PHARMACOKINETICS, PHARMACODYNAMICS, METABOLISM, TRANSPORT, AND RESISTANCE STUDIES OF A NOVEL HISTONE DEACETYLASE INHIBITOR FK228 (FR901228, NSC630176)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Jin Xiao, M.S.

The Ohio State University
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Dissertation Committee:

Dr. Kenneth K. Chan, Adviser
Dr. John C. Byrd
Dr. William L. Hayton
Dr. James T. Dalton

Approved by
Adviser
Graduate program in Pharmacy
ABSTRACT

Depsipotide FK228 (FR901228, NSC630176), a promising histone deacetylase (HDAC) inhibitor, is currently undergoing clinical evaluation against various malignancies.

Pharmacokinetic study of FK228 in the rat was first conducted with a focus on its pharmacokinetic properties and dose recovery. FK228 was found to be removed rapidly from the circulation with a total body clearance higher than the rat cardiac output, suggesting extensive metabolism in the blood. Dose recovery of FK228 was low (<15%) and there was an involvement of glutathione in FK228 elimination. Based on these results, we conducted in vitro metabolism studies, which led to identification of four glutathione conjugates and two thiols from rat and human blood incubations. Purification of these major metabolites followed by HDAC inhibition assays indicated that FK228 is a prodrug, with three major metabolites being more potent HDAC inhibitors than FK228 itself.

A clinical pharmacokinetic study in AML and CLL patients was conducted. A pharmacokinetic-pharmacodynamic correlation study showed that HDAC inhibitory activity was inversely correlated with FK228 systemic exposure. This provided the first clinical evidence that FK228 was a prodrug.
FK228 transport and uptake was investigated. It was found that FK228 is a substrate for both MDR1 and MRP1 by transport and cytotoxicity studies using specific inhibitors of the membrane transporters. In order to study the roles of MDR1 and MRP1 in acquired FK228 resistance, four FK228 resistant cell lines were established and characterized. We found that upregulation of MDR1, but not MRP1 or other ABC transporters, was responsible for the acquired resistance. The maintenance of acquired FK228 resistance depended on continuous drug exposure. No deregulation or impairment of the histone acetylation machinery was found. The MDR1 upregulation was further found to be via a reversible induction procedure, in which FK228 first inhibited HDACs and then caused hyperacetylation at the MDR1 promoter region to form the euchromatin structure ready for transcription.

Overall, the dissertation work, involving both preclinical and clinical studies, provided valuable information of pharmacokinetics, bioactivation, transport and uptake, and resistance of the novel anticancer drug FK228.
DEDICATION

To my dear wife Sunny

For her love and support
ACKNOWLEDGMENTS

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VITA

December 25, 1973………………...Born in Beijing, P.R. China

1992 – 1996………………...B.S. Pharmacy, Beijing Medical University

1996 - 1999………………...M.S. Pharmacy
Institute of Materia Medica
Chinese Academy of Medical Sciences

1999 - Present………………...Graduate Teaching and Research Associate,
The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Pharmacy

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<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>Gene name for P-glycoprotein or Pgp</td>
</tr>
<tr>
<td>ABCC1</td>
<td>Gene name for MRP1</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AP→BL</td>
<td>Apical to basolateral</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the plasma concentration-time curve</td>
</tr>
<tr>
<td>BL→AP</td>
<td>Basolateral to apical</td>
</tr>
<tr>
<td>BMLP</td>
<td>N-t-Boc-Met-Leu-Phe</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
</tr>
<tr>
<td>Caco 2</td>
<td>Human colorectal adenocarcinoma cell line Caco 2</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CCV</td>
<td>Constant Coefficient of Variation</td>
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<tr>
<td>CDKs</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CTEP</td>
<td>Cancer Therapy Evaluation Program</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DEM</td>
<td>Diethyl maleate</td>
</tr>
<tr>
<td>df</td>
<td>Degree of freedom</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPS or ε</td>
<td>Intra-individual error</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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xvi
<table>
<thead>
<tr>
<th>Acronym</th>
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<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ETA or η</td>
<td>Inter-individual variability of pharmacokinetic parameters</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
</tr>
<tr>
<td>FTIs</td>
<td>Farnesyl transferase inhibitors</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
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<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HCT-15</td>
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<td>Hda1</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HIF-1</td>
<td>Hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HL60</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatograph</td>
</tr>
<tr>
<td>IGROV1</td>
<td>Human ovarian carcinoma cell line IGROV1</td>
</tr>
<tr>
<td>ILACUC</td>
<td>Institutional Laboratory Animal Care and Use Committee</td>
</tr>
<tr>
<td>IRBs</td>
<td>Institutional Review Boards</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
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<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>K562</td>
<td>Human chronic myelogenous leukemia cell line K562</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass over charge ratio</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human breast carcinoma cell line MCF7</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi-drug resistance protein 1</td>
</tr>
<tr>
<td>MeCP2</td>
<td>Methyl-CpG-binding protein 2</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MRP1</td>
<td>Multidrug resistant-associated protein 1</td>
</tr>
<tr>
<td>MS^n</td>
<td>Multiple stage mass spectrometry</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NCI DTP</td>
<td>National Cancer Institute, Developmental Therapeutics Program</td>
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<tr>
<td>N-CoR</td>
<td>Nuclear co-repressor</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acid</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
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<td>NIH</td>
<td>National Institute of Health</td>
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Papp    Apparent permeability coefficient
PBS    Phosphate-buffered saline
pCAF   P300/CBP associated factor
PDGFR  Platelet derived growth factor receptor
PDK1   Phosphoinositide-dependent protein kinase-1
Pgp    P-glycoprotein
PI3    Phosphoinositide 3
PKB    Protein kinase B
popPK  Population pharmacokinetics
Q      Inter-compartment clearance
θ      Fixed effect parameters in population pharmacokinetic modeling
RARα   Retinoic acid receptor α
RARα-RXR Retinoic acid receptor α-retinoid-X receptor heterodimer
RB     Retinoblastoma tumor suppressor protein
RBC    Red blood cells
RTKs   Receptor Tyrosine Kinase
RXR    Retinoid-X receptor
σ²     Variance of EPS
SAHA   Suberoylanilide hydroxamic acid
SIM    Single Ion Monitoring
SIR2   Silent information regulator 2
siRNA  Small interfering RNA
SMRT   Silencing mediator of retinoic acid and thyroid hormone receptor
T1/2,α Half life of α phase
T1/2,β Half life of β phase
TEER   Transepithelial electrical resistance
TIC    Total ion chromatogram
Topo   Topoisomerases
tPAs   Tissue plasminogen activators
TR     Thyroid receptor
TSA    Trichostatin A
Uex%   Urinary excretion recovery%
ULAR  University Laboratory Animal Resources
uPAs  Urokinase-type plasminogen activators
V1     Central compartment distribution volume
V2     Peripheral compartment distribution volume
Vdss   Apparent steady state volume of distribution
VEGF  Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
ω²  Variance of ETA
WHO  World Health Organization
XIC  Extracted ion chromatogram
CR  Complete remission
CHAPTER 1

INTRODUCTION

1.1 Literature Review

Decades of efforts in search of effective cures of cancers have not yet been totally successful, while scientists began to realize and appreciate the ‘smartness’ of cancer cells. We have to admit sometimes “in its own microscopic way, becoming cancerous is about the most glamorous and successful thing a cell can do” (Garrett, Walton et al. 2003).

Anticancer chemotherapy emerged in the 1940s from toxicological studies of nitrogen mustard-based war gas, which led to the discovery of effective alkylating agents (i.e., chlorambucil, cyclophosphomide, ifosfamide, melphalan, cisplatin), some of which are still being used clinically (Baumann and Preiss 2001). This first generation of anticancer drugs was developed based on their ‘anti-proliferative’ rather than ‘anticancer’ properties, and was inevitably associated with low selectivity for tumor versus normal cells.

The second wave of chemotherapy was witnessed by development of antimetabolite drugs such as analogs of nucleotides (e.g., purine analogs 6-
mercaptopurine and 6-thioguanine, and pyrimidine analogs fluorouracil and cytarabine) and folic acid (e.g., methotrexate, dichloromethotrexate, and aminopterin) in the 1960s and 1970s on the basis of an understanding of key enzyme steps in nucleotide biosynthesis and sensitivity of tumor cells to alterations in these pathways (Gibbs 2000; Gmeiner 2002; McGuire 2003). New anticancer drugs of this family, such as OGT 719, a novel nucleoside analog (Mok, Leung et al. 2004), continue to emerge and show promise in the management of some cancers (i.e., liver and colon cancers). Even though the second wave of chemotherapy was thought to be the start of the mechanism-based approach, identification of anticancer agents was empirical due to lack of knowledge and tools on target identification, target characterization, target-drug interactions and unavailability of appropriate models. Due to limited understanding of the genetic abnormalities of cancers, it was difficult to learn from successes and failures and to understand why different tumor types have different susceptibilities (Gibbs 2000).

This empirical strategy as featured by cytotoxicity screening in tumor cells, although not efficient, has led to discovery of several successful anticancer drugs (i.e., doxorubicin and taxanes), whose biochemical mechanisms were identified several years after their discoveries (Gibbs 2000). With the availability of combinational chemistry, extensive structure modifications of known lead compounds have greatly expanded the chemical library (e.g., the repository of more than 600,000 compounds at the Developmental Therapeutics Program,
application of modern structure analytical tools, such as X-ray crystallography
(Neidle 1994), nuclear magnetic resonance (Stubbs 1999), mass spectrometry
(Burkitt, Derrick et al. 2003), and docking computation (Glen and Allen 2003),
has provided accurate information about target binding site conformations and
drug-target interactions, and made it possible to screen anticancer drugs in a
more rational and efficient way (i.e., structure-activity relationship analysis).
Together with advances in high throughput pharmacokinetics/ADME,
pharmacodynamics and bioinformatics, these powerful tools have formed the
basis for innovative drug discovery and development for a given target.

During the past 20 years, we have experienced an explosion of knowledge
in genetic based initiation, progression and metastasis of cancers, which has
brought forward the third wave of molecular-based anticancer development
(Gibbs 2000; Garrett, Walton et al. 2003). The first feature of this stage is the
close collaborative and expanding network that involves scientists in cell biology,
biochemistry, molecular genetics, pathology, medicinal chemistry,
pharmaceutics, clinical medicines, bioengineering and many other related fields.
Another important feature is the rapid validation of molecular targets that are
potentially tractable in drug development (Garrett, Walton et al. 2003). This has
greatly changed the drug development strategy from empiricism to a more
rational basis, where the mechanism of a lead drug under development may be
elucidated even before the drug is actually synthesized. Moreover, the concept of
cancer treatment has changed to individualized diagnosis and treatments based on cancer pathology and the known mechanisms of anticancer drugs. Some excellent results have been obtained in curing several childhood cancers (Pui 2004) and certain adult malignancies such as lymphoma and leukemia (Uzuka and Saito 2003; Tallmann 2004). In the following section, I attempt to summarize identification and targeting of novel molecular targets in anticancer therapies.

1.1.1 Identification of novel molecular targets in anticancer therapies

Since many malignant processes are related to gene defects, identification of normal gene functions and determination of gene deregulation in cancer cells are key initial steps towards targeted chemotherapy. The successful sequencing of the human genome has opened the door of genome-wide elucidation of gene functions, and is thus expected to accelerate the development of targeted chemotherapy (Makalowski 2001). However, only a small fraction (~1%) of the genes is transcriptionally deregulated in cancer cells (Zhang, Zhou et al. 1997). Surprisingly, the principle disparities in gene transcriptional activity between cancerous and normal cells are not in oncogenes as previously expected, but rather in genes associated with protein synthesis (Zhang, Zhou et al. 1997; Chen and Ioannou 1999), ribosomal proteins (Chen and Ioannou 1999), protein elongation factors (i.e., eEF1A2) (Thornton, Anand et al. 2003), glycolysis (Fernie, Carrari et al. 2004), and also differentiation markers (i.e., lymphocyte differentiation marker CD38, and melanocytic differentiation marker Melan-A) (Shubinsky and Schlesinger 1997; Busam and Jungbluth 1999). Moreover, gene
deregulation patterns are cancer-type dependent and may change over time due to the instability of the cancer genome (Shadan and Koziol 2000). Consequently, identification of gene targets, assignment of biological function to specific genes, and validation of chemotherapy targets are becoming primary tasks, and sometimes the rate-limiting steps in anticancer drug discovery and development (Szekeres and Novotny 2002; Novotny and Szekeres 2003).

Identification and validation of anticancer targets has been greatly accelerated by gene array technologies (Debouck and Goodfellow 1999), protein technologies (i.e., proteomics) (Page, Amess et al. 1999), high throughput screenings (Bevan, Ryder et al. 1995; Archer 1999; Dove 1999), advances in animal models (knock-out mice) (Gu, Marth et al. 1994), and recently the knock-down techniques using siRNAs (Montgomery 2004), as well as progress in related fields. Some excellent reviews on molecular targets have been published (Gibbs 2000; Buolamwini and Assefa 2003; Garrett, Walton et al. 2003) and are summarized in Table 1.1 and 1.2.

It is of note that for each given molecular target, its function may be modified (i.e., inhibited) in several ways to induce cytotoxicity. For example, small molecule ATP competitors such as olomoucine and roscovitine that block the co-substrate (ATP) binding site can be used to ablate the function of cyclin-dependent kinases (CDKs) (Gray, Wodicka et al. 1998; Gray, Detivaud et al. 1999). Additionally, synthetic peptides mimicking \textit{in vivo} CDK inhibitors such as p16INK4A and p21CIP1/WAF1 (Fahraeus, Paramio et al. 1996; Ball, Lain et al.
1997) or CDK substrate peptides that block the substrate recognition site may exert similar CDK inhibition (Colas, Cohen et al. 1996). Moreover, there are so called ‘indirect’ CDK inhibitors, such as rapamycin (Albers, Williams et al. 1993), lovastatin (Poon, Toyoshima et al. 1995), or antisense against CDKs or cyclins (Morishita, Gibbons et al. 1994). These compounds can lead to loss of CDK activity by modulation of the upstream pathways required for CDK activation.

1.1.1.1 Signal transduction pathways

Abnormal signal transduction pathways are well-recognized targets for anticancer chemotherapy (Garrett and Workman 1999). For the purpose of cancer intervention, two signal transduction pathways have drawn enormous attention as potential targets for anticancer chemotherapy.

The Receptor Tyrosine Kinase (RTK)$\rightarrow$Ras$\rightarrow$Raf-1$\rightarrow$MEK$\rightarrow$ERK signal transduction pathway has been intensively studied. It was one of the earliest and best elucidated pathways, and its deregulation is frequently associated with human cancers (Hilger, Scheulen et al. 2002; Leirdal and Sioud 2002) (Figure 1.1). The first level targets are RTKs. For example, the erbB2/HER-2 receptor is commonly over-expressed in human breast and ovarian cancers (Slamon, Godolphin et al. 1989). As important is epidermal growth factor receptor (EGFR) deregulation (Fischer, Hart et al. 2003). A list of inhibitors of RTKs already on the market, in clinical trials, and in preclinical development is included in Table 1.1. These agents are targeted to EGFR, HER-2, platelet-derived growth-factor
receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and also RTK-associated tyrosine kinases such as Src (Zwick, Bange et al. 2001).

Downstream of RTK is Ras, which has been found to mutate frequently in human cancers (Hancock, Magee et al. 1989; Kiaris, Spandidos et al. 1995; Spandidos, Glarakis et al. 1995). Activating point mutations of the small GTPase Ras are present in about 30% of all human tumors (Duursma and Agami 2003). Constitutively active Ras induces growth factor independent cell proliferation and cell survival. The oncogene nature of Ras was shown by malignant transformation of normal epithelial cells by mutant Ras (i.e., Harvey Ras) (Marshall 1995; Hahn, Counter et al. 1999). Several agents have been tested clinically to block Ras. Ras antisense oligonucleotides block Ras translation (Cowsert 1997), while farnesyl transferase inhibitors (FTIs) prevent Ras from proper post-translational modification (i.e., prenylation) and activation (Crul, de Klerk et al. 2001).

Further downstream in the RTK→Ras→Raf-1→MEK→ERK signal transduction pathway is Raf-1. Efforts for blocking Raf-1 led to discovery of a small molecule Raf-1 kinase inhibitor BAY 43-9006 (Wilhelm and Chien 2002), which is now in a phase I clinical trial in patients with advanced solid tumors (Hotte and Hirte 2002). Raf-1 Antisense ISIS 5132 (CGP 69846A) has entered phase II clinical trials in patients with advanced colon and lung cancers (Coudert, Anthoney et al. 2001; Cripps, Figueredo et al. 2002). A MEK inhibitor CI-1040 (PD184352) has showed promising preclinical pharmacologic results and has
entered a phase I clinical trial for safety evaluation (Allen, Sebolt-Leopold et al. 2003; Sausville, Elsayed et al. 2003). Mitogen-activated protein kinases (MAPKs) are over-expressed in certain cancers such as human head and neck squamous cell carcinomas (Gottschlich, Folz et al. 1998; Gorogh, Berwig et al. 2004). This led to targeting MAPKS, such as ERK, c-Jun N-terminal kinase (JNK) and p38 for anticancer chemotherapy (Boldt and Kolch 2004). One example is ERK inhibitor PD98059, which has shown synergism with docetaxel in inducing apoptosis of androgen-independent human prostate cancer cells (Zelivianski, Spellman et al. 2003). JNK inhibitor SP600125 and JNK antisense molecules reduce proliferation of all breast cancer cell lines (Mingo-Sion, Marietta et al. 2004). Moreover, inhibitors of p38 (i.e., PD169316, SB203580 and SB202190) can augment bisphosphonate (BPs)-induced growth inhibition of breast carcinoma (Merrell, Suarez-Cuervo et al. 2003).

The other important signal transduction pathway serving as a molecular target for anticancer chemotherapy is the phosphoinositide 3 (PI3) kinase pathway (Garrett and Workman 1999; Stein and Waterfield 2000) (Figure 1.1). PI3 kinases phosphorylate the hydroxyl group at the 3-position on the inositol ring of phosphor-inositides, and generate phospholipids as second messengers, which in turn play key roles in the regulation of many cellular processes (Stein and Waterfield 2000). PI3 kinases are divided into 3 classes on the basis of sequence homology and substrate preference. Class I PI3 kinases have been extensively characterized (Stein and Waterfield 2000; Cantley 2002). However,
the most well known PI3 kinase in relation to cancer is the Class IA form of p110α, which is activated by RTKs via its associated adapter protein p85 (Garrett and Workman 1999; Stein and Waterfield 2000). PI3 kinase deregulation has been found in various tumors. For example, p110α, coded by gene PIK3CA, is amplified and over-expressed in certain ovarian cancer (Garrett and Workman 1999), and is now recognized as an oncogene. On the other hand, the tumor suppressor gene PTEN acts as a lipid phosphatase that reverses the PI3 kinase reaction. This has suggested the use of PI3 kinase inhibitors (e.g., LY294002 and demethoxyviridin) as anticancer agents (Garrett and Workman 1999).

Downstream of PI3 kinase is phosphoinositide-dependent protein kinase-1 (PDK1), which in turn activates protein kinase B (PKB). PKB, also known as AKT, is the mammalian homologue of the oncogene v-AKT (Stein and Waterfield 2000). Downstream of PKB/AKT are a number of effector enzymes responsible for cellular activities and cell survival (Stein and Waterfield 2000) (Figure 1.1). Members in this pathway have been used as molecular targets for anticancer therapy. UCN-01 (7-hydroxystaurosporine), a specific PDK1 inhibitor, is currently undergoing clinical trials for the treatment of advanced cancer (Komander, Kular et al. 2003). In addition, efforts to target PKB/AKT for anticancer chemotherapy led to discovery of a class of PKB/AKT inhibitors (i.e., NL-71-101), which show promising activity against in vitro cancer models (Hill and Hemmings 2002; Reuveni, Livnah et al. 2002).
Reciprocal t(9:22) chromosomal translocation results in expression of a chimeric gene encoding the unregulated Bcr-Abl tyrosine kinase, Bcr-Abl-mediated tyrosine phosphorylation promotes transformation of hematopoietic progenitor cells into chronic myeloid and acute lymphocytic leukemia (Donato, Wu et al. 2004). Bcr-Abl fusion protein serves as an excellent molecular target for anticancer chemotherapy. STI571 (Gleevec) targeting Bcr-Abl has entered phase III clinical trials with promising results in chronic leukemia patients (Fabbro, Ruetz et al. 2002).

1.1.1.2 Cell cycle and apoptosis

Division of mammalian cells is a tightly regulated process consisting of four coordinated processes: G1, S, G2 and M phases. A detailed review of the cell cycle and regulation is beyond the scope of this introduction. However, it is well established that cancer is closely associated with abnormalities in cell cycle control. More specifically, cyclin-dependent kinases (CDKs) play a key role in cell division regulation by sending signals to push the cell to go through cell cycle check points (i.e., G1 and G2 check points). For full activity, each CDK protein must associate with a regulatory subunit from the cyclin family of proteins (Garrett and Workman 1999). This complex then phosphorylates a number of substrate proteins, including the retinoblastoma tumor suppressor protein (Rb), so as to regulate cell cycle progression. Since CDK deregulation is frequently found in cancers, CDKs and cyclins serve as either direct or indirect targets for
anticancer therapy (Bartkova, Lukas et al. 1997). A list of CDK inhibitors is shown in Table 1.1.

Another important member of cell cycle regulators is p53. The p53 tumor suppressor gene plays an indispensable role in two key processes: to induce cell cycle arrest at the G1/S transition along with DNA repair, or if DNA repair is impossible, to activate apoptosis (Lane 1992; Garrett and Workman 1999). The p53 protein functions through transcriptional activation of various downstream effector genes (i.e., CDK inhibitor p21^{CIP1/WAF}), which have promoters containing p53-specific binding sites. Mutation of p53 is observed in over half of all sporadic cancers, making p53 mutations the most common genetic change in human cancers (Lane 1992; Garrett and Workman 1999). The first strategy targeting p53 is via MDM2, an oncogene that antagonizes p53 function by either repressing transcription of p53 or by induction of ubiquitin-mediated proteolysis of p53 (Prives 1998). Peptide inhibitors (Bottger, Bottger et al. 1997) and antisense oligonucleotides against MDM2 (Mu, Hachem et al. 2004) showed positive results in model cancer cells. Another strategy is to re activates p53 in tumor cells. This includes gene delivery using adenovirus (ONYX-015), which can only replicate in mutant p53 cells (McCormick 2003). Small molecules that stabilize p53 (i.e., CP-31398) have also received clinical interest (Wischhusen, Naumann et al. 2003).

Apoptosis of cancer cells is induced by many chemotherapeutic agents. Progress in elucidation of apoptosis machinery and mechanism has provided
numerous potential molecular targets for anticancer therapy. One important family of apoptosis proteins is the BCL2 family, which is reviewed elsewhere (Burlacu 2003; Kuwana and Newmeyer 2003). BCL2, an oncoprotein, has become an important target for anticancer chemotherapy since BCL2 deregulation has been frequently associated with malignant transformation and acquisition of drug resistance (Sartorius and Krammer 2002). One approach to turn off BCL2 is by antisense oligonucleotides such as G3139, which is now under phase II/III clinical trials (Gutierrez-Puente, Zapata-Benavides et al. 2002; Marcucci, Byrd et al. 2003). As an alternative, small interfering RNA (siRNA) against BCL2 decreased BCL2 transcription and showed synergism when combined with cisplatin (Wacheck, Losert et al. 2003).

1.1.1.3 Protein translation, post-translational modification and degradation

Protein synthesis, including translation, folding and modification, is a critical process during cell growth, especially during the G1 phase (Brooks 1977). In certain forms of cancers, deregulation of this process may occur (Zimmer, DeBenedetti et al. 2000). One established molecular target during protein translation is eIF-4E. As an mRNA cap-binding protein, eIF-4E is a translation initiation factor. The biological function of eIF-4E is reviewed elsewhere (Zimmer, DeBenedetti et al. 2000). The utility of eIF-4E as a molecular target was suggested by the fact that eIF-4E over-expression induces transformation and is found in breast, head and neck, and prostate tumors (De Benedetti and Harris 1999). Numerous approaches have been proposed to reverse eIF4E over-
expression. EIF4E antisense (DeFatta, Nathan et al. 2000) and stimulation of 4E-BP, a translational factor that sequesters eIF4E (Miron, Verdu et al. 2001), show prominent results. Rapamycin, an eIF4E inhibitor, has been used as a novel agent to treat cancers as reviewed recently (Chan 2004).

Protein folding is an essential post-translational process mediated by a class of proteins (e.g., TRiC/CCT, Hsp70, and Hsp90). Deregulation of this process is associated with tumorigenesis as reviewed recently (Scott and Frydman 2003). As one of the established molecular targets, heat shock protein 90 (Hsp90) is required for the correct folding, stability and function of a range of oncoproteins that are mutated or over-expressed in cancers (Workman 2002). Hsp90 inhibition by 17-allyamino-17-demethoxygeldanamycin (17AAG) demonstrated activity in various cancer models (Chung, Troy et al. 2003; Braga-Basaria, Hardy et al. 2004).

Another post-translational event, protein modification, is also involved in tumorigenesis. Several kinases responsible for (de)phosphorylation and activation of oncogenes have been targeted for anticancer therapy. These kinases include protein kinases A (Miller 2002) and C (Lahn, Sundell et al. 2003; Hanauske, Sundell et al. 2004), and protein phosphatase Cdc25 (Kristjansdottir and Rudolph 2004). Some typical inhibitors that possess anticancer activity are summarized in Table 1.1.
In addition to protein synthesis and modification, proteolysis is also implicated in tumorigenesis. Ubiquitin-mediated proteolysis is a multi-step reaction, involving ubiquitin tagging of target proteins and subsequent degradation mediated by the 26S proteasome. This proteolysis pathway is responsible for rapid turnover of many regulatory proteins (e.g., p53, cyclin E and D, p27) (Garrett and Workman 1999). Indeed, all proteins that are subject to this pathway show deregulation in cancer cells (Spataro, Norbury et al. 1998). A list of the 26S proteasome inhibitors with promising anticancer cytotoxicity is presented in Table 1.1 (Wang, Figueiredo Pereira et al. 1999; Pasquini, Paez et al. 2003; Lara, Davies et al. 2004).

1.1.1.4 Angiogenesis and Metastasis

Pathological neovascularization around the tumor plays a key role in tumor growth and metastasis (Hoekman and Pinedo 2004; Kini 2004). Therefore, inhibition of angiogenesis has become one of the most promising strategies that might lead to the development of novel anticancer therapy (Zhang, Wang et al. 2003). Reviews on the molecular pathology of angiogenesis and targeting angiogenesis for anticancer chemotherapy have been published (Gupta and Qin 2003; Zhang, Wang et al. 2003).

Angiogenesis is a complex process that involves the activation, proliferation, and migration of endothelial cells, disruption of vascular basement membranes, formation of vascular tubes and networks, and linkage to the pre-
existing vascular networks (Zhang, Wang et al. 2003). One strategy to inhibit angiogenesis is to suppress proangiogenic signals. Some proteolytic enzymes that are involved in basement membrane breakdown, the first step in angiogenesis, have been targeted. Examples include matrix metalloproteinases (MMPs), urokinase-type plasminogen activators (uPAs), and tissue plasminogen activators (tPAs) (Chu, Chiou et al. 2004).

The breakdown of basement membrane is followed by migration and proliferation of vascular endothelial cells, which are stimulated by a variety of growth factors. These growth factors are now being targeted clinically for anticancer therapy. Vascular endothelial growth factor (VEGF) binds to specific tyrosine kinase receptors (KDR and flt-1) on the membrane of proliferating endothelial cells to stimulate vascular growth. Antibodies and drugs targeting VEGF receptors have been developed (Table 1.2).

The fibroblast growth factor (FGF) family includes more than 20 structurally related proteins. Two important members, acidic FGF (aFGF) and basic FGF (bFGF-2), stimulate vascular formation during angiogenesis and have been extensively studied (Zhang, Wang et al. 2003). Inhibition of FGF mitogenic activity has been suggested as a crucial target for the development of antiangiogenic cancer treatment. For example, suramin, suradistas and their derivatives were discovered and are currently tested in clinical trials (Botta, Manetti et al. 2000).
Angiogenin (ANG), another target originally isolated as a human tumor-derived angiogenesis factor (Fett, Strydom et al. 1985), was also identified as a potentially important target for anticancer therapy. Mouse monoclonal antibody 26-2F (Piccoli, Olson et al. 1998) and an antisense oligonucleotide JF2S (Olson, Byers et al. 2001) directed against ANG are highly effective in athymic mice models. Another ANG antagonist, an 11-amino acid peptide chANG, dramatically reduces liver metastases in mice injected with human colorectal carcinoma cells (Gho, Yoon et al. 2002).

Some endogenous angiogenesis inhibitors (e.g., angiostatin and endostatin) have been used clinically against tumor angiogenesis and metastasis. Angiostatin is a proteolytic fragment of plasminogen (Folkman 1995) and endostatin is a proteolytic fragment of collagen type XVIII (O'Reilly, Boehm et al. 1997). Clinical studies showed positive results when they were used either alone or in combination with other chemotherapeutic agents (Caceres and Gonzalez 2003).

Another family of important molecular targets comprises the integrin, a family of cell adhesion proteins that promote the attachment and migration of cells on the surrounding extracellular matrix (ECM) during angiogenesis (Jin and Varner 2004). Some integrin inhibitors (e.g., Vitaxin, Sch221153, EMD-121974) showed promising results in vitro and in tumor bearing animals (Zhang, Wang et al. 2003).
1.1.1.5 Replication and transcription

Tumorigenesis is a process of accumulation of gene abnormalities (i.e., mutation, deletion etc.), as well as deregulation of DNA replication and transcription. Some transcription factors play essential roles in various tumors. NFκB is a pivotal transcription factor in prostate cancer and is thought to be responsible for metastasis to bone (Andela, Gordon et al. 2003). It exerts tumorigenic function by regulating the transcription of cytokines, adhesion molecules, and other mediators (i.e., MMPs) (Sun and Andersson 2002; Andela, Gordon et al. 2003). Some strategies of NFκB inhibition have been reviewed (Sun and Andersson 2002), and some inhibitors are listed in Table 1.2.

Deregulated expression of the c-Myc proto-oncogene contributes to malignant progression of a variety of tumors. The c-Myc protein (or Myc) is a transcription factor that positively or negatively regulates expression of distinct sets of target genes (Amati, Frank et al. 2001). To date, the only promising strategy of antagonizing c-Myc is by antisense oligonucleotides such as AVI-4126, which has entered phase I clinical trials (Iversen, Arora et al. 2003).

Anti-estrogens are central to the treatment of breast cancer of all stages. We now understand that the action of estrogen is mediated by estrogen receptors (ER), which belong to the nuclear receptor family of ligand-regulated transcription factors (Hanstein, Djahansouzi et al. 2004). Targeting to ER has led
to excellent clinical outcomes (Chan 2002), and some ER antagonists are listed in Table 1.2.

DNA transaction that replicates and maintains a stable human genome is essential for healthy growth of proliferation of eukaryotic cells. Emerging information on the properties and functions of human DNA polymerases, with emphasis on connections between DNA polymerase functions and cancer, has been reviewed (Kunkel 2003). Although deregulation of some RNA polymerases (White 2004) and DNA polymerases (Kunkel 2003) in cancers suggests their potential as anticancer targets, the most recognized molecular targets involved in polymerization are topoisomerases I and II (Topo I and II). Topo I (Murphy, Cmelak et al. 2001) and Topo II (Larsen, Skladanowski et al. 1996) are essential for survival of all eukaryotic cells. Topoisomerases are both enzymes and structural components of the nuclear matrix. They regulate the topological states of DNA by transient cleavage, strand passing and re-ligation of double-stranded DNA resulting in decatenation of intertwined DNA molecules and relaxation of supercoiled DNA (Larsen, Skladanowski et al. 1996). Topoisomerases play an important role in DNA replication and are required for condensation and segregation of chromosomes (Larsen, Skladanowski et al. 1996). The expression of topoisomerases is cell cycle dependent with both protein levels and catalytic activity peaking at G2/M. Deregulation of Topo enzymes has been found in tumors, and both classes of topoisomerases serve as important molecular
targets in anticancer therapy (Walker and Nitiss 2002; Kuppens, Beijnen et al. 2004). Some typical Topo enzyme inhibitors are listed in Table 1.2.

Telomeres are the ends of linear chromosomes, shortened with each round of DNA replication. Loss of telomeric DNA can lead to senescence, a state in which cells no longer divide, and crisis, which triggers cell death (Ulaner 2004). To prevent these phenomena, cancer cells must maintain their telomeres, for example, by expressing telomerase, an enzyme that can extend telomeres (Ulaner 2004). For this reason, telomerasenases have become a molecular target of chemotherapy against tumors (i.e., gastrointestinal, bladder and breast carcinomas) (Yoshida and Toge 2004).

As our knowledge accumulates about tumorigenesis, it appears that epigenetic deregulation plays as important a role as does the genetic abnormality (Jones 2003; Vigushin and Coombes 2004). DNA methylation, its regulation and how its deregulation is related to cancer have been reviewed recently (Jones 2003). DNA methylation is mediated by a family of enzymes called DNA methyltransferases. Some small molecule inhibitors (e.g., 5-azacytidine and 5-aza-2'-deoxycytidine) that target DNA methyltransferases have demonstrated encouraging activity against leukemia but not solid tumors (Goffin and Eisenhauer 2002). The antisense drug MG98, which is directed against the 3' untranslated region of the DNA methyltransferase-1 enzyme mRNA, is now under phase II study (Goffin and Eisenhauer 2002). Another emerging
epigenetic target is a family of enzymes called histone deacetylases, which is discussed below.
Among the labeled enzymes, RAS, PI3 kinase, PKB/AKT are oncogenes, while PTEN is a tumor suppressor gene. Abbreviations: AGC, group of protein kinases including protein kinases A, B, C, G and p70S6 kinase; BAD, BCL2 antagonist of cell death; BTK, Bruton’s tyrosine kinase; ERK, extracellular signal regulated kinase or mitogen activated kinase; FKHLR1, forkhead transcription factor; GSK-3, glycogen synthase kinase 3; IKK, IκB kinase; MEK, ERK kinase, PDK1, phosphoinositide dependent kinase; PLC, phospholipase C (Stein and Waterfield 2000).
### Table 1. Summary of novel molecular targets for anticancer chemotherapy (Part I).

<table>
<thead>
<tr>
<th>Categories</th>
<th>Molecular targets (drugs or lead therapeutics)</th>
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<tbody>
<tr>
<td><strong>Signal transduction</strong></td>
<td></td>
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<tr>
<td>RTK-RAS-ERK pathway</td>
<td>EGFR (Iressa®, Cetuximab, ABX-EGF), HER-2 (Herceptin®, Geldanamycin), PDGFR (Gleevec®), bFGFR (suramin, PD145709, SU5402), c-kit (Gleevec®)</td>
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<td></td>
<td>Ras (antisense, ras farnesyl transferase inhibitors), Grb2*, Raf-1 (antisense, BAY-43-9006), MEK (PD98059, CI-1040), MAPK (PD98059, SP600125, PD169316), Src (2-substituted aminopyrido pyrimidinones), bcr-abl (Gleevec®), Ick (dihydroxyisoquinolines)</td>
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<tr>
<td>PI3 kinase pathway</td>
<td>PI3 kinases (LY294002, demethoxyvinidin), PDK1 (UCN-01), PKB/AKT (NL-71-101)</td>
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<td><strong>Cell cycle and apoptosis</strong></td>
<td></td>
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<tr>
<td>CDKs&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CDK1, 2, 4, 6 and 9 (flavopiridol, olumoucine, roscovitine, purvalanols, paulones, aminothiazoles)</td>
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<tr>
<td>P53 pathway</td>
<td>MDM2 (peptide inhibitors, antisense), P53 (gene delivery, SCH58500, CP-31398)</td>
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<tr>
<td>Apoptosis</td>
<td>bcl2 (antisense, siRNA),</td>
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<tr>
<td><strong>Protein synthesis</strong></td>
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<tr>
<td>26S protease</td>
<td>26S protease (lactacystin, aclarubicin, PS-341)</td>
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<tr>
<td><strong>Protein translation</strong></td>
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<tr>
<td>Hsp90</td>
<td>Hsp90 (17-Allyamino-17-demethoxygeldanamycin)</td>
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<tr>
<td><strong>Protein modification</strong></td>
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<tr>
<td>PKA (8-chloro-cyclic AMP, antisense), PKC (UCN-01, safingol, CGP53506), protein phosphatase Cdc25 (BN82002), protein farnesyltransferase (R115777, Zarnestra)</td>
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<sup>a</sup> CDK inhibitors are generally not selective.
<table>
<thead>
<tr>
<th>Categories</th>
<th>Molecular targets (drugs or lead therapeutics)</th>
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<tbody>
<tr>
<td><strong>Angiogenesis</strong></td>
<td>VEGFR (vatalanib, bevacizumab, SU5416), FGFs (suramin, suradistas and derivatives), angiogenin (antibody, antisense, peptide), Endogenous angiogenesis inhibitors(^a) (angiostatin, endostatin, AG-1470)</td>
</tr>
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<td>and metastasis</td>
<td></td>
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<tr>
<td>Basement membrane breakdown</td>
<td>Matrix metalloproteinases (Marimastat, AG-3340, CGS-27023A), uPAs and tPAs (B623, PAI-1)</td>
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<tr>
<td>Cell adhesion molecules</td>
<td>Integrins (Vitaxin, Sch221153, EMD121974, EMD-121974, IS20), cadherins, selectins, mucins, hyaluronan, gangliosides (RGD-containing synthetic peptides)</td>
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<tr>
<td><strong>Replication</strong></td>
<td>c-Myc (antisense), NF-(\kappa)B (Celecoxib, epoxyquinomicin C derivatives), estrogen receptor (tamoxifen, SERMs)</td>
</tr>
<tr>
<td>and Transcription</td>
<td>Topoisomerase I (camptothecin, topotecan, irinotecan), topoisomerase II (Elsamicin A, XK469), ribonucleotide reductase R2 gene (antisense)</td>
</tr>
<tr>
<td>DNA polymerization</td>
<td>Telomerase (porphyrins, anthraquinones, 7-deaza-nucleotide analogs)</td>
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<tr>
<td>Telomere elongation</td>
<td>Promoter DNA CpG hypermethylation (5-azacytidine, Decitabine, antisense MG98), histone hypoacetylation (depsipeptide, SAHA, valproic acid, MS275)</td>
</tr>
</tbody>
</table>

\(^a\) Endogenous angiogenesis inhibitors are not targets. They are used as angiogenesis antagonists.

Table 1. 2. Summary of novel molecular targets for anticancer chemotherapy (Part II).
1.1.2 Histone deacetylase inhibitors as anticancer drugs

1.1.2.1 Histone acetyltransferases

The eukaryotic nucleosome consists of 146 base pairs of DNA wrapped on a histone octamer core, which are arranged as a (H3-H4)2 tetramer and two H2A-H2B dimers (Davie 1997; Davie and Chadee 1998; Davie and Spencer 1999). Under physiological conditions, the lysines on histone proteins bear positive charges and bind to the negatively charged phosphates on the DNA backbone. It has been 40 years since it was proposed that transcriptional competence is governed by histone acetylation status, which is determined by two families of enzymes, histone acetyltransferases (HATs) (Grant and Berger 1999) and HDACs (Cress and Seto 2000; Marmorstein 2001). HATs, once recruited by transcriptional factors bound to the target gene sequence, promote acetylation of the lysine groups on histone proteins. This interferes with folding of the histone N-terminal tails and destabilizes the electrostatic bonds between DNA phosphates and histone lysines, resulting in an open DNA conformation (euchromatin) for gene transcription (Figure 1.2 and 1.3) (Davie and Chadee 1998; Davie and Spencer 1999). On the other hand, HDACs promote histone deacetylation (heterochromatin) and result in gene silencing (Johnson and Turner 1999) (Figure 1.4).
HDACs are divided into 3 classes on the basis of their homology to yeast histone deacetylases (Gray and Ekstrom 2001; Gray and Teh 2001) as summarized in Table 1.3. These HDACs differ in their gene coding sequences, intracellular localizations, substrate specificities and cofactor requirements. The structures of the Class I HDACs resemble RPD3, a yeast transcriptional regulator with HDAC activity. This class includes HDAC1, 2, 3 and 8 (Gray and Ekstrom 2001). All four members of this class reside in the nucleus (de Ruijter, van Gennip et al. 2003) and are sensitive to most known HDAC inhibitors (Gray and Ekstrom 2001). Class II HDACs are yeast histone deacetylase-A1 (Hda1)-like and include at least HDAC 4, 5, 6, 7, 9, 10 and 11 (de Ruijter, van Gennip et al. 2003). The Class II HDACs are able to shuttle in and out of the nucleus in response to certain cellular signals (de Ruijter, van Gennip et al. 2003) and are sensitive to HDAC inhibitors as well. Class III HDACs are silent information regulator 2 (SIR2)-like proteins and include at least 7 members (SIRT1 to SIRT7). In contrast to the other two classes, SIRTs are NAD+ dependent and are insensitive to HDAC inhibitors (Gray and Ekstrom 2001). HDACs play essential roles in embryo development (Segev, Memili et al. 2001; Kantor, Makedonski et al. 2003). It has been estimated that depending on the cell type, inhibition of HDACs in cancer cells can lead to transcriptional activation and silencing of about 2% of human genes (Weidle and Grossmann 2000).
<table>
<thead>
<tr>
<th>HDACs</th>
<th>Other names</th>
<th>Chromosomal location</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I: RPD3-like histone deacetylases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDAC1</td>
<td>RPD3L, HD1</td>
<td>1p34.1</td>
<td>U50079, D50405</td>
</tr>
<tr>
<td>HDAC2</td>
<td>RPD3</td>
<td>6q21</td>
<td>U31814</td>
</tr>
<tr>
<td>HDAC3</td>
<td></td>
<td>5q31.1-5q31.3</td>
<td>U75697, U75696, AF005482,</td>
</tr>
<tr>
<td>HDAC8</td>
<td></td>
<td>Xq21.2-Xq21.3, or Xq13</td>
<td>AF230097, AF245664,</td>
</tr>
<tr>
<td>Class II: Had1-like histone deacetylases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDAC4</td>
<td>HDAC-A</td>
<td>2q37</td>
<td>AB006626, AF132607</td>
</tr>
<tr>
<td>HDAC5</td>
<td>HDAC-B</td>
<td>17</td>
<td>AF039691, AB011172,</td>
</tr>
<tr>
<td>HDAC6</td>
<td>JM21</td>
<td>Xp11.23</td>
<td>AJ011972, AB020708,</td>
</tr>
<tr>
<td>HDAC7</td>
<td>HDAC-D</td>
<td>12q13.1</td>
<td>AC004466, AF239243</td>
</tr>
<tr>
<td>Class III: SIR2-like histone deacetylases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIRT1</td>
<td></td>
<td>10</td>
<td>AF083106</td>
</tr>
<tr>
<td>SIRT2</td>
<td></td>
<td>19q13</td>
<td>AF083107, AF095714</td>
</tr>
<tr>
<td>SIRT3</td>
<td></td>
<td>11p15.5</td>
<td>AF083108</td>
</tr>
<tr>
<td>SIRT4</td>
<td></td>
<td>12q</td>
<td>AF083109</td>
</tr>
<tr>
<td>SIRT5</td>
<td></td>
<td></td>
<td>AF083110</td>
</tr>
<tr>
<td>SIRT6</td>
<td></td>
<td>19p13.3</td>
<td>AF233396</td>
</tr>
<tr>
<td>SIRT7</td>
<td></td>
<td>17q</td>
<td>AF233395</td>
</tr>
</tbody>
</table>

Table 1. Summary of the human histone deacetylase family. (Gray and Ekstrem 2001)
Similar to HATs, HDACs do not bind to DNA sequences directly, but are recruited as a complex with other transcription co-repressors. Different HDACs may form different complexes. The best-characterized HDAC complex involves Rpd3-like Class I HDACs (i.e., HDAC 1 and 2) as shown in Figure 1.4. First, certain sequence-specific transcriptional repressors, such as the Mad-Max heterodimer, unliganded retinoic acid receptor α-retinoid-X receptor heterodimer (RARα-RXR), and thyroid receptor (TR), bind to their target DNA promoter sequences (Johnson and Turner 1999). These repressors then exert their silencing function by recruiting the HDAC complex. The complex that contains HDAC1 or HDAC2 in association with the co-repressors Sin3A/B may further interact with nuclear co-repressor (N-CoR) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Fenrick and Hiebert 1998; Johnson and Turner 1999). N-CoR and SMRT are two silencing mediators for unliganded retinoid and thyroid hormone receptors (Johnson and Turner 1999). Moreover, a synergism of histone deacetylation and DNA methylation in transcriptional repression is achieved by interaction of the HDAC complex with sequence non-specific repressor methyl-CpG-binding protein 2 (MeCP-2) as shown in Figure 1.4 (Nan, Ng et al. 1998). Excellent reviews on the interaction of DNA methylation and histone deacetylation are available (Nan, Ng et al. 1998; Zhu, Lakshmanan et al. 2001). Another HDAC-recruiting transcription factor is unphosphorylated Retinoblastoma (Rb) (Takaki, Fukasawa et al. 2004). This is mediated by the Rb-associated proteins RbAp46 and RbAp48 (Johnson and
Turner 1999). Rb retains the G1/S transition in the cell cycle by binding to E2F transcription factor and recruiting HDACs to silence E2F’s target genes. Upon Rb phosphorylation by CDKs, Rb can no longer bind to either HDAC or E2F, and thus the cell enters into the S phase (Takaki, Fukasawa et al. 2004). Another complex for HDAC1/2 is the Mi2 complex (Cress and Seto 2000). This complex utilizes another set of cofactors (i.e., Mi2, MTA2 and MBD3), and is thought to interact with some repressors. However, the function of the Mi2 complex is only partially elucidated. Other proteins interacting with HDACs are reviewed by Cress et al. (Cress and Seto 2000).

The activity of HDACs is subject to post-translational modification. Phosphorylation of HDACs leads to increased deacetylation activity of HDACs including at least HDAC1 (Pflum, Tong et al. 2001) and HDAC2 (Tsai and Seto 2002). On the other hand, the specificity of HDACs may be altered upon phosphorylation as suggested by studies of yeast histone deacetylase HD1 (Johnson and Turner 1999).

HDACs are not only responsible for epigenetic modulation by deacetylating histone proteins, but are also involved in post-translational modification of non-histone proteins. For example, it has been reported that p53 exists in both the active acetylated form and the inactive deacetylated forms (Luo, Li et al. 2004). Acetylated p53 shows dramatically higher binding affinity to its target sequence than does the unmodified form. The deacetylation of p53 is mediated by HDACs (Luo, Li et al. 2004).
Histone acetylation occurs at the $\varepsilon$ amino groups of evolutionarily conserved lysine residues located at the N-termini. All core histones can be acetylated \textit{in vivo} (Davie and Spencer 1999). However, modifications of histones H3 and H4 are much more extensively characterized than those of H2A and H2B (de Ruijter, van Gennip et al. 2003). Important positions for acetylation are Lys9 and Lys14 on histone H3, and Lys5, Lys8, Lys12 and Lys16 on histone H4 (Figure 1.2) (de Ruijter, van Gennip et al. 2003; Zhang, Freitas et al. 2004). Approximately 15% of the core histone proteins undergo rapid acetylation and deacetylation (Davie and Spencer 1999). Since both HATs and HDACs need to be recruited to their target histones by transcription factors and complexes, which are generally associated with responsive elements at target gene promoters, it is expected that histone proteins with rapid (de)acetylation dynamics are also in proximity to gene promoter regions.

It is important to realize that post-translational modification of chromatin histones involves not only (de)acetylation, but also methylation, phosphorylation, ubiquitylation, and ADP ribosylation, as reviewed by Davie et al. (Davie and Chadee 1998; Davie and Spencer 1999). Although the above modifications play important roles in transcriptional regulation as well as in histone degradation, a detailed discussion is beyond the scope of my study, and they are not addressed in the following chapters.
Figure 1. Various aspects of the transcription process and its regulation by histone modification.

(a) Schematic representation of a nucleosome. (b) Transcriptional repression and activation in chromatin. Transcriptional activation mediated by HAT (upper), and transcription repression mediated by HDAC.
Figure 1.3. Regulation of histone lysine acetylation.

An acetate group is transferred from acetyl-CoA to the lysine-amino group by histone HATs and can be removed by HDACs. Attachment of the acetate group removes the positive charge of the amino group at physiological pH.
Figure 1. 4. Model of a histone deacetylase complex.

The hypothetical complex shown contains either HDAC1 or HDAC2. See text for details.
Recent advances in cancer pathophysiology indicate that tumorigenesis may be associated with repression of transcription through abnormal recruitment (Cress and Seto 2000; Somech, Izraeli et al. 2004). This can be demonstrated by the malfunction of retinoic acid receptor $\alpha$ (RAR$\alpha$) in leukemia. RAR$\alpha$ is a nuclear hormone receptor that binds to DNA as a heterodimer with a retinoid-X receptor (RXR). Normally, RAR$\alpha$ represses target genes by tethering co-repressors such as nuclear co-repressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Fenrick and Hiebert 1998). These co-repressors are now believed to form a complex with HDACs as discussed earlier. However, in the presence of RAR$\alpha$ ligand (i.e., retinoic acid), the RXR-RAR$\alpha$ heterodimer undergoes a conformational change, and then acts as a transcriptional activator by releasing N-CoR/SMRT and recruiting co-activators such as p300, CREB-binding protein (CBP), and p300/CBP associated factor (pCAF) (Fenrick and Hiebert 1998). All these co-activators have HAT activity and are transcriptional activators. In acute promyelocytic leukemia (APL), fusion proteins PML-RAR$\alpha$ and PLZF-RAR$\alpha$ are formed by translocation t(15;17) and t(11;17), respectively. As a result, these fusion proteins no longer respond to physiological levels of retinoic acid and act as repressors to genes that promote myeloid differentiation. This results in a clonal expansion of cells arrested in the promyelocyte stage of development, a hallmark feature of APL (Fenrick and Hiebert 1998). Similar cases include AML1-ETO fusion protein in acute myeloid leukemia (AML) by translocation t(8;21) (Fenrick and Hiebert 1998). This
translocation disturbs the function of AML-1 protein as a differentiation-promoting factor, which transcriptionally activates AML-1-dependent myeloid differentiation genes by recruiting p300, CBP and pCAF. Moreover, since ETO recruits corepressors such as Sin3A and N-CoR, the fusion protein actually further silences the AML-1-dependent genes (Fenrick and Hiebert 1998).

Recently, HDAC upregulation has been found in prostate cancer (Halkidou, Gaughan et al. 2004) and breast cancer (Zhu, Martin et al. 2004). This suggests that HDAC deregulation may be directly involved in tumorigenesis.

1.1.2.2 HDAC inhibitors

Some representative HDAC inhibitors are summarized in Table 1.4 (Marks, Richon et al. 2000; Secrist, Zhou et al. 2003). These inhibitors can be divided into several classes based on their structures, including short-chain fatty acids (butyrates and Valproic acid), hydroxamic acids (Trichostatin A and SAHA), cyclic tetrapeptides (depsipeptide), benzamides (MS-27-275), and epoxide-containing agents (trapoxin). Most of them inhibit HDACs in a reversible manner except trapoxin, which possesses an epoxide group capable of irreversibly alkylating HDACs. The reversible inhibitors generally have a long aliphatic tail containing a nucleophilic end, such as –SH or –OH, which interacts with the active zinc center located on the bottom of HDAC active binding pocket (Finnin, Donigian et al. 1999; Marmorstein 2001). Other HDAC inhibitors are emerging based on modification of the listed structures. Most of the new agents are
derivatives of hydroxamic acids, including amide analogues of Trichostatin A (TSA) (Kapustin, Fejer et al. 2003; Suzuki, Kouketsu et al. 2004) and thio/phosphorus-based SAHA (Kapustin, Fejer et al. 2003; Suzuki, Kouketsu et al. 2004). Replacement of the amide linkage in MS-27-275 structure with a sulfonamide led to discovery of a new class of potent HDAC inhibitors (Fournel, Trachy-Bourget et al. 2002). Promising HDAC inhibitors that have entered clinical trials include hydroxamic acid derivative LAQ824 (Remiszewski 2003), butyric acid derivative Titan (Hobdy and Murren 2004), Valproic acid (Gottlicher 2004), MS-27-275 (ongoing), SAHA (Kelly, Richon et al. 2003), CI-994 (Piekarz and Bates 2004), and depsipeptide FK228 (Piekarz and Bates 2004).

HDAC inhibitors cause transcriptional activation or repression of target genes (i.e., upregulation of p21 and downregulation of c-Myc), cell cycle arrest, differentiation, and apoptosis in cancer cells (Kim, Kim et al. 2003). Detailed pharmacological properties of HDAC inhibitors are discussed in the following section with a novel HDAC inhibitor FK228 as an example. However, it is likely that different HDAC inhibitors have different pharmacological properties. This is suggested by differences in their inhibition mechanism and specificity. For example, FK228 specifically inhibits Class I HDACs, while TSA and its derivatives inhibit of both Class I and Class II HDACs (Furumai, Matsuyama et al. 2002). Moreover, some HDAC inhibitors may cause apoptosis in cancer cells by mechanisms that are not apparently linked to their HDAC inhibitory activity. As discussed in the following section, FK228 causes cancer cell apoptosis by its
angiostatic and microtubule-disturbing properties, which have not been reported for other HDAC inhibitors. This suggests that HDAC inhibitors, although grouped as a single class of anticancer agents due to their HDAC inhibitory activity, may cause different pharmacological effects in cancer cells.

1.2 Depsipeptide FK228, a novel HDAC inhibitor

1.2.1 Chemistry

Depsipeptide FK228 (FR901228, NSC630176) was first isolated from Chromobacterium violaceum by the Fujisawa Company during a screening for microbial metabolites that induce transcriptional activation of the SV40 promoter (Ueda, Manda et al. 1994; Ueda, Nakajima et al. 1994). Its composition is \( \text{C}_{24}\text{H}_{36}\text{N}_{4}\text{O}_{6}\text{S}_{2} \) with molecular weight 540. Its structure (Table 1.4) was elucidated to be, \((E)-(1S,4S,10S,21R)-7-[((Z)-ethyldiene]-4,21-diisopropyl-2-oxz-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone, \) a bicyclic tetra-peptide with a disulfide linkage (Shigematsu, Ueda et al. 1994). Since FK228 production in bacteria is of trace amount, a fermentation method for production of highly pure FK228 was developed (Otsuka, Fujita et al. 1992), and improved later (Ueda, Watamoto et al. 2002). A 14-step total synthesis method of FK228 was also developed (Li, Wu et al. 1996).

FK228 is highly hydrophobic and has very poor water solubility. Early work was done with FK228 in DMSO solution (Chan, Bakhtiar et al. 1997; Li and Chan 2000). Due to the toxicity of DMSO, a new solution system was developed during
clinical evaluation (Marshall, Rizvi et al. 2002; Sandor, Bakke et al. 2002). The special diluent consists of 40% propylene glycol, and 10% ethanol in saline and is diluted further with normal saline before use. FK228 is stable in this solution with a shelf life more than 1 month at room temperature.

FK228 has a unique cyclic structure when compared with other HDAC inhibitors. It is the only one that does not possess a tail-like aliphatic chain and a nucleophilic functional group that are thought to be required to block the active binding pocket of HDAC proteins as discussed earlier in this chapter (Finnin, Donigian et al. 1999). This raises the question whether FK228 blocks HDACs by the same mechanism as other reversible HDAC inhibitors, since it seems impossible for its bulky structure to fit in the narrow HDAC binding pocket. Indeed, the uniqueness of its structure, as well as its pharmacokinetic behavior, led to discovery of FK228 as a prodrug as discussed in the following chapters (Furumai, Matsuyama et al. 2002; Xiao, Byrd et al. 2003). Based on these findings, potent derivatives of the metabolites mimicking the structures of FK228 metabolite have been synthesized (Furumai, Komatsu et al. 2001).
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric acid</td>
<td><img src="image" alt="Butyric acid structure" /></td>
</tr>
<tr>
<td>MS-27-275</td>
<td><img src="image" alt="MS-27-275 structure" /></td>
</tr>
<tr>
<td>SAHA</td>
<td><img src="image" alt="SAHA structure" /></td>
</tr>
<tr>
<td>Trichostatin A</td>
<td><img src="image" alt="Trichostatin A structure" /></td>
</tr>
<tr>
<td>Apicidin</td>
<td><img src="image" alt="Apicidin structure" /></td>
</tr>
<tr>
<td>Oxamflatin</td>
<td><img src="image" alt="Oxamflatin structure" /></td>
</tr>
<tr>
<td>FK228</td>
<td><img src="image" alt="FK228 structure" /></td>
</tr>
<tr>
<td>Trapoxin</td>
<td><img src="image" alt="Trapoxin structure" /></td>
</tr>
</tbody>
</table>

Table 1. 4. Histone deacetylase inhibitors. (Marks, Richon et al. 2000)
1.2.2 Pharmacology

Shortly after its isolation, FK228 was shown to reverse Ha-ras transformed cells and down regulate c-myc expression (Ueda, Manda et al. 1994; Wang, Brunner et al. 1998). FK228 was also found to be highly toxic against various cancer cell lines during the In Vitro Cell Line Screening project at the National Cancer Institute, Developmental Therapeutics Program (NCI DTP). Later on, FK228 was found to be a potent histone deacetylase inhibitor (Nakajima, Kim et al. 1998; Yoshida and Horinouchi 1999; Yoshida and Horinouchi 2000; Yoshida, Furumai et al. 2001). FK228 induces differential accumulation of acetylated histones, especially lysines at H3 and H4 subunits, promotes specific gene expression, restores cell differentiation and/or induces apoptosis at low nanomolar concentrations (Rajgolikar, Chan et al. 1998; Yoshida, Furumai et al. 2001).

FK228 can cause G1 and G2/M cell cycle arrest. The G1 arrest is by down-regulation of cyclin D1 and induction of p53-independent p21Waf1 and 16INK4A, which results in Retinoblastoma (Rb) hypophosphorylation (Sandor, Senderowicz et al. 2000; Ju and Muller 2003). The mechanism of G2/M arrest is probably via formation of aberrant spindles by interfering with chromosome attachment, causing mitotic accumulation without affecting mitotic microtubules (Sandor, Senderowicz et al. 2000).
FK228 induces apoptosis in cancer cells by various mechanisms. Apoptosis via the TNF-receptor pathway (extrinsic) has been observed in non-proliferating CLL cells by down-regulation of FLICE-inhibitory protein (FLIP) (Aron, Parthun et al. 2003). FLIP acts downstream of Fas to inhibit TNF-receptor mediated apoptosis by binding to adaptor protein FADD and caspase 8, and thus interfering with caspase 8 activation (Aron, Parthun et al. 2003). FK228 also causes apoptosis via the extrinsic pathway by induction of Fas ligand in various osteosarcoma cells (Imai, Adachi et al. 2003). However, FK228-induced apoptosis also occur via mitochondrial (intrinsic) pathways as evidenced by mitochondrial membrane damage after FK228 treatment (Peart, Tainton et al. 2003). Therefore, it seems that the primary mechanism of FK228-induced apoptosis may vary among different cancer cells. FK228’s toxicity is highly selective towards cancer cells, and its IC50 ratios between normal blood mononuclear cells and peripheral CLL blast cells from patients were between 20~50 (n=10) (Byrd, Shinn et al. 1999).

FK228 inhibits hypoxia-stimulated tumor angiogenesis in vivo by suppression of various angiogenic growth factors (Kwon, Kim et al. 2002; Mie Lee, Kim et al. 2003; Sasakawa, Naoe et al. 2003). Hypoxia-inducible factor-1 (HIF-1) plays a pivotal role in cellular response (i.e., angiogenesis) to low oxygen concentrations. FK228 was found to inhibit HIF-1 expression in hypoxic tumors (Mie Lee, Kim et al. 2003). Under hypoxia, induction of another important angiogenic growth factor, vascular endothelial growth factor (VEGF), can also be
blocked by FK228 treatment (Mie Lee, Kim et al. 2003; Sasakawa, Naoe et al. 2003). Moreover, FK228 inactivates the transcription of basic fibroblast growth factor (bFGF) in PC3 xenografts (Sasakawa, Naoe et al. 2003). Interestingly, FK228 exerts its angiostatic activity at low nanomolar concentrations, which is of the same order of concentration required for its cytotoxicity (Kwon, Kim et al. 2002). This suggests that FK228’s antitumor activity is, at least in part, due to its anti-angiogenesis properties.

As an epigenetic modulator, FK228 is also a promising agent for gene therapy. FK228 enhances adenovirus transgenic expression in several malignant cell lines (Yamano, Ura et al. 2000; Kitazono, Goldsmith et al. 2001; Kitazono, Robey et al. 2001; Kitazono, Rao et al. 2002), as well as increases expression of the Na+/I- symporter and iodine accumulation in four poorly differentiated thyroid carcinoma cell lines (Kitazono, Chuman et al. 2001; Kitazono, Robey et al. 2001; Kitazono, Chuman et al. 2002). FK228 is now under clinical evaluation to treat thyroid carcinomas that are unable to trap iodine, as an adjunct to radioiodine therapy.

Due to its promising activity, in vivo efficacy of FK228 has been extensively tested in mice bearing murine ascitic tumors, namely, P388 and L1210 leukemias, B16 melanoma, Colon 38 carcinoma, M5076 reticulum cell sarcoma, and Meth A fibrosarcoma (Ueda, Manda et al. 1994), as well as human tumor xenografts including Lu-65 and LC-6 lung carcinomas, and SC-6 stomach adenocarcinoma (Ueda, Manda et al. 1994). Significant tumor shrinkage was
observed especially against murine Meth A fibrosarcoma and human SC-6 stomach adenocarcinoma. Moreover, FK228 is effective against P388 leukemias that are resistant to mitomycin C, cyclophosphamide, vincristine and 5-fluorouracil (Ueda, Manda et al. 1994). The high antitumor activity and the lack of cross-resistance to other anticancer agents identify FK228 as a promising new drug. Several phase I trials were completed and showed partial responses among patients with advanced or refractory cancers (Marshall, Rizvi et al. 2002; Sandor, Bakke et al. 2002). Ongoing clinical trials of FK228 aim to investigate FK228 efficacy in patients with thyroid tumor, acute myeloid leukemia, Chronic Lymphocytic Leukemia, cutaneous T cell lymphoma, peripheral T cell lymphoma, follicular lymphoma, non-Hodgkin's lymphoma, anaplastic large cell lymphoma, mycosis fungoides and Sezary syndrome. A phase I trial was initiated in children with advanced cancers for toxicity evaluation. Moreover, clinical trials combining FK228 with other therapeutic agents (i.e., Rituximab and Fludarabine) in treating patients with relapsed or refractory low-grade B-cell Non-Hodgkin's lymphoma were initiated. (clinical trial information available at http://www.clinicaltrials.gov/ by searching depsipeptide)

1.2.3 Analytical methodology

FK228 concentrations were determined initially by HPLC/UV. However, FK228 lacks UV absorbance, and thus the quantification was achievable only above the micromolar range. This was insufficient for adequate plasma concentration determination and pharmacokinetic study, since FK228 is such a
potent HDAC inhibitor and a toxic agent. Our group established a sensitive FK228 HPLC/MS/MS method with a detection limit of 0.1 ng/ml (Chan, Bakhtiar et al. 1997; Li and Chan 2000). This analytical method has provided the basis for pharmacokinetic studies in both animals (Li and Chan 2000; Berg, Stone et al. 2004) and patients (Sandor, Bakke et al. 2002). The detailed method is described in following chapters.

1.2.4 Preclinical pharmacokinetics and toxicity

Preliminary FK228 rat pharmacokinetic studies showed that FK228 follows a two-compartment model after i.v. bolus dosing with high total body clearance ($425.3 \pm 117.7$ ml/min for 250 g rats) and large distribution volume ($22.3 \pm 7.3$ L/kg for 250 g rats) (Chan, Bakhtiar et al. 1997; Li and Chan 2000). Oral bioavailability study in the rat showed that FK228 is poorly absorbed after oral dosing (Li and Chan 2000). Later on, FK228 was found to be a P-glycoprotein substrate (Pgp) (Scala, Akhmed et al. 1997), suggesting Pgp may play an important role in FK228 absorption and elimination. For example, Pgp-mediated efflux of FK228 back to intestinal lumen may result in poor intestinal absorption, and Pgp-mediated liver/renal excretion may be associated with the fast FK228 elimination. However, the role of Pgp in determining FK228 pharmacokinetic behavior is yet to be studied. Interestingly, the total body clearance of FK228 from the rat is significantly higher than the rat cardiac output rate ($74$ ml/min for a 250 g rat) (Davies and Morris 1993). This blood-flow independent clearance suggests extensive metabolism in the blood (and maybe the endothelial
membrane of blood vessels). This is because for a drug-eliminating organ, the upper limit of clearance is its blood flow rate. Theoretically, the total body clearance is limited by the cardiac output, which is the sum of blood flow rates to all organs, provided the drug is eliminated by organs. Blood is the only tissue that may contribute to the apparent total body clearance higher than the cardiac output. A list of drugs that are metabolized in blood (e.g., red blood cells) has been reviewed (Cossum 1988). Drug metabolism pathways in the RBC include reduction, methylation, acetylation, and glutathione conjugation. However, metabolism of FK228 is yet to be studied.

In order to find out the role of Pgp and RBC in FK228 disposition/elimination, and the effect of different dosing regimens on FK228 pharmacokinetics, we designed and conducted a series of pharmacokinetic studies in the rat (Chapter 2). Results suggest that the glutathione (GSH) level in the RBC is a determinant of FK228 elimination in rats. In vitro blood incubation studies subsequently demonstrated formation of seven FK228 reduction and GSH conjugates, which were then identified by HPLC/multi-stage MS (Chapter 3). Purification and in vitro HDAC inhibition assays showed that some of the metabolites are more potent HDAC inhibitors compared with the parent drug, suggesting that FK228 is a prodrug. This seems consistent with results from other studies. For example, FK228's HDAC inhibitory effect is still observable after FK228 is quickly eliminated from circulation (Sandor, Bakke et al. 2002). Recently, Furumai et al. found FK228, after reduction by dithiothreitol (DTT),
showed a 40-fold increase in HDAC inhibitory activity (Furumai, Matsuyama et al.
2002). The structure of the active reduction product was found to be identical to one of the metabolites identified in Chapter 3.

Fatigue was the primary side effect of FK228 in the rat (Chan, Bakhtiar et al. 1997). Cardiac toxicity, including elevations in cardiac enzymes and necrosis, with chronic inflammation or neutrophilic infiltration of cardiac muscle has been observed in some dosing schedules (Sandor, Bakke et al. 2002). In addition, local inflammation and necrosis were noted at catheter insertion sites (Sandor, Bakke et al. 2002). The toxicity was dosing schedule-related, with short infusions (<4 min) and prolonged infusions (>24 hr) causing higher toxicity as compared with infusions of 1-4 hr (Sandor, Bakke et al. 2002).

1.2.5 Clinical pharmacokinetics and toxicity

In a phase I trial, FK228 was given to patients as a 4-hr infusion at doses beginning at 1 mg/m², which represented one-third of the minimum toxic dose in dogs (Sandor, Bakke et al. 2002). The plasma concentration-time profile of FK228 was found to follow a two-compartment model and showed linear pharmacokinetics at doses between 1-24.9 mg/m² (Sandor, Bakke et al. 2002). The maximum tolerated dose (MTD) was found to be 17.8 mg/m² (Sandor, Bakke et al. 2002). However, due to large variations among patients, as well as a small patient number at each dose, the calculated pharmacokinetic parameters were not reliable. In order to gain a better characterization of FK228 pharmacokinetics
and to seek potential clinical covariates that account for the inter-individual variations, we conducted a pharmacokinetic study in 20 AML and CLL patients receiving FK228 at 13 mg/m² given by a 4-hr i.v. infusion. In conjunction with the clinical evaluation, a pharmacokinetics-pharmacodynamics correlation (Chapter 4) and a population-base pharmacokinetic study were also conducted (Chapter 5).

1.2.6 Transport and uptake

Even though FK228 was found to be a Pgp substrate during the NCI screening (Scala, Akhmed et al. 1997), no kinetic information of FK228 transport in the presence or absence of Pgp inhibitors was available. In addition, there is no information available about whether FK228 also possesses affinity to other membrane transporters, or whether Pgp and other transporters play a role in acquired FK228 resistance. In the current project, we studied FK228 transport and uptake kinetics using multiple cellular models. FK228 was a substrate for both Pgp and multidrug resistant-associated protein 1 (MRP1) (Chapter 6). By developing multiple FK228 resistant cell lines, we established for the first time that Pgp induction is the primary mechanism for the acquired FK228 resistance in cancer cells by histone hyperacetylation at the promoter region of Pgp (Chapter 7).
CHAPTER 2

PHARMACOKINETICS OF FK228 IN THE RAT

2.1 Abstract

The objective of this study was to characterize the disposition and dose recovery of FK228, to determine its pharmacokinetics with different dosing regimens, and the effects of MDR1 inhibition and glutathione depletion on FK228 pharmacokinetics in the rat. Male Sprague-Dawley rats received either an i.v. bolus alone, a 4-hr i.v. infusion, or an i.v. bolus following pretreatment with MDR1 inhibitor Cyclosporin A (5 mg/kg) or GSH-depleting agent diethyl maleate (0.8 mL/kg). The same dose of FK228 (2 mg/kg) was used for all studies. Following an i.v. bolus dose, FK228 was found to follow a two-compartment pharmacokinetic model with a high total body clearance exceeding the rat cardiac output. Following the i.v. infusion, FK228 pharmacokinetics showed a deceased distribution volume as compared with that in the i.v. bolus group. Cyclosporin A pretreatment caused little change in pharmacokinetics in the rat, while pretreatment with diethyl maleate resulted in both a decreased distribution volume and an increased initial plasma concentration. A dose recovery study for the intact drug showed that biliary and urinary excretion accounted for 4.8% and
5.9% of the dose, respectively. Total recovery of the intact FK228 from brain, heart, lung, kidney, testes, muscle, liver, fat and spleen were only $2.57 \pm 1.26\%$ and $0.59 \pm 0.34\%$ at 6 hr and 24 hr post dose, respectively. Little FK228 was recovered from lysed red blood cells. The overall dose recovery as the intact drug from all sources was <15%. Our data suggested that FK228 is extensively metabolized in the blood, which may involve GSH. MDR1-mediated excretion played a minor role in FK228 disposition and elimination.
2.2 Introduction

Depsipeptide FK228 (FR 901228, NSC 630176) was first isolated from Chromobacterium violaceum in 1994 (Ueda, Manda et al. 1994; Ueda, Nakajima et al. 1994). It is a bicyclic tetra-peptide with a disulfide linkage (Shigematsu, Ueda et al. 1994). Depsipeptide is a potent HDAC inhibitor, which causes cancer cell cycle arrests and apoptosis, and is now in phase I/II clinical trials against a variety of cancers (Sandor, Senderowicz et al. 2000; Marshall, Rizvi et al. 2002).

We previously characterized the pharmacokinetic properties of FK228 in the rat, using a HPLC/MS/MS method developed in our laboratory (Chan, Bakhtiar et al. 1997; Li and Chan 2000). FK228 was found to follow a two-compartment model after an i.v. bolus dosing with a large volume of distribution at steady state ($V_{ds}$) of $970 \pm 317$ L/m$^2$ (or $22.3 \pm 7.3$ L/kg) and total body clearance ($CL_{total}$) of $1109 \pm 307$ L/hr/m$^2$ (or $425.3 \pm 117.7$ mL/min), with the latter higher than the rat cardiac output rate of 74 mL/min for a 250 g rat (Davies and Morris 1993). However, these data was preliminary in nature and left several questions to be answered. First, the rat pharmacokinetics profile was very different from that of patients receiving a 4 hr infusion of FK228 as will be discussed in Chapter 4 (Sandor, Bakke et al. 2002), where $V_{ds}$ (43.8 l/m$^2$) and $CL_{total}$ (16.8 l/hr/m$^2$) were much smaller after normalization to body surface area. It was not clear if this difference was due to interspecies difference or difference in dose regimes. Second, it has been reported that FK228 is a MDR1 (P-
glycoprotein or Pgp) substrate (Scala, Akhmed et al. 1997), but the role of MDR1 in FK228’s distribution and elimination was unclear. Moreover, the disulfide bond in the structure of FK228 suggests that it may react with glutathione (GSH) in red blood cells (RBC) (extensively studied in Chapter 3). It was unclear, however, whether the GSH level in RBC is a determinant of FK228 elimination. To answer these questions, we designed a series of pharmacokinetic experiments, where rats received FK228 at a dose 2 mg/kg by either i.v. bolus, 4 hr i.v. infusion, or i.v. bolus after treatment with MDR1 inhibitor Cyclosporin A (5 mg/kg), or after treatment with GSH-depleting agent diethyl maleate (DEM, 0.8 mL/kg). The dose was chosen according to our previous pharmacokinetic results (Chan, Bakhtiar et al. 1997; Li and Chan 2000).

2.3 Experimental procedures

2.3.1 Drug and chemicals

Depsipptide FK228 (purity>99%) was provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD, USA). FK228 dosing solution (1.8 mM) was prepared in 40% propylene glycol, 10% ethanol (USP) and 50% normal saline. N-ethylmaleimide (NEM), diethyl maleate (DEM), cyclosporin A (CsA) and N-t-Boc-Met-Leu-Phe (BMLP, purity >97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium phthalate buffer (pH 4, 50 mM) was obtained from Van Water and Roger Scientific (Chicago, IL, USA). HPLC-grade water (>18 mΩ) was generated from an E-pure water purification system (Barnstead, Dubuque, IA, USA). Acetonitrile (ACN, HPLC
grade), acetic acid and ethyl acetate (reagent grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Drug-free heparinized rat and human plasma was purchased from Harlan Bioproducts for Science (Indianapolis, IN, USA). Human albumin (Fraction V, 96~99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3.2 Animal protocols

The animal protocols were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) of the Ohio State University Laboratory Animal Resources (ULAR), and adhered to the guideline and “Principles of Laboratory Animal Care” by National Institute of Health (NIH).

2.3.2.1 Right jugular vein cannulation

Rats were anesthetized with an i.m. injection of ketamine (Bedford Laboratory, Bedford, OH) at a dose of 100 mg/kg body weight. The right jugular vein of each rat was catheterized with a PE-50 cannula (Boulanger, Delva et al. 1976; Cushieri, Baker et al. 1983). Briefly, the fur around the ventral right side of the neck was shaved. The skin was sterilized with 70% alcohol. A 1.5 cm incision was made, and the neck muscle was gently separated to expose the right jugular vein. A small nick was made, and a 15 cm long beveled-end piece of PE-50 tubing (ID 0.58mm, OD 0.965 mm, Clay Adams brand, Becton Dickinson, Sparks, MD) was inserted and pushed into the right atrium. The cannula was anchored with suture thread and the cannula was exteriorized through the skin at
the back of the neck. The muscle and skin were then closed with suture. The blood flow was examined and the tubing was filled with heparinized (50 IU/ml) normal saline solution. The rats were kept in cages and allowed to recover overnight before dosing with food and water given *ad libitum*.

2.3.2.2 Tail vein cannulation

Rats were anesthetized with ketamine, and the tail vein was dilated by applying a tourniquet around the base of the tail. The vein was cannulated by first piercing it with a 20G needle (Angiocath™, Becton Dickinson, Sparks, MD) and a PE-10 tubing (ID 0.28 mm, OD 0.61 mm, Clay Adams brand, Becton Dickinson, Sparks, MD) was inserted into the barrel of needle. The needle was then withdrawn, leaving the catheter in the vein. The catheter was filled with heparinized normal saline solution prior to the insertion. A small volume of normal saline solution was injected and withdrawn to examine the success of the *iv* cannulation. To anchor the catheter, a piece of adhesive tape was wrapped around the catheter and the tail. A flexible sheath was then wrapped around the tail to protect the insertion point and a 30 cm section of compression spring was threaded over the catheter and taped to the sheath to prevent the rat from chewing the catheter. The rat was then put into a metabolic cage with access to food and water *ad libitum* for 2 hr before *i.v.* infusion dosing. The spring and the catheter were passed through the top of the cage. The catheter was then connected to a swivel (Harvard Apparatus, Holliston, MA) which was also connected to an infusion pump (model KDS200, Fisher, Pittsburgh, PA) and the
spring was fixed to a cross rod on the top of the cage to prevent the catheter from pulling back into the cage and also to allow the animal 360° mobility within the cage.

2.3.2.3 Bile duct cannulation

Rats were anesthetized with an *i.m.* injection of ketamine at a dose of 100 mg/kg body weight, and maintained unconscious with diethyl ether throughout the experiment. The bile duct was cannulated using a PE10 tube as previously described (Liles and Flecknell 1993). Briefly, the rat abdomen was opened and the bile duct was separated from surrounding tissues. A small cut was made on the duct using scissors, and a PE10 tube was carefully inserted into the bile duct and fixed by sutures. The rats were allowed to recover for 2 hr before *i.v.* bolus dosing of FK228 at 2 mg/kg via the right jugular cannula with body temperature kept at 37°C. Pre-warmed normal saline was given to the rats by *i.v.* infusion via the jugular cannula at 1 mL/hr. Bile was collected for up to 6 hr.

2.3.3 Dosing and sample collection

Twenty-three male S.D. rats weighing approximately 250-300 g were divided into 5 groups. In the first group of six rats in metabolism cages, FK228 was given by *i.v.* bolus to each rat at a dose of 2 mg/kg through the right jugular vein cannula followed by flushing the cannula with 2 volumes of normal saline. At the time schedule of predose, 5, 10, 20, 45, 60, 120, 180, and 300 min after dosing, blood samples of 0.2 mL each were withdrawn from the same cannula,
and the lost fluid was replaced by flushing the cannula with an equal volume of normal saline. Plasma was separated immediately by centrifugation (10,000 g × 0.5 min). Urine and feces were collected up to 24 hr. At 24 hr post dose, rat blood was drained via the right jugular catheter and the rats were sacrificed. Major organ tissues, namely, heart, lung, liver, kidney, brain, spleen, testes, muscle, fat, and intestinal contents were removed. In the second group, six rats were given an i.v. infusion of FK228 by the tail vein over 4 hr at the same dose (1 mL/hr). Blood samples were withdrawn and processed as in the first group at the time schedule of predose, 30, 60, 120, 180, 240, 245, 250, 260, 285, 300, 360 and 420 min. In the third group, bile duct cannulas were implanted in three animals. Animals were allowed to recover for 2 hr, and then given FK228 by i.v. bolus through the jugular cannula at the same dose. The bile fluid was collected up to 6 hr, and the rats were sacrificed by exsanguinations. The organ tissues were then removed as in the first group. In the fourth group, three rats were given CsA (5 mg/kg) (Shitara, Hirano et al. 2004) by the right jugular vein 10 min before the i.v. bolus dosing of FK228 at 2 mg/kg. Blood samples were collected and processed as in the i.v. bolus group up to 300 min. In the last group of five rats, DEM was given at 0.8 mL/kg i.p. 60 min before FK228 i.v. bolus dosing to deplete the RBC GSH. Blood samples were collected and centrifuged immediately as in the first group, and both the RBC and plasma fractions were saved for assays. All samples were kept under –80°C until analysis. The RBC intracellular GSH levels in the DEM pretreated rats were determined using the
Bioxytech GSH-400™ assay kit (Oxis, Portland, OR) to evaluate the extent of in vivo RBC GSH depletion.

2.3.4 Preparation of blood, urine, bile and organ tissue samples

A liquid-liquid extraction method (Chan, Bakhtiar et al. 1997; Li and Chan 2000) was used. To an appropriate volume of plasma (0.1 mL), urine (0.2 mL) or bile (0.1 mL), an aliquot of internal standard (BMLP, 1 µg) and 0.4× volume of pH4 potassium phthalate buffer were added. The solution was extracted with 10× volume ethyl acetate. After centrifugation, the organic layer was transferred to a clean conical Falcon™ polypropylene tube, and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 µL 70% ACN, and 40 µl was injected to HPLC/MS for FK228 determination.

Weighed wet organ tissue samples were homogenized in appropriate volumes of blank human plasma (total volumes recorded) with an Ultra-Turrax SDT1610 electric homogenizer (Tekmar, Cincinnati, Ohio) at the highest speed setting for 10 seconds × 4 times while immersed in an ice bath. Human plasma was used to assure FK228 stability. FK228 is stable in human plasma and binds over 90% to plasma proteins (Chan, Bakhtiar et al. 1997; Li and Chan 2000). Thus the addition of human plasma was expected to facilitate drug removal from organ tissues. The homogenates were then extracted as plasma samples. Feces samples were also weighed and homogenized in blank human plasma similarly to the above. After centrifugation, the supernatant was extracted as described for plasma samples.
2.3.5 HPLC/MS analysis

FK228 concentrations in samples were analyzed using our previously published LC/MS/MS method (Chan, Bakhtiar et al. 1997; Li and Chan 2000). Briefly, the LC/MS/MS system consisted of a Perkin-Elmer Sciex API 300 triple-quadruple mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The HPLC separation was achieved on a 50 × 2 mm BetaBasic C8 5-μm column with isocratic elution consisting of 70% ACN and 0.1% acetic acid (v:v) at a flow rate of 0.2 mL/min. The split ratio was 20:1 so that the eluate was introduced into the API source at 10 μL/min. The mass spectrometer was equipped with an electrospray ionization (ESI) source. The instrument parameters used were re-tuned based on those published before according to the manufacturer’s manual. Multiple reaction monitoring (MRM) was used to monitor the precursor/product ion pairs of FK228 (m/z 541.2/424) and the internal standard BMLP (m/z 510.0/217). This HPLC/MS/MS method was re-validated by the operator for plasma sample determinations (Appendix Figure A.1, Table A 1).

All tissue samples were determined by individual calibration curves constructed using specific blank tissue matrix (Appendix Figure A.2). The FK228 dose recoveries from heart, lung, liver, kidney, brain, spleen, and testes were calculated based on the homogenate FK228 concentrations and the whole organ homogenate volumes. The recoveries from rat muscle and fat samples were further normalized to whole body weight by assuming that muscle and fat
compose 40 and 4% of the rat total body weight, respectively (Davies and Morris 1993).

2.3.6 Pharmacokinetic data analysis

The concentration-time data were analyzed using WinNonlin (version 4.0, Pharsight Corporation, Mountain View, CA). Two-compartment models with either i.v. bolus or i.v. infusion dosing were applied to the appropriate data sets. Detailed descriptions of the pharmacokinetic models are included in Appendix Schemes A.1 and A.2. The mean values and standard deviations of pharmacokinetic parameters in each rat group were calculated. Data from each group were compared by paired Student’s t-test.

2.4 Results

2.4.1 Plasma FK228 pharmacokinetics in the rat

Relevant pharmacokinetic parameters (i.e., AUC, $C_{\text{max}}$, $V_{\text{dss}}$, $C_{\text{total}}$) are listed in Table 2.1. The i.v. bolus group showed PK parameters similar to the previously published data (Chan, Bakhtiar et al. 1997; Li and Chan 2000). FK228 showed a large distribution volume ($32.7 \pm 4.5$ l/kg) and a rapid total body clearance ($237 \pm 58$ mL/min). I.v. infusion led to a significant decrease in distribution volume ($10.8 \pm 2.7$ L/kg, $p<0.001$) and an increase in AUC ($6194 \pm 881$ nM⋅min, $p<0.002$), while an insignificant decrease in $CL_{\text{total}}$ was observed ($178.6 \pm 27.5$ mL/min, $p = 0.06$) as compared with those in the i.v. bous group. CsA pretreatment caused no change of the pharmacokinetics parameters as
compared with i.v. bolus group. DEM pretreatment resulted in 50% depletion of RBC GSH (Figure 2.1), and a much higher $C_0$ ($1185 \pm 637$ nM, $p < 0.05$) than that of the i.v. bolus group, although other parameters (AUC, Vd and CLtotal) were not changed significantly ($p>0.1$). The DEM treated group showed large variation of pharmacokinetic parameters, probably due to inconsistent *in vivo* GSH depletions. The role of GSH in FK228 metabolism will be further studied in Chapter 3. The FK228 plasma concentration-time profiles after different dosing regimen are shown in Appendix Figure A.3-6.
<table>
<thead>
<tr>
<th>PK parameters</th>
<th>I.v. bolus (n = 6)</th>
<th>I.v. infusion (n = 6)</th>
<th>I.v. bolus with CsA pretreatment (n = 3)</th>
<th>I.v. bolus with DEM pretreatment (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{\infty}$ (nM·min)</td>
<td>4097 ± 937$^a$</td>
<td>6194 ± 881</td>
<td>4004 ± 249</td>
<td>5419 ± 1450</td>
</tr>
<tr>
<td>C$_{\max}$ (nM)</td>
<td>490 ± 164</td>
<td>25.3 ± 3.7</td>
<td>548 ± 249</td>
<td>1185 ± 637</td>
</tr>
<tr>
<td>Vd$_{ss}$ (L/kg)</td>
<td>32.7 ± 4.5</td>
<td>10.8 ± 2.7</td>
<td>29.0 ± 3.2</td>
<td>22.4 ± 13</td>
</tr>
<tr>
<td>$\text{Cl}_{\text{total}}$ (mL/min)</td>
<td>237 ± 58</td>
<td>178.6 ± 27.5</td>
<td>249 ± 18</td>
<td>276 ± 152</td>
</tr>
</tbody>
</table>

$^a$ All values are shown as mean ± SD

Table 2. 1. Relevant pharmacokinetic parameters of FK228 in rats following different dose regimens and treatments.

The rats received FK228 (2 mg/kg) by either i.v. bolus, i.v. infusion over 4 hr, i.v. bolus after pretreatment with CsA (5 mg/kg) or DEM (0.8 mL/kg). Parameters were fitted by a two-compartmental pharmacokinetic model with first-order elimination from the central compartment.
Figure 2. 1. In vivo Glutathione depletion in rat blood by DEM.

Rats were given DEM *i.p.* at 0.8 ml/kg 60 min before FK228 dosing. The GSH levels in RBC were monitored throughout the experiment. GSH depletion by 50% or higher was achieved by the time of FK228 dosing and maintained relatively stable throughout the experiment. Time 0 corresponds to the FK228 dosing time.
Figure 2. FK228 recovery as the intact drug from 9 major organ tissues at 6 and 24 hr after an i.v. bolus of 2 mg/kg FK228 (n = 3).

Liver and muscle showed the highest FK228 recoveries among the monitored organs. However, the overall recovery from all organs was low: 2.5 ± 1.26 % at 6 hr and 0.56 ± 0.3 % at 24 hr post dosing.
Figure 2.3. Dose recovery of intact FK228 in urine, feces and intestinal contents, and bile.

Recoveries from cumulative urine, feces and intestinal contents, and bile were 5.9 ± 1.6% (24 hr n = 6), 0.28 ± 0.08% (24 hr, n = 6), and 4.76 ± 0.34% (6 hr, n = 3), respectively.
Figure 2. 4. Total dose recovery of intact FK228.

Total recovery of intact FK228 was calculated from plasma (6 hr, n = 6), bile (6 hr, n = 3), nine major organ tissues (6 hr, n = 3), and cumulative urine (24 hr, n = 6). The total dose recovery monitored was less than 15%.
2.4.2 Dose recovery

Following *i.v.* bolus dosing, dose recovery was measured from the 9 major organ tissues, namely, brain, heart, lung, kidney, testes, muscle, liver, fat and spleen. Liver showed the highest dose recovery of $1.5 \pm 0.77\%$ at 6 hr and $0.23 \pm 0.16\%$ at 24 hr. Recovery from muscle accounted for $0.87 \pm 0.51\%$ at 6 hr and $0.31 \pm 0.18\%$ at 24 hr after dosing. However, the total recoveries of intact FK228 from these organ tissues were low, only $2.5 \pm 1.3\%$ and $0.56 \pm 0.3\%$ at 6 hr and 24 hr, respectively (Figure 2.2). Dose recoveries from 24 hr cumulative urine, 24 hr pooled feces and intestinal contents, and 6 hr bile were $5.9 \pm 1.6\%$ ($n = 6$), $0.23 \pm 0.079\%$ ($n = 6$) and $4.76 \pm 0.34\%$ ($n = 3$), respectively (Figure 2.3). The total amount of FK228 recovered from plasma, various major organs and tissues, urine and bile was less than 15% at 6 hr after dosing (Figure 2.4).

2.5 Discussion

2.5.1 Plasma pharmacokinetics of FK228

FK228 is now undergoing phase I/II clinical evaluation (Marshall, Rizvi *et al.* 2002; Sandor, Bakke *et al.* 2002). Although FK228 pharmacokinetics were studied in rats and patients, little is known about the mechanism of FK228's disposition and elimination. The current pharmacokinetic study in the rat confirms a large $CL_{\text{total}}$ (Chan, Bakhtiar *et al.* 1997; Li and Chan 2000), higher than the rat cardiac output (74 mL/min) (Davies and Morris 1993). The high $CL_{\text{total}}$ in the rat
suggested extensive metabolism of FK228 in blood, which could cause apparent blood-flow-independent drug elimination.

FK228 was reported to be a substrate of Pgp (Scala, Akhmed et al. 1997), which is highly expressed in the rat kidney, liver and the apical lining of intestine lumen. Thus Pgp is likely to be involved in FK228 renal, biliary and intestinal excretion and elimination. However, the total amount of FK228 in 24-hr urine and 6 hr bile together accounted for less than 11% of the dose, and no apparent major metabolite was found in urine and bile by HPLC/MS analysis, so there are probably other elimination pathway(s). The amount of FK228 in feces (0.23%) was much lower than excreted in bile, probably due to degradation of FK228 in intestinal contents, since FK228 is poorly absorbed in intestine (Li and Chan 2000). The low overall FK228 recovery as the intact drug (15%) further suggested extensive metabolism in blood.

CsA pretreatment resulted in little change of the FK228 plasma concentration-time profile (Table 1.1). This suggests that Pgp is not a major factor in FK228 distribution and elimination. This is consistent with the low excretion by rat kidney and liver, which express functional Pgp. This provides valuable information for future combination studies of FK228 with a Pgp inhibitor. One prerequisite requirement for such combinations is that the pharmacokinetic profile of the drug should not be dramatically changed in combination with the Pgp inhibitor, so as to avoid abnormal increases in blood concentrations and
associated toxicities (Gonzalez, Colombo et al. 1995). Our data suggests that the pharmacokinetic drug-drug interaction may be minimal in this combination.

I.v. infusion in the rat showed a significant decrease in $V_{d_{ss}}$ when compared with that following i.v. bolus at the same dose. This could be due to the existence of a saturable efflux transporter in tissues, which effluxes FK228 back to plasma effectively at low FK228 plasma concentrations, but becomes saturated at high FK228 concentrations. Following i.v. infusion, the plasma FK228 concentrations were low (<20 nM), which led to an effective efflux and trapping of FK228 in the plasma pool. This hypothesis will be further tested in Chapter 3, where MRP1 protein on RBC membrane was demonstrated to serve as an efflux pump for FK228.

DEM pretreatment significantly decreased RBC intracellular GSH levels, and resulted in a higher $C_0$ ($p<0.05$). This suggests possible metabolism in the red blood cells and that the GSH level may be a determinant of FK228 pharmacokinetics.

2.5.2 FK228 recovery as the intact drug

Determination of FK228 in rat organ tissues provided distribution profiles of FK228 in the rat. Even though FK228 is a lipophilic drug and thus expected to distribute into organs, especially adipose tissues, as suggested by its apparent large body distribution volume, FK228 recovery as the intact drug from the monitored organ tissues was low. Due to the lack of radio-labeled FK228,
determination of FK228 mass balance was not possible. However, the low dose recovery of intact FK228 (<15%) and the extremely high body clearance indicated extensive metabolism in the blood.
CHAPTER 3

METABOLISM OF FK228 IN THE BLOOD

3.1 Abstract

The objective of this study was to investigate the existence of extensive metabolism of FK228 in rat and human blood, and to identify major FK228 metabolites. A series of \textit{in vitro} blood incubations was conducted to determine FK228 uptake/metabolism by red blood cells. HPLC/MS\textsuperscript{n} was used to identify the structures of six metabolites, including four GSH conjugates and two thiols. Four major metabolites were purified and three of them, which possess an aliphatic spacer and a free thiol end, showed significantly higher HDAC inhibitory activities than did FK228. The same six metabolites were also detected in human blood incubation. The results not only confirm extensive metabolism of FK228 in the blood, but also provide strong evidences for FK228 being a prodrug.
3.2 Introduction

As shown in the previous chapter, the pharmacokinetic study showed that FK228 followed a two-compartment pharmacokinetic model in the rat with a large steady state distribution volume. The total body clearance exceeded the rat cardiac output, while no accumulation of FK228 into organ tissues was found. This suggests extensive metabolism in the blood. Elevated C₀ of FK228 after glutathione depletion in the blood further suggests the involvement of glutathione in FK228 metabolism.

Typical reversible HDAC inhibitors (e.g., Trichostatin A and SAHA) possess a long aliphatic tail with a nucleophilic end, such as –SH or –OH. X-ray crystallographic structure and computational docking analysis revealed that the long aliphatic tail acts as a spacer, and the nucleophilic end interacts with the active zinc ion located deep in the catalytic pocket of HDACs (Finnin, Donigian et al. 1999). On the other hand, FK228, has a unique bicyclic structure and its nearly spherical structure does not appear to fit into the catalytic pocket. However, the disulfide linkage in FK228 suggests a potentially labile site that could result in a ring-open structure.

In this chapter, we studied FK228 uptake/metabolism by red blood cells, and use HPLC/multi-stage MS (HPLC/MSⁿ) to identify metabolites from such incubations. Following purification of four major metabolites, we also determined their HDAC inhibitory activities.
3.3 Experimental procedures

3.3.1 Materials

3.3.1.1 Chemicals and reagents

Depsipeptide FK228 (purity >99%) was provided by the Drug Synthesis and Chemistry Branch, the National Cancer Institute (Bethesda, MD, USA). N-ethylmaleimide (NEM). Potassium Phthalate buffer (pH 4, 50 mM) was obtained from Van Water and Roger Scientific (Chicago, IL, USA). The HPLC-grade water (>18 mΩ) was generated from an E-pure water purification system (Barnstead, Dubuque, IA, USA). Acetonitrile (ACN, HPLC grade), acetic acid and ethyl acetate (reagent grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Drug-free heparinized rat and human plasma was purchased from Harlan Bioproducts for Science (Indianapolis, IN, USA). Human albumin (Fraction V, 96~99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat blood was obtained from Sprague-Dawley rats following jugular vein cannulation. Human blood was provided from volunteer donors.

3.3.1.2 Instrumentation

The LC/API 300 MS system consisted of a Perkin-Elmer Sciex API 300 triple-quadruple mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The mass spectrometer was equipped with an electrospray ionization (ESI) source. The instrument was tuned based on those published before (Li and Chan 2000) and according to the manufacturer’s manual.
The LC/LCQ MS system consisted of a Shimadzu HPLC system (Shimadzu, Columbia, MD) and a LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) coupled with an electrospray ionization (ESI) source. The positive ion mode was used, and the general system parameters were tuned with the parent compound FK228 except for those used in collision-induced dissociation (CID), where the collision energy and collision time for each ion were optimized individually. The temperature of the heated capillary was set at 200°C and the spray voltage was 5 kV.

The LC/Q-TOF MS consisted of a Waters Alliance 2690 Separation Module (Waters, Milford, MA) and a Micromass Q-TOF™ II (Micromass, Wythenshawe, UK) equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode. Sodium iodide was used for mass calibration for a range of m/z 100-2000. Optimal ESI conditions were: capillary voltage 3000V, source temperature 110°C, and a cone voltage of 55V. Nitrogen was used as the ESI gas and data were acquired in continuum mode during the LC run.

3.3.2 Analytical methods

FK228 determination of plasma samples was conducted using the LC/API 300 MS system. The HPLC separation was achieved on a 50×2 mm BetaBasic C8 5-µm column with isocratic elution consisting of 70% ACN and 0.1% acetic acid (v:v) at a flow rate of 0.2 mL/min. The split ratio was 20:1 so that the eluate was introduced into the API source at 10 µL/min. Multiple reaction monitoring (MRM) was used to monitor the precursor/product ion pairs of FK228 (m/z
and the internal standard BMLP (m/z 510.0/217). This HPLC/MS/MS determination method for plasma samples was re-validated by each operator. Plasma samples were extracted as described in Chapter 2. Purification of FK228 metabolites for HDAC inhibition assay was achieved with a preparative Waters C18 preparation column (250×10 mm, 5 µm). The gradient was initiated at 25% ACN in 0.1% acetic acid for 5 min, followed by a linear increase to 70% ACN over 5 min, which was kept constant for 15 min. Then the mobile phase was linearly returned to the original condition over 3 min, and was maintained for 7 min.

Structure identification of FK228 and its metabolites was performed by the LC/LCQ MS system. The metabolites were separated by a Spherisorb S3 ODS1 column (2 mm ID×10 cm, particle size 5 µm, Waters Corporation, Milford, MA, USA) with a gradient elution. The gradient was initiated with 25% ACN in 0.1% acetic acid for 5 min, followed by a linear increase to 70% ACN over 5 min, which was kept constant for 15 min. Then the mobile phase was linearly returned to the original condition over 3 min, and was maintained for 7 min. The cycle time for each analysis was 35 min.

Accurate mass determination was performed using the LC/Q-TOF MS. For HPLC separation, the same HPLC gradient elution program as for the LCQ instrument was used. The effluent was split post column using a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) with ≈20 µL/min to the ESI source.
3.3.3  

3.3.3.1  

**In vitro FK228 uptake by red blood cells**

Fresh whole blood obtained from male S.D. rats was heparinized (14 U/mL) and the hematocrit measured (Chen, Abdelhameed *et al.* 1992). The blood was spiked with FK228 (10×) to make final concentrations of 0.18, 1.8, and 18 µM, and then incubated at 37°C in a water bath with agitation. Aliquots of 0.2 mL were removed at pre-selected time points of 2, 4, 6, 8, 10, 12, 16, 20, 30, 45, and 60 min. The samples were centrifuged, plasma was separated, and kept frozen at -80°C until analysis. Following a 1-hour incubation, one mL aliquot of blood was centrifuged and the RBC fraction was washed three times with 0.5 mL ice-cold drug-free rat plasma, 1 min each time. The plasma washings were kept frozen at -80°C until analysis. Then, the RBC fraction was mixed in 1 mL pre-warmed drug-free rat plasma and incubated again at 37°C. Aliquots of 0.2 mL each were removed at pre-selected time points of 5, 10, 20, 40, and 60 min. The samples were centrifuged and plasma was separated and kept frozen at -80°C until analysis. Since the uptake/removal of FK228 from plasma was rapid, the time-0 plasma samples were prepared by spiking FK228 stock solution into appropriate volumes of plasma corresponding to 1 mL blood, which was calculated according to: plasma volume of 1 mL blood = (1 – H) + 0.09 × H, where H is the hematocrit value, and 0.09 × H accounts for the residual plasma volume in the packed RBC (Chen, Abdelhameed *et al.* 1992).
The same procedure was repeated following pretreatment of rat blood with 10 mM NEM, a GSH depleting agent, at room temperature for 30 min. Saline pretreatment was used as the control. FK228 removal due to uptake/metabolism was calculated.

3.3.3.2 Sample preparation

All the above samples were extracted as plasma samples according to the procedure described in Chapter 2. Briefly, an appropriate volume of sample (0.1 mL) was spiked with 1 µg internal standard BMLP, 0.4× volume of pH 4 potassium phthalate buffer, and the solution was extracted with 10× volume ethyl acetate. After centrifugation, the organic layer was transferred to a clean conical Falcon polypropylene tube, and evaporated under a stream of nitrogen. The residue was dissolved in 100 µl 70% ACN, and 40 µl was injected for FK228 determination. The LC/API 300 MS system was used for this purpose.

3.3.4 In vitro metabolism

3.3.4.1 FK228 stability test

The stability of FK228 at 18 µM, 37°C was tested in rat plasma alone, rat plasma containing 10 mM GSH, or rat plasma containing 10 mM GSH but pretreated with 10 mM NEM for 30 min. FK228 stability in RBC cytosol and normal saline was also studied. Aliquots of 0.2 mL were removed at pre-selected time points up to 1 hr and determined as before.
3.3.4.2 FK228 recovery after incubation in rat blood

A FK228 recovery study was conducted, where 2 mL fresh heparinized rat whole blood containing 18 µM FK228 was incubated at 37°C for 1 hr. After centrifugation, the plasma was taken out and the RBC fraction was washed 8 times with 2 mL saline each. Then drug-free rat plasma was added to the RBCs to restore the original volume. The reconstituted blood was homogenized on ice with a glass tissue grinder (400 strokes) followed by centrifugation at 16,000 g for 15 min, and 0.95 mL supernatant was removed. The RBC debris was further washed with 1 mL ACN each for 8 times. FK228 concentrations in the plasma, saline washings, lysis supernatant, and ACN washings were determined using the LC/API 300 MS as described in section 2.3.5, and the cumulative recovery of FK228 was calculated.

3.3.4.3 Identification of FK228 metabolites

Incubations of FK228 at 180 µM in 0.5 mL heparinized rat blood (14 units/mL) and in 0.2 mL heparinized human blood at 37°C were performed. After 30 min, the blood was homogenized (400 strokes), using a glass dounce tissue grinder (Wheaton, NJ) in an ice-bath. Then two volumes of ACN were added to the homogenate to precipitate the proteins, and the contents were centrifuged at 1600g for 1 min. The supernatant was transferred to a clean vial and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 0.5 mL or 0.2 mL 25% ACN in 0.1% acetic acid for rat and human blood homogenates, respectively, and a 30 µL aliquot was analyzed by LC/LCQ MS as described in
Section 3.3.1.2 and Section 3.3.2. multistage MS (MS^n) was used in structural identification of FK228 metabolites. The parent drug FK228 was first characterized by sequential fragmentation to establish its fragmentation profile, to which the fragmentation profiles of the metabolites were compared. In some cases, the identities of metabolites were confirmed by high-resolution mass determinations obtained from the LC/Q-TOF MS.

3.3.4.4 Formation kinetics of FK228 metabolites

FK228 (180 \( \mu \text{M} \)) was incubated in rat plasma containing 10 mM GSH at 37°C. Serial samples were removed at pre-selected time points up to 4 hr. The plasma samples were prepared as described in Section 3.3.4.3. FK228 and its major metabolites were monitored by HPLC/ API 300 ESI MS with BMLP as the internal standard. Single Ion Monitoring (SIM) mode was used and FK228, reduced FK228, FK228 GSH conjugate and a reduced FK228 GSH conjugate were monitored at \( m/z \) 541, 543, 848 and 850, respectively. Due to the lack of metabolite standards, the concentrations of FK228 metabolites were semi-quantified under the assumption that the system responses to FK228 and its metabolites were the same.

3.3.4.5 Translocation of FK228 metabolites

One mL rat blood containing 180 \( \mu \text{M} \) of FK228 was incubated at 37°C for 60 min. The blood was centrifuged to remove plasma. To the RBC fraction was added blank rat plasma to restore the initial volume, and the content was
homogenized on ice with a glass tissue grinder (400 strokes). The plasma and RBC homogenate were analyzed by HPLC/LCQ ESI MS as described in Section 3.3.2.

3.3.5 HDAC inhibitory activity determination

3.3.5.1 Preparation and purification of FK228 metabolites

FK228 metabolites were prepared in rat plasma incubation containing 10 mM GSH and 180 µM FK228 at 37°C in a water bath with agitation for 60 min. Two volumes of ACN was added to precipitate the proteins followed by centrifugation. The ACN layer was transferred to a clean Falcon polypropylene tube and evaporated to dryness under a stream of nitrogen. The metabolites were separated and monitored by HPLC/API 300 MS as described in Section 3.3.2. The chromatogram is shown in Appendix Figure A.7. The eluates were collected according to the retention times of individual peaks, and then freeze-dried. The identity and purity of each metabolite were determined by API 300 MS.

3.3.5.2 HDAC activity assay

The HDAC inhibitory activities of the purified FK228 metabolites were assayed by using the Fluor de LysTM HDAC Fluorescent Activity Assay/Drug Discovery Kit (BioMol, Plymouth Meeting, PA). The metabolites were weighed and dissolved and serially diluted in the working buffer provided with the kit to obtain a range of concentrations. The assay was performed according to the
manufacturer’s protocol and the IC50 values were calculated using the inhibitory effect sigmoid model with four parameters, $E_{\text{max}}$, $E_0$, $\gamma$ and IC50 as defined in Appendix Figure A.8.

### 3.4 Results

#### 3.4.1 FK228 uptake/metabolism by rat red blood cells

We first performed the *in vitro* incubation of FK228 in rat blood at 37°C. By monitoring plasma FK228 concentrations over time, we found that the drug disappeared from the plasma rapidly, with over 80% removed within 2 min incubation, in an apparent concentration-independent manner through the concentration range 0.18 - 18 µM (Figure 3.1). The RBC was then separated by centrifugation and washed with ice-cold normal saline, followed by post-wash incubation in drug free rat plasma at 37°C. Little was recovered from RBC (Figure 3.1). Since FK228 is stable in rat plasma alone, this result suggested either an entrapment or metabolism of FK228 by rat RBC.

To further evaluate whether the uptake/metabolism of FK228 was reversible, we determined the recovery of FK228 from the rat blood incubation system (Figure 3.2). The cumulative recovery of FK228 was less than 35%, suggesting significant metabolism.

Pretreatment with NEM (10 mM), an agent known to deplete intracellular GSH, not only significantly decreased the rate and extent of FK228
uptake/metabolism, but also increased the amount recoverable from RBC in the

*in vitro* blood incubation (Figure 3.3).
Figure 3. 1. In vitro rat RBC uptake/metabolism of FK228.

Fresh heparinized rat whole blood spiked with FK228 and incubated at 37°C. Removal of FK228 from plasma was followed up to 60 min. RBC fraction was washed with ice-cold drug-free rat plasma three time, and then incubated in drug-free rat plasma at 37°C. Flux of FK228 back to plasma was followed for 60 min. FK228 uptake/metabolism was calculated based on sample volumes and hematocrits. (a) More than 80% of FK228 was removed from plasma within 2 min in an irreversible manner. The triple washes and the post-wash incubation caused little reflux of FK228 to plasma. (b) Rat RBC uptake of FK228 appeared to be concentration-independent.
Figure 3. 2. FK228 recovery as the intact drug from the in vitro whole rat blood incubation.

After incubation, FK228 was recovered from plasma, saline washing solutions, cytosol and extensive ACN washing solutions. Less than 35% of drug was recovered.
Figure 3. Effect of N-ethylmaleimide (NEM) on FK228 uptake/metabolism by rat RBC.

Fresh heparinized rat whole blood was pretreated with 10 mM NEM at room temperature for 30 min (○), with normal saline pretreated rat blood as control (●). After pretreatment, the RBCs were resuspended in warm drug-free rat plasma, spiked with FK228 to make a final blood concentration of 1.8 μM, and incubated at 37°C for 60 min, followed by washing and post-washing incubation as described in Figure 3.1. NEM pretreatment significantly decreased the RBC uptake rate and extent. It also made the uptake process reversible with more FK228 washed out during triple washing.
3.4.2 FK228 stability

To investigate what factors are involved in the metabolic reactions, a series of stability tests was done (Figure 3.4). FK228 was stable in rat plasma, saline, or saline containing 10 mM GSH at 37°C for at least 4 hr. Only in the presence of plasma containing 10 mM GSH or in the presence of RBC cytosol, FK228 was unstable over time. Again, this reaction was completely ablated after 10 mM NEM pretreatment. These suggest both GSH and plasma/cytosol proteins are involved in the metabolism.

3.4.3 Identification of major FK228 metabolites in the blood
3.4.3.1 Screening of FK228 metabolites

When FK228 was incubated with GSH in the presence of rat plasma, the LC/MS total ion profile for the acetonitrile extract showed a total of 6 peaks of various intensities (Figure 3.5b). These peaks were absent in the blank plasma extract (Figure 3.5a). Initial scan of these peaks for molecular ions revealed FK228 with MH+ at m/z 541 at the peak with retention time of 20.52 min (Figure 3.5c). Two peaks (Figure 3.5d) with m/z 543 were detected at 19.56 (A) and 20.54 min (B); the latter overlapped with the parent compound on the chromatograph, but was not due to the isotope distribution of the parent compound because of its higher intensity than that of the parent compound. Thus, the MH+ ion of FK228 was observed with an intensity of 1.58x10^8 counts; the calculated intensity of its isotope peak at m/z 543 is 2.2x10^7 counts (14%).
and the observed intensity for component B was $1.89 \times 10^8$ counts. Three other peaks at retention times of 21.41 and 22.32, and 26.7 min, all with MH$^+$ ions at $m/z$ 848 (Figure 3.5e), were also detected, with last of these peaks showing the lowest intensity. The peak at retention time 23.05 min gave a MH$^+$ at $m/z$ 850 (Figure 3.5f). FK228 was identified as the peak at 20.52 min, based on its ESI MS$^2$-MS$^4$ scans of the appropriate fragment ions as indicated in Figure 3.6, and the mass spectra were identical in every respect with those of the authentic sample. (To provide a better quality of the mass spectra, Figure 3.6 recorded those of the authentic compound.) The major fragment ions in the various MS$^n$ spectra were readily rationalized by the proposed fragmentation pathways (Scheme 3.1).

3.4.3.2 Fragmentation of the parent drug FK228

Collision induced dissociation (CID) of FK228 proceeded by initial cleavage at the peptide bond at N5-C6,10 followed by elimination of the L-valine to form a high intensity fragment at $m/z$ 424 (MS$^2$, Figure 3.6b). Cleavage of the peptide bond at N8-C9 with the elimination of both dehydrobutyrine and the L-valine generated a rather abundant fragment at $m/z$ 341 (MS$^2$, Figure 3.6b). Sequential elimination of CO generated an abundant ion at $m/z$ 313 (MS$^3$, Figure 3.6d). This fragment further underwent ring contraction by a loss of CO to give a fragment at $m/z$ 285. CID of the ion at $m/z$ 424 caused a major fission of the bond at C9 and C10 and a loss of CH$_3$CN to give rise to a major fragment at $m/z$ 272 (MS$^3$, Figure 3.6c). Loss of HCO from the fragment at $m/z$ 272 gave a major
fragment at m/z 244 (MS^3, Figure 3.6e). The proposed fragmentation pathways (including those in the subsequent Schemes) represent some of the energetically feasible cleavages and may not be the only possibilities.

3.4.3.3 Metabolite A

Peak A (retention time 19.56 min) gave a mass spectrum with MH^+ at m/z 543.0 (Figure 3.7a), suggesting it to be a reduced product of FK228. Its MS2 spectrum showed a base fragment peak at m/z 426 (Figure 3.7b), consistent with the cleavage of N5-C6 bond (numbering system based on FK228 structure) and parallel to that of FK228 (Scheme 3.2). Further elimination of H2S from S12 gave a rather abundant fragment at m/z 392 (Figure 3.7c), providing further support for the reduced thiol structure. Ring closure between the remaining thiol (S13) and the D-valine peptide bond, with an elimination of the residual cystine peptide fragment, gave rise to an abundant fragment peak at m/z 240 (MS^3, Figure 3.6d; Scheme 3.2). Further ring contraction with expulsion of CO generated a base fragment at m/z 212.0 (MS^5, Figure 3.7e; Scheme 3.2). This fragmentation scheme is consistent with the assigned reduced thiol structure (Scheme 3.2, II).
Figure 3. 4. FK228 (1 µg/mL) stability in different solutions.

FK228 was stable in rat plasma (♦), normal saline containing 10 mM GSH (■), and rat plasma containing 10 mM GSH, but pretreated with 15 mM NEM at 37°C for 30 min (▲). FK228 disappeared very fast when incubated in rat plasma containing 10 mM GSH (×), and rat RBC cytosol extract (○).
Figure 3.5. HPLC/ESI-MS chromatograms of extracts from the in vitro incubation of rat plasma incubated with 100 µg/ml FK228 and 10 mM GSH.

(a) Total ion chromatogram (TIC) of blank rat plasma extract; (b) TIC of the extract from the in vitro incubation; (c) Extracted ion chromatogram (XIC) of the parent drug FK228 at m/z 541; (d) XIC of two reduction products A and B at m/z 543; (d) XIC of three single GSH-FK228 conjugates C, D and E at m/z 848; and (f) XIC of one reduced GSH-FK228 conjugate F at m/z 850.
Figure 3.6. HPLC/ESI-MSn spectra of the authentic parent drug FK228.

(a) ESI-MS\textsuperscript{1} of Peak FK228 overlapping with Peak B; (b) MS\textsuperscript{2} scan of FK228 at m/z 541; (c) MS\textsuperscript{3} scan of FK228’s daughter ion at m/z 424; (d) MS\textsuperscript{3} scan of FK228’s daughter ion at m/z 341; and (e) MS\textsuperscript{4} scan of the ion at m/z 272.
Scheme 3. 1. Proposed major fragmentation pathways of FK228 accounting for the MS2-MS4 spectra of FK228.
3.4.3.4 Metabolite B

Peak B (retention time 20.54 min) overlapped with FK228, but little interference from FK228 was found since the second isotope peak of FK228 at $m/z$ 543 only contributes only 14% of the total intensity. The totally different CID pathways of peak B from those of FK228 further confirmed this independent structure. Peak B gave a MH$^+$ ion at $m/z$ 543 (Figure 3.8a), and thus was also tentatively assigned as a reduced thiol structure. Upon CID, the molecular ion eliminated mass 239 Da (Scheme 3.3) to give rise to a single base fragment at $m/z$ 304 (MS$^2$, Figure 3.8b). The formation of this ion could be rationalized if ring formation between the S12 thiol and the α, β unsaturation of the dehydrobutyrine was invoked (Scheme 3.3, III). Cleavage of the D-valine with the elimination of the thiol-containing moiety (S13) would give rise to the ion at $m/z$ 304 as depicted, with reasonable stability. Further elimination of NH$_3^+$ (Scheme 3.3) was consistent with the observed major fragment at $m/z$ 287 (MS$^3$, Figure 3.8c). Further elimination of HCOOH was also consistent with the observed base peak at $m/z$ 241 (MS$^4$, Figure 3.8d; Scheme 3). This elimination of formic acid also generated a minor fragment at $m/z$ 259 (MS$^3$, Figure 3.8c; Scheme 3.3). CID of the ion at $m/z$ 241 (MS$^4$, Figure 3.8e) gave rise to fragment ions at $m/z$ 170 and 213 (Scheme 3.3). Thus, the ion at $m/z$ 304 appeared to be diagnostic for this thiol structure for the conjugate (Peak D) as shown later.
Figure 3. 7. HPLC/ESI-MSn spectra of the reduced FK228 peak A.

(a) ESI-MS$^1$ spectrum of A; (b) MS$^2$ scan of A at m/z 543; (c) MS$^3$ scan of A's daughter ion at m/z 426; (d) MS$^4$ scan of the ion at m/z 392; (e) MS$^5$ scan of the ion at m/z 240.
Scheme 3. 2. Proposed major fragmentation pathways accounting for the MS2–MS5 spectra of Peak A.
Figure 3. 8. HPLC/ESI-MSn spectra of the reduced FK228 peak B.

(a) ESI-MS\textsuperscript{1} spectrum of the reduced FK228 B; (b) MS\textsuperscript{2} scan of B at \textit{m/z} 543; (c) MS\textsuperscript{3} scan of B’s daughter ion at \textit{m/z} 426; (d) MS\textsuperscript{4} scan of the ion at \textit{m/z} 392; (e) MS\textsuperscript{5} scan of the ion at \textit{m/z} 241.
Scheme 3. Proposed major fragmentation pathways accounting for the MS2–MS5 spectra of Peak B.
3.4.3.5 Metabolites C, D and E

On the basis of the structure of FK228, the known chemistry of GSH, and the nominal masses (Figures 3.9a, 3.10a, 3.11a), Peaks C, D, and E were tentatively assigned as GSH conjugates of FK228 formed at different locations. The existence of GSH conjugates (Peaks C and D) was confirmed by accurate mass measurement, which gave $m/z$ values of 848.2992 and 848.2984, respectively, with 1 ppm deviation from the theoretical values. The similar retention times between peaks C and D suggested their structural similarity. Therefore, they were attributed to formation of GSH conjugates upon fission of the disulfide linkage (Scheme 3.4, IV and Scheme 3.5, V, respectively). On the other hand, Peak E was possibly a GSH conjugate formed through the nucleophilic addition of the $\alpha, \beta$-unsaturation at the Z-dehyrobutyrine portion of FK228 (Scheme 3.6, VI), and this tentative assignment was consistent with the rather different polarity and low abundance of this adduct. The structural assignments of these GSH conjugates were derived from the MS$^n$ analysis. Common to all of these GSH conjugates, cleavages of each of the peptide bonds of glycine and glutamine, on the GSH side chain gave fragmentation ions at $m/z$ 773 and $m/z$ 719, respectively, at high intensities ($MS^2$, Figures 3.9b, 3.10b, and 3.11b, and Schemes 3.4-3.6). CID of the ion at $m/z$ 719 ($MS^3$) discerned between Peaks C and D. The former produced intense fragment ions at $m/z$ 527, 498.9, and 415.8 (Figure 3.9d), whereas the latter generated fragment ions at $m/z$ 625.9, 415.9, and the base ion at $m/z$ 303.9 (Figure 3.10e). These
differences led to the tentative assignment of Peak C as a GSH conjugate at the sulfur atom of cystine (S12), following the scission of the disulfide bond (Scheme 3.4, IV), while Peak D was assigned as the conjugate formed at S13 (Scheme 3.5, V). Following scission of the disulfide bond, the free SH (S12) group of cystine could presumably form a rather favorable seven-membered ring through the nucleophilic addition with the Z-dehydrobutyryne. This tentative assignment was supported by the observation of an abundant fragment ion at \( m/z \) 304 (MS\(^4\), Figure 3.10d), which was probably formed from cleavage of a major peptide fragment (–415 Da) through the peptide bond of the D-valine between C22 and N23, followed by fission of the ester bond at C1 and O2 (Scheme 3.5). This predominant fragment is consistent with that as generated by the thiol (Peak B) shown earlier. CID of the ion at \( m/z \) 773 could be rationalized in terms of the formation of a fragment ion at \( m/z \) 543 (MS3, Figure 3.10c) with the cleavage of the remainder of the GSH conjugate (Scheme 3.5). Elimination of a neutral fragment of mass 304 Da also occurred, with the detection of an abundant fragment at \( m/z \) 416 (Figure 3.10e and Scheme 3.5).

For Peak C, elimination of the L-Valine from the ion at \( m/z \) 719 would give rise to the ion at \( m/z \) 602 (MS\(^3\), Figure 3.9d) for which the assigned structure is depicted in Scheme 3.4; further loss of a glycine (Scheme 3.4) would give rise to a fragment ion at \( m/z \) 527 (MS\(^3\), Figure 3.9d). Subsequent cleavage of the disulfide bond at S12 (Scheme 3.4) would give rise to the fragment ion at \( m/z \) 426 (MS\(^4\), Figures 3.9e). Further CID of the ion at \( m/z \) 527 would also give rise to a major fragment ion at \( m/z \) 499 (MS\(^4\), Figure 3.9e), and CID of the ion at \( m/z \)
499 generated a fragment ion at $m/z$ 240 (MS$^5$, Figure 3.9f) as depicted (Scheme 3.4). CID of the ion at $m/z$ 773 generated a reasonably abundant ion at $m/z$ 565 (Figure 3.9c). These fragmentation patterns were consistent with the assigned structures of Peak C.

For conjugate E, in addition to the common fragmentation of the GSH conjugate (Scheme 3.6), MS$^3$ of the ion at $m/z$ 719 showed that elimination of the L-valine to form an abundant ion at $m/z$ 573 (Figure 3.11c) appeared to be a favorable process. MS$^4$ of the ion at $m/z$ 573 (Figure 3.11d) showed cleavage of the bond between N8 and C9 (Scheme 3.6) to give rise to an ion at $m/z$ 341 (Figure 3.11d) and sequential losses of CO and CH3CN gave the ion at $m/z$ 272 (Figure 3.11d). Loss of glycine from the ion at $m/z$ 573 also gave an intense ion at $m/z$ 456. The assigned structure of Peak E was therefore VI, which is consistent with the proposed fragmentation pathways (Scheme 3.6).
Figure 3. HPLC/ESI-MSn spectra of the GSH-FK228 conjugate peak C.

(a) ESI-MS$^1$ scan of C; (b) MS$^2$ scan of C at m/z 848; (c) MS$^3$ scan of C’s daughter ion at m/z 773; (d) MS$^3$ scan of C’s daughter ion at m/z 719; (e) MS$^4$ scan of the ion at m/z 527; (f) MS$^5$ scan of the ion at m/z 499.
Scheme 3.4. Proposed major fragmentation pathways accounting for the MS2–MS5 spectra of Peak C.
Figure 3. 10. HPLC/ESI-MSn spectra of the GSH-FK228 conjugate peak D.

(a) ESI-MS\(^1\) scan of D; (b) MS\(^2\) scan of C at \(m/z\) 848; (c) MS\(^3\) scan of D's daughter ion at \(m/z\) 773; (d) MS\(^4\) scan of the ion at \(m/z\) 543; (e) MS\(^3\) scan of D's daughter ion at \(m/z\) 719.
Scheme 3. 5. Proposed major fragmentation pathways accounting for the MS2-MS4 spectra of Peak D.
Figure 3. 11. HPLC/ESI-MSn spectra of the GSH-FK228 conjugate peak E.

(a) ESI-MS$^1$ scan of E; (b) MS$^2$ scan of E at $m/z$ 848; (c) MS$^3$ scan of E’s daughter ion at $m/z$ 719; (d) MS$^4$ scan of the ion at $m/z$ 573.
Scheme 3. 6. Proposed major fragmentation pathways accounting for the MS2-MS4 spectra of Peak E.
3.4.3.6 Metabolite F

Having assigned the structures of Peaks A-E, Peak F, which gave MH$^+$ at $m/z$ 850 (Figure 3.12a), could be assigned as the thiol-GSH conjugate formed on the side chain following reduction (Scheme 3.7, VII). This structural assignment was supported by cleavages of glycine and glutamine to give rise to major fragments at $m/z$ 775 and 721, respectively (Figure 3.12b), both two Da higher than the previously described unreduced GSH conjugates (Schemes 3.4 - 3.6). In this structure we have proposed that the thiol at S12 formed a seven membered ring with the carbonyl at C6, similar to the ring structure of the thiol of Peak B (Scheme 3.7, III). This proposed ring structure was supported by the ready cleavage of the D-valine and the connected aliphatic thiol fragment (Scheme 3.7), generating a major fragment ion at $m/z$ 482 (MS$^3$, Figure 3.12c) upon CID of ion at $m/z$ 721 (Scheme 3.7). Further loss of the L-valine generated another intense fragment at $m/z$ 365 (MS$^4$, Figure 3.12d). Loss of NH3 (Scheme 3.7) generated a stable ion at $m/z$ 348 (MS4, Figure 3.12e) and ring contraction with a loss of HCO (Scheme 3.7) generated a major ion at $m/z$ 336 (MS$^4$, Figure 3.12d). Elimination of glycine from ion at $m/z$ 365 generated a major ion at $m/z$ 290 (MS$^5$, Figure 3.12e) and from the ion at $m/z$ 336 (Scheme 3.7) generated a reasonably intense ion at $m/z$ 262 (MS$^4$, Figure 3.12e), further supporting the proposed ring structure.
Figure 3. 12. HPLC/ESI-MSn spectrums of the reduced GSH-FK228 conjugate peak F.

(a) ESI-MS\(^1\) scan of F; (b) MS\(^2\) scan of F at m/z 850; (c) MS\(^3\) scan of F's daughter ion at m/z 721; (d) MS\(^4\) scan of the ion at m/z 482; (e) MS\(^5\) scan of the ion at m/z 365.
Scheme 3. 7. Proposed major fragmentation pathways accounting for the MS2-MS4 spectra of Peak F.
3.4.3.7 FK228 Metabolites in human blood

These same products were also detected in plasma extract, when human plasma was incubated with FK228 in the presence of 10 mM GSH; FK228 was unstable in the incubation mixture, and only 18% of the parent compound was found at the end of a 1 hr incubation period. A similar result was found in human serum albumin in the presence of GSH (data not shown). However, FK228 was found to be stable in normal saline, in 10 mM GSH solution alone, in rat plasma, and in 5% human serum albumin alone for at least 1 hr, with essentially no change in concentration. In human and rat red blood cells, following incubation with FK228, all of these products were also found in the homogenates (Figures 3.14a and b). There was a slight change of the retention times, probably due to small variations in the HPLC conditions. All these products were detected using LC/MS. This suggested that plasma or cellular proteins plus GSH were required for the formation of these FK228 products; the proteins possibly catalyzed the formation with GSH. A possible scheme for the formation of these conjugates is proposed in Scheme 3.8.
Figure 3.13. Summary of the structures of FK228 and its metabolites.
Figure 3. 14. The formation of thiols and GSH conjugates of FK228.

FK228 (100 µg/mL) was incubated in rat or human whole blood at 37°C for 1 hr, followed by homogenization of the blood and detection of the metabolites. (a) Formation of FK228 metabolites in rat whole blood. (b) Formation of FK228 metabolites in human whole blood.
Scheme 3. 8. A proposed scheme for the formation of the thiols and GSH conjugates.

The formation of FK228 metabolites involves proteins. R and R' refer to the ring structure on both sides of the disulfide bond of FK228. Pro-SH represents thiol-containing proteins.
3.4.4 Formation kinetics of major FK228 metabolites

The fates of FK228 and its metabolites in rat plasma containing 10 mM GSH were followed by HPLC/MS. Due to lack of the metabolite reference standards at the time we conducted this experiment, we could only determine the relative amounts of metabolites by normalizing their peak areas to that of FK228 at time 0. Upon mixing of FK228 at 180 µM with rat plasma containing 10 mM GSH, FK228 concentration decreased to about 30% within 2 min, and remained essentially constant thereafter (Figure 3.15). The two reduced thiol metabolites A and B reached their peak concentration rapidly, followed by approximately parallel decays with terminal half lives of 18.5 min and 14.4 min, respectively. Concentration levels of GSH conjugates C reached a plateau after 1 hr, followed by a two-phase decay. Conjugate D reached its maximum concentration at 1 hr followed by a slow decrease. Concentrations of conjugate E increased over time and reached its plateau at 2 hr. Due to the sensitivity issue, it was hard to follow the profile of F.
Figure 3. 15. Formation and degradation kinetics of FK228’s metabolites following incubation of FK228 (100 mg/mL) in plasma containing 10 mM GSH at 37°C.
3.4.5 Translocation of FK228 metabolites in rat red blood cells

Following a 1 hr incubation of 180 µM FK228 in fresh heparinized rat whole blood at 37°C, FK228 and its metabolites A, B, C, D and F were detected in plasma. In RBC cytosol, only FK228 and its metabolites B and D were detected. In both fractions, metabolite E was found to be a minor peak (Figure 3.16).

3.4.6 HDAC inhibitory activity of major metabolites

Four most abundant FK228 metabolites, namely, A, B, C and D were prepared in rat plasma and purified by HPLC. The purity of metabolites was examined by MS and MS² (Appendix Figure A.9-11). The HDAC inhibitory activities of the metabolites were determined (Figure 3.17). The IC50s of FK228, A, B, C and D were determined to be 76.5 ± 5.9, 1.65 ± 0.24, 0.94 ± 0.14, 4.0 ± 1.9 nM and 15.9 ± 2.7 nM, respectively (n = 3). The apparent HDAC inhibitory activity of FK228 and metabolite D, which do not possess the aliphatic tail and the free –SH, may be due to the GSH activity contained in the assay kit. The presence of GSH is expected to reduce FK228 to form the active metabolites and also react with metabolite D to form metabolite B. Follow-up determination of the GSH activity in the HDAC inhibition kit using the Bioxytech GSH-400™ assay kit (Oxis, Portland, OR) revealed the GSH concentration was as high as 24 mM.
Figure 3. Distribution of FK228 metabolites in rat RBCs after their formations.

FK228, metabolites A, B, C, D and F were detected in the plasma fraction (a) while FK228, metabolites B, D and E were detected in the RBC homogenate (b).
Figure 3. Dose-response curves of FK228 and its metabolites A, B, C and D.

The HDAC inhibitory activity was determined using the Fluor de LysTM HDAC Fluorescent Activity Assay/Drug Discovery Kit (n = 3). The IC50s were: FK228 76.5 ± 5.9 nM, A 1.65 ± 0.24 nM, B 0.94 ± 0.14 nM, C 4.0 ± 1.9 nM, and D 15.9 ± 2.7 nM.
3.5 Discussion

3.5.1 Prodrug theory for FK228

Very recently, Furumai et al. reported that FK228 is a prodrug for a reduced thiol (Furumai, Matsuyama et al. 2002). When FK228 was incubated in extracts of several tumor cell lines, a reduced thiol formed by reduction of the disulfide linkage was detected and the structure was verified by chemical synthesis. This reduction product, which is structurally identical to the proposed reduced thiol A, was shown to be 40-fold more active than FK228 itself for the inhibition of histone deacetylases (HDACs) in vitro. The free –SH group on the longer aliphatic tail was proposed to be involved in the HDAC inhibitory activity. Similar to our finding, Furumai et al. also demonstrated that GSH and cytosolic proteins were required for the intracellular reduction of FK228 (Furumai, Matsuyama et al. 2002).

Using the HPLC/ESI-MS\textsuperscript{n} technique, the structures of the 6 potential metabolites of FK228 have been identified in both rat and human blood based on extensive MS\textsuperscript{n} evidence, rationale of their formation, and in some cases accurate mass measurement. Among the metabolites, A, B, C and F all possess a free –SH on the longer aliphatic chain, suggesting potential HDAC inhibitory activities. Purification of A, B, C and D, and subsequent HDAC inhibition assay revealed that A, B and C are more potent HDAC inhibitors as compared with the parent drug FK228.
These findings may provide valuable information for development of new HDAC inhibitors. The identification of active metabolites not only confirmed the HDAC inhibition model (Finnin, Donigian et al. 1999), in which an aliphatic chain and a nucleophilic group are required for the HDAC inhibitor. In addition, the current study suggests the importance of the ‘bulky cap’ as possessed by FK228 after bioactivation. It is possible that this cap binds to the entrance of the active HDAC binding pocket with high affinity, and thus results in more potent HDAC inhibition. In contrast, linear HDAC inhibitors that do not have a similar ‘bulky cap’ (i.e., butyric acid) are found to be much less potent. Indeed, compounds that possess both the linear and bulky structures have been synthesized and shown promising HDAC inhibitory activity (Furumai, Komatsu et al. 2001).

3.5.2 Implication in FK228 pharmacokinetics in the rat

More attention has recently been drawn to the role of RBC as a determinant of drugs’ pharmacokinetic behaviors. For most drugs, the partition between RBC and plasma is approximately equal and reversible. In these cases, depending on the partition rate, RBC may modulate drug distribution volume (Hinderling 1997), or delays drug elimination by trapping the drug in RBCs when blood flows through eliminating organs (i.e., liver) (Chen, Abdelhameed et al. 1992; Hinderling 1997). In some other cases, however, the drug uptake by RBC appears irreversible by either tight non-covalent binding or chemical modification (Hinderling 1997). In this case, RBC either serves as compartment where drugs accumulate, or as an elimination pathway. In the present study, rat RBC
uptake/metabolism of FK228 is rapid and irreversible. This confirms the hypothesis raised from Chapter 2 that extensive metabolism is responsible for the enormously high total body clearance of FK228 in the rat. Plasma/cytosol proteins and GSH are involved in the formation of the 6 metabolites. A possible reaction scheme has recently been proposed (Xiao, Byrd et al. 2003) (Scheme 3.8).

Human RBC, on the other hand, showed FK228 concentration and hematocrit-dependent uptake/metabolism of FK228 (Chapter 6). It is known that rat and human RBC contain similar concentrations of GSH (up to 10 mM), but in rat RBC cytosol, there are even higher concentrations of more reactive thiol-containing proteins (Di Simplicio, Cacace et al. 1998; Rossi, Barra et al. 1998). After FK228 molecule diffuses across RBC membrane, it can diffuse back to plasma or reacts with GSH or free thiols on cytosol proteins. In rat RBC, it is more likely that FK228 molecules react with thiols or GSH, and thereby are trapped inside RBC by formation of metabolites than in human RBC. And as a result, rat RBC uptakes/metabolizes FK228 more completely and rapidly than human RBC (For human RBC uptake data, see Chapter 6).

What makes things more complex is the MRP1-mediated efflux of FK228. It has been reported that MRP1 is highly expressed on both human and rat RBC membrane (Rychlik, Pulaski et al. 2000). Recent data from a series of in vitro transport studies (Chapter 6) indicates that FK228 is a MRP1 substrate. The
effect of MRP1 on FK228 elimination by rat RBC is further discussed in Chapter 6.
CHAPTER 4

PHARMACOKINETICS AND PHARMACODYNAMICS OF FK228 IN CANCER PATIENTS

4.1 Abstract

Pre-clinical studies of the histone deacetylase (HDAC) inhibitor FK228 in Chronic Lymphocytic Leukemia (CLL) and Acute Myeloid Leukemia (AML) cell lines have demonstrated that it effectively induces apoptosis at concentrations where HDAC inhibition occurs. In this study, we aimed to study FK228 pharmacokinetics in CLL and AML patients receiving i.v. infusion of FK228 at the previously established maximum tolerated dose (MTD), 13 mg/m². Ten CLL and ten AML patients were treated with FK228 on days 1, 8, and 15 of therapy. FK228 concentrations in plasma and urine samples collected following dosing on Day 1 were determined by a validated HPLC/MS/MS method. FK228 was found to follow a two-compartment pharmacokinetic model, with AUC of $6.02 \pm 2.93 \, \mu\text{M} \cdot \text{hr}$, $CL_{\text{total}}$ of $4.81 \pm 2.02 \, \text{L/hr/m}^2$, and $V_d_{\text{ss}}$ of $3.29 \pm 0.94 \, \text{L/m}^2$. These pharmacokinetic parameters were consistent with the results from a previous phase I FK228 pharmacokinetic study. However, dramatic differences were
found when comparing human and rat pharmacokinetics of FK228 after BSA-based normalization. In patients, increases in HDAC inhibition and H3 and H4 acetylation were noted following treatment, and a good correlation was found between \( \text{CL}_{\text{total}} \) of FK228 and the total histone H4 acetylation status, consistent with FK228 being a prodrug.
4.2 Introduction

Acute Myeloid Leukemia (AML) and Chronic Lymphocytic Leukemia (CLL) are two of the most common types of leukemia diagnosed in adults (Jemal, Thomas et al. 2002). Most of the patients diagnosed with AML present with bone marrow failure require immediate treatment. Standard induction therapy for patients with AML, consisting of daunorubicin and ara-C in conventional doses, results in a complete remission (CR) rate of 50-60% in an unselected population and a long-term survival of about 10-20% (Kimby, Nygren et al. 2001). However, the relapse rate after the first complete remission is high and patients’ responses to further treatment are poor (Leopold and Willemze 2002). Among young patients with AML, however, only 40 to 50 percent will attain a long-term disease-free survival (Ravindranath 2003). The chance for cure with conventional therapies is even smaller in high-risk AML patients such as the elderly (Jackson and Taylor 2002), patients with high-risk cytogenetics or patients with treatment-related AML (Stone 2002).

CLL represents 22-30% of all leukemia cases with a worldwide incidence projected to be between 1 and 5.5 per 100,000 people (Redaelli, Laskin et al. 2004). Adult chronic lymphocytic leukemia is found at higher rates in males than in females and in whites than in blacks. Median age at diagnosis is 64-70 years. Five-year survival rate in the USA is 83% for those <65 years old and 68% for those >65 years old (Redaelli, Laskin et al. 2004). While the introduction of fludarabine (Schmitt, Wendtner et al. 2002) and more recently rituximab (Plosker
and Figgitt 2003) has improved the overall success of CLL treatment, but the therapy is still palliative with no curative potential. Identification of new therapies for both CLL and AML therefore remains a high priority.

One promising new therapeutic strategy is aimed at reversing tumor-related alterations in chromatin structure and concomitant transcriptional silencing. Epigenetic silencing or down-modulation of important tumor suppressor and differentiation genes can lead to neoplastic transformation and disease progression (Claus and Lubbert 2003; Nephew and Huang 2003).

Transcriptional silencing also occurs in tumors through mutation or deletions, but these changes are not easily targeted by pharmacologic intervention as the gene sequence itself is permanently altered. However epigenetic silencing, as occurs through deacetylation of histone proteins, is a reversible process. Importantly, epigenetic silencing involving histone deacetylation has been demonstrated to be active in the pathogenesis of AML and CLL (Moe-Behrens and Pandolfi 2003; Zhang, Freitas et al. 2004), suggesting potential use of HDAC inhibitors as therapeutic agents against AML and CLL. FK228 is one of the HDAC inhibitors currently under clinical development. Previous work demonstrated that FK228 promotes apoptosis in both primary CLL and AML tumor cells in vitro at concentrations corresponding to those at which H3 and H4 acetylation and HDAC inhibition occurs (Byrd, Shinn et al. 1999; Klisovic, Maghraby et al. 2003). These studies provide a strong rationale for pursuit of clinical trials with FK228 in AML and CLL.
Up to date, two phase I studies of FK228 have been completed in patients with refractory solid tumor malignancies (Marshall, Rizvi et al. 2002; Sandor, Bakke et al. 2002). While these trials established the safety of depsipeptide in solid tumors, they did not include patients with AML or CLL who have impaired marrow reserve and predisposition for infection.

In these trials, patients received escalating doses of FK228 by a 4-hr i.v. infusion. FK228 was found to follow a two-compartment pharmacokinetic model with apparent dose-independent pharmacokinetic parameters. However, no urinary excretion data was provided. Additionally, since the patient number at each dose level was small, large variations were observed for the fitted pharmacokinetic parameters, preventing further analysis and comparison. Furthermore, no clinical pharmacokinetics-pharmacodynamics correlation of FK228 was provided and such information is crucial for future clinical research. Therefore, in the current clinical trial, we conducted a FK228 pharmacokinetic study in AML and CLL patients receiving an i.v. infusion of FK228 at 13 mg/m². The plasma and urinary FK228 concentrations were determined by a validated HPLC/MS/MS method. Pharmacokinetic analysis in these patients was performed. The pharmacokinetic parameters obtained were compared with those obtained from the previous report (Sandor, Bakke et al. 2002). A pharmacokinetics-pharmacodynamics correlation was then performed with total histone H4 acetylation status as a surrogate marker.
4.3 Patients and methods

4.3.1 Formulation

FK228 was supplied by the Division of Cancer Treatment and Diagnosis, National Cancer Institute (DCTD, NCI) in a dual pack with special diluent. A sterile, single-use vial contained 10 mg of lyophilized FK228 and 20 mg of the bulking agent, povidone, USP. The second vial contained sterile diluent consisting of 20% ethanol, USP, in propylene glycol, USP. The dosing solution was prepared by first dissolving the FK228 with 2 mL of the special diluent. This provided a 5 mg/mL solution. This stock solution was further diluted with 0.9% Sodium Chloride Injection, USP, to a final concentration in the range of 0.02 to 0.1 mg/mL. The dilute solution is compatible with both glass bottles and polyvinyl chloride plastic (PVC) i.v. infusion bags and is chemically stable for at least 24 hr when stored at room temperature.

4.3.2 Patients

This study included two cohorts of patients. Cohort 1 included patients with CLL and small lymphoma as defined by the World Health Organization (WHO) classification (Genevieve, Delisle et al. 2001). These patients were required to have received at least one prior therapy that included fludarabine, cladribine, or pentostatin. Patients with contraindication to receiving purine analog therapy were allowed to enroll. Cohort 2 included patients with primary,
refractory, or relapsed AML (<1 year) who were not candidates for allogeneic or autologous stem cell transplant. In addition, previously untreated patients with poor risk leukemia, defined as having any of the following: age 65 years or older, poor risk cytogenetics as defined by Cancer and Leukemia Group B (CALGB) (Wetzler, Dodge *et al.* 1999), or poor candidates for aggressive chemotherapy, could enroll in this study without receipt of prior chemotherapy for AML. Patients in cohort 2 were also required to have a normal leukocyte count (equal to or below 10 x 10^9/L) or a stable count (equal to or below 40 x 10^9/L for greater than one week). Patients were allowed to receive hydroxyurea prior to starting therapy and during the first week of therapy, if clinically indicated to maintain the leukocyte count equal to or below 40 x 10^9/L.

Other eligibility criteria for patients in both cohorts included: age greater than 18, Eastern Cooperative Oncology Group (ECOG) performance status less than or equal to 2, total bilirubin less than or equal to 1.5 mg/dL, creatinine less than 2.0 mg/dL, and ALT/AST less than or equal to three times the upper limit of normal. Given the potential for cardiac toxicity with FK228, patients were required to have a cardiac ejection of at least 50% and no history of a myocardial infarction or unstable angina within the past six months. In addition, patients were required to have had no recent major surgery, radiotherapy or chemotherapy (except hydroxyurea) within 28 days of treatment, and could not have an active infection requiring oral or intravenous antibiotics. Patients with active autoimmune processes and HIV infection were also ineligible.
4.3.3 Dosing regimen

All patients underwent a screening assessment (history, physical examination, bone marrow aspirate and biopsy, cardiac ejection fraction, and electrocardiogram) within ten days of beginning treatment. FK228 (13 mg/m²) was administered intravenously through a central line over four hours on days 1, 8, and 15 of every four-week cycle. All patients received granisetron hydrochloride 2 mg (or equivalent) for anti-emetic prophylaxis. Other anti-emetic prophylaxis was administered based upon patient symptoms. All patients were treated for a minimum of two months in the absence of progression of disease or unacceptable toxicity. Extensive cardiac monitoring, including serial electrocardiograms, MUGA scans, troponin levels (pre and post-therapy) and non-invasive monitoring, were performed throughout treatment. Response was assessed using the previously reported criteria for CLL (Cheson, Bennett et al. 1988) and AML (Cheson, Cassileth et al. 1990).

4.3.4 Plasma and urine sample collection

Plasma samples were obtained at the following time points: pretreatment; 30, 60, 120, and 180 minutes prior to the end of infusion and 15, 30 minutes and 1, 3, 6, and 12 hrs after the end of infusion. Urine samples were collected during treatment and for 24 hours after completion of therapy.
4.3.5 FK228 determination using HPLC/MS

Depsipeptide concentrations in the plasma and urine samples were measured using the HPLC/MS/MS method as previously described in Section 3.3.2. This method provided a limit of quantification of 0.18 nM with 0.5 mL human plasma samples used.

4.3.6 Standard two-stage pharmacokinetic and statistical analysis

A standard two-stage clinical pharmacokinetics analysis (Sheiner 1984) was performed using a two-compartment model (EQ 4.1, WinNonlin, Version 4.0, model #9, Pharsight Corp., Mountain View, CA), in which the pharmacokinetic parameters of individual patients were first fitted by regression, and then the parameters of different patients were analyzed and the mean values and standard deviations were calculated when appropriate.

\[ C = A_1(e^{-\alpha t} - e^{-\alpha t'}) + A_2(e^{-\beta t} - e^{-\beta t'}) \]  

EQ 4.1

A detailed description of the pharmacokinetic model is included in Appendix Scheme A.2. The results were compared with those from another FK228 clinical trial (Sandor, Bakke et al. 2002), as well as our data from rat pharmacokinetic study after normalization (Chapter 2).

4.3.7 Histone Protein Acetylation and Immunoblot Studies

Histone protein acetylation was determined for the pharmacodynamics-pharmacokinetics correlation study. The determination was conducted in
collaboration Dr. Mark R. Parthun’s group (The Ohio State University). Blood cells were obtained from blood samples of patients before treatment and at 4 and 24 hours following FK228 administration. Histone proteins were isolated as previously described (Aron, Parthun et al. 2003). Total protein in each sample was quantified by the BCA method (Pierce, Rockford, IL). Lysates or extracted histone proteins were analyzed by SDS-PAGE/immunoblotting with antibodies specific for various histone acetylation or methylation sites (Upstate, Lake Placid, NY). Gel loading equivalence was confirmed by coomassie blue staining of gels. Species-specific secondary antibodies conjugated to horseradish peroxidase were purchased from BioRad (Hercules, CA). Blots were developed with chemiluminescent substrate (Pierce Super-Signal, Pierce) and autoradiography was performed using X-OMAT film (Kodak, Rochester, NY). Protein bands were quantified using a ChemiDoc instrument with Quantity One software (BioRad).

4.3.8 Pharmacokinetics-pharmacodynamics correlation

Pharmacokinetics parameters AUC, Cmax, Cl, Vdss and MRT were plotted against total acetylation of histone H3 and H4 as well as against acetylation of H4 on individual lysines K5, K12 and K8. Correlation coefficient $r^2$ was used an indicator of goodness of linear regressions.
4.4 Results

4.4.1 Clinical pharmacokinetics of FK228

The pharmacokinetics of Fk228 was studied in all twenty patients enrolled in this trial. Only samples following the four-hour i.v. infusion on Day 1 were determined for FK228 concentrations (Appendix Table A.2) and analyzed using WinNonLin. All the plasma concentration-time profiles are shown in Figure 4.1 and fitted with the two-compartment model with zero order input and first order elimination (Appendix Scheme A.1). Initially, pharmacokinetic parameters for the two patient populations (AML and CLL) were separately estimated, but were found to be statistically equivalent (Appendix Table A.3). Therefore, these two patient populations were grouped together, although the dose was slightly higher.

The results of this analysis is shown in Table 4.1, together with the results from a previous FK228 clinical trial (Sandor, Bakke et al. 2002). Our results confirmed a two-compartment pharmacokinetic model for FK228, but showed a lower clearance and a smaller Vdss as compared with the previous report, possibly due to differences between patient populations. However, due to the large standard deviations in the previous study, no statistically significant difference was found between these two studies. It is of note that our study had a larger number of patients and smaller standard deviations for the calculated pharmacokinetic parameters.
Figure 4. 1. FK228 plasma concentration-time profiles for patients 1-20.
Similar comparison between human and rat pharmacokinetics after body surface area normalization is shown in Table 4.2. In contrast to the high CL value in the rat (466 ± 72 L/hr/m²), which exceeded the rat cardiac output (193 L/hr/m²), patients showed much lower CL (4.81 ± 2.02 L/hr/m²), which is smaller than human cardiac output (336 L/hr with body weight 70 kg, or 186 L/hr/m² with body surface area of about 1.81 m² for a 70 kg, 170 cm man). A significantly larger Vdss in the rat (117 ± 29 L/m²) than that in the patients (3.29 ± 0.94 L/m²) was also observed.

4.4.2 Pharmacokinetics-pharmacodynamics correlation

Histone acetylation status of leukemia cells from 16 patients was determined in Dr. Mark R. Parthun’s laboratory. However, due to the assay sensitivity and sample availability, Histone H4 acetylation was determined in a subset of 11 patients (Table 4.3). A typical Western blot is shown in Figure 4.2. FK228 treatment caused a time-dependent change in histone acetylation status on specific lysine (K) residues. Increases in acetylation were noted in histone H4 K5, H4 K12, H4 K8 and histone H3 K9 as previously observed in our in vitro work (Aron, Parthun et al. 2003). Interestingly, we also detected increases in H3 K14 and H4 K16 acetylation that we had not previously seen. For PK-PD correlation purpose, relevant pharmacokinetic parameters (AUC, Cmax, CL and Vdss) for the corresponding 16 patients are summarized in Table 4.3. Histone acetylation status in leukemia cells from patients at 4 hr and 24 hr after initiation of the i.v.
infusion is summarized in Tables 4.4 and 4.5 respectively. Patient # 8 developed renal failure associated with abnormally high FK228 concentrations, and was excluded from the analysis.

The pharmacokinetic parameters were then plotted individually against specific and total histone lysine acetylation status. Significant correlations \((r^2 > 0.4)\) were found between the pharmacokinetic parameters and the total histone H4 acetylation at 4 hr and 24 hr (Figure 4.3 and 4.4). AUC and \(C_{\text{max}}\) showed normal correlation with histone H4 acetylation, while CL and \(V_{\text{dss}}\) showed an inverse correlation with total histone H4 acetylation. On the other hand, no correlation was found between total histone H3 acetylation and these pharmacokinetic parameters (Appendix Figure A.12 and A.13). Plotting of histone H4 acetylation on specific lysines showed that H4 K5 and H4 K16 were poorly correlated to the pharmacokinetic parameters. H4 K8 showed similar trend as compared with the total histone H4. Interestingly, H4 K12 showed the opposite correlation (Appendix Figure A.12 and A.13). The reason for this difference remains unclear.
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<th>Parameter</th>
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<td>Dose (mg/m²)</td>
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<td>AUC (µM · hr)</td>
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<tr>
<td>C_{max} (µM)</td>
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<td>CL (L/hr/m²)</td>
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<td>Vdss (L/m²)</td>
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<td>Uex, % dose</td>
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<td>T_{1/2, α} (hr)</td>
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<td>3.67 (2.3 – 6.5)</td>
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Table 4. 1. Comparison of relevant pharmacokinetic parameters of depsipeptide between current study and a previous phase I study (Sandor, Bakke et al. 2002).
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<th>Parameter</th>
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<td>1.39 ± 0.69</td>
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<td>Vdss (L/m²)</td>
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<td>T1/2, β (hr)</td>
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Table 4. 2. Comparison of relevant pharmacokinetic parameters of depsipeptide between patients and rats after normalization by the body surface area.
Figure 4. 2. Biologic effects of depsipeptide in CLL Patient Cells.

FK228 treatment caused a time-dependent increase in lysine-specific acetylation of H3 and H4 that is maximal at completion of treatment.
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Table 4. 3. Summary of relevant pharmacokinetic parameters for the pharmacokinetics-pharmacodynamics correlation.
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</tr>
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</tbody>
</table>

<sup>a</sup> Patient 5 developed renal failure and showed abnormal pharmacokinetic, pharmacodynamic profiles of FK228, and was dropped from the study.

Table 4. Summary of histone acetylation status in patients at the end of 4 hr infusion of FK228.

H3, histone H3 acetylation; H4, histone H4 acetylation; K, lysine residue.
Table 4. 5. Summary of histone acetylation status in patients at 24 hr after initiation of the 4 hr infusion of FK228.

H3, histone H3 acetylation; H4, histone H4 acetylation; K, lysine residue.
Figure 4. 3. Correlation between the pharmacokinetic parameters and the total histone H4 acetylation at the end of 4 hr i.v. infusion.

AUC and Cmax show an inverse correlation with the total histone H4 acetylation, while CL and Vdss show a normal correlation with the total histone H4 acetylation.
Figure 4.4. Correlation between the pharmacokinetic parameters and the total histone H4 acetylation at 24 hr after initiation of the 4 hr i.v. infusion.

Similar to the results shown in Figure 4.3, AUC and Cmax show an inverse correlation with the total histone H4 acetylation, while CL and Vdss show a normal correlation with the total histone H4 acetylation.
4.5 Discussion

4.5.1 Comparison of FK228 pharmacokinetics

Comparison between our clinical pharmacokinetic study with the previous report (Sandor, Bakke et al. 2002) revealed some differences in the calculated pharmacokinetic parameters. However, due to the small patient number for each dose level in the previous study, it is reasonable to believe that our results, which are associated with larger population and much smaller deviations, should provide better estimates of the pharmacokinetic parameters.

Although it is generally believed that small animals eliminate drugs faster than human, the dramatic FK228 CL difference between in rats (466 ± 72 L/hr/m²) and in patients (4.81 ± 2.02 L/hr/m²) after body surface area (BSA) normalization is still unexpected (Table 4.2). The reason for this discrepancy is yet to be studied.

4.5.2 Clinical evidence for the prodrug theory of FK228

As discussed in Chapter 3, typical reversible HDAC inhibitors (i.e., Trichostatin A and SAHA) possess a long aliphatic tail with a nucleophilic end, such as –SH or –OH. The long aliphatic tail acts as a spacer, and the nucleophilic end interacts with the active zinc ion located deep in the catalytic pocket of HDACs (Finnin, Donigian et al. 1999). FK228, on the other hand, has a
unique bicyclic structure and its nearly spherical structure does not appear to fit into the catalytic pocket.

In Chapter 3, we demonstrated formation of at least 3 active metabolites of FK228 from \textit{in vitro} blood incubation. However, there has not been \textit{in vivo} evidence to support FK228 being a prodrug. Our pharmacokinetics-pharmacodynamics correlation in the patients revealed existence of significant inverse correlation between FK228 system exposure (AUC) and total histone H4 acetylation status with $r^2 = 0.7768$ and 0.5529 for 4 hr and 24 hr after initiation of the infusion, respectively. On the other hand, total histone H4 acetylation status was found proportional to CL with $r^2 = 0.7751$ and 0.9137 at the two sampling times, respectively. These clinical correlations suggest that FK228 is a prodrug and FK228 bioactivation is a major pathway of FK228 elimination.
CHAPTER 5

POPULATION-BASED PHARMACOKINETICS OF FK228 IN AML AND CLL PATIENTS

5.1 Abstract

This study aimed to develop a population pharmacokinetic (popPK) model for FK228 analysis, so as to examine the correlation of its pharmacokinetics (PK) with clinical covariates and to improve prediction of FK228 PK profiles in patients under different physiological conditions. Plasma concentration-time data of FK228 obtained in Chapter 4 was used in this study. A two-compartment model with zero order input and first order elimination from the central compartment was chosen as the basic structural model. During model development, BSA did not show a significant correlation with V1 or CL as indicated by the objective function and scatter plots, and was therefore not included in the final popPK model. The final pharmacokinetic model, incorporating the covariates sex and RBC counts, showed significant improvement in the objective function. However, its prediction performance was unsatisfactory, and this could be due to the limited data size or a lack of correlation between pharmacokinetic parameters and covariates. At the
same time, there may exist other determinants of FK228 pharmacokinetics such as the RBC intracellular GSH level.
5.2 Introduction

Depsipeptide FK228 is a potent HDAC inhibitor with selective in vitro cytotoxicity against cancer cells (Byrd, Shinn et al. 1999). FK228 was evaluated in several phase I clinical trials against various malignancies (Marshall, Rizvi et al. 2002; Sandor, Bakke et al. 2002). Patients were given FK228 by a 4-hr i.v. infusion. The maximum tolerated dose (MTD) was found to be either 13.3 mg/m² when FK228 was given on Days 1, 7, and 21 of a 4-week cycle (Marshall, Rizvi et al. 2002) or 17.8 mg/m² when patients received FK228 on Days 1 and 5 of a 21-day cycle (Sandor, Bakke et al. 2002). The dose-limiting toxicities included thrombocytopenia, fatigue, nausea, vomiting, and cardiac arrhythmia.

In the previous clinical pharmacokinetic study (Sandor, Bakke et al. 2002), FK228 followed a dose-independent two-compartment pharmacokinetic model at doses ranging from 1 to 24.9 mg/m². However, patients showed large variation in FK228 plasma concentrations and calculated pharmacokinetic parameters.

In our current clinical trial, a total of 20 AML and CLL patients received a 4-hr i.v. infusion of FK228 at a dose of 13 mg/m², and FK228 pharmacokinetics was studied. The dose calculation was under the assumption that FK228 clearance values in patients were proportional to BSA values of patients. One patient, who developed renal failure, showed significantly higher FK228 plasma concentrations (Patient 5). Leukemia cells from the same patient also showed
abnormal histone acetylation status (Tables 4.4 and 4.5). Pharmacokinetic data from this patient was excluded from the PK/PD analysis in Chapter 4. The large pharmacokinetic variation among patients and treatment-associated side effects (i.e., acute or progressively worsening chronic nausea, anorexia, and fatigue) would suggest the use of real-time pharmacokinetic studies to identify the FK228 dose in the second or later courses. While real-time pharmacokinetics may provide adequate information to explain and predict FK228 exposure and possible side effects in patients, it is costly, time-sensitive, and in most cases requires an initial sub-optimal dose since FK228 pharmacokinetics in the particular patient is unknown. Due to its time-sensitive nature, this method cannot be readily implemented in settings where a clinical pharmacokinetic facility with rapid turnover is not available.

Population pharmacokinetics (popPK) is the study of the sources of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of a drug of interest (Aarons 1991). PopPK, a rapidly evolving area of drug development and regulation, has been widely used in analysis of pharmacokinetics, safety and efficacy of drugs during clinical trials. The advantages of popPK strategy have been recognized by US Food and Drug Administration (FDA), and ‘Guideline Industry Population Pharmacokinetics’ was issued by FDA in 1999 (available at http://www.fda.gov/cder/guidance/1852fnl.pdf).
In a popPK study, certain patient demographical, pathophysiological, and therapeutical features, such as age, sex, body weight, excretory and metabolic functions, and the presence of other therapies, can regularly alter dose-concentration relationships. PopPK seeks to identify the measurable pathophysiologic factors (so called covariates) that cause changes in the dose-concentration relationship and the extent of these changes. If such changes are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified. Using the popPK approach in drug development offers the possibility of gaining integrated information of pharmacokinetics, not only from relatively dense data obtained from subjects (i.e., phase I trials), but also from relatively sparse data or a combination of sparse and dense data (i.e., phase III trials). The population PK approach allows the analysis of data from a variety of unbalanced designs as well as from studies that are normally excluded, such as concentration data obtained from pediatric and elderly patients, or data obtained during the evaluation of the relationships between dose/concentration and efficacy/safety.

The objective of this study was to first identify the sources of the variability, identify clinical covariates that affect FK228 pharmacokinetics and then to develop a popPK model, that might be used to predict FK228 pharmacokinetics in previously untreated patients. For model validation purpose, a standard cross-validation was used, where patients were randomly divided into 10 groups (2 in each), and the popPK model, obtained from 18 patients, was used to predict pharmacokinetic model parameters for the remaining two
patients. This procedure was repeated for all 10 combinations. Bias and precision of parameter prediction for the final popPK models were compared with the results from individual fitting.

5.3 Materials and methods

5.3.1 Patient treatment protocol

Ten AML patients and ten CLL patients were enrolled in this phase I trial. Patient eligibility and exclusion criteria, and treatment protocol were described in Chapter 4. These studies were approved by both the Cancer Therapy Evaluation Program (CTEP) at the National Cancer Institute, and by the Institutional Review Boards (IRBs) of The Ohio State University. The patients received a 4-hr i.v. infusion at a dose of 13 mg/m$^2$ through a central line. Plasma samples were obtained at the following time points: pretreatment; 30, 60, 120, and 180 minutes prior to the end of infusion and 15, 30 minutes and 1, 3, 6, and 12 hrs after the end of infusion. FK228 concentration-time data are included in Appendix Table A.2.

5.3.2 Pharmacokinetic analysis

As detailed in Chapter 4, the pharmacokinetic data of FK228 was previously analyzed by the standard two-step method using WinNonLin version 4.0 (Pharsight Corporation, Mountain View, CA). The results indicated that FK228 disposition was adequately described with a two-compartment model with FK228 eliminated from the central compartment.
5.3.3 Population-based pharmacokinetic analysis

Plasma concentration-time data from all 20 patients were used to establish the popPK model. PopPK analysis was performed using WinNonMix version 2.0.1 (Pharsight Corporation, Mountain View, CA) to identify the sources of inter-individual variability in pharmacokinetic parameters in a stepwise manner. The first step was to define the appropriate base model consisting of a structural model and an error model. The second step was to screen covariates contributing to inter-individual variability and develop a full model. Covariates are patient specific factors, such as weight, age and gender that might affect the pharmacokinetics of a drug. Thirdly, to ascertain that the selected covariates were the critical determinants of the inter-individual variability and to eliminate redundant covariates (i.e., covariates that are highly correlated with each other but do not contribute to reducing variability), a backward elimination procedure was performed by determining whether elimination of individual covariates affected the performance of the full model. Only the covariates whose elimination resulted in significant deterioration in the model performance were included in the final model. These steps are detailed as follows.

5.3.4 Model building: Basic models

The structural model was a two-compartment open model with zero-order i.v. infusion input and first-order elimination from the central compartment.

\[ C_{i,j} = A_j \cdot (e^{-\alpha_i} - e^{-\alpha_j}) + B_j \cdot (e^{-\beta_i} - e^{-\beta_j}) \]  

EQ 5.1
where $C_{i,j}$ is the predicted plasma concentration at a particular time $i$ for a patient $j$. Detailed description of this model is included in Appendix Scheme A.2. This pharmacokinetic model has been compiled as pharmacokinetic model 9 in WinNonMix software.

In WinNonMix analysis, random inter-individual deviations of pharmacokinetic parameters between model-predicted and observed data (individual fitting) are described by the first-level error ETA (or $\eta$). Equations 5.2-5.5 define the first-level error terms used in the current study. The reason that each pharmacokinetic parameter had an $\eta$ is justified by the ‘rich’ nature of the data set, where each patient has 12 observations of FK228 plasma concentrations in the present study. This enabled estimation of all four pharmacokinetic parameters for each patient.

\[
CL_j = \hat{CL} \cdot \exp(\eta_{\hat{CL}}) \quad \text{EQ 5.2}
\]

\[
V1_j = \hat{V1} \cdot \exp(\eta_{\hat{V1}}) \quad \text{EQ 5.3}
\]

\[
V2_j = \hat{V2} \cdot \exp(\eta_{\hat{V2}}) \quad \text{EQ 5.4}
\]

\[
Q_j = \hat{Q} \cdot \exp(\eta_{\hat{Q}}) \quad \text{EQ 5.5}
\]

where left-side variables $CL_j$, $V1_j$, $V2_j$ and $Q_j$ are observed values (individual fittings) of total body clearance, central compartment distribution volume,
peripheral compartment distribution volume, and inter-compartment clearance in
patient \( j \), respectively. And the \( \hat{CL} \), \( \hat{V}_1 \), \( \hat{V}_2 \) and \( \hat{Q} \) on the right side are the
predicted typical values in the population. Lognormal distribution was assumed
for all four pharmacokinetic parameters to guarantee a non-negative distribution.
This is defined mathematically by using the exponential error terms \( \exp(\eta) \),
where the \( \eta \) values in this structure model are inter-individual variability normally
distributed around a mean of zero with a variance of \( \omega^2 \). Decreasing the \( \omega^2 \)
values by incorporating covariates is a direct goal of popPK analysis.

Even with the inclusion of \( \eta \)s to account for inter-individual errors, the
predicted observations (plasma concentrations of FK228) in the present study
still showed deviation as compared with the model predicted values. This kind of
second-level intra-individual errors is depicted by EPS (or \( \varepsilon \)) as shown in EQ 5.6.

\[
Y_{ij} = \hat{C}_{ij} \cdot (1 + \epsilon_{ij})
\]

EQ 5.6

where \( Y_{ij} \) and \( \hat{C}_{ij} \) are the observed and predicted concentrations of FK228 of the
\( j^{th} \) individual at the \( i^{th} \) sampling time. In the error term, \( \epsilon_{ij} \) is the residual error
normally distributed with a mean of zero and a variance of \( \sigma^2 \). The specific error
form as defined by EQ 5.6 is called a Constant Coefficient of Variation (CCV)
model, assuming the variance of an observation error (\( V_{error} \)) is proportional to the
squared prediction \( (\hat{C}_{ij})^2 \) with \( \omega^2 \) being the proportionality factor (i.e., \( V_{error} = \).
\[ \omega^2 \cdot C_{ij}^2. \] This follows that the coefficient of variation (CV) of the error is equal to a constant value of \( \omega \) since \( CV = \sqrt{V_{error} / C_{ij}}. \)

5.3.5 Model building: Identification of significant covariates

The next step is to identify covariates that could account for the inter-individual variability in FK228 pharmacokinetics. Previously, we plotted CL, AUC, C\text{max}, V\text{d}_{ss} and MRT values obtained by individual fitting (Chapter 4) against available covariates, such as sex (1 for male and 0 for female), age, body weight, body surface area, creatinine clearance, serum albumin level, serum glucose level, and red blood cell count (Appendix Table A.4). Linear regression analysis was first used to examine the relationships between the pharmacokinetic parameters and the potential covariates. However, no significant correlation was found for any covariate \( (r^2 > 0.4) \). This may be because of either a lack of correlation or the concomitant effects of multiple covariates that mask the tested correlation. For example, increasing plasma creatinine level and increasing BSA may have conflicting effects on the CL. At the same time, it is important to realize that even though a high \( r^2 \) usually indicates a good correlation, the reverse is not valid and \( r^2 < 0.4 \) does not necessarily mean a poor correlation.

For this reason, available covariates were used as carryalong variables during the WinNonMix fitting using the base model. The chart output of ‘individual \( \eta \) vs carryalong variable’ was used to identify potential correlations between covariates and pharmacokinetic parameters. These plots are especially useful
since they not only predict whether inclusion of a covariate into the popPK model would reduce the residual errors ($\eta$) by the plotted covariate, but also show whether there is room for model improvement with other covariates after inclusion of this particular covariate. A non-zero slope suggests a potential correlation. The promising covariates were added into the base model one by one. The covariates were kept in the model only if they showed improvement in the objective function, variance of estimated parameters and scatter plots in the output charts. The model that contains all these covariates is called the full model.

The objective function ‘-2 log likelihood’ (-2LL) as defined in EQ 5.7

$$-2 \times \log(L) = n \cdot \log(2\pi) + \sum_{i=1}^{n} \left[ \log(\sigma^2_{i}) + \frac{(Y_i - \hat{Y}_i)^2}{\sigma^2_{i}} \right]$$

EQ 5.7

where $L$ is the likelihood (probability) of the observation, $\sigma^2$ is the variance of the model as defined earlier, $n$ is the number of observations, $Y_i$ is an observation, and $\hat{Y}_i$ is the predicted value. Equation 5.8 gives an example of the incorporation of a covariate into the model to account for the variability of CL.

$$\hat{CL} = a + b \cdot \text{Covariate}$$

EQ 5.8

where $a$ and $b$ are fixed effect parameters ($\theta$s), and the covariate is proportional to the typical value of CL ($\hat{CL}$). To determine whether a covariate offered
improvement over the base model, the log likelihood ratio test as defined by EQ 5.9 was used. The log(L1/L2) follows a $\chi^2$ distribution with degree of freedom being the difference in the number of parameters between two nested models under comparison. A nested model is obtained from another by fixing one parameter. In this case, the base model is a nested model of a single-covariate model such as EQ 5.8.

$$-2 \times \log(L1/L2) = -2 \times [\log(L1) - \log(L2)] \quad \text{EQ 5.9}$$

A reduction in the objective function value of more than 3.9 is associated with $p < 0.05$ for a decrease in degree of freedom (df) by 1 or addition of a single covariate according to EQ 5.8, and is required for inclusion of the covariate into the popPK model. Here, the difference in df between the two models under comparison is ‘1’, since ‘df = number of data points – number of parameters’ for each model, and therefore addition of a single covariate results in a decrease of df by ‘1’. The full model included all covariates that met the above requirement.

To ascertain if each of the conceived covariates play an indispensable role in the model performance, any insignificant covariate should be removed in a backward elimination process in order to obtain the final model. In this process, a more restrictive criterion was used. To eliminate a parameter from the full model, an increase of the objective function of more than 7.9 after setting that parameter to zero (elimination of the covariate term) was required ($\chi^2$ value associated with $p < 0.005$ with 1 degree of freedom). The final model was established using data from all 20 patients.
5.3.6 Validation of the final model

The performance of the final popPK model was examined by a standard cross-validation as specified in “Guidance for Industry Population Pharmacokinetics” issued by US FDA in 1999 (available online at http://www.fda.gov/cder/guidance/index.htm). For this purpose, the 20 patients were randomly divided into 10 groups (2 in each). Final model with parameters determined from 18 patients were used to predict the pharmacokinetic parameters for the remaining two patients. This procedure was repeated for each of the ten combinations so that predicted parameters were calculated for each patient using the final model. Correlation plots between the observed (individual fitting) and the popPK model predicted values were used to test the prediction performance of the final model. Since the SD% of observed V1 and CL values among the patients were rather small (<15%, Table 5.4). The final popPK model, if successful, is expected to give prediction error less than 10%.

5.4 Results

5.4.1 Model building process

Table 5.1 summarizes the demographic and clinical characteristics of the 20 patients receiving depsipeptide FK228 by a 4-hr i.v. infusion. Data from all 20 patients was used for development of the popPK model. Figure 5.2 shows results from fitting the data using the base model. Population prediction showed much higher deviation in concentration from the observed values as compared with that
of the individual prediction. This suggested an improvement window for the popPK analysis.

Using the strategy described earlier, two covariates for V1 were identified. Incorporation of BSA as a covariate in the V1 equation (EQ 5.8) decreased the objective function by 3.85. Similarly, sex was found to affect V1. Inclusion of sex in V1 (EQ 5.8) showed a promising drop in the objective function by 6.13, where the categorical variable sex was set equal to 1 for males and 0 for females. Several covariates were found to improve prediction of CL. BSA, creatinine level, and RBC, when individually added to the model caused a decrease in the objective function by 5.82, 9.79 and 12.57, respectively. No covariate model was tested for the other two pharmacokinetic parameters, V2 and Q. These two have less physiological meaning, making any apparent correlations with covariates are difficult to interpret physiologically. However, the rich data set allowed fitting of an individual $\eta$ for each pharmacokinetic parameter, thus estimation of individual parameterization of all 4 pharmacokinetic parameters was achieved. Some representative covariate plots are included in Figure 5.2.

It was noticed that some of the covariates were correlated with each other, since incorporation of one covariate might exclude another with the objective function as a criterion. For example, plasma creatinine level, when added as a covariate for CL, showed a decrease in the objective function by 7.9, but caused a minor change in the objective function after ‘Sex’ was included in the V1 term. Additionally, since there may be correlation between pharmacokinetic
parameters (e.g., CL and V1), one covariate may show correlation to more than one parameter. For example, plasma creatinine decreased the objective function significantly when included in either CL or V1. While with the aid of the 'individual \( \eta \) vs carryalong variable' plots, it was apparent that incorporation of BSA to one of the two pharmacokinetic parameters diminished the correlation for the other.
<table>
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<th></th>
<th>Normal (range)</th>
<th>Population data set n = 20 Mean (range)</th>
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</tr>
<tr>
<td><strong>Females</strong></td>
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<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>62 (47 – 81)</td>
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<tr>
<td>Weight (kg)</td>
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<td>172 (155 – 185)</td>
</tr>
<tr>
<td>BSA (m²)</td>
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<td>Serum Creatinine (mg/dL)</td>
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<td>1.07 (0.7 – 1.4)</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
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<td>3.8 (3.4 – 4.4)</td>
</tr>
<tr>
<td>White cell count (x 10⁹/L)</td>
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<td>42 (0.8 – 306)</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>13 – 18</td>
<td>10.6 (8.3 – 13.9)</td>
</tr>
<tr>
<td>Platelets (x 10⁹/L)</td>
<td>150 – 400</td>
<td>85 (10 – 232)</td>
</tr>
<tr>
<td>FK228 CL (L/hr/m²)</td>
<td>NA</td>
<td>4.7 (1.7 – 8.6)</td>
</tr>
<tr>
<td>Vdss (L/m²)</td>
<td>NA</td>
<td>3.9 (1.6-12.2)</td>
</tr>
</tbody>
</table>

Table 5.1. Summary of demographic and clinical characteristics and FK228 pharmacokinetics in patients.
Figure 5.1. Plots of observed concentrations versus predicted concentrations using the base model.

(a) Predicted concentrations are based on pharmacokinetic parameters of population mean. (b) Predicted concentrations are based on pharmacokinetic parameters of individual patients.
Figure 5. 2. Some scatter plots of $\eta$'s of V1 and CL against covariates.

Data was first fit to the base model. ETA ($\eta$) values of CL1 and V1 were then plotted against a list of covariates. Even though FK228 dose was normalized to patient BSA, BSA only shows minor correlation with CL$\_\text{ETA}$. 
A full model including promising covariates was developed. Fixed effect parameters (θs) were used to define the pharmacokinetic parameters required in the model (Table 5.2). Four ηs were included in the model. Simplification of the full model was carried out by eliminating the covariates that did not significantly contribute to the model performance. As shown in Table 5.2, elimination of the coefficient of BSA (θ3) or the error term for Q (ηQ) resulted in minimal change in the objective function, indicating that BSA and ηQ should be dropped from the model. On the other hand, fixing any other parameters to zero resulted in dramatic increases in the objective function. Thus the final popPK model was developed with the parameter estimates and the estimated SE% summarized in Table 5.3. Compared with the base model, the final model has two more fixed effect parameters (θs), but one less ω error term associated with inter-individual variation η for the inter-compartment clearance Q. So the difference in degree of freedom (df = 1) between these two models is one. The final model causes a decrease in the objective function by 32 (p < 0.005 for ∆df = 1), indicating a significant improvement in data fitting. In addition, allowing WinNonMix to estimate covariance among ηs rather than fixing the off-diagonal covariance to zero offered a further decrease in the objective function by 22. For this reason, a full variance-covariance matrix was added to the final model.
5.4.2 Validation of the final model

The final model had reasonably small SE% for the fixed-effect model parameter ($\theta$s) and random effect parameters ($\omega^2$s and $\sigma^2$). However, the overall performance of the final model did not seem to account for inter-individual variability as suggested by Figure 5.3 in comparison with the base model (Figure 5.1). This mandated a validation to test the prediction performance of the final model. As described in Section 5.3.6, a standard cross-validation was performed for this purpose, and the results are graphically shown in Figure 5.4. Despite a significant decrease in the objective function, the popPK model predicted values still differ significantly from individually fitted values of the pharmacokinetic parameters. Consistently, Table 5.4 shows some severe biases in prediction. Only V1 and CL were tested in this validation because V2 and Q had no covariate in their equations. The V1 values predicted by the final popPK model gave a reasonable averaged error% of 12.65%. While this is mainly because of the narrow range of V1 among the population (SD% = 12.65%), rather than accurate prediction by the model. CL values predicted by the final popPK model showed a large averaged error% of 33.17%, especially for patients 5 and 9. All these indicate that the popPK model developed from the available data cannot be used for prediction of FK228 exposure in patients.
Full Model

\[
\begin{align*}
CL &= (\theta_1 + \theta_2 \times \text{RBC} + \theta_3 \times \text{BSA}) \times \exp(\eta_{CL}) \\
V1 &= (\theta_4 + \theta_5 \times \text{SEX}) \times \exp(\eta_{V1}) \\
Q &= \theta_6 \times \exp(\eta_{Q}) \\
V2 &= \theta_7 \times \exp(\eta_{V2}) \\
Y_{ij} &= C_{ij} \cdot (1 + \varepsilon_{ij})
\end{align*}
\]

<table>
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<tr>
<th>Fixed parameters</th>
<th>Increase in the objective function</th>
<th>P values</th>
</tr>
</thead>
<tbody>
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<td>NA</td>
</tr>
<tr>
<td>(\theta_1 = 0)</td>
<td>30</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>(\theta_2 = 0)</td>
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<td>&lt; 0.005</td>
</tr>
<tr>
<td>(\theta_3 = 0)</td>
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</tr>
<tr>
<td>(\theta_4 = 0)</td>
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<td>&lt; 0.005</td>
</tr>
<tr>
<td>(\theta_5 = 0)</td>
<td>20.5</td>
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</tr>
<tr>
<td>(\eta_{CL} = 0)</td>
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<td>&lt; 0.005</td>
</tr>
<tr>
<td>(\eta_{V1} = 0)</td>
<td>319</td>
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Table 5.2. Development of the full population pharmacokinetic model.

Fixed effect parameter \(\theta_3\) and random error parameter \(\eta_{Q}\) should be dropped from the model based on changes in the objective function \(p>0.05\). The fixed effect parameters \(\theta_6\) and \(\theta_7\) cannot be dropped since they stand for the population mean values of \(Q\) and \(V2\), respectively.
The final model includes two covariates sex and RBC, and causes a decrease in the objective function by 54 as compared with the base model with $\Delta \text{df} = 1$. 

Table 5.3. Development of the final population pharmacokinetic model.
Figure 5. 3. Performance of the final model developed with pharmacokinetic data from all 20 patients.

(a) The circled points are severely underestimated and cannot be accounted for by adding covariates largely due to abnormally high concentrations from Patients (b) 5 and (c) 9. Patient 5 developed renal failure after FK228 treatment.
The prediction performance of the final popPK model was tested by a cross-validation. The pharmacokinetic parameters, (a) V1 and (b) CL, were predicted by the final popPK model, and plotted against the parameter values obtained by individual fitting. The overall prediction performance of the final model was unsatisfactory.

Figure 5.4. Cross-validation of the final population model.
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\[ a \text{ Prediction error}\% = \left( \frac{\text{Individually predicted value} - \text{Final model predicted value}}{\text{Individually predicted value}} \right) \times 100\%; \]

\[ b \text{ The average of the absolute values of error}\% \]

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\(^a\) Prediction error\% = (Individually predicted value – Final model predicted value)/Individually predicted value × 100%;
\(^b\) The average of the absolute values of error\% 

Table 5.4. Summary of prediction performance of the final population pharmacokinetic model using cross-validation.

V1 and CL values predicted by the final model show higher SD\% than those from individual fitting. In addition, the final popPK model gave averaged error\% values of 12.65\% and 33.17\% for V1 and CL, respectively. Note the significant overestimation of V1 and CL are found for patients 5 and 9.
5.5 Discussion

Pharmacokinetics analysis conducted in Chapter 4 showed that abnormal pharmacokinetic profiles appear to be associated with toxicities, sometimes life threatening. For example, data from Patient 5 who developed renal failure showed a much higher AUC (14 $\mu$M·hr) than the population mean (6 $\mu$M·hr). The current study aimed to identify clinical covariates that can be used to explain and predict FK228 pharmacokinetic profiles in patients receiving a 4 hr i.v. infusion of FK228 at 13 mg/m$^2$. Successful development of the popPK model was expected to provide statistical support to justify the BSA-based FK228 regimen, and to maximizing its anticancer effect while minimize associated toxicities.

Even though the optimized final popPK model significantly improved the model fitting as evidenced by the decreases in the objective function, its predictive performance was found to be unsatisfactory as indicated by the cross-validation.

Unexpectedly, BSA did not show significant correlation to CL or V1, and was therefore not a determinant for FK228 pharmacokinetics in patients. This suggests that the current BSA-based FK228 dosing, under the assumption that CL of FK228 in patients is proportional to the patient body surface area, may not provide accurate dose estimation for targeted FK228 exposure.
We have previously suggested that the GSH level in RBC may be a determinant for FK228 elimination (Chapter 3), and RBC counts did show promising correlation with CL. However, GSH levels may vary in patients with the same RBC counts. Collection of new clinical information from patients, such as the GSH level in RBC, in future FK228 clinical trials may provide a chance for performing further popPK study with FK228.
CHAPTER 6

FK228 TRANSPORT AND UPTAKE

6.1 Abstract

Depsipeptide FK228, a novel histone deacetylase (HDAC) inhibitor, was previously reported to be a P-glycoprotein (Pgp, MDR1) substrate. We now expand the investigation to demonstrate whether FK228 is a substrate for both Pgp and multidrug resistance-associated protein 1 (MRP1). Transport of FK228 across the Caco-2 cell monolayer in both apical to basolateral (AP→BL) and basolateral to apical (BL→AP) directions in the absence and presence of Pgp and MRP inhibitors was investigated. An in vitro uptake study in human red blood cells (RBC) and a cytotoxicity assay in MRP1(-) HL60 and MRP1(+) HL60Adr cells were conducted and the data showed that FK228 is a MRP1 substrate. FK228 showed a nearly unidirectional flux across the Caco-2 cell monolayer, with the BL→AP apparent permeability coefficient 32x that of AP→BL without apparent saturation. Both Pgp and MRP inhibitions decreased the BL→AP $P_{\text{app}}$ and increased the AP→BL $P_{\text{app}}$. Human RBC showed a concentration-dependent uptake and a saturable efflux of FK228. HL60Adr cells were 4-fold more resistant to FK228 than HL60 cells and the resistance was reversed by MRP inhibition.
Transport of FK228 across the Caco2 monolayer in the presence of MRP inhibitors further suggested FK228 being a substrate for other MRP transporters (i.e., MRP2). In conclusion, FK228 is a substrate for Pgp and MRP1, both of which are associated with FK228 resistance.
6.2 Introduction

Depsipeptide FK228, formerly FR901228 and NSC 630176 (Fig. 1), is a novel, naturally occurring bicyclic peptide with a disulfide linkage isolated as a fermentation product of *Chromobacterium violaceum* (Shigematsu, Ueda *et al.* 1994; Ueda, Manda *et al.* 1994; Ueda, Nakajima *et al.* 1994). It was originally developed as an anti-ras compound (Ueda, Nakajima *et al.* 1994; Wang, Brunner *et al.* 1998), later found to interfere with mitogen induced signaling pathways (Rajgolikar, Chan *et al.* 1998; Sandor, Robbins *et al.* 2000; Sandor, Senderowicz *et al.* 2000), and more recently has been found to be a potent histone deacetylase (HDAC) inhibitor (Nakajima, Kim *et al.* 1998; Yoshida and Horinouchi 1999; Yoshida, Furumai *et al.* 2001). Because of its observed preclinical antitumor activity, selectivity, and passage in its preclinical toxicology evaluation, FK228 has entered into several phase I clinical trials against various refractory cancers (Marshall, Rizvi *et al.* 2002; Sandor, Bakke *et al.* 2002), and now being planned into phase II clinical trials.

Preclinical pharmacokinetics studies in rats showed a low oral bioavailability of FK228 (Chan, Bakhtiari *et al.* 1997; Li and Chan 2000). Later, it was reported that FK228 is a P-glycoprotein (Pgp) substrate, but not a Pgp inhibitor, during a NCI screening project (Scala, Akhmed *et al.* 1997). However, due to the screening nature of the study, no kinetic or quantitative transport data of FK228 was reported.
Multidrug resistance-associated protein 1 (MRP1) is highly expressed in RBC membrane, responsible for the efflux of oxidized GSH (GSSG) to maintain a reducing intracellular environment (Rychlik, Pulaski et al. 2000). MRP1 is also associated with drug resistance and functions as an efflux pump in cancer cells for drug GSH conjugates and a variety of structurally unrelated drugs, including some neutral compounds (Paul, Belinsky et al. 1996; Rychlik, Pulaski et al. 2000; Wijnholds 2002). The overlap of substrates between Pgp and MRP1 (van Zuylen, Nooter et al. 2000) suggests the possibility of FK228 being a MRP1 substrate. Moreover, expression of MRP2 on the apical membrane of the Caco 2 monolayer (Hirohashi, Suzuki et al. 2000; Sun, Lennernas et al. 2002; Cooper, Moore et al. 2004) provides a model to investigate if FK228 shares affinity for MRP2.

Herein, we aimed to further characterize Pgp and/or MRP1 mediated FK228 transport and uptake kinetics using multiple models, including the Caco 2 cell monolayer, human RBC (Rychlik, Pulaski et al. 2000), and Pgp(-)/MRP1(-) HL60 and Pgp(-)/MRP1(+) HL60Adr cell lines (Gollapudi and Gupta 1992).

6.3 Experimental procedures

6.3.1 Chemicals and reagents

Non-formulated FK228 (purity >99%) was supplied by the Drug Synthesis and Chemistry Branch, the National Cancer Institute (Bethesda, MD) and used without further purification. N-t-Boc-Met-Leu-Phe (BMLP, purity >97%) and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO). Potassium phthalate buffer (pH 4, 50 mM) was obtained from Van Water and
Rogers Scientific (Chicago, IL). MK571, a MRP inhibitor, was purchased from BioMol Research Lab., Inc. (Plymouth Meeting, PA). Cyclosporin A (CsA) and (±)-verapamil hydrochloride (Ver) and Lucifer Yellow CH were purchased from Sigma. All organic solvents were obtained from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. HPLC-Grade water (>18 mΩ) was generated with an E-pure water purification system (Barnstead, Dubuque, IA). RPMI 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM) non-essential amino acids (NEAA), sodium penicillin G, Streptomycin, HEPES buffer, Hanks’ balanced salt solution (HBSS), D-PBS and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY).

6.3.2 FK228 transport and uptake

6.3.2.1 Cell culture

Caco 2 and HCT-15 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HL60 and HL60Adr cells were a generous gift from Dr. Hans Hinderman, Roswell Park, NY. K562/Dox cell line was a gift from J.P. Marie, INSERM, E9912, University of Paris 6, France. LLC-PK1 and LMDR1 cells were a generous gift from Dr. James Dalton, College of Pharmacy, the Ohio State University. Caco 2 cells were cultured in high glucose DMEM, supplemented with 10% FBS, 25 mM HEPES, 0.1 mM NEAA, 4 mM L-glutamine, 100 U/mL sodium penicillin G and 180 μM streptomycin. MRP1(-) HL60, MRP1(+) HL60Adr, MDR1(-) K562 and MDR1(+) K562/Dox cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL sodium penicillin G
and 180 µM streptomycin. LLC-PK1 and LMDR1 cells were cultured in M119 medium containing 10% FBS, 100 U/mL sodium penicillin G and 100 µg/mL streptomycin. Cells were grown in 75-cm² tissue culture flasks at 37°C in a 5% CO₂ atmosphere, and the culture medium was replaced every other day. The adhesive cells were harvested at 80% confluence by exposure to a trypsin-EDTA solution (0.25% trypsin and 0.002% EDTA in Hanks’ balanced salt solution). For transport studies, Caco-2 Cells of passages 44 - 46 were seeded on collagen-coated 6-well Transwell inserts (0.4-µm pore size, 4.7 cm² growth area, Corning Costar Corp., Cambridge, MA) at densities of 5×10⁴ cells/cm². The medium was changed every other day for the first week and every day thereafter until the transport study (24-27 days post-seeding).

6.3.2.2 FK228 transport protocols

The Caco-2 cell monolayers were washed twice with warm (37°C) Dulbecco’s phosphate-buffered saline (D-PBS, 1×, pH 7.4). For FK228 transport studies without Pgp inhibition, the D-PBS was replaced by the warm HBSS bathing solution (1× HBSS containing 25 mM D-glucose and 10 mM HEPES, pH 7.4). The volumes of HBSS bathing solution at the apical and the basolateral sides of the cell layer were 1.5 mL and 2.5 mL, respectively. The Transwell plates were returned to the incubator to equilibrate at 37°C for 30 min. The HBSS bathing solution was removed and the Transwell inserts were transferred to a clean 6-well plate. Warm HBSS bathing solution alone or HBSS bathing solutions containing FK228 at appropriate concentrations were added to the acceptor and
donor chamber, with 1.5 mL on the apical side and 2.5 mL on the basolateral side, respectively, followed by incubation of the plate at 37°C. A 200 µL sample was taken from the acceptor side at pre-selected time points of 0, 15, 30, 45, 60, 90, 120, 150 and 180 min, and 200 µL warm blank HBSS bathing solution was added each time to the acceptor side to maintain the constant volume. FK228 concentrations in the donor-chamber evaluated for both apical to basolateral (AP→BL) and basolateral to apical (BL→AP) transport were 0.5, 1, 2, 5, 10 and 20 µM. This concentration range is similar to the plasma steady state concentrations seen in patients receiving 4 hr FK228 infusion at 13 mg/m² (Sandor, Bakke et al. 2002). Three inserts were used for each treatment. The integrity of the cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) prior to and after the transport study. Only monolayers with TEER values >800 Ω·cm² were utilized. Lucifer Yellow CH was used as a paracellular transport marker during the experiments, and no leak was observed. For FK228 transport with pretreatment and co-incubation with the Pgp (CsA and Ver) or MRP1 (MK571 and indomethacin) inhibitor, the same procedures as described above were followed, except that the HBSS bathing solutions contained one of the following Pgp/MRP1 inhibitors 5 µM CsA, 100 µM Ver, 50 µM MK571, or 20 and 40 µM indomethacin during both the pretreatment and transport study. FK228 concentrations were determined by the HPLC/MS/MS method as described previously (Li and Chan 2000).
6.3.2.3 Calculation of Apparent Permeability Coefficient $P_{app}$

Apparent permeability coefficients $P_{app}$ were calculated from concentration-time profiles as measured in the receiver compartment according to Fick’s first law using the following equation:

$$P_{app} = \left( \frac{dC}{dt} \right) \cdot \frac{V}{A \cdot C_0}$$

EQ 6.1

where $dC/dt$ represents the appearance of FK228 in the receiver chamber (pmol/mL/min), $V$ is the volume of the receiver compartment (mL), $A$ is the cross-sectional area, and $C_0$ is the initial donor concentration (pmol/mL) at time $= 0$.

The flux across the monolayer was determined by linear regression from individual FK228 concentration versus time curves. Comparison of $P_{app}$ in the BL→AP direction ($P_{app, BL→AP}$) to $P_{app}$ in the AP→BL direction ($P_{app, AP→BL}$) was used to assess efflux transport efficiency (\(T_{eff} = \frac{P_{app, BL→AP}}{P_{app, AP→BL}}\)).

6.3.3 FK228 uptake

6.3.3.1 Rhodamine 123 uptake inhibition assay

To test FK228’s ability to inhibit Pgp, an uptake study was performed with Rhodamine 123, a specific fluorescent MDR1 substrate. LLC-PK1 and LMDR1 cells were seeded on 24-well plates at $5 \times 10^4$ cells/well and allowed to reach confluence in 48 hr. Cells were washed with HBSS 3 times and then incubated in 200 µL HBSS at 37°C for 30 min to equilibrate. The differences in Rhodamine 123 uptake between LLC-PK1 and LMDR1 cells were first characterized by a
Rhodamine 123 uptake experiment at concentrations 0, 0.2, 0.5, 1, 2, 5, 10 and 20 µM in the absence of FK228. After 60 min of incubation, the cells were washed with ice-cold HBSS 5 times, and lysed in 300 µL 1% (w/v) Triton X-100. Two hundred µL of the lysate was used to determine intracellular levels of Rhodamine on a Fluorocount™ microplate fluorometer (Packard Instrument Company, Meriden, CT) at excitation/emission wavelengths of 540/590 nm; and 50 µL lysate was used for protein assay using the BCA protein assay kit (Pierce, Rockford, IL). The Rhodamine 123 uptake was normalized to the protein level.

Then the individual inhibitory effect of CsA and FK228 on 10 µM Rhodamine 123 uptake by LLC-PK1 and LMDR1 cells was examined following the same procedure, except that the HBSS contained indicated levels of CsA or FK228.

6.3.3.2 Uptake by human red blood cells

Freshly collected heparinized blood samples from 8 healthy volunteers were used to test FK228 uptake at two concentrations, 1.8 µM and 18 µM. At 1.8 µM of FK228 the uptake study in the presence of 50 µM MRP inhibitor MK571 was also carried out. Following addition of appropriate drugs, the blood samples were incubated at 37°C in a shaking water bath, and an aliquot of blood sample was taken out at preselected time points of 2, 5, 10, 20, 30, 45 and 60 min. The blood was centrifuged at 16,000 g for 30 seconds. The plasma FK228
concentrations were determined by the validated HPLC/MS/MS method as described in Section 2.3.5.

6.3.4 FK228 cytotoxicity assays

MRP1(-) HL60 and MRP1(+) HL60Adr cells were also seeded on 96-well plates at 5000 cells/well, and treated with FK228 at appropriate concentrations in the absence or presence of 50 µM MK571 continuously for 72 hr. The cytotoxicity was determined by standard XTT assay, which is generally used for suspension cells (Goodwin, Holt et al. 1995). The IC50 was calculated using the inhibitory effect sigmoid model provided with WinNonLin 4.0 (Appendix Figure A.7). The IC50 was defined as the concentrations of the tested compounds, at which the number of living cancer cells was reduced by 50% as compared with the untreated controls.

6.4 Results

6.4.1 FK228 efflux by P-glycoprotein

6.4.1.1 FK228 transport

AP→BL and BL→AP transports of FK228 were investigated at concentrations ranging from 0.5 to 20 µM. Figure 6.1 shows the accumulative amount of FK228 transported across Caco-2 monolayers over time for both AP→BL and BL→AP directions (Figure 6.1 a). For AP→BL transport at 0.5 µM of FK228, only trace amounts of FK228 were found, and the levels were below the quantification limit and thus not included in the calculation. FK228 transport was
found to be linear with time for up to 180 min for both directions. The flux (J) was found to be proportional to the donor-side FK228 concentration (Figure 6.1.b). The calculated $P_{app}$ values were $4.07 \pm 0.74 \times 10^{-6}$ cm/s ($n = 18$) and $1.27 \pm 0.73 \times 10^{-7}$ cm/s ($n = 15$) for BL→AP and AP→BL, respectively. The BL→AP transport was 32 times faster than that of AP→BL. No apparent Pgp saturation was observed. The figures of transport at other concentrations are shown in Appendix Figure A.14.

6.4.1.2 Effects of P-glycoprotein inhibition on FK228 transport

In the inhibition studies, pretreatment and co-incubation with CsA (5 µM) or Ver (100 µM) caused dramatic decreases in the BL→AP transport rate (Figure 6.2). Between the two inhibitors, CsA showed higher inhibition, causing a 13.6-fold decrease in $P_{app}$, as compared with 8.4 times by Ver. For AP→BL direction, both inhibitors caused small but significant increases of $P_{app}$ ($p<0.05$). In the presence of the inhibitors, the $T_{eff}$ ratios decreased from 32 to 1.65 and 2.47 for CsA and Ver, respectively, suggesting essentially complete Pgp inhibitions.
Figure 6.1. Transepithelial flux of FK228 across the Caco-2 cell monolayer.

(a) FK228 flux was linear with time up to 180 min for both BL→AP and AP→BL directions as measured at 5 μM (n = 3). (b) Flux was proportional to the FK228 concentration throughout the concentration range with no apparent saturation for both BL→AP and AP→BL directions (n = 3).
Figure 6. 2. Effects of Cyclosporin A (CsA) and Verapamil (Ver) on FK228 flux across the Caco-2 cell monolayer.

Transport of FK228 was studied with pretreatment and co-incubation of 5 μM CsA or 100 μM Ver. Transport of FK228 alone was used as a control. For AP→BL direction, inhibitors caused a small but significant increase in P_app (p < 0.05); For BL→AP direction, the inhibitors caused a dramatic drop in P_app (p < 0.005).
6.4.1.3 Cytotoxicity of FK228 in K562 and K562/Dox cells

Cytotoxicity of FK228 in Pgp(-) K562 and Pgp(+) K562/Dox cells is shown in Figure 6.3. In the absence of the specific Pgp inhibitor CsA, the IC50s of FK228 were 2.74 ± 0.19 nM and 2274 ± 164 nM for K562 and K562/Dox cells, respectively. Addition of CsA at 5 µM caused little change in IC50 in K562 cells (1.93 ± 0.07 nM), while the same treatment caused a dramatic decrease in IC50 in K562/Dox cells (117 ± 9.5 nM). The decrease in IC50 in the presence of 5 µM CsA is about 95%.

6.4.2 FK228 efflux by MRPs

6.4.2.1 Human blood incubation

To investigate the contribution of MRP1 to FK228 transport and uptake, FK228 was incubated in human blood from 8 healthy volunteers. FK228 showed a concentration dependent uptake (Figure 6.4) and was taken up by RBC more rapidly and extensively at the higher concentration (18 µM) than at the lower concentration (1.8 µM). MRP1 inhibition by MK571 at 50 µM in the blood significantly increased the rate of FK228 removal from the plasma at 1.8 µM.
Figure 6. 3. Cytotoxicity assays of FK228 in Pgp(-) K562 and Pgp(+) K562/Dox cells.

(a) cytotoxicity curves; (b) comparison of IC50s of FK228 between K562 and K562/Dox cells in the presence or absence of CsA. The IC50 values in (b) is shown in logarithmic units.
6.4.2.2 Cytotoxicity of FK228 in HL60 and HL60/Adr cells

HL60 cells are Pgp(-)/MRP1(-), while the derivative HL60Adr cells are Pgp(-)/MRP1(+) (Bhalla, Hindenburg et al. 1985; Gollapudi and Gupta 1992). If FK228 is indeed a MRP1 substrate, HL60Adr cells should be more resistant to FK228 than HL60 cells. For this reason we conducted cytotoxicity assays in these two cell lines. Our results showed that in the absence of the MRP1 inhibitor MK571, the IC50 values of FK228 were 5.6 ± 0.56 and 19.4 ± 1.8 nM for HL60 and HL60Adr cells, respectively. In the presence of MK571, the IC50 did not change appreciably for HL60 cells (5.3 ± 0.59 nM), but for HL60Adr cells, it was reduced by more than 3 folds (6.5 ± 0.46 nM) (Figure 6.5). This strongly suggested that FK228 is a MRP1 substrate.
Figure 6.4. Human RBC uptake/metabolism of FK228.

Blood samples from 8 healthy volunteers were spiked with FK228, incubated at 37°C, and the RBC uptake/metabolism was followed over time up to 60 min. RBCs appeared to uptake/metabolize FK228 more rapidly at higher FK228 concentrations. Treatment of blood with a MRP inhibitor MK571 at 50 µM significantly increased the rate of FK228 removal from plasma.
Figure 6.5. FK228 cytotoxicity assays in Pgp(-)/MRP1(-) HL60 and Pgp(-)/MRP1(+) HL60/Adr cells.

(a) cytotoxicity curves; (b) comparison of IC50s of FK228 between HL60 and HL60/Adr cells in the presence or absence of MK571.
Figure 6. Effects of MRP inhibition on FK228 transport across the Caco 2 cell monolayer in AP→BL and BL→AP directions.

MRP inhibitor MK571 at 50 μM increased AP→BL transport as well as decreased BL→AP transport of FK228, while MRP inhibitor indomethacin (IN) had less influence on FK228 transport at either 20 or 40 μM.
6.4.2.3 Effects of MRP inhibitors on FK228 transport

To further investigate whether FK228 is also a substrate of another major member of MRP family, MRP2, the effects of MRP inhibitors MK571 and indomethacin on FK228 transport across the Caco 2 cell monolayer were studied. MK571 at 50 \( \mu \text{M} \) significantly decreased BL\( \rightarrow \)AP transport, but increased AP\( \rightarrow \)BL transport across the Caco 2 monolayer, while indomethacin at 20 or 40 \( \mu \text{M} \) showed similar but less significant effects (Figure 6.6). The change in FK228 transport in the presence of MRP inhibitors was consistent with the apical localization of MRP2 in the differentiated Caco 2 cell monolayer (Hirohashi, Suzuki et al. 2000; Sun, Lennernas et al. 2002; Cooper, Moore et al. 2004).

6.4.3 Inhibition of Rhodamine123 uptake by FK228

Rhodamine 123 uptake by LLC-PK1 and LMDR1 cells without Pgp inhibition indicated that LLC-PK1 cells accumulated Rhodamine 123 to a greater extent than LMDR1 cells at various Rhodamine 123 concentrations (Figure 6.7). This is consistent with higher MDR1 levels in LMDR1 cells. CsA significantly increased Rhodamine 123 uptake by both cells (Figure 6.8), while FK228 at up to 100 \( \mu \text{M} \) failed to change Rhodamine 123 uptake by either cells, confirming that FK228 is not a Pgp inhibitor. The change in LLC-PK1 cell uptake by CsA was probably due to the intrinsic Pgp-like activity in these cells (e.g., MRP proteins).
Figure 6. 7. Rhodamine 123 uptake by LLC-PK1 and LMDR1 cells.

The Rhodamine 123 uptake was normalized to the total protein level and showed linear uptake with Rhodamine 123 concentrations. LLC-PK1 cells showed higher intracellular accumulation of Rhodamine 123 at various concentrations.
Figure 6.8. Effect of FK228 on Rhodamine 123 uptake by LLC-PK1 and LMDR1 cells.

Rhodamine 123 uptake was normalized to the total protein level. Rhodamine 123 uptake by untreated cells were used as blank controls. The cells treated with 5 µM CsA were used as positive controls. FK228 at various concentrations failed to increase Rhodamine 123 uptake by the Pgp-expressing LMDR1 cells.
6.5 Discussion

Pgp and MRP1 are associated with resistance of many anticancer drugs (Lee 2000). An NCI screening project showed that FK228 is a Pgp substrate but not a Pgp inhibitor (Scala, Akhmed et al. 1997). However, no quantitative data regarding FK228 transport kinetics are available, and it was not clear if other membrane transporters are associated with FK228 efflux.

Our current study confirmed that FK228 is a Pgp substrate, with BL→AP transport more than 30 times faster than AP→BL direction across the Caco 2 cell monolayer. The low AP→BL P_{app} values helps to explain the low oral bioavailability of FK228 (Chan, Bakhtiar et al. 1997; Li and Chan 2000). However, no apparent Pgp saturation was achieved throughout the rather wide FK228 concentration range from 0.5 to 20 µM. A possible explanation would be that FK228 is not a strong Pgp substrate and its Km value for Pgp binding is higher than 20 µM, the highest concentration we tested. This explanation is consistent with the data published by Scala et al. (Scala, Akhmed et al. 1997), which showed that FK228 did not competitively inhibit cellular efflux of several model Pgp substrates by SW620 Ad300 cells. On the other hand, CsA at 5 µM significantly blocked FK228 BL→AP transport at 5 µM in the donor chamber, and the resulting transport rates for both directions became similar, with T_{eff} ratio of 1.65 (Figure 6.2). This could be readily explained if FK228 is only a fair Pgp
substrate, since CsA, as a moderate competitive Pgp inhibitor at the same concentration, seemed to bind Pgp more efficiently than FK228.

Paradoxically, being a relatively weak Pgp substrate, FK228’s cellular efflux is expected to be minimal; however, our data showed an almost unidirectional FK228 transport across Caco 2 monolayers. We hypothesize that FK228, being highly lipophilic (Chan, Bakhtiar et al. 1997), is likely to be trapped within the cell membrane. It has been reported that Pgp works as a flippase or a ‘hydrophobic vacuum cleaner’, which pumps out substrate drugs from within the inner layer of cell membrane rather than from cytosol (Teodori, Dei et al. 2002). Thus, although FK228 is a relatively weak Pgp substrate, its high concentration within the cell membrane may increase the efficiency of its Pgp-mediated efflux. This efflux is expected to decrease the FK228 concentration in the inner layer of cell membrane, as well as the FK228 concentration gradient between the inner membrane and cytosol. Since the concentration gradient is the driving force for FK228 to diffuse into the cell, Pgp-mediated efflux is thus expected to result in significantly decreased FK228 concentration in cytosol.

The above findings may have clinical significance. FK228 may be potentially used against Pgp positive cancers in combination with Pgp inhibitor(s), since Pgp-related efflux can be readily reversed by Pgp inhibition.

The possibility of FK228 functioning as a MRP1 substrate was first established by a human RBC uptake study (Figure 6.4). Human RBC not only expresses high concentrations of MRP1 (Rychlik, Pulaski et al. 2000), but also
the membrane structure is relatively simple and contains fewer interfering membrane transporters (e.g., P-gp). For these reasons, RBC is a good model to study MRP1-related uptakes (Zaman, Cnubben et al. 1996; Evers, Cnubben et al. 1997; Klokouzas, Barrand et al. 2001). The saturable RBC uptake kinetics and the effect of MRP1 inhibition on the uptake are consistent with FK228 being a MRP1 substrate (Figure 6.4). The Pgp(-)/MRP1(-) HL60 and Pgp(-)/MRP1(+) HL60/Adr cell pair serves as another good system to study MRP1-mediated cellular uptake, and showed an inverse correlation between MRP1 expression and FK228 cytotoxicity (Figure 6.5). Taken together, these evidences indicated that FK228 is a MRP1 substrate.

Caco 2 cell monolayer is widely used to study Pgp related transport. However, its use to study MRP-mediated transport and uptake has been limited due to debates on whether differentiated Caco 2 cells express functional MRP proteins. Recently, it was reported that MRP2 is the major functional MRP transporter on the apical membrane of Caco 2 cell, while MRP1 expression is minimal (Hirohashi, Suzuki et al. 2000; Sun, Lennernas et al. 2002; Cooper, Moore et al. 2004). Using Caco 2 cell monolayer as a model, we found that MK571 at 50 µM significantly increased FK228 AP→BL but decreased BL→AP transport (Figure 6.6), suggesting that FK228 is also a MRP2 substrate. Another commonly used MRP inhibitor, indomethacin, at either 20 or 40 µM, showed similar but lower effect on FK228 transport profile across the Caco 2 cell monolayer, probably due to its weaker intrinsic binding affinity as compared with MK571.
The finding that FK228 is a MRP1 substrate together with its metabolism in RBC may provide a possible explanation for the pharmacokinetics differences between rats (Chapter 2) and patients (Chapter 4). MRP1-mediated FK228 efflux by RBC is a competing procedure with FK228 metabolism by RBC. As discussed in Chapter 3, higher GSH activity in rat RBC leads to more rapid FK228 metabolism as compared with in the case of human RBC. This helps to explain, at least in part, the much higher total body clearance of FK228 in rats (Chapter 2) than in patients (Chapter 4). If MRP1 indeed plays a role in FK228 \textit{in vivo} elimination, it is expected that MRP1-mediated efflux is more efficient at low concentrations of FK228, while this process may be saturated at higher concentrations. This turns out to be consistent with the FK228 pharmacokinetics data in rats (Chapter 2), where \textit{i.v} infusion is associated with much lower FK228 plasma concentrations and significantly increased AUC as compared with the \textit{i.v.} Bolus.

In conclusion, we demonstrated in this chapter that FK228 is a substrate for both Pgp and MRP1, and probably also MRP2 and other MRP transporters. Expression of both Pgp and MRP1 are associated with FK228 resistance.
CHAPTER 7

DEVELOPMENT AND CHARACTERIZATION OF FK228-RESISTANT CANCER CELL LINES

7.1 Abstract

Histone acetylation status, an epigenetic determinant of gene transcription, is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The potent HDAC inhibitor FK228 is a substrate for MDR1 and MRP1, both of which mediate the in vitro FK228 resistance as shown in Chapter 8. To determine the mechanisms underlying acquired FK228 resistance, we developed four FK228 resistant cell lines from HCT-15, IGROV1, MCF7 and K562 cells by stepwise increments of FK228 exposures. Parent and resistant cells were characterized using a 70-oligomer cDNA microarray, real time RT-PCR, Western blot, and cytotoxicity assays. At both mRNA and protein levels, MDR1, but not MRP1 or other potential resistance genes, was strongly upregulated in all resistant cell lines. HAT or HDAC activities were unaffected by FK228 pretreatment, consistent with a lack of cross-resistance to HDAC inhibitors that are not MDR1 substrates. Real time RT-PCR, Western blot, and
chromatin immunoprecipitation indicated that FK228 reversibly induced MDR1 expression by HDAC inhibition and subsequent histone hyperacetylation at the MDR1 promoter. This study reveals a significant role of histone acetylation in MDR1 transcription, which appears to mediate FK228 resistance.
7.2 Introduction

The eukaryotic nucleosome consists of 146 base pairs of DNA wrapped around a histone octamer core, which is arranged as a \((H_3-H_4)_2\) tetramer and two \(H_{2A}-H_{2B}\) dimers (Davie and Chadee 1998; Davie and Spencer 1999). Transcriptional competence is governed by histone acetylation status, which is determined by two families of enzymes, histone acetyltransferases (HATs) (Grant and Berger 1999) and histone deacetylases (HDACs) (Cress and Seto 2000; Marmorstein 2001). HATs promote acetylation of lysines on histone proteins, and thereby, destabilize the electrostatic bonds between DNA phosphates and histones, resulting in an open DNA conformation for gene transcription. On the other hand, HDACs promote histone deacetylation, which results in gene silencing. Depending on the cell type, inhibition of HDACs in cancer cells can lead to transcriptional activation and silencing of ~2% of human genes (Weidle and Grossmann 2000). HDAC-mediated silencing of specific tumor suppressor genes appears to play a role in cancer pathophysiology (Cress and Seto 2000). Moreover, HDACs work synergistically with promoter DNA methyltransferases, both of which result in gene silencing (Weiser, Guo et al. 2001; Zhu, Lakshmanan et al. 2001; Klisovic, Maghraby et al. 2003).

FK228 (FR901228, depsipeptide) is a potent histone deacetylase (HDAC) inhibitor (Nakajima, Kim et al. 1998; Yoshida, Furumai et al. 2001) in phase I/II clinical trials against various malignancies (Marshall, Rizvi et al. 2002; Sandor,
FK228, after intracellular bioactivation (Furumai, Matsuyama et al. 2002; Xiao, Byrd et al. 2003), specifically inhibits class I HDAC enzymes (Furumai, Matsuyama et al. 2002), and results in up or down-regulation of various genes (Ueda, Nakajima et al. 1994; Wang, Brunner et al. 1998; Sandor, Senderowicz et al. 2000). *In vitro* screening conducted at the National Cancer Institute (NCI) indicated that FK228 is an MDR1 (P-glycoprotein, ABCB1) substrate (Scala, Akhmed et al. 1997). Recently, we have shown that FK228 is a substrate of both MDR1 and multidrug resistance-associated protein 1 (MRP1 or ABCC1) (Xiao, Foraker et al. 2003). Cancer cells with either MDR1 or MRP1 over-expression were significantly more resistant to FK228 (Xiao, Foraker et al. 2003).

MDR1 and MRP1 are well-characterized ATP-binding cassette (ABC) transporters responsible for multi-drug resistance (Tan, Piwnica-Worms et al. 2000). While they show overlapping substrate specificity (Seelig, Blatter et al. 2000), their expression appears to be controlled by different mechanisms. This is suggested by their different tissue distribution profiles and unsynchronized expressions in cancer cells (Efferth, Gebhart et al. 2003; Huang and Sadee 2003; Anderle, Huang et al. 2004). It has been reported that MDR1 expression is dominantly controlled by promoter DNA methylation status, while histone acetylation status appears to serve only as a secondary control mechanism (Jin and Scotto 1998; Nakayama, Wada et al. 1998; Baker and El-Osta 2003). On the other hand, effects of DNA methylation and histone acetylation on MRP1 expression has yet to be studied.
The overall clinical response rate to FK228 has been low, although primary or secondary clinical FK228 resistance factors has yet to be demonstrated (Marshall, Rizvi et al. 2002; Sandor, Bakke et al. 2002). We found that HCT-15 colon carcinoma cells readily acquired FK228 resistance, which can be reversed by MDR1 inhibition. This raised the following questions: 1) whether FK228 can induce resistance to itself in cancer cells of various origins through MDR1 and/or MRP1 upregulation or other mechanisms; 2) whether the resistance is due to selection of sub-populations of cancer cells with pre-existing, high MDR1 and/or MRP1 expressions, as reported for paclitaxel and doxorubicin. Alternatively, this may be due to rapid MDR1 and/or MRP1 induction by FK228-mediated HDAC inhibition; 3) if there is induction of gene expression, what would be the molecular mechanism.

To address these questions, we developed three additional FK228-resistant cell lines from MCF7, IGROV1 and K562 parental cells, all of which are MDR1(-)/MRP1(-) based on microarray analysis (Huang, Anderle et al. 2004). The parental and resistant cells were subsequently characterized using a 70-oligomer cDNA microarray, real time RT-PCR, Western blot, cytotoxicity assays and HDAC and HAT activity assays. Moreover, the association between MDR1 promoter histone hyperacetylation and MDR1 induction was established by chromatin immunoprecipitation (ChIP). Our data indicate that MDR1 induction is the major mechanism for acquired FK228 resistance, suggesting that the importance of promoter histone acetylation in regulating MDR1 transcription might have been underestimated. Moreover, histone hyperacetylation, which has
been used as a surrogate biomarker, may not provide good correlation to HDAC-inhibitor-induced cytotoxicity.

7.3 Experimental procedures

7.3.1 Cell culture and development of FK228 resistance

Human colon carcinoma cell line HCT-15, human breast carcinoma cell line MCF7, human ovarian carcinoma cell line IGROV1 and human chronic myelogenous leukemia cell line K562 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in RPMI 1640 medium containing 25 mM HEPES buffer and L-glutamine, supplemented with 10% fetal bovine serum, 100 U/mL sodium penicillin G and 180 µM streptomycin at 37°C under 5% CO₂ atmosphere. The cells were rendered resistant to FK228 by stepwise exposures to FK228 of increasing concentrations. Within 4 weeks, all cell lines developed FK228 resistance and were designated as HCT-15/FK228, MCF7/FK228, IGROV1/FK228 and K562/FK228 cells, respectively. IGROV1/FK228 cells were routinely cultured in medium containing 750 nM FK228 and the other FK228 resistant cell lines in medium containing 1000 nM FK228. K562/Dox cell line (a gift from J.P. Marie, INSERM, E9912, University of Paris 6, France) was also cultured as the parental cell lines and stimulated with 0.1 µM doxorubicin once a month.
7.3.2 Characterization of the resistant daughter cell lines

7.3.2.1 Custom 70-mer microarray

We used a 70-oligomer custom microarray previously developed in our laboratories (Anderle, Huang et al. 2004; Huang, Anderle et al. 2004), which comprises of 1070 probes targeting 640 transporter genes (including 48 known ABC transporters) and ion channel genes, as well as 430 genes belonging to families of growth factors and receptors, cell adhesion molecules and signal transduction (the list of included genes is available upon request). For some genes of special interest (e.g., MDR1 and MRP1), two different probes were designed and printed onto the arrays. Total RNA was extracted from the parental and daughter cells with TRIzol (Invitrogen, Carlsbad, CA, USA), and further purified using the Rneasy Mini column (Qiagen, Carlsbad, CA, USA). Eighteen µg total RNA was used for cDNA synthesis and labeled with Cy5 (green fluorescence) or Cy3 (red fluorescence) dyes by amino-allyl coupling. The protocol is available at [http://derisilab.ucsf.edu/pdfs/ami no-allyl-protocol.pdf](http://derisilab.ucsf.edu/pdfs/amino-allyl-protocol.pdf). A paired and dye-swap design was applied, in which cDNA samples of parental and resistant cell lines were first labeled with Cy3 dye and Cy5 dye, respectively; and then labeled in the reversed order in the dye-swap experimental group. The Cy3 and Cy5-labeled samples were then mixed and hybridized to the array slides for 16 h at 65°C. Slides were washed, dried and scanned in an Affymetrix 428 scanner to detect Cy3 and Cy5 fluorescence.
7.3.2.2 Microarray data analysis

Background subtraction and calculation of medians of pixel measurements per spot was carried out using GenePix Software 3.0 (Foster City, California). Spots were filtered out if they had both red and green intensity < 500 units after subtraction of the background, or if they were flagged for any visual reason (odd shapes, background noise, etc). Data normalization was carried out using the statistical software package R (www.r-project.org). The plot of $M = \log_2 \frac{R}{G}$ vs. $A = \log_2 \sqrt{R \times G}$ shows dependence of the log ratio $M$ on overall spot intensity $A$, where $R$ is the red fluorescence intensity of Cy5 and $G$ is the green fluorescence intensity of Cy3. To correct intensity- and dye-bias, we used location and scale normalization methods, which are based on robust, locally linear fits, implemented in the SMA R package (Yang, Dudoit et al. 2002; Huang, Anderle et al. 2004). This method is based on transformations:

$$R/G \rightarrow \log_2 \frac{R}{G} - c_j(a) = \log_2 \frac{R}{k_j(a)} \times G \rightarrow (1/a_j)^* \log_2 \frac{R}{k_j(a)} \times G,$$

where $c_j(a)$ is the Lowess fit of the $M$ vs. $A$ plot for spots on the $j^{th}$ grid of each slide, and $a_j$ is the scale factor for the $j^{th}$ grid (to obtain equal variances along individual slides).

We calculated the differential MDR1 mRNA levels based on the $R/G$ ratios for each primer (4 prints/primer) and for each dye-swap group (2 groups/primer). The values were averaged and standard deviation calculated. In cases when 2
primers were used for one gene, the MDR1 mRNA levels were individually calculated for each primer.

7.3.2.3 Real time RT PCR

Total RNA was extracted from sample cells by TRIsol. The RNA was precipitated by isopropyl alcohol and rinsed with 70% ethanol. Single-strand cDNA was prepared from the purified RNA using oligo-dT priming (Thermoscript RT Kit, invitrogen, Carlsbad, CA, USA) as described previously (Anderle, Huang et al. 2004), followed by CYBR-green real time PCR (ABI Prism 7700 Sequence Detection System, Foster City, CA). The primers for MDR1 were 5’-CAGCAAAAGGAGCCCAACATAC-3’, and 5’-TGAGGCTGTCTAACAAGGGCA-3’. The primers for β-actin were 5’-CCTGGCACCCAGCACAAT-3’ and 5’-GCCGATCCACACCGAGTACT-3’. The threshold cycle for PCR products was defined as the cycle at which the SYBR-green fluorescent signal was 20 standard deviations above background. Relative quantification of gene expression was performed using the comparative CT method (or DDCT, method available in the user bulletin of ABI Prism 7700 Sequence Detection System) with beta-actin as the control. K562/Dox cell line was used as a MDR1(+) control. Melting dissociation was performed to evaluate the purity of the PCR product.

7.3.2.4 Western blot

For Western blot analysis, cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 950 mM Tris-HCl, 250
mM NaCl, 5 mM EDTA, 50 mM NaF, 0.15% Igepal CA-630 and 1.5 mM PMSF). Equal amounts of proteins (100 µg) were size fractionated on 6% or 15% SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked with blocking buffer (5% Nonfat MILK, 200 mM NaCl, 50 mM Tris and 0.05% Tween-20) at room temperature for 2 h. The blocked membrane was then incubated with primary antibodies at 4°C overnight. After washing the membrane with TBS-T buffer (20 mM Tris, 500 mM NaCl and 0.05% Tween-20) for 3 × 15 min, the membrane was incubated with secondary antibody at room temperature for 1 h. The detection of specific protein binding was performed with the ECL chemiluminescent Western blotting detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were mouse monoclonal JSB-1 anti-human P-glycoprotein antibody (Research Diagnostics Inc., 1:50), mouse monoclonal AC-15 anti-β-actin antibody (Abcam Inc., 1:5000), and peroxidase-conjugated AffiniPure donkey anti-mouse IgG (Research Diagnostics Inc., 1:10,000), rabbit polyclonal anti acetylated histone H3 antibody (Upstate, 1:500), rabbit anti acetylated histone H4 antibody ChIP grade (Upstate, 1:500), peroxidase-conjugated donkey anti-rabbit IgG (Upstate, 1:2000).

7.3.2.5 Cytotoxicity assays

To determine the role of MDR1 in the acquired FK228 resistance, cytotoxicity assays were conducted. Adhesive HCT-15, HCT-1/FK228, MCF7, MCF7/FK228, IGROV1, and IGORV1/FK228 cells were seeded in 96-well plates
at 5000 cells/well in 100 µL medium, and allowed 24 h for adhesion at 37°C. Fifty µL dosing solutions (4×) of either FK228, HDAC inhibitors TSA or SAHA made by serial dilutions were added to the wells. Also added was 50 µL 20 µM CsA solution (4×) or 50 µL blank medium, so that the total volume in each well was 200 µL. The plates were then incubated continuously for 72 h followed by SRB assay, which provides robust results for adhesive cells (Skehan, Storeng et al. 1990). K562, K562/FK228 and K562/Dox cells were evaluated under the similar procedure at a cell seeding density of 2000 cells/well by XTT method, which is convenient for suspension cells (Goodwin, Holt et al. 1995). All resistant cells were cultured in FK228-free medium for 3 days before the cytotoxicity assays to avoid possible FK228 accumulation in cells. The 3-day culture in FK228-free medium should not lead to significant decrease of MDR1 expression as discussed later.

7.3.2.6 Histone deacetylase and histone acetyltransferase activity assays

Nuclear contents were extracted from the $8 \times 10^6$ cells of interest using a nuclear extract kit (Upstate Biotechnology, Inc., Waltham, MA) according to the manufacturer’s protocol. The resistant cells were cultured in FK228-free medium for 3 days before nuclear extraction. The HAT and HDAC activities contained in the nuclear extracts were determined using non-radioactive colorimetric and fluorescent kits, respectively (Upstate Biotechnology, Inc., Waltham, MA). The experiments were conducted according to the manufacturer’s protocols.
7.3.2.7 MDR1 Induction Kinetics

MDR1(-)/MRP1(-) IGROV1, MCF7 and K562 cells were treated with 1, 10 and 100 nM FK228 for 4, 8, 24, and 48 h, followed by real time RT-PCR to determine MDR1 induction as described above. K562 cells were treated with two other non-MDR1-substrate HDAC inhibitors (TSA and SAHA) at 100 and 1000 nM for 8, 24 and 48 h, followed by real time RT-PCR to determine MDR1 induction. Untreated cells were used as controls. Similarly, MDR1 induction was determined in IGROV1, MCF7 and K562 parental cells after treatment with 2.5 µM of 5-azacytidine, a hypomethylating agent, for 24 h.

7.3.2.8 Chromatin immunoprecipitation (ChIP)

K562/FK228 cells and K562 cells treated with 10 nM FK228 for 0, 2, 4, 8, and 24 h were collected. ChIP was performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Inc., Waltham, MA) according to the manufacturer's protocol with the antibodies to acetyl-histone H3 and acetyl-histone H4 (Upstate Biotechnology, Inc.). Immunoprecipitated chromatin was analyzed by PCR with primers specific for MDR1 promoter: 5’-ACAGCCGCTTCGCTCTTTTGG-3’ and 5’-AGAAGCCCTTCTCCCGTGAAG-3’. The cycle number and the amount of templates were varied to ensure that results were within the linear range of the PCR.
7.4 Results

7.4.1 Development of FK228 resistant cell lines.

Exposure to stepwise increase in concentrations of FK228 resulted in acquisition of FK228 resistance in all four cell lines within a month. The FK228-resistant daughter cell lines were designated as HCT-1/FK228, MCF7/FK228, IGROV1/FK228 and K562/FK228, and were cultured in medium containing high concentrations of FK228. The resistant cells appeared to grow well, with no observable change in their proliferation rates as compared with their parental cells. No apparent morphological change was observed under microscope for the resistant cell lines.

7.4.2 MDR1 upregulation in resistant cell lines

To characterize the resistant cell lines, we first used a custom 70-oligomer cDNA microarray to identify mRNAs that were differentially expressed between the parental and daughter cell lines. Figure 7.1 shows that MDR1 (ABCB1), but not MRP1 (ABCC1) or other genes, is predominantly upregulated among all four resistant cell lines. This suggests that MDR1 upregulation plays a central role in the acquired FK228 resistance. For this reason, in the subsequent studies, we focused on MDR1 only. To confirm the microarray results, we performed real time RT-PCR in all resistant-parental cell line pairs. Table 7.1 summarizes the relative upregulation of MDR1 mRNA in all FK228 resistant cell lines as compared with their parental counterparts. The MDR1 mRNA level in MCF7 cells (the lowest among all) was arbitrarily defined as unity. A MDR1(+)
K562/Dox cell line was included as a positive control. As shown, the resistant cells showed 4 to 18,728 folds increases in the MDR1 mRNA level as compared with their parental cell lines. K562/Dox cells was included as an MDR1(+) control, and showed high MDR1 mRNA level. The MDR1 upregulation was further confirmed by Western blot (Figure 7.2). Little MDR1 expression was detected in HCT-15, MCF7, IGROV1 and K562 parental cells, while thick bands were detected for their resistant daughter cell lines. A clear but relatively weaker band was detected for the K562/Dox cells. This is in contrast with the high MDR1 mRNA level in K562/Dox cells (Table 7.1). Since the real time RT PCR and the Western blot were conducted at different times, this inconsistency may be due to the periodical doxorubicin stimulation and thus fluctuating MDR1 expressions.

7.4.3 Correlation of MDR1 transcription and FK228 cytotoxicity

To investigate the effects of MDR1 expression on the cytotoxicity of FK228, we conducted a series of cytotoxicity assays (Table 7.2). All MDR1(-)/MRP1(-) parental cell lines showed low IC50 values of FK228 at the low nanomolar ranges. MDR1(+) parental HCT-15 cells showed higher IC50 values. All resistant daughter cell lines showed much higher IC50 values of FK228 than their parental counterparts. MDR1 inhibitor CsA at 5 µM reversed FK228 resistance in all resistant daughter cell lines, indicating that MDR1 upregulation is a major mechanism for the acquired FK228 resistance. This is further supported by the linear correlation between the IC50 values and the MDR1 transcription levels (Figure 7.3).
Figure 7. 1. The mRNA expression levels in FK228 resistant and parental cell lines as determined by a custom 70-oligomer cDNA microarray.

MDR1 is the only gene that is consistently upregulated at the mRNA level in all three resistant cell lines.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Condition</th>
<th>Relative MDR1 mRNA</th>
<th>Folds of upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-15</td>
<td>Parental</td>
<td>2894</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>FK228/FK228</td>
<td>12292</td>
<td>4</td>
</tr>
<tr>
<td>MCF7</td>
<td>Parental</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>FK228/FK228</td>
<td>18728</td>
<td>18728</td>
</tr>
<tr>
<td>IGROV1</td>
<td>Parental</td>
<td>11</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>FK228/FK228</td>
<td>8991</td>
<td>789</td>
</tr>
<tr>
<td>K562</td>
<td>Parental</td>
<td>38</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>FK228/FK228</td>
<td>19982</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>FK228/Dox</td>
<td>19161</td>
<td>503</td>
</tr>
</tbody>
</table>

Table 7.1: Determinations of MDR1 mRNA levels in the parental and the resistant cell lines by real time RT-PCR.
Figure 7.2. MDR1 expression levels in the resistant and parental cell lines as determined by Western blot.

All resistant cell lines have significantly higher MDR1 expressions than their parental counterparts as determined by Western immunoanalysis. (a) HCT15 (lane 1) and HCT-1R (lane 2). (b) MCF7 (lane 1); MCF7/FK228 (lane 2); IGROV1 (lane 3); IGROV1/FK228 (lane 4); K562 (lane 5); K562/FK228 (lane 6); MDR1(+) K562/Dox (lane 7). Beta actin was used as the loading control.
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Mean IC50 (nM)</th>
<th>SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parental cells, no CsA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT-15</td>
<td>378</td>
<td>49</td>
</tr>
<tr>
<td>MCF7</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>IGROV1</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>K562</td>
<td>2.7</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Parental cells, with CsA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT-15</td>
<td>7.4</td>
<td>1.4</td>
</tr>
<tr>
<td>MCF7</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>IGROV1</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>K562</td>
<td>1.9</td>
<td>0.1</td>
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<tr>
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<tr>
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<td>89</td>
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<tr>
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<tr>
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Table 7. 2. Summary of IC50 values of FK228 in the parental and the resistant cell lines in the absence and presence of 5 µM CsA.
Figure 7. 3. Correlation between the MDR1 mRNA level and the FK228 IC50 values.

MDR1 mRNA levels of the 4 parental and the 4 resistant cell lines showed linear correlation with their IC50 values of FK228 on the log-log plot. K562/Dox cell line was not included due to their time-dependent MDR1 expression.
7.4.4 HAT and HDAC activities unchanged in the resistant cells

The lack of complete reversal in MCF7/FK228 and K562/FK228 cells (Table 7.2) could be due to their very high MDR1 expressions, since even residual MDR1 levels would result in a significant drug efflux. On the other hand, it is possible that the resistance may be due to some defect in the transcription machinery. For this reason, we determined the cytotoxicity of two non-MDR1-substrate HDAC inhibitors TSA and SAHA (Margueron, Licznar et al. 2003) in these cell lines. No cross-resistance was found in the resistant daughter cell lines in comparison to their parental counterparts (Figure 7.4), suggesting that the target of HDAC inhibitors, histone acetylation/deacetylation machinery, remained intact. To further confirm this, we determined HAT and HDAC activities (Figure 7.5) in these paired cell lines, and the results showed that there was no significant change in HAT and HDAC activities.

7.4.5 Global histone hyperacetylation in resistant cell lines

Global histone acetylation status in the parental and the resistant cells was determined (Figure 7.6). All resistant cell lines showed significant histone H3 and H4 hyperacetylation as compared with the parental counterparts.
Figure 7.4. Cytotoxicity of other HDAC inhibitors.

(a) TSA and (b) SAHA in the parental and the resistant cells \((n = 3)\). No consistent cross-resistance to these two non-MDR1-substrate HDAC inhibitors was found in the resistant cell lines. IGROV1/FK228 cells showed cross-resistance to SAHA \((p < 0.001)\), but not to TSA.
Figure 7. 5. Determination of HAT and HDAC activities in parental and resistant cells.

Neither the HAT (a) nor the HDAC activity (b) is significantly changed in resistant cell lines as compared with their parental counterparts.
Figure 7. Global histone acetylation status in the parental and resistant cells as determined by Western blot.

Resistant cells showed significant global hyperacetylation of histone H3 and H4 as compared with their parental cell lines. Lanes 1-6 are MCF7, MCF7/FK228, IGROV1, IGROV1/FK228, K562, and K562/FK228 cells, respectively.
7.4.6 Reversible MDR1 induction

We studied the reversibility of MDR1 induction by FK228. K562/FK228 cells were cultured either in medium containing 1000 nM FK228 or in FK228-free medium for 1 to 6 weeks. The MDR1 expressions were then determined by Western blot. Consistent with FK228 being a reversible HDAC inhibitor (Furumai, Matsuyama et al. 2002), and histone acetylation status being an epigenetic modulator, the MDR1 protein level decreased over time in K562/FK228 cells in the absence of FK228 treatment (Figure 7.7). However, the decrease of MDR1 is rather slow. Since the histone acetylation turnover time is rapid (Waterborg 2002), the slow decrease is presumably because of slow turnover of Pgp.

It has been reported that short-time treatment with MDR1-substrate drugs, such as paclitaxel (Schondorf, Neumann et al. 2003) and doxorubicin (Abolhoda, Wilson et al. 1999), may result in rapid MDR1 upregulation. FK228 is both a MDR1 substrate and HDAC inhibitor, and the acquisition of FK228 resistance in selected cell lines was rapid. This raises a question if the MDR1 upregulation is due to selection of sub-populations of cancer cells with pre-existing high MDR1 and/or MRP1 expressions, or due to rapid MDR1 and/or MRP1 induction by FK228-mediated HDAC inhibition. To answer this question, we monitored the progress of MDR1 induction using real time RT-PCR. It was found that FK228 readily increased MDR1 mRNA levels in a concentration and time dependent manner (Figure 7.8).
To investigate if FK228 treatment is associated with histone hyperacetylation, we first determined global histone acetylation profiles in the paired cell lines (Figure 7.6). All resistant cell lines showed significant histone H3 and H4 hyperacetylation as compared with the parental counterparts. In order to find out if the MDR1 induction is caused by histone hyperacetylation at the MDR1 promoter region, we conducted a ChIP study using K562 and K562/FK228 cell pair as a model. We first found significant histone H3 and H4 hyperacetylation at the MDR1 promoter region in K562/FK228 cells as compared with that in K562 parental cells (Figure 7.9a). Additionally, we found that a short time exposure of K562 cells to 10 nM FK228 (up to 24 hr) caused increases in histone H3 and H4 acetylation at the MDR1 promoter (Figure 7.9b). This suggests that FK228 induces MDR1 via its HDAC inhibitory activity, which leads to HDAC inhibition, histone hyperacetylation at the MDR1 promoter region, and formation of MDR1 euchromatin ready for transcription.

That FK228 induces MDR1 by its HDAC inhibitory activity was further confirmed by similar induction in K562 parental cells following treatments with HDAC inhibitors TSA and SAHA, which are not MDR1 substrates (Figure 7.10). Both compounds were able to induce MDR1 within 8 h at either 100 or 1000 nM.
Figure 7. Reversible MDR1 induction by FK228 as determined by Western blot.

K562/FK228 cells were either cultured in medium containing 1000 nM FK228 (lane 1) or in the absence of FK228 for 1 to 6 weeks (lanes 2-7). The MDR1 expression decreased over time and was barely detectable at the end of 6 weeks. Beta actin was used as the loading control.
Figure 7.8. MDR1 induction in IGROV1, MCF7 and K562 parental cells as measured by real time RT-PCR after FK228 treatments.

Cells were exposed to FK228 at 1, 10 or 100 nM for 4, 8, 24 or 48 h, followed by real time RT-PCR. FK228 readily induced MDR1 in a concentration and time dependent manner. MDR1 mRNA levels in the parental cell lines were arbitrarily defined as unity.
Figure 7. MDR1 promoter hyperacetylation in K562/FK228 cells and in K562 cells treated with 10 nM FK228 as determined by ChIP.

(a) K562/FK228 (lane 1) showed significant hyperacetylation of both histone H3 and H4 on the MDR1 promoter as compared with untreated K562 (lane 2) cells. (b) FK228 induced histone H3 and H4 hyperacetylation in K562 cells at the MDR1 promoter in a time dependent manner. The inputs total DNA without immunoprecipitation were used as loading controls.
Figure 7. 10. MDR1 induction in K562 cells as measured at the mRNA level by real time RT-PCR after TSA and SAHA treatments.

Both TSA and SAHA induced MDR1 in a concentration and time dependent manner. The MDR1 mRNA level in the parental K562 cells was arbitrarily defined as unit.
7.5 Discussion

Methylation of cytosine residues in DNA CpG islands on the promoter region (Graff, Herman et al. 1997; Baylin, Herman et al. 1998) and histone deacetylation (Nan, Cross et al. 1998; Nan, Ng et al. 1998; Ng, Zhang et al. 1999) are two important epigenetic mechanisms in gene expression control. Scotto et al. first reported that the transcriptional regulation of the MDR1 gene by histone deacetylase inhibitor TSA in the human colon carcinoma cell line SW620 (Abolhoda, Wilson et al. 1999). However, transfection with single constructed reporter gene (XmaI-NheI MDR1 promoter-luciferase) did not predict the significance of this mechanism in other types of cancers. Later on, several groups found that MDR1 expression is controlled by MDR1 promoter methylation (Kusaba, Nakayama et al. 1999; Tada, Wada et al. 2000; El-Osta, Baker et al. 2001; El-Osta and Wolfe 2001). Recently, El-Osta et al. reported that promoter methylation is the predominant mechanism for MDR1 transcriptional control (El-Osta, Kantharidis et al. 2002). Promoter hypermethylation inhibits MDR1 transcription by either directly interrupting the binding of transcription factors to the promoter, or by recruiting HDACs through methyl DNA binding proteins (MBPs) (Baker and El-Osta 2003). Thus, HDAC inhibition was thought to cause MDR1 expression only when the MDR1 promoter is hypomethylated (El-Osta, Kantharidis et al. 2002; Baker and El-Osta 2003). Unfortunately, this study used only MDR1(-) CEM-CCRF and its derivative MDR1(+) CEM-A7R. It is not clear how important these two mechanisms are in suppressing MDR1 transcription in
MDR1(-) cancer cell lines, since these two mechanisms seem to be overlapping and either one can suppress MDR1 expression by itself. For these reasons, studies using multiple cancer cell lines may provide more insight in the role of HDACs and histone deacetylation on MDR1 transcription.

By developing several model cell lines resistant to FK228, we demonstrated that the rapid acquisition of the resistance was due to induction of MDR1. It seems that even though promoter hypomethylation is believed to be an upstream event to histone acetylation for MDR1 transcription, the MDR1 repression in all tested MDR1(-) cancer cells of various origins can be induced by HDAC inhibitors. This suggests that the role of histone acetylation in MDR1 transcriptional control have been underestimated. Moreover, recent progresses in MDR1 promoter methylation mapping (David, Yegnasubramanian et al. 2004) revealed that MCF7 cells have hypermethylated MDR1 promoter. This is interesting since according to the literature, HDAC inhibition should not result in MDR1 induction in cell lines with hypermethylated MDR1 promoters. This suggests that promoter histone acetylation status may not only be a secondary mechanism for MDR1 transcription control. Instead, dynamic interactions between DNA methylation and histone deacetylation may work together to repress MDR1 transcription. To find out if IGROV1 and K562 cells have hypermethylated MDR1 promoter, we treated MCF7, IGROV1 and K562 cells with 2.5 μM of 5-azacytidine, a hypomethylating agent, for 24 h. The MDR1 mRNA level was determined by real time RT-PCR. Surprisingly, MCF7, IGROV1 and K562 cells showed 4, 701, and 437-fold increases in MDR1 transcription.
after the treatment, respectively, suggesting that MDR1 transcription is also suppressed by promoter hypermethylation in all three MDR1(-) cell lines. The fact that MDR1 can be induced in MDR1(-) cell lines by both hypomethylating agents and HDAC inhibitors strongly suggests that there are dynamic interactions between these two mechanisms and the role of MDR1 promoter acetylation on controlling MDR1 transcription and expression may have been underestimated.

Our finding may have clinical significance. 1) Frequent FK228 treatments with short intervals could result in MDR1 upregulation and FK228 resistance; 2) patients refractory to FK228 should not be treated with drugs being MDR1 substrates, such as paclitaxel or doxorubicin; 3) For future combination therapies, FK228 may not be suitable to pre-treat patients receiving anticancer drugs that are MDR1 substrate due to the possible rapid MDR1 induction. Indeed, some recent combination studies showed sequence-dependent outcomes. Combination of paclitaxel and FK228 produced synergistic effect against human prostate DU-145 carcinoma cells, when used simultaneously or sequentially with paclitaxel dosing first; however, sequential exposure to FK228 followed by paclitaxel showed antagonistic effect (Naoe, Inoue et al. 2004); 4) Since MDR1 induction is shared by other HDAC inhibitors, future HDAC inhibitors may have similar concerns.

Since the acquired FK228 resistance is mainly due to elevated MDR1-mediated efflux, it would be expected that the intracellular exposure to FK228 in the resistant cells would be minimal even though the media contained
prohibitively high FK228 concentrations. Paradoxically, all resistant cells showed significant global hyperacetylation on both histones H3 and H4 (Figure 7.6), suggesting effective intracellular exposures. On the other hand, this is consistent with the fact that FK228 is a reversible HDAC inhibitor, and sustained intracellular exposure of FK228 is required to maintain the high transcription level of MDR1.

Contrary to the well-accepted association between HDAC inhibition-caused histone hyperacetylation and cytotoxicity (Vigushin and Coombes 2002), the resistant cells were still living well with global histone hyperacetylation. There may be two possible ways to explain this phenomenon. 1) Pathways downstream to histone hyperacetylation that lead to apoptosis may be blocked in the resistant cells, and 2) FK228 induced cancer cell apoptosis is not associated with histone hyperacetylation. The lack of cross-resistance to TSA and SAHA in the FK228-resistant cells (Figure 7.4) suggests that the downstream pathways were not blocked, assuming that all these HDAC inhibitors kill cancer cells by a shared apoptosis pathway. Therefore, it seems that FK228-induced apoptosis is not triggered by global histone hyperacetylation. Indeed, recent data in HDAC inhibition has suggested that other mechanisms, such as abnormal mitosis and G2/M arrest (Sandor, Robbins et al. 2000) and anti-angiogenesis (Kwon, Kim et al. 2002), may play important roles in HDAC inhibition induced apoptosis. At the same time, it seems necessary to re-examine the assumption that all HDAC inhibitors cause cancer cell apoptosis by their HDAC inhibitory activity. Further studies can be done to dissect FK228-induced histone hyperacetylation from
FK228-induced apoptosis by transfecting cancer cells with HATs to maintain treatment-independent histone hyperacetylation.

It was interesting to note that MDR1 was the major gene upregulated out of more than 1000 genes according to our microarray study. Assuming that expressions of 2% of genes are governed by histone acetylation/deacetylation status (Weidle and Grossmann 2000), we would expect about 20 genes differentially expressed on each array, which was clearly not the case (Figure 7.1). One possible explanation would be that altered expression of genes other than MDR1 would not lead to survival advantage in FK228-containing medium. Therefore, only cells with upregulation of favorable genes (i.e., MDR1) survive. We did not find any MRP1 upregulation in all four resistant cell lines. This suggests that MRP1 may not be suppressed by histone hypoacetylation.

In conclusion, we developed and characterized four FK228-resistant cell lines. The acquired resistance is due to a reversible MDR1 induction caused by FK228-induced histone hyperacetylation at the MDR1 promoter. Since FK228 is a MDR1 substrate, the rate and extent of induction may be important for clinical prognosis of patients receiving FK228 treatments. In addition, the role of HDACs in silencing MDR1 may have been underestimated. Moreover, the use of histone hyperacetylation as a surrogate marker for HDAC-inhibitor-induced cytotoxicity needs to be re-considered.
CHAPTER 8

FUTURE STUDIES

Pharmacokinetics, metabolism, transport and acquired resistance of depsipeptide FK228, a novel, potent HDAC inhibitor, has been characterized in previous chapters. Two major contributions to the development of FK228 and related agents have been made. First, FK228 was demonstrated to be a prodrug, as supported by both in vitro and in vivo evidences. This information has resolved the paradox that FK228 lacks the required structure as a reversible HDAC inhibitor, but has potent HDAC inhibitory activity, and thus may provide the basis for future structural modifications in order to improve drug stability and potency based on the structures of active metabolites. Second, It has been established that FK228 readily induces resistance in selected cancer cell lines. This resistance is via reversible induction of MDR1 (Pgp) by FK228’s HDAC inhibitory activity, as evidenced by the concomitant histone hyperacetylation at the MDR1 promoter region after FK228 treatment.

However, there is still work to be done in order to fully characterize this novel drug. For example, due to the difficulty in obtaining pure standards of the unstable metabolites, we could not develop a sensitive method to determine the
metabolites in vivo. Consequently, only preliminary pharmacokinetics-pharmacodynamics correlation could be obtained, even though the correlation between CL and histone acetylation status is in consistent with FK228 being a prodrug. No direct evidence has been provided to show that the histone hyperacetylation is due to the presence of effective concentrations of active metabolites following the FK228 treatment.

In addition, while efforts have been made to characterize FK228 disposition and elimination in rats, no definitive conclusion concerning its mass balance can be made without the aid of radio-labeled FK228. This is especially important, since FK228 is a prodrug. The distribution and elimination of the active metabolites deserve further investigations.

The population pharmacokinetic study aimed to identify common covariates for FK228 pharmacokinetics and to explain and predict pharmacokinetic profiles in patients was not successful. This may be due to the limited patient population or lack of real pharmacokinetic covariates that correlate with FK228’s distribution, metabolism or elimination in patients. Based on the results of FK228 uptake and metabolism studies, we proposed some new potential covariates (i.e., GSH level in RBCs) that need to be collected in future clinical trials in order to continue the population pharmacokinetic study.

Furthermore, it is now well known that FK228 is a Pgp substrate, and the Pgp expression level in cancer cells is a major determinant of FK228 antitumor activity; however, no study has addressed to the relationship between Pgp
expression in patients’ tumors and the outcome of clinical treatment with FK228. Moreover, whether the 4-hr infusion of FK228 in patients can lead to Pgp induction and FK228 resistance remains to be demonstrated. The interaction between the two epigenetic modulators, CpG island methylation and histone acetylation status at the MDR1 promoter region, in controlling MDR1 transcription \textit{in vivo} remains unclear.

Indeed, in order to answer these questions, a new clinical trial in leukemia patients has been proposed by Dr. G. Marcucci et al. at the Ohio State University Comprehensive Center. Pharmacokinetic study of this trial will focus on the determination of FK228 metabolites in patient blood samples, as well as searching for more potential clinical covariates for popPK study. Determination of Pgp expressions on isolated leukemia cells both before and after drug treatments will be conducted to reveal its potential correlation with clinical outcome.

In Chapter 7, we demonstrated that global histone acetylation status might not be a good surrogate marker for HDAC inhibitor-induced apoptosis in the clinical settings. This contention was supported by the FK228 resistant cells cultured in FK228 containing medium, which showed global histone hyperacetylation with no apoptosis, but were still sensitive to other HDAC inhibitors TSA an SAHA. This suggests that the FK228-induced histone acetylation and apoptosis may be dissectible; in other words, inhibition of HDACs and histone acetylation might not be the necessary initial step for apoptosis. Consistent with this is the discovery of other mechanisms by which FK228
causes cell killing such as anti-angiogenesis (Kwon, Kim et al. 2002; Mie Lee, Kim et al. 2003; Sasakawa, Naoe et al. 2003). It seems that the global histone hyperacetylation may be achievable at low FK228 concentrations that do not cause cell killing. However, dissection of these two processes may be difficult. One plausible way is to develop a HAT-over-expressing cell line, which has treatment-independent global histone hyperacetylation; alternatively, an HDAC (e.g., HDAC1 or HDAC2) null cell line could be developed, which does not have a primary molecular target for FK228, and thus should have sustained global histone hyperacetylation. Testing FK228 cytotoxicity in these cells may demonstrate if FK228 causes apoptosis without altering histone acetylation status. This method may appear straightforward; however, whether the cells can survive after the genetic perturbation is not clear, since HDACs and HATs may play indispensable roles in cell survival. Additionally, since there are multiple HATs and HDACs in cancer cells, it is difficult to identify a single essential enzyme that is essential to work with. Even after successful development of the cell line, cautions must be made to determine if other HATs or HDACs are upregulated in response to the genetic perturbation.
APPENDIX A

TABLES AND FIGURES NOT PRESENTED IN THE PREVIOUS CHAPTERS
### Between day variation

FK228 concentrations (ng/mL)  
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<th>Average ± SD&lt;sup&gt;b&lt;/sup&gt;</th>
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### Within day variation

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<sup>a</sup> 0.1 mL of rat plasma was used;  
<sup>b</sup> Arithmetic mean ± SD

Table A. 1. Validation of the HPLC/MS/MS determination method of FK228
## Table A. 2. FK228 determination in patient plasma samples.

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Table A. 3. Comparison of pharmacokinetic parameters of FK228 between AML and CLL patients.

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<th>P (two-tail)</th>
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<td>5471 ± 3227</td>
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<td>Cmax (nM)</td>
<td>1433 ± 764</td>
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<td>CL (mL/hr)</td>
<td>9553 ± 3946</td>
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<td>Vss (mL)</td>
<td>7231 ± 3842</td>
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<td>MRT (hr)</td>
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*a The missing value is replaced by population mean during popPK modeling.

Table A. 4. Some clinical parameters used for population pharmacokinetic modeling.
Figure A. 1. A representative HPLC/MS/MS chromatogram of FK228 and BMLP.

Extract from 0.1 mL rat plasma spiked with 0.5 µM each of FK228 and BMLP was injected for the HPLC/MS/MS analysis.
Figure A. 2. FK228 calibration curves for bile, testes, urine, lung, brain, muscle, feces, kidney, heart, liver, fat and plasma samples.

BMLP is used as the internal standard.
Figure A. 3. FK228 plasma concentration-time profiles of the rat following i.v. bolus dosing of FK228 at 2 mg/kg.
Figure A. 4. FK228 plasma concentration-time profiles of the rats following a 4 hr i.v. infusion dosing of FK228 at 2 mg/kg.
Figure A. 5. FK228 plasma concentration-time profiles of the rats following CsA pretreatment and i.v. bolus dosing of FK228 at 2 mg/kg.
Figure A. 6. FK228 plasma concentration-time profiles of the rats following DEM pretreatment and i.v. bolus dosing of FK228 at 2 mg/kg.
Figure A. 7. HPLC/MS Chromatogram of FK228 and its major metabolites.

Peaks of FK228 and identified metabolites A, B, C, D and E are labeled in the figure. Single-ion monitoring (SIM) was used with m/z channels corresponding to individual the MH⁺ ions.
Inhibitory Effect Sigmoid Emax, C=0 at Emax, C=\infty at E_0.

\[ E = E_{max} - (E_{max} - E_0) \times \left( \frac{C}{C + EC_{50}} \right) \]

Figure A. 8. Inhibitory Effect Sigmoid Emax model.

This model is encoded in WinNonLin v.4.0 as Pharmacodynamics model 108, and used for calculations of HDAC inhibitory activity of FK228 metabolites (Chapter 3) and cytotoxicity (Chapter 6 and 7).
Figure A. 9. Purity test of metabolite A by infusion-MS.

Purified metabolite A was induced into API 300 MS by infusion and detected by a) full scan and b) product scan of MH+ ion at m/z 543.
Figure A. 10. Purity test of metabolite B by infusion-MS.

Purified metabolite B was induced into API 300 MS by infusion and detected by a) full scan and b) product scan of MH$^+$ ion at $m/z$ 543.
Figure A. 11. Purity test of metabolites C and D by infusion-MS.

Only full scans of (a) C and (b) D are given due to the lack of characteristic product scan on the API 300 system. Metabolites C and D did not give product ions on the available API 300 mass spectrometer using N₂ as the collision gas.
Figure A. 12. Scatter plots of selected pharmacokinetic parameters and acetylation status of total histone H3 and specific lysines on histone H4 (K5, K8, K12 and K16) at end of the 4-hr i.v. infusion.
Figure A. 13. Scatter plots of selected pharmacokinetic parameters and acetylation status of total histone H3 and specific lysines on histone H4 (K5, K8, K12 and K16) at 24 hr after initiation of the 4-hr i.v. infusion.

H4 K8 and H4 K12 showed opposite trends. The reason for this is unknown.
Figure A. 14. FK228 transport across the Caco 2 monolayer.

(a) BL→AP and (b) AP→BL direction at tested FK228 concentrations of 0.5, 1, 2, 5, 10, 20 μM on the donor-side.
\[ C_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \]

where

\[ A = \frac{D}{V} \frac{(\alpha - K21)}{(\alpha - \beta)} \]
\[ B = -\frac{D}{V} \frac{(\beta - K21)}{(\alpha - \beta)} \]

and \( \alpha \) and \( \beta \) (\( \alpha > \beta \)) are the roots of the quadratic equation:

\[ r^2 + (K12 + K21 + K10) \cdot r + K21 \cdot K10 = 0 \]
\[ k21 \cdot k10 = \alpha \cdot \beta \]
\[ k10 + k12 + k21 = \alpha + \beta \]

Scheme A. 1. A two-compartment pharmacokinetic model provided by WinNonLin 4.0 with i.v. bolus dosing, micro-constants, no lag time, and 1st order elimination from the central compartment.
$C_T = A \cdot (e^{-\alpha T} - e^{-\alpha T^*}) + B \cdot (e^{-\beta T^*} - e^{-\beta T})$

**WHERE** $T_{INF} =$ LENGTH OF INFUSION, $T^* = T - T_{INF}$ IF $T > T_{INF}$, AND $T^* = 0$ IF $T < T_{INF}$

$A = \frac{D}{T_{INF} \cdot V_1} \cdot \frac{(K_{21} - \alpha)}{(\alpha - \beta) \cdot \alpha}$

$B = \frac{-D}{T_{INF} \cdot V_1} \cdot \frac{(K_{21} - \beta)}{(\alpha - \beta) \cdot \beta}$

**AND** $\alpha$ AND $\beta$ ($\alpha > \beta$) ARE THE ROOTS OF THE QUADRATIC EQUATION:

$r^2 + (K_{12} + K_{21} + K_{10}) \cdot r + K_{21} \cdot K_{10} = 0$

$CL = k_{10} \cdot V_1$

$Q = k_{12} \cdot V_1$

$V_2 = Q / k_{21}$

**Scheme A. 2.** A two-compartment pharmacokinetic model provided by WinNonMix 2.0.1 with zero order i.v. Infusion, micro-constants, no lag time, and 1st order elimination from the central compartment.
BIBLIOGRAPHY


is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells." Leukemia 17(2): 350-8.


