB-231 cells were treated with varying concentrations of FLLL32 for 24 hours before the transcriptional activity was evaluated. Consistent with the results
obtained from the STAT3 DNA binding activity assay, FLLL32 was able to potently inhibit STAT3-dependent transcriptional activity in a dose-dependent manner. At a concentration of just 2.5 µM, FLLL32 was able to reduce the STAT3-dependent transcriptional activity to under 20% relative to control. At a concentration of 5 µM, FLLL32 was able to nearly abolish STAT3-dependent transcription.

![Graph showing STAT3-dependent transcriptional activity in MDA-MB-231 cells following treatment with FLLL32.](image)

**Figure 71.** STAT3-dependent transcriptional activity in MDA-MB-231 cells following treatment with FLLL32. (This data was obtained in the lab of Dr. Jiayuh Lin.)

In addition, FLLL3s 31 and 32 were shown to inhibit JAK2 activity, down-regulate STAT3 phosphorylation, inhibit cell viability and cell invasion, and to act synergistically with doxorubicin. Since its initial evaluation, numerous papers on the STAT3
inhibitory and anti-cancer activities of FLLL32 have been published. 624,638,639,640,641,642,643,644,645,646,647

6.3 Replacement with an Oxygen-Containing Spiro-Tetrahydropyranyl Ring (FLLL62)

The activities of FLLL32 were promising and are continuing to be researched. However, one of the major drawbacks to FLLL32 is its limited aqueous solubility, which limits its therapeutic relevance. FLLL62 was designed in an attempt to increase aqueous solubility. The only difference between FLLL32 and FLLL62 is that FLLL62 contains an ether, while FLLL32 does not (Figure 72). The hope was that FLLL62 would maintain the same potency and specificity of FLLL32.

Figure 72. Structures of FLLL32 and FLLL62.

6.3.1 Computational Modeling of FLLL32 and FLLL62

Computational modeling indicated that the binding modes of FLLL32 and FLLL62 to the STAT3 SH2 domain were nearly identical (Figure 73). Like FLLL32, FLLL62 was predicted to bind to a hydrophobic side pocket in addition to the Tyr\(^705\) and Leu\(^706\) sites. The only minor difference in binding was predicted to occur at the Leu\(^
The binding free energies of FLLLs 32 and 62 were predicted to be -7.9 and -8.0 kcal/mol, respectively.

Figure 73. Binding modes of FLLL32 and FLLL62 to the STAT3 SH2 domain. (This work was performed in the lab of Dr. Chenglong Li and this figure was originally published in PLoS ONE.)

6.3.2 The Improved Solubility Profile of FLLL62

The structural difference between FLLL32 and FLLL62 might seem minor, but the small change was enough to lower the calculated logP by about 1.5 (calculated with ChemDraw Ultra 11.0). To evaluate if FLLL62 did indeed have an improved solubility
profile, increasing concentrations of FLLLs 32 or 62 were added to cell culture media, and the amount of solubilized compound was quantified (Figure 74). It was found that FLLL32 was completely soluble at concentrations that were less than or equal to 5 µM. FLLL62 was completely soluble at concentrations that were less than or equal to 10 µM. At concentrations above those indicated, neither FLLL32 nor FLLL62 went into solution in proportion to the amount of compound that was added. However, in general, FLLL62 was roughly twice as soluble as FLLL32. FLLL62 did have a significantly better solubility profile than FLLL32.

Figure 74. Comparison of the solubility profiles of FLLLs 32 and 62 in cell culture media. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
6.3.3 Biological Evaluation of FLLL62

In a panel of renal cell carcinoma cell lines, FLLLs 32 and 62 were both found to induce apoptosis and to inhibit STAT3 phosphorylation. Following annexin V/propidium iodide staining, flow cytometry was used to quantify the pro-apoptotic effects of FLLLs 32 and 62. Absolute IC\textsubscript{50} values were not achieved in SK-RC-45 or Caki cell lines. However, the compounds were only evaluated at concentrations up to 30 μM. In contrast, both FLLLs 32 and 62 achieved IC\textsubscript{50} values of 4.0 to 5.8 μM in ACHN and SK-RC-54 cells (Figure 75). FLLL62 was more potent in SK-RC-54 cells. It achieved an IC\textsubscript{50} of 4.6 μM, while FLLL32 achieved an IC\textsubscript{50} of 5.8 μM.

![Figure 75. Pro-apoptotic effects of FLLLs 32 and 62 in renal cell carcinoma cell lines. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)](image)

To confirm the pro-apoptotic activity of FLLLs 32 and 62, immunoblots were obtained after incubating the indicated renal cell carcinoma (SK-RC-45, Caki, ACHN and SK-RC-54) or melanoma (A375 and Hs294T) cell lines with FLLLs 32 or 62 at the
indicated concentrations for 24 hours (Figure 76-80). FLLLs 32 and 62 were both found to potently inhibit STAT3 phosphorylation at Tyr$^{705}$ in a dose-dependent manner in all cell lines in which they were evaluated. In all cell lines and in a dose-dependent manner, they also both induced the cleavage of PARP. FLLL32 was also shown to induce the processing of caspase-3. Both of these events are biomarkers for apoptosis. In addition, FLLL32 was shown to dose-dependently inhibit the expression of cyclin D1, a STAT3-regulated protein. Together, these data demonstrate that FLLLs 32 and 62 act as inhibitors of STAT3, and that they are able to induce apoptosis in cancer cell lines.

Figure 76. Immunoblot analysis of PARP, STAT3, cyclin D1 and caspase-3 in SK-RC-45 and SK-RC-54 cell lines following a 24 hour treatment with FLLL32 at the indicated concentrations. C: 20 µM curcumin. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

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Figure 77. Immunoblot analysis of PARP and STAT3 in SK-RC-45 and SK-RC-54 cell lines following a 24 hour treatment with FLLL62 at the indicated concentrations. C: 20 µM curcumin. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

Figure 78. Immunoblot analysis of PARP, STAT3, cyclin D1 and caspase-3 in ACHN and Caki cell lines following a 24 hour treatment with FLLL32 at the indicated concentrations. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
Figure 79. Immunoblot analysis of PARP and STAT3 in ACHN and Caki cell lines following a 24 hour treatment with FLLL62 at the indicated concentrations. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

Figure 80. Immunoblot analysis of PARP and STAT3 in A375 and Hs294T cell lines following a 24 hour treatment with FLLL62 at the indicated concentrations. C: 20 µM curcumin. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
FLLLs 32 and 62 were also evaluated in SK-RC-45 and SK-RC-54 cell lines for their activities against other targets, including p38 MAPK, Akt and ERK. Analyzing the activities of FLLLs 32 and 62 against these targets is critical for determining specificity, especially since curcumin is known to act against them. FLLLs 32 and 62 were fairly specific in this regard, although they did down-regulate pERK at higher concentrations (Figures 81 and 82). This was found to be especially problematic for FLLL62. FLLL32 did not down-regulate pERK in SK-RC-45 cells at the tested concentrations, but it did down-regulate pERK in SK-RC-54 cells at concentrations of 6 µM and above. In contrast, FLLL62 down-regulated pERK in both cell lines. At a concentration of 6 µM, it resulted in almost undetectable levels of pERK in SK-RC-54 cells.

![Figure 81. Immunoblot analysis of p38 MAPK, ERK and Akt in SK-RC-45 and SK-RC-54 cell lines following a 24 hour treatment with FLLL32 at the indicated concentrations. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)](image)

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Figure 82. Immunoblot analysis of P38 MAPK, ERK and Akt in SK-RC-45 and SK-RC-54 cell lines following a 24 hour treatment with FLLL62 at the indicated concentrations. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

To further determine the specificity of the analogues for STAT3 over STAT1, FLLL32, FLLL62 and curcumin were evaluated for their abilities to inhibit IFNγ-induced signaling, which involves STAT1. ACHN or A375 cells were pre-treated with the indicated compounds for 16 hours prior to a 15 minute stimulation with 10 ng/mL IFNγ. In all cases, pSTAT3 was down-regulated (Figures 83 and 84). In contrast, treatment with FLLL32 or FLLL62 did not result in an adverse down-regulation of pSTAT1. Treatment with FLLL32 did not appear to down-regulate pSTAT1 at all, while treatment with FLLL62 only resulted in a slight down-regulation of pSTAT1 in ACHN cells. However, the down-regulation was only apparent when the cells had been treated with 8 µM FLLL62.
Figure 83. Immunoblot analysis of STAT3 and STAT1 in ACHN cells. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

Figure 84. Immunoblot analysis of STAT3 and STAT1 in A375 cells. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
Next, real time polymerase chain reaction (rtPCR) was utilized to determine the effects of FLLLS 32 and 62 on the IFNγ-induced gene expression of interferon-regulatory factor-1 (IRF1) in ACHN cells. This allows for further evaluation of selectivity, as IRF1 transcription has been shown to be activated by the binding of STAT1 homodimers to a GAS element. Cells were pre-treated for one hour with either DMSO (vehicle) or compounds, and then stimulated with either 10 ng/mL IFNγ or PBS (vehicle) for four hours prior to determining the expression of IRF1 via rtPCR. FLLL32 did not affect the expression of IRF1, while curcumin down-regulated its expression in a dose-dependent manner (Figure 85). Unfortunately, FLLL62 also dose-dependently down-regulated the expression of IRF1. This confirmed the immunoblot data, which suggested that FLLL32 is fairly selective for STAT3, and that the structural modification in FLLL62 resulted in a significant loss in selectivity.

FLLLS 32 and 62 were also evaluated for their abilities to inhibit vascular endothelial growth factor (VEGF) secretion in SK-RC-54 and SK-RC-45 cell lines. VEGF is a pro-angiogenic factor that is induced by STAT3; it plays a major role in the progression of renal cell carcinoma. After a 24 hour incubation with FLLL32 or FLLL62 at the indicated concentrations, the amount of soluble VEGF was measured in SK-RC-45 and SK-RC-54 cells (Figures 86 and 87). Treatment with FLLL32 at concentrations of 2 to 8 µM resulted in a significant decrease in VEGF secretion. FLLL62 was slightly less active in this regard, but treatment with FLLL62 at concentrations of 4 to 8 µM also resulted in a significant decrease in VEGF secretion. The compounds were also evaluated after a 12 hour treatment, and even with the shorter
duration of time they both still significantly reduced the amount of VEGF secretion (Figures 88 and 89). Their effects on ACHN and Caki cells could not be measured due to low basal levels of VEGF secretion.

Figure 85. IFNγ-induced gene expressions of IRF1 in ACHN cells. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

STAT3 is also capable of inducing the differentiation of early myeloid cells into myeloid-derived suppressor cells. Through this mechanism, STAT3 is capable of
promoting immunosuppression in hosts that have tumors. As a result, FLLLs 32 and 62 were evaluated in their abilities to inhibit the generation of myeloid-derived suppressor cells from peripheral blood mononuclear cells. The peripheral blood mononuclear cells were cultured with GM-CSF and IL-6, and the results were analyzed in vitro, as it has been previously described. The generated myeloid-derived suppressor cells were confirmed to be suppressive against T cells derived from the same host, which were stimulated to proliferate via ligation of CD28 and CD3. At a concentration of 6 µM, FLLLs 32 and 62 were found to inhibit CD33⁺CD11b⁺ myeloid-derived suppressor cell generation in 7-day differentiation cultures. The degree of inhibition was found to be beyond significant. (This work was performed in the lab of Dr. Gregory B. Lesinski and this data was originally published in PLoS ONE.)

Figure 86. Amount of VEGF secretion in SK-RC-45 cells following a 24 hour treatment with the indicated compounds. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
Figure 87. Amount of VEGF secretion in SK-RC-54 cells following a 24 hour treatment with the indicated compounds. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

Figure 88. Amount of VEGF secretion in SK-RC-45 cells following a 12 hour treatment with the indicated compounds. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)
Figure 89. Amount of VEGF secretion in SK-RC-54 cells following a 12 hour treatment with the indicated compounds. (This work was performed in the lab of Dr. Gregory B. Lesinski and this figure was originally published in PLoS ONE.)

FLLLS 32 and 62 were evaluated in the STAT3 FP assay to evaluate their abilities to inhibit STAT3 dimerization in vitro. Without an inhibitor present, 5-FAM-SpYLPQTV (4 nM) bound to STAT3 with high affinity. After a 24 hour incubation at room temperature, the $K_d$ was derived to be 146.7 nM. The presence of 50 µM FLLL32 resulted in the saturation curve shifting to the right (Figure 90). The $K_d$ was derived to be 274.0 nM when 50 µM FLLL32 was present. The presence of 50 µM FLLL62 had similar effects to those of FLLL32, although not as much of a shift in the curve was observed. The $K_d$ when 50 µM FLLL62 was present was derived to be 204.8 nM. This signifies that both FLLL32 and FLLL62 are able to bind to the STAT3 SH2 domain, but that FLLL32 is able to do so with a higher affinity. The $K_d$ values were calculated via Scatchard plot analysis (Figure 91).
Figure 90. STAT3 fluorescence polarization saturation curves when 4 nM 5-FAM-SPYLPQTV was incubated with increasing concentrations of STAT3 in the presence and absence of 50 µM FLLL32.

Figure 91. Scatchard plots of the data from Figure 90. The slope of each data set equals the negative reciprocal of the $K_d$. $B_{\text{max}}$ is equal to the x-intercept.
A Hill plot was also constructed, and the slopes of the plot were 0.9334, 0.9658 and 0.9608 when no inhibitor, 50 µM FLLL32 and 50 µM FLLL62 were present, respectively (Figure 92). All of these values are close to 1. This is expected since a slope of 1 signifies non-cooperative binding.

![Hill plots](image)

**Figure 92.** Hill plots of the three curves that were obtained via the STAT3 FP assay. An increase in the $K_d$ can be seen by a shift to the right. Non-cooperative binding is indicated by a slope of 1.

Neither FLLL32 nor FLLL62 was able to produce a shift of the magnitude that resulted from the presence of 8 nM of the competitor peptide, SpYLPQTV. However, this is expected due to the extremely high binding affinities of the probe and corresponding competitor peptide. In summary, consistent with what has been predicted through computational modeling, both FLLL32 and FLLL62 are able to bind to the STAT3 SH2 domain in vitro.
6.4 Summary of FLLLs 31, 32 and 62

In summary, the stories of FLLLs 31, 32 and 62 have been described. All three of these analogues are significantly more potent than curcumin (Table 15). FLLLs 31 and 32 were synthesized based on the predictions of computational modeling. Both of them were found to be extremely active, but FLLL32 displayed slightly higher activity. FLLL32 is predicted to interact with three sites of the STAT3 SH2 domain, and the interactions with these sites are likely the reason for its high level of activity against STAT3.

![Table 15. Activities of curcumin, FLLL31, FLLL32 and FLLL62 in various cancer cell lines.](image)

FLLL32 has been the primary lead compound; many analogues have been synthesized based on its structure. FLLL62 was one of those compounds. It displays similar activities to FLLL32, but also has a significantly improved solubility profile. As can be seen, FLLL62 achieved a sub-micromolar IC_{50} value in MDA-MB-231 cells.

However, the main drawback to FLLL62 is that it lacks selectivity relative to FLLL32. Such a simple substitution in the molecular scaffold of FLLL32 resulted in increased activity against STAT1, which is not desired. As a result, further improvements
were needed. In the next chapter, two series of analogues will be described. One of those analogues is FLLL100P, a water soluble phosphate-containing analogue. The development of FLLL100P marks another critical step in the development of these STAT3 inhibitors.
Chapter 7: SAR Studies of Two Series of Curcumin Analogues

7.1 Introduction

Many compounds have been synthesized in order to further determine the SARs of curcumin-related compounds. In this section, two series of analogues will be discussed. The main objective of these studies was to analyze the effects of altering the central region of curcumin. However, the effects of different substitution patterns on the aromatic rings were also evaluated.

The first series of analogues feature the replacement of the central β-diketone of curcumin with functional groups that constrict the conformation of the analogues to the keto-enol form. The second series of analogues contains a few compounds that are able to tautomerize. However, the majority of the compounds in the second series do not have any protons on the central carbon. They are not capable of undergoing tautomerization, and as a result, they mimic the diketone form of curcumin.

7.2 Synthesis of Analogues

Symmetric curcumin analogues were synthesized via aldol condensation between 2,4-pentanedione and specified aldehydes, using the procedure established by Venkateswarlu and colleagues,\textsuperscript{573} as it has been discussed in preceding chapters. The alkylation of the central carbon was also carried out as it has been described elsewhere (Chapter 6, Scheme 6). The syntheses of all of the keto-enol mimics, as well as the
alkylated analogues, were performed by Eric Schwartz, a graduate student in the lab of Dr. James Fuchs.

Eric is also to credit for the syntheses of the unsymmetrical curcumin analogues. The unsymmetrical analogues were synthesized by one of two methods, both of which involve acylation chemistry. In the first method, a simple ketone that already contained the desired alkyl substitution acted as the starting material. It was first subjected to an aldol condensation with the specified benzaldehyde. The enone-containing intermediate was then reacted with the specified acid chloride to produce the corresponding analogue (Scheme 7).

In the second method, the specified enone was first deprotonated. It was then added into the specified acid chloride, which produced the corresponding unsymmetrical curcumin analogue (Scheme 8). In this case, the compounds were then alkylated as described above.

Scheme 7. First method for generating unsymmetrical curcumin analogues.
Scheme 8. Second method for generating unsymmetrical curcumin analogues.

7.3 Biological Activities of the Keto-Enol Mimics

The keto-enol mimics all lack a β-diketone moiety. Instead, they contain either pyrazole (FLLLs 7, 8, 68 and 70), isoxazole (FLLLs 67 and 69), pyrimidine (FLLLs 58 and 59) or benzene (FLLLs 60, 98 and 99) rings. These replacements lock the structures of these compounds into the planar keto-enol form. It should be noted that the computational modeling, which has been discussed, predicted that the keto-enol form of curcumin was not able to bind STAT3, but that it is able to bind JAK2 with similar affinity to the diketone form. With that being said, the general activities of these compounds were evaluated with the knowledge that they would probably not be binding to the STAT3 SH2 domain.

The structures of the analogues and their antiproliferative activities in DU145 cells are shown (Table 16). The first thing apparent is that, in general, the pyrazole-containing analogues exhibit respectable potency, while the benzene-containing analogues lack activity. The isoxazole and pyrimidine analogues appear to have moderate levels of activity. It is interesting that of the two isoxazole-containing compounds, the analogue containing the same substitution pattern as curcumin, FLLL67, is nearly three times as potent as curcumin (30.8 ± 1.9 µM) in DU145 cells, while the analogue
containing the 3,4-dimethoxy substitution pattern, FLLL69, lacked activity. In contrast, the pyrimidine analogue containing the 3,4-dimethoxy substitution pattern, FLLL59, was active, although not as much so as FLLL67. Removal of the 3-methoxy substituent resulted in a complete loss of activity (FLLL58).

Table 16. Structures of the keto-enol mimics and their antiproliferative activities in DU145 cells.
The SARs of the pyrazole analogues were also intriguing. FLLL7 features the same substitution pattern as curcumin. However, it is approximately four times more potent than curcumin. N-methylation (FLLL8) or methylation of the central carbon (FLLL70) resulted in similar losses in activity. The resulting compounds were roughly half as potent as FLLL7, but still about twice as potent as curcumin. Protecting the hydroxyl substituents as methoxy groups had little effect for these compounds, as FLLL68 and FLLL7 had similar activities.

The antiproliferative activities of the compounds were also evaluated in DU145, HT29, MDA-MB-231 and PC3 cancer cell lines at concentrations of 0.5, 5 and 50 µM. The data in DU-145 cells is in line with the derived IC\textsubscript{50} values (Figure 93). None of the compounds inhibited cell growth by more than 20% at concentrations of 0.5 or 5 µM. Only FLLLs 7, 8, 59, 67, 68 and 70 had significant antiproliferative activities at 50 µM. FLLLs 58, 69 and 99 did not have an observable effect on cell growth at any concentration, while FLLLs 60 and 98 resulted in only minor decreases in cell growth at a concentration of 50 µM.

The activities of the compounds in HT29 cells were similar (Figure 94). However, none of the compounds resulted in any inhibition of cell growth at concentrations of 0.5 or 5 µM in HT29 cells. FLLLs 7, 8, 67, 68 and 70 still exhibited antiproliferative activities at concentrations of 50 µM, reducing cell growth by 70 to 90% when compared to controls. The major observed difference is that FLLL59 had significant antiproliferative activities in DU145 cells at a concentration of 50 µM, but in HT29 cells it only inhibited cell growth by a little over 10% at that concentration.
Figure 93. Antiproliferative activities of keto-enol mimics in DU145 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.

Figure 94. Antiproliferative activities of keto-enol mimics in HT29 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.
MDA-MB-231 cells appear to be the least susceptible to these compounds (Figure 95). Similar to what was observed in HT29 cells, FLLL59 was not able to achieve a 50% inhibition of cell growth in MDA-MB-231 cells at a concentration of 50 µM. FLLLs 68 and 70 were also not able to achieve that type of inhibition in MDA-MB-231 cells. FLLLs 7, 8 and 67 were the only compounds that inhibited cell growth by more than 50%, and they only did so when present at a concentration of 50 µM.

![Figure 95. Antiproliferative activities of keto-enol mimics in MDA-MB-231 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.](image)

In contrast, PC-3 cells appear to be the most susceptible to the keto-enol mimics (Figure 96). FLLLs 8, 59 and 98 did not achieve significantly different degrees of growth inhibition when comparing them at concentrations of 0.5 and 5 µM. In contrast, for all of
the other analogues there was a substantial difference in the inhibition of cell growth that they resulted in when they were present at concentrations of 0.5 and 5 µM. Still, the general trends that were observed were comparable to the trends that were observed in DU145 cells. FLLLs 7, 8, 59, 67, 68 and 70 resulted in greater than 50% inhibition of cell growth when they were present at a concentration of 50 µM; all other compounds did not display these types of inhibitory activities.

![Graph](image)

**Figure 96.** Antiproliferative activities of keto-enol mimics in PC3 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.

Since it unclear what pathway(s) the keto-enol mimics are targeting, it is difficult to rationalize the different inhibitory activities that they displayed in different cell lines. However, the pyrazole-containing compounds, FLLLs 7 and 8, and the isoxazole-
containing analogue, FLLL67, exhibited noteworthy antiproliferative activities in all cell lines in which they were evaluated. FLLLs 68 and 70, which also contain a pyrazole moiety, displayed notable activities in all but MDA-MB-231 cells, and the pyrimidine-containing analogue, FLLL59, displayed high levels of antiproliferative activity at a concentration of 50 µM in two out of four cell lines. The benzene-containing analogues were relatively inactive in all four cell lines, which suggests that the presence of a hydrogen-bond acceptor in that region of the molecular scaffold is necessary for activity.

7.4 Biological Activities of the Diketone Mimics

The majority of the diketone mimics are alkylated at the central carbon and do not contain any protons at this location. As a result, they exist exclusively in the diketone form, since they are not capable of undergoing tautomerization. The exceptions are FLLLs 10, 18 and 24, along with curcumin, which are capable of tautomerizing and likely exist predominantly in the keto-enol form.

FLLL31 is dimethylated at the central carbon, while FLLL45 contains a cyclopentyl moiety. FLLLs 64, 77, 78, 85-88, 100 and 100P all contain a cyclohexyl moiety, and are analogues of the lead compound, FLLL32. However, FLLLs 85 and 88 feature a vinyl group that has been saturated, which abrogates the conjugation with the corresponding ketone and allows for greater rotational flexibility for half of the molecule. The structures of all 16 compounds and their antiproliferative activities in DU145 cells are shown (Table 17).
Table 17. Structures of the diketone mimics and their antiproliferative activities in DU145 cells.
Out of the 15 compounds, 11 of them of them are between 3 to 20 times more potent than curcumin. As it was observed for FLLL58 of the keto-enol series, lacking a substituent at the third position of the aromatic rings resulted in a total abrogation of activity (FLLL24). Interestingly, two other compounds that exhibited activity similar to curcumin were FLLLS 85 and 88, which lack conjugation between an aromatic moiety and a carbonyl group. This suggests that the increased rotational flexibility in their scaffolds is detrimental to activity. They are roughly one tenth to one twentieth as active as the corresponding analogues, FLLLs 32 and 86, which are unsaturated at those carbon atoms. It is also interesting that the unsymmetrical analogue, FLLL88, which only lacks methoxy substituents on one of the aromatic rings, was more active than FLLL85, which features methoxy substituents on both sides.

FLLL64 is more than 10 times less active than FLLL32. This is the result of a single substitution at the third position of the aromatic ring. This suggests that the isobutoxy substituent might disrupt the favorable interaction with the Tyr$^{705}$ or Leu$^{706}$ sites of STAT3. Protecting one of the 4-hydroxy substituents as a sulfamate (FLLL78) only led to an approximate two-fold reduction in activity when compared to FLLL32. However, FLLL78 was still slightly more active than the analogue which featured one unprotected hydroxy substituent, FLLL77.

FLLLs 31 and 45 displayed only slightly reduced activities when compared to FLLL32. The only structural difference between them and FLLL32 is the identity of the central alkyl substituent(s). All three of the symmetrical compounds that feature 3,4-dimethoxy substituents, FLLLs 31, 32 and 45, are significantly more active than FLLL10,
which also contains 3,4-dimethoxy substituents, but is able to tautomerize. This supports the predictions of computational modeling, which suggest that the diketone form of curcumin is able to bind to the STAT3 SH2 domain, while the keto-enol form is not. FLLL32 might be more active than FLLLS 31 and 45 due to more favorable interactions between its central alkyl moiety and the hydrophobic side pocket of the STAT3 SH2 domain, as predicted by computational modeling.

Interestingly, exchanging the hydroxy and methoxy substituents of curcumin did not result in a significant decrease in activity in DU145 cells (FLLL18). In fact, the IC$_{50}$ values achieved by FLLL10 and FLLL18 in DU145 cells were not significantly different. In all four cell lines that were evaluated, FLLL18 displayed inhibitory activities that were similar to that of curcumin at all three concentrations that were tested. In contrast, in all but MDA-MB-231 cells, FLLL10 was significantly more active than FLLL18 at a concentration of 5 µM (Figures 97-100).

FLLLS 86, 87, 100 and 100P are all unsymmetrical analogues that contain the cyclohexyl moiety and lack a substituent at the third position on one of the aromatic rings. They all achieved IC$_{50}$ values of less than 10 µM in DU145 cells, which signifies that it is not necessary for both rings to contain substituents at the third and fourth positions. FLLLS 86 and 87 are unsubstituted at both positions of one of the rings, and replacing the 4-methoxy group with a 4-hydroxy substituent resulted in only a slight decrease in activity in DU145 cells. Relative to FLLL86, FLLL100 contains an additional 4-hydroxyl substituent, which only lowered its activity slightly. If that group is replaced with a phosphate, an approximate two to three-fold loss in activity is observed.
(FLLL100P). However, FLLL100P is intended to be a prodrug, as its solubility profile is vastly improved when compared to FLLL32. In theory, it should be cleaved to FLLL100 \textit{in vivo}, which is only approximately three-fold less potent than FLLL32.

The data lined up fairly well with the derived IC$_{50}$ values when the compounds were evaluated at three concentrations in DU145 cells (Figure 97). FLLL24 was the only compound that did not achieve at least a 50% inhibition of cell growth at a concentration of 50 µM. FLLL10 was the only compound capable of undergoing tautomerization that was able to inhibit cell growth by nearly 50% at a concentration of 5 µM. In contrast, all analogues that were structurally restricted to the diketone form were able to inhibit cell growth by approximately 50% or more at a concentration of 5 µM, except for the more flexible compounds, FLLLs 85 and 88, and the prodrug, FLLL100P.

Similar results were obtained for HT29 cells (Figure 98). However, a few notable differences in activities were observed. FLLL10 was not as active in HT29 cells, as it inhibited cell growth by less than 30% at a concentration of 5 µM. The isobutoxy-containing analogue, FLLL64, was also less active at this concentration, as it only inhibited cell growth by less than 20%. The rotationally flexible analogues, FLLLs 85 and 88, were also less active in HT29 cells. From the cell data, their estimated IC$_{50}$ values in HT29 cells would be along the lines of 50 µM or greater. This supports the idea that a certain amount of rigidity is required for the curcumin analogues to adopt the appropriate confirmation for binding to the STAT3 SH2 domain. Interestingly, the unsymmetrical analogue, FLLL77, was more active in HT29 cells, inhibiting cell growth by almost 70% at a concentration of 5 µM.
Figure 97. Antiproliferative activities of diketone mimics in DU145 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.

Figure 98. Antiproliferative activities of diketone mimics in HT29 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.
In general, the diketone analogues were less active in MDA-MB-231 cells, although the general trends were the same (Figure 99). FLLL24 was the least active of all analogues evaluated; it did not inhibit cell growth at a concentration of 50 µM. FLLL64 displayed less activity in MDA-MB-231 cells, only inhibiting cell growth by about 50% at a concentration of 50 µM. The unsymmetrical analogue, FLLL87, also displayed less activity in MDA-MB-231 cells. At a concentration of 5 µM, it did not inhibit cell growth by even 50%, where it had in DU145 and HT29 cells. Curcumin, as well as FLLLs 10, 18, 24, 64, 77, 85, 87, 88 and 100P did not inhibit cell growth by more than 30% at a concentration of 5 µM.

![Graph](image)

**Figure 99.** Antiproliferative activities of diketone mimics in MDA-MB-231 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.
Consistent with the data from DU145 and HT29 cells, FLLLs 24, 85 and 88 were the least active in PC3 cells. They did not inhibit cell growth by even 50% at a concentration of 50 µM (Figure 100). FLLL10 also only achieved an approximate 30% reduction in cell growth at a concentration of 5 µM, which was similar to its activity in HT29 and MDA-MB-231 cells. As was the case in MDA-MB-231 cells, FLLL64 only inhibited cell growth by about 50% at a concentration of 50 µM, and FLLL87 did not inhibit cell growth by 50% at a concentration of 5 µM in PC3 cells.

![Figure 100. Antiproliferative activities of diketone mimics in PC3 cells. Absorbances are proportionate to the number of living cells and are shown as percentages relative to controls.](image)

The activities of all of the compounds were quite consistent across cell lines. FLLLs 31, 32, 45, 78 and 86 all inhibited cell proliferation by around or greater than 50%
at a concentration of 5 µM in all four cell lines. FLLLs 87 and 100 also did so, with the exceptions of PC3 cells. FLLL77 accomplished this in two out of the four evaluated cell lines (DU145 and HT29). In contrast, FLLL68 was the only keto-enol mimic that displayed that type of activity, and it only did so in PC3 cells.

Western blots were obtained in order to confirm that compounds from the diketone series were exerting their antiproliferative activities by, at least in part, down-regulating the phosphorylation of STAT3. At concentrations of just 5 µM, and for a treatment time of only 12 hours, several diketone analogues significantly down-regulated the phosphorylation of STAT3 at Tyr705 in DU145 cells (Figure 101).

Treatment with FLLLs 32 and 86 led to nearly undetectable levels of pSTAT3. FLLLs 87 and 100P also down-regulated the phosphorylation of STAT3, but not to the same extent as FLLLs 32 and 86. Interestingly, despite having antiproliferative activities that are relatively similar to FLLL32, FLLLs 45 and 77 did not have an appreciable effect on the phosphorylation of STAT3; perhaps this was due to a relatively short treatment time. However, the possibility that these compounds are also targeting other pathways cannot be ruled out.

Curcumin and FLLL10 also had only minor impacts on the level of STAT3 phosphorylation. Interestingly, FLLL88 did not result in the reduction of pSTAT3 levels. Perhaps this is a result of it being less restricted in conformation. The Western blot data, along with the high IC50 value for FLLL88 in DU145 cells, are evidence for the suggestion that both enones might be necessary for tight binding to the STAT3 SH2 domain. It is likely that FLLL88’s antiproliferative activity is a result of it affecting other
pathways, perhaps one or more of those that curcumin is known to target. However, FLLL88 does not appear to significantly affect the STAT3 pathway.

![Image](image_url)

**Figure 101. Inhibition of STAT3 phosphorylation in DU145 cells by curcumin and analogues.** Cells were treated with 5 µM of the indicated compounds for 12 hours. The numbers of the FLLL compounds are listed. C: curcumin.

Selected diketone analogues were also evaluated in the STAT3 FP assay to demonstrate that they could directly bind to the STAT3 SH2 domain. As it has been discussed, the limited solubility of test compounds in the FP buffer is a problem that has been often encountered. Curcumin and related analogues have significant problems in this regard.

Unfortunately, in order to show significant activity in the STAT3 FP assay, a compound has to have a high level of aqueous solubility in addition to having the ability to bind the STAT3 SH2 domain. Take FLLLs 32, 45, 87 and 100P. Their calculated logP values are 5.11, 4.7, 5.1 and 2.81, respectively (ChemDraw Ultra 12.0). Out of these
compounds, FLLL32 exhibited the highest level of antiproliferative activity in cell studies, and it also led to the largest decrease in pSTAT3. However, it is also the least soluble in the FP buffer. It only showed activity in the STAT3 FP assay that was similar to/slightly better than FLLL87, which has a nearly identical logP (Figure 102).

![Figure 102. Percent inhibition of the STAT3 FP signal resulting from the presence of selected diketone analogues (500 µM), SpYLPQTV (100 µM), or SYLPQTV (100 µM).](image)

In contrast, FLLL45 is more soluble in the FP buffer, and is significantly more active than FLLL32 in the STAT3 FP assay. FLLL100P is significantly more soluble than all of the other curcumin analogues and demonstrates a drastically higher level of activity in the assay. Even at half of the concentration that is shown above, FLLL100P
inhibited the FP signal by $45.8 \pm 2.7\%$. So at 250 $\mu$M, FLLL100P still appears to be significantly more active than the other compounds at 500 $\mu$M. However, in DU145 cells, FLLL100P is roughly one seventh as potent as FLLL32.

These data emphasize how critical it is for test compounds to have high levels of aqueous solubility in order for them to be accurately evaluated in the FP assay. If a compound has a low level of aqueous solubility, it might not show high levels of inhibition in the STAT3 FP assay, despite being able to bind to the STAT3 SH2 domain with a high affinity. The discrepancies between activities in the cellular and FP assays are apparent, and presumably result from differences in conditions. The inhibition of cell proliferation and the induction of apoptosis depend on numerous mechanisms, while the fluorescent polarization assay is used solely to determine a molecule’s ability to bind to the STAT3 SH2 domain. The tight binding of the fluorescent probe used in the FP experiments, along with other factors, such as the differences between cellular STAT3 concentrations and the concentrations used in the FP assay, could result in there being different effective concentrations of test compounds in the different assays. Regardless, the main objective of the FP experiments were to validate that the diketone analogues are able inhibit the phosphorylation of STAT3 by binding to its SH2 domain. The inhibitory activity of the water soluble analogue, FLLL100P, validates the binding of the diketone analogues to the proposed molecular target.
7.5 Summary of SAR Studies

Some valuable information can be learned from these SAR studies. As a whole, the diketone analogues were significantly more potent than the keto-enol mimics. Only one compound from the keto-enol series, FLLL68, achieved an IC\textsubscript{50} value that was under 7 µM in DU145 cells. In contrast, 8 out of 12 compounds that were restricted to the diketone form achieved IC\textsubscript{50} values that were under 7 µM.

Certain diketone analogues were shown to potently inhibit STAT3 phosphorylation in DU145 cells. In addition, the diketone analogues, FLLL100P in particular, were shown to bind the STAT3 SH2 domain in an \textit{in vitro} assay. These data confirm that the STAT3 pathway is a primary pathway that is targeted by these compounds. However, the possibility that they might also target other pathways cannot be ruled out.

The activity of the water soluble analogue, FLLL100P, offers some future direction. The limited aqueous solubility of FLLL32 limits its therapeutic effectiveness. Although FLLL100P is nearly seven times less potent than FLLL32, it is expected to be cleaved to FLLL100 \textit{in vivo}, which is only two to three times less potent than FLLL32. Regardless, the synthesis of phosphate-contain curcumin derivatives appears to be a viable approach. If a phosphate-containing derivative that is both potent and water soluble can be discovered, it could possibly have the properties that are necessary to be a therapeutically relevant STAT3 inhibitor.
Chapter 8: Additional Curcumin and Monocarbonyl Analogues

8.1 Introduction

There have been a number of other compounds that have been synthesized and evaluated in cellular assays that were not included in the studies that have been discussed up to this point. They include a number of monocarbonyl compounds, compounds which have been monoalkylated at the central carbon of curcumin’s scaffold, and FLLL31- and FLLL32-related compounds. In the next chapter, the more recent studies of the effects of electron withdrawing substituents on the aromatic rings of the curcumin scaffold will be discussed. Here, the data obtained for the remaining compounds will be presented.

8.2 Additional Monocarbonyl Compounds

The additional monocarbonyl compounds can be broken down into three groups. Compounds from the first group can be roughly classified as AG490 analogues. Compounds from the second group are related to those that were discussed in chapter 4. The third group of compounds is really not a group at all, but consists of several compounds that don’t fit into any other group.

8.2.1 AG490-Related Compounds

The three compounds that will be briefly discussed in this section are structurally related to AG490. However, they feature some noticeable differences (Table 18). The
compounds do contain the cyano group of AG490, but they all lack the amide nitrogen.

All but one of them also lacks the bromopyridine moiety that AG490 features.

![Chemical structures of AG490-related compounds]

<table>
<thead>
<tr>
<th>Compound</th>
<th>DU145</th>
<th>MDA-MB-468</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL44</td>
<td>ND</td>
<td>18.0 ± 4.6</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL46</td>
<td>11.7 ± 0.1</td>
<td>11.2 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL56</td>
<td>9.46 ± 0.56</td>
<td>ND</td>
<td>15.1 ± 7.3</td>
</tr>
</tbody>
</table>

Table 18. Structures and antiproliferative activities of AG490-related compounds. (These compounds were synthesized by Dr. Dalia Abdelhamid in the lab of Dr. James Fuchs.)

It is interesting that the 3,4-dimethoxybenzene containing compound, FLLL46, displays moderate levels of activity. As it was discussed earlier, the AG490 analogues that contained the same substitution pattern were almost completely inactive (FLLLs 124, 127, 130, 136 and 142). However, it should be noted that FLLL46 is nearly five times less potent than FLLL14, which contains the 3,4-dimethoxybenzene substitution pattern on both sides and lacks the cyano group. A direct comparison can be made between FLLL14 and FLLL44, and the results suggest that the addition of the cyano group at the specified position of the monocarbonyl scaffold leads to a decrease in activity. The
presence of the 3,4-dimethoxybenzene moiety opposite the bromopyridine also leads to a decrease in potency relative to the other compounds that have been evaluated. Collectively, the data obtained from evaluating these compounds, the AG490 analogues, and the initial monocarbonyl compounds suggest that exchanging the moieties of the AG490 analogues with the curcumin-related monocarbonyl derivatives does not lead to an increase in potency.

8.2.2 Additional Pentadienone Compounds

In our initial SAR studies of curcumin derivatives, a number of potent pentadienone compounds were identified. Among these were several symmetrical compounds that contained only methoxy substituents; they featured two or three methoxy substituents on each side (Table 19). FLLL22, featuring the 1,3,5-trimethoxybenzene substitution pattern, was the most potent compound identified (Table 20).

![Structure of pentadienone compounds](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL13</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>FLLL14</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>FLLL22</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>FLLL23</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

Table 19. Structures of the initial pentadienone compounds that featured methoxy substituents only.
<table>
<thead>
<tr>
<th>Compound</th>
<th>PC3</th>
<th>LNCaP</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL13</td>
<td>2.5 ± 0.5</td>
<td>2.1 ± 0.9</td>
<td>2.7 ± 0.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>FLLL14</td>
<td>2.9 ± 0.6</td>
<td>2.2 ± 0.5</td>
<td>2.5 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>FLLL22</td>
<td>2.1 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>FLLL23</td>
<td>4.6 ± 0.2</td>
<td>1.7 ± 0.6</td>
<td>2.4 ± 1.0</td>
<td>2.4 ± 0.4</td>
</tr>
</tbody>
</table>

Table 20. Antiproliferative activities of the initial pentadienone compounds that featured methoxy substituents only.

In order to further elucidate the SARs of the methoxy-substituted pentadienone compounds, additional analogues were synthesized and evaluated. The new series consisted of compounds that were mono-, di-, or tri-substituted (Table 21). Unfortunately, no compound was identified that was more potent than FLLL22 (Table 22). However, the evaluation of the additional compounds was helpful in determining the SARs.

![Diagram of pentadienone compounds]

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL33</td>
<td>H</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>FLLL40</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>FLLL41</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>FLLL42</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>FLLL43</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>FLLL47</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OCH₃</td>
</tr>
<tr>
<td>FLLL52</td>
<td>H</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

Table 21. Additional pentadienone compounds featuring only methoxy substituents. (These compounds were synthesized by Eric Schwartz and Dr. Dalia Abdelhamid.)
Table 22. Antiproliferative activities of additional pentadienone compounds featuring only methoxy substituents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DU145</th>
<th>MDA-MB-468</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL33</td>
<td>20.70 ± 0.35</td>
<td>23.0 ± 4.7</td>
<td>21.8 ± 0.6</td>
</tr>
<tr>
<td>FLLL40</td>
<td>6.40 ± 1.65</td>
<td>7.58 ± 2.05</td>
<td>4.43 ± 0.71</td>
</tr>
<tr>
<td>FLLL41</td>
<td>ND</td>
<td>9.85 ± 2.46</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL42</td>
<td>1.92 ± 0.28</td>
<td>1.27 ± 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL43</td>
<td>4.57 ± 0.13</td>
<td>4.86 ± 0.35</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL47</td>
<td>2.69 ± 0.47</td>
<td>2.66 ± 0.66</td>
<td>4.15 ± 0.56</td>
</tr>
<tr>
<td>FLLL52</td>
<td>1.33 ± 0.83</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The one compound that was monosubstituted was the least active in all cell lines (FLLL33). All of the disubstituted compounds of both the initial compounds and the additional compounds were significantly more active. This data suggests that, in general, the disubstituted compounds are more active than the monosubstituted compounds. However, additional monosubstituted derivatives would not need to be evaluated in order for this to be fully validated.

Only minor differences in activity were observed between the di- and tri-substituted analogues. The 1,2,3-trisubstituted analogue, FLLL43, was noticeably less potent than the other trisubstituted analogues. However, in general, all of the di- and tri-substituted compounds had similar potencies. None of the additional compounds were as potent as the initially identified FLLL22.
8.2.3 *Unique Monocarbonyl Compounds*

Several additional monocarbonyl compounds were also evaluated (Figure 103). FLLL34 features the same aromatic substitution pattern as FLLL22, but it differs in the central portion of the molecule, as it features a piperidin-4-one moiety. FLLL35 features the same aromatic substitution pattern as FLLL14, but it also has a fluorine substituent at the same location that AG490 features a cyano group. FLLL53 also resembles FLLL14, but it features cyclic acetal substituents. FLLL57 has 3,4-dimethoxybenzene substituents as well, but it also features methyl ester moieties that are attached to the alpha carbon atoms. Lastly, FLLL61 consists of the basic pentadienone scaffold linked to two pyridine substituents on both sides.

Figure 103. Unique monocarbonyl compounds. (These compounds were synthesized by Eric Schwartz and Dr. Dalia Abdelhamid.)
These compounds were evaluated for their antiproliferative activities (Table 23). FLLL34 was approximately 10 times less potent than FLLL14; the addition of the piperidine moiety was clearly detrimental to activity. The addition of a fluorine to an alpha carbon (FLLL35) also resulted in a reduction in activity, although it wasn’t as significant. A large reduction in activity was also observed when the methyl esters were added to the alpha carbon (FLLL57). The methyl ester moieties are large enough that they could significantly alter the preferred conformation of the compound relative to FLLL14, so the loss in activity is not surprising. However, the reduced activity of FLLLSs 34 and 35 are telling. Together, these data suggest that the pentadienone portion of the scaffold acts as more than just a linker; the addition of various substituents to this portion of the molecule invariably led to a decrease in activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM) DU145</th>
<th>IC₅₀ (µM) MDA-MB-468</th>
<th>IC₅₀ (µM) MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL34</td>
<td>23.8 ± 3.8</td>
<td>14.2 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL35</td>
<td>7.18 ± 0.73</td>
<td>3.77 ± 0.38</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL53</td>
<td>&gt;100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL57</td>
<td>39.9 ± 4.9</td>
<td>ND</td>
<td>11.7 ± 4.7</td>
</tr>
<tr>
<td>FLLL61</td>
<td>0.959 ± 0.423</td>
<td>ND</td>
<td>0.251 ± 0.230</td>
</tr>
</tbody>
</table>

Table 23. Antiproliferative activities of unique monocarbonyl compounds.

Interestingly, the conversion of the dimethoxy substituents to acetal moieties led to a total loss of activity (FLLL53). This data suggests that the two methyl groups are critical for activity. Lastly, the incredible potency of the pyridine-containing analogue, FLLL61, is intriguing. FLLL61 is the most potent of any monocarbonyl or diketone
curcumin analogue that was evaluated. Its relatively empty scaffold leads plenty of room for adding additional substituents that might lead to increased potency. Unfortunately, in the synthesis of FLLL61, the condensation did not work well with the piperidine-containing picolinaldehyde and acetone. An optimized procedure will need to be established in order to be able to synthesize additional analogues in sufficient quantities.

8.3 Additional Heptadienedione Analogues

Additional curcumin derivatives have also been evaluated. They consist of curcumin analogues which feature a central monoalkylation, an additional keto-enol mimic (FLLL39), and a number of compounds which were synthesized based on the structures of FLLLs 31 and 32. The structural alterations and resulting impacts on antiproliferative activities of the compounds will be discussed in this section.

8.3.1 Curcumin Analogues Featuring a Central Monoalkylation

The improvements in activity resulting from the absence of both protons attached to the central carbon of curcumin have already been discussed. This modification locks the analogues into the more active diketone form. However, several other analogues were synthesized and evaluated in order to also investigate the loss of just one of the central protons.

FLLL19 consists of the aromatic substitution pattern of curcumin, and is also monomethylated at the central carbon. FLLL19S is the same as FLLL19, except that the hydroxyl substituents are protected as sulfamates. FLLLs 36 and 37 consist of the same
aromatic substitution pattern as FLLL31, but they are monomethylated (FLLL36) or monoethylated (FLLL37) at the central carbon. The structures and antiproliferative activities of the compounds are shown (Table 24).

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM) DU145</th>
<th>MDA-MB-468</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>30.8 ± 1.9</td>
<td>23.9 ± 3.2</td>
<td>28.4 ± 4.3</td>
</tr>
<tr>
<td>FLLL31</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>CH₃</td>
<td>2.70 ± 0.82</td>
<td>1.12 ± 0.27</td>
<td>1.54 ± 0.14</td>
</tr>
<tr>
<td>FLLL19</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
<td>ND</td>
<td>8.20 ± 4.47</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL19S</td>
<td>OSO₂NH₂</td>
<td>CH₃</td>
<td>H</td>
<td>ND</td>
<td>7.75 ± 2.23</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL36</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>H</td>
<td>4.56 ± 0.59</td>
<td>2.94 ± 0.64</td>
<td>2.47 ± 0.33</td>
</tr>
<tr>
<td>FLLL37</td>
<td>OCH₃</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>3.13 ± 0.42</td>
<td>1.62 ± 0.04</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 24. Structures and antiproliferative activities of curcumin analogues that are monoaalkylated at the central carbon. (Curcumin, FLLL31, FLLL19 and FLLL19S were synthesized by Eric Schwartz and Dr. James Fuchs. FLLLS 36 and 37 were synthesized by condensing 3-methyl-2,4-pentanedione or 3-ethyl-2,4-pentanedione with 3,4-dimethoxybenzaldehyde, using the procedure established by Venkateswarlu and colleagues.)

Interestingly, the monomethylated analogue of curcumin, FLLL19, was two to three times more potent than curcumin. This is surprising since the monoaalkylated analogues are still able to undergo tautomerization, and the keto-enol forms are likely preferred. For the non-alkylated analogues, the protection of the hydroxyl substituents as sulfamates had led to a significant increase in activity. Here, the sulfamate derivative, FLLL19S, had only similar potency when compared to FLLL19.
FLLL36 is the monomethylated version of FLLL31. It was slightly more potent than the unmethylated analogue, FLLL10. However, it was approximately half as potent as FLLL31, which is not capable of undergoing tautomerization. Interestingly, the monoethylated analogue, FLLL37, was nearly as potent as FLLL31. It was also slightly more active than FLLL36 in both DU145 and MDA-MB-468 cells.

As expected, the monomethylated derivatives were not as active as the dimethylated analogue, FLLL31. However, the monoethylated analogue, FLLL37, did exhibit similar potency to FLLL31. It is unlikely that the diethylated analogue would be as potent as some of the FLLL32 analogues that have been evaluated, but it would be interesting to compare the potencies of the diethylated analogue and FLLL31.

8.3.2 Additional Curcumin Analogues

A number of additional analogues related to curcumin, FLLL31 and FLLL32 have also been synthesized (Figure 104). In some cases, the synthesized analogues were expected to lack potency. In these cases, the low level of potency helps to confirm what has been predicted by computational modeling and validates the SARs of the compounds.

For example, FLLL39 is alkylated at the enol oxygen, which locks it into the keto-enol form. Despite featuring the 3,4-dimethoxybenzene substitution pattern, which is common to many of the active analogues, it lacks antiproliferative activity (Table 25). Thus, its low level of activity is expected, as it is similar to many of the other keto-enol mimics.
Figure 104. Various curcumin, FLLL31 and FLLL32 analogues. (These compounds were synthesized by Eric Schwartz in the lab of Dr. James Fuchs.)

FLLL50 also features the 3,4-dimethoxybenzene substitution pattern and cyclohexyl moiety of FLLL32, but it lacks the olefin double bonds. This alteration also led to a total loss of activity. The previously discussed FLLL85 lacked only one of the olefin double bonds and was approximately 20 times less potent than FLLL32. In light of this, it was expected that FLLL50 would not exhibit activity, and this data helps to confirm that the olefin double bonds are required for antiproliferative activity.

FLLLs 45 and 64 have already been discussed, but additional cell data is presented here, which helps to confirm what has been stated earlier. FLLL45 is approximately two times less active than FLLL32 in DU145 and MDA-MB-231 cells. In
MDA-MB-468 cells, it was as active as FLLL32. This confirms that the presence of the cyclopentyl moiety leads to a high level of activity, but that, in general, the cyclohexyl moiety leads to a higher level of activity. This has been predicted by computational modeling. The isobutoxy-containing analogue, FLLL64, has a similar IC$_{50}$ value in MDA-MB-231 and DU145 cells. It is about 10 times less potent than FLLL32. This further confirms that the bulkiness of the isobutoxy moiety hinders the binding to STAT3. This is in contrast to what was suggested by computational modeling, as the isobutoxy moiety is predicted to bind to the Leu$^{706}$ site of STAT3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DU145</th>
<th>MDA-MB-468</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL31</td>
<td>2.70 ± 0.82</td>
<td>1.12 ± 0.27</td>
<td>1.54 ± 0.14</td>
</tr>
<tr>
<td>FLLL32</td>
<td>1.41 ± 0.06</td>
<td>1.36 ± 0.14</td>
<td>1.29 ± 0.16</td>
</tr>
<tr>
<td>FLLL5</td>
<td>5.18 ± 0.56</td>
<td>ND</td>
<td>5.12 ± 0.13</td>
</tr>
<tr>
<td>FLLL39</td>
<td>&gt;150</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>FLLL45</td>
<td>2.83 ± 0.20</td>
<td>1.33 ± 0.05</td>
<td>2.02 ± 0.12</td>
</tr>
<tr>
<td>FLLL50</td>
<td>&gt;150</td>
<td>ND</td>
<td>&gt;150</td>
</tr>
<tr>
<td>FLLL51</td>
<td>2.58 ± 0.41</td>
<td>ND</td>
<td>2.96 ± 0.00</td>
</tr>
<tr>
<td>FLLL63</td>
<td>4.24 ± 1.27</td>
<td>ND</td>
<td>4.66 ± 0.54</td>
</tr>
<tr>
<td>FLLL64</td>
<td>15.3 ± 1.4</td>
<td>ND</td>
<td>17.9 ± 1.4</td>
</tr>
<tr>
<td>FLLL65</td>
<td>4.55 ± 0.18</td>
<td>ND</td>
<td>1.93 ± 0.34</td>
</tr>
</tbody>
</table>

Table 25. Antiproliferative activities of various curcumin, FLLL31 and FLLL32 analogues.

FLLL51, which features a methyl and a butyl substituent at the central carbon, is about as active as FLLL31, but only about half as active as FLLL32. It would be rational to think that the butyl substituent could extend further into the hydrophobic pocket of STAT3, and that this interaction would contribute significantly to binding. However, its
reduced potency when compared to FLLL32 suggests that the cyclohexyl moiety at this location is superior in its ability to interact with STAT3.

FLLLs 63 and 65 of the FLLL31 series are homologous to FLLLs 77 and 78 of the FLLL32 series. Given the relatively similar potencies of FLLL31 and FLLL32, it is not surprising that FLLLs 63 and 65 have similar potencies to FLLLs 77 and 78. Protecting the lone hydroxyl substituent as a sulfamate (FLLL65) did not result in significant differences in activity. Again, this was also observed for FLLLs 77 and 78. FLLL5, which features two free hydroxyl substituents, also had relatively similar potencies to FLLLs 63 and 65. None of these compounds were found to be quite as active as FLLL32, but this data suggest that these types of analogues, which are more water soluble, are a viable option.

8.3.3 Importance of the Cyclohexyl Moiety

In order to demonstrate just how crucial the cyclohexyl moiety is for binding to STAT3 and for a high level of activity, two series of analogues will be compared. Curcumin, FLLL10, FLLL18, JE46 and FLLL24 all lack the cyclohexyl moiety (Table 26). Their corresponding cyclohexyl-containing analogues are FLLL49, FLLL32, JE48, JE47 and JE39, respectively. FLLLs 10 and 18 are significantly more active than curcumin, while JE46 and FLLL24 more or less lack antiproliferative activity.

It should be noted that JE46 is the BOC-protected analogue of FLLL18. Initially, protection was attempted with chloromethyl ethyl ether. However, an unsymmetrical product was obtained, which signified that chloromethyl ethyl ether was reacting with the
deprotonated 1,3-diketone in addition to the phenoxide substituents. This is not surprising since both moieties have similar pK\textsubscript{a} values (\approx 9 in H\textsubscript{2}O). Fortunately, when the reaction was attempted with di-\textit{tert}-butyl dicarbonate, the symmetrical BOC-protected product was obtained in greater than 90% yield, as it was previously reported with curcumin.\textsuperscript{654} It is suspected that di-\textit{tert}-butyl dicarbonate was sufficiently sterically hindered to result in the exclusive formation of the desired product. Regardless, due to the large substituents, JE46 was not expected to be active. However, since we ultimately had in our possession both JE46 and the alkylated analogue, JE47, we thought that it would be interesting to see if the addition of the cyclohexyl moiety would be enough to allow JE47 to exhibit some level of activity.

\begin{center}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{R\textsubscript{1}} & \textbf{R\textsubscript{2}} & \textbf{DU145} & \textbf{HT29} & \textbf{MDA-MB-231} \\
\hline
Curcumin & OCH\textsubscript{3} & OH & 30.8 ± 1.9 & 10.1 ± 4.2 & 28.4 ± 4.3 \\
FLLL10 & OCH\textsubscript{3} & OCH\textsubscript{3} & 6.94 ± 0.12 & ND & 4.9 ± 0.9 \\
FLLL18 & OH & OCH\textsubscript{3} & 7.47 ± 0.95 & 7.23 ± 1.70 & 3.95 ± 0.61 \\
JE46 & OCO\textsubscript{2}C(CH\textsubscript{3})\textsubscript{3} & OCH\textsubscript{3} & >150 & >150 & >150 \\
FLLL24 & H & OCH\textsubscript{3} & >50 & >150 & >150 \\
\hline
\end{tabular}
\end{center}

\begin{center}
Table 26. Structures and antiproliferative activities of curcumin analogues lacking the cyclohexyl moiety. (Curcumin was synthesized by Dr. James Fuchs. Dr. Bulbul Pandit obtained the IC\textsubscript{50} value for FLLL10 in MDA-MB-231 cells.)
\end{center}
In all cases, the analogues containing the cyclohexyl moiety had dramatically increased potency (Table 27). The increases in activity are less dramatic for FLLL49, FLLL32 and JE48, since the parent compounds, curcumin, FLLL10 and FLLL18, all display moderate levels of activities. However, the addition of the cyclohexyl moiety to the BOC-protected analogue resulted in an IC\textsubscript{50} value of less than 8 µM in MDA-MB-231 cells, and the increased activity of JE39 is even more telling. FLLL24 did not achieve an IC\textsubscript{50} of under 50 µM in any cancer cell line, while the cyclohexyl-containing analogue, JE39, achieved IC\textsubscript{50} values of less than 15 µM in HT29 cells, and less than 7 µM in DU145 and MDA-MB-231 cells. Together, these data demonstrate that the presence of the cyclohexyl moiety leads to significantly enhanced activity. Similar effects will also be seen in the next chapter on FLLL32 analogues which feature electron withdrawing substituents. It should be noted that JE47 was relatively inactive in DU145 and HT29 cells, confirming that the bulky BOC substituents are not ideal.

It should also be noted that the relative activities of compounds within each series are the same. The compounds containing the BOC substituents, JEs 46 and 47, were the least active. Those containing the 4-methoxy substituents were the next least active (FLLL24 and JE39). The vanillin-containing compounds, curcumin and FLLL49, displayed moderate levels of activity. In contrast, for each of the two series, the compounds containing either the isovanillin or 3,4-dimethoxybenzene substitution patterns were the most potent, and had similar IC\textsubscript{50} values in all cell lines.
Table 27. Structures and antiproliferative activities of curcumin analogues featuring the cyclohexyl moiety. (FLLLs 32 and 49 were synthesized by Eric Schwartz in the lab of Dr. James Fuchs.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>DU145</th>
<th>HT29</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL49</td>
<td>OCH₃</td>
<td>OH</td>
<td>6.20 ± 0.96</td>
<td>ND</td>
<td>9.82 ± 1.12</td>
</tr>
<tr>
<td>FLLL32</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>1.41 ± 0.06</td>
<td>2.31 ± 0.36</td>
<td>1.29 ± 0.16</td>
</tr>
<tr>
<td>JE48</td>
<td>OH</td>
<td>OCH₃</td>
<td>1.47 ± 0.24</td>
<td>2.07 ± 0.44</td>
<td>1.09 ± 0.01</td>
</tr>
<tr>
<td>JE47</td>
<td>OCO₂C(CH₃)₃</td>
<td>OCH₃</td>
<td>&gt;50</td>
<td>&gt;150</td>
<td>7.22 ± 0.67</td>
</tr>
<tr>
<td>JE39</td>
<td>H</td>
<td>OCH₃</td>
<td>6.73 ± 0.12</td>
<td>14.3 ± 2.2</td>
<td>5.20 ± 0.14</td>
</tr>
</tbody>
</table>

It should also be noted that from the initial studies of curcumin analogues, it was thought that FLLL18 was inactive. However, after reevaluating FLLL18 numerous times, it seems to be clear that its interpreted inactivity was due to high absorbance readings that resulted from the presence of FLLL18 at high concentrations, not living cells. Many times the resulting curves that were obtained were inaccurate, as extremely high absorbances were recorded at high concentrations of FLLL18, low absorbances were recorded when the concentration of FLLL18 was low enough to not produce high absorbance readings but still high enough to exert antiproliferative effects, and high absorbance readings were recorded at low concentrations of FLLL18, where it was not able to inhibit cell growth.
8.4 Summary of the Data Obtained for the Additional Analogues

In summary, numerous curcumin analogues have been synthesized and evaluated, which has provided a wealth of information about the SARs of these compounds. Importantly, the SARs of the monocarbonyl and dicarbonyl analogues are very different. Many of the compounds discussed in this chapter were also evaluated in MDA-MB-468 and DU145 cells at concentrations of 10 and 50 µM (Figures 105 and 106). Without exception, the activities observed in those studies line up well with the IC$_{50}$ values that have been discussed. The data is also helpful in verifying the activity of certain compounds that were not tested across a range of doses in the process of obtaining an IC$_{50}$ value. For example, an IC$_{50}$ value was not derived for FLLL41 in DU145 cells, but its inhibitory activities at 10 and 50 µM in DU145 cells can still be seen.

While the quest has been to develop anticancer compounds in general, a particular emphasis has been placed on developing STAT3 inhibitors. A curcumin-derived scaffold has been successfully identified. It has allowed for the development of potent inhibitors of STAT3. There have been many unique compounds that have been synthesized along the way, which have helped to confirm what has been predicted by both computational modeling and the other cell studies. Hopefully, using the FLLL32 scaffold for future studies will lead to the identification a potent STAT3 inhibitor with an improved pharmacokinetic profile. In the next chapter, the most recent curcumin and FLLL32 analogues will be discussed, which contain electron withdrawing substituents. As it will be seen, the data obtained for those compounds are encouraging, and their structures
leave room for improvements to be made as far as both potency and aqueous solubility are concerned.

Figure 105. Antiproliferative activities of selected compounds in DU145 cells at concentrations of 10 and 50 µM.
Figure 106. Antiproliferative activities of selected compounds in MDA-MB-468 cells at concentrations of 10 and 50 µM.
Chapter 9: SAR Studies of Curcumin and FLLL32 Analogues Featuring Electron Withdrawing Substituents

9.1 Introduction

Many curcumin and FLLL32 analogues have been synthesized and evaluated. However, only recently have the effects that the presence of electron withdrawing substituents on the aromatic rings have on STAT3 inhibition and cellular activity been investigated. All previous analogues featured electron donating groups; most contained hydroxyl and methoxy substituents in particular.

Curcumin analogues were synthesized as it has been previously described, using the procedure established by Venkateswarlu and coworkers. FLLL32 analogues were synthesized by alkylating the central carbon of the 1,3-diketone. Deprotonation was accomplished with $K_2CO_3$, and subsequent inter- and intra-molecular $S_N2$ reactions with 1,5-diiodopentane produced the desired FLLL32 analogues.

Seven curcumin analogues featuring electron withdrawing substituents were synthesized. They consist of analogues featuring fluorine, chlorine, nitro or cyano substituents at the 2, 3 or 4 positions of the aromatic rings. FLLL32 analogues of the fluorine- and chlorine-containing compounds were also synthesized. Unfortunately, the nitro- and cyano-containing curcumin analogues were not obtained in large enough yields to take on to the next step.
The analogues were evaluated for their antiproliferative activities in DU145, HT29 and MDA-MB-231 cancer cell lines. The molecular targets of selected analogues were also evaluated via Western blot analysis. Furthermore, selected compounds were evaluated in the STAT3 FP assay in order to determine their abilities to bind to the STAT3 SH2 domain.

9.2 Antiproliferative Activities of Curcumin and FLLL32 Analogues

The curcumin analogues exhibited activities that were relatively similar to those of curcumin (Table 28). The analogues that were para-substituted with chlorine or fluorine were the least active. They demonstrated no antiproliferative activities. The compounds that were para-substituted with nitro or cyano substituents also exhibited modest levels of activity. The cyano-containing analogue, JE33, was more active than the nitro-containing analogue, JE32, in all three cell lines. JE33 was approximately twice as potent as curcumin in MDA-MB-231 cells, but it was four times less potent than curcumin in HT29 cells. In DU145 cells, JE33 and curcumin had similar potencies.

Compounds that were meta-substituted with fluorine or chlorine displayed intermediate levels of activity. Both JE35 and JE38 were significantly more potent than curcumin in DU145 and MDA-MB-231 cells, but significantly less potent than curcumin in HT29 cells. The ortho-substituted analogue, JE31, had detectable levels of antiproliferative activities, but it was less potent than curcumin in all three cell lines.
Concerning the halogen-containing analogues, the meta-substituted derivatives were the most active, the para-substituted derivatives were the least active, and the ortho-substituted derivative exhibited an intermediate level of antiproliferative activity. It is likely that the position of the halogen on the rings affects the electronics of the compounds and the acidity of the central protons. Perhaps this could affect the equilibriums between the keto-enol and diketone forms and play a role in the resulting activities of the compounds. However, it is more likely that the position of the substituents significantly affects the molecular interactions between the compounds and molecular targets. FLLls 3 and 24, which feature para-hydroxyl or -methoxy substituents, but lack a meta-substituent, exhibit similar (FLLL3) or greatly reduced (FLLL24) potencies when compared to curcumin. Either way, the data presented here
supports the conclusion that a substituent at the *meta* position is critical for activity, as it concerns curcumin analogues.

The *meta*-substituted analogues, JEs 42 and 44, were also the most potent compounds in the FLLL32 series (**Table 29**). JE44 was close to as active as FLLL32 in DU145 cells, as active as FLLL32 in HT29 cells, and more active than FLLL32 in MDA-MB-231 cells. JE42 was as active as FLLL32 in DU145 and HT29 cell lines, and more active than FLLL32 in MDA-MB-231 cells.

![Chemical structure](image)

**Table 29.** Structures and antiproliferative activities of FLLL32 analogues featuring electron withdrawing substituents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>DU145</th>
<th>HT29</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLLL32</td>
<td>H</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.41 ± 0.06</td>
<td>2.31 ± 0.36</td>
<td>1.29 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>JE41</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>3.10 ± 0.24</td>
<td>4.78 ± 0.58</td>
<td>1.45 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>JE42</td>
<td>H</td>
<td>F</td>
<td>H</td>
<td>1.45 ± 0.17</td>
<td>2.26 ± 0.61</td>
<td>0.647 ± 0.060</td>
<td></td>
</tr>
<tr>
<td>JE40</td>
<td>H</td>
<td>H</td>
<td>F</td>
<td>2.07 ± 0.32</td>
<td>3.76 ± 0.73</td>
<td>1.14 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>JE44</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>2.11 ± 0.38</td>
<td>2.66 ± 0.22</td>
<td>0.710 ± 0.076</td>
<td></td>
</tr>
<tr>
<td>JE43</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>2.56 ± 0.09</td>
<td>3.91 ± 0.54</td>
<td>1.69 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>

The fluorine- and chlorine-containing compounds had similar activities, but in general, the analogues featuring fluorine substituents were slightly more potent than the corresponding chlorine-containing analogues. Within the fluorine series, the *meta*-substituted analogue, JE42, was the most active; the *para*-substituted analogue, JE40, was almost as potent; and the *ortho*-substituted analogue, JE41, was the least active of the
three. This is in contrast to the curcumin analogues, where the ortho-substituted analogue, JE31, was more active than both para-substituted derivatives (JEs 30 and 36). However, all of the FLLL32 derivatives were extremely active in all cell lines. In MDA-MB-231 cells, all of them achieved IC\textsubscript{50} values of 1.69 µM or below.

The importance of the cyclohexyl moiety for these analogues is quite apparent. The curcumin analogues that featured para-substituents did not have any detectable antiproliferative activities, while the corresponding FLLL32 analogues achieved IC\textsubscript{50} values of 4.78 µM or below across all cell lines. Overall, the addition of the cyclohexyl moiety increased the potency of all curcumin analogues by 10-fold or more.

9.3 Inhibition of STAT3 Phosphorylation by FLLL32 Analogues

Western blot analysis was utilized in order to confirm that the potent FLLL32 analogues were exerting their antiproliferative activities through the inhibition of STAT3 signaling (Figure 107). The expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL, which are two target proteins of STAT3, were also monitored.\textsuperscript{33,169} In order to determine selectivity, the levels of Akt and mTor expression were evaluated as well.

The FLLL32 analogues were found to be potent inhibitors of STAT3 phosphorylation. JEs 41 and 42 were able to down-regulate pSTAT3 in a dose-dependent manner. Consistent with the determined IC\textsubscript{50} values in MDA-MB-231 cells, JE42 was the most potent inhibitor of the FLLL32 analogues. It down-regulated pSTAT3 similarly to FLLL32 at a concentrations of 2.5 µM, but was superior in its inhibitory activity at a concentration of 5 µM.
Figure 107. Mediated cellular effects by FLLL32 and analogues in MDA-MB-231 cells, as determined by Western blot analysis. Cells were treated with the indicated concentrations of compounds for 24 hours.
JE43 was the least potent analogue in MDA-MB-231 cells. Consistent with this, among the analogues evaluated, it also resulted in the smallest down-regulation of pSTAT3 at a concentration of 5 µM. However, all analogues significantly down-regulated pSTAT3 at a concentration of 5 µM without drastically affecting the overall STAT3 levels. JE42 achieved the lowest IC₅₀ values in the cell lines, and it was also the most potent inhibitor of STAT3 phosphorylation.

The analogues also effectively reduced the expression of Bcl-2, a STAT3 target gene. At a concentration of 5 µM, the JE compounds down-regulated Bcl-2 in a manner similar to what was observed for FLLL32. At a concentration of 2.5 µM, JE42 was the most effective at reducing Bcl-2 levels, as it did so significantly more than FLLL32. With the exception of JE43, the analogues also down-regulated Bcl-xL. Again, JE42 was slightly more effective in doing so than FLLL32.

JEs 41-43 also demonstrated selectivity. These compounds did not significantly down-regulate mTor or the phosphorylation at Ser²⁴⁴⁸. The levels of Akt and phosphorylation at Thr³⁰⁸ were also unaffected. However, JEs 40 and 44 did noticeably down-regulate the phosphorylation of Akt at Ser⁴⁷³. These results were unexpected. In particular, JE44 appears to be a potent inhibitor of the phosphorylation of Akt at Ser⁴⁷³, although the mechanism of its inhibition was not pursued. However, the parent compound, curcumin, has been shown to inhibit Akt phosphorylation in several cell lines.⁵⁸⁶,⁵⁸⁹
9.4 Evaluation of Analogues in the STAT3 FP Assay

The analogues were further evaluated in the STAT3 FP assay in order to evaluate their abilities to bind to the STAT3 SH2 domain. SpYLPQTV and SYLPQTV were used at a concentration of 10 µM as positive and negative controls, respectively. As expected, the presence of 10 µM SpYLPQTV led to 93.2 ± 2.0% inhibition of the FP signal, while the same amount of the unphosphorylated peptide, SYLPQTV, did not result in any inhibition of the FP signal.

In general, the analogues were able to inhibit the FP signal in a dose-dependent manner (Figure 108). Consistent with the cell data, JE42 proved to have the most activity in the FP assay, still inhibiting the FP signal by greater than 25% at 250 µM. JE44 also achieved notable inhibitory activity at this concentration. JE40 exhibited slightly reduced activity, and JE43 was even less active. JE41 displayed the least activity, both at 500 and 250 µM. This is again in line with the obtained cell data.

9.5 Discussion

A series of potent FLLL32 analogues has successfully been identified. Across three cell lines the analogues achieved IC_{50} values of 0.647 to 4.78 µM. Overall, JE42 was found to be the most potent analogue in the cell studies, as it exhibited to similar to increased potencies when compared to FLLL32. JE42 was also more effective than FLLL32 at down-regulating pSTAT3, Bcl-2 and Bcl-xL in MDA-MB-231 cells. JE42 was selective in targeting STAT3, as it did not down-regulate mTor or Akt levels at the evaluated concentrations.
Figure 108. Inhibitory activities of FLLL32 analogues in the STAT3 FP assay.

The data demonstrates that all of the FLLL32 analogues exert their effects, at least in part, by inhibiting the phosphorylation of STAT3 at Tyr\(^{705}\). JEs 41-43 were all selective for STAT3, and did not affect Akt or mTor levels. However, two of the compounds, JEs 40 and 44, also appear to down-regulate the phosphorylation of Akt at Ser\(^{473}\) without affecting mTor, which is interesting in light of the relationship between the two.

The Akt pathway has been implicated in the phosphorylation of mTor,\(^{655}\) and it has even been suggested that Akt is directly responsible for phosphorylating mTor at Thr\(^{2446}\) and Ser\(^{2448}\).\(^{656,657}\) However, through the evaluation of mTor mutants exhibiting alanine substitutions at these positions, it was found that these phosphorylations were not
required for the activation of p70 ribosomal S6 protein kinase 1 (S6K1) or the phosphorylation of eukaryotic initiation factor 4E binding protein 1 (4EBP1), which are two downstream targets of mTorC1. As a result, the Akt-mediated activation of mTorC1 is not thought to be dependent on the phosphorylation of mTor at Ser^{2448}. In contrast, rapamycin, which has been shown to be an inhibitor of mTor function, blocks the phosphorylation at Ser^{2448} of mTor without affecting Akt phosphorylation at Ser^{473}. There is evidence that ribosomal S6 kinase 1 (S6K1), which is a substrate of mTor, is responsible for the phosphorylation of mTor at Thr^{2446} and Ser^{2448}. Ser^{2448}-phosphorylated mTor has been found to be a component of both mTor complexes, but the down-regulation of Ser^{2448} phosphorylation has been associated with lowered mTorC1 activity, but not altered mTorC2 activity. Consistent with this discrepancy, it was found in another study that mTor autophosphorylated at Ser^{2481} is the predominant component of mTorC2. Regardless, if S6K1 is both activated by mTor and responsible for phosphorylating mTor at Ser^{2448}, then the level of Ser^{2448} phosphorylation can act as an indicator of the amount of mTor signaling that is occurring in cells.

Wortmannin is a PI3K inhibitor. In the presence of wortmannin, the phosphorylation of Akt at Thr^{308} and Ser^{473} is blocked. Wortmannin also blocks the phosphorylation of mTor at Ser^{2448}. These data suggest that the activation of Akt and mTor are linked, but the fact that mTor phosphorylation at Ser^{2448} is not highly correlated with Akt activation suggests otherwise. PDK1 is also activated by PI3K, and is responsible for phosphorylating Thr^{229} in the activation loop of S6K1.
As a result, wortmannin could be inhibiting Ser\textsuperscript{2448} phosphorylation independently of Akt activation.

Our data supports this notion, as JEs 40 and 44 significantly down-regulated Akt phosphorylation at Ser\textsuperscript{473}, but did not affect the level of mTor phosphorylation at Ser\textsuperscript{2448}. Since inhibition of PI3K would also presumably lead to decreased phosphorylation at both Ser\textsuperscript{2448} of mTor and Ser\textsuperscript{473} of Akt, it is not likely that they are acting by directly inhibiting PI3K. The compounds could be directly binding to Akt. They could also be inhibiting an upstream kinase which is responsible for phosphorylating Akt at Ser\textsuperscript{473}, such as ILK\textsuperscript{194,195} or DNA-PK\textsuperscript{196,197}. PDK1 is also thought to be capable of gaining Ser\textsuperscript{473} kinase activity\textsuperscript{198}, but if the compounds were inhibiting PDK1, a reduction in the phosphorylation of mTor at Ser\textsuperscript{2448} and Akt at Thr\textsuperscript{308} should also be observed\textsuperscript{192}.

More studies will have to be carried out to determine their mechanism(s) of down-regulating phosphorylation at Ser\textsuperscript{473} of Akt. It is peculiar that the two analogues that inhibited Akt phosphorylation featured halogen substituents at different positions. Why these compounds targeted Akt while the other analogues did not is unknown. However, the hope was that these compounds would be potent and selective inhibitors of STAT3 phosphorylation, and the majority of the FLLL32 analogues accomplished this selectivity, at least among the molecular targets that were evaluated.

Concerning the FP assay, it has been mentioned that problems with low aqueous solubilities of test compounds and high FP readings resulting from the presence of test compounds has been encountered. The analogues evaluated here were no exception. Curcumin-derived compounds seem to be exceptionally problematic in this regard; the
presence of JEs 31, 35 and 38 resulted in extremely high FP readings at concentrations of 500 to 62.5 µM even when only the probe was present. They were not fit for the assay. These high FP readings can result from the probe non-specifically binding to aggregates of compounds, light scattering resulting from the low aqueous solubilities of the compounds and the nature of the precipitate, or the autofluorescence of aggregate-forming fluorescent compounds which effectively become much higher molecular weight compounds. 618,627,629,630,631,632

JEs 30, 33 and 36 also had significant problems in this regard, although not to the same extent as the previously mentioned compounds. The 4-nitro analogue, JE32, was the only curcumin-derived analogue that did not result in high and anomalous FP readings when only compound and probe were present in buffer; this is likely a result of increased solubility in the aqueous buffer due to the presence of the polar nitro groups.

In contrast, FLLL32-related analogues are still not highly soluble in aqueous solutions, but tend to not be problematic in regards to light scattering or aggregate formation. Incorporation of 10% DMSO in the FP buffer helped to improve the solubility of test compounds. However, for certain compounds, insolubility in the FP buffer remains a significant problem.

As a result, it can be difficult to compare compounds’ abilities to bind the SH2 domain based on FP data, since solubility appears to play such a major factor. The major drawback is that high concentrations of test compounds are often necessary in FP assays. 627 This insolubility of test compounds at concentrations required for the FP assay
can often lead to false negatives, or at the least, the observation of inaccurately low inhibitory activities.

Here, the abilities of the compounds to bind to the STAT3 SH2 domain do correlate well with the cellular activities, at least among the compounds evaluated. This is logical in light of the structural similarities between these compounds; the position of or difference in the halogen on the aromatic rings would not be expected to significantly alter their solubility profiles. In support of this, the calculated logP value of all three analogues containing fluorine substituents is 5.94, while the calculated logP value for the chlorine-containing analogues is 6.74 (ChemDraw Ultra 12.0). Still, the discrepancies in the cellular and FP assays are apparent, and are likely due to the relatively low aqueous solubilities of the compounds.

Similar to our results, Cpd 188 has been reported to have EC$_{50}$ values of 0.73 and 3.96 µM in MDA-MB-468 and MDA-MB-231 cells, respectively. However, in a SPR assay its IC$_{50}$ for inhibiting the binding of a phosphotyrosine peptide ligand to STAT3 was significantly higher (20 µM).\textsuperscript{554} It should be noted that when analyzing peptidomimetics, the opposite has been observed. For example, 14aa was found to have a $K_i$ of 5 ± 1 µM in the FP assay. However, in several cancer cell lines it only achieved IC$_{50}$ values of roughly 50 µM, which the authors attributed to poor cell permeability and metabolic stability.\textsuperscript{551}

The possibility that the compounds presented here are also acting through other mechanisms, in addition to inhibiting STAT3, can’t be ruled out. JEs 40 and 44 appear to inhibit Akt activation, and the parent compound, curcumin, is known to have numerous
targets. However, the abilities of the compounds to abrogate Tyr$^{705}$ phosphorylation signifies that the potencies of the compounds presented here are largely a result of targeting STAT3.

The presence of the cyclohexyl moiety was found to be critical to the activities of these compounds. It is able to lock the analogues into the diketone conformation, which is predicted to bind to STAT3. In addition, it is predicted to interact with an additional pocket of STAT3 when compared to curcumin, which is also predicted to increase the activities of these compounds. The presence of this moiety led to dramatic increases in activity, as JEs 30 and 36 did not exhibit any antiproliferative activity, while the corresponding FLLL32 analogues achieved IC$_{50}$ values below 4 µM in all cell lines in which they were evaluated.

The quest for potent STAT3 inhibitors that are both potent and have therapeutically relevant pharmacokinetic profiles is ongoing. However, the results presented here are encouraging. In chapter 4, which presented the SAR studies of curcumin analogues, it was demonstrated that analogues that were substituted at both the meta and para positions were more potent than compounds that were monosubstituted.$^{574}$ The most potent compounds presented here, JE42 and JE44, are only substituted at the meta position. It would stand to reason that an additional substituent at the para position, such as a methoxy, hydroxyl, or phosphoryl substituent, could dramatically increase the potency and/or aqueous solubility of these FLLL32 derivatives. This could potentially result in the discovery of more therapeutically relevant compounds with improved
pharmacokinetic profiles. However, additional analogues will first need to be synthesized and evaluated.
Chapter 10: SAR Studies of LLL Analogue

10.1 Introduction

The first synthesized LLL compounds were based on STA-21.\textsuperscript{552,561} STA-21 was identified through virtual screening and was predicted to bind the STAT3 SH2 domain.\textsuperscript{552} It was the first reported small molecule inhibitor of the STAT3 SH2 domain.\textsuperscript{552}

STA-21 is structurally complex, making the production of STA-21 analogues for SAR studies difficult.\textsuperscript{561} As a result, the structure was simplified in the production of the initial LLL compounds.\textsuperscript{561} The anthraquinone moiety was retained, while the fourth ring was discarded.\textsuperscript{561} This most potent analogue identified was LLL3, which was subsequently evaluated in glioblastoma.\textsuperscript{563} Further studies led to the discovery of LLL12 (Figure 109), which is the most potent STAT3 inhibitor that has been identified to date.\textsuperscript{562} Over the past few years LLL12 has been extensively studied.\textsuperscript{489,504,641,645,667,668,669,670,671,672,673}

![Figure 109. Structures of STA-21, LLL3 and LLL12.](image)

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10.2 Additional LLL Compounds and Synthetic Procedures

Additional analogues were synthesized and evaluated in order to further elucidate the SARs of these compounds and develop more potent STAT3 inhibitors. The majority of the compounds feature the anthraquinone moiety (Table 30). These analogues feature different substituents at position 1 of the anthraquinone ring, although all of them except for LLL15 contain either a carbonyl or a sulfonamide moiety directly adjacent to position 1. All of the analogues that are substituted at this position feature electron withdrawing substituents, which include esters, amides, sulfonamides, acids or a methyl ketone moiety.

Positions 5 and 8 of the anthraquinone moiety were also substituted in order to understand how the capability of hydrogen bonding at the two positions correlates with activity. All of the analogues feature substituents at either the 5 or 8 position, but none of them have substituents at both positions. The substituents at these positions consist of hydroxyl, propyl ether or benzyl ether groups.

There were also a number of intermediates that were analyzed. In the process of the syntheses of LLLs 3, 5, 6 and 12, the intermediates, termed LLLs 3.1, 5.1, 6.1 and 12.1, were generated (Table 31). These intermediates are 1,4-naphthoquinones, which were formed prior to the completion of the anthraquinone structures. These compounds are structurally simpler than the corresponding final analogues, as they lack the third ring. By studying them, the contribution of only the substituted 1,4-naphthoquinone portions can be evaluated.
Table 30. Structures of LLL compounds. (These analogues were synthesized by Dr. Deepak Bhasin and Somsundaram Chettiar in the lab of Dr. Pui-Kai Li.)

The synthetic procedures for several of the LLL compounds have been reported (LLLs 3, 3a, 3.1, 12 and 12.1).\textsuperscript{561,562} Many of the novel analogues presented here were synthesized in a similar fashion. The condensation of methyl crotonate with bis(dimethylamino)methoxymethane yielded \textit{tert}-aminodienylester, according to an established procedure (\textbf{Scheme 9}).\textsuperscript{674} Combination of the intermediate with juglone produced LLL5 exclusively. The ester was then hydrolyzed to produce LLL4.
Table 31. Structures of evaluated LLL intermediates and juglone. (These analogues were synthesized by Dr. Deepak Bhasin and Somsundaram Chettiar in the lab of Dr. Pui-Kai Li.)

Scheme 9. Syntheses of LLLs 4 and 5.

The oxidation of commercially available 1-naphthalene carboxamide produced LLL6.1 (Scheme 10). 3-Hydroxy-2-pyrone then underwent a Diels-Alder type reaction to
regioselectively produce LLL6. It is postulated that hydrogen bonding occurs between the protons of the carboxamide and the carbonyl group, and that this resulted in the observed regioselectivity.

Scheme 10. Syntheses of LLLs 6 and 6.1.

DCC was used in the coupling of LLL4 with β-alanine ethyl ester hydrochloride or glycine methyl ester hydrochloride, which produced LLL7ester or LLL9ester, respectively. Hydrolysis with lithium hydroxide monohydrate then produced LLLs 7 and 9. LLLs 11, 11a, 13 and 13a were produced by the substitution reactions between LLL3 or LLL3a with propyl iodide or benzyl bromide (Scheme 11).

Scheme 11. Syntheses of LLLs 11, 11a, 13 and 13a. The syntheses of LLLs 11 and 13 are shown as examples.
Treatment of naphthalene-1-sulfonyl chloride with methyl amine hydrochloride produced \(N\)-methylnaphthalene-1-sulfonamide (Scheme 12). Oxidation and subsequent addition using the methods shown for the synthesis of LLL6 were then utilized to produce LLL14. Similarly to the synthesis of LLL7, DCC was used in the coupling of \(\beta\)-alanine ethyl ester hydrochloride with anthraquinone-2-carboxylic acid. Hydrolysis of the ester intermediate yielded LLL15. LLL5.1 was synthesized by esterification and subsequent oxidation. 1-Naphthoic acid was first reacted with methanol in the presence of a catalytic amount of sulfuric acid to produce methyl 1-naphthoate. Subsequent oxidation yielded LLL5.1.


10.3 Biological Evaluation of LLL Compounds

10.3.1 Antiproliferative Activities of LLL Compounds

The analogues were first evaluated in MDA-MB-231, PC3, HT29 and DU145 cell lines at concentrations of 0.5, 5 and 50 \(\mu\)M. The relative potencies of the compounds were fairly consistent across all cell lines (Figures 110-113). However, there were a few exceptions. At a concentration of 50 \(\mu\)M, LLLs 9 and 10 were significantly more active in DU145 and HT29 cells than in PC3 and MDA-MB-231 cells. At a concentration of 5
µM, this trend was also observed for LLL14. LLLs 13 and 12.1 were less active in HT29 cells than in the other cell lines. Some compounds, including LLLs 4, 7ester, 7, 11, 11a and 15, showed minimal activity in all cell lines at all tested concentrations. In contrast, with the exception of LLL12.1 in HT29 cells, all of the substituted 1,4-naphthoquinones (LLLs 3.1, 5.1, 6.1 and 12.1) nearly abrogated cell growth at a concentration of 50 µM. LLL12 was by far the most active compound in all cell lines.

The IC$_{50}$ values of all of the analogues were also determined in HT29 and DU145 cells, while the values of selected analogues were also derived in MDA-MB-231 cells (Table 32). The derived values were in line with the observed activities at 0.5, 5 and 50 µM. LLL12 was the most active compound in both cell lines, achieving IC$_{50}$ values of less than 200 nM. LLL14 was the next most potent compound of those that featured an anthraquinone moiety. However, the methylation of the sulfonamide is clearly not advantageous, as LLL14 was roughly 40 to 60 times less potent than LLL12.

It is interesting that LLLs 3 and 3a exhibited similar potencies in both cell lines. This suggests that there is flexibility as to the position of the hydroxyl substituent, at least as it concerns the methyl ketone analogues. However, the presence of a hydroxyl substituent is clearly important, as LLLs 11, 11a, 13 and 13a displayed no antiproliferative activity. No antiproliferative activity was observed regardless of whether the ether was at the 5 or 8 position, or the identity of the substituent was a propyl ether or benzyl ether.

In comparing LLLs 3-6, 7, 7ester, 9, 9ester and 12 it is clear that besides the sulfonamide moiety, the methyl ketone moiety (LLL3) leads to the most activity out of
the substituents analyzed. Substituting with a carboxylic acid at this position resulted in a total loss of activity (LLL4). The presence of a methyl ester at this location was somewhat tolerated, but LLL5 was still roughly four times less potent than LLL3. Replacement with the primary amide was the most tolerated, as LLL6 was roughly half as potent as LLL3. Extending the amide of LLL6 with an acetic acid moiety did not appear to alter activity (LLL9), while extension with a methyl acetate moiety resulted in a three-fold reduction in activity (LLL9ester). The presence of larger substituents resulted in a total loss of activity (LLLs 7 and 7ester).

The activities of the four 1,4-naphthoquinone derivatives did not follow the same pattern as the anthraquinone derivatives. The major observed difference was that the methyl ketone and methyl ester derivatives were the most active (LLLs 3.1 and 5.1) of the 1,4-naphthoquinones, while the sulfonamide-containing LLL12.1 was among the least active. Also, LLL6 was more active than LLL5, while LLL5.1 was more active than LLL6.1. LLL6.1, which contains a primary amide substituent, had similar activities to LLL12.1. Interestingly, it also had similar activities to LLL6.

In contrast, LLL3.1 was roughly twice as potent as LLL3, and LLL5.1 was four to seven times as potent as LLL5. 1,4-Naphthoquinone derivatives of LLLs 4, 7, 7ester, 9, 9ester, 14 and 15 were not evaluated. LLLs 9 and 14 were the only compounds of these that demonstrated noteworthy antiproliferative activities. However, it would be interesting to see if the corresponding 1,4-naphthoquinone derivatives would also lack activity, since identical trends were not observed for the analogues of the two series.
Figure 110. Antiproliferative activities of LLL compounds in MDA-MB-231 cells. (These values were obtained by Dr. May Mok in the lab of Dr. Pui-Kai Li.)

Figure 111. Antiproliferative activities of LLL compounds in PC3 cells. (These values were obtained by Dr. May Mok in the lab of Dr. Pui-Kai Li.)
Figure 112. Antiproliferative activities of LLL compounds in HT29 cells. (These values were obtained by Dr. May Mok in the lab of Dr. Pui-Kai Li.)

Figure 113. Antiproliferative activities of LLL compounds in DU145 cells. (These values were obtained by Dr. May Mok in the lab of Dr. Pui-Kai Li.)
<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (µM)</th>
<th>IC_{50} (µM)</th>
<th>IC_{50} (µM)</th>
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<tr>
<td></td>
<td>DU145</td>
<td>HT29</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td></td>
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<td></td>
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<tr>
<td>LLL3</td>
<td>11.5 ± 1.8</td>
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<td>10.2 ± 2.4</td>
<td>8.5 ± 0.6</td>
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<td>&gt;100</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
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<td>45.7 ± 7.5</td>
<td>37.3 ± 1.9</td>
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<td>LLL11</td>
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<td>&gt;100</td>
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<tr>
<td>LLL12</td>
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<td>LLL13</td>
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<tr>
<td>LLL14</td>
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<td>LLL15</td>
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</tr>
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</table>

Table 32. Antiproliferative activities of LLL compounds in DU145, HT29 and MDA-MB-231 cells. (These values were obtained by Jonathan Etter and Dr. May Mok in the lab of Dr. Pui-Kai Li.)

10.3.2 Evaluation of LLLs 3.1, 5.1 and 12.1 in the STAT3 FP Assay

Since LLL12 is known to inhibit STAT3 phosphorylation, it was evaluated in the FP assay along with LLLs 3.1, 5.1 and 12.1. As predicted, LLL12 inhibited the FP signal considerably. Unfortunately, the lone presence of LLL12 in the FP buffer also resulted in
relatively high FP readings, making it difficult to quantify its ability to inhibit the FP signal.

LLLs 3.1, 5.1 and 12.1 all inhibited the FP signal in a similar fashion (Figure 114). These data demonstrate that the evaluated 1,4-naphthoquinone derivatives are able to bind to the STAT3 SH2 domain. However, their similar activities in the assay make it difficult to determine the SARs of these compounds in regards to STAT3 inhibition.

It should be noted that the calculated logP values for these compounds are all 0.73 or below (ChemDraw Ultra 12.0). As a result, it was predicted that solubility in the aqueous FP buffer would not be a limiting factor in the evaluation of these 1,4-napthoquinones. This proved to be the case.

**Figure 114.** Inhibitory activities of LLL compounds in the STAT3 FP assay at a concentration of 500 μM. SpYLPQTV and SYLPQTV were used as controls at a concentration of 100 μM.
10.4 Discussion

The differences in the structural characteristics that led to activity in the anthraquinone and 1,4-naphthoquinone series are intriguing. These data suggest that the analogues of the two series are binding to biological targets through different interactions. None of the analogues were nearly as potent as the previously known LLL12. However, LLLs 3.1 and 5.1 were found to be relatively potent, which is encouraging. It is possible that by using LLLs 3.1 and 5.1 as lead compounds, the discovery of more potent 1,4-naphthoquinones could result. There are still several positions of the 1,4-naphthoquinone core of LLLs 3.1 and 5.1 that could be functionalized, and in comparing the potencies of LLL12.1 and PDs 13-15, which will be presented in the next chapter, it is clear that additional substitutions can be advantageous. In addition, further functionalization of the LLL12 core could potentially result in more potent anthraquinone derivatives as well.

The sulfonamide portion of LLL12 is predicted to bind to the STAT3 Tyr^{705} site in an interaction than involves at least three hydrogen bonds. The sulfonamide moiety is able to act as both a hydrogen bond acceptor and donor. In contrast, the substituents of the analogues not containing a sulfonamide at this position are only able to act as hydrogen bond acceptors.

Still, it is perplexing that LLL12.1 is not the most potent of the 1,4-naphthoquinones, at least if one is assuming that the 1,4-naphthoquinone analogues bind to STAT3 in the same way. Based on the data obtained from the FP assay, the conclusion can be made that both LLL12 and the 1,4-naphthoquinone analogues are able to bind to
the STAT3 SH2 domain. Nonetheless, it is possible that the smaller sizes of the 1,4-naphthoquinone analogues results in them binding to the SH2 domain with a slightly different geometry. This could make the sulfonamide-containing compounds no longer the tightest binding of the monosubstituted 1,4-naphthoquinone analogues. However, the 1,4-naphthoquinone derivatives could also be acting through additional MOAs. Their resulting effects on cellular signaling in cancer cell lines will need to be determined to make further conclusions. Additional studies will hopefully result in the discovery of more potent analogues and further elucidation of their MOA(s).
Chapter 11: SAR Studies of 1,4-Naphthoquinones as STAT3 Inhibitors

11.1 Introduction

Substituted 1,4-naphtoquinones were introduced in the preceding chapter. Here, the SARs of substituted 1,4-naphtoquinones, as it concerns anticancer activity and STAT3 inhibition, will be discussed. Many members of the quinone family are 1,4-naphtoquinones. These compounds are known to produce reactive oxygen species, including hydroxyl and superoxide radicals. They can also act as Michael acceptors, and are able to react with a number of biological targets. They are known to have a number of biological activities, as they have been shown to have anti-inflammatory, antifungal, antiarthrosclerotic, antiviral, antibacterial, and anticancer effects.

Juglone is a component of Juglans mandshuria, whose fruit has been termed “Qing Long Yi,” which has been used to treat cancer in traditional Chinese medicine. Other 1,4-naphtoquinones, like plumbagin and menadione, are also being investigated for their potential as anticancer therapeutics. Plumbagin is the major component of Plumbago species. It has been investigated for its anti-parasitic and anticancer properties. Interestingly, STAT3 signaling has been shown to be a target of plumbagin. Plumbagin is able to inhibit STAT3 phosphorylation and DNA binding. It has been shown to induce the expression of SHP1 in U266 cells, suggesting that the up-regulation of this PTP is a probable MOA. Menadione, which is
also called vitamin K3, has also been shown to have anticancer effects, as have other vitamin K forms. The structures of the basic 1,4-naphthoquinone, the three derivatives that have been mentioned, and LLL12.1 are shown (Figure 115).

![Structures of 1,4-naphthoquinones](image)

**Figure 115. Structures of 1,4-naphthoquinones.**

In an effort to discover 1,4-naphthoquinone derivatives that have more potent anticancer properties, and to study the SARs of these types of compounds, a series of analogues were synthesized based on the structures of 1,4-naphthoquinone, juglone, plumbagin, menadione and LLL12.1. The compounds listed above, as well as 18 additional compounds, termed PD compounds, were evaluated in DU145, HT29 and MDA-MB-231 cancer cell lines. Additionally, the majority of the compounds were also evaluated in the STAT3 FP assay. It should be mentioned that much of the discussion of the 1,4-naphthoquinones and the data presented in this chapter were originally published in Bioorganic & Medicinal Chemistry. Bhasin, D.; Chettiar, S. N.; Etter, J. P.; Mok, M.; Li, P.-K. *Bioorganic & Medicinal Chemistry*. 2013; 21:4662-9. © Elsevier Ltd.
11.2 Synthesis of Substituted 1,4-Naphthoquinones

The syntheses of seven of the PD compounds have been previously reported. These include PDs 1-4, 6, 8 and 18. The structure of 1,4-naphthoquinone inspired the design of PDs 1 and 2. PDs 3-5, 9 and 10 are based on juglone; PDs 6-8 are menadione analogues; PDs 11-15 are LLL12.1 derivatives; PDs 16-18 are derived from plumbagin. The structures of the parent compounds and the analogues are shown (Table 33).

PDs 1-5 and 13-15 were synthesized via the amination of 1,4-naphthoquinone, juglone or LLL12.1 with aniline, m-anisidine or p-anisidine (Scheme 13). The reaction conditions were similar to a reported procedure. However, the Lewis acid catalyst, cerium(III) chloride, was not used in these reactions.

PDs 6, 7, 16 and 17 were prepared by the oxidative coupling of plumbagin or menadione with m- or p-anisidine in acetic acid (Scheme 14). Copper acetate was used as a catalyst at a concentration of 10%. The reactions were carried out according to a reported procedure, where the presence of copper acetate in acetic acid was shown to promote the oxidative coupling.

PDs 8-12 and 18 were prepared by reacting juglone, menadione, plumbagin or LLL12.2 with 2-thioethanol in the presence of 10% copper(II) sulfate in a solvent mixture of 2-propanol and methanol (Scheme 15). The presence of copper(II) sulfate has previously been shown to increase the yields of these types of reactions.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>R\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plumbagin</td>
<td>OH</td>
<td>CH\textsubscript{3}</td>
<td>H</td>
</tr>
<tr>
<td>Juglone</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>1,4-Naphthoquinone</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Menadione</td>
<td>H</td>
<td>CH\textsubscript{3}</td>
<td>H</td>
</tr>
<tr>
<td>LLL12.1</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>PD1</td>
<td>H</td>
<td>H</td>
<td>NHPH</td>
</tr>
<tr>
<td>PD2</td>
<td>H</td>
<td>H</td>
<td>NHPH(4-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD3</td>
<td>OH</td>
<td>H</td>
<td>NHPH</td>
</tr>
<tr>
<td>PD4</td>
<td>OH</td>
<td>H</td>
<td>NHPH(4-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD5</td>
<td>OH</td>
<td>H</td>
<td>NHPH(3-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD6</td>
<td>H</td>
<td>CH\textsubscript{3}</td>
<td>NHPH(4-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD7</td>
<td>H</td>
<td>CH\textsubscript{3}</td>
<td>NHPH(3-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD8</td>
<td>H</td>
<td>CH\textsubscript{3}</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
</tr>
<tr>
<td>PD9</td>
<td>OH</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
<td>H</td>
</tr>
<tr>
<td>PD10</td>
<td>OH</td>
<td>H</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
</tr>
<tr>
<td>PD11</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
<td>H</td>
</tr>
<tr>
<td>PD12</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
</tr>
<tr>
<td>PD13</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>H</td>
<td>NHPH(3-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD14</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>H</td>
<td>NHPH</td>
</tr>
<tr>
<td>PD15</td>
<td>SO\textsubscript{2}NH\textsubscript{2}</td>
<td>H</td>
<td>NHPH(4-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD16</td>
<td>OH</td>
<td>CH\textsubscript{3}</td>
<td>NHPH(4-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD17</td>
<td>OH</td>
<td>CH\textsubscript{3}</td>
<td>NHPH(3-OCH\textsubscript{3})</td>
</tr>
<tr>
<td>PD18</td>
<td>OH</td>
<td>CH\textsubscript{3}</td>
<td>S(CH\textsubscript{2})\textsubscript{2}OH</td>
</tr>
</tbody>
</table>

Table 33. Structures of the 1,4-naphthoquinone parent compounds and derivatives. (These analogues were synthesized by Dr. Deepak Bhasin and Somsundaram Chettiar in the lab of Dr. Pui-Kai Li.)

Scheme 14. Syntheses of PDs 6, 7, 16 and 17.

Scheme 15. Syntheses of PDs 8-12 and 18.
Theoretically, many of these reactions could result in isomeric mixtures. However, in general, the reactions involving juglone have been shown to favor a predominant isomer, which varies depending on the reactant and reaction conditions. For the majority of the reactions reported here, a predominant isomer was formed, while the other isomer was present in trace amounts. An exception to this was the reaction between juglone and 2-thioethanol, where PDs 9 and 10 were obtained in almost equal quantities. The reaction of LLL12.1 with 2-thioethanol yielded both a mono- and di- substituted product. 2D NMR was utilized in order to make the proper structural assignments.

11.3 Biological Evaluation of Substituted 1,4-Naphthoquinones

11.3.1 Antiproliferative Activities of Substituted 1,4-Naphthoquinones

The 1,4-naphthoquinone analogues were evaluated for their antiproliferative activities in DU145, HT29 and MDA-MB-231 cancer cell lines (Table 34). 1,4-Naphthoquinone achieved IC$_{50}$ values of 4.48 to 12.76 µM. The addition of a methyl substituent at the R$_2$ position (menadione), a hydroxyl substituent at the R$_1$ position (juglone), or both (plumbagin) did not seem to greatly alter activity. Consistent with the data reported in the last chapter for 1,4-naphthoquinone analogues, the presence of a sulfonamide substituent at the R$_1$ position resulted in a decrease in activity (LLL12.1).

The presence of an aniline or p-anisidine moiety at the R$_3$ position (PDs 1 and 2) did not result in major differences in activity relative to 1,4-naphthoquinone, except that they lacked activity in MDA-MB-231 cells. The presence of an aniline, m-anisidine or p-
anisidine moiety at the R₃ position of the juglone core resulted in a general increase in activity (PDs 3-5), particularly in DU145 cells. For these compounds, the absence of a substituent at the R₁ position and the presence of a methyl substituent at the R₂ position (the menadione core) resulted in a decrease in activity; PDs 6 and 7 were less active than PDs 4 and 5 in all cell lines.

When menadione was substituted with 2-thioethanol at the R₃ position a general loss in activity was observed, with the exception of MDA-MB-231 cells, where PD8 was found to be roughly four times as potent as menadione. In contrast, the addition of the 2-thioethanol substituent at the R₂ or R₃ position of the juglone core resulted in increases in activity in all cell lines. PDs 9 and 10 achieved IC₅₀ values of 3.62 µM or below.

The presence of the sulfonamide substituent at the R₁ position resulted in the most potent compounds (PDs 13-15). PDs 13-15 also contained an aromatic amine at the R₃ position and lacked an R₂ substituent. PDs 13-15 showed vastly increased activity relative to LLL12.1. The identity of the aromatic amine did not seem to matter greatly. However, PD15, which contains a p-anisidine moiety at the R₃ position, was the most potent in all cell lines. It achieved IC₅₀ values of 1.05 to 1.73 µM. In contrast, the addition of the 2-thioethanol moiety at the R₂ or R₃ positions did not lead to compounds with comparable potencies (PDs 11 and 12).

The addition of aromatic amines to the R₃ position of the plumbagin core resulted in general decreases in activity (PDs 16 and 17). In contrast, with the exception of DU145 cells, the addition of a 2-thioethanol substituent at the R₃ position resulted in an increase in activity (PD18).
<table>
<thead>
<tr>
<th>Compound</th>
<th>DU145 (µM)</th>
<th>HT29 (µM)</th>
<th>MDA-MB-231 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plumbagin</td>
<td>5.23 ± 0.24</td>
<td>4.19 ± 0.06</td>
<td>21.24 ± 0.51</td>
</tr>
<tr>
<td>Juglone</td>
<td>11.67 ± 0.05</td>
<td>7.71 ± 0.88</td>
<td>7.15 ± 1.43</td>
</tr>
<tr>
<td>1,4-Naphthoquinone</td>
<td>10.89 ± 2.29</td>
<td>12.76 ± 1.12</td>
<td>4.48 ± 0.11</td>
</tr>
<tr>
<td>Menadione</td>
<td>9.86 ± 0.22</td>
<td>9.69 ± 0.42</td>
<td>12.68 ± 0.50</td>
</tr>
<tr>
<td>LLL12.1</td>
<td>14.39 ± 0.61</td>
<td>24.62 ± 1.40</td>
<td>16.13 ± 0.17</td>
</tr>
<tr>
<td>PD1</td>
<td>5.52 ± 1.25</td>
<td>31.25 ± 3.95</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>PD2</td>
<td>6.02 ± 1.37</td>
<td>12.64 ± 0.14</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>PD3</td>
<td>3.13 ± 0.70</td>
<td>10.02 ± 0.99</td>
<td>7.41 ± 0.06</td>
</tr>
<tr>
<td>PD4</td>
<td>2.85 ± 0.43</td>
<td>6.88 ± 0.30</td>
<td>2.67 ± 0.14</td>
</tr>
<tr>
<td>PD5</td>
<td>2.27 ± 0.38</td>
<td>5.51 ± 0.36</td>
<td>3.31 ± 0.03</td>
</tr>
<tr>
<td>PD6</td>
<td>8.12 ± 0.77</td>
<td>19.0 ± 6.6</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>PD7</td>
<td>15.16 ± 1.78</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>PD8</td>
<td>22.30 ± 1.55</td>
<td>17.5 ± 2.8</td>
<td>3.27 ± 1.09</td>
</tr>
<tr>
<td>PD9</td>
<td>3.62 ± 0.05</td>
<td>2.17 ± 0.59</td>
<td>2.66 ± 0.29</td>
</tr>
<tr>
<td>PD10</td>
<td>3.37 ± 0.29</td>
<td>2.11 ± 0.78</td>
<td>1.69 ± 0.05</td>
</tr>
<tr>
<td>PD11</td>
<td>2.85 ± 0.17</td>
<td>9.68 ± 1.73</td>
<td>9.68 ± 1.50</td>
</tr>
<tr>
<td>PD12</td>
<td>19.50 ± 4.75</td>
<td>26.6 ± 2.5</td>
<td>22.90 ± 0.70</td>
</tr>
<tr>
<td>PD13</td>
<td>1.47 ± 0.08</td>
<td>1.89 ± 0.14</td>
<td>2.22 ± 0.03</td>
</tr>
<tr>
<td>PD14</td>
<td>2.44 ± 0.23</td>
<td>1.97 ± 0.05</td>
<td>3.39 ± 0.64</td>
</tr>
<tr>
<td>PD15</td>
<td>1.22 ± 0.01</td>
<td>1.05 ± 0.08</td>
<td>1.73 ± 0.26</td>
</tr>
<tr>
<td>PD16</td>
<td>14.22 ± 2.28</td>
<td>15.65 ± 11.30</td>
<td>21.40 ± 1.64</td>
</tr>
<tr>
<td>PD17</td>
<td>10.28 ± 1.64</td>
<td>&gt; 100</td>
<td>20.51 ± 0.86</td>
</tr>
<tr>
<td>PD18</td>
<td>8.16 ± 2.35</td>
<td>2.16 ± 0.50</td>
<td>5.09 ± 1.21</td>
</tr>
</tbody>
</table>

Table 34. Antiproliferative activities of 1,4-naphthoquinones. (These values were obtained by Dr. May Mok in the lab of Dr. Pui-Kai Li.)
11.3.2 Evaluation of 1,4-Naphthoquinones in the STAT3 FP Assay

Due to the structural resemblance of the PD compounds, particularly PDs 13-15, to the known STAT3 inhibitor, LLL12, the 1,4-naphtoquinones were evaluated in the STAT3 FP assay. The compounds were initially evaluated at a 500 µM concentration in order to see if any of them warranted further evaluation (Figure 116). The parent compounds, plumbagin and juglone, were able to inhibit the FP signal by 58.1 and 76.4%, respectively. Additionally, PDs 9, 10, 11 and 18 demonstrated similar inhibitory activities.

Figure 116. Inhibitory activities of 1,4-naphthoquinones in the STAT3 FP assay at a concentration of 500 µM. SpYLPQTV and SYLPQTV were used as controls at a concentration of 100 µM.
Due to their suitability for and their high inhibitory activity in the STAT3 FP assay, PDs 9 and 18 were further investigated. Their \( K_i \) values were derived to be 13.3 ± 0.6 and 18.2 ± 4.8 µM, respectively. This is of similar potency to a number of other highly active compounds that have been reported on through the use of the assay.\(^{553,558,560,621}\)

### 11.4 Discussion

In consideration of their cellular activities, it is interesting that PDs 9 and 18 were the most active in the FP assay. These compounds had comparable cellular activities to PDs 4 and 5, while PDs 13-15 were generally more potent. However, PDs 4, 5, 13, 14 and 15 all contain an additional aromatic ring relative to PDs 9 and 18. As a result, their aqueous solubilities were limited. PDs 13, 14 and 15 inhibited the FP signal by less than 50%, even at concentrations of 500 µM. PDs 4 and 5, which also lack a sulfamoyl group relative to PDs 13-15, were almost entirely insoluble in the FP buffer, and achieved less than 12% inhibition of the FP signal at a concentration of 500 µM. As a result, it is possible that their reduced activities in the FP assay could be due to insolubility, and not an inability to bind to the STAT3 SH2 domain. However, the possibility that these compounds are exerting their antiproliferative effects through additional mechanisms cannot be ruled out.

In summary, the SARs of substituted 1,4-naphthoquinones have been evaluated. Several of the compounds displayed enhanced antiproliferative activities when compared to the parent compounds. PDs 9, 10, 13, 14 and 15 were found to be the most active in
the cell assays. These compounds were also all more potent than the LLL analogues presented in the preceding chapter. PDs 9 and 18 were well suited for and had the most activity in the FP assay, which suggests that STAT3 inhibition is a probable MOA.

It is interesting that PDs 13-15 all contain a sulfonamide moiety. Of the 1,4-naphthoquinones discussed in the preceding chapter, LLL12.1 was not the most potent. However, the data for the analogues presented here demonstrate that sulfonamide-containing 1,4-naphthoquinones can exhibit potent antiproliferative activities. Assuming that STAT3 is a primary target of both the 1,4-naphthoquinones and anthraquinones, which the FP data supports, one could reason that the lack of activity of LLL12.1 relative to LLLs 3.1 and 5.1 could be due to differences in interactions with STAT3. Perhaps the addition of the m-anisidine, p-anisidine or aniline moiety at the R₃ position results in more favorable interactions with STAT3, which are more comparable to those of LLL12. This could have led to the observed activities of PDs 13-15. However, it would be interesting to see if the addition of an m-anisidine, p-anisidine or aniline moiety at the R₃ position of the LLL3.1 or LLL5.1 core could lead to more potent 1,4-naphthoquinones as well. For that to be determined, additional analogues will first need to be synthesized and evaluated.
Chapter 12: Experimental Section

12.1 Synthesis of Curcumin Analogues

12.1.1 General Procedure for the Synthesis of Curcumin Analogues

Curcumin analogues were synthesized according to the procedure established by Venkateswarlu and coworkers.\textsuperscript{573} 2,4-Pentanedione (1.0012 g, 1.03 mL, 10 mmol) was added to a solution of boron oxide (0.6961 g, 10 mmol) in DMF (2.0 mL). Tributyl borate (4.6030 g, 5.40 mL, 20 mmol) was then added and the mixture was heated at 65°C for 15 minutes. The desired aldehyde (20 mmol) was then added and the mixture was stirred for 5 minutes. A mixture of 1,2,3,4-tetrahydroquinoline (0.2 mL) and acetic acid (0.6 mL) in DMF (2 mL) was then added and the reaction was heated at 95°C for 4 hours under N\textsubscript{2}. The reaction mixture was then cooled to 15°C and 20% acetic acid (100 mL) was added. The mixture was then heated at 70°C for 1 hour. Then after cooling to 15°C, the crude
product was collected via vacuum filtration. The methods of purification and the scales of the reactions varied slightly, and these differences will be noted below.

12.1.2 FLLL3 (1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione)

The reaction was conducted using 5 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (chloroform-methanol, 95:5) and subsequent recrystallization from chloroform-methanol. The pure product (0.3786 g, 24.6%) was a bright orange solid: $^1$H NMR (250 MHz, DMSO-d$_6$) $\delta$ 16.35 (s, 1H), 10.06 (s, 2H), 7.57 (d, $J = 8.7$ Hz, 4H), 7.54 (d, $J = 16.0$ Hz, 2H), 6.82 (d, $J = 8.7$ Hz, 4H), 6.70 (d, $J = 15.9$ Hz, 2H), 6.04 (s, 1H).

12.1.3 FLLL4 (1,7-bis(3-methoxyphenyl)hepta-1,6-diene-3,5-dione)
The reaction was conducted using 5 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (CH₂Cl₂-hexanes, 3:2). The pure product (0.5466 g, 32.5%) was a bright yellow solid: 

\[ ^1H \text{ NMR (250 MHz, CDCl}_3 \] \( \delta \) 15.87 (s, 1H), 7.64 (d, \( J = 15.9 \text{ Hz}, 2\text{H} \)), 7.37 – 7.29 (m, 2H), 7.19 – 7.13 (m, 2H), 7.09 – 7.07 (m, 2H), 6.97 – 6.91 (m, 2H), 6.62 (d, \( J = 15.9 \text{ Hz}, 2\text{H} \)), 5.86 (s, 1H), 3.85 (s, 6H).

\[ 12.1.4 \text{ FLLL10 (1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione)} \]

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (EtOAc-CH₂Cl₂, 1:4) and subsequent recrystallization from benzene. The pure product was a bright orange solid:  

mp 128-129°C; 

\[ ^1H \text{ NMR (250 MHz, CDCl}_3 \] \( \delta \) 16.03 (s, 1H), 7.61 (d, \( J = 15.8 \text{ Hz}, 2\text{H} \)), 7.15 (d, \( J = 8.7 \text{ Hz}, 2\text{H} \)), 7.08 (s, 2H), 6.89 (d, \( J = 8.3 \text{ Hz}, 2\text{H} \)), 6.50 (d, \( J = 15.8 \text{ Hz}, 2\text{H} \)), 5.82 (s, 1H), 3.94 (s, 6H), 3.93 (s, 6H).
12.1.5 FLLL18 (1,7-bis(3-hydroxy-4-methoxyphenyl)hepta-1,6-diene-3,5-dione)

![Chemical structure of FLLL18](image)

The reaction was conducted using 20 mmol of 2,4-Pentanedione. The crude product was purified by recrystallization from ethanol to give the pure product (2.9256 g, 39.7%) as bright yellow-orange flakes: mp 191-192°C; $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 16.26 (s, 1H), 9.24 (s, 2H), 7.50 (d, $J = 15.8$ Hz, 2H), 7.18 -- 7.09 (m, 4H), 6.98 (d, $J = 8.7$ Hz, 2H), 6.65 (d, $J = 15.8$ Hz, 2H), 6.11 (s, 1H), 3.82 (s, 6H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 183.11, 150.05, 146.75, 140.44, 127.63, 121.65, 121.43, 114.10, 112.05, 101.17, 55.65; IR (cm$^{-1}$) 3402, 3205, 2962, 2933, 2838, 1625, 1588, 1511, 1436, 1363, 1267, 1203, 1187, 1129, 1021, 973, 962, 928, 885, 870, 839, 802, 762, 741, 611; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{21}$H$_{20}$O$_6$ 391.1158, found 391.0977.

12.1.6 FLLL24 (1,7-bis(4-methoxyphenyl)hepta-1,6-diene-3,5-dione)

![Chemical structure of FLLL24](image)

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The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by recrystallization from ethanol to give the pure product (1.4282 g, 42.5%) as pumpkin orange crystals: mp 159-160°C; $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 16.22 (s, 1H), 7.69 (d, $J$ = 8.5 Hz, 4H), 7.60 (d, $J$ = 15.8 Hz, 2H), 7.00 (d, $J$ = 8.5 Hz, 4H), 6.79 (d, $J$ = 15.9 Hz, 2H), 6.09 (s, 1H), 3.81 (s, 6H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 183.20, 161.08, 140.02, 130.14, 127.34, 121.83, 114.50, 101.21, 55.36; IR (cm$^{-1}$) 2993, 2962, 2934, 2836, 1626, 1600, 1572, 1510, 1460, 1439, 1421, 1305, 1251, 1172, 1135, 1110, 1028, 972, 826; MS (HR-ESI) $m/z$ (M + Na)$^+$ calcd for C$_{21}$H$_{20}$O$_4$ 359.1259, found 359.1213.

12.1.7 FLLL36 (1,7-bis(3,4-dimethoxyphenyl)-4-methylhepta-1,6-diene-3,5-dione)

![Chemical Structure](image_url)

The reaction was conducted using 5 mmol of 3-methyl-2,4-Pentanedione. The crude product (1.4876 g, 72.5%) was purified by recrystallization from benzene to give the pure product (0.8530 g, 41.6%) as ruby red crystals: $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.70 (d, $J$ = 15.4 Hz, 2H), 7.19 (dd, $J$ = 8.4, 2.2 Hz, 2H), 7.10 (d, $J$ = 1.9 Hz, 2H), 6.98 (d, $J$ = 15.5 Hz, 2H), 6.89 (d, $J$ = 8.3 Hz, 2H), 3.95 (s, 6H), 3.93 (s, 6H), 2.19 (s, 3H).
12.1.8 FLLL37 (1,7-bis(3,4-dimethoxyphenyl)-4-ethylhepta-1,6-diene-3,5-dione)

The reaction was conducted using 5 mmol of 3-ethyl-2,4-Pentanedione. The crude product (2.0482 g, 96.5%) was purified by recrystallization from benzene to give the pure product (1.1344 g, 53.5%) as orange-red crystals: mp 173-174°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.72 (d, $J$ = 15.4 Hz, 2H), 7.20 (dd, $J$ = 8.4, 2.0 Hz, 2H), 7.08 (d, $J$ = 1.9 Hz, 2H), 6.95 (d, $J$ = 15.3 Hz, 2H), 6.90 (d, $J$ = 8.3 Hz, 2H), 3.95 (s, 6H), 3.93 (s, 6H), 2.61 (q, $J$ = 7.3 Hz, 2H), 1.23 (t, $J$ = 7.5 Hz, 3H).

12.1.9 JE30 (1,7-bis(4-fluorophenyl)hepta-1,6-diene-3,5-dione)

The reaction was conducted using 10 mmol of 2,4-Pentanodione. The crude product was purified by recrystallization from ethanol to give the pure product (0.5975 g, 19.1%) as reddish brown crystals: mp 162-163°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 15.91 (s, 1H), 7.62 (d, $J$ = 15.9 Hz, 2H), 7.54 (dd, $J$ = 8.2, 5.6 Hz, 4H), 7.09 (dd, $J$ = 8.5, 8.5
Hz, 4H), 6.54 (d, $J = 15.8$ Hz, 2H), 5.81 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 183.35, 164.01 (d, $J = 251.4$ Hz), 139.59, 131.41 (d, $J = 3.4$ Hz), 130.16 (d, $J = 8.5$ Hz), 123.90 (d, $J = 2.4$ Hz), 116.30 (d, $J = 21.9$ Hz), 102.03; IR (cm$^{-1}$) 3076, 3045, 1631, 1594, 1509, 1414, 1314, 1272, 1232, 1159, 1139, 1096, 1012, 967, 831, 732, 513, 467; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{19}$H$_{14}$F$_2$O$_2$ 335.0860, found 335.0700.

$^{12.1.10}$JE31 $(1,7$-bis(2-fluorophenyl)hepta-1,6-diene-3,5-dione)

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (EtOAc-hexanes, 3:7) and subsequent recrystallization from ethanol to give the pure product (0.4358 g, 14.0%) as fine light orangish yellow crystals: mp 110-111°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 15.80 (s, 1H), 7.78 (d, $J = 16.1$ Hz, 2H), 7.57 (ddd, $J = 7.6$, 7.6, 1.5 Hz, 2H), 7.41 – 7.30 (m, 2H), 7.22 – 7.07 (m, 4H), 6.75 (d, $J = 16.1$ Hz, 2H), 5.89 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 183.49, 161.68 (d, $J = 254.0$ Hz), 133.64 (d, $J = 2.6$ Hz), 131.67 (d, $J = 8.8$ Hz), 129.45 (d, $J = 3.1$ Hz), 126.80 (d, $J = 7.1$ Hz), 124.69 (d, $J = 3.6$ Hz), 123.27 (d, $J = 11.5$ Hz), 116.46 (d, $J = 22.0$ Hz), 102.36; IR (cm$^{-1}$) 3085, 3058, 3034, 1635, 1577, 1485, 1458, 1319, 1285, 1266, 1230, 1179, 1145, 1108, 1093, 1033, 975, 889, 869, 878, 823,
798, 760, 740, 704, 578, 529, 499, 464, 426; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{19}$H$_{14}$F$_2$O$_2$ 335.0860, found 335.0600.

12.1.11 JE32 (1,7-bis(4-nitrophenyl)hepta-1,6-diene-3,5-dione)

![Chemical structure of JE32](image)

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (EtOAc-hexanes, 3:7) and subsequent recrystallization from ethanol to give the pure product (0.0042 g, <1%) in low yield as an orangish brown solid: mp >240°C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 15.61 (s, 1H), 8.27 (d, $J = 8.4$ Hz, 4H), 7.80 – 7.66 (m, 6H), 6.76 (dd, $J = 16.0$ Hz, 2H), 5.93 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 182.74, 148.58, 141.16, 138.41, 128.89, 127.94, 124.48, 103.41; IR (cm$^{-1}$) 3110, 3078, 2925, 2852, 1632, 1597, 1518, 1458, 1413, 1344, 1193, 1109, 1013, 977, 853, 736, 700.

12.1.12 JE33 (4,4'-(3,5-dioxohepta-1,6-diene-1,7-diyl)dibazonitrile)

![Chemical structure of JE33](image)
The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (EtOAc-hexanes, 3:7) and subsequent recrystallization from ethanol to give the pure product (0.0439 g, 1.4%) in low yield as a dark pumpkin orange powder: mp 205-207°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 15.65 (s, 1H), 7.71 – 7.62 (m, 10H), 6.71 (d, \(J = 15.9\) Hz, 2H), 5.90 (s, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 182.80, 139.34, 138.83, 132.89, 128.63, 127.30, 118.60, 113.44, 103.13; IR (cm\(^{-1}\)) 3053, 2930, 2226, 1629, 1604, 1502, 1410, 1299, 1202, 1134, 1018, 974, 825, 733, 547, 447; MS (HR-ESI) \(m/z\) (M + Na)\(^+\) calcd for C\(_{21}\)H\(_{14}\)N\(_2\)O\(_2\) 349.0953, found 349.0934.

12.1.13 JE35 (1,7-bis(3-fluorophenyl)hepta-1,6-diene-3,5-dione)

![Diagram](image)

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by silica gel column chromatography (EtOAc-hexanes, 3:7) and subsequent recrystallization from ethanol to give the pure product (0.3121 g, 10.0%) as a bright yellow solid: mp 109-110°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 15.80 (s, 1H), 7.64 (d, \(J = 15.8\) Hz, 2H), 7.46 – 7.23 (m, 6H), 7.10 (dd, \(J = 8.0, 8.0\) Hz, 2H), 6.64 (d, \(J = 15.9\) Hz, 2H), 5.88 (s, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 183.20, 163.27 (d, \(J = 246.7\) Hz), 139.61 (d, \(J = 2.8\) Hz), 137.40 (d, \(J = 7.7\) Hz), 130.68 (d, \(J = 8.3\) Hz), 125.40, 124.43 (d,
$J = 2.9 \text{ Hz}, 117.21 \text{ (d, } J = 21.4 \text{ Hz)}, 114.40 \text{ (d, } J = 21.9 \text{ Hz)}, 102.45; \text{ IR (cm}^{-1}) 3067, 3041, 1630, 1583, 1490, 1447, 1434, 1284, 1238, 1223, 1168, 1142, 1076, 983, 971, 941, 884, 861, 798, 780, 724, 694, 677, 660, 518, 489, 457, 450; \text{ MS (HR-ESI) } m/z (M + Na)^+ \text{ calcd for C}_{19}H_{14}F_{2}O_{2} \text{ 335.0860, found 335.0695.}

12.1.14 JE36 (1,7-bis(4-chlorophenyl)hepta-1,6-diene-3,5-dione)

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by recrystallization from ethanol to give the pure product (0.5367 g, 15.5%) as a yellowish orange solid: mp 198-200$^\circ$C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 15.84 (s, 1H), 7.61 (d, $J = 15.9$ Hz, 2H), 7.48 (d, $J = 8.5$ Hz, 4H), 7.37 (d, $J = 8.5$ Hz, 4H), 6.59 (d, $J = 15.9$ Hz, 2H), 5.82 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.25, 139.53, 136.25, 133.65, 129.47, 129.43, 124.66, 102.29; IR (cm$^{-1}$) 1637, 1584, 1561, 1489, 1403, 1271, 1138, 1091, 1010, 972, 822, 787; MS (HR-ESI) $m/z (M + Na)^+$ calcd for C$_{19}$H$_{14}$Cl$_2$O$_2$ 367.0269, found 367.0157.
12.1.15 JE38 (1,7-bis(3-chlorophenyl)hepta-1,6-diene-3,5-dione)

![Chemical Structure](image)

The reaction was conducted using 10 mmol of 2,4-Pentanedione. The crude product was purified by recrystallization from acetone or silica gel column chromatography (EtOAc-hexanes, 3:7) and subsequent recrystallization from ethanol to give the pure product (0.3302 g, 10.0%) as bright yellowish orange crystals: mp 144-145°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 15.77 (s, 1H), 7.59 (d, \(J = 15.9\) Hz, 2H), 7.55 (s, 2H), 7.46 – 7.38 (m, 2H), 7.37 – 7.28 (m, 4H), 6.62 (d, \(J = 15.8\) Hz, 2H), 5.84 (s, 1H); \(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 183.15, 139.40, 136.96, 135.17, 130.39, 130.20, 127.88, 126.67, 125.45, 102.51; IR (cm\(^{-1}\)) 3091, 3065, 3022, 1637, 1563, 1474, 1411, 1301, 1276, 1203, 1166, 1139, 1098, 1075, 970, 905, 863, 796, 719, 695, 679, 486, 442; MS (HR-ESI) \(m/z\) (M + Na)\(^+\) calcd for C\(_{19}\)H\(_{14}\)Cl\(_2\)O\(_2\) 367.0269, found 367.0229.
12.2 BOC Protection and Deprotection

12.2.1 Procedure for the BOC Protection of FLLL18

The BOC protection was conducted according to the procedure reported by Lee and coworkers.\textsuperscript{654} FLLL18 (0.7500 g, 2.04 mmol) was dissolved in methanol (29.93 mL). Sodium hydroxide (0.2452 g, 6.13 mmol) and di-\textit{tert}-butyl dicarbonate (1.7774 g, 1.87 mL, 8.14 mmol) were then added and the reaction was stirred under N\textsubscript{2} for three hours at room temperature. The pure product (1.0664 g, 92.1\%) was then collected via vacuum filtration.
12.2.2 JE46 (di-tert-butyl ((3,5-dioxohepta-1,6-diene-1,7-diyl)bis(2-methoxy-5,1-phenylene)) dicarbonate)

The pure product was a bright yellow solid: mp 164-165°C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 15.94 (s, 1H), 7.56 (d, $J = 15.8$ Hz, 2H), 7.45 – 7.30 (m, 4H), 6.96 (d, $J = 9.0$ Hz, 2H), 6.47 (d, $J = 15.8$ Hz, 2H), 5.76 (s, 1H), 3.88 (s, 6H), 1.56 (s, 18H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 183.31, 153.09, 151.48, 140.72, 139.53, 128.39, 127.99, 123.03, 121.64, 112.65, 101.87, 83.92, 56.26, 27.82; IR (cm$^{-1}$) 3053, 2981, 2936, 2842, 1760, 1629, 1609, 1581, 1512, 1459, 1440, 1395, 1370, 1274, 1215, 1151, 1129, 1026, 969, 874, 814, 739, 457; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{31}$H$_{36}$O$_{10}$ 591.2206, found 591.2239.
12.2.3 Procedure for the BOC Deprotection of JE47

JE47 (0.1074 g, 0.17 mmol) was placed in a small vial and stirred and heated at 145-155°C until both BOC groups had been removed, which was determined by TLC (EtOAc-hexanes, 1:1). The reaction was complete after two hours. The product was purified by silica gel column chromatography (EtOAc-hexanes, 2:3 to 1:1) to give the pure product (0.0649 g, 88.2%).
12.2.4 JE48 (1,1’-(cyclohexane-1,1-diyl)bis(3-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one))

The pure product was a light yellow solid: mp 190-192°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.60 (d, \(J = 15.5\) Hz, 2H), 7.12 (d, \(J = 1.9\) Hz, 2H), 7.04 (dd, \(J = 8.3, 1.8\) Hz, 2H), 6.80 (d, \(J = 8.4\) Hz, 2H), 6.74 (d, \(J = 15.5\) Hz, 2H), 5.61 (s, 2H), 3.90 (s, 6H), 2.13 – 2.02 (m, 4H), 1.61 – 1.52 (m, 4H), 1.46 – 1.39 (m, 2H); \(^1^3\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 197.97, 149.06, 145.98, 144.06, 128.26, 122.87, 119.94, 113.66, 110.66, 66.01, 56.21, 30.36, 25.82, 22.92; IR (cm\(^{-1}\)) 3411, 2935, 2852, 1707, 1662, 1593, 1509, 1440, 1359, 1267, 1164, 1130, 1094, 1080, 1047, 1025, 982, 803, 762, 505; MS (HR-ESI) \(m/z\) (M + Na\(^+\)) calcd for C\(_{26}\)H\(_{28}\)O\(_6\) 459.1784, found 459.1664.
12.3 Synthesis of FLLL32 Analogues

12.3.1 General Procedure for the Synthesis of FLLL32 Analogues

The desired curcumin analogue (1 mmol) was dissolved in reagent grade DMF (10 mL) and potassium carbonate (0.6911 g, 5 mmol) was added. Water (0.1 mL) was then added and the reaction mixture was stirred under N₂ for one hour at room temperature. 1,5-Diodopentane (0.4859 g, 0.22 mL, 1.5 mmol) was then added and the reaction was stirred overnight. The reaction was then quenched with water (20 mL) and the product was extracted three to four times with EtOAc. The crude product was then purified by silica gel column chromatography (EtOAc-CH₂Cl₂-hexanes). The ratios of solvents used for the purifications and the scales of the reactions varied slightly, and these differences will be noted below.
12.3.2 JE39 (1,1’-(cyclohexane-1,1-diyl)bis(3-(4-methoxyphenyl)prop-2-en-1-one))

The reaction was conducted using 0.8377 mmol of FLLL24. The crude product was purified by silica gel column chromatography (EtOAc-CH₂Cl₂-hexanes, 1:3:36) to give the pure product (0.2193 g, 64.7%) as a yellowish white solid: mp 118-121°C; ¹H NMR (300 MHz, CDCl₃) δ 7.65 (d, J = 15.5 Hz, 2H), 7.44 (d, J = 8.7 Hz, 4H), 6.82 (d, J = 8.7 Hz, 4H), 6.77 (d, J = 15.6 Hz, 2H), 3.76 (s, 6H), 2.13 – 2.01 (m, 4H), 1.61 – 1.50 (m, 4H), 1.44 – 1.36 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) δ 197.78, 161.83, 143.77, 130.48, 127.16, 119.23, 114.37, 65.85, 55.40, 30.27, 25.71, 22.81; IR (cm⁻¹) 3052, 3004, 2934, 2853, 2835, 1724, 1666, 1593, 1573, 1512, 1462, 1423, 1327, 1305, 1288, 1255, 1209, 1172, 1133, 1094, 1080, 1032, 986, 934, 907, 877, 825, 802, 781, 736, 707, 638, 554, 534, 431; MS (HR-ESI) m/z (M + Na)⁺ calcd for C₂₆H₂₈O₄ 427.1885, found 427.1808.

12.3.3 JE40 (1,1’-(cyclohexane-1,1-diyl)bis(3-(4-fluorophenyl)prop-2-en-1-one))

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The reaction was conducted using 0.6583 mmol of JE30. The crude product was purified by silica gel column chromatography (EtOAc-CH₂Cl₂-hexanes, 1:2:97 to 1.5:3.5:95) to give the pure product (0.1177 g, 47.0%) as an off-white solid: mp 137-138°C; ¹H NMR (300 MHz, CDCl₃) δ 7.65 (d, J = 15.6 Hz, 2H), 7.50 (dd, J = 8.7, 5.4 Hz, 4H), 7.02 (dd, J = 8.6, 8.6 Hz, 4H), 6.81 (d, J = 15.6 Hz, 2H), 2.15 – 2.04 (m, 4H), 1.63 – 1.51 (m, 4H), 1.47 – 1.37 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) δ 197.67, 164.34 (d, J = 252.3 Hz), 143.04, 130.76 (d, J = 8.6 Hz), 130.76 (d, J = 3.4 Hz), 121.11 (d, J = 2.4 Hz), 116.23 (d, J = 22.0 Hz), 66.22, 30.23, 25.70, 22.82; IR (cm⁻¹) 3069, 3056, 2937, 2855, 1670, 1589, 1509, 1452, 1414, 1317, 1298, 1279, 1237, 1159, 1134, 1097, 1080, 1045, 1032, 1015, 987, 937, 907, 860, 826, 812, 790, 737, 723, 705, 511, 470, 437; MS (HR-ESI) m/z (M + Na)⁺ calcd for C₂₄H₂₂F₂O₂ 403.1486, found 403.1442.

12.3.4 JE41 (1,1'-(cyclohexane-1,1-diyl)bis(3-(2-fluorophenyl)prop-2-en-1-one))

The reaction was conducted using 0.6209 mmol of JE31. The crude product was purified by silica gel column chromatography (EtOAc-CH₂Cl₂-hexanes, 1:2:97 to 2.5:7.5:90) to give the pure product (0.1117 g, 47.3%) as a light yellow solid: mp 101-104°C; ¹H NMR (300 MHz, CDCl₃) δ 7.83 (d, J = 15.8 Hz, 2H), 7.56 – 7.45 (m, 2H), 7.38 – 7.27 (m, 2H), 7.16 – 7.02 (m, 4H), 6.98 (d, J = 15.8 Hz, 2H), 2.16 – 2.05 (m, 4H), 1.47 – 1.37 (m, 2H); ¹³C NMR (75 MHz, CDCl₃) δ 197.67, 164.34 (d, J = 252.3 Hz), 143.04, 130.76 (d, J = 8.6 Hz), 130.76 (d, J = 3.4 Hz), 121.11 (d, J = 2.4 Hz), 116.23 (d, J = 22.0 Hz), 66.22, 30.23, 25.70, 22.82; IR (cm⁻¹) 3069, 3056, 2937, 2855, 1670, 1589, 1509, 1452, 1414, 1317, 1298, 1279, 1237, 1159, 1134, 1097, 1080, 1045, 1032, 1015, 987, 937, 907, 860, 826, 812, 790, 737, 723, 705, 511, 470, 437; MS (HR-ESI) m/z (M + Na)⁺ calcd for C₂₄H₂₂F₂O₂ 403.1486, found 403.1442.
1.63 – 1.51 (m, 4H), 1.47 – 1.38 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 197.77, 161.93 (d, $J = 254.9$ Hz), 136.76 (d, $J = 3.0$ Hz), 132.26 (d, $J = 8.8$ Hz), 129.48 (d, $J = 2.8$ Hz), 124.55 (d, $J = 3.7$ Hz), 123.65 (d, $J = 6.3$ Hz), 122.65 (d, $J = 11.5$ Hz), 116.35 (d, $J = 21.9$ Hz), 66.25, 30.13, 25.67, 22.77; IR (cm$^{-1}$) 3063, 3042, 2926, 2860, 1673, 1608, 1577, 1487, 1456, 1320, 1279, 1232, 1174, 1152, 1136, 1097, 1080, 1046, 1029, 1014, 985, 936, 907, 864, 843, 832, 816, 798, 754, 716, 587, 499, 457; MS (HR-ESI) $m/z$ (M + Na)$^+$ calcd for C$_{24}$H$_{22}$F$_2$O$_2$ 403.1486, found 403.1432.

12.3.5 JE42 (1,1'-(cyclohexane-1,1-diyl)bis(3-(3-fluorophenyl)prop-2-en-1-one))

![Chemical Structure]

The reaction was conducted using 0.7249 mmol of JE35. The crude product was purified by silica gel column chromatography (EtOAc-CH$_2$Cl$_2$-hexanes, 1:2:97) to give the pure product (0.1104 g, 40.0%) as a light beige solid: mp 101-102°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.64 (d, $J = 15.6$ Hz, 2H), 7.36 – 7.24 (m, 4H), 7.20 (br d, $J = 10.2$ Hz, 2H), 7.09 – 6.99 (m, 2H), 6.87 (d, $J = 15.6$ Hz, 2H), 2.17 – 2.02 (m, 4H), 1.64 – 1.49 (m, 4H), 1.48 – 1.38 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 197.52, 163.10 (d, $J = 247.1$ Hz), 142.98 (d, $J = 2.8$ Hz), 136.71 (d, $J = 7.7$ Hz), 130.58 (d, $J = 8.2$ Hz), 124.81 (d, $J = 2.9$ Hz), 122.45, 117.75 (d, $J = 21.4$ Hz), 114.90 (d, $J = 21.9$ Hz), 66.33, 30.12, 25.65, 22.77; IR (cm$^{-1}$) 3066, 2937, 2855, 1673, 1605, 1581, 1485, 1448, 1320, 1273, 1240, 303
1174, 1146, 1080, 1046, 1016, 1000, 984, 946, 933, 906, 879, 844, 782, 737, 718, 675, 584, 565, 520, 502, 483, 448; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{24}$H$_{22}$F$_2$O$_2$ 403.1486, found 403.1410.

12.3.6 JE43 (1,1'-(cyclohexane-1,1-diyl)bis(3-(4-chlorophenyl)prop-2-en-1-one))

The reaction was conducted using 0.6390 mmol of JE36. The crude product was purified by silica gel column chromatography (EtOAc-CH$_2$Cl$_2$-hexanes, 1:2:97) to give the pure product (0.1404 g, 53.2%) as a fluffy off-white solid: mp 162-164°C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.64 (d, $J = 15.6$ Hz, 2H), 7.45 (d, $J = 8.7$ Hz, 4H), 7.32 (d, $J = 8.6$ Hz, 4H), 6.85 (d, $J = 15.6$ Hz, 2H), 2.16 – 2.01 (m, 4H), 1.62 – 1.53 (m, 4H), 1.49 – 1.39 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 197.65, 142.98, 136.92, 132.98, 129.98, 129.38, 121.78, 66.34, 30.22, 25.70, 22.83; IR (cm$^{-1}$) 2920, 2851, 1666, 1604, 1564, 1490, 1405, 1313, 1091, 1079, 1046, 1033, 1011, 988, 816, 784, 726; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{24}$H$_{22}$Cl$_2$O$_2$ 435.0895, found 435.0862.
12.3.7 JE44 (1,1’-(cyclohexane-1,1-diy)bis(3-(3-chlorophenyl)prop-2-en-1-one))

The reaction was conducted using 0.5918 mmol of JE38. The crude product was purified by silica gel column chromatography (EtOAc-CH$_2$Cl$_2$-hexanes, 1:2:97) to give the pure product (0.0916 g, 37.5%) as an off-white solid: mp 96-98°C; $^1$H NMR (300 MHz, CDCl$_3$) δ 7.64 (d, $J = 15.6$ Hz, 2H), 7.51 (s, 2H), 7.39 (d, $J = 7.2$ Hz, 2H), 7.36 – 7.27 (m, 4H), 6.89 (d, $J = 15.6$ Hz, 2H), 2.19 – 2.06 (m, 4H), 1.65 – 1.53 (m, 4H), 1.50 – 1.40 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 197.48, 142.81, 136.27, 135.09, 130.73, 130.27, 128.28, 127.10, 122.47, 66.35, 30.12, 25.65, 22.77; IR (cm$^{-1}$) 3061, 2935, 2854, 1672, 1606, 1564, 1473, 1451, 1429, 1309, 1264, 1201, 1172, 1134, 1095, 1079, 1046, 1032, 1014, 984, 935, 907, 847, 829, 782, 761, 738, 690, 571, 480, 435, 417; MS (HR-ESI) $m/z$ (M + Na)$^+$ calcd for C$_{24}$H$_{22}$Cl$_2$O$_2$ 435.0895, found 435.0845.
12.3.8 JE47 (di-tert-butyl ((cyclohexane-1,1-diylbis(3-oxoprop-1-ene-3,1-diyl))bis(2-methoxy-5,1-phenylene)) dicarbonate)

The reaction was conducted using 0.6546 mmol of JE46. The crude product was purified by silica gel column chromatography (EtOAc-CH$_2$Cl$_2$-hexanes, 2.5:7.5:90 to 7:13:80) to give the pure product (0.2051 g, 49.2%) as a light yellow solid: mp 110-112$^\circ$C; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.60 (d, $J = 15.5$ Hz, 2H), 7.34 (d, $J = 8.7$ Hz, 2H), 7.31 (s, 2H), 6.90 (d, $J = 8.4$ Hz, 2H), 6.71 (d, $J = 15.5$ Hz, 2H), 3.84 (s, 6H), 2.10 – 2.02 (m, 4H), 1.58 – 1.51 (m, 4H), 1.53 (s, 18H), 1.44 – 1.38 (m, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 197.67, 153.60, 151.36, 143.18, 140.56, 128.70, 127.64, 122.16, 120.19, 112.48, 83.84, 66.03, 56.21, 30.26, 27.77, 25.74, 22.83; IR (cm$^{-1}$) 3058, 2979, 2935, 2854, 1762, 1668, 1597, 1513, 1440, 1395, 1370, 1305, 1275, 1216, 1152, 1131, 1095, 1080, 1047, 1031, 985, 896, 874, 811, 781, 737, 704, 624, 537, 511, 455; MS (HR-ESI) m/z (M + Na)$^+$ calcd for C$_{36}$H$_{44}$O$_{10}$ 659.2832, found 659.2823.
12.4 Cell Assays

12.4.1 Cell Culture

PC3, HT29 and DU145 cells were cultured in RPMI 1640 medium (Gibco). MDA-MB-231 cells were cultured in either DMEM/F12 (Gibco) or DMEM (Gibco) medium. MDA-MB-468 cells were cultured in DMEM medium as well. The medium was supplemented with 10% heat inactivated fetal bovine serum (Gibco), 50 units/mL of penicillin, and 50 µg/mL of streptomycin (Gibco). If the medium did not contain L-glutamine, then GlutaMAX™ (Gibco) was also added to a final concentration of 2 mM. Cells were routinely grown in 25 or 75 cm² tissue culture flasks (BD Falcon) and were passaged 1:3 to 1:8 multiple times each week. Cells were cultured at 37°C in a 5% CO₂ atmosphere.

12.4.2 Cell Proliferation Assays

MDA-MB-468 (breast), MDA-MB-231 (breast), DU145 (prostate), PC3 (prostate) and HT29 (colon) cancer cell lines were used in cell proliferation assays due to the role that STAT3 signaling plays in each of them. MDA-MB-468, MDA-MB-231, DU145, and PC3 cells all exhibit IL-6 autocrine loops. Inhibiting STAT3 can suppress growth and induce apoptosis. STAT3 activation in HT29 (colon) cancer cells is associated with enhanced cell invasion, proliferation and survival. Inhibition of STAT3 in HT29 cells induces cell-cycle arrest and apoptosis, and also reduces tumor cell invasion.
Cell proliferation assays were performed in 96 well tissue culture plates (BD Falcon). Cells were counted using a hemocytometer and trypan blue (Sigma) to exclude non-viable cells. Cells were plated at 2,500 cells per well and allowed to incubate overnight. Test compound were diluted in cell medium from 20 or 30 mM DMSO stock solutions. Test compounds were then added to the plates at concentrations of 2X and volumes of 100 µL.

In determining IC₅₀ values, a three-fold serial dilution of the test compounds in cell medium was carried out prior to the addition of the test compounds to the wells. Cells were then subjected to eight concentrations of test compounds, ranging from 150 µM to 69 nM. If compounds were too potent to be evaluated at these concentrations, then they were evaluated over a less concentrated range, starting at 1.5 µM. The maximum amount of DMSO that was present in any well was 1.67%.

The cells were then incubated with the test compounds for 72 hours. Following the incubation, 100 µL of medium was removed from each well and 20 µL of 20:1 MTS:PMS (Promega) was added to each well. The cells were then generally incubated at 37°C for 1 to 4 hours prior to taking readings. If the absorbances were not sufficiently high, then the incubation time was increased. Absorbances were recorded at 490 nM using a SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA) microplate reader. All data were analyzed with SoftMax Pro (Molecular Devices, Sunnyvale, CA). IC₅₀ values were determined using a standard 4-parameter logistic fit.

To determine IC₅₀ values, compounds were tested twice in quadruplicate. However, it should be noted that the values in this document for FLLLs 33, 118, 120,
121, 122, and 123 in DU145 cells, FLLLs 114-116 in MDA-MB-468 cells, and FLLLs 118-123 in MDA-MB-231 cells were determined from testing once in quadruplicate. In evaluating compounds at concentrations of 10 and 50 µM, the compounds were evaluated twice in sextuplicate. The only exceptions were curcumin and FLLL116 in DU145 cells, which were evaluated once in sextuplicate. In evaluating compounds at 0.5, 5 and 50 µM, the compounds were tested twice in triplicate.

12.4.3 Western Blot Analysis

Cells were grown to approximately 70% confluency before being dosed with the indicated compounds at the indicated concentrations. DU145 cells were treated with test compounds for 12 hours, while MDA-MB-231 cells were treated for 24 hours. Protease (P8340, Sigma, 1:100) and phosphatase (PhosSTOP, Roche, 1 tablet per 10 mL) inhibitor cocktails were added to M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), which was used to extract the protein from the cells. The BCA Protein Assay Kit (Thermo Scientific) was used to determine protein concentrations.

SDS-PAGE was used to separate the proteins, which were run at 10 µg/lane. Proteins were then transferred to PVDF membranes (GE Healthcare Life Sciences). The blots were generally incubated with primary antibodies (1:1000) overnight at 4°C and secondary antibodies (1:3000) for 1 hour at room temperature. Protein levels were detected with LumiGLO (CST). All antibodies were purchased from CST.
12.5 STAT3 Fluorescence Polarization Assay

12.5.1 Materials and Reagents

**STAT3:**

STAT3 protein (>90% purity) was purchased from Abcam (Cambridge, MA).

**Fluorescent Peptide:**

The fluorescent peptide with the amino acid sequence 5-carboxyfluorescein-SpYLPQTV (5-FAM-SpYLPQTV) (>95% purity) was prepared by GenScript (Piscataway, NJ).

**Unlabeled Peptide:**

The unlabeled peptide with the amino acid sequence SpYLPQTV (≥95% purity) was prepared by GenScript (Piscataway, NJ).

**Unlabeled Negative Control:**

The unlabeled peptide with the amino acid sequence SYLPQTV (98.4% purity) was prepared by GenScript (Piscataway, NJ).

**Initial Buffer Conditions:**

The initial FP buffer conditions were 10 mM HEPES, 50 mM NaCl, 1 mM EDTA, 0.1% NP-40, 2 mM DTT, pH 7.6. These conditions were used for the ‘shift in K_d’ studies.

**Altered Buffer Conditions:**

Following the publication in PLoS ONE, the FP buffer conditions were 10 mM HEPES, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% DMSO, pH 7.6.
12.5.2 Methods

General (Applies to all FP Assays):

The assays were performed in black 384-well microplates (Perkin Elmer, Waltham, MA) in total volumes of 25 μL per well. Measurements were taken with either FlexStation 3 or SpectraMax M5 microplate readers (Molecular Devices, Sunnyvale, CA). The fluorescence polarization values were recorded using an excitation filter at 480 nm and an emission filter at 530 nm. Specific binding was defined as the contribution to the signal from the bound 5-FAM-SpYLPQTV.

Assays Designed to Observe a Shift in K_d:

Due to solubility issues with FLLL32 and high FP signals resulting from FLLL62 at higher concentrations, the IC_{50} values were not able to be determined. However, the competitive efficiencies of FLLL32 and FLLL62 were assessed by simultaneously running three saturation curves (up to 1020 nM STAT3). One curve was obtained by incubating STAT3 with 5-FAM-SpYLPQTV (final concentration of 4 nM) when no inhibitor was present. The two other curves were obtained by incubating STAT3 and 5-FAM-SpYLPQTV with 50 µM FLLL32 or 50 µM FLLL62. This is similar to the methods of Hao and colleagues. Compounds were diluted from 20 mM DMSO stock solutions in FP buffer. The final concentration of DMSO was 0.25% by volume. In order to assure complete equilibration, mixtures were incubated for 24 hours prior to readings being taken. The consistency in observed K_d values for STAT3 and a fluorescent probe over a 24 hour-period has previously been demonstrated. The K_d values resulting from
the presence of no inhibitor, 50 µM FLLL32 or 50 µM FLLL62 were determined via Scatchard plot analysis. They were compared in order to analyze the competitive efficiencies of FLLL32 and FLLL62. Microsoft Excel (Microsoft Corporation) was used to analyze experimental data. The data obtained from these studies was initially published in PLoS ONE, and was presented in chapter 6.

**All Other Assays:**

Final concentrations of 10 nM probe and 150 nM STAT3 are the most commonly utilized conditions for the STAT3 FP assay. These concentrations were used for the remainder of the studies. In evaluating STAT3 inhibitors, test compounds were diluted in FP buffer from 20 mM DMSO stock solutions. STAT3 was added and the mixtures were incubated at room temperature for at least 60 minutes. 5-FAM-SpYLPQTV was then added, and the mixtures were incubated for roughly 30 minutes before measurements were taken. Inhibitory activities were calculated relative to controls.

Multiple concentrations (500 to less than 2 µM) of test compounds, using a 1:2 serial dilution, were utilized to determine IC₅₀ values. IC₅₀ values were determined with SoftMax Pro version 5.4.4 (Molecular Devices, Sunnyvale, CA), using a standard 4-parameter logistic fit. Kᵢ values were derived according to the equation, Kᵢ = IC₅₀/(1 + [STAT3]/Kₐ). The peptides SpYLPQTV and SYLPQTV were used at concentrations of 10 or 100 µM as positive and negative controls, respectively. In evaluating compounds, all experiments were performed at least twice in triplicate. In
evaluating STAT3 decoys, all experiments were performed once in triplicate at the indicated concentrations. The standard curves presented in chapter 5, which were used to obtain the $K_d$ under the new buffer conditions, were obtained by using STAT3 at concentrations of 0 to 1200 nM. These experiments were performed twice in duplicate.
References


(7) Jones, S. A.; Rose-John, S. *Biochimica et biophysica acta* 2002, 1592, 251–63.


(179) Roskoski, R. Biochemical and biophysical research communications 2010, 399, 313–7.

(180) Roskoski, R. Biochemical and biophysical research communications 2012, 417, 5–10.

(181) Yoon, S.; Seger, R. Growth factors (Chur, Switzerland) 2006, 24, 21–44.


(201) Sparks, C. A.; Guertin, D. A. Oncogene 2010, 29, 3733–44.


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(325) Ji, Q.; Chattopadhyay, A.; Vecchi, M.; Carpenter, G. Molecular and cellular biology 1999, 19, 4961–70.


(351) Traynor, K. American journal of health-system pharmacy 2102, 69, 2120.


(369) Bercovich, D.; Ganmore, I.; Scott, L. M.; Wainreb, G.; Birger, Y.; Elimelech, A.; Shochat, C.; Cazzaniga, G.; Biondi, A.; Basso, G.; Cario, G.; Schrappe, M.;


343


(441) Yang, J.; Stark, G. R. Cell research 2008, 18, 443–51.


(444) Ng, I. H. W.; Ng, D. C. H.; Jans, D. A.; Bogoyevitch, M. A. Biochemical journal 2012, 447, 125–36.


349


(496) Bromberg, J.; Darnell, J. E. Oncogene 2000, 19, 2468–73.


(504) Ball, S.; Li, C.; Li, P.-K.; Lin, J. PloS one 2011, 6, e18820.


Weidler, M.; Rether, J.; Anke, T.; Erkel, G. *FEBS letters* 2000, 484, 1–6.


Sen, M.; Tosca, P. J.; Zwyer, C.; Ryan, M. J.; Johnson, J. D.; Knostman, K. A. B.; Giclas, P. C.; Peggins, J. O.; Tomaszewski, J. E.; McMurray, T. P.; Li, C.;


(540) McMurray, J. S. *Biopolymers* **2008**, *90*, 69–79.


(559) Zhang, X.; Sun, Y.; Pireddu, R.; Yang, H.; Urlam, M. K.; Lawrence, H. R.; Guida, W. C.; Lawrence, N. J.; Sebti, S. M. Cancer research 2013, 73, 1922–33.


(634) Quitschke, W. W. *BMC biotechnology* 2008, 8, 84.


(694) Blackhall, A.; Thomson, R. H. *Journal of the chemical society (Resumed)* **1953**, **1138–43**.


(703) Borovkov, V. V.; Filippovich, E. I.; Evstigneeva, R. P. Chemistry of heterocyclic compounds 1988, 24, 494–501.


Appendix A: $^1$H NMR Spectra
1,7-bis(3-methoxyphenyl)hepta-1,6-diene-3,5-dione

\[ \text{FLLL4} \]
FLLL10
1,7-bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione

$^1$H NMR
FLLL24
1,7-bis(4-methoxyphenyl)hepta-1,5-diene-3,5-dione

$^1$H NMR
JE36
1,7-bis(4-chlorophenyl)hepta-1,5-diene-3,5-dione

$^1H$ NMR
1H NMR

1,1'-(cyclohexane-1,1-diyl)bis(3-(4-fluorophenyl)prop-2-en-1-one)
1H NMR

1,1-(cyclohexane-1,1-diyl)bis[3-(3-fluorophenyl)prop-2-en-1-one]

385
1,1'-[(cyclohexane-1,1-diyl)bis(3-(4-chlorophenyl))prop-2-en-1-one]

$^1$H NMR
(R)-tert-butyl (cyclohexane-1,1-diylbis(3-oxoprop-1-ene-3,1-diyl))bis(2-methoxy-5,1-phenylene) dicarbonate
Appendix B: $^{13}$C NMR Spectra
JE32
1,7-bis(4-nitrophenyl)hepta-1,6-diene-3,5-dione

$^{13}C$ NMR
$^{13}$C NMR

JE33

4,4'-(3,5-dioctoexa-1,5-diene-1,7-diyi) dibenzonitrile
1,7-bis(3-fluorophenyl)hepta-1,6-diene-3,5-dione

$^{13}$C NMR
JE38
17-bis(3-chlorophenyl)hepta-1,6-diene-3,5-dione
1,1'-cyclohexane-1,1-diy)bism(3-(4-methoxyphenyl)prop-2-en-1-one)
1,1'-((cyclohexane-1,1-diyl)bis(3-(3-fluorophenyl)prop-2-en-1-one))

$^{13}$C NMR
1,1'-[(cyclohexane-1,1-diyl)bis(3-(3-chlorophenyl)prop-2-yl)en]one
JE46

di-tert-butyl tri(3,5-dioxohepta-1,6-diene-1,7-diy)bis(2-methoxy-5,1-phenylene) dicarbonate
di-tert-butyl ((cyclohexane-1,1-diyl)bis(3-oxoprop-1-ene-3,1-diyl))bis(2-methoxy-5,1-phenylene)) dicarbonate
Appendix C: HRMS Spectra
HRMS

1,7-bis(3-hydroxy-4-methoxyphenyl)hepta-1,6-diene-3,5-dione

TOF MS ES+
3.30e4
HRMS

1,7-bis(4-methoxyphenyl)hepta-1,6-diene-3,5-dione

LLL24

359.1213
322.7773
360.1280

m/z
HRMS

1,7-bis(4-fluorophenyl)hepta-1,6-diene-3,5-dione

JE30

335.0700  4.70e4

336.0817

322.7719
JE31
1,7-bis(2-fluorophenyl)hepta-1,6-diene-3,5-dione
HRMS

1,7-bis(4-chlorophenyl)hepta-1,6-diene-3,5-dione
JE38
1,7-bis(3-chlorophenyl)hepta-1,6-diene-3,5-dione
HRMS

JE39

1,1'- (cyclohexane-1,1-diyl) bis(3-(4-methoxyphenyl)prop-2-en-1-one)

m/z
1,1'-cyclohexane-1,1-diy[(3-4-fluorophenyl)prop-2-en-1-one]
1,1'-cyclohexane-1,1-diy)b(is(3-(2-fluorophenyl))prop-2-en-1-one)
1,1'-cydihexane-1,1'-diylbis(3-(3-fluorophenyl)prop-2-en-1-one)
HRMS

Je44

1,1'-{cyclohexane-1,1-diyl}bis(3-(3-chlorophenyl)prop-2-en-1-one)
je46

di-tert-butyl ([(3,5-dioxohepta-1,6-diene-1,7-diyl)bis(2-methoxy-5,1-phenylene)] dicarbonate

HRMS
HRMS

di-tert-butyl ((cyclohexane-1,1-diylbis(3-oxoprop-1-ene-3,1-diyl))bis(2-methoxy-5,1-phenylene)) dicarbonate
1,1'-(cyclohexane-1,1-diyl)bis(3-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one)