STUDY OF STRUCTURE ACTIVITY RELATIONSHIP OF ANALOGS DERIVED FROM SU-5416 AND THALIDOMIDE AND MECHANISM OF ANTIPROLIFERATIVE ACTIVITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

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By

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ABSTRACT

SU-5416, a VEGF-receptor tyrosine kinase inhibitor and Thalidomide demonstrate anti-angiogenic activity in different model systems. Two series of compounds, 1,3-dihydro-indole-2-one analogs based on SU-5416 and iso-indole-1,3-dione based on Thalidomide were evaluated for their anti-cancer activity.

SU5416, a potent ATP competitive, VEGF receptor tyrosine kinase inhibitor was used as a template for the design of the first series. A series of 1,3-dihydro-indole-2-one compounds were evaluated for anti-proliferative activities on cancer cells. The most potent analog was compound 145E with a tri-methoxy benzylidene substituted on position 3 of 6-methoxy-1,3-dihydro-indole-2-one. It caused a G2/M phase arrest in the cell cycle. It inhibited the polymerization of tubulin to polymeric microtubule and induced apoptosis following twenty-four hour incubation demonstrated by three different assays. Structurally, analog 145E resembles colchicine site agents. Additional SAR was conducted to evaluate the effect of the number of methoxy groups on the benzylidene ring; position of the methoxy groups; nature of substituents on position 6 of the core ring. Two structural features, namely the 6-OCH3 group in the indolinone ring and the trimethoxy group in the benzylidene ring, are essential for potent cytotoxicity and inhibition of tubulin polymerization.
The next series of compounds were derived from Thalidomide. In an search for potent thalidomide analogs, anti-angiogenic analog 5-hydroxy-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione 5HPP-33 was identified. In addition to its anti-angiogenic activity, 5HPP-33 demonstrated potent anti-proliferative activity (low micromolar range) in vitro against nine tumor cell lines. Flow cytometric analyses reveal that 5HPP-33 induced cell cycle arrest in G2/M phase in tumor cells. Cells treated with compound 5HPP-33 exhibit a breakdown of nucleus into micronuclei. 5HPP-33 enhances tubulin polymerization in vitro tubulin polymerization assay and the polymers thus formed are resistant to cold. It demonstrated inhibition of NFκB activation and stabilized the degradation of inhibitory protein IκB. Following the mechanistic study of 5HPP-33, a series of structural analogs were evaluated for their cytotoxicity in tumor cells. The analogs included modifications on the rings A and C of 5HPP-33. Two structural features, namely the 5-substitution on ring A and 2, 6-diisopropyl group in the ring C, are essential for potent cytotoxicity and induction of tubulin polymerization.
Dedicated to my parents
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VITA

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LIST OF ABBREVIATIONS

α  alpha
Abs absorbance
Ac acetyl
β  beta
Bu butyl
°C degrees Celsius
CDK cyclin dependant kinase
DAPI 4',6-diamidino-2-phenylindole
DNA Deoxyribonucleic acid
DMSO dimethylsulfoxide
Et ethyl
ELISA Enzyme-Linked ImmunoSorbent Assay
FBS fetal bovine serum
FGF fibroblast growth factor
γ  gamma
g gram(s)
G₀/G₁ quiescence/ growth phase 1 (cell cycle analysis)
G2/M  growth phase 2/ mitosis
GTP  guanosine triphosphate
h  hour(s)
IC50  inhibitory concentration for half of maximal activity
IκB  Inhibitor of NFκB
IKK  IκB kinase
L  liter(s)
μ  micro
μM  micromoles per liter
Me  methyl
min  minute(s)
mol  mole(s)
MT  microtubule
MTS  (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
sulfophenyl)-2H-tetrazolium,
NFκB  Nuclear factor kappa-B
NMR  nuclear magnetic resonance
PARP  poly(ADP- ribose) polymerase
PBS  phosphate buffered saline
PDGF  platelet derived growth factor
PI  propidium iodide
PIPECES  1,4 piperazine-diethanesulfonic acid)
PC-3  Prostate carcinoma
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<td>PGA</td>
<td>Pthaloyl glutamic acid</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidyl serine</td>
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<tr>
<td>R</td>
<td>alkyl group</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>SAR</td>
<td>structure activity relationship</td>
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<td>Taxol</td>
<td>Paclitaxel</td>
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<td>TRIS</td>
<td>trishydroxymethylaminomethane</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER 1

MICROTUBULES AND ANGIOGENESIS IN CANCER

1.1 Background

Cancer is a group of diseases in which the body's cells become abnormal, divide without control and may invade nearby tissues. Tumor formation is a complex process, which involves many steps including genetic defects in tumor cells leading to hyperproliferation, loss of contact mediated growth inhibition, recruitment of new blood supply to support immense growth and metastasis of tumor cells to distant sites.

Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Once cells become malignant, they multiply more rapidly than usual. Then they often form masses which invade nearby tissue by metastasis and interfere with their functions. Cancer develops when mutations occur in genes that normally control cell division. These mutations prompt the cell to divide inappropriately.

The uncontrolled proliferation of tumor cells may arise from increased mitogenic signaling and/or decreased threshold required for cell-cycle commitment. Therefore, understanding how cell cycle progression and cell death are regulated can provide
important insights in anti-cancer drug discovery. Other cancer-promoting genetic abnormalities may be randomly acquired through errors in DNA replication, or are inherited, and thus present in all cells from birth. In the U.S. and other developed countries, cancer is presently responsible for about 25% of all deaths. The statistics below are for adults in the United States.

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<td>Pancreatic cancer (5%)</td>
<td>Endometrial cancer (6%)</td>
<td>Ovarian cancer (6%)</td>
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<td>Leukemia (4%)</td>
<td>Non-Hodgkin lymphoma (4%)</td>
<td>Pancreatic cancer (6%)</td>
</tr>
</tbody>
</table>

Table 1.1 American Cancer Society data

Traditional chemotherapy uses anticancer drugs that target the hyperproliferative cancer cells. These chemotherapeutic drugs traditionally interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target rapidly dividing cells. Among the cellular structures necessary to maintain the growth and function of normal and malignant cells, microtubules play a pivotal role exceeded only by that of DNA as the template for transcription. Microtubules are extremely important in the process of
mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage into two daughter cells. Their importance in mitosis and cell division makes microtubules an exciting target for anticancer drugs. Microtubules and their dynamics are the targets of a chemically diverse group of antimitotic drugs that have been used with great success in the treatment of cancer. In view of the success of this class of drugs, it can be argued that microtubules represent one of the best cancer target to be identified thus far$^{2,3}$.

In addition to targeting the hyperproliferative nature of a tumor, research efforts are also being focused on novel-mechanism based and target–based approaches specific for pathways that lead to or are specific for cancer. One such approach is targeting the formation of blood vessels of a growing tumor called angiogenesis.

Angiogenesis is the process by which new vasculature is recruited from pre-existing vessels, and it is particularly relevant to the pathology of most, if not all, human tumors. Data on the growth and metastasis of various solid tumors strongly suggest that angiogenesis is a crucial prerequisite for progression to advanced disease$^4$. The angiogenic process in solid tumors is now known to be crucial for advanced tumor growth and progression to a metastatic state. Microvessel density is an indicator of biological aggressiveness and metastatic potential in many primary tumors. Developing tumors require new vasculature as they grow in order to ensure a constant supply of required nutrients and oxygen while allowing for the elimination of metabolic waste.
1.2 Cell Cycle

The cell cycle, or cell-division cycle, is the series of events that take place in a eukaryotic cell between its formation and the moment it self replicates. These events can be divided in two main parts: interphase (in between divisions phase grouping G₁ phase, S phase, G₂ phase), during which the cell is forming and carries on with its normal metabolic functions; the mitotic phase (M mitosis), during which the cell is dividing itself. M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell's cytoplasm physically divides. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G₀ phase, while cells that have permanently stopped dividing due to age or accumulated DNA damage are said to be senescent.

Proper progression through the cell cycle alternation of S and M phases, and coordination of growth and division is assured by ‘‘checkpoints’’ that guard crucial transitions in the chromosome cycle. The G₁ checkpoint controls entry into S phase, while the G₂ checkpoint guards entry in mitosis\(^5,6\). The metaphase checkpoint guards the metaphase-to anaphase transition. If problems arise in assembling the spindle or aligning the chromosomes, the metaphase checkpoint blocks activation of the mitotic exit network. The master regulator molecules of the cell cycle are enzymes called cyclin-dependent protein kinases (CDKs)\(^7\). As their name implies, CDKs require a cyclin partner to be active. When associated with appropriate cyclins, CDKs trigger major events of the chromosome cycle (DNA replication, nuclear envelope breakdown, chromosome condensation, spindle assembly) by phosphorylating serine-threonine residues on certain
target proteins. Cyclin protein levels rise and fall during the cell cycle and in this way they periodically activate CDK. Different cyclins are required at different phases of the cell cycle. The three D type cyclins (cyclin D1, D2 and D3) bind to CDK4 and to CDK6 and CDK-cyclin D complexes are essential for entry in G₁. Another G₁ cyclin is cyclin E which associates with CDK2 to regulate progression from G₁ into S phase.

Figure 1.1 A schematic representation of the mammalian cell cycle

1.3 Mitosis

Mitosis is the process in which a cell divides its chromosomes to generate two identical cells. It is generally followed by cytokinesis which divides the cytoplasm and cell membrane. During the process of mitosis the pairs of chromosomes condense and
attach to spindle fibers that pull the sister chromatids to opposite sides of the cell. Involved in this step, is the mitotic spindle, which consists of microtubules and many proteins that interact with the chromosomes and facilitate segregation. Highly dynamic microtubules in the spindle are required for the various stages of mitosis. ATP hydrolysis is a driving force for the movement of microtubules.

The process of mitosis can be classified into five stages: interphase, prophase, prometaphase, metaphase and anaphase\(^8\) (Figure 1.2). During prophase, the nuclear envelope breaks down, chromosomes are condensed, and centrosome separation occurs. Minus-ends of microtubules are tethered at the spindle poles with the plus-ends extending away. Some of the microtubules plus-ends can attach to the kinetochores of the chromosomes and facilitate the translocation of chromosomes within the spindle\(^9\). Several kinesin motor proteins also coordinate the construction of a mitotic spindle.

At prometaphase, the microtubules capture the kinetochores of the chromosomes thereby leading to the bi-orientation of the chromosomes and congression at the center of the microtubule array\(^10,11\). Chromosomes are aligned during the metaphase. Once the chromosomes are oriented and aligned, they go into the anaphase and chromatid segregation and spindle separation occurs. The cyclins A and B are complexed with CDK1 in mitosis. In late \(G_2\) and early M, cyclin A complexes with CDK1 to promote entry into M. Mitosis is further regulated by cyclin B in complex with CDK1.
1.4 Microtubules

Microtubules, the key components of the cytoskeleton, are long, filamentous tube shaped protein polymers that are essential for all eukaryotic cells. They are crucial in development and maintenance of cell shape, transport of vesicles, mitochondria, cell signaling, cell division and mitosis. Microtubules are composed of α and β-tubulin.
heterodimers. Each has a molecular weight of approximately 55 kilo-Dalton (kDa). The α and β tubulin present in eukaryotic cells have a high degree of homology\textsuperscript{12, 13}. The tubulin dimers polymerize end to end in protofilaments. The protofilaments then bundle in hollow cylindrical filaments. Typically, the protofilaments arrange themselves in an imperfect helix with one turn of the helix containing 13 tubulin dimers each from a different protofilament.

Highly dynamic mitotic-spindle microtubules are extremely important in the process of mitosis. Their importance in mitosis and cell division makes microtubules an desirable target for anticancer drugs\textsuperscript{2, 14}.

\textbf{Figure 1.3} Microtubule structure\textsuperscript{2}
1.4.1 Microtubule polymerization and dynamic instability

The polymerization of microtubules occurs by a nucleation-elongation mechanism in which the relatively slow formation of a short microtubule ‘nucleus’ is followed by rapid elongation of the microtubule at its ends by the reversible, non-covalent addition of tubulin dimers.

It is important to emphasize that microtubules are not simple equilibrium polymers. Each tubulin subunit binds to two molecules of GTP. The GTP bound to the $\alpha$ tubulin subunit is locked at the interface of the two subunits and is non-exchangeable. On the other hand the GTP bound to the $\beta$ subunit (exchangeable-E site) undergoes hydrolysis during polymerization. The resulting E-site GDP does not exchange, while the $\beta$ tubulin remains in the polymer. Upon depolymerization, the released tubulin subunits can exchange E-site GDP for GTP and undergo another round of polymerization.

The tubulin dimers show complex polymerization dynamics that use energy provided by the hydrolysis of GTP at the time that tubulin with bound GTP adds to the microtubule ends; these dynamics are crucial to their cellular functions. The correct movements of the chromosomes and their proper segregation to daughter cells require extremely rapid and precise tubulin dynamics, making mitosis exquisitely sensitive to microtubule-targeted drugs. The biological functions of microtubules in all cells are determined and regulated in large part by their polymerization dynamics.\textsuperscript{10} Microtubules show two kinds of non-equilibrium dynamics, both with purified microtubule systems \textit{in vitro} and in cells.

One kind of dynamic behavior that is highly prominent in cells, called ‘dynamic instability’, is a process in which the individual microtubule ends switch between phases
of growth and shortening. “Dynamic instability” refers to the continuous switching between microtubule polymerization and depolymerization which leads to the rapid switching between growth and shrinkage of microtubules. The second dynamic behavior, called ‘treadmilling’ is net growth at one microtubule end and balanced net shortening at the opposite end. It involves the intrinsic flow of tubulin subunits from the plus end of the microtubule to the minus end and is created by differences in the critical subunit concentrations at the opposite microtubule ends. This behavior occurs in cells as well as in vitro, and might be particularly important in mitosis. Treadmilling and dynamic instability are compatible behaviors, and a specific microtubule population can show primarily treadmilling behavior, dynamic-instability behavior or some mixture of both.

1.4.2 Antimitotic tubulin ligands

Numerous tubulin ligands with antimitotic properties and anticancer activity have been found in recent years. These compounds inhibit proliferation of tumor cells by acting on the polymerization dynamics of spindle microtubules. Proper spindle function in mitosis has a requirement of highly dynamic microtubules. First, for the correct attachment of chromosomes at their kinetochores to the spindle, second, for complex movements of chromosomes that bring them to properly aligned position at the metaphase plate, finally, for the separation of chromosomes after metaphase-anaphase checkpoint is complete. The anti-mitotic agents can bind either on soluble tubulin or directly to tubulin in the microtubules. These agents can be classified into three main groups according to their binding sites: those that bind to the colchicine-binding site;
those that bind to the vinca site, and those that bind to the paclitaxel (taxol) site. The many drugs that modulate microtubule dynamics might be mimicking the actions of numerous regulators that control microtubule dynamics. 18, 19

Vinca site and colchicine site agents are commonly classified as inhibitors of microtubule polymerization or microtubule destabilizing agents. Agents that belong to the taxol site are known as microtubule stabilizing agents and stimulate microtubule polymerization. The classification of drugs as stabilizers or inhibitors of assembly is simplistic. The reason being drugs that increase or decrease microtubule polymerization at high concentrations can powerfully suppress microtubule dynamics at 10-100 fold lower concentration. The dynamics of spindle microtubules is suppressed without change in the polymer mass, sufficient to slow or block mitosis and induce apoptosis. This property is responsible for the high efficacy of anti-microtubule agents for cancer treatment.

1.4.2.1 Colchicines Site

Colchicine, isolated from Colchicum autumnale, has played a fundamental role in studies of mitosis 20. It was found to destroy the mitotic spindles by binding to protein molecules. Tubulin was initially purified using 3H colchicine as a biochemical marker.

The effect of colchicine on microtubule dynamics is concentration dependant. At high concentrations it inhibits microtubule polymerization and depolymerizes pre-formed microtubules. At low concentration its can inhibit the dynamics of microtubules without reduction in polymer mass. Colchicine binds to soluble tubulin to form a tubulin-colchicine complex (TC), which gets incorporated into the microtubule ends as it gets
polymerized. At low TC the incorporation of complex disrupts the tubulin lattice at or near the end such that the efficiency of new tubulin addition gets impaired. At high TC concentration the addition of tubulin at the microtubule ends is blocked completely. Colchicine blocks mitosis at the metaphase/anaphase transition in cells inducing accumulation of blocked spindles in a metaphase like state\textsuperscript{21, 22}. Compounds that bind to the colchicine site are structurally simpler than agents that bind to the vinca or taxol site but are more diverse.

A large number of naturally occurring and synthetic antimitotic agents which bind to the colchicine site have recently been discovered. These include E7010 (\(N\)-[2-[(4-hydroxyphenyl) amino]-3-pyridinyl]-4-methoxybenzenesulfonamide), T138067 (\(N\)-(3-fluoro-4-methoxyphenyl)pentafluorobenzenesulfonamide), steganacin, indonacaine, podophyllotoxin, combretastatin etc\textsuperscript{23-27}.

Combretastatin A-4 (CA-4) and A-1 (CA-1) are tubulin binding agents structurally related to colchicine. These bind on to the colchicine site on tubulin and induce depolymerization of the microtubule protein\textsuperscript{28}. When CA-4-P is added to cultures of endothelial cells (at 0.1–1 µM) the microtubules rapidly depolymerize, the cells become round within minutes, undergo blebbing and detachment from the substrate and actin stress fibers form. These changes increase vascular permeability and disrupt tumor blood flow\textsuperscript{29}. In experimental tumors, anti-vascular effects are seen within minutes of drug administration and rapidly lead to extensive ischemic necrosis in areas that are often resistant to conventional anti-cancer treatments.
1.4.2.2 Vinblastine Site

Vinblastine and vincristine are alkaloids found in the Madagascar periwinkle, *Catharanthus roseus*. Vinca alkaloids are a class of potent antitumor drugs used in cancer chemotherapy. Vinblastine is mainly useful for treating Hodgkin's disease, advanced testicular cancer and advanced breast cancer. Vincristine is mainly used to treat acute leukemia and other lymphomas. Vinblastine and vincristine bind to tubulin at a distinct site called the Vinca-binding domain\(^{30}\).

Vinblastine potently blocks or slows mitosis at the metaphase/anaphase transition in a number of cell types. It was thought to act primarily as a microtubule depolymerizer. However at low concentrations it exhibits stabilizing effect on microtubule dynamics in the absence of depolymerization. In contrast to colchicine, vinblastine binds directly to microtubules without first forming a complex with soluble tubulin\(^{31}\). It binds to microtubule ends with high affinity but also has low affinity binding sites along the sides
of microtubule cylinders$^{32}$. It strongly reduces the rate and extent of microtubule growth and shortening while increasing the time microtubules spend in an attenuated state. Binding of a vinca site agent on tubulin inhibits guanine nucleotide exchange known to occur on the $\beta$ tubulin$^{30}$. A fluorescent vinblastine derivative, vinblastine-4’-anthranilate formed a covalent adduct on $\beta$ tubulin following irradiation. The binding site was conclusively demonstrated by the tryptic digestion of the covalent adduct. The hydrolysis identified a single fluorescent $\beta$ peptide coinciding with the residues 175-213$^{33}$. Vinblastine depolymerizes microtubules and destroys mitotic spindles at concentration of 10-100nM, thereby leaving the dividing cancer cells blocked in mitosis with condensed chromosomes. At a low, but clinically relevant concentrations (0.8 nM in Hela cells), vinblastine does not depolymerize spindle microtubules, yet blocks mitosis which eventually induces apoptosis$^{14}$. Additionally, binding of vinblastine to tubulin induces a conformational change in the protein, which appears to be responsible for the self-association of tubulin in the presence of high concentration of drug in the micromolar range. The ability of vinblastine/vincristine to increase the affinity of tubulin for itself plays a major role in the ability of the drug to stabilize microtubules kinetically.
1.4.2.3 Taxol Site

Taxol (Paclitaxel) was discovered at Research Triangle Institute (RTI) in 1967 when Monroe E. Wall and Mansukh C. Wani isolated the compound from the bark of the Pacific yew tree, *Taxus brevifolia*, and noted its antitumor activity in a broad range of rodent tumors. Paclitaxel has since become an effective tool for patients with lung, ovarian, breast cancer, and advanced forms of sarcoma. Taxol binds to microtubules rather than soluble tubulin. A photolabeled taxol derivative was demonstrated to bind specifically to a peptide at the N terminal 1-31 amino acids and also at residues 217-231 following formic acid, cyanogen bromide and trypsin induced cleavage of β tubulin\textsuperscript{34-36}. The exact location of taxol binding on tubulin is known with precision due to determination of the electron crystal structure of tubulin carried out with taxol complexed to it\textsuperscript{37, 38}. It gains access to binding site by diffusing through small openings in the microtubules or fluctuations of the lattice. In contrast to vinblastine and colchicine, taxol enhances microtubule polymerization in vitro promoting both the nucleation and elongation phases of polymerization and reduces the critical tubulin subunit concentration.
(soluble tubulin concentration at steady state). Microtubules formed in the presence of taxol are extremely stable and resist depolymerization by low temperatures, calcium ions and anti-mitotic drugs. Suppression of microtubule dynamics by taxol leads to mitotic block in the absence of microtubule bundling. In Hela cells, mitosis is half maximally blocked at 8 nm taxol, where as there is no increase in microtubule-polymer mass below 10 nM39. The suppression of spindle microtubule dynamics prevents the dividing cancer cells from progressing from metaphase to anaphase and cells die by apoptosis. In addition to taxol, docetaxel (Taxotere), its semi-synthetic analogue, has also been approved in treatment of prostate, brain and lung tumors. Recent development of taxol analogues have attempted to address limitations inherent to taxol. The limitations include resistance caused by mutation in β tubulin; P-gp based multi-drug resistance and undesirable side effects. In addition to taxol and its analogues, several promising compounds such as epithilones, discodermolide, laulimalide and sarcodictyins have demonstrated to enhance microtubule polymerization. Treatment of prostate cancer cell lines expressing bcl-2 with taxol induces bcl-2 phosphorylation and programmed cell death40, 41. Bcl-2 phosphorylation seems to inhibit its binding to bax, a pro-apoptotic protein.

Figure 1.6 The structure of Paclitaxel (Taxol)
1.5 Angiogenesis

Angiogenesis is the process of forming new blood vessels from pre-existing ones\(^4, 42, 43\). It is a complex process that is tightly regulated by pro- and anti-angiogenic growth factors such as the vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF). Some of these factors are highly specific for the endothelium, such as VEGF, while others have a wide range of activities, such as the matrix metalloproteinase (MMP).

Angiogenesis occurs in a series of complex and interrelated steps. First, diseased (tumorigenic) or injured cells release pro-angiogenic growth factors, such as VEGF, FGF, or PDGF, into the surrounding tissue. Angiogenic growth factors are released in response to secreted proteins in the microenvironment (e.g., epidermal growth factor, fibroblast growth factor, insulin-like growth factor, interleukins, platelet-derived growth factor), hypoxia, hypoglycemia, mechanical stress (e.g., increased pressure caused by proliferating tumor cells), release of inflammatory proteins (e.g., cyclooxygenase-2, prostaglandins, mast cell activation), and genetic alterations.

The released growth factors (such as the VEGF which plays central role in tumor angiogenesis) bind to thereby activating endothelial cells that form the walls of nearby blood vessels. There are currently six known members of the VEGF family. The biological effects of VEGF are mediated by two receptor tyrosine kinases (RTKs); VEGFR-1 (Flt-1 or \textit{fms}-like-tyrosine kinase) and VEGFR-2 (Flk-1/KDR). The expression of these receptors is largely restricted to the vascular endothelium and the effects of VEGF on vascular endothelium are mediated by these receptors. VEGFR2 is thought to be the dominant signal transduction pathway regulating angiogenesis. When VEGF binds
to its receptor, the pro-angiogenic signal is transmitted by the RTK to downstream proteins, initiating a signal cascade.

Activated endothelial cells signal their nucleus to produce enzymes, such as MMPs. These enzymes break down the extracellular matrix of the blood vessel, allowing endothelial cells to invade the matrix and to divide in response to tumor-derived growth factors. The proliferating endothelial cells migrate through the holes made by the enzymes toward the growth factor stimulus. Adhesion molecules, or integrins mediate the migration of the new endothelial cells toward the growth factor stimulus and additional enzymes are released to dissolve the surrounding tissue. The adhesion receptor integrin αvβ3, present on the surface of activated endothelial cells, is required for the differentiation, maturation, and survival of blood vessels.

1.6 Role of angiogenesis in cancer

Without developing blood supply, a tumor can only reach the size of 1-2 mm\textsuperscript{44}, which is small enough to be physically removed or treated with conventional cytotoxic agents. VEGF plays a central role in tumor angiogenesis; it is expressed in most tumors often at substantially increased levels. VEGF expression in tumor cells is stimulated by hypoxia and oncogenes such as ras or by inactivation of tumor suppressor genes and other cytokines. VEGF mRNA is markedly up-regulated in most human tumors. Whereas VEGF mRNA is up-regulated in tumor, KDR mRNA are up-regulated in the endothelial cells associated with the tumor.

There is a correlation between tumors with higher densities of blood vessels and metastasis and poor clinical outcome. Anti-angiogenic therapy has demonstrated to
decrease the growth of primary tumors and secondary tumor metastases. In addition to uncontrolled tumor cell proliferation, angiogenesis is a major target of cancer therapy. Angiogenic inhibitors may be most effective when combined with traditional cytotoxic chemotherapy. There are several reasons to support this hypothesis. The cellular target for the angiogenesis inhibitors differs from that of cytotoxic agents; therefore, combination therapy should lead to reduced tumor burden without overlap in patterns of resistance. In fact, since angiogenic inhibitors target normal, genetically stable endothelial cells, resistance to these agents may not develop. The side effect profiles of cytostatic and cytotoxic agents can be very different. For example, angiogenesis inhibitors often do not cause myelosuppression and, therefore, should allow for administration of the maximal dose of the cytotoxic agent without fear of additive toxicity.

Rhu MAb-VEGF bevacizumab (Avastin, Genentech, San Francisco, CA) is a humanized monoclonal antibody, and was the first commercially available angiogenic inhibitor. RhuMAb-VEGF has been shown to reduce VEGF levels. Selective inhibition of VEGF receptor 2 (KDR/Flk-1) using a RhuMAb-VEGF (2C3) blocks tumor growth in mice. It was approved by the Food and drug administration (FDA) in February 2004 for use in colorectal cancer along with standard chemotherapy.

VEGF and its receptors are good targets for cancer therapy because VEGF receptors are highly specific for VEGF and are expressed in increased numbers primarily during periods of tumor growth. Several different strategies have been used to inhibit VEGF, including anti-VEGF monoclonal antibodies, coupling a toxin to VEGF, soluble
VEGF receptors, peptides that interfere with VEGF binding and agents that block VEGF receptor signaling. A number of VEGF receptor tyrosine kinase inhibitors were developed to inhibit the angiogenesis associated with tumors. A series of substituted indolin-2-ones$^{45-47}$, anilinoquinazolines$^{48, 49}$, pyridine carboxamide (Sorafenib) and phthalazines have been synthesized as tyrosine kinase inhibitors with potential anti-angiogenic activity.

1.6.1 SU-5416

Compounds containing the indole-2-one pharmacophore with a pyrrole ring at the C-3 position of the core have been developed and studied extensively as catalytic inhibitors of the vascular endothelial growth factor, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) RTKs by SUGEN Inc.

Semaxinib or SU5416 is an indolin-2-one that binds in the ATP binding site of the VEGF RTK for VEGFR-1 and VEGFR-2. In addition, it binds to the PDGF receptor, which is also involved in the transduction of angiogenesis signals. Finally, it binds to c-kit, a related kinase receptor for stem cell factor.

*In vitro* studies, it exerts a potent and rapid antiproliferative effect on endothelial cells without directly affecting tumor cell growth in culture$^{50}$. When administered to mice, it inhibited the growth of tumor cells in a variety of tumor models$^{51, 52}$. In a model of AML, SU5416 inhibited the stem cell factor-induced proliferation of MO7e cells. Incubation of MO7e cells with SU5416 induced apoptosis through activation of caspase-3 and increased poly(ADP-ribose) polymerase cleavage. The direct effect of SU5416 on tumor angiogenesis was demonstrated in a C6 rat glioma model. The newly formed
microvasculature within and around the tumor was evaluated. A reduction in the total and functional vascular density and vascular leakage was reported. It produces a dose-dependent inhibition of tumor growth in a variety of xenograft models, including malignant melanoma, glioma, fibrosarcoma, and carcinomas of the lung, breast, prostate, and skin. In mice, SU5416 has been shown to reverse tumor resistance to radiotherapy\textsuperscript{98} and in a mouse metastasis model, to inhibit the growth of metastases. In a human colon cancer xenograft model, it inhibits tumor metastases, microvessel formation, and proliferation. Additionally clinical studies are being performed by using SU 5416 on solid tumors.\textsuperscript{50, 53, 54}

Another tyrosine kinase inhibitor developed with indoline-2-one pharmacophore was SU6668. It inhibited the binding to receptors of vascular endothelial (VEGF), platelet-derived (PDGF) and fibroblast growth factor (FGF), involved in the formation of new blood vessels. Molecular modeling of SU6668 in the ATP binding pockets of the Flk-1/KDR and PDGF receptor kinases provided insight to explain the relative potency and selectivity of SU6668. Oral or intra peritoneal administration of SU6668 in athymic mice results in significant growth inhibition of a diverse panel of human tumor xenografts of glioma, melanoma, lung, colon, ovarian, and epidermoid origin and is being studied in clinical studies\textsuperscript{50, 55-58}.

Sutent, previously known as SU-11248 or sunitinib malate is also a tyrosine kinase inhibitor\textsuperscript{59, 60}. The cellular targets of this drug include KIT, PDGRFA, PDGFRB, all three VEGF receptors, FLT-3, and RET. In gastrointestinal stromal tumor (GIST), it acts not only by inhibiting KIT and PDGFRA (like imatinib or Gleevec) but also by inhibiting blood vessel growth (angiogenesis) to the tumor. Sunitinib is thought to block
more mutant forms of KIT than does imatinib. It was approved by the FDA for imatinib-resistant and imatinib-intolerant GIST and for renal cell carcinoma on January 26, 2006.

Figure 1.7 Structures of SU-5416, 6668 and 11248

1.6.2 Thalidomide

Thalidomide is a drug discovered in the mid-1950s marketed as a safe, non-addictive sedative-hypnotic drug with good antiemetic activity. The drug was used extensively for morning sickness during pregnancy until it was found to cause significant fetal abnormalities. Today, Thalidomide is being studied for a number of conditions including HIV ulcers and wasting, Crohn’s disease, rheumatoid arthritis, chronic host versus graft’s disease, Behcet’s vasculitis and cancer. It has been approved in the US for chronic treatment of erythema nodosum leprosum (ENL).

The observed bioactivity of Thalidomide in ENL and other disorders inspired research into potential antiangiogenic activity. Thalidomide demonstrated to be a potent...
anti-angiogenic agent based on studies using rabbit cornea micro pocket assay and a mouse cornea model of neovascularization, by inhibition of bFGF and VEGF\textsuperscript{68, 69}. Animal studies support this hypothesis since Thalidomide treatment can decrease vascular density in granulation tissue. Studies suggest that one or more of the Thalidomide metabolites may be responsible for its anti-angiogenic effects, and their generation may be species dependent. For example, Thalidomide inhibits microvessel formation in the rat aortic ring assay and slows human aortic endothelial cell proliferation in the presence of human or rabbit liver microsomes, but not in the presence of rat liver microsomes. In the absence of liver microsomes, Thalidomide had no effect on either microvessel formation or cell proliferation. Furthermore, a decrease of cyclooxygenase-2 leading to a diminished prostaglandin synthesis may contribute as prostaglandins stimulate angiogenesis. The anticancer activity of Thalidomide has been explored most intensely in myeloma where it is highly active, inducing clinically meaningful response in patients at various stages of the disease.

Besides the anti-angiogenic effect, Thalidomide induces stimulation of immune-mediated responses, reduction of growth stimulating factors, and attenuation of the metastatic potential. The most widely noted bioactivity of Thalidomide is its inhibitory effect on tumor necrosis factor alpha (TNF-\(\alpha\)), an angiogenic cytokine that is overproduced in several malignancies. An inhibition of human monocyte TNF-\(\alpha\) production in lipopolysaccharide-induced human monocytes has been demonstrated. Inhibition of TNF-\(\alpha\) production was later shown to correlate with Thalidomide-induced TNF-\(\alpha\) mRNA degradation rate. It also inhibits the IKK\(\beta\) induced phosphorylation of
IκB and subsequently activation of NFκB.\textsuperscript{70, 71} A wide range of activities of Thalidomide may be explained to a great extent by its effects on nuclear factor-κB (NF-κB) activity.

![Structure of Thalidomide](image)

**Figure 1.8 Structure of Thalidomide**

SU-5416 and Thalidomide that demonstrate anti-angiogenic activity were used as the lead compounds for designing potent analogs in this study. The benefits of anti-angiogenic agents have been discussed in the last section. Additionally several studies have indicated that combining an anti-angiogenic agent such as SU5416 with traditional cytotoxic agents\textsuperscript{72-74} is a promising therapeutic strategy. A series of substituted 1,3-dihydro-indole-2-one analogs based on SU-5416 and iso-indole-1,3-diones based on Thalidomide with structural modifications were designed to possess anti-angiogenic and anti-proliferative properties. Dual acting compounds could be powerful tools in inhibiting the tumor cells and blood supply to tumor cells.

**In short the specific aims of this research project were:**

1. Identify active compounds with anti-cancer activity based on the indoline-2-one pharmacophore (Chapter 2)
   - Evaluate the potent cytotoxic analogs by measuring the IC\textsubscript{50} value on \textit{in vitro} tumor cell based assays.
• In vitro high throughput screening of the library of indoline-2-one analogs by using 96-well plate cytotoxicity and anti-microtubule assays to establish a structure activity relationship.

• Identify lead compounds and elucidate the mechanism of action behind the cytotoxic activity.

• Study the effect on cell cycle, anti-microtubule effect, apoptosis induction.

2. Identify and evaluate the activity of synthetic analogs of Thalidomide (Chapter 3)

• Elucidation of mechanism of action of cytotoxic activity of 5-hydroxy-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (5HPP-33)

• Structure activity relationship study on analogs with aromatic ring replacing the glutaramide ring of Thalidomide to identify potent anti-cancer agents.

• Identify potent anti-angiogenic analogs of 5HPP-33 with additional effects on tumor cells

• Validate NFκβ as a target for 5HPP-33 and evaluate other active analogs of 5HPP-33.
REFERENCES


CHAPTER 2

DESIGN, SAR AND MECHANISM OF ACTION OF ANTIMICROTUBULE INDOLINE-2-ONE ANALOGS

2.1 Background

2.1.1 Compounds with 1,3-dihydroindole-2-one pharmacophore

3-substituted 1,3-dihydroindole-2-ones with a pyrrole ring at the C-3 position of the core have been developed and studied extensively as catalytic inhibitors of the vascular endothelial growth factor, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) receptor tyrosine kinases by SUGEN Inc. These compounds are ATP competitive inhibitors in which 1,3-dihydroindole-2-one core occupies the adenine binding site of ATP.

Semaxinib or SU5416 is an 1,3-dihydroindole-2-one that binds in the ATP binding site of the VEGF RTK for VEGFR-1 and VEGFR-2. In addition, it binds to the PDGF receptor, which is also involved in the transduction of angiogenesis signals. Finally, it binds to c-kit, a related kinase receptor for stem cell factor. \textit{In vitro} studies show a potent and rapid antiproliferative effect on endothelial cells without directly affecting tumor cell growth in culture. When administered to mice, it inhibits the growth
of tumor cells in a variety of tumor models.\textsuperscript{1-4} It produces a dose-dependent inhibition of tumor growth in a variety of xenograft models, including malignant melanoma, glioma, fibrosarcoma, and carcinomas of the lung, breast, prostate and skin. Clinical studies are being performed by using SU 5416 on solid tumors\textsuperscript{5-7}.

Another tyrosine kinase inhibitor developed with the 1,3-dihydroindole-2-one pharmacophore was SU6668. It inhibited the binding to receptors of vascular endothelial (VEGF), platelet-derived (PDGF) and fibroblast growth factor (FGF), involved in the formation of new blood vessels. Molecular modeling of SU6668 in the ATP binding pockets of the Flk-1/KDR and PDGF receptor kinases provided insight to explain the relative potency and selectivity of SU6668. Oral or i.p. administration of SU6668 in athymic mice results in significant growth inhibition of a diverse panel of human tumor xenografts of glioma, melanoma, lung, colon, ovarian, and epidermoid origin. It is currently being studied in clinical trials\textsuperscript{1,7-10}.

Sutent, previously known as SU-11248 or sunitinib malate, is also a tyrosine kinase inhibitor. The cellular targets of this drug include KIT, PDGRFA, PDGFRB, all three VEGF receptors, FLT-3, and RET. In gastrointestinal stromal tumor (GIST), it acts not only by inhibiting KIT and PDGFRA (like imatinib or Gleevec) but also by inhibiting blood vessel growth (angiogenesis) to the tumor. It was approved by the FDA for imatinib-resistant and imatinib-intolerant GIST and for renal cell carcinoma on January 26, 2006.\textsuperscript{11,12}
In addition to the tyrosine kinase inhibitors, 1,3-dihydroindole-2-one core is part of compounds with diverse pharmacological activities.

**A-432411** is a small molecule anti-mitotic agent that inhibits the proliferation of tumor cells by destabilization of microtubule dynamics.\(^\text{13}\) A-432411 competes with the colchicine-binding site on tubulin and inhibits microtubule polymerization. It activates the spindle checkpoint by inhibiting phospho-cdc2 at Tyr15 (cdc2-Y15).

**TAS-301**, is an inhibitor of smooth muscle cell migration and proliferation and is an inhibitor of post angioplasty intimal thickening.\(^\text{14, 15}\)

**Methisazone** [N-methylisatin β-thiosemicarbazone] is an anti-viral that interferes with the translation of mRNA messages to protein synthesis on the cell ribosome.\(^\text{16}\)

**SU9516** is a selective cyclin dependent kinase-2 inhibitor which also induces cytochrome c release, bax mitochondrial translocation, and apoptosis in association with pronounced down-regulation of the antiapoptotic protein Mcl-1\(^\text{17}\).
Figure 2.2 Pharmacologically active compounds with 1,3-dihydro-indole-2-one nucleus
2.2 Results and discussion

![Indoline-2-one Pharmacophore](image)

**Figure 2.3** Indoline-2-one Pharmacophore

In our search for new anti-cancer agents, the 1,3-dihydroindole-2-one pharmacophore based on SU-5416 was used. Analogs were designed by substituting $R_1$ with different functional groups either at positions 5 or 6 of the core and the $R_2$ position with substituted phenyl or pyrrole rings.

Analogs were systematically synthesized with modifications at $R_1$ and $R_2$. We were interested in evaluating their anti-proliferative activity. The anti-proliferative activity on tumor cells was determined with a preliminary screen of the compounds. In the preliminary screen the ability to inhibit the growth of PC-3 prostate carcinoma cells at 30 $\mu$M concentration was measured. Compounds that inhibited the viability of the PC-3 cells by 50% or greater at 30 $\mu$M concentration were evaluated for further biological activity. An initial structure activity relationship (SAR) was developed to identify structural features contributing to biological activity.
2.2.1 Preliminary screen of 3- substituted 1,3-dihydroindole-2-one analogs

2.2.1.1 Analogs with substitution on the 3-(substituted benzylidene) ring of the 1,3-dihydroindole-2-one

The initial screen was performed on compounds with substituted 3-benzylidene rings on the 1,3-dihydro-indole-2-one core for their ability to inhibit the proliferation of PC-3 cells at 30 µM concentration. The compounds in this group included a hydroxy, methoxy, dimethylamine, sulfamate or bromo substituent at 2’, 3’ or 4’ position of the 3-benzylidene ring and a methoxy or hydroxy group at position 6 of the core structure.

The presence of a substituent at the 3’ position exhibited anti-proliferative activity with more than 50% inhibition among the compounds with 6-hydroxy 1,3-dihydroindole-2-one screened while compounds with a substituent either on position 2’ or 4’ demonstrated less than 50% inhibition of proliferation.

It is clear from the screen that the presence of a methoxy group at the 6 position translated into strong anti-proliferative activity. Except for the compound with the bromo substituent at the 4’ position, other analogs exhibited a 50-80% inhibition of proliferation. The presence of a bromo group at 4’position led to no inhibition in either of the compounds with 6-hydroxy or methoxy 1,3-dihydroindole-2-one. Table 2.1 summarizes the percent inhibition of proliferation for the screen.
Table 2.1 Inhibition of growth of PC-3 cells by 30 µM substituted 3- benzylidene-1,3-dihydro-indole-2-one. Data are the mean of three or more experiments and are reported as means ± standard deviation.
The next screen had analogs with a sulfamate group either on position 5 or 6 of the 1,3-dihydroindole-2-one assayed with modifications on the 3-benzylidene ring. The modifications on the 3-benzylidene ring included sulfamate, methoxy and dimethylamine on the 3’ or 4’ positions. Among the four compounds evaluated compound 108 exhibited a percent inhibition of 64%. The other compounds inhibited proliferation at less than 40%.

The next class of analogs screened had no substitution on the 1,3-dihydroindole-2-one core. The 3-benzylidene ring had substituents on positions 3’ and 4’. Compound 120 with a dimethyl amine group at 4’ position on the 3-benzylidene ring demonstrated anti-proliferative activity of 66.3±7.60. Other analogs of this class had little anti-proliferative activity. Table 2.2 summarizes the percent inhibition data.
Table 2.2 Inhibition of growth of PC-3 cells by substituted 3-Benzylidene-1,3-dihydro-indole-2-one. Data are mean of three or more experiments and are reported as means ± standard deviation.
2.2.1.2 Analogs with - (pyrrol-2-ylmethylene) substituent on 1,3-dihydroindole-2-one core

Eight compounds with a pyrrole ring on the 1,3-dihydroindole-2-one core were evaluated in the next screen. Compounds 128 and 133 with a hydroxy group on the position 5 and 6 of the 1,3-dihydroindole-2-one ring and compound 130 with no substitution on the 1,3-dihydroindole-2-one ring demonstrated strong antiproliferative activity with greater than 50% inhibition. Compound 129 (SU-5416), that inhibits the KDR and VEGFR-1 tyrosine kinase, did not inhibit the proliferation of prostate carcinoma cells as expected. Table 2.3 summarizes the percent inhibition data for this screen. Additionally compounds 102 with a sulfamate group on position-6, 131 with a 6-OCH₂COOH, 132 with a 6-methoxy group exhibited poor inhibitory activity. Compound 103 with a sulfamate group on position 5 demonstrated inhibition of 40.7±3.40.
Table 2.3 Inhibition of growth of PC-3 cells by 5 or 6-substituted 3-(1H-pyrrol-2-ylmethylene)-1,3-dihydro-indole-2-one. Data are mean of three or more experiments and are reported as means ± standard deviation.
2.2.2 Identification of the lead compound

Following an initial anti-proliferative screen 3-benzylidene substituted, 1,3-dihydroindole-2-one analogs that inhibited cell viability by greater than 50% in the single concentration screen were further evaluated. A concentration dependent study was performed to quantitatively determine the IC$_{50}$ value of the active analogs on prostate and breast carcinoma cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>PC-3 IC$_{50}$ µM</th>
<th>MDA-MB-231 IC$_{50}$ µM</th>
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</thead>
<tbody>
<tr>
<td>108</td>
<td>6-OSO$_2$NH$_2$</td>
<td>3’- OSO$_2$NH$_2$</td>
<td>0.66±0.17</td>
<td>2.10±0.70</td>
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<td>109</td>
<td>6-OCH$_3$</td>
<td>4’-OSO$_2$NH$_2$</td>
<td>1.23±0.54</td>
<td>3.12±0.90</td>
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<td>111</td>
<td>6-OCH$_3$</td>
<td>3’-OH</td>
<td>0.54±0.23</td>
<td>0.46±0.20</td>
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<td>114</td>
<td>6-OH</td>
<td>3’-OCH$_3$</td>
<td>31.6±1.95</td>
<td>11.2±1.40</td>
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<tr>
<td>120</td>
<td>-</td>
<td>4’-N(CH$_3$)$_2$</td>
<td>21.9±5.78</td>
<td>8.02±2.80</td>
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<tr>
<td>124</td>
<td>6-OCH$_3$</td>
<td>4’-N(CH$_3$)$_2$</td>
<td>9.50±4.20</td>
<td>1.81±0.50</td>
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<tr>
<td>126</td>
<td>6-OCH$_3$</td>
<td>4’-OH</td>
<td>0.64±0.25</td>
<td>0.55±0.30</td>
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<tr>
<td>136</td>
<td>6-OH</td>
<td>3’-OH</td>
<td>19.6±3.70</td>
<td>7.10±0.50</td>
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</tbody>
</table>

Table 2.4 IC$_{50}$ values in µM of active analogs determined from screen. Data are mean of three or more experiments and are reported as means ± standard deviation.
A few structural features were identified that correlated with a high anti-proliferative activity. The presence of a 6-methoxy group on the 2-1,3-dihydroindole-2-one ring and a hydroxy group on the 3’ or 4’ positions of 3-benzylidene ring were two structural features that translated to compounds with the highest anti-proliferative activity. Compounds 111 and 126 with a methoxy group at position 6 exhibited highest anti-proliferative activity on the tumor cell lines tested. Additionally, compound 108 with sulfamate groups at position 6 on the core and 3’ position of benzylidene ring also demonstrated cytotoxic activity with IC_{50} value of 0.66±0.20 µM and 2.09± 0.70 µM on PC-3 and MDA-MB231 cells. Compounds 120 and 124 with a dimethylamine group at 4’ position of 3-benzylidene differ by a methoxy group at 6 position. The presence of a 6-methoxy group reduces the IC_{50} value by 3-4 fold.

2.2.2.1 Significance of 6-methoxy group.

The presence of a methoxy group at position 6 of the 1,3-dihydroindole-2-one core demonstrated potent anti-proliferative activity. In order to evaluate the significance of the methoxy group on position 6 an additional SAR study was performed. Replacement of the methoxy group with a more hydrophilic 6-OH hydroxy group resulted in a drop of 22 to 86 fold against PC-3 and MCF-7 cells, while a slight reduction of cytotoxicity against the MDA-MB-231 cells was observed. Removal of the methoxy group and replacement with a 6-H decreased the anti-proliferative activity by 10 fold against MDA-MB-231 cells and by almost 100 fold against the PC-3 and MCF-7 cells. Compounds 116, 117 and 118 had demonstrated a poor inhibition profile in the preliminary screen and demonstrated high IC_{50} values, as expected.
Based on its potent IC$_{50}$ values, compound 111 was identified as the lead for the determination of anti-proliferative activity and further mechanistic studies. Table 2.5 demonstrates the inhibitory activity of compounds with methoxy, hydroxyl or a hydrogen at carbon-6 of 1,3-dihydroindole-2-one core.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>PC-3 IC$_{50}$ µM</th>
<th>MDA-MB-231 IC$_{50}$ µM</th>
<th>MCF-7 IC$_{50}$ µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>6-OCH$_3$</td>
<td>3'-OH</td>
<td>0.54±0.20</td>
<td>0.46±0.20</td>
<td>0.39±0.20</td>
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<tr>
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<td>6-OCH$_3$</td>
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<td>0.55±0.30</td>
<td>2.37±0.80</td>
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<td>117</td>
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<td>31.4±7.60</td>
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<tr>
<td>118</td>
<td>6-H</td>
<td>4'-OH</td>
<td>&gt;50</td>
<td>25.7±3.50</td>
<td>17.5±3.54</td>
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<tr>
<td>136</td>
<td>6-OH</td>
<td>3'-OH</td>
<td>19.6±3.70</td>
<td>7.10±0.50</td>
<td>9.43±1.30</td>
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<tr>
<td>116</td>
<td>6-OH</td>
<td>4'-OH</td>
<td>44.7±6.10</td>
<td>9.12±1.30</td>
<td>19.7±3.80</td>
</tr>
</tbody>
</table>

Table 2.5 IC$_{50}$ values of analogs to demonstrate the significance of the 6- methoxy group. Data are mean of three or more experiments and are reported as means ± standard deviation.
2.2.2.2 Cell Cycle Analysis

Compounds 111 and 126 exhibited potent cytotoxic activity with IC₅₀ values of 0.23±0.10 to 2.37±0.80 µM. In order to understand the effect of compounds on the cell cycle progression, cell cycle analysis was performed on PC-3 cells. The cells were treated with compound 111 for 6, 12 and 24 hours and a media only (vehicle) control following which the cells were harvested as described in the experimental section. The results from the flow cytometric analysis demonstrated an increase in the percent of cells in the G₂-M phase following compound treatment. Cells in the control group were distributed with 33.9-35.5% percent of cells in G₀/G₁ stage, 19.7-24.7% of cells in S phase and 28.9-34.8% of cells in the G₂/M phase. On the other hand, in compound 111 treated samples the percent of cells that accumulated in the G₂-M phase increased in a time dependent manner. Following twenty four hour incubation with 1 µM compound 111, 93.7% of the cells were arrested in G₂-M phase, as demonstrated in Figure 2.4.
Flow Cytometric Analysis-PC3 Cells Control/DMSO
Time dependent Study

0 hours
G0/G1 35.5%
G2/M 34.8%
S 19.7%

6 hours
G0/G1 35.1%
G2/M 33.0%
S 21.3%

12 hours
G0/G1 33.9%
G2/M 28.9%
S 24.7%

24 hours
G0/G1 34.7%
G2/M 33.1%
S 20.6%

Flow Cytometric analysis Compound 111
PC3 Cells
Time dependent Study

6 hours
G0/G1 20.5%
G2/M 36.0%
S 43.4%

12 hours
G0/G1 0.30%
G2/M 60.4%
S 39.1%

24 hours
G0/G1 0.32%
G2/M 93.7%
S 5.9%

Figure 2.4 Cell cycle analysis of PC-3 cells incubated with 1 μM 111
2.2.2.3 Effect on Tubulin Assembly

Mitosis is the last step during cell cycle where chromosomes segregate and the parent cell is divided into two daughter cells. The mitotic spindle is involved in this step, which consists of microtubules and other proteins that interact with the chromosomes and facilitate segregation. Anti-mitotic agents that induce G₂-M phase arrest often interfere with the dynamic equilibrium of microtubule protein by either inhibiting tubulin polymerization or blocking microtubule disassembly.

The effect of compound 111 was tested on the GTP-induced assembly of purified porcine brain tubulin. The assay was performed in the absence of microtubule associated protein (MAP) and the control demonstrated an increase in absorbance over time. Samples containing compound 111 exhibited a decrease in the absorbance of the reaction solution in a dose dependent manner. Quantitative measurement of the extent of disassembly was performed over a period of 30 minutes. The IC₅₀ value calculated for Compound 111 was 9.10±0.69 μM. Compound 126 inhibited the assembly of microtubules with IC₅₀ value of 22.9±2.26μM. Based on their effect on tubulin in the polymerization assay compounds 111 and 126 can be classified as inhibitors of tubulin polymerization.
Figure 2.5 Inhibition of tubulin polymerization by compound 111. Each point on the graph is the mean of 3 independent experiments.
Compound 118, which demonstrated poor anti-proliferative profile on the tumor cell lines, caused no inhibition of tubulin assembly. Compound 111 caused more than 70% inhibition at 20 µM concentration while samples containing 118 demonstrated an assembly profile similar to control (Figure 2.6). Compounds 111 and 118 differ structurally by a methoxy group. Tubulin polymerization in the presence of compound 118 further illustrates the importance of 6-methoxy group as suggested in the previous section and demonstrates correlation between the cytotoxicity of the compound and its ability to inhibit tubulin polymerization.

![Inhibition of Tubulin Polymerization](image)

**Figure 2.6** Comparison of the effect of 20 µM of compounds 111 and 118 on tubulin polymerization. Each point on the graph is the mean of 3 independent experiments.
2.2.2.4 Binding Site Study on Tubulin

Compound 111 inhibited the assembly of microtubules in the tubulin polymerization assay. In general, compounds that inhibit tubulin polymerization belong either to the colchicine site agents or the vinca site agents. The binding site of compound 111 was identified by using a competitive fluorescence assay. The fluorescence of AC (analog of colchicine with rings A and C of colchicine) increases significantly after addition of tubulin\textsuperscript{19}. The fluorescence of AC was measured in the presence of compound 111, 118, taxol and the vinca site agent cryptophycin. Decreased fluorescence is an indication of a colchicine site agent inhibiting the binding of AC.

The values in Table 2.6 demonstrate the percentage of control fluorescence, which indicates AC fluorescence remaining after the addition of test agent to AC+ tubulin assay mixture. Compound 111 was identified as a colchicine site agent. It has a response similar to podophyllotoxin, a known colchicine site agent that reduced the fluorescence of the AC-tubulin mixture. Cryptophycin and taxol do not compete with AC for the colchicine site and have fluorescence in the range of 95-100% of control. The binding site experiment was performed in the lab of Dr. DL.Sackett.
### AC-Colchicine

<table>
<thead>
<tr>
<th>Agents</th>
<th>AC binding (% of control)</th>
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<tr>
<td></td>
<td>6 µM agent</td>
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<tr>
<td>Compounds</td>
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<td>111</td>
<td>46</td>
</tr>
<tr>
<td>118</td>
<td>93</td>
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<tr>
<td>Colchicine site binding agent</td>
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<td>Podophyllotoxin</td>
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<tr>
<td>Vinca site compound</td>
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<tr>
<td>Cryptophycin</td>
<td>97</td>
</tr>
<tr>
<td>Taxol</td>
<td>105</td>
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</table>

**Table 2.6** Competitive fluorescence inhibition study to identify binding site of compound 111. The binding site experiment was performed in the laboratory of Dr. DL. Sackett.
2.2.3 Design and evaluation of conformationally restricted analogs of compound 111

As discussed earlier, in general there are three classes of anti-microtubule agents, the taxol-site agents, the colchicine-site agents and the vinca-site agents. These agents are structurally simple, however the most diverse group among the three classes of tubulin binding agents. Some of the prototypical colchicine site inhibitors demonstrate a few structural features similar to colchicine. Compounds in this group have three features: (1) a diaryl system, (2) a trimethoxy phenyl (TMP) moiety, and (3) a constrained conformation\textsuperscript{20, 21}. The compounds that belong to the prototypical colchicine like compounds include podophyllotoxin, combretastatin, indonacaine, etc. The TMP moiety has been demonstrated to be buried in the $\beta$ tubulin structure\textsuperscript{21}.

Compound 111 includes a diaryl system. In an effort to increase the activity of compound 111, a tri-methoxy group was introduced on the benzylidene ring resulting in a trimethoxy phenyl group, a structural feature of colchicine like compounds. Various analogs of 111 were synthesized with the tri-methoxy group on various positions of the phenyl ring and further evaluated for anti-proliferative and anti-microtubule activity. Compound 145 was designed and synthesized as a conformationally restricted analog of compound 111 following introduction of the methoxy group at 3’, 4’ and 5’ positions of the benzylidene ring. This resulted in increasing the restriction of rotation around the double bond compared to 111.

In an effort to validate the structural similarity of compound 145 (3-3’,4’,5’-tri-methoxy-benzylidene-1,3-dihydroindole-2-one) to colchicine like compounds, overlapping of the energy minimized structures of compound 145 E and Combretastatin A-4 was performed using sybyl 6.9. The structures of 145 E and combretastatin-A-4 were
minimized using Tripos force field and Gasteiger–Huckel charges, using Powell method and an energy gradient of 0.005 kcal mol\(^{-1}\) Å\(^{-1}\). Conformational search of the combretastatin A-4 molecule with a 30° rotation increment of the C=C–C bonds produced 6 conformers in the energy range of 44.34–34.59 kcal mol\(^{-1}\). Among the conformers, the lowest energy conformer of combretastatin A-4 was aligned with compound 145E using sybyl 6.9. The minimized structures of 145E and the lowest energy conformation of combretastatin A-4 demonstrated good alignment.

![Figure 2.7 Illustration of the structural similarity between the minimized structure of 145E and lowest energy conformation of combretastatin A-4 using sybyl 6.9.](image)

Following the design of compound 145, various analogs were synthesized with methoxy groups on different positions and different numbers on the 3-benzylidene ring and evaluated for cytotoxicity. The 6-methoxy-3-benzylidene-1,3-dihydroindole-2-one were synthesized by coupling 6-methoxy-1,3-dihydroindole-2-one and substituted
benzaldehydes in the presence of a secondary amine\textsuperscript{22}. In general, the E isomers were synthesized as the major product, and only in three cases (compounds 145, 152 and 149), Z isomers were also isolated. The E configuration of the compounds was assigned based upon the chemical shifts of the protons at the C-2’ and C’-6’ positions in the phenyl ring at the C-3 position or 2D NOE analysis. It has been demonstrated through NOE experiment that the chemical shift of the C-2’ and C-6’ protons of 3-(substituted benzylidene)-1,3-dihydroindole-2-one was approximately 7.45-7.84 for the E isomers and 7.85-8.53 for the Z isomers.\textsuperscript{23}

\subsection*{2.2.3.1 145 E Inhibits Growth of \textit{In vitro} Tumor Cells}

In the anti-proliferative MTS assay done to assess the cytotoxic activity of 145 E we found it had IC\textsubscript{50} values of 8.10±2.30 nM against PC-3 cells and 0.83±0.07 nM against MDA-MB231 cells. The improvement of cytotoxicity was almost 70 fold in the PC-3 cells while more than 100 fold in the MDA-MB231 cells as compared to compound 111. The cytotoxic activity of 145E was comparable to various colchicine agents such as colchicine, podophyllotoxin and combretastatin A-4 (IC\textsubscript{50} values of 10-20 nM). The anti-proliferative profile of compound 145 E was then evaluated at the National Cancer Institute (NCI) against 52 human cancer cell lines. The results are shown in Table 2.7. Compound 145E was extremely potent, with GI\textsubscript{50} values below 10 nM towards 45 out of 53 human cancer cell lines tested. The compound is active in all colon, CNS, prostate and renal cancer cell lines tested (<10 nM). An interesting observation from this screen was that compound 145E was equally effective on Adriamycin resistant NCI/ADR-RES cell line. GI\textsubscript{50} is the concentration that inhibits cell growth by 50%. As mentioned earlier, the
Z isomer of compound 145 was also isolated. It demonstrated anti-proliferative activity in low nanomolar range (34.10±10.00 and 3.57±1.08 nM), represented in Table 2.9. The rate and extent of isomerization of compounds 145E and Z will be discussed in section 2.2.4.4.
<table>
<thead>
<tr>
<th>CELL-LINE</th>
<th>pGI$_{50}$</th>
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*Table 2.7 –log GI$_{50}$ NCI-60 cell line screen of 145E*
2.2.3.2 Cell cycle analysis of 145E

The effect of compound 145 E on the cell cycle progression of proliferating tumor cells was studied in a concentration dependent cell cycle analysis. Compound 145 E caused a G₂/M phase arrest in PC-3 cells following 24 hours of incubation in a concentration dependent manner. Table 2.8 lists the percentage of cells arrested in the G₂/M phase. Podophyllotoxin, a known anti-mitotic, anti-microtubule agent was used as positive control.

<table>
<thead>
<tr>
<th>Compound</th>
<th>%(G₀-G₁)</th>
<th>%S</th>
<th>%G₂-M</th>
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<td>145E 50 nM</td>
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<tr>
<td>145E 100 nM</td>
<td>2.47</td>
<td>5.86</td>
<td>92.8</td>
</tr>
<tr>
<td>Podophyllotoxin 50 nM</td>
<td>0.14</td>
<td>10.7</td>
<td>89.2</td>
</tr>
</tbody>
</table>

**Table 2.8** Percentage of PC-3 cells in different phases of the cell cycle. Values are the average of two experiments.
2.2.3.3 Effect on tubulin polymerization

To investigate whether the cytotoxicity of compound 145E has direct correlation with its ability to inhibit tubulin polymerization, it was evaluated for inhibition of tubulin polymerization using purified porcine tubulin (Figure 2.8). It inhibited tubulin polymerization with an IC$_{50}$ of 4.52±1.07 µM. The anti-microtubule activity of 145E was only two fold better than compound 111. However, it was 70-100 fold better than 111 in terms of its anti-proliferative activity. One possible explanation for this phenomenon is that 145E has molecular targets in addition to tubulin resulting in strong antiproliferative activity. Another explanation is that the concentration of compound that can decrease the actual tubulin polymer mass (to induce depolymerization and decrease the turbidity in the assay) is generally several fold higher than the concentration that can alter the dynamic instability of microtubule, sufficient to induce mitotic arrest and eventually cell death.

2.2.3.4 Effect of 145 E and Z isomers on tubulin polymerization

While compound 145E acts as an inhibitor of tubulin polymerization, its Z isomer does not inhibit tubulin polymerization to any extent (Figure 2.8 and Table 2.9). This might be due to stereochemical requirement for binding to tubulin and requires further investigation. However, 145Z still possesses potent cytotoxicity in the low nM range (Table 2.9). The cytotoxicity of compound 145Z may result from its isomerization to the E form in solution. The timeline for tubulin polymerization assay is thirty minutes, while incubation of cells with compounds lasts for seventy two hours in the anti-proliferation assay. NMR analysis of the compounds in solution (DMSO-in the presence of light between $^1$H NMR analysis) showed that compound 145Z gradually isomerizes to its E
form and attains equilibrium after 7 days with a ratio of E to Z about 3:1. A similar ratio was also obtained if the NMR analysis begins with pure compound 145E in solution (Fig 2.10). Interestingly, a similar process is also observed between cis and trans isomers of combretastatin A-4, a colchicine site agent.24

Figure 2.8 Effect of E vs. Z isomer of 145 on tubulin polymerization. Each point on the graph is the mean of 3 independent experiments.
Figure 2.9 Equilibrium ratio of 145 E and Z.
Figure 2.10 Isomerization and equilibration of 145 E and Z over 7 day period.
2.2.3.5 Apoptosis Induction by 145 E

PC-3, prostate cancer cells treated with 145E demonstrated distinct morphological changes at IC₅₀ and higher concentrations as compared to control cells (Figure 2.11). The cells appeared to be rounded off and smaller. To evaluate apoptotic cell death, three methods were used: PARP protein cleavage, annexin V/PI staining and an ELISA based DNA fragmentation.

One of the earliest events to occur during apoptosis is the externalization of phosphatidylserine (PS), a phospholipid normally restricted to the inner leaflet of the plasma membrane. This apoptotic event can be monitored using Annexin V, a PS-specific binding protein. Flow cytometric analysis showed that the extent of 145 E induced apoptotic death (annexin V positive cells) was 63.47 % with 50 nM, 88.60% with 100nM and 88.07% at 500nm following incubation for twenty four hours (Figure 2.12). The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (annexin V⁻PI⁻), the cells in early apoptosis are in the lower right quadrant (annexin V⁻PI⁺), and the ones in late apoptosis are in the upper right quadrant (annexin V⁺PI⁺).

145 E induced PARP cleavage in prostate cancer cell lines (PC-3 and DU-145) in a time dependent manner. Bands corresponding to cleaved PARP fragment were visible after 24 hours. The intensity of the bands increased substantially following 48 hours of treatment. In control cells the band corresponding to cleaved PARP protein was not visible indicating the absence of apoptosis (Figure 2.13).

The third assay was an ELISA based assay that quantifies cytoplasmic histone-associated DNA fragments that result from induction of apoptosis. The result of the
ELISA is shown in Figure 2.14. The increase in absorbance at 405 nM corresponds to an increase in apoptosis and 145 E induced apoptosis in a dose dependent manner.

**Figure 2.11** Effect on cell morphology following treatment with 145E for 24 hours
Figure 2.12 Induction of Apoptosis in PC-3 cells by 145 E measured by annexin V/PI
Figure 2.13 PARP cleavage by 50 and 100nM 145E following 24 and 48 hours of incubation. (C-control sample)

Figure 2.14 Representation of Cell death detection ELISA induced by 145E.
2.2.4 Structure activity relationship of 145 analogs

2.2.4.1 Effect of the substitution pattern based on position of substituents on benzylidene ring

Compound 145 E contains the 3,4,5-trimethoxy group on the benzylidene moiety which is a common structural feature of colchicine, CA-4, podophyllotoxins and others. Following identification of the impressive activity profile of 145E, the first structure-activity-relationship study was to investigate the substitution pattern of the trimethoxy groups. The location of the trimethoxy groups on the benzylidene ring plays a critical role for the compound to exhibit potent cytotoxicity. Transferring the 3’,4’,5’-trimethoxy groups in compound 145E to the 2’,3’,4’ position as in 151E decreases the cytotoxicity by 80-2000 fold (Table 2.9). It is interesting to note that a similar substitution pattern (2’,3’,4’-trimethoxy to replace 3’,4’,5’-trimethoxy) in a combretastatin analog also resulted in a dramatic decrease in cytotoxicity\(^{25}\). Interestingly, rotating the 3’,4’,5’-trimethoxy groups in compound 145E to the 2’,4’,5’ (compound 150) or 2’,4’,6’ (compound 149E) positions retained the potent cytotoxicity Table 2.9.

2.2.4.2 Effect of the substitution pattern based on nature of substituents on benzylidene ring

The substitution pattern of the trimethoxy groups is not the only factor that determines the cytotoxicity of the compounds. The nature of the substituents also plays a significant role. Substituting the 2’,4’,6’-trimethoxy groups in compound 149E with 2’,4’,6’-trimethyl or 2’,4’,6’-triethyl groups in compounds 190E and 191E, result in a decrease in cytotoxicity by 73 – 500 fold. This might be due to loss of the hydrogen
bonding ability in compounds 190 and 191. However, the N-1 hydrogen does not seem to be critical for its high cytotoxicity. Substituting the N1-H with a methyl group (compound 200) retains cytotoxicity with IC₅₀ values of 31.04±0.40 and 37.56±1.12 nM on PC-3 and MDA-MB-231 cells respectively. Compound 200 inhibited the polymerization of microtubules with an IC₅₀ value of 13.43±3.12 µM. This suggests that the N-1 hydrogen is not crucial for hydrogen bonding with the protein target. (Table 2.9)
Table 2.9 Anti-proliferative and anti-microtubule activity of compounds with tri-methoxy or tri-alkyl groups at different positions on 3-benzylidene-6-methoxy-1,3-dihydroindole-2-one core. Data are mean of three or more experiments and are reported as means ± standard deviation.
2.2.4.3 Effect of the substitution pattern based on number of substituents on benzylidene

The next study was to investigate the importance of the number of methoxy groups on the benzylidene ring with respect to the cytotoxicity and anti-microtubule activity. Analogs were synthesized with three, two and one methoxy groups. Based on the activity of the compounds the study demonstrated that the trimethoxy group is optimum for activity. Reducing the number of methoxy groups from 3 (compound 145) to 2 (compounds 152-163) reduced the cytotoxicity (Table 2.10). The reduction ranges from 6 – 500 fold in PC-3 cells and 80 – 2,400 fold in MDA-MB-231 cells.

The most dramatic reduction in cytotoxicity was observed when the dimethoxy groups are at the 2’ and 3’ positions as in compound 154E (500 and 2400 fold decrease in cytotoxicity in PC-3 and MDA-MB-231 cells respectively). Compound 154E, an analog of compound 151E which also contains 2’ and 3’ methoxy groups in the benzylidene nucleus, as discussed in the previous section exhibits 500-2000 fold decrease in cytotoxicity as compared to compound 145E. Based on the results, it can be postulated that the substitution of methoxy groups on the 2’ and 3’ positions is detrimental to the cytotoxicity of the compounds.

Reducing the number of methoxy groups from 2 to 1 (compound 161 – 163) also decreases the cytotoxicity of the compounds further by 2 – 3 orders of magnitude. The order of cytotoxicity is based on the number of methoxy groups on benzylidene ring, with 3>2>1. The anti-microtubule activity correlated with the cytotoxic activity.
Table 2.10 Anti-proliferative and anti-microtubule activity of compounds 153-163, with three, two and one methoxy group at different positions on 3-benzylidene-6-methoxy-1,3-dihydroindole-2-one. Data are the mean of three or more experiments and are reported as means ± standard deviation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>PC-3 (IC₅₀ nM)</th>
<th>MDA-MB-231 (IC₅₀ nM)</th>
<th>Tubulin Polymerization (IC₅₀ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145 (E)</td>
<td>3’,4’,5’- OCH₃</td>
<td>8.1±2.3</td>
<td>0.88±0.07</td>
<td>4.5±1.07</td>
</tr>
<tr>
<td>152 (E)</td>
<td>3’,5’- OCH₃</td>
<td>275±20</td>
<td>89.7±3.1</td>
<td>22.2±2.1</td>
</tr>
<tr>
<td>153 (E)</td>
<td>3’,4’- OCH₃</td>
<td>736±18</td>
<td>856±11</td>
<td>&gt;40</td>
</tr>
<tr>
<td>153 (Z)</td>
<td>3’,4’- OCH₃</td>
<td>410±155</td>
<td>3100±67</td>
<td>&gt;40</td>
</tr>
<tr>
<td>154 (E)</td>
<td>2’,3’- OCH₃</td>
<td>4200±600</td>
<td>2206±339</td>
<td>&gt;40</td>
</tr>
<tr>
<td>155 (E)</td>
<td>2’,4’- OCH₃</td>
<td>49.5±7.05</td>
<td>84±5.04</td>
<td>27.2±5.1</td>
</tr>
<tr>
<td>159 (E)</td>
<td>2’,5’- OCH₃</td>
<td>66.7±12.1</td>
<td>74.7±10.40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>160 (E)</td>
<td>2’,6’- OCH₃</td>
<td>640±9</td>
<td>179±35.1</td>
<td>16±4.2</td>
</tr>
<tr>
<td>161 (E)</td>
<td>2’- OCH₃</td>
<td>1063±172</td>
<td>734.3±80.1</td>
<td>&gt;40</td>
</tr>
<tr>
<td>162 (E)</td>
<td>3’- OCH₃</td>
<td>1382±144</td>
<td>812±53</td>
<td>&gt;40</td>
</tr>
<tr>
<td>163 (E)</td>
<td>4’- OCH₃</td>
<td>12790±838</td>
<td>8650±840</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>-</td>
<td>13.2±1.56</td>
<td>8.12±3.11</td>
<td>1.56±0.41</td>
</tr>
</tbody>
</table>
2.2.4.4 Effect of the substitution pattern based on the nature of substituents on 1,3-dihydroindole-2-one

Next the significance of the 6-OCH$_3$ group on the 1,3-dihydroindole-2-one ring based on the size of the substituent was investigated. At the beginning of the chapter the methoxy group at position 6 was replaced with a hydrophilic hydroxy group or a hydrogen atom. In the present study, the effect of increasing the size of the substituent (bulkiness) at position six was investigated.

Six analogs of compound 145E (compounds 146-156) were synthesized with different substituents at the 6 position. Replacing the 6-OCH$_3$ group in compound 145E with H (compound 156) resulted in the elimination of cytotoxicity (Table 2.11). A similar trend was observed between compounds 111 and 117 (Table 2.5).

Next the OCH$_3$ methoxy group was replaced with alkoxy groups of varying sizes. The alkoxy groups contain both the straight chains (OEt ethoxy - compound 146; OPr Propoxy compound 147; and OBu Butoxy – compound 148) and the branched chain analogs (OiPr Isopropoxy– compound 157; and OiBu Isobutoxy – compound 158). In the straight chain series, replacing the 6-OCH$_3$ in compound 145E with an OEt group retains the potent cytotoxicity. As the length of the alkoxy group increases, the activity decreases slightly. The reduction in activity from the methoxy to OPr was 5-10 fold. The extent of reduction is similar if the alkoxy group is switched from a straight to branched chains.
**Table 2.11** Anti-proliferative and anti-microtubule activity of compounds with increasing bulk at the 6-position. Data are the mean of three or more experiments and are reported as means ± standard deviation.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>R</th>
<th>PC-3 (IC₅₀ nM)</th>
<th>MDA-MB-231 (IC₅₀ nM)</th>
<th>Tubulin Polymerization (IC₅₀ µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145 (E)</td>
<td>OCH₃</td>
<td>8.21±2.3</td>
<td>0.88±0.07</td>
<td>4.10±0.81</td>
</tr>
<tr>
<td>145 (Z)</td>
<td>OCH₃</td>
<td>34.2±10.1</td>
<td>3.62±1.10</td>
<td>&gt;40</td>
</tr>
<tr>
<td>146 (E)</td>
<td>OCH₂CH₃</td>
<td>6.51±2.06</td>
<td>8.57±2.06</td>
<td>6.32±1.20</td>
</tr>
<tr>
<td>147 (E)</td>
<td>O(CH₂)₂CH₃</td>
<td>35.4±4.1</td>
<td>69.5±6.1</td>
<td>&gt;20</td>
</tr>
<tr>
<td>148 (E)</td>
<td>O(CH₂)₃CH₃</td>
<td>14.5±3.1</td>
<td>1616±43</td>
<td>&gt;40</td>
</tr>
<tr>
<td>157 (E)</td>
<td>OCH(CH₃)₂</td>
<td>34.7±7.2</td>
<td>805±110</td>
<td>&gt;40</td>
</tr>
<tr>
<td>158 (E)</td>
<td>OCH₂CH(CH₃)₂</td>
<td>233±6</td>
<td>1187±330</td>
<td>&gt;40</td>
</tr>
<tr>
<td>156 (Z)</td>
<td>H</td>
<td>27000</td>
<td>9400±89</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td></td>
<td>13.2±1.6</td>
<td>8.12±3.11</td>
<td>1.56±0.41</td>
</tr>
</tbody>
</table>
2.2.4.5 Effect of the substitution pattern based on nature of heterocyclic ring system

The last study in this class of compounds was to investigate the effect of replacing the pyrrolidin-2-one ring of the core 1,3-dihydroindole-2-one with other heterocyclic rings. The heterocyclic rings in this study included 2-methyl-4,5-dihydro-1H-imidazole, oxazolidin-2-one, imidazolidin-2-one, pyrrolidine-2,3-dione and pyrrolidin-2-one with the nitrogen connecting the benzyl group. The modification of the rings resulted in reduction of the methylene bridge that connected the TMP and 1,3-dihydroindole-2-one in 145E and avoided the problems associated with isomerization. Based on the activity of the compounds, the carbonyl group is required for activity. Replacing the carbonyl in the pyrrolidin-2-one with a methyl group and introduction of a double bond in compound 169 resulted in complete loss of activity. The introduction of an additional nitrogen in compound 168 (imidazolidin-2-one) resulted in loss of cytotoxicity by 5-50 fold as compared to 145E. However, 168 inhibited microtubule polymerization with IC50 value of 16.14±5.09 μM. Compound 178, with an additional carbonyl group on the linker connecting the two aryl ring systems, also demonstrated cytotoxic activity with 2-3 fold decrease as compared to 168. Compound 181, with a 3-methyl-4,5-dihydro-1H-imidazole ring, was an analog of 168 with a methyl group. It demonstrated a 10-100 fold decrease in cytotoxic activity as compared to 168. Next the oxazolidin-2-one ring was introduced which resulted in a dramatic decrease in activity as compared to 145E. Compound 183, in which the pyrrolidin-2-one nitrogen was rotated, caused a reduction in cytotoxic activity by 20-240 fold.
Table 2.12 Anti-proliferative and anti-microtubule activity of compounds with different heterocyclic rings. Data are the mean of three or more experiments and are reported as means ± standard deviation. *Compound 182 had poor solubility in DMSO.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>PC-3 (IC&lt;sub&gt;50&lt;/sub&gt; nM)</th>
<th>MDA-MB-231 (IC&lt;sub&gt;50&lt;/sub&gt; nM)</th>
<th>Tubulin Polymerization (IC&lt;sub&gt;50&lt;/sub&gt; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145 (E)</td>
<td>8.21±2.31</td>
<td>0.88±0.07</td>
<td>4.10±0.81</td>
</tr>
<tr>
<td>164</td>
<td>4486±560</td>
<td>4520±342</td>
<td>&gt;40</td>
</tr>
<tr>
<td>177</td>
<td>&gt;20000</td>
<td>&gt;20000</td>
<td>n.d</td>
</tr>
<tr>
<td>168</td>
<td>44.2±10.2</td>
<td>52.7±12.8</td>
<td>16.5±2.90</td>
</tr>
<tr>
<td>178</td>
<td>125±12</td>
<td>108±6.8</td>
<td>19.1±5.1</td>
</tr>
<tr>
<td>169</td>
<td>&gt;40000</td>
<td>&gt;40000</td>
<td>&gt;40</td>
</tr>
<tr>
<td>181</td>
<td>555±24</td>
<td>3027±45</td>
<td>&gt;40</td>
</tr>
<tr>
<td>182*</td>
<td>318±5</td>
<td>934±50</td>
<td>n.d</td>
</tr>
<tr>
<td>183</td>
<td>165±3</td>
<td>193±25</td>
<td>31.2±7.75</td>
</tr>
</tbody>
</table>

Podophyllotoxin 13.2±1.56 8.12±3.11 1.56±0.41
2.2.5 Conclusion and future directions

In summary, a detailed SAR has been established on a series of 3-benzylidene-2-1,3-dihydroindole-2-one analogs tested for cytotoxicity on tumor cells and for inhibition of tubulin polymerization.

A single concentration anti-proliferation screen was used to identify 1,3-dihydroindole-2-one analogs with anti-proliferative activity. Compounds that inhibited the proliferation of cells by 50% or higher were further evaluated. Compound 111 was identified as the lead based on its anti-proliferative profile. It demonstrated anti-proliferative activity in sub-micromolar range. To investigate the mechanism of action cell cycle analysis was performed. It caused a G2/M phase arrest in the cell cycle following 24 hour incubation. It inhibited polymerization of tubulin and was identified as a colchicine site agent (binding to β tubulin). In an effort to further its activity, modifications were made to compound 111. Colchicine and colchicine site agents have a common structural feature, the tri-methoxy phenyl (TMP) group. Analogs with methoxy groups on the benzylidene ring were synthesized and evaluated.

The most potent of the analogs was 145E with a very impressive cytotoxic activity profile. The NCI 60 cell line screen showed that 145E is extremely cytotoxic with GI50 values below 10 nM toward 45 out of 53 human cancer cell lines tested. It caused a G2/M phase arrest in the cell cycle following 24 hour incubation. It also demonstrated structural similarity to combretastatin A-4. Additionally 145E induced apoptosis in PC-3 and DU-145 prostate cancer cells following twenty four and forty eight hours of incubation.
Additional SAR was carried out to evaluate the effect of the number of methoxy groups on the benzylidene ring, nature of the groups on the benzylidene ring, position of the methoxy groups and nature of substituents on position 6 of the core ring. It is clear from the results that two structural features, namely the 6-OCH$_3$ group in the 1,3-dihydroindole-2-one ring and the trimethoxy group in the benzylidene, are essential for potent cytotoxicity and inhibition of tubulin polymerization. The study contributes to the knowledge about the structure-activity-relationship of 3-benzylidene-1,3-dihydroindole-2-one analogs as promising anti-microtubule anti-cancer agents.

Since the compounds are derived from the KDR inhibitor SU5416, studies would be carried out to examine if the compounds also bind to the receptor. The effect on the growth and migration of endothelial cells should be studied. Potentially, some of the compounds could be dual antitubulin-antiangiogenic agents.

The anti-microtubule activity of the most active compounds 145 and 146 E translated well to the anti-proliferative activity. They exhibited inhibition of anti-microtubule effect similar to podophyllotoxin, a colchicine site agent. However, compounds 145E, 146E could have additional molecular targets which should be evaluated. Further studies should be performed to identify the molecular targets involved in the cell-death mediated activity.
REFERENCES


16. Rohde, W.; Cordell, B.; Webster, R.; Levinson, W. Inhibition of amino acyl tRNA synthetase activity by copper complexes of two metal binding ligands. N-Methyl


CHAPTER 3

ELUCIDATION OF THE MECHANISM OF ACTION OF ISO-INDOLE-1,3 DIONE ANALOGS

3.1 Role of Thalidomide

Thalidomide is a derivative of glutamic acid that exists as an equal mixture of enantiomers which rapidly interconvert at physiological pH. The drug undergoes rapid pH dependent, spontaneous, non-enzymatic hydrolysis to multiple metabolites which are rapidly excreted. It was discovered in the mid-1950 and marketed as a safe, non addictive sedative-hypnotic drug with good antiemetic activity. The drug was used extensively for morning sickness during pregnancy until it was found to cause significant fetal abnormalities\(^1\). Today, thalidomide is being studied for a number of conditions including HIV ulcers and wasting, Crohn’s disease, rheumatoid arthritis, chronic host versus graft’s disease, Behcet’s vasculitis and cancer\(^2\-7\). It has been approved in the US for chronic treatment of erythema nodosum leprosum (ENL).
The mechanism of teratogenicity due to thalidomide has been not fully understood. It is hypothesized that the putative thalidomide free-radical may generate highly reactive oxygen species such as \( \cdot \text{OH} \) or superoxide anion radical (\( \text{O}_2 \cdot \)), which is consistent with the \textit{in vivo} oxidation of glutathione by thalidomide. If not detoxified, these reactive oxygen species can irreversibly modify DNA in many ways, including the formation of 8-OH-2’-dG, which can be mutagenic and probably have other effects on gene expression\(^8,9\). Species specific metabolic activation of thalidomide is required for teratogenicity. When given orally (PO), it is virtually non-toxic to rats, where as in mice and rabbits it initiates embryonic DNA oxidation and teratogenicity. Intraperitoneal (IP) administration of thalidomide also leads to teratogenic effects in rats\(^9\). It was believed that since development of fetal limb depends strongly on formation of new blood vessels, the antiangiogenic activity was behind the teratogenic nature of the drug. Since then, many studies have been performed to understand the bio-chemical mechanism of thalidomide and separate the teratogenic and anti-angiogenic effects, though the mechanism of anti-angiogenic activity of thalidomide is still not well understood.

In fact in oncology it is one of the few drugs for which so many molecular targets have been identified. D’Amato et al. first reported that thalidomide inhibits angiogenesis by interrupting processes mediated by basic fibroblast growth factor (bFGF) and/or vascular endothelial growth factor (VEGF). Its effects on corneal angiogenesis induced by VEGF have been reported.\(^{10,11}\) Additionally, thalidomide has demonstrated inhibitory effects on angiogenesis in the bFGF\(^{11}\) induced rabbit corneal micropocket assay and orally in mice models. Studies suggest that one or more of the thalidomide metabolites may be responsible for its anti-angiogenic effects, and their generation may be species
dependent. For example, thalidomide inhibits microvessel formation in the rat aortic ring assay and slows human aortic endothelial cell proliferation in the presence of human or rabbit liver microsomes, but not in the presence of rat liver microsomes. In the absence of liver microsomes, thalidomide had no effect on either microvessel formation or cell proliferation.

Thalidomide inhibits the production of inflammatory cytokines TNF-α by enhancing its mRNA degradation in lipopolysaccharide (LPS) induced macrophages and monocytes. It induces a dose dependent inhibition of growth factor IL-6 and interferon γ in mononuclear cells. It inhibits the growth-factor mediated activation of αvβ3 thus preventing stimulation of angiogenesis in developing limb buds. Thalidomide inhibits the IKKβ induced phosphorylation of IκBα and subsequently activation of NFκB. Many effects exhibited by thalidomide are due to inhibition of NFκB activation. It prevents induction of hypoxia inducible factor HIF-1α and activation of inflammatory and angiogenic genes. It is also demonstrated to inhibit LPS mediated induction of cyclooxygenase (COX-2) by destabilizing its mRNA and subsequent prostaglandin (PGE2) biosynthesis. Inhibition of PGE2 also explains its anti-angiogenic activity as PGE2 induces pro-angiogenic growth factor production.

Thalidomide affects apoptosis by reducing levels of the anti-apoptotic members of the Bcl-2 family and by down regulating proteins conferring resistance against Fas- or TRAIL-mediated apoptosis. Thalidomide and its analogues have various effects on the immune system. By acting as a costimulator, thalidomide increases the response of T-lymphocytes to T-cell receptor-mediated stimulation, yielding increased proliferation and
greater production of IL-2 and interferon-γ\textsuperscript{16}. In addition, it increases the number of natural killer cells, while these cells exhibit augmented cytotoxic activity against tumor cells. Furthermore, the increased sensitization to Fas- and TRAIL-induced apoptosis after exposure to thalidomide renders tumor cells more prone to immune responses since cellular immunity exerts anti-tumor effects partially through Fas and TRAIL. An additional mechanism underlying the anti-tumor activity of thalidomide is attenuation of metastatic potential of tumor cells by reducing TNF-α-induced upregulation of adhesion molecules on endothelial cells such as intracellular adhesion molecule-1 (ICAM-1), vascular-cell adhesion molecule-1 (V-CAM-1), and E-selectins.

It has shown to reduce tumor growth in model systems and in patients. As a monotherapy in the clinic it has fairly weak effects in patients with solid tumors\textsuperscript{17-23}, however, in a few patients with AIDS related Kaposi’s sarcoma\textsuperscript{4}, hepatocellular carcinoma and renal cell carcinoma durable progression-free survival has been reported. In addition it has been demonstrated to reduce the prostate-specific antigen level in hormone refractory prostate cancer\textsuperscript{22}. The anticancer activity of thalidomide has been explored most intensely in myeloma where it induces clinically meaningful responses in patients at various stages\textsuperscript{24} of the disease.

Thalidomide has been studied in combination in several phase II studies. It has demonstrated some favorable outcomes in combination with carmustine, temozolomide and IL-2 in high grade glioma, melanoma and renal cell carcinoma\textsuperscript{19, 25-27}. In 2006, thalidomide in combination with dexamethasone received accelerated approval for the treatment of newly diagnosed multiple myeloma (MM) patients.
3.1.1 Immunomodulatory drugs (IMiD)

Following the discovery of anti-angiogenic and anti-inflammatory properties of Thalidomide, a series of immunomodulatory compounds were developed. Thalidomide was used as the lead compound and pthaloyl substituted analogs were designed and synthesized that could improve the immunological and anti-cancer properties, while reducing the toxic side effects. Because of the structural similarity to thalidomide these compounds demonstrate inhibition of angiogenesis and co-stimulation of killer T cells. The 4-amino analogs in which an amino group was added to the fourth carbon of thalidomide exhibited TNF-α inhibiting activity almost 50,000 times more potent than Thalidomide. Revlimid and Actimid are two such IMiDs that are being tested in clinical trials. Revlimid was approved for patients with low or intermediate-1 risk myelodysplastic syndromes (MDS) with del 5q. Del 5q is one of the most common cytogenetic abnormalities found in de-novo and therapy-related myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). It was also approved for use in combination with dexamethasone in patients with multiple myeloma who have received at least one prior therapy.

![Figure 3.1 Structures of Thalidomide, Actimid and Revlimid](image)
3.1.2 NFκB in cancer

NF-κB, which was discovered as a factor in the nucleus of B cells, binds to the enhancer of the kappa light chain of immunoglobulin. It translocates to the nucleus only when activated, where it regulates the expression of over 200 genes that control the immune system, growth and inflammation. NF-κB is present in all cells in a resting state in the cytoplasm; only when it is activated and translocated to the nucleus is the usual sequence of events generated. Currently, NF-κB consists of a family of Rel-domain-containing proteins; Rel A (also called p65), Rel B, c-Rel, p50 (also called NF-κB1), and p52 (also called NF-κB2). While p100 undergoes phosphorylation-dependent cleavage to form p52 product, p105 is cleaved to form p50.

Similarly, a family of anchorin-domain-containing proteins has been identified, that keep NF-κB in its inactive state within the cytoplasm. These include IκB-α, IκB-β, IκB-γ, bcl-3, p105 and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65 and IκBα in the cytoplasm. Upon cell triggering by a wide spectrum of stimuli, IκB molecules are phosphorylated on critical serine residues by 2 highly related specific kinases, IKK1(α), or, and IKK2(β), which together with the scaffold protein IKK/NFκB essential modulator (NEMO) form the IKK complex that integrates signals for NF-κB activation. Serine phosphorylation results in polyubiquitination of IκB and its subsequent degradation by the proteasome. Active dimeric NF-κB (usually p50-p65) then translocates to the nucleus and binds its specific consensus sequence. Several lines of evidence show that NF-κB plays a role in cancer. Its activity has been found constitutively elevated in many types of human tumors from either hematological or solid
origin, such as melanomas, breast, prostate ovarian, pancreatic, colon and thyroid carcinomas.\textsuperscript{35-40} NF-κB regulates the expression and the function of a wide spectrum of genes involved in the control of the cell cycle, apoptosis, cell growth, tissue invasiveness and inflammation. NF-κB activity is also involved in the regulation of angiogenesis. Vascular endothelial growth factor (VEGF), which is the main member of angiogenic factors family, is under the transcriptional control of NF-κB. The finding that NF-κB is a key player in cancer has prompted researchers to focus their efforts in looking for drugs able to suppress NF-κB activity in malignant cells. NF-κB activation could be blocked at different levels targeting the various components of its signaling cascade, such as the IKK complex, the IκB-α inhibitory protein, the p65 subunit of the transcriptionally active heterodimer or the proteasome. Therefore, an increasing number of compounds able to block NF-κB by inhibiting one or more of the molecules involved in the pathway activating it have been tested and have shown to suppress the growth of cancer cells\textsuperscript{41}. Many of these drugs have given promising results in preclinical models for NF-κB dependent tumors but their clinical efficacy has been disappointing. Actually, the only pharmacological inhibitors of NF-κB activation approved for clinical use are represented by proteasome inhibitors for treatment of some hematological malignancies, such as multiple myeloma, or adult T-cell leukemia\textsuperscript{42,43}. 

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3.2 Results and Discussion

3.2.1 Identification and evaluation of anti-cancer activity of 5HPP-33

Thalidomide is a pro-drug and metabolism is crucial for its activity. It can be metabolized by hydrolysis at the glutaramide ring and or the pthalimide ring. Hydroxylation of thalidomide in the 5 position of the pthalimide ring with the glutaramide ring in either open or closed form demonstrates anti-angiogenic activity. It has been reported that the glutaramide ring of thalidomide is not essential for antimetastatic activity against B16BL6 melanoma cells, since a completely hydrolyzed glutaramide group does not render the molecule inactive. That evidence, coupled with the numerous published examples of various N-phenylphthalimides that possess increased TNF-α regulatory activity over thalidomide, lends support to the notion that structural simplification of the glutaramide moiety produces biologically active analogues.

Thalidomide demonstrates anti-angiogenic activity and the anti-angiogenic activity is likely due to an active metabolite or metabolites. Recently, the antiangiogenic activities of several putative metabolites and analogs of thalidomide were reported by our lab. 5-hydroxy-(2,6-diisopropylphenyl)-1H-isodiol-1,3-dione (5HPP-33), a synthetic analog of thalidomide was identified in the search for potent antiangiogenic compounds. The glutaramide ring of thalidomide is replaced with a substituted aniline (2,6-diisopropyl aniline) in 5HPP-33. It reduced the blood vessel density in VEGF/bFGF stimulated CAM following 10 µg treatment for 48 hours. It has been reported to have tumor necrosis factor-α inhibitory activity in vitro.

Thalidomide does not inhibit the proliferation of PC-3 tumor cells in vitro at 300µM. However it has been reported that thalidomide weakly inhibits the proliferation
of MDA-MB-231 breast cancer cells. Additionally activation of thalidomide by microsomes does not enhance the anti-proliferative activity\textsuperscript{44}. The cytotoxic activity of 5HPP-33 on tumor cell lines was evaluated. In addition to the key target of thalidomide, NFκB, other molecular targets of 5HPP-33 were identified and studied.

3.2.1.1 5HPP-33 Inhibits Growth in a Variety of Tumor Cell Types \textit{In vitro}

5HPP-33 and thalidomide were examined for their antiproliferative activities against nine human cancer cell lines derived from six different tissues. The cancer cells were subjected to 72 hours of exposure to the drugs. IC\textsubscript{50} values of the antiproliferative activities on these cell lines ranged from 1.65 μM (LNCaP) to 11.06 μM (Hs Sultan; Table 3.1). Interestingly, comparison of the IC\textsubscript{50} values of 5HPP-33 on the prostate cancer cell lines suggests that 5HPP-33 is effective against prostate cancer cell lines irrespective of their p53 status (LNCaP, wild-type p53; PC-3 and DU-145, mutant p53).

In addition, the antiproliferative activities of 5HPP-33 on cancer cell lines were independent of their hormone dependence. Both androgen-dependent (LNCaP) and androgen-independent (PC-3 and DU-145) prostate cancer cell lines and estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) breast cancer cell lines were equally susceptible to 5HPP-33. LNCap cells demonstrated greater sensitivity to 5HPP-33 with IC50 of $1.65\pm0.21$ μM which implies that 5HPP-33 might act on a target specific to LNCap and should be investigated. Thalidomide, on the other hand, had little anti-proliferative activity with IC\textsubscript{50} values of $>300$ μM in all cell lines.
Table 3.1 5HPP-33 showed inhibition of cell proliferation tumor cells. Data are the mean of three experiments and are reported as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>5HPP-33 (Compound 1) IC₅₀ (µM)</th>
<th>Thalidomide IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>6.20 ± 0.41 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>5.26 ± 0.32 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>HT-29</td>
<td>5.09 ± 1.41 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>TCCSUP</td>
<td>6.52 ± 0.97 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>LNCap</td>
<td>1.65 ± 0.21 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>DU-145</td>
<td>5.74 ± 0.23 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>PC-3</td>
<td>8.28 ± 0.81 µM</td>
<td>&gt; 100 µM</td>
</tr>
<tr>
<td>Hs Sultan</td>
<td>11.1 ± 1.7 µM</td>
<td>&gt; 100 µM</td>
</tr>
</tbody>
</table>

3.2.1.2 Effect of 5HPP-33 on Cell Cycle

The effect of 5HPP-33 on cell cycle progression was studied. Cell cycle analysis was performed using propidium iodide to stain the DNA through flow cytometry. 5HPP-33 treated cells were analyzed with varying concentrations in which it caused a significant cell cycle arrest in the G₂-M phase (Fig. 3.2). Subsequently two-variable flow cytometric analysis was performed on compound-treated 1A9 ovarian carcinoma cells at
the laboratory of Dr DL Sackett in NICHD. For each drug concentration, two plots were produced (Fig. 3.3 A–F), arranged as vertical pairs. The bottom plots present single-variable DNA content data, which allowed calculation of cell distribution among G1, S, and G2-M phases of the cell cycle. The top plots show the number of cells reacting with a mitosis-specific antibody TG-3 (Y axis) as a function of DNA content (X axis), which permitted quantitation of M-phase cells separately from those in G2. Flow cytometry has demonstrated that TG-3 immunofluorescence is >50-fold more intense in mitotic cells (M) than in interphase cells. In Western blots, the antibody reacts with a phosphorylated form of nucleolin Mr 105,000 protein that is present in abundance in extracts of cells treated for 20 h with an antimitotic agent but present at only low levels in extracts from cycling MCF-7 cells. At 10 µM, 96% of the cells were in G2-M phase consistent with that shown to be antiproliferative (Table 3.2). At higher concentrations, however, cells appeared to be arrested at other points in the cell cycle.
Figure 3.2 Cell Cycle Analysis on PC-3 cells following 24 hours treatment with 5HPP-33
Figure 3.3 Two-parameter flow cytometric analysis of the effect of 5HPP-33 on the cell cycle distribution of 1A9 cells. Experiments performed in the laboratory of Dr. DL Sackett at the NICHD.
<table>
<thead>
<tr>
<th>Sample</th>
<th>% G2-M</th>
<th>% M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Taxol (10 nM)</td>
<td>81</td>
<td>64</td>
</tr>
<tr>
<td>3 µM (5HPP-33)</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>10 µM (5HPP-33)</td>
<td>96</td>
<td>70</td>
</tr>
<tr>
<td>30 µM (5HPP-33)</td>
<td>68</td>
<td>18</td>
</tr>
<tr>
<td>100 µM (5HPP-33)</td>
<td>47</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 3.2 % G2-M and % mitotic cells in taxol and 5HPP-33 treated 1A9 cells. The major accumulation of mitotic cells peaks at 10 µM for 5HPP-33 following 24 hour incubation.

### 3.2.1.3 Immunofluorescent Microscopy

Many antimitotic agents exert their effects by perturbing microtubules by either inhibiting tubulin polymerization (such as colchicine or nocadazole) or hyperstabilizing the microtubules (such as taxol).

The anti-microtubule effect of 5HPP-33 was studied by immunofluorescent visualization of microtubules performed by incubating treated and fixed cells with anti-α tubulin antibody followed by FITC-labeled secondary antibody. Control cells in interphase are presented because their well-spread microtubule arrays are easily visualized. Microtubules in the interphase are stable and turn over relatively slowly with
half-time ranging from several minutes to hours. In the control cells, microtubules can be clearly seen, and individual microtubules often appear long and relatively straight. Taxol treatment seemed to result in a larger number of microtubules, which appear shorter than and not as straight as in the control cells. Nocodazole treatment caused the loss of content of most microtubules. 5HPP-33 treatment caused changes similar to those of taxol, in that microtubules seemed more abundant and appeared shorter than in the control cells. However, in 5HPP-33-treated cells, the short microtubules appeared to be straighter than those observed following taxol treatment (Figure 3.4).

By comparing the nuclear staining of the control cells, the cells treated with 5HPP-33 exhibited multiple mitotic spindles and break down of nucleus into micronuclei after only two hours (Figure 3.5). Formation of micronuclei is also a hallmark of taxol and taxol like compounds.
Figure 3.4 Effect of 5HPP-33, paclitaxel, and nocadazole exposure on microtubules in MCF-7 cells
Figure 3.5 Effects of 5HPP-33 (10 μM) on cellular microtubules and nuclear structure in PC-3 cells. Untreated cells exhibited typical bipolar spindle poles [A]. 5HPP-33 treated cells exhibited multiple mitotic spindles and micronuclei [B].

3.2.1.4 Stimulation of Tubulin Polymerization \textit{In vitro} by 5HPP-33 and Taxol

As suggested in the previous section, based on the immunofluorescence visualization of tubulin, 5HPP-33 demonstrates more abundant MT with bundling (similar to taxol). In the next assay, the incubation of purified porcine tubulin with varying concentrations of 5HPP-33 was performed in an \textit{in vitro} assay. 5HPP-33 induced tubulin polymerization was determined by spectrophotometric measurement of the increase in absorbance (turbidity $\Delta A$) at 351 nm under conditions that do not promote control polymerization. It induced a concentration dependant increase in the rate and
extent of microtubule assembly. 5HPP-33 treated tubulin samples had a shorter lag period compared with taxol treatment (Figure 3.6 A). In addition, the polymers formed were resistant to cold. The control (no drug) microtubules depolymerized by 80% within 10 minutes of incubation on ice, whereas 5HPP-33-treated samples showed no cold-induced depolymerization. Thus, we concluded that 5HPP-33 stabilized microtubules and favored their polymerization.
Figure 3.6 Effect of 5HPP-33 on tubulin polymerization.
A. Control, 20 and 40 μM 5HPP-33
B. Control, 5 μM Taxol and 40 μM 5HPP-33
3.2.1.5 5HPP-33 overcomes drug resistance

Currently, taxol is approved for the treatment of ovarian, breast, non–small cell lung cancers and AIDS-related Kaposi’s sarcoma. Despite the clinical success of taxol, drug resistance has been shown in the laboratory and in the clinic. Mechanisms of taxol resistance include the over expression of the drug efflux pump P-glycoprotein (Pgp-MDR1) and alteration to tubulin structure including differential expression of β-tubulin isotypes, point mutations, decreased expression of tubulin, acetylation of α-tubulin etc. The effects of these drug resistance mechanisms on the antitumor efficacy of 5HPP-33 were investigated in two model systems. First, the sensitivity to 5HPP-33 on two NIH3T3 cell lines transfected with the wild-type pHaMDR1/A (NIH3T3-MDR-G185) and mutant pHaMDR1/A (NIH3T3-V185) retroviral vectors were evaluated that demonstrated two forms of MDR. It was clearly demonstrated that the cells expressing either form of MDR (NIH3T3-G185 and NIH3T3-V185) were substantially more resistant to taxol than the nontransfected counterpart (parental NIH3T3) and all of the cells, regardless of MDR status, were equally sensitive to the antiproliferative effects of 5HPP-33. The relative resistance, RR = (ratio of the IC₅₀ of transfected cells) / (IC₅₀ of the nontransfected cells) was 57 and 25 for taxol against the NIH3T3-G185 and NIH3T3-V185 respectively. In contrast, 5HPP-33 exhibited RR values of 1.2 and 1.5 on the two resistant cells. Colchicine also tested in the assay exhibited RR values of 6 and 87 respectively and might be a substrate for the Pgp pump. The results indicate that 5HPP-33 is not affected by the presence of P-glycoprotein pump. Data is demonstrated in Table 3.3.
In the second model, taxol-resistant 1A9 ovarian cancer cell lines which possess mutant β-tubulin M40 isotype with modification at nucleotide, 810 (T to G; Phe270 to Val) in 1A9PTX10 cells and nucleotide 1092 (G to A; Ala364 to Thr) in 1A9PTX22 cells were studied (1A9 cell line is a clone of the human ovarian carcinoma cell line, A2780)\textsuperscript{50}. The cells with mutant β tubulin exhibit impaired taxol-driven tubulin polymerization possibly due to impaired binding of taxol to its binding site. Taxol exhibited RR values of 150 and 78 respectively on the 1A9PTX10 and 1A9PTX22 cells. 5HPP-33 was equally effective against the parental 1A9 cells and the two taxol-resistant cell lines with RR of 1.1 and 0.7 (Table 3.4). These experiments were performed in the laboratory of Dr. DL Sackett at the NICHD.

This data suggests that 5HPP-33 binds to/interacts with a different site on tubulin compared to taxol and is a potentially effective microtubule-targeted agent for the treatment of taxol-resistant tumors.
### Table 3.3 Cytotoxicity of 5HPP-33, taxol and colchicine on NIH3T3 cells alone or transfected with retroviral vectors expressing MDR. Experiment performed in the laboratory of Dr. DL Sackett at the NICHD.

<table>
<thead>
<tr>
<th>Drug</th>
<th>NIH 3T3 IC₅₀ (µM)</th>
<th>NIH3T3G185 IC₅₀ (µM)</th>
<th>NIH3T3V185 IC₅₀ (µM)</th>
<th>Relative Resistance RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HPP-33</td>
<td>3</td>
<td>3.6</td>
<td>4.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.04</td>
<td>2.28</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.06</td>
<td>0.4</td>
<td>5.2</td>
<td>87</td>
</tr>
</tbody>
</table>

### Table 3.4 Cytotoxicity of 5HPP-33 in Taxol-resistant 1A9 ovarian carcinoma cells. All data are means of triplicate determination (RR= (ratio of the IC₅₀ of transfected cells/IC₅₀ of the untransfected cells). * Data from reference^50. Experiment performed in the laboratory of Dr. DL Sackett at the NICHD.

<table>
<thead>
<tr>
<th>Drug</th>
<th>1A9 Parental IC₅₀ (µM)</th>
<th>PTX10 Relative Resistance RR</th>
<th>PTX22 Relative Resistance RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HPP-33</td>
<td>2.1 ± 0.1</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.002</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.004*</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>
3.2.1.6 Inhibition of NFκB translocation

Thalidomide has been reported to be an inhibitor of NF-κB activation. In fact a wide range of activities of thalidomide are attributed to its inhibition of NFκB activation. The predominant form of NFκB is a heterodimer of p50 and p65. It is involved in the transcription regulation of many genes and as a result NFκB plays a key role in proliferation, angiogenesis, tumor growth, prevention from apoptosis and immunological responses. Normally, NF-κB is present in the cytoplasm as homo- or heterodimer with members of the IκB inhibitor family. Upon phosphorylation and degradation of IκB by IκB kinase-β (IKK) the nuclear localization sequence becomes accessible and NFκB translocates to the nucleus. Thus, translocation from the cytoplasm to the nucleus is a definitive measure of NFκB activation.

5HPP-33 was assayed for inhibitory activity in an IL-1 induced NF-κB activation cell based assay. A quantitative NFκB nuclear translocation assay was used to measure the inhibitory activity toward IL-1 induced in HeLa cells, with (E)-3-(t-butylphenylsulfonyl)-2-propenenitrile (BAY-11-7085), a potent IKK inhibitor, as the positive control. (structure in Table 3.11)

Immunofluorescence staining of p65 in HeLa cells (unstimulated, PBS control) shows that p65 mainly located in cytoplasm with a minimal amount in the nucleus. Upon stimulation with IL-1 for 30 minutes, a significant amount of the p65 gets translocated into the nucleus. In the IL-1 stimulated cells, the amount of nuclear fluorescence is increased, while the cytoplasmic fluorescence markedly decreases. The results are shown in Figure 3.6. The inhibitory activities of the compounds (IC_{50}) were calculated as the
concentration of the compounds to reduce 50% of the nuclear minus cytoplasmic fluorescence in the cells. The IC$_{50}$ of thalidomide was 2.04 µM. In addition, it was demonstrated for the first time that Actimid, a thalidomide analog currently in clinical trial for multiple myeloma, also inhibited NF-κB activation with a slightly higher potency than thalidomide (IC$_{50}$ – 1.27 µM). 5HPP-33 demonstrates 4 times more potent activity than thalidomide as an inhibitor of NF-κB activation with IC$_{50}$ of 0.53 µM.

Additionally compound 163 was identified as a potent inhibitor of NF-κB activation with IC$_{50}$ of 0.43 µM. Compound 163 is a analog of 5HPP-33 with a bromo substituent at the 2’,6’ positions of ring C (Table 3.11).
3.2.1.7 Inhibition of TNF-α induced IκB phosphorylation and degradation

The activation of NF-κB by TNF-α or any other cytokine requires phosphorylation followed by proteosomal degradation of its inhibitor IκBα. To determine if the inhibition of NF-κB activation (measured by translocation to the nucleus) was due to inhibition of IκBα degradation; Hela cells were pretreated with 40 µM Thalidomide, 20 and 40 µM 5HPP-33 and 40 µM 2’,6’ dibromo-analog (compound 163) for 2 hours. BAY-11-7085, a potent IKK inhibitor, was used as the positive
control\textsuperscript{60}. Following pre-treatment with test compounds the cells were induced by
20ng/mL of TNF-\(\alpha\), lysed and evaluated for the \(\text{IkB}\alpha\) levels by western blot. In the TNF-
\(\alpha\) activated control cells (DMSO treated) degradation of the \(\text{IkB}\alpha\) was indicated by
absence of a band. Cells that were pretreated with Thalidomide, 5HPP-33 and its 2’,6’-
dibromo analog inhibited \(\text{IkB}\alpha\) degradation. Thalidomide and the 2’,6’-dibromo analog
exhibited inhibition of degradation at 40\(\mu\)M concentration, while 5HPP-33 inhibited the
degradation in a dose dependant manner at 20 and 40 \(\mu\)M. TNF-\(\alpha\) induction leads to
\(\text{IkB}\alpha\) phosphorylation by \(\text{IkB}\) kinase(IKK) causing its ubiquitination followed by
proteosomal degradation. This allows the nuclear localization sequence to become
accessible and NF\(\kappa\)B translocation to the nucleus. The inhibition of \(\text{IkB}\alpha\) degradation is
consistent with the inhibition of NF\(\kappa\)B translocation as previously demonstrated but a
much higher concentration of the test compounds was required to inhibit
\(\text{IkB}\alpha\) degradation.
20ng/mL TNFα - + + + +
Thalidomide 40µM - - - + +

0 5 15 5 15 minutes

A. Inhibition of degradation of IκB-α in samples pre-treated with Thalidomide

<table>
<thead>
<tr>
<th>Compound#</th>
<th>5HPP33</th>
<th>163</th>
<th>BAY11-7085</th>
</tr>
</thead>
</table>

B. Inhibition of degradation of IκB-α in samples pre-treated with 5HPP-33, BAY11-7085. (BL-Blank)

**Figure 3.8** Effect of TNF-α induced IκB degradation
3.2.1.8 Inhibition of \( \text{IkB} \) phosphorylation by purified IKK\( \beta \) in a ELISA assay

An essential prerequisite for induced \( \text{IkB} \alpha \) degradation is the \textit{de novo} phosphorylation of conserved serine residues on the N-terminal domain of \( \text{IkB} \alpha \) catalyzed by the \( \text{IkB} \) kinase complex. The effect of compounds on inhibition of \( \text{IkB} \alpha \) phosphorylation was studied by ELISA based IKK-\( \beta \) kit. It is an assay in which human recombinant IKK-\( \beta \) is incubated with its substrate (50-amino acid) GST \( \text{IkB} \alpha \) that has two phosphorylation sites Ser-32 and Ser-36. Decrease in absorbance with respect to the control is an indication of inhibition of IKK-\( \beta \) activity. The concentration of IKK-\( \beta \) was 2ng in the assays. 1\( \mu \)M of inhibitor provided in the kit caused a 96.3% inhibition of \( \text{IkB} \alpha \) phosphorylation, while 20\( \mu \)M of Bay 11-7085 (IKK inhibitor) inhibited \( \text{IkB} \alpha \) phosphorylation by 90.5% as compared to control.

With 20\( \mu \)M of 5HPP-33 and thalidomide no inhibition was observed in two experiments. Absorbance recorded for both 5HPP-33 and thalidomide was similar to control. In the previous section inhibition of \( \text{IkB} \alpha \) degradation was demonstrated by 5HPP-33 and Thalidomide; however by this assay inhibition of phosphorylation of \( \text{IkB} \alpha \) by purified IKK\( \beta \) did not take place. This observation suggests that 5HPP-33 and thalidomide do not have a direct influence on IKK\( \beta \) enzyme activity rather act on factors regulating or activating the kinase activity and should be further investigated.
<table>
<thead>
<tr>
<th>Compound #</th>
<th>20 µM</th>
<th>100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay-11-7085</td>
<td>90.5%</td>
<td>98.66%</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>No Inhibition</td>
<td>No Inhibition</td>
</tr>
<tr>
<td>Actimid</td>
<td>No Inhibition</td>
<td>No Inhibition</td>
</tr>
<tr>
<td>5HPP-33</td>
<td>No Inhibition</td>
<td>No Inhibition</td>
</tr>
<tr>
<td>Compound 163</td>
<td>No Inhibition</td>
<td>No Inhibition</td>
</tr>
</tbody>
</table>

**Table 3.6** Inhibition of IKKβ by BAY-11-7085, Thalidomide and 5HPP-33. Percentages are the average of two experiments.

### 3.2.2 Structure Activity Relationship

As described in the previous section, 5HPP-33 was identified as a potent *in vitro* anti-cancer agent. It demonstrated anti-cancer activity against nine tumor cell lines with IC$_{50}$ values of 2-12 µM and induced cell cycle arrest in the mitotic phase. It demonstrated anti-microtubule activity with a taxol like mode of action. Additionally it was effective against four taxol resistant cell lines. Because of its impressive activity profile, identification and understanding of its structure activity relationship may reveal new compounds with activity greater than or equal to 5HPP-33. An SAR would be useful in identifying desirable structural features associated with improved activity.

In an effort to identify more potent cytotoxic analogs of 5HPP-33, a structure activity relationship was determined by separating the core structure of 5HPP-33 into three parts, A, B and C. Each part was individually synthetically modified and an initial
SAR was established by using a single concentration (30 µM) anti-proliferative screen on PC-3 cells. 

A ring substitution

Compounds with substitution at the 5-position on ring A give greater activity than at the 4 position in the anti-proliferative screen. 5-amino substituents showed the greatest anti-proliferative activity based on a single concentration screen. Additionally the presence of the hydroxyl group was not required for activity. Compound 51, which lacks a hydroxyl group, inhibited the proliferation of PC-3 cells by 57.74% at 30 µM concentration.

B ring substitution

Variation in the B ring had a significant effect on activity. Substituting one carbonyl group with hydroxyl, methoxy or ethoxy group, that changed the sp² carbon to sp³, led to a decrease in activity. However replacing one of the carbonyl oxygen atoms with sulfur, which retained sp³ configuration, increased the activity. Additionally, the presence of sulfur in place of one carbonyl oxygen to form a thiolactam was able to compensate for removal of one carbonyl group.
C ring substitution

In the single concentration screen assay it was established that a non aromatic ring in region C resulted in complete loss of anti-proliferative activity. Mono-substitution on the C ring, regardless of nature (lipophilic, electronic and steric) or location of substituents, resulted in a poor activity profile. On the other hand it was demonstrated that the presence of di-substitution at the 2 and 6 positions on the C ring with bulky substituents, such as the 2,6-diisopropyl group is essential for anti-proliferative activity. There was a direct correlation between the size of 2’,6’ substituents and anti-proliferative activity. 2’,6’-dimethyl substituent demonstrated a percent inhibition of 2.27±2.57%, while 2’,6’-diethyl substituent demonstrated inhibition of 24.7±13.9% and the 2’,6’-diisopropyl substituent inhibited the proliferation by 57.7±8.42%.

3.2.2.1 Effect of nature of substitution on ring A

The initial SAR was utilized and expanded by performing IC\textsubscript{50} determinations following concentration dependant assays on tumor cells. IC\textsubscript{50} determination was also done on the human umbilical vein endothelial cells (HUVEC) also to identify the anti-angiogenic potential of the 5HPP-33 analogs.

In the first SAR study analogs with substitutions on ring A were evaluated. Ring B and C were maintained as in 5HPP-33. Compounds with substitution on position 5 demonstrated a trend of increased cytotoxicity as compared to their position 4 analogs on ring A, best observed between compounds 5HPP-33/104. Compounds with a substituent on position 5 show IC\textsubscript{50} values between 1.72±0.34 and 16.83±4.12 µM. The nature of the
substituent can be electron withdrawing or donating, hydrophobic or hydrophilic and does not seem to affect the activity. Compound 126 with a nitro group demonstrated poor cytotoxic activity. On the other hand compound 51, with no substitution on ring A demonstrated anti-proliferative activity similar to other 5-substituted analogs.

This leads to the understanding that a substitution at position 5 is well tolerated but not required. A substituent on position 4 decreases the activity. Compounds 51, 111, 129 and 130 were identified as potent cytotoxic analogs of 5HPP-33 (all demonstrating IC$_{50}$ values of $\leq$10 $\mu$M), while 129 with an amino group at position 5 demonstrated the most potent cytotoxic activity profile on the cells tested. Additionally compounds also demonstrated potent cytotoxic activity against HUVEC cells suggesting their anti-angiogenic potential. All of the data are consistent with the single concentration PC-3 cell screen$^{61}$. Table 3.7 demonstrates the anti-proliferative activity of the compounds.
Table 3.7 Anti-proliferative activity of compounds with modification on ring A. IC\textsubscript{50} values are the mean of three experiments and reported as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>PC-3 IC\textsubscript{50} (µM)</th>
<th>DU-145 IC\textsubscript{50} (µM)</th>
<th>MDA-MB231 IC\textsubscript{50} (µM)</th>
<th>HS-SULTAN IC\textsubscript{50} (µM)</th>
<th>HUVEC IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>----</td>
<td>4.01±0.78</td>
<td>7.12±0.25</td>
<td>4.12±1.02</td>
<td>6.66±1.45</td>
<td>3.02±1.19</td>
</tr>
<tr>
<td>97</td>
<td>5-COOH</td>
<td>6.40±1.12</td>
<td>9.16±0.82</td>
<td>5.56±1.04</td>
<td>&gt;50</td>
<td>2.76±1.03</td>
</tr>
<tr>
<td>5HPP-33</td>
<td>5-OH</td>
<td>9.31±0.81</td>
<td>5.74±0.23</td>
<td>5.35±0.34</td>
<td>8.46±1.86</td>
<td>3.32±0.22</td>
</tr>
<tr>
<td>104</td>
<td>4-OH</td>
<td>45.2±4.32</td>
<td>45.6±3.31</td>
<td>15.±1.92</td>
<td>30±3.48</td>
<td>8.99±1.40</td>
</tr>
<tr>
<td>108</td>
<td>5-Cl</td>
<td>5.52±1.12</td>
<td>14.1±1.52</td>
<td>10.6±2.39</td>
<td>16.8±4.12</td>
<td>3.94±0.97</td>
</tr>
<tr>
<td>111</td>
<td>5-CH\textsubscript{3}</td>
<td>8.02±0.78</td>
<td>6.62±1.51</td>
<td>4.85±1.12</td>
<td>3.47±2.43</td>
<td>6.45±0.43</td>
</tr>
<tr>
<td>125</td>
<td>4-NO\textsubscript{2}</td>
<td>38.3±2.65</td>
<td>&gt;50</td>
<td>37.7±3.4</td>
<td>&gt;50</td>
<td>16.9±1.3</td>
</tr>
<tr>
<td>126</td>
<td>5-NO\textsubscript{2}</td>
<td>n.d</td>
<td>15±4.81</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>16.6±0.9</td>
</tr>
<tr>
<td>127</td>
<td>5-COOCH\textsubscript{3}</td>
<td>21.8±2.13</td>
<td>24.6±3.22</td>
<td>6.87±2.75</td>
<td>&gt;50</td>
<td>6.99±1.14</td>
</tr>
<tr>
<td>129</td>
<td>5-NH\textsubscript{2}</td>
<td>2.07±0.28</td>
<td>3.94±0.56</td>
<td>7.24±2.40</td>
<td>6.58±1.21</td>
<td>2.08±0.56</td>
</tr>
<tr>
<td>130</td>
<td>4-NH\textsubscript{2}</td>
<td>6.26±2.43</td>
<td>8.14±0.68</td>
<td>7.24±1.20</td>
<td>1.72±0.34</td>
<td>2.18±0.08</td>
</tr>
<tr>
<td>160</td>
<td>5-OH</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>n.d</td>
</tr>
<tr>
<td>161</td>
<td>5-OH</td>
<td>&gt;50</td>
<td>45.9±6.70</td>
<td>29.2±3.91</td>
<td>24.6±1.54</td>
<td>n.d</td>
</tr>
</tbody>
</table>

R= methyl - 160
ethyl - 161
3.2.2.2 Effect of bulkiness of substitution on ring A

In the next SAR study analogs of 5HPP-33 were synthesized by increasing the size of substituents at the 5- position in ring A. Table 3.8 demonstrates the antiproliferative activity of compounds with bulky groups on the A ring. The cytotoxicity did not change as compared to 5HPP-33 and analogs with bulky groups at 5- position had a similar IC$_{50}$ profile compared to smaller groups such as amino or hydroxy (Table 3.7). However, some of the bulky groups included reactive functional groups of affinity analogs 157, 158, 172 and 179 synthesized to study the potential binding of 5HPP-33 to tubulin. The cytotoxic activity of these compounds could be due to the reactive electrophilic groups such as COCH$_2$Cl that form covalent bonds (by alkylating) with nucleophilic groups on various proteins and other bio-molecules in cells.

This study illustrates that the binding pocket occupied by the ring A of 5HPP-33, tolerated bulky groups and seems to be fairly large. At the same time it should be remembered that compound 51 without any substituent on the A ring demonstrates potent activity suggesting that the pocket does not require a bulky group. While bulky groups are well tolerated, compounds 191 and 192 with a very bulky hydrophobic aromatic side chain 5-N(SO$_2$-p-Cl-C$_6$H$_5$)$_2$ and 5-N(SO$_2$-p-CH$_3$-C$_6$H$_5$)$_2$, demonstrated little or no cytotoxicity against various tumor cells.
Table 3.8 Anti-proliferative activity of compounds with bulky modification on ring A. IC\textsubscript{50} values are the mean of three experiments and reported as mean ± standard deviation.
3.2.2.3 Effect of substitution on ring C

The next study was done to see the effect of increasing the bulk on the C ring of 5HPP-33. The di-isopropyl phenyl ring was replaced with a tricyclic acridine ring. Table 3.9 demonstrates the anti-proliferative activity of compounds substituted with acridine as the ring C. The A ring included analogs with hydroxy, nitro and amino groups. All the compounds (228-233) evaluated in this series demonstrated potent cytotoxic activity. The IC₅₀ values range from 1.55±1.10 to 5.88±2.09 on the breast and prostate tumor cells tested. The nitro/amino/hydroxy groups could not render any differences in the activity. The antiproliferative activity seems to come from the tri-cyclic acridine ring that can bind and intercalate both the DNA and RNA. However, like 5HPP-33, the tri-cyclic C ring is forced perpendicular to the rest of the molecule.

The next study also evaluated the effect of increasing bulk on the ring C of 5HPP-33. The diisopropyl phenyl ring was replaced with 2-yl-biphenyl, 4-yl-biphenyl and 3-benzoyl phenyl. The A ring was substituted with hydroxy and amino groups. The results are represented in Table 3.10. None of the compounds studied demonstrated any cytotoxicity on the tumor cells and had IC₅₀ values of >50 µM. The data from this study suggests that the C ring does not tolerate bulky substituents (Table 3.10).
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>R₁</th>
<th>PC-3 IC₅₀ (µM)</th>
<th>MDA-MB231 IC₅₀ (µM)</th>
<th>DU-145 IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>228</td>
<td>4-NO₂</td>
<td>1.71±0.80</td>
<td>1.57±0.45</td>
<td>n.d.</td>
</tr>
<tr>
<td>229</td>
<td>4-NH₂</td>
<td>2.20±1.03</td>
<td>5.88±2.09</td>
<td>1.55±1.10</td>
</tr>
<tr>
<td>230</td>
<td>4-OH</td>
<td>2.42±0.54</td>
<td>3.51±0.75</td>
<td>2.60±1.10</td>
</tr>
<tr>
<td>231</td>
<td>5-NO₂</td>
<td>2.47±1.77</td>
<td>2.56±1.15</td>
<td>2.07±0.54</td>
</tr>
<tr>
<td>233</td>
<td>5-OH</td>
<td>2.16±0.66</td>
<td>4.74±0.44</td>
<td>1.94±0.44</td>
</tr>
</tbody>
</table>

Table 3.9 Anti-proliferative activity of compounds with bulky modification on ring C. IC₅₀ values are the mean of three experiments and reported as the mean ± standard deviation.
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>PC-3 IC$_{50}$ ($\mu$M)</th>
<th>MDA-MB231 IC$_{50}$ ($\mu$M)</th>
<th>DU-145 IC$_{50}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>219</td>
<td>4-NH$_2$</td>
<td>2'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>220</td>
<td>4-OH</td>
<td>2'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>221</td>
<td>5-OH</td>
<td>2'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>224</td>
<td>4-OH</td>
<td>4'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>225</td>
<td>5-OH</td>
<td>4'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>234</td>
<td>5-NH$_2$</td>
<td>4'-Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>235</td>
<td>4-NH$_2$</td>
<td>3'-CO- Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>236</td>
<td>4-OH</td>
<td>3'-CO- Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>237</td>
<td>5-NH$_2$</td>
<td>3'-CO- Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>238</td>
<td>5-OH</td>
<td>3'-CO- Phenyl</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

**Table 3.10** Anti-proliferative activity of compounds with bulky modification on ring C. IC$_{50}$ values are the mean of three experiments and reported as the mean ± standard deviation.
3.3.2.4 Effect of 5HPP-33 analogs on tubulin polymerization

To further validate tubulin as a target for anti-proliferative activity of 5HPP-33 analogs, tubulin polymerization inducing ability was then tested using one concentration (50 μM) and reported as ΔA. Compound 156 and 159 gave a ΔA of 0.05 and 0.06 respectively (Figure 3.9). Compound 129 was another active analog with ΔA of 0.05. Compound 104 with 4-OH group on ring A gave ΔA of 0.026. Some other cytotoxic analogs such as compounds 51, 111, 126, 130 did not induce tubulin polymerization. The requirement of 2,6-diisopropyl group was again confirmed by comparing the MT stabilizing activity of compounds 160 and 161 with 2,6-dimethyl and di-ethyl groups respectively on ring C to 5HPP-33. At 50 μM 5HPP-33 gave a ΔA (351 nm) of 0.055 which was greater than 161 which had ΔA of 0.043 and 160 which caused no increase in ΔA (Figure 3.10).
Figure 3.9 Effect of 5HPP-33 and A ring analogs on the assembly of purified porcine brain tubulin in a 50 µM screen. Each point on the graph is the mean of three independent experiments.
**Figure 3.10** Effect of compound 5HPP-33, 160 and 161 on the assembly of purified porcine brain tubulin in a 50 µM assay. Each point on the graph is the mean of three independent experiments.

3.3.2.4 Effect of 5HPP-33 analogs on NFκB activation

Additionally, a structure-activity-relationship study of 5HPP-33 analogs was conducted in regard to their ability to inhibit NF-κB activation. A quantitative NFκB nuclear translocation assay was used to measure the inhibitory activities of the compounds. BAY-11-7085 (an IKK inhibitor) was used as a positive control. Twenty
5HPP-33 analogs with modifications on the 2,6-diisopropyl phenyl (C-ring) or on the phthalimide ring (A-ring) were evaluated (Table 3.11). Actimid, an immunomodulatory analog of thalidomide demonstrated inhibition of NF-κB activation. The 2,6-diisopropyl groups in 5HPP-33 are necessary but not essential for activity. Substituting the 2,6-diisopropyl groups with Me, Et, F or Cl (compounds) resulted in elimination of inhibitory activity. However, 2,6-dibromo analog of 5HPP-33 (compound 163) is as active as 5HPP-33 (IC$_{50}$ – 0.43 µM, Table 3.11) with 4 fold more activity than thalidomide as inhibitor of NF-κB activation. The 5-OH group in 5HPP-33 is essential for activity. Substituting the 5-OH group with H (compound 51), COOH (compound 97), CO$_2$CH$_3$ (compound 128), Cl (compound 108), CH$_3$ (compound 111), NO$_2$ (compound 126), OSO$_2$NH$_2$ (compound 156) and NHCOCH$_3$ (compound 159) all resulted in elimination of inhibitory activity. The inhibitory activity is decreased by 500 folds when the 5-OH is substituted with a 5-NH$_2$ group (compound 129). Transferring the OH group of 5HPP-33 from 5 to 4 position (compound 104) resulted in ten fold reduction in inhibitory activity. Substituting the 4-OH in compound 104 with NO$_2$ or NH$_2$ all resulted in the elimination of the inhibitory activity of NF-κB.

It is interesting to note that compounds 51, 108 and 111 that inhibited the proliferation of tumor cells, exhibit little to no inhibition of NF-κB activation which suggests additional targets of these compounds.
Table 3.11 Inhibitory activity of Thalidomide, Actimid, BAY-11-7085, 5HPP-33 and its analogs on IL-1 induced nuclear translocation and DNA binding of NF-κB in HeLa cells
3.3.2.6 Inhibition of proliferation and migration of endothelial cells

For evaluation of the anti-angiogenic activity, selected analogs were tested for their inhibitory activity on the migration of human umbilical vein endothelial (HUVEC) and human micro-vascular endothelial cells (HMEC) in addition to inhibition of growth of HUVEC. Analogs 51, 97 and 5HPP-33 inhibited migration of both HUVEC and HMEC with equal potency. Analog 126 and thalidomide caused no inhibition of migration. Thalidomide requires metabolic activation for its anti-angiogenic activity. All analogs tested included a ring A substitution.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>HUVEC IC$_{50}$ (µM)</th>
<th>Inhibition of HUVEC Migration IC$_{50}$ (µM)</th>
<th>Inhibition of HMEC Migration IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>3.02±1.19</td>
<td>4.37±1.32</td>
<td>1.41±0.65</td>
</tr>
<tr>
<td>97</td>
<td>2.76±1.03</td>
<td>4.81±2.43</td>
<td>5.01±1.59</td>
</tr>
<tr>
<td>5HPP-33</td>
<td>3.32±0.22</td>
<td>4.60±1.87</td>
<td>4.85±2.43</td>
</tr>
<tr>
<td>126</td>
<td>16.9±1.32</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>&gt;50</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 3.12 Inhibitory effect on growth and migration of endothelial (HUVEC and HMEC) cells. IC$_{50}$ values are mean of three experiments and reported as mean ±standard deviation.
CONCLUSION AND FUTURE WORK

Thalidomide and its analogues have demonstrated efficacy for the treatment of inflammatory, autoimmune diseases and cancer. A number of molecular targets of thalidomide have been demonstrated. We have identified a synthetic thalidomide analogue, 5HPP-33 previously demonstrated to have anti-angiogenic activity with potent in vitro activity against a variety of tumor cell lines (both solid tumor and lymphoma). The antiproliferative activity on tumor cell lines ranged from 1.65 µmol (LNCaP) to 11.06 µmol (Hs Sultan). The antiproliferative effect of 5HPP-33 was attributed to cell cycle arrest in the G2-M phase in PC-3 and A19 cells. It induced changes similar to those caused by taxol i.e., production of more abundant and shorter microtubules which was demonstrated by immunofluorescent visualization of microtubules. It stabilized microtubules in a cell-free tubulin polymerization assay under conditions that do not promote polymerization in the control sample. Additionally it demonstrated effectiveness against four different paclitaxel-resistant cell lines. Being a synthetic analog of thalidomide, 5HPP-33 was evaluated for and demonstrated inhibition of NFκB activation. It inhibited the degradation of IκBα protein which is a cellular NFκB inhibitor. However the exact mechanism of inhibition is not clear. A detailed western blot is desired to look at the effect of 5HPP-33 on various proteins involved in the NFκB activation pathway. The level of IKK-α, IKK-β, cytoplasmic p65 and proteins further upstream that phosphorylate and activate IKK such as the Akt, MEKK1 should be evaluated following 5HPP-33 treatment. In summary 5HPP-33 in addition to previously demonstrated anti-angiogenic activity has anti-microtubule and anti-NFκB property.
Based on its impressive activity profile; the next logical step is to look at its in vivo activity. An initial study to look at the pharmacokinetic profile of 5HPP-33 and activity in vivo tumor model has already been performed in a melanoma model (B16-F10, experiments ongoing).

Following the mechanistic study of 5HPP-33, a series of structural analogs were evaluated for their cytotoxicity in tumor cells. The analogs included modifications on the rings A and C of 5HPP-33. A detailed SAR was established based on the anti-proliferative activity of the congeners. It was demonstrated that two structural features, namely the 5-substitution on ring A and the 2’,6’-diisopropyl group in the ring C, are essential for potent cytotoxicity. The 5-substitution on ring A tolerated bulky groups and seems to bind to a large and flexible pocket. The 2’,6’-diisopropyl group forces the C ring in a perpendicular orientation to phthalimide ring and seems to be crucial for cytotoxic activity of the analogs investigated in this study. Additionally analogs with an acridine ring substituted for the C ring were evaluated for the anti-proliferative activity and demonstrated IC$_{50}$ values between 2-10 µM. Their potent activity might stem from their intercalating activity. Experiments to evaluate their mechanism need to be carried out in the future.

The active analogs identified from cytotoxicity screening were then tested for their ability to induce polymerization of mammalian tubulin. The structural features associated with potent anti-proliferative activity were also found to be significant for the microtubule polymerizing ability. The anti-angiogenic activity of some analogs was determined based on their cytotoxic activity on HUVEC cells and inhibition of migration of HUVEC and HMEC cells. Finally the anti-NFκB activity of analogs was determined
by measuring the inhibition of nuclear translocation. Analogs with no anti-proliferative activity demonstrated inhibition of NFκB activation suggesting that the two activities were independent of each other.

The study contributes to the knowledge about the structure-activity-relationship and mechanism of the biological activity of 2-(2,6-diisopropyl-phenyl)-isoindole-1,3-dione analogs. In fact, 5HPP-33 and its analogs are one of the few synthetic classes that induce and stabilize the polymerization of tubulin to microtubules. However, like thalidomide these compounds may demonstrate multiple molecular targets currently unidentified.
REFERENCES


51. Cardarelli, C. O.; Aksentijevich, I.; Pastan, I.; Gottesman, M. M. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. *Cancer Res* 1995, 55, 1086-91.


CHAPTER 4
MATERIAL AND METHODS

4.1 Cell Lines and Culture

Tumor cell lines (breast: MDA-MB-231; prostate: PC-3 and DU-145; Burkitt’s lymphoma: Hs Sultan; colon: HT-29; bladder: TCCSUP), and HUVEC cells were originally obtained from American Type Culture Collection. PC-3, DU-145 and Hs-Sultan cells were cultured in complete RPMI 1640 (Gibco, Invitrogen, Grand Island, NY) + 10% FBS (GIBCO, Grand Island, NY), 2mM L-glutamine (GIBCO, Grand Island, NY) and 1% gentamicin (GIBCO, Grand Island, NY). TCCSUP was grown in Minimum Essential Medium (Eagle) in Earle's BSS containing 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino acids supplemented with 10% FBS. HT-29 was grown in McCoy's 5a Medium containing 2 mM L-glutamine supplemented with 10% FBS. Breast cancer cell line (MDA-MB-231) was cultured in DMEM F-12 + 10% FBS, 2mM L-glutamine and 1% gentamicin. Hela cells were a kind gift from Dr. Carcache-de-Blanco and were cultured in high glucose DMEM medium.
4.2 Cytotoxicity Assays

Cells were grown as adherent cultures in T-75 flasks at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated into 96-well plates at a cell density of 1,000-5000 cells per well in 100 µL of media and were allowed to attach overnight. Varying concentrations of compounds were added to the cells with a total final volume per well of 200 µl. The treated cells were incubated at 37°C, 5% CO₂ for 72 hours. Cell viability was determined by Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation assay (MTS) (Promega, Madison, WI). IC₅₀ values were calculated using the SOFTmax Pro plate reader program. Each compound was run at least 3 times in triplicate. For the drug treatment of HUVECs, test compounds were added in the presence of the low-serum growth supplement (Cascade Biologics). HUVECs were plated into 96-well plates at 1.5 x 10³ cells per well in 100 µL of the aforementioned medium. The MTS cytotoxicity assay, which is sold in kit form by Promega (Madison, WI; CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay), is based on the conversion by viable cells, but not by dead cells, of the tetrazolium salt, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), into a water-soluble colored formazan which is detected by spectrophotometry. A SpectraMax Plus microplate reader (Molecular Devices) was then used to measure optical densities at 490 nm. IC₅₀ values, the concentration of the compound that inhibited cell growth by 50% compared to untreated control, were determined with the aid of the software program SoftMax Pro (Molecular Devices). This program uses the dose-response equation $y = ((a$
\[-d/(1 + (x/c)^b)) + d,\] where \(x\) is the drug concentration, \(y\) is absorbance at 490 nm, \(a\) is the upper asymptote, \(b\) is the slope, \(c\) is IC\(_{50}\), and \(d\) is the lower asymptote.

### 4.3 Endothelial migration Assays.
HUVEC and HMEC-1 were harvested at appropriate cell density. Cells were seeded at 5 \(x10^4\) /insert in EBM-2 containing 0.1% BSA in multiwell inserts. Chemotactant, 5% FBS, in EBM-2 was added to 24-well plates into which multiwell inserts were placed. The assembled assays were allowed to proceed for 6 hours in 37\(^\circ\)C incubator with 5% CO\(_2\) before Calcein AM (Molecular Probe) labeling. For inhibitor studies, 10 \(\mu\)M of indicated compounds (10mM stock in DMSO) were added to cell suspensions and chemottractant compartment prior to assay assembly. 0.1% DMSO was used as a control. After the single concentration studies, several inhibitors were selected for IC\(_{50}\) determination using various inhibitor concentrations (0.01 – 100 \(\mu\)M). At the end of an assay, inserts were transferred to 24-well plates containing fluorescent dye Calcein AM (Molecular Probes) at 4 \(\mu\)g/ml in Hank’s Balance Salt Solution and incubated in 37\(^\circ\)C incubator at 5% CO\(_2\) for 1.5 hours. Plates were read at excitation 485 nm/emission 530 nm in CytoFluor multiwell plate reader. Data from each well were collected and analyzed.

### 4.4 Cell Cycle Analysis
Mitotic cells were quantitated by two-variable flow analysis using propidium iodide and the mitotic antibody TG3\(^{66}\) (a kind gift of Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY). Trypsinized, washed cells (1A9) were resuspended in
500 µL RPMI 1640; 500 µL FCS and 3 mL ice-cold 70% ethanol were added drop wise. The cells were kept at 4°C for at least 30 minutes, after which they were centrifuged and resuspended in blocking solution (2% bovine serum albumin in PBS) and incubated at 4°C overnight. The cells were then centrifuged and resuspended in blocking solution with TG3 antibody (hybridoma cell culture medium), diluted 1:10, and allowed to incubate for 30 minutes. After washing with blocking solution, the cells were stained with FITC-conjugated goat anti-mouse secondary antibody for 30 minutes. The cells were then centrifuged and resuspended in 450 µL PBS, 450 µL propidium iodide solution (50 µg/mL in PBS), and 50 µL RNase solution (5 mg/mL in PBS) and allowed to incubate for 30 minutes. The suspension was then passed through a nylon mesh filter and analyzed on a FACSsort flow cytometer (Becton Dickinson, San Jose, CA). FITC fluorescence was monitored with a 525 nm bandpass filter and propidium iodide with a 585 bandpass filter. For apoptotic cell determination, after exposure to drug treatment, cells were harvested, washed with cold PBS and resuspended in 1X annexin binding buffer at a concentration of 1 X 10^6 cells/ml. One hundred microliters of the solution (1 X 10^5 cells) was transferred to a 5 ml culture tube followed by addition of 50 µl of Annexin V-PE and 450 µl of propidium iodide. The cells were then gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Four hundred microliters of 1 X binding buffer was added to each tube and flow cytometry was performed within one hour. For flow cytometric analysis, a FACSCalibur flow cytometer (Becton Dickinson, NJ) equipped with a single argon ion laser was used. DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system.
4.5 Porcine Tubulin Purification

Porcine brains, freshly obtained from a local slaughterhouse, were sliced into approximately 1 cm sections, frozen on dry ice, and stored at -80 °C until use. Frozen brain (approximately 100 g) was broken into small pieces and thawed in 100 mL PME + DTT buffer (0.1 M PIPES, pH 6.9, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT). This brain tissue was homogenized in a blender at 4°C using 3 × 5 s bursts at high power. A 150 mL portion of the homogenate was centrifuged in a Ti-70 rotor at 100,000g for 40 min at 4 °C, and then the supernatant was loaded on an 11 mL column containing DEAE-Sepharose Fast Flow matrix (Amersham Pharmacia) equilibrated with PME + DTT. The DEAE-Sepharose column was washed with two column volumes of PME + DTT, four volumes of PME containing 0.3 M glutamate, and four volumes of PME + DTT containing 1 M glutamate. PME + DTT containing 0.75 M glutamate and 0.3 M KCl was then used to elute tubulin from the column. Tubulin-rich fractions (consisting of approximately 10 mL total) were pooled, and then GTP and DMSO were added slowly with gentle vortexing to bring the final concentrations of these components to 1 mM and 8% (v/v), respectively. This solution was incubated at 37 °C for 40 min. After this 37°C incubation, the solution became cloudy because of the formation of microtubules, which were collected by centrifugation at 100,000g at 35° C for 30 min. The pellet was resuspended in 2.3 mL of PME on ice and sonicated once using a Microson XL2000 sonicator (Misonix, Farmingdale, NY) at an output power of 10 W to completely redissolve the pellet. Following an additional 30 min of incubation on ice, this solution was spun at 80,000g at 4°C for 20 min. The supernatant containing porcine tubulin was
stored at -80°C in 80 µL aliquots and concentration determined by the Bradford assay (Bio-Rad, Hercules, CA).

4.6 Tubulin Polymerization Assay

Assembly reactions were performed at 37°C in PME buffer [0.1 M PIPES (pH 6.9) 1mM MgCl₂, 1mM EGTA] at a protein concentration of 1mg/ml (10 µM) in a 96 well half-area plate. With the plate kept on ice, PME was added to each well followed by GTP to a 1mM final concentration. This was followed by various drug dilutions and controls into different wells. Tubulin was added to the wells and assembly initiated by simultaneous addition of glutamate (final concentration 0.4M) at 37°C. Polymerization was monitored by the increase in absorbance at 351nm using a Spectra Plus micro plate reader (Molecular Device Corp. Sunnyvale, CA). In each assay 5 µM taxol was used as a positive control. The ∆A values were calculated by the difference between absorbance of the sample at a particular time point and absorbance at time 0 at 351nm.

4.7 Immunofluorescence Microscopy

MCF-7 cells were cultured in chamber slides and treated with compounds for twenty hours. After treatment, cells were fixed by immersion in –20°C absolute methanol for 10 minutes. Slides were then washed with PBS and blocked with blocking buffer consisting of 4% bovine serum albumin in PBS for 10 minutes at room temperature. Slides were then incubated with monoclonal anti-α-tubulin antibody (DM1A, Sigma Chemical Co., St. Louis, MO), diluted 1:200 in blocking buffer for 1 hour, washed thrice
with PBS, and then incubated for 1 hour with FITC-labeled anti-mouse antibody (Vector, Burlingame, CA) diluted 1:500 in blocking buffer. The slides were washed again with PBS, DNA was stained with 4',6-diamidino-2-phenylindole, and slides were mounted with Fluoromount G. Slides were observed with a Zeiss LSM 410 scanning confocal microscope.

4.8 Colchicine competitive fluorescence binding Assay

3 µM of purified porcine tubulin was incubated with excess AC (20 µM). The fluorescence of AC was measured after addition of tubulin. 6 and 20 µM of test compounds were added to the assay mixture. The fluorescence of AC was measured again. The decrease in fluorescence is an indication of colchicine site agent inhibiting the binding of AC. The fluorescence was monitored at an excitation wave length of 365 nm and a Kodak 2A emission filter (cutoff at 400 nm) was used. The kinetics were corrected for free drug fluorescence.

4.9 Apoptosis detection by an enzyme-linked immunosorbent assay (ELISA)

Induction of apoptosis was also assessed by using a Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany) by following the manufacturer's instructions. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief, 2.5 x 10^6 PC-3 cells were cultured in a T-75 flask 24 hours before the experiment. Cells were treated with a test agent or the DMSO vehicle for
various time points. Both floating and adherent cells were collected, and cell lysates equivalent to $10^4$ cells were used in the ELISA.

**4.10 NFκβ translocation assay**

This immunofluorescence-based reagent kit enables the measurement of the cytoplasm to nucleus translocation of NFκB. The assay was performed by following the manufacturer's instructions. HeLa cells were incubated with the test compound and/or IL-1α at 10ng/mL for 30 minutes at 37 °C in a CO₂ incubator. Cell fixation was performed by incubation for 10 minutes at room temperature with 37% formaldehyde. Following fixation cells were blocked with blocking solution after which the primary antibody (anti NFκβ-p65) solution was added to each well, incubated for 1 hour, and washed. Secondary antibody solution was also added to each well, incubated for 1 hour protected from light; then discarded. Plates were read in a plate reader Fluostar Optima from BMG LabTech and cells were observed under an inverted phase-contrast Axiovert 40 CFL fluorescence microscope. The nuclear minus cytoplasmic fluorescence was measured. The inhibitory activities of the compounds (IC₅₀) are designated as the concentration of the compounds to reduce 50% of the nuclear minus cytoplasmic fluorescence in the cells.

**4.11 Western blot**

Trypsinized cells were collected by centrifugation and discarding the supernatant. The cell pellet was lysed with cell lysis buffer (Cell Signaling 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton 2.5 mM sodium
pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 µg/ml leupeptin) containing protein phosphatase inhibitor cocktail (cantharidin protein phosphatase 2A inhibitor {PP-2A}, bromotetramisole alkaline phosphatase inhibitor microcystin LR protein phosphatases 1 and 2A inhibitor {PP-1 and PP-2A}). 1mM PMSF was added to the lysates followed by incubation on ice for 15 minutes, brief sonication and centrifugation for 12,000 x g for 15 minutes. The supernatants were collected and protein concentration was determined by using the Bradford assay (Bio-Rad, Hercules, CA). A standard curve was first prepared by using BSA. 50 mg of protein was loaded into Ready Gel Tris-HCl precast gels (Bio-Rad) and separated by electrophoresis. After transferring proteins to PVDF membrane (Amersham), proteins were blocked in 5% non-fat dried milk or BSA and blotted with desired antibodies.

4.12 IKK-β ELISA

The IKK-β kit is an ELISA based assay and was performed according to the manufacturer’s instructions. Human recombinant IKK-β, a serine-threonine kinase was incubated with its substrate (50-amino acid) GST Iκ-β that has two IKK-β phosphorylation sites Ser32 and Ser36. IKK-β, its substrate Iκ-β and ATP were incubated in presence/absence of IKK-β inhibitors in a glutathione-coated plate. The phosphorylated substrate GST Iκ-β was detected by anti-phospho Iκ-β (Ser32 and Ser36) antibody, followed by HRP- conjugate and chromogenic TMB substrate. The absorbance was read at 450 nM. In the first assay a standard curve was prepared using 2, 1 and 0.2 mg/mL of purified enzyme without inhibitor and one well of background TMB
absorbance without enzyme. Following the IKK-β standard curve assay, inhibitor screening was performed using the in-house inhibitor provided with the kit as the positive control and no inhibitor enzyme as the negative control. Decrease in absorbance with respect to the control was an indication of inhibition of IKK-β activity. The concentration of IKK-β was 2ng in the assays.
APPENDIX

Structures

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125 126 128 129 130
131 132 133 136
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Chapter 3


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