PHARMACOKINETICS, PHARMACODYNAMICS AND METABOLISM OF BCL-2
ANTISENSE PHOSPHOROTHIOATE OLIGONUCLEOTIDE G3139 (GENASENSE®)

DISSERTATION

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

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2005

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ABSTRACT

Overexpression of the anti-apoptotic protein Bcl-2 has been found in about half of human cancers. G3139 is an 18-mer phosphorothioate antisense oligonucleotide designed to bind to the first six codons of the open reading frame of the human Bcl-2 mRNA. In this project, the preclinical and clinical pharmacokinetics, pharmacodynamics and metabolism of this novel therapeutics were investigated.

A novel, ultrasensitive, nonradioactive hybridization ELISA method has been developed and validated for quantification of G3139. Plasma pharmacokinetics of G3139 in acute myeloid leukemia (AML) patients was characterized and found to fit a two-compartment open infusion model. The mean total body clearance was 7.1 L/hr and the $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 0.4 hr and 4.3 hr, respectively. There was no major pharmacokinetic interaction between G3139 and concomitant chemotherapeutic agents. Robust intracellular concentrations (ICs) of G3139 in bone marrow (BM) and PBMC obtained from treated AML patients were achieved and a correlation between the Bcl-2 mRNA/protein down-regulation and disease response was found. A higher median ICs of G3139 was detected in the complete responders as compared with non-responders.
Cellular uptake and distribution of G3139 was studied in K562 cells. When exposed to free G3139, only low intracellular concentrations of G3139 were found in the cells with no significant suppression of Bcl-2 mRNA. In contrast, a 10 to 25-fold increase of the intracellular G3139 was observed when G3139 was delivered with cationic lipids. Dose-response curve shows that G3139 concentration that produces 50% down-regulation was 0.29 µM. Two in vitro PK/PD models were developed for AML cells NB4, which describe the relationship between drug exposure and target down-regulation reasonably well.

A novel ESI LC/MS/MS method has been developed. Using this method, several chain-shortened G3139 metabolites were identified in mice, rats and humans, implicating the involvement of 3’-exonuclease in G3139 metabolism. In the mouse, tissue distribution is extensive and the highest concentrations were found in kidneys, liver, spleen and BM. Metabolite kinetics were modeled and they appear to follow formation-limited kinetics.

The results from these studies have provided a better characterization and understanding of disposition and pharmacological roles of G3139 and other antisense therapeutics.
Dedicated to my parents and to my wife
ACKNOWLEDGMENTS

It is with deep gratitude that I thank my advisor, Dr. Kenneth K Chan, for his intellectual guidance, unending support, constant encouragement, patience and stimulation during my graduate studies. Appreciation also goes to my Committee members, Drs. William L Hayton, Robert J Lee, and Guido Marcucci for their time, constructive suggestions and comments on this project. I also thank Drs Dale Hoyt, John Byrd, and Michael A Caligiuri for their support and invaluable comments on our published manuscripts.

The work with G3139 project was made possible from the significant contribution and support by Dr. Guido Marcucci and his laboratory at Division of Human Cancer Genetics, College of Medicine. A special acknowledgment goes to Dr. Tiansheng Shen for her technical assistance in extraction and quantitation of Bcl-2 mRNA, imaging analysis and her continuous friendship which carried me through the difficult time. Thank Dr. Marko Klisovic for the many hours he spent collecting and organizing patients’ samples for both phase I studies involved. I extend appreciation to Ms. Sharon Chiu and Dr. Robert Lee for providing me with the liposomal G3139. Additional thanks go to Dr. Shujun Liu for his assistance on flow cytometry analysis, western blotting and numerous experiments, to Dr. Zhongfa Liu for his assistance on operation of LCQ and
encouragement, to Dr. Yilong Zhang for his assistance with animal experiments and discussion of modeling, and to Ms. Xiaohui Wei for her kind assistance with sample preparation and in setting up LC/MS experiments. I am also grateful to Ms. Kathy Brooks and Ms. Joy Scott for their administrative assistance and their encouragement and friendship. I am indebted to many fellow graduate students and colleagues, whom I cannot name them all, for their valuable scientific discussion and friendship.

I am eternally grateful for the support of my parents and my brother who never held me back and encouraged me to embrace the future with the most positive attitude. Finally I would like to thank my wife, Yanxing Zhao, for her unwavering support both domestically and scientifically.

This work was supported by grant NIH/NCI R21 CA 94552, NIH/NCI U01-CA 76576, and by BioMedical Mass Spectrometry Laboratory at College of Pharmacy, The Ohio State University.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ABL</td>
<td>Abelson leukemia oncogene</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>CIVI</td>
<td>continuous intravenous infusion</td>
</tr>
<tr>
<td>CL</td>
<td>Total body clearance</td>
</tr>
<tr>
<td>CL_R</td>
<td>Renal clearance</td>
</tr>
<tr>
<td>C_max</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>C_ss</td>
<td>Steady state concentration</td>
</tr>
<tr>
<td>CVs</td>
<td>coefficients of variations</td>
</tr>
<tr>
<td>Dig</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>E_max</td>
<td>Maximum effect</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ESI</td>
<td>Electrospray soft ionization</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>The drug concentration at 50% inhibitory</td>
</tr>
<tr>
<td>IC</td>
<td>Intracellular Concentration</td>
</tr>
<tr>
<td>IR</td>
<td>Incomplete remission</td>
</tr>
<tr>
<td>IR-RP-HPLC</td>
<td>Ion-pair reversed phase HPLC chromatography</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>MNCs</td>
<td>mononuclear cells</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MRT</td>
<td>Mean residence time</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NR</td>
<td>No response</td>
</tr>
<tr>
<td>ODNs</td>
<td>oligodeoxynucleotides</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PK/PD</td>
<td>Pharmacokinetics/Pharmacodynamics</td>
</tr>
<tr>
<td>PS ODN</td>
<td>phosphorothioate oligodeoxynucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RNase-H</td>
<td>ribonuclease H</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOS</td>
<td>Simple Oligonucleotide Sequencer</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>t_{1/2}</td>
<td>Half-life</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TEAA</td>
<td>Triethylammonium acetate</td>
</tr>
<tr>
<td>TEAB</td>
<td>Triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>UPN</td>
<td>Unique patient number</td>
</tr>
<tr>
<td>V_{ss}</td>
<td>Steady-state volume of distribution</td>
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CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 Background

The antisense oligonucleotides (ODNs) are sequences of 16 to 26 bases of single-stranded DNA or RNA that hybridize to specific genes or their mRNA products by Watson-Crick base pairing, resulting in a disruption of their function. Technology in antisense ODNs field has grown at an unprecedentedly rapid rate over the past decade. The driving force of antisense therapy is the Human Genome Project which provides about 40,000 gene sequences and 100,000 mRNAs as tools to validate candidate genes for antisense therapeutic purposes (Human Genome Project, link: http://www.ornl.gov/sci/techresources/Human_Genome). Antisense therapeutics represents a new class of drugs. Similar to small molecule drugs/ligands bind to their receptors, antisense drugs bind to RNA in a sequence specific manner and serve as potent inhibitors of translation of specific gene. The specificity and selectivity of antisense therapeutics make antisense ODNs attractive as therapeutics to selectively inhibit the expression of genes differentially expressed in diseased tissue. Although the oligonucleotides containing natural backbone, i.e. phosphodiester linkages, are capable to selectively inhibit the expression of genes, they are not suitable as drugs because they are
rapidly degraded by ubiquitous exonucleases and endonucleases before they reach their intended targets in the body. Therefore, to be an effective antisense agent, the oligonucleotide must be chemically modified to increase its stability against nuclease enzymes. Phosphorothioate (PS) modified oligonucleotides became the choice for the first generation of antisense molecules in which one of the oxygen on the phosphate backbone is replaced with a sulfur (Figure 1.1). Over a dozen of antisense oligonucleotide drugs are undergoing clinical trials for the treatment of infections, cancers, and AIDS. The first drug made by ISIS pharmaceuticals, formivirsen, was approved by FDA for the treatment of cytomegalovirus (CMV) retinitis (Table 1.1). Although phosphorothioate oligonucleotides possess all the required properties to be antisense agents, their application as drugs \textit{in vivo} has been hampered because of their unfavorable pharmacokinetic properties, unexpected toxicity and poor cellular uptake. In fact, the concept for antisense therapy reflects a physiological event and numerous studies \textit{in vitro} have demonstrated the power of this approach. However, there remain some questions whether one can translate it into clinical studies and in a more defined way how we can improve its pharmacokinetic and pharmacological properties by better chemistry and better dosing design? Only limited success in clinical trials has been demonstrated for some antisense drugs. A detailed understanding of antisense mechanism and pharmacology are critical for successful translational research and further development of this approach. Molecular mechanism of action, non-antisense effects, pharmacokinetics/pharmacodynamics (PK/PD) and metabolism of first generation antisense drugs will be reviewed in this chapter.
One of the most intense therapeutic areas for antisense therapy is probably cancer therapy (1). Although chemotherapy has been partially successful, novel selective anticancer drugs with high selectivity and less cytotoxic side effects than conventional chemotherapy still need to be developed. The increasing number of molecular targets identified in the last decade and the problem of selectivity of standard chemotherapy have generated great interest in the development of new generations of anti-cancer agents, which have enhanced molecular targeting for malignant cells and reduced cytotoxic side effects toward normal cells (2). A great number of molecular intervention approaches and gene therapy that selectively target the function and expression of abnormal genes have shown efficacy in preclinical models as well as in early clinical studies. Several approaches are available to specifically manipulate gene expression at transcriptional or translational level of protein synthesis. Gene therapy can be used to reconstruct defective genes or block the effects of unwanted genes by introducing another gene. Gene expression can also be altered at the transcriptional level using single-strand antisense oligonucleotides or catalytic ribozymes to modify gene expression before or at the translational step. From rational drug design point of view, antisense is attractive in term of lead optimization. Generally, the drug discovery and development process starts with identification of an appropriate molecular target responsible for a certain disease followed by the in vitro screening of a panel of thousands of chemicals. A lead chemical will be selected with specific recognition and binding affinity to that target. However, additional time and resources are needed to modify the chemical structure of the lead compound to obtain desired potency, pharmacokinetic profile and toxicity profile. In contrast, the specificity of Watson-Crick base-paring rule is the basis for rational drug
design of antisense oligonucleotides, leading to a new class of selective protein synthesis inhibitors. The process of lead optimization can be expedited from 5-10 years in conventional way to < 1 years (3). Furthermore, similar pharmacokinetic and toxicological characteristics of antisense drugs with different sequences facilitate its development from bench to clinic. The elucidation of the pathogenetic role of individual target proteins for certain diseases is rapidly progressing in basic cancer research over the past decade. The antisense strategy might be used for target validation of these novel molecular targets that can be readily used for discovery of other small molecular inhibitors (4). Collectively, the number of clinical trials ongoing represents a growing interest in antisense technology (Table 1.1). In this chapter, current status of pathogenesis and chemotherapy of acute myeloid leukemia (AML) will be discussed. Discovery, preclinical and clinical experience of Bcl-2 antisense G3139 (Table 1.1) will be reviewed.

1.2 Introduction

1.2.1 Principle of antisense therapy

Antisense therapy is generally intended as a therapeutic approach that modifies the expression of a specific gene by inhibiting processing or translation of its messenger RNA (mRNA). Antisense oligonucleotides are generally chemically modified and 16–25 nucleotides in length. Considerable effort has been focused on the backbone modifications of oligonucleotides. The original phosphodiester linkage can be replaced by phosphorothioate, methylphosphonate, N3’->P5’ phosphoramidate, or neutral peptide linkages (Figure 1.1). Among them, the phosphorothioate derivative is the most widely studied analog. Antisense ODNs are specifically designed to hybridize to its targeting
mRNA by Watson-Crick base-pairing rules. The relatively short length of these ODN sequences is a necessary feature that facilitates cell internalization and increases the hybridization efficiency by reducing base-mismatch errors. In human genome, there are ca. 40,000 genes and 3.16x10⁹ nucleotides (Human Genome Project, link: http://www.ornl.gov/sci/techresources/Human_Genome). Assuming a random distribution of four nucleotide (A, T, G, C), a sequence of 17 nucleotides is expected to occur only once (frequency is 1 out of 1.7 x 10¹⁰) within the human genome (5).

1.2.1.1 Molecular mechanisms of antisense therapy

Antisense oligonucleotides inhibit mRNA function in several ways: 1) interfering the processing of mRNA by sterical inhibition of spliceosomes, and/or regulation of proteins; 2) facilitating degradation of RNA/DNA hybrids by activation of RNase H; 3) binding to DNA, resulting in the formation of DNA triplexes which, in turn, prevents transcription; 4) disrupting ribosome assembly or blocking initiation codon by translational arrest (6, 7). Of these, cleavage of mRNA by RNase H is the most important mechanism. Once the hybridization between antisense oligonucleotides and its target mRNA has occurred, the ODN-mRNA duplex becomes a substrate for intracellular ribonuclease H (RNase-H) that catalyzes mRNA degradation, while allowing the antisense oligonucleotide to recycle for another base-pairing event with the next available mRNA molecule. The net result of this process is a sustained decrease in target mRNA translation and, ultimately, a reduced level of the corresponding oncogenic protein synthesis (8, 9). RNase H is a ubiquitous endonuclease involved in DNA replication. It is found both in the cytoplasm and the nucleus although it enriches in the nucleus (10).
RNase H only cleaves the RNA strand of a DNA-RNA duplex. Recently, human RNase H genes have been cloned and their properties, enzymatic activity as well as cleavage preference have been characterized (11-13). However, the precise recognition elements for RNase are largely unknown. Oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (14). Changes in the sugar component influence activation of RNase H. RNA-like oligonucleotides with sugar modifications are not substrates for RNase H (15). Further, backbone modifications also affect the ability of oligonucleotides to activate RNase H. Methylphosphonates do not activate RNase H (16) (Figure 1.1) and phosphorothioates are excellent substrates for this enzyme (1, 17). In addition, second-generation antisense oligonucleotides or chimeric oligonucleotides (Figure 1.2) have been extensively studied as model for activation of RNase H. For second-generation antisense drugs, efforts has been focused on activation of RNase H, an increase in binding affinity with target mRNA, and enhancing the binding specificity (18). One of the common designs for second-generation antisense drugs is modification of deoxy-ribose with 2’-O-(2-methoxyethyl)(2’-MOE) at 3’ and 5’ terminals for the purpose of enhancing affinity for the target RNA in combination with a phosphorothioate oligodeoxynucleotide gap to serve as a substrate for RNase H (Figure 1.2) (19, 20). For example, ISIS 104838, which is oligonucleotides comprising wings of five 2’ MOE modified nucleotide at both terminal and a ten-base gap of phosphorothioate oligodeoxynucleotide, binds to its target mRNA with greater affinity than all phosphorothioate analog and elicit antisense effect through activation of RNase H (21, 22).
Binding of antisense molecules to its target mRNA at the translation initiation codon also causes translational arrest. It has been shown that appropriate sugar modified oligonucleotide could indeed inhibit translation through RNase H independent mechanism (23). Further, interference with post-transcriptional processing of mRNA could result in potent antisense action. A key step in the process of mRNA is the excision of introns. Antisense oligonucleotides could interfere with spliceosomes which in turn inhibit mRNA production. Additionally, 5’-capping and 3’-adenylation could also be a possible mechanism for antisense molecules (24). Another yet important molecular mechanism is activation of double-strand RNase which will be discussed in Section 1.2.1.3, since it is mostly associated with a new class of antisense molecules, siRNA.

1.2.1.2 Non antisense effect of oligonucleotides

Antisense drugs are large molecules with molecular weight ranging from ca. 5000 –7500 Da and are highly charged due to phosphate linkage (Figure 1.1). They are capable of triggering non-antisense effects that can be both sequence specific and sequence independent. Sequence-specific effects include potential immune stimulation by sequences such as CpG motifs or presence of three or more consecutive guanine residues which can form G quartet structure. It is widely accepted that immune recognition of CpG motifs triggers protective pathways similar to those activated by endotoxins or other microbial products (25). Safety studies in experimental animals have shown that repeated administration of phosphorothioate oligonucleotides containing repeated CpG dinucleotide motifs in a particular base context or G quartets provoke adverse side effects due to cytokine release, decreased platelet counts and hepatotoxicity as a result of
immune stimulation. Non-sequence effects mainly come from its interaction with protein, for example, serum albumin. Phosphorothioate oligonucleotides are more prone to albumin binding than its phosphodiester counterpart. However, the affinity of such interaction with albumin is low with $K_d$ around $200 \, \mu\text{M}$ while capacity is quite high with 95-99 % protein bound in human plasma (26). It also binds much more avidly to various cell membrane proteins for example, interferon-γ receptor, and CD4. Sequence independent mechanism of antisense drugs is responsible for reduced cell proliferation, thrombocytopenia, complement activation which can also be caused by other charged macromolecules such as heparin. Although antisense inhibition seems to be the predominant mechanism responsible for antitumour activity of ODNs, as confirmed in numerous ongoing clinical trials, it is worth noting that the overall response rate could be a summation of both antisense mechanism and non-antisense mechanism.

1.2.1.3 Other nucleic acid based therapeutics

Gene silencing mediated by double-strand RNA (dsRNA) through the process of RNA interference (RNAi) is sequence-specific and highly conserved mechanism in eukaryotic cells (27). Once in the cell, the dsRNAs are processed into short, 21–22 nucleotide dsRNAs termed small interfering RNAs (siRNAs) that is used by the cell in a sequence-specific manner to recognize and destroy complementary mRNA. More recently, Tuschl and co-workers developed a form of 21–22 nucleotide synthetic siRNAs that are too small to activate the interferon pathway in mammals but are capable of activating the RNAi pathway (28). Thus, siRNA has become the most exciting area in the antisense field.
The hallmark of siRNA is that it activates double strand RNase intracellularly which is distinct from antisense mechanism of action (see section 1.2.1.1). Although they differ mechanistically from each other, antisense and siRNA technologies have many similarities. Both molecules work at the post-transcriptional level to reduce the level of a target protein. In cell-culture applications, the cellular delivery and intracellular distribution issues for antisense oligonucleotides and siRNAs are similar. Recently, a comparative study of antisense oligonucleotides and siRNA was conducted (29). The potency, maximal effectiveness, duration of action, and sequence specificity of antisense PS oligonucleotides and siRNA oligonucleotide duplexes were evaluated and found to be comparable. In addition, it was found that the activity of both siRNA oligonucleotides and antisense oligonucleotides are affected by the secondary structure of the target mRNA (29). Nevertheless, antisense technology is a more mature technology with respect to understanding of molecular mechanism, chemical modification, target validation and other properties than the technology of siRNA. Further, strict controls have been established for antisense experiments, for example, mismatch, reverse control, and scramble control, to avoid misleading results. In contrast, there is no consistent control for siRNA experiments. Antisense oligonucleotides accumulate in the nucleus and therefore can be used to alter splicing of precursor mRNAs. However, siRNAs have its distinct advantage over antisense in that lower concentrations are needed to achieve levels of knockdown that are comparable to antisense reagents. Further, siRNAs can achieve stable knockdown of a target gene, while antisense oligonucleotide strategy only produce temporary down-regulation of target mRNA (30).
1.2.2 Pharmacokinetics (PK), metabolism and Pharmacodynamics (PD) characteristic of the first generation antisense drugs

One of the fundamental prerequisites for the successful development of antisense strategy is metabolic stability. The second generation antisense drugs are chimeric oligonucleotides which possess high affinity to mRNA and activate RNase H to degrade RNA. The second generation antisense analogs possess improved physicochemical properties and significantly different pharmacokinetics/metabolism from the first generation drugs. The focus of this dissertation will be on the first generation, i.e., phosphorothioate (PS) oligonucleotide G3139.

1.2.2.1 Uptake and delivery of antisense in cells \textit{in vitro}

Phosphorothioate oligonucleotides are taken up by a wide range of cells \textit{in vitro}. Cellular uptake is time-, concentration- and temperature-dependent. It also depends on cell type, cell-culture conditions, media (with FBS versus without FBS), and length of the oligonucleotides (31). Factors governing differences in uptake of different antisense oligonucleotide were poorly understood. Oligonucleotides are not likely to diffuse across lipid membranes due to their high molecular weight and negative charge. Several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular uptake (32). The mechanism of oligonucleotide cellular uptake and responsible membrane transporters or proteins remain to be fully elucidated. A putative receptor called nucleic acid binding receptor-1 (NABR-1) has been identified that binds to the end-modified oligo(dT) in HL-60 and fibroblast L929 cells (33). Another important oligonucleotide binding protein, Mac-1 has also been described (34). This protein was
located predominantly on the surface of human neutrophils, natural killer cells and macrophages. The binding was competitive with other polyanions, including PS oligonucleotides, suramin, heparin. Pinocytosis may also be involved in oligonucleotide cellular uptake in HL-60 cells (31). Furthermore, the mechanism of oligonucleotide cellular uptake may be cell type dependent because the intracellular distribution of fluorescently labeled oligonucleotides differs among cell types (35). Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (59, 72). Nevertheless, it is not surprising that cellular uptake was inefficient in most studies, which might impede the use and development of therapeutic oligonucleotides.

Cationic lipids have been widely used to enhance cellular uptake of phosphorothioate oligonucleotides in vitro (36). Other approaches to enhance intracellular uptake in vitro have included streptolysin-O treatment of cells (37), polymer (PAMAM dendrimers) (38), and protamine (39, 40).

1.2.2.2 Pharmacokinetics (PK) of antisense therapeutics

Discouraging results obtained from in vitro cell culture studies using radiolabeled oligonucleotides (see Section 1.2.2.1) led to a conclusion that free antisense drugs were not adequately internalized by target cells or tissues and therefore the desired pharmacological activity was not expected. However, researchers shortly after found that phosphorothioate oligonucleotide can be effectively taken up by various tissues with high concentration when given in saline solution (41).
Phosphorothioate oligonucleotides bind nonspecifically to various protein in plasma in a greater affinity than their phosphodiester counterpart. At clinically relevant concentration, more than 96% of PS oligonucleotides in plasma are associated with plasma protein in mice, rats, monkey and humans (42, 43). The primary oligonucleotide binding proteins are albumin and α2-macroglobulin. The apparent affinity for albumin ranges from 150 to 400 µM and the binding affinity to α2-macroglobulin was found to be greater than albumin with less binding capacity. In contrast, binding to α1-acid glycoprotein was negligible. PS oligonucleotide also exhibit high nonspecific binding in tissues (43). The high propensity of PS oligonucleotide drugs bound nonspecifically to proteins may explain in part pharmacokinetics and biodistribution properties of the first generation antisense drugs in animals and humans. Protein-bound drugs serve as a reservoir of circulating oligonucleotides in blood and prevent rapid renal excretion. Excretion of intact drugs in urine is a minor elimination pathway since protein binding prevent it from glomerular filtration (44).

In blood, PS oligonucleotides distribute quickly into tissue with initial disappearance half-life less then 0.5 hr. Plasma clearance exhibit multi-exponential decay pattern with terminal elimination half-life of 5 to 70 hr, depending on the bioanalytical methods used for pharmacokinetic characterization (45-48). It is also worth noting that terminal elimination phase in plasma as detected by radiolabeled method is not specific for parent drug and does not discriminate from chain shorten metabolites. Following repeated dosing at 20 mg/kg in mice and monkeys, nonlinear kinetics were observed as evident by greater than proportional increase of AUC and decrease in clearance. Saturation of tissue uptake, especially liver and kidneys, might contribute to the nonlinear
kinetics. The uptake of drug by tissues, especially, liver and kidneys is the predominant factor in plasma clearance, although metabolism by exonucleases plays a much less role. Urinary excretion seems not to play an important role in plasma clearance. The clearance is also species independent in the rat, dog, monkey and human (49, 50). In contrast, mouse tend to eliminate the PS oligonucleotides faster than the other species with plasma clearance ranging from 8 to 14 mL/min/kg (51).

PS oligonucleotides distribute widely to all peripheral tissues. The highest amount of antisense drugs in all species was found to be liver, kidneys, spleen, bone marrow, lymph nodes, and other tissues have measurable amount of the drug except the brain (48, 52). The liver is one of the major organs for drug accumulation. The highest concentration was often found within the cytoplasm of the non-parenchymal and endothelial cells. In contract, parenchymal cells (hepatocytes) contained significantly less drug. Kidneys contain the highest concentrations per gram of tissue in comparison to the liver (52). Oligonucleotide drugs are reabsorbed in epithelial cells in the glomerular capsule and proximal tubule epithelia. It is critical to evaluate tissue clearance since plasma clearance is not a determinant for overall disposition. Studies have shown that tissue clearance of antisense drugs is slow. By 24 hr, steady-state concentration was achieved after i.v. administration. Parent drugs exhibit longer elimination half-life in kidney, liver, spleen and lymph nodes. Half-life in mouse liver is around 2.8 days while it is longer in monkey liver (50). Tissue exposure was dose-dependent but not linear relationship. Tissue concentrations in liver and kidneys increased less than proportional to dose as the dose increase from 2 to 20 mg/kg.
Excretion of antisense drugs from body is not consistent in previous publications. The main reason is the analytical method used for mass balance studies. Some earlier studies using $^{14}$C-labeled drugs indicated that 15% of total dose was excreted through urinary elimination (48). Another study showed that urinary elimination represented 26% of total $^{35}$S-labeled GEM 91 over 24 hr and 58% over 10 days after administration (46). Clearance studies with radiolabeled drugs may be misleading since catabolites of parent drug containing the radiolabel does not represent the true disposition of the parent drug. For instance, if 5’-end $^{35}$S-labeled oligonucleotides were used for the clearance studies, single nucleotide deletion from the 5'-end may liberate $^{35}$S-labeled mononucleotide that is likely to be excreted rapidly in urine therefore higher dose recovery via urine. More recently, non-radioactivity methods have been utilized to attain more accurate disposition profile of antisense drugs in animals. Assay specific to parent drugs has shown that urinary excretion is dose-dependent and accounts for less than 0.5% of total dose. At higher dose up to 100 mg/kg, it was increased to 6% of total dose over 24 hrs (44). Fecal excretion is a minor elimination pathway which only account for less than 5% of the total dose, while bile excretion was even lower (less than 1% of dose) (45, 53).

1.2.2.3 Metabolism of antisense therapeutics

The principal metabolic pathway for PS oligonucleotide is cleavage via endonucleases and exonucleases (Figure 1.3). The patterns of metabolites suggests that the exonucleolytic cleavage by 3’-exonucleases is the major route of metabolism followed by 5’-exonuclease pathway and endonucleases pathway is probably minor one. PS oligonucleotides are competitive inhibitors of nucleases. At high concentration, PS ODNs
inhibited degradation mediated by both endonucleases and exonucleases. In most cases, there is a pattern of oligonucleotide metabolism which consists of progressively chain-shortened oligonucleotide from the parent compound. In plasma, ESI LC/MS/MS method shows the removal of bases from the 3'-end of the oligonucleotide is the major metabolic pathway. Analysis of $^{35}$S labeled oligonucleotide in plasma also proved the presence of degraded forms of parent compound (41). Both 5' and 3' exonuclease metabolism occurred in liver and kidneys. Using capillary gel electrophoresis (CGE)/UV and ESI HPLC/MS/MS, Gaus and coworkers identified extensive degraded metabolites from the 3'-end in kidneys. There was less extensive degradation in liver. The 5' truncated metabolites represented the predominant species in liver (54). To further identify the metabolic mechanism in tissues, Agrawol synthesized various 3' end-capped, 5'-end capped and 3', 5'-end capped PS oligonucleotide (Figure 1.4) (55). Results showed that 3'-end capped and 3', 5'-end capped were more stable than the 5'-end capped or uncapped oligonucleotides, suggesting that 3' end shortened metabolites are more common than metabolites generated by 5'-exonuclease. These results were further confirmed by placing two or three methylthiophosphonate linkages at the 3'-end of a PS oligonucleotide (Figure 1.1). These 3'-end modified oligonucleotide showed increased in vivo stability (56). These patterns of 3'-end shortened metabolism have been reported for other antisense drugs as well as many species from the mouse to monkey, suggesting that the metabolic pathways are similar and might be sequence independent (50, 53). On the other hand, endonuclease mediated degradation of PS oligonucleotides is a minor pathway and generally not observed. The dominance of the 3'-exonucleolytic activity in
vivo and in vitro liver homogenates suggest that proper modifications on the 3'-end can potentially stabilize antisense drugs in vivo (57).

Although cleavage by the nucleases (either exo- or endo-) are expected to be major metabolic pathways, other types of ODNs metabolites were also observed in animal studies (58). It was suggested that the observed higher molecular weight metabolites might be Phase II conjugates. But Cummins et al. utilized a ESI HPLC/MS/MS method and proved that these unknown metabolites with slower electrophoretic mobility than the parent drug were actually the addition of a ribonucleotide or a phosphorothioate deoxyribonucleotide to the parent compound (54). These results were consistent with another study that has shown that N+1 metabolites were generated from addition of a riboguanosine monophosphate to either 3’ or 5’ terminus. Attempt to de novo sequencing of these N+1 metabolites was not successful (58).

There has been some speculation that PS ODNs undergo metabolic oxidation, i.e., sulfur for oxygen oxidation at the phosphorothioate linkages in vivo via the CYP450s or flavin-containing monooxygenases (59). Nicklin et al. (60) have detected minor products consistent with sulfur for oxygen exchange by the ESI/MS/MS method. However, another study demonstrated that oxidation did not contribute to metabolism of PS ODNs in whole liver homogenates over an 8 hr period (61). The discrepancy could be explained by different processing and handling of tissue samples.
1.2.3 Acute Myeloid Leukemia in the Elderly Patients

Cancer is the second overall leading cause of death in the United States for people over the age of 65, with more than 60% of all new cancers occurring in this population. Acute leukemia is a heterogeneous group of neoplasm composed of blasts or minimally differentiated cells. Acute leukemia is broadly divided into myeloid and lymphoid categories based on the cell origin. Similar to other types of cancers, the incidence of AML increases with age with one case per 100,000 people per year under age of 30 years old, while the incidence reaches 17 per 100,000 by age 75 year. The median age of AML patients is in the range of 65 to 70 years (62). There were 10,600 estimated new cases of AML in the United States in 2002, with 50% led to death. Of note, although a number of clinical and biological factors have been used to predict prognosis in AML, age and cytogenetics have been shown to be the most useful indicators for clinical outcome. Cytogenetic abnormalities such as 11q23 abnormalities, deletions of chromosomes 5 or 7, other unbalanced translocations, complex karyotype (5 or greater abnormalities), or primary chromosome additions that have been shown to predict short remission durations and survival, occurred more frequently in older AML patients (age >60) than in younger AML patients (63). Recent studies have shown that the currently available treatments with conventional chemotherapy are not effective in elderly AML patients (64). Unfortunately, new treatment strategies (e.g., high dose chemotherapy with autologous/allogeneic stem cell transplantation) have not been feasible for the majority of this patient population because of high incidence of treatment-related toxicity with increasing age. About 50 to 80% of the AML patients who achieve CR experienced disease relapse. The main therapeutic challenge in the treatment of AML is the ability of
therapeutic strategies to overcome chemoresistance and to treat relapsed patients successfully. As the biology of elderly leukemogenesis becomes better understood and the geriatric population continues its rapid growth, it becomes imperative to design innovative therapeutic strategies that might finally address the question how to improve the clinic outcome of elderly AML.

1.2.4  Bcl-2 family: its role in chemoresistance in AML

1.2.4.1 Bcl-2 family and its role in apoptosis and survival signaling pathway

Bcl-2 stands for B cell lymphoma/leukemia 2 and is a human proto-oncogene located on chromosome 18. In cancer cells, the bcl-2 gene is translocated from its normal chromosomal position at 18q21 into close proximity to the immunoglobulin heavy chain (IgH) enhancer on chromosome 14. This translocation places Bcl-2 gene close to the heavy chain enhancer and result in transcriptional activation of Bcl-2 gene. Overexpression of Bcl-2 is solely responsible for the lymphoma phenotype. The Bcl-2 family of proteins is central regulators for apoptosis. There are at least 16 other proteins in the Bcl-2 family (65). These proteins can be divided into two groups: pro-death or pro-apoptotic proteins and pro-survival or anti-apoptotic proteins. For example, Bcl-2, Bad, Bcl-XL, and Mcl-1 are anti-apoptotic members, while Bax, Bak Bid belong to the pro-apoptotic group (Figure 1.5). Bcl-2 gene is an anti-apoptotic gene that plays a major role in prevention of apoptosis in cancer. As a proto-oncogene, its product is an integral membrane protein located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membrane of the mitochondria (66). Bcl-2 has intracellular half-life of about 14 hr and molecular mass of 26 kD (67).
Mitochondria are the primary site of action of Bcl-2 protein family. A variety of physiological death signals and pathology cellular insults trigger the apoptosis. Dysregulation of apoptosis machinery can promote cell accumulation by slowing the rate of cell turnover and providing neoplastic clones with a selective survival advantage relative to normal cells. Anti-apoptotic processes contribute significantly to cancer progression and metastasis by providing a germane environment that cause oncogene activation, genetic instability, and metastasis could prevail over normal cell cycle and mitosis. Apoptosis manifests in two major execution programs downstream of the death signal: the caspase pathway and organelle dysfunction. The fundamental steps of apoptosis are primarily regulated by 3 key families: Bcl-2, tumor necrosis factors (TNF) and caspase. During the apoptosis, there is a change in mitochondrial membrane potential, production of reactive oxygen species (ROS), opening of the permeability transition pore (PTP), and the release of cytochrome c. Released cytochrome c binds and activates Apaf-1, which in turn activates a downstream caspase-9 and caspase-3 execution program, i.e., intrinsic apoptosis pathway. Caspases could be activated through apoptotic protease-activating factor 1 (Apaf-1)/cytochrome c or directly by activation of cell surface death receptors through caspase-8 or extrinsic apoptosis pathway. Finally, The activated caspase-3 cleaves death substrates poly-ADP ribosylpolymerase (PARP), which ultimately leads to cell death (Figure 1.5).

Bcl-2 family regulates intrinsic apoptosis pathway. Bcl-2 and anti-apoptotic proteins work in a manner to stabilize the mitochondrial membrane such that cytochrome c is not released from mitochondria when cell death stimuli present. Thus the initiation of intrinsic apoptosis pathway is blocked. In contrast, Bax migrates to mitochondria
membrane and form homodimer on the membrane and trigger cytochrome c release to cytosol to activate caspase-9 and Apaf-1. But Bcl-2 or other anti-apoptotic proteins can form heterodimer with Bax and cancel its pro-apoptotic effect (Figure 1.5). Several other soluble molecules are released from mitochondria to the cytosol or nucleus in response to apoptosis stimuli. One of them is apoptosis inducing factor (AIF) which causes large scale DNA fragmentation, peripheral chromatin condensation and loss of mitochondrial membrane potential. Overexpression of Bcl-2 can block its release from mitochondria (Figure 1.5). Bcl-2 protein offers cancer cells a higher apoptosis threshold against chemotherapy-induced stress by stabilizing the mitochondrial membrane thus blocking the intrinsic apoptosis pathway.

1.2.4.2 Bcl-2 overexpression: an important chemoresistance mechanism in AML

Abnormally elevated bcl-2 production has been found in roughly half of human cancers, including melanoma, prostate cancer, breast cancer, lung cancer, AML, chronic lymphocytic leukemia (CLL), and lymphoma, suggesting that the deregulated expression of this proto-oncogene represents one of the most common events associated with human malignancies (68). About 50 to 80 % of the AML patients who achieve complete remission experienced disease relapse. The low remission rate and the high incidence of relapse in elderly AML patients are likely related to chemoresistance. The main therapeutic challenge in the treatment of AML is the ability of therapeutic strategies to overcome chemoresistance without increased dose-related toxicity. Leukemogenesis is a complex process involving multiple genetic alterations that result in uncontrolled cell proliferation and abnormal differentiation. Many biological mechanisms have been found
to be involved in resistance of myeloid blasts to therapy. Among them, anti-apoptotic processes contribute significantly to cancer progression and metastasis. Therefore, AML appears to result not only from uncontrolled cell proliferation but also from defect apoptosis. Thus, great interest has emerged in developing novel therapeutic strategies for modulating the key factors of apoptosis in the past few years (69, 70).

Many chemotherapeutic drugs exert their effects through apoptosis pathway that can be blocked by over-expressed Bcl-2 protein. This ultimately results in a lower response rate to chemotherapy and earlier relapse of disease. The checkpoint of apoptosis is delicately controlled by the ratio of pro-apoptotic and anti-apoptotic members (71). On the basis of these data, a direct involvement of Bcl-2 overexpression as a mechanism for chemoresistance in leukemia has been suggested (65). It has been reported that expression of Bcl-2 is generally lower in AML patients with the favorable t(8:21) karyotype in comparison with patients with other chromosome aberrations (72). It was also noted that pediatric and elderly patients express high level of Bcl-2 when compared with young adults with favorable t(8:21) AML. Overexpression of Mcl-1, another anti-apoptotic protein, was also identified in relapsed AML as a negative prognostic factor (73).

Based on the role of Bcl-2 protein in regulating intrinsic apoptosis pathway and its frequency of overexpression in many cancers, they could be used as novel molecular targets for either direct cytotoxic effect or chemosensitization of tumor cells to conventional chemotherapy. Bcl-2 itself is an attractive target since the protein is overexpressed in AML and other hematological cancers and associated with
chemoresistance to cytotoxic drugs. One of the most exciting strategies would be antisense therapy (see section 1.2.5). Other approaches also generate excitement in the past few years, including peptides or small molecules mimic BH3 domain of Bcl-2 to affect Bcl-2 regulated apoptosis pathway (74).

1.2.5 Bcl-2 antisense therapy: facilitating apoptosis in leukemia

1.2.5.1 Discovery of G3139

G3139, an 18-mer fully phosphorothioate antisense ODN with sequence of 5’-TCTCCAGCGTCGCCAT-3’ designed to bind to the first six codons of the open reading frame (ORF) of the human Bcl-2 mRNA, is being investigated in several phase I to III clinic trials (75-77). As high levels of the Bcl-2 were found associated with chemoresistance in malignant cells, the main hypothesis of these studies is that antisense down-regulation of its target would decrease the apoptosis threshold and induce chemotherapy sensitivity in otherwise chemoresistant disease. The initial efforts of many investigators have led to the synthesis of G3139 (78). During the drug screening process, 40 phosphorothioate ODNs were designed to target sites from 750 bases upstream of the initiation codon to the 3'-end of the Bcl-2 cDNA sequence (Figure 1.6). Secondary structure of Bcl-2 mRNA based on RNAdraw algorithm (a software to calculate secondary structure and melting curve of mRNA) (79) is rather complicated as shown in Figure 1.7. Local secondary and tertiary structure around the hybridizing sequence in Bcl-2 mRNA could sterically inhibit accessibility of antisense molecules to its target. Therefore, an ideal antisense drug should bind to the open sites of Bcl-2 mRNA without self-hybridization. Among them, G3139 was proved the most biologically active
antisense compound although two other ODNs also demonstrated similar antisense effect (position 422 and 717) (80). It is not surprising that G3139 is the most active ODN among many other ODNs, since Bcl-2 mRNA is single stranded at the AUG site to allow ribosomal binding to mRNA, thus more accessible to antisense molecules (81). This compound has demonstrated a potent antisense activity \textit{in vitro} by downregulating Bcl-2 expression and lowering the apoptotic threshold in resistant tumor cells treated with cytotoxic agents or chemosensitization effect. Treatment of B-cell lymphoma cell lines carrying the t(14:18) translocation with G3139 resulted in down-regulation of Bcl-2 and a significant reduction in cell viability (82). Further, \textit{in vivo} administration of the G3139 to SCID mice inoculated with lymphoma xenografts has been shown to eliminate malignant lymphoproliferation, as measured by PCR for the t(14:18) translocation. Of 60 mice treated with 5 mg/kg of G3139 daily for 2 weeks, 83% achieved complete abolition of lymphoma (82). G3139 was also evaluated \textit{in vitro} and in mice with human 518A2 melanoma xenografts. In SCID mice with human melanoma, a dose of 5 mg/kg/day of G3139 for 14 days led to significantly lower mean tumor weight while reverse or mismatch control ODNs failed to show benefit.

1.2.5.2 Preclinical pharmacokinetics and biodistribution

The preclinical pharmacokinetics of G3139 has only been studied in BALB/c mice using radiolabeled G3139 (Table 1.2). Studies in mice demonstrate that G3139 is rapidly distributed and slowly eliminated from plasma with low blood levels 6 hr after administration of single i.v. bolus of 5 mg/kg (83). The pharmacokinetic profiles of G3139 follow i.v. administration was fitted with a three-compartment model with $\alpha$, $\beta$
and $\gamma$ half-lives of 5 min, 37 min and 11 hr, respectively. It is highly protein bound (98% at 5 minutes) (83), consistent with the property of other PS ODNs (see Section 1.2.3.2). Tissues to plasma ratios were 87 for kidney, 17 for liver, 5 for spleen, 2.5 for heart and lung and 3.5 for gut. In contrast to widely distribute to various tissues, G3139 does not cross the blood-brain barrier (BBB). When G3139 was administered via slow subcutaneous (s.c.) infusion in mice, plasma steady state was reached by day 3 and terminal elimination half-life was 22 hrs (83). Following s.c. infusion, about 50 % of the parent compound was protein bound at steady state, with significantly more parent drug reaching the tissues and bone marrow. The primary elimination route following both i.v. and s.c. administration in mice appears to be renal, with greater metabolism and elimination of the parent compound occurring with i.v. bolus administration than that with sc infusion. G3139 was metabolized to at least three different products in plasma, liver and kidney. After both routes of administration, most of the radioactivity was eliminated in the urine and to a less extent in the feces. Significantly less drug was excreted in the urine after s.c. infusion than that with i.v. administration (7% versus 33 % by 24hrs) (83).

Another study using 5'-$^3$H-labeled G3139 was conducted in combination with doxorubicin (Dox) in SCID mice bearing a human breast cancer xenograft. G3139 administered i.v. at 5 mg/kg dose revealed a biexponential decay with $\alpha$ and $\beta$ half-lives of 18 min and 9.8 hr, respectively (84). The plasma clearance was 10.5 mL/hr in comparison with 3.2 mL/hr when given as i.p. administration. The bioavailability of i.p. administration was 84 % compared to i.v. administration. No change in clearance after
three prior i.p. doses of G3139 was observed. Concomitant Dox had significant effects on the PK of G3139. C\textsubscript{max} was increased about 1.5 fold and higher AUC and a 9-fold lower plasma clearance were observed. The rate of G3139 accumulation in organs was dependent upon the presence of doxorubicin (84). Significant accumulation of G3139 was observed in solid tumors, with peak levels of 5 µg/g. Tumor exposure to Dox was increased two to three fold without alteration in Dox pharmacokinetics in the presence of G3139.

However, the results from these studies suffer from a lack of specificity due to the bioanalytical method used. The $^{35}$S-labeled at 5'-end of G3139 could generate significant radioactivity due to degradation products/metabolites in the \textit{in vivo} study (83). Similar problem also exist for 5'- $^3$H-labeled G3139 used in the second study (84). Direct scintillation counting used in this study to quantify drug concentration only provides information regarding to the disposition of the radioactivity of tritium rather than that of G3139. The drug-drug interaction, biodistribution, and metabolism results are neither informative nor conclusive.

1.2.5.3 Clinical pharmacokinetics and cancer pharmacology

High Bcl-2 expression has been demonstrated in up to 55% of patients with large-cell non-non-Hodgkin lymphoma (NHL). In a phase I study the safety and activity of G3139 as a single agent in patients with NHL were evaluated. Twenty-one patients received a 14-day s.c. infusion of G3139 with doses ranging from 4.6 to 195.8 mg/m\textsuperscript{2}/day) (Table 1.3) (85). There was a linear correlation between C\textsubscript{ss} and dose (p=0.002). Renal function significantly affect C\textsubscript{ss} (p=0.044). The mean elimination half-life was 7.46
hr (SD: 4.32 hr) (Table 1.3), which was different from that reported for other PS oligonucleotides. The minimally effective plasma concentration appears to be 1 µg/mL, which was consistent with preclinical tumor-bearing animal model. The maximum tolerated dose (MTD) in this trial was 147.2 mg/m$^2$/ day equivalent to 4.1 mg/kg/day. Dose limiting toxicities included thrombocytopenia, hypotension, fever, and asthenia. There was 1 complete response (CR), 2 minor responses, and 9 patients who experienced stable disease (SD). Bcl-2 protein was reduced in 7 of 16 assessable patients. This trial demonstrated that G3139 can be safely given to patients with NHL with promising antitumor activity.

A phase I clinical study combining G3139 with decarbazine, which is the standard chemotherapy for malignant melanoma, was evaluated in 24 patients with advanced malignant melanoma, including patients with disease resistant to decarbazine and other first-line regimens (86). G3139 was administered as continuous i.v. infusion (CIVI) for 14 days ranging from 0.6 mg/kg/d to 6.5 mg/kg/d, with dacarbazine 200 mg/m$^2$ per day given for 5 days beginning on day 5. A 6.5 mg/kg/d dose led to mean C$_{ss}$ of 6.47 µg/mL (SD=2.51 µg/mL). All combinations were well tolerated. Hematologic toxicities were mild or moderate in severity and were similar to those observed with single-agent decarbazine therapy. Antitumor activity was reported in 6 of 14 patients with disease stabilization for at least 1 year and a median overall survival of 17 months which favorably compares with 4–5 month overall survival time generally reported in patients with advanced melanoma who have failed first-line systemic chemotherapy (86). All patients demonstrated Bcl-2 protein expression in melanoma metastases at the baseline. There was a 75% decrease of Bcl-2 protein during G3139 administration in 10 out of 12
evaluable patients whose plasma G3139 concentrations were greater than 1 µg/ml. These results establish proof of principle in human subjects of downregulation of the target protein following systemic administration of G3139. Based on this promising result, a multicenter, randomized phase III trial was conducted to assess the effectiveness of decarbazine with or without G3139 in the treatment of stage III/IV malignant melanoma patients (87). A 5-day CIVI of G3139 7 mg/kg/day plus decarbazine 1000 mg/m² was compared with decarbazine 1000 mg/m² alone. A total of 771 patients were randomized at 139 study sites in 9 countries. The primary endpoint of overall survival was not achieved in this study. However, the results of the statistical analysis favored the G3139 plus decarbazine because all secondary endpoints statistically significantly supported the response benefit achievable with G3139 plus decarbazine. Progression-free survival time (PFS) was significantly longer in the G3139 plus decarbazine group than decarbazine alone (median: 74 versus 49 days, p=0.0003). The antitumor response rate for G3139 plus decarbazine was 11.7% versus 6.8% for decarbazine alone (p=0.019).

These results suggest that G3139 should be tested in combination with chemotherapy and other approaches, such as monoclonal antibody therapy. More recently, pharmacokinetics and safety of G3139 in combination with weekly paclitaxel was evaluated in patients with advanced cancer (88). The maximum serum concentration and AUC increased with dose. The terminal plasma half-life of G3139 was 2 h. The C_{ss} was achieved at 24 hr and remained constant throughout the infusion. The mean C_{ss} increased linearly with doses up to 5.3 mg/kg/d. However, at 6.9 mg/kg/day, the plasma level was higher than projected. In combination with mitoxantrone, G3139 reaches C_{ss} at 24 hr. Mean C_{ss} was 2.82 µg/mL (SD=0.66) for 3 mg/kg/d and 4.29 µg/mL (SD=0.52) for
5 mg/kg/d. Bcl-2 protein levels in lymphocyte were decreased in 5 of 5 patients as assessed by flow cytometry(89). Rudin et al. (90) conducted a phase I trial evaluating G3139 (5 and 7 mg/kg/d, CIVI on days 1–8) plus carboplatin and etoposide on day 6-8 in patients with chemorefractory small cell lung cancer. Average G3139 plasma concentration at G3139 dose of 5 mg/kg/d was 2.01 µg/mL (SD=1.12), 3.03 µg/mL (SD=1.79) at the dose of 7 mg/kg/d. Post infusion G3139 half-life on day 8 was estimated to be 1.86 hours (SD=1.70), suggesting that carboplatin and etoposide do not significantly alter G3139 metabolism and clearance (Table 1.3). Of 14 patients assessable for response, partial response was documented in 12 patients (86%), and stable disease in two. Dose selection of G3139 in clinical studies was based on previous extensive preclinical PK and PD data in tumor-bearing animal model which have shown that G3139 concentrations above 1 µg/mL were sufficient to achieve downregulation of intracellular Bcl-2 protein in vivo. This concentration (1 µg/mL) was established as a minimum target concentration.

1.2.6 Challenge in analysis of antisense drugs in biological matrix

1.2.6.1 Current method used for quantification of PS oligonucleotides in biological matrices

As a fairly new class of therapeutics, antisense oligonucleotide compounds lack an established bioanalytical method for studying PK, PK/PD correlations and metabolism. This poses a serious challenge for better understanding of the therapeutic role of this class of compounds as a single agent or in combination with other drugs. Early studies mainly used radiotracer or anion-exchange chromatography (AX-HPLC)
with radiolabel oligonucleotides followed by radioisotopic detector to study the disposition of oligonucleotides (48, 83). These methods, however, are not specific to antisense compounds nor able to resolve closely spaced chain shortened metabolites. Additionally, radiolabeled drugs are not suitable for use in clinical settings. Furthermore, previous data generated by radiolabeled oligonucleotides were inconsistent due to differences in radiolabel and labeled positions. Most radiolabeled drugs utilized either 5'-end or 3'-end radiolabels (\(^{35}\)S or \(^{3}\)H) which could explain many of discrepancy of published pharmacokinetic and metabolism data. Uniformly labeled \(^{35}\)S, \(^{14}\)C or \(^{3}\)H seem to be superior to only one label on either end of oligonucleotide. However, for \(^{3}\)H label, there was a report that tritium label could actually exchange with water during the extensive sample preparation. Therefore, in using radiolabeled drugs, it is critical to consider if the radiolabel applied is stable, if the label location is subjective to metabolic attack, and if the separation method (i.e. HPLC or AX-HPLC chromatography) is adequate.

More recently, capillary gel electrophoresis (CGE) has been successfully used as a standard bioanalytical tool for quantification of antisense ODN. Leeds et al demonstrated that CGE/UV coupled with a unique two-step solid phase extraction met nearly all requirements of linearity, accuracy and precision for quantification of ODN (91). However, the sensitivity of this assay was only 10 nM, which is not sufficient for defining either the elimination phase of ODNs in plasma or intracellular exposure after drug treatment. Additionally, these methods require extensive sample preparation and in the most cases, recovery was not reported. Other methods like fluorescent end-labeling technique combined CGE/laser-induced fluorescence (LIF) detection may provide good
sensitivity (LOD was 1 ng/ml) and selectivity (only 3’ deletion metabolites be captured by ligation), but evidence for reproducible quantification in biological matrix especially intracellular drug levels was not reported (59).

Although ESI LC/MS/MS technology has been widely used for quantitative analysis of small organic molecules, it has been a challenging task to quantitatively analyze antisense drugs in biological matrices. This is probably due to the inherent differences in analysis between small molecule drugs and antisense drugs (Table 1.4) with respect to sample preparation, mass spectrometry fragmentation pattern, charge state distribution, and chromatography, for example, fragmentation of antisense ODNs under MS² is complicated while it is usually straightforward for small molecules. Antisense drugs are not easily electrosprayed due to cation adduction, which requires special mobile phase additive to enhance mass spectrometry sensitivity. To date, there was only one published paper using LC-MS for quantification of antisense drugs in biological samples (58). ESI LC/MS/MS appears to be the ideal bioanalytical tools for antisense drugs in terms of assay selectivity, sensitivity, robustness, and accuracy. However, quantification methods using ESI LC/MS/MS still remain to be fully explored.

The nature of hybridization between two oligonucleotides has attracted some attentions in the past 4 years as a useful approach in sample preparation. The binding affinity between two single strand oligonucleotides in length of 16-27 nucleotides is on the order of 10⁻⁸ M. Thus, theoretical sensitivity (low nM ranges) should be much higher than UV related chromatography and might be comparable to radioactivity methods. However, a competitive hybridization based ELISA only achieved a limit of detection (LOD) of 4 nM (92). Another hybridization based ELISA method provided good
sensitivity and selectivity but evidence for reproducible quantification in cellular matrix was not reported (93).

1.2.6.2 Challenge in metabolism studies of antisense drugs using LC/MS/MS

Electrospray ionization Mass spectrometry (ESI MS) has become an indispensable tool for analysis of macromolecules such as DNA, RNA, protein and peptides, and synthetic oligonucleotides. ESI mass spectra of oligonucleotides are usually obtained under negative ESI conditions and often suffer from both low intense multiply charged ions and Na$^+$ and K$^+$ adduct signals with a Gaussian like distribution. Characterization of oligonucleotides by ESI MS has been rather difficult due to the lower sensitivity and several analytical problems associated with this type of molecules, such as

1) inadequate chromatographic separation in either reversed phase or anion exchange chromatography, 2) lack of suitable ion-pairing reagents for both MS sensitivity and HPLC chromatography, and 3) extensive adduction with ubiquitous cations, such as sodium or potassium ions, in biological samples (94). Reduction of sodium adducts is critical to obtain high quality spectra thus accurate mass measurement. One breakthrough study of effect of solvent additive on MS sensitivity of oligonucleotides was reported by Greig and Griffey (95). It was shown in their study that organic bases such as Triethylamine (TEA) and piperidine reduce the signals from bound sodium most effectively, but also decrease the total ion current from oligonucleotides. Imidazole provides modest suppression of cation adducts with improved MS sensitivity. Another strategy for reduction of cation adduction is the post-column addition of sheath liquid which offers both the reduction of cation adducts and flexibility of shifting the charge
state distribution of multiply charged ions including oligonucleotides (96). It has been shown that very few cation adducts of oligonucleotides were observed in the mass spectra when post-column sheath liquid such as methanol, acetonitrile, iso-propanol, with or without triethylamine or hexafluoroisopropanol was applied. However, organic acids and bases in the sheath liquid generally deteriorated the signal-to-noise ratios in the chromatograms and mass spectra mainly because of increased background noise.

Although ESI MS provide reasonable mass accuracy for simple mixture of oligonucleotide, additional dimension of separation is usually advantageous to investigate more complex biological samples, for example, plasma or animal tissue extracts, where there are extensive adduct formation and compounding oligonucleotides with similar sequence. It appears the problem of mobile phase incompatibilities between HPLC and ESI/MS has not been well solved in the field of oligonucleotide. As suggested by Apffel et al that optimized mobile phase composition for ion pair reverse phase HPLC typically compromises the MS sensitivity (97), several solvent systems were reported to overcome the problem of incompatible HPLC mobile phase to electrospray for oligonucleotides. Huber et al. used trimethylammonium bicarbonate (TEAB) with addition of post-column acetonitrile as sheath liquid and achieved sensitivity of 104 fmole for (dT)16 by using a capillary column (98). Same group later reported another stronger mobile phase additive butyl-dimethylammonium bicarbonate (DMBA) at 25 mM increased detection sensitivity by a factor of 10 as compared with TEA additive (99). However, to date, utility of these mobile phase systems in study of oligonucleotide metabolism has not been reported.

Sample preparation is of paramount importance for the success of metabolism studies. General methods used are based on precipitation of ODNs from ammonium
acetate solutions or acetonitrile but low recovery remains a matter of concern. Ultrafiltration and size-exclusion spin columns do not provide a sufficient degree of desalt. Dialysis allows for a high degree of desalting, but it is difficult and costly to large number of samples. In fact, due to the major difference in analysis between small molecules and antisense drugs (Table 1.4), there was only one published paper showing the power of LC/MS/MS methods in identification of antisense drugs in biological matrix (58).

1.3 Rational for the current investigation

Bcl-2 is currently a frequently investigated novel molecular target for anticancer therapeutics (see section 1.2.4). Over-expression of this antiapoptotic protein appears to contribute to chemoresistance to a variety of agents and radiation through apoptosis pathway. Differential expression of this protein in tumor tissues in contrast to normal tissues makes Bcl-2 attractive molecular target to restore the chemosensitivity of resistant tumor cells. Bcl-2 antisense strategy, i.e. G3139, is currently being investigated in several phase I to III clinical trials for both solid tumors and hematologic malignancies (see Section 1.2.5). In leukemia, Bcl-2 antisense is a rational strategy since normal early hematopoietic stem cells do not express Bcl-2 while AML blasts express higher levels of Bcl-2 than normal progenitor cells. The working hypothesis to these studies is that by down-regulating Bcl-2, G3139 could decease the apoptosis threshold, thereby re-inducing chemotherapeutic sensitivity in otherwise resistant cells. However, a recurrent motif in the G3139 clinical trials has been the lack of correlations between plasma drug levels, Bcl-2 down-regulation and disease response. This problem stems in part from the low
sensitivity of the previous reported analytical methods, the uncertainty of whether significant levels of G3139 uptake occur in malignant cells and how the intracellular distribution of the drug related to down-regulation of its target. As such, a novel and highly sensitive assay is needed to address these questions.

The practical utility of pharmacokinetic studies is based on the assumption that drug exposure and/or pharmacokinetic parameters are correlated with pharmacological effects, and ultimately with clinical response/outcomes. Pharmacodynamic or pharmacology study is critical for better understanding of a novel therapeutics. There has been no detailed pharmacodynamic data reported for G3139 to date. Therefore, it is necessary to identify appropriate biomarker(s) of pharmacological response for G3139. Most of the time, those biological data are indirect measures of underlying physiological phenomena which cannot be quantified directly. Thus, biomarkers allow us to begin to probe the complexity of molecular targeted therapeutics such as G3139. Specifically, the Bcl-2 mRNA and protein as well as other Bcl-2 family protein/mRNA will be evaluated as biomarkers for pharmacological effect of G3139 and correlation between Bcl-2 (or other factors) downregulation after treatment with G3139, pharmacokinetics parameters and disease response will be explored in vitro and in vivo.

There is no information available regarding to metabolism of G3139 in vivo. Further, only limited pharmacokinetics of G3139 was reported previously. Identifying both the metabolism and pharmacokinetics of G3139 will provide information for potential molecular improvement to enhance the cellular uptake of G3139 in leukemia blasts. Characterization of disposition of G3139 in vivo may also facilitate combination strategies with conventional chemotherapy in AML such as Ara-C or daunorubicin.
1.4 Specific Aims

The purpose of this study was to investigate the cellular uptake, pharmacokinetics, pharmacodynamics and metabolism of G3139. It was accomplished by the following nine specific aims:

a) To develop and validate a novel, sensitive and specific hybridization ELISA assay for G3139 in various biological matrixes.

b) To measure intracellular drug concentration, evaluate cellular uptake, intracellular localization and pharmacology of G3139 in cultured leukemia cell lines and leukemia blasts obtained from patients.

c) To develop an ESI LC/MS method coupled to ion-pair reverse phase HPLC (IR-RP-HPLC) chromatography for separation and characterization of G3139.

d) To investigate the metabolic profile of G3139 in mouse, rat and human, and to identify the major metabolites of G3139 by the ESI LC/MS/MS method.

e) To determine metabolite kinetics of G3139 and its major metabolites in the rat.

f) To study the biodistribution and PK of G3139 in mouse.

g) To correlate down-regulation of Bcl-2 mRNA/protein with intracellular concentrations of G3139 in AML cell lines through PK/PD modeling.

h) To determine the clinical PK and PD of G3139, to investigate the PK/PD and clinical response correlation of G3139.

i) To guide the ongoing phase III and future clinical studies of G3139 in combination with chemotherapy with the above results.
1.5 Overview of Dissertation

This dissertation consists of eight chapters.

Chapter 1 described the principle and molecular mechanisms of antisense therapy, PK/PD characteristics of first generation antisense drugs, current knowledge of Bcl-2 antisense PS oligonucleotide G3139. Major issues about pharmacokinetics, pharmacodynamics, and metabolism of G3139 were proposed. The objectives of current investigation study were outlined.

Chapters 2-8 present the main body of this dissertation. In Chapter 2, development and validation of a novel, sensitive and specific hybridization ELISA assay for G3139 in various biological matrices are discussed. This novel assay lays a solid foundation for the following PK/PD and metabolism study of G3139 in vitro and in vivo.

Chapter 3 described the cellular uptake study on cell lines and leukemia blasts. G3139 induced cytotoxicity and apoptosis on cell lines were evaluated. How the intracellular drug levels correlated to down-regulation of Bcl-2 was discussed.

In Chapter 4, the clinical activity, pharmacokinetics and safety of G3139 was presented.

In Chapter 5, a novel ion-pair reverse phase ESI LC/MS/MS method was developed for identification and quantification of G3139 and metabolites in vivo. Detailed methodology and new findings were presented.

Chapter 6 detailed biodistribution and pharmacokinetics of G3139 in mouse. Metabolite kinetics of G3139 and three major metabolites in the rat were studies.

Chapter 7 two in vitro PK/PD models were proposed to describe the dynamic perturbation of Bcl-2 mRNA and protein by G3139.
In the last chapter, Chapter 8, the results from these studies were summarized, and implications for future clinical studies were proposed.
Table 1.1: Summary of recently published clinical studies with antisense therapy
<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Indication</th>
<th>Type of study</th>
<th>Dose ranges</th>
<th>Treatment duration</th>
<th>Administration</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitravene</td>
<td>CMVIE2</td>
<td>Cytomegalovirus induced retinitis</td>
<td>FDA approved</td>
<td>N/A</td>
<td>21 days every 4 weeks</td>
<td>Intravitreal injection</td>
<td>(100)</td>
</tr>
<tr>
<td>ISIS 3521</td>
<td>Protein Kinase C-α</td>
<td>Non-small cell lung cancer (NSCLC) and other advanced solid tumors</td>
<td>Phase I/II</td>
<td>0.5 - 3 mg/kg/day</td>
<td>Continuous i.v. infusion</td>
<td>(101)</td>
<td></td>
</tr>
<tr>
<td>ISIS 5132</td>
<td>e-raf kinase</td>
<td>Small-cell lung cancer and NSCLC</td>
<td>Phase II</td>
<td>2 mg/kg/day</td>
<td>21 days every 4 weeks</td>
<td>Continuous i.v. infusion</td>
<td>(102)</td>
</tr>
<tr>
<td>ISIS 2503</td>
<td>H-ras</td>
<td>Advanced cancer</td>
<td>Phase II</td>
<td>0.5 – 10 mg/kg/d</td>
<td>14 days every 3 weeks</td>
<td>Continuous i.v. infusion</td>
<td>(103)</td>
</tr>
<tr>
<td>G3139</td>
<td>Bel-2</td>
<td>Advance malignant melanoma</td>
<td>Phase II</td>
<td>0.6 -6.5 mg/kg/d</td>
<td>14 days</td>
<td>s.c. infusion</td>
<td>(86)</td>
</tr>
<tr>
<td>GTI-2040</td>
<td>Ribonucleotide reductase R2 unit</td>
<td>Advanced cancers</td>
<td>Phase I</td>
<td>18.5-222 mg/m²/d</td>
<td>21 days</td>
<td>Continuous iv infusion</td>
<td>(104)</td>
</tr>
<tr>
<td>MG 98</td>
<td>DNA methytransferase</td>
<td>Advanced cancers</td>
<td>Phase I/II</td>
<td>40 - 480 mg/m²</td>
<td>2 hr twice weekly</td>
<td>Short i.v. infusion</td>
<td>(105)</td>
</tr>
<tr>
<td>Dose (mg/kg/d)/dosing route</td>
<td>Duration (days)</td>
<td>AUC (µg·h/mL)</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>C&lt;sub&gt;ss&lt;/sub&gt; (µg/ml)</td>
<td>Terminal t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>CL (ml/hr)</td>
<td>MRT (hr)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>----------------------</td>
<td>-----------------------------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>5, i.v.</td>
<td>Single</td>
<td>10</td>
<td>15 ±1.0</td>
<td>N/A</td>
<td>11</td>
<td>7.7</td>
<td>4.4</td>
</tr>
<tr>
<td>5, s.c.</td>
<td>7</td>
<td>160</td>
<td>1.2 ±0.2</td>
<td>1 ±0.1</td>
<td>22</td>
<td>NR</td>
<td>3.6</td>
</tr>
<tr>
<td>5, i.p.</td>
<td>3</td>
<td>17.4 ±2.2</td>
<td>6.9 ±1.9</td>
<td>NR</td>
<td>3.5</td>
<td>0.4</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR: not reported; N/A: not apply.

Table 1.2: Summary of published preclinical pharmacokinetics of G3139
| Dose (mg/kg/day) | dosing route | Duration (days) | AUC (µg.h/mL) | C<sub>max</sub> (µg/mL) | C<sub>ss</sub> (µg/ml) | Terminal t<sub>1/2</sub> (h) | CL (L/hr) | MRT (hr) | V<sub>ss</sub> (L) | Reference |
|----------------|--------------|----------------|---------------|----------------|----------------|----------------|--------------|----------|-----------|--------------|-----------|
| 4              | CIVI         | 10             | 856±361       | NR             | 3.19±1.29      | 0.63 (0.36-1.8) | 4.35±1.85    | NR       | 7.45±4.91 | (75)       |
| 7              | CIVI         | 10             | 1546±659      | NR             | 5.47±2.16      | 0.52 (0.33-1.13) | 3.89±1.48    | NR       | 4.70±4.27 | (75)       |
| 4.6-196 mg/m²  | s.c.         | 14             | 107-1200      | NR             | 0.45-5.63<sup>b</sup> | 7.46±4.32       | NR          | NR       | NR        | (85)      |
| 5 and 7        | CIVI         | 8              | NR            | NR             | 2.01±1.12; 3.031.79<sup>a</sup> | 1.86±1.70       | 9.3±4.28     | NR       | NR        | (90)      |
| 0.6-6.5        | CIVI         | 14             | NR            | NR             | 1.8<sup>b</sup> | NR          | NR          | NR       | NR        | (86)      |
| 4.1            | CIVI         | 14 or 21       | 894±102       | 2.66±0.3       | 2.66±0.3     | 3.16       | 2           | NR       | NR        | (88)      |
| 5.3            | CIVI         | 14 or 21       | 1443±441      | 4.28±1.34     | 4.28±1.34   | 4.6        | 2.08        | NR       | NR        | (88)      |
| 6.9            | CIVI         | 14 or 21       | 2550±350      | 7.58±1.07     | 7.58±1.07   | 7.67       | 2.1         | NR       | NR        | (89)      |
| 3; 5           | CIVI         | 14             | NR            | NR             | 2.82±0.66; 4.29±0.52 | NR          | NR          | NR       | NR        | (89)      |

<sup>a</sup> C<sub>ss</sub> of 7 mg/kg/day group; <sup>b</sup> C<sub>ss</sub> increase linearly with dose; CIVI: Continuous iv infusion; NR: not reported; N/A: not apply.

**Table 1.3:** Summary of published pharmacokinetics of G3139 in human
Table 1.4: Major characteristics of analysis of antisense drugs in biological matrix using LC/MS/MS in comparison with small molecule drugs.
Figure 1.1: Chemical structures of important first and second generation antisense molecules
Figure 1.2: Examples of second generation antisense drugs. Underlined nucleotide are modified at the 2’ ribose sugar position with 2’-O-(2-methoxyethyl) (2’-MOE).
Figure 1.3: Possible degradation pathways for PS oligonucleotides in vivo. a) 5’-exonuclease; b) 3’-end exonuclease; c) endonuclease cleavage would generate random shorter oligomers.
<table>
<thead>
<tr>
<th>Chemical Modification</th>
<th>Sequence (from 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncapped PS ODN</td>
<td>ACACCAATTCTGAAAATGG</td>
</tr>
<tr>
<td>5’-end capped PS ODN</td>
<td>X-ACACCAATTCTGAAAATGG</td>
</tr>
<tr>
<td>3’-end capped PS ODN</td>
<td>ACACCAATTCTGAAAATGG-X</td>
</tr>
<tr>
<td>3’,5’-end capped PS ODN</td>
<td>X-ACACCAATTCTGAAAATGG-X</td>
</tr>
</tbody>
</table>

Where $X = \begin{align*}
&\text{O} \quad \text{O} \\
&\text{S}^{-} \quad \text{OH} \\
&\text{NH}_{2}
\end{align*}$

**Figure 1.4**: Structures and sequences of chemically modified oligonucleotides for metabolism study
**Figure 1.5**: Model of apoptotic and survival signal pathway: the role of Bcl-2 family
Figure 1.6: Gene-walking analysis of Bcl-2 mRNA. Data were shown as % of inhibition of Bcl-2 mRNA relative to that of G3139 (100%) and plot as the 5’-end binding position along the Bcl-2 mRNA of each ODN
Figure 1.7: secondary structure of Bcl-2 mRNA as predicted by RNAdraw program. RNAdraw program is software to calculate secondary structure and melting curve of mRNA. Bcl-2 mRNA contains 5086 nucleotides and G3139 targets to base number of 1459 to 1476.
REFERENCE


CHAPTER 2

DEVELOPMENT AND VALIDATION OF A NOVEL, SENSITIVE AND SPECIFIC HYBRIDIZATION ELISA ASSAY FOR G3139 IN VARIOUS BIOLOGICAL MATRICES

2.1 Introduction

There are a growing number of phosphorothioate oligonucleotides in preclinical development and clinical studies for use as therapeutics. Quantification of phosphorothioate oligonucleotides in biological fluids is important to understand the pharmacokinetics behavior, pharmacological activity, toxicity, relationship between pharmacokinetics and efficacy/disease response. Therefore, development of a specific, sensitive, reproducible and accurate analytical method for antisense oligonucleotides in biological matrices are essential to successful preclinical and clinical development of antisense drugs. Early studies mainly used radiotracer (1) or anion-exchange chromatograph of radiolabel oligonucleotides followed by radioisotopic detector to study the disposition and mass balance of oligonucleotides (2, 3). These methods, however, are neither selective to N-1 or N-2 metabolites of antisense compounds nor suitable for measuring intracellular drug levels in clinical samples. Further, radiolabeled parent drugs suffer from enzymatic cleavage of phosphatase, exonucleases and endonucleases. Similar
problems of sensitivity and selectivity were also observed with HPLC-based assays. More recently, capillary gel electrophoresis (CGE) has been successfully used as a standard bioanalytical tool for quantification of antisense ODN. Leeds et al. (4) demonstrated that CGE/UV coupled with a unique two-step solid phase extraction met nearly all requirements of linearity, accuracy and precision for quantification of ODN in plasma, but the sensitivity of this assay was only 10 nM, which is not sufficient for defining either the elimination phase of ODN in plasma or intracellular exposure after drug treatment. Further, it requires extensive sample preparation prior to analysis. Recently, hybridization based assay was proposed as quantification methods for phosphorothioate oligonucleotides (5-7). The main advantage of this approach is high sensitivity, specificity against potential metabolites and high throughput with minimal sample clean-up. Thus, a novel homogeneous competitive hybridization assay for quantification of a 15mer antisense oligonucleotide in plasma has been reported (8). However, the cross reactivity of this study ranged from 27 to 55%, suggesting one step homogeneous competitive hybridization is not adequate to distinguish parent drug from 3' and 5'-deletion oligomers. To date, no hybridization based assay for Bcl-2 antisense G3139 in plasma or intracellular drug levels in clinical samples has been reported.

Although previously reported (9), pharmacokinetics of G3139 in the OSU 9977 study was truncated to 2 hours following the termination of the infusion due to the low sensitivity of the high-performance liquid chromatography (HPLC) assay utilized. This truncation prevented an accurate definition of the antisense plasma concentration profile over time and quantification of the drug concentrations achieved in different biological
matrices including blood and bone marrow (BM) cell lysate. Further, the lack of specificity made it impossible to distinguish the parent compound from its chain-shortened metabolites preventing a reliable correlation between plasma levels, target down-regulation and disease response. This deficiency has been a common problem for many of the antisense studies reported to date. In order to overcome these problems, we have developed a novel, highly sensitive, and specific hybridization ELISA assay for quantification of Bcl-2 antisense G3139 in various biological matrices, including plasma and cell lysates. This assay has allowed us to follow the terminal elimination of the drug in plasma over a longer period of time and to achieve an accurate definition of the antisense plasma concentration profile, and quantification of the intracellular drug in samples obtained from AML patients.

2.2 Materials and Methods

2.2.1 Antisense and reagents.

G3139 (Table 2.1) was supplied by the National Cancer Institute (Bethesda, MD). The putative metabolites shorter of 1, 2, or 3 nucleotides (N-1, N-2 or N-3) were obtained as follows: 3'-N-1 of G3139 was a gift from Dr. William Tong (Memorial Sloan-Kettering Cancer Center, New York); 3' N-2, 3' N-3, and 5' N-2 (Table 2.1) were purchased from Integrated DNA Technologies (Coralville, Iowa). The capture ODN 5'-GAATAG CGA ATGGCGACGCTGGGAGA/Biotin/-3') and the probe ODN (5'-TCGCTATTC-3' phosphorylated at the 5'-end and digoxigenin modified at the 3'-end) were purchased from Integrated DNA Technologies (Coralville, Iowa). The purity (>95%) and identity of each ODN was examined by elution sequence of Capillary Gel
Electrophoresis (CGE) and by HPLC/UV/Mass spectrometry (Finnigan LCQ, San Jose, CA). The assay buffer contains 60 mM phosphate buffer, pH 7.4, 1.0 M NaCl, 5 mM EDTA and 0.3% Tween 20. T4 DNA ligase was purchased from Amersham Biosciences (Piscataway, NJ). S1 nuclease was purchased from Invitrogen (Carlsbad, CA). The black NeutrAvidin-coated 96 well plate and superblock buffer in Tris buffered saline (TBS) were obtained from Pierce (Rockford, IL). The ligation buffer contained 66 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP. The nuclease reaction buffer was prepared in 30 mM NaAc (pH=4.6), 1 mM ZnAC, 150 mM NaCl and 5% glycerol. Attophos® substrate and reconstitution buffer were purchased from Promega (Madison, WI).

2.2.2 Fluorogenic ELISA assay procedures

The assay principle is illustrated in Figure 2.1. The simplified procedure was depicted in Figure 2.2. The capture ODN was first diluted in assay buffer at a concentration of 200 nM, heated at 95°C for 5 min and mixed with plasma or cell lysates containing G3139. The mixture was then incubated at 42°C for 2 hr. The analyte complex was captured by binding to a black NeutrAvidin-coated 96 well plate, which was subsequently washed with warm washing buffer (TBS in 0.1% Tween 20) at 30°C. The probe ODN at a concentration of 50 nM was diluted with ligation buffer containing 5 U/ml T4 DNA ligase. One hundred fifty µL of the mixture was dispensed into each well of the 96-well plate, and incubated at 18°C overnight. The excess ODN probe was removed with addition of 30 units of S1 nuclease in nuclease reaction buffer into each well. After enzymatic hydrolysis at 37°C for 60 min, the plate was washed five times.
with washing buffer and three times with ddH$_2$O, and blocked with 1:1 Superblock buffer in TBS. Subsequently, 150 µL anti-digoxigenin-alkaline phosphatase (AP) diluted 1:2500 with super BSA block buffer in TBS was added into each well. Following 0.5 hr incubation at 37°C, the plate was washed with washing buffer. One hundred fifty µL of the Attophos® substrate in diethanolamine buffer prepared as recommended by the manufacturer was added to each well. The fluorescence intensity was measured at Ex 430/EM 560 (filter=550 nm) following incubation at 25°C for 30 min using Gemini XS plate reader (Molecular Devices, CA).

2.2.3 Validation studies

Serial dilutions of G3139 in human plasma or cell lysate ranging from 25 to 4000 pM were used to construct a standard curve, against which samples with unknown concentrations of the antisense could be measured. Each standard sample was quantified in duplicate using the procedures as described in section 2.2.2, and the mean fluorescence signal was plotted against G3139 concentrations. The within- and between-day assay accuracy and precision of the method as assessed from blank human plasma, mouse plasma, and K562 cell lysate at concentrations of 50 pM (limit of quantification, LOQ), 100 pM (low quality control (QC)), 500 pM (medium QC) and 2000 pM (high QC). The specificity of the assay was evaluated using human plasma from three healthy donors (Red Cross, Columbus, OH) to assess possible interference from endogenous substances, and by cross-reactivity studies with putative metabolites (see below).

2.2.4 Cross reactivity analysis
To evaluate the cross reactivity of the assay with putative metabolites, various concentration of N-1, N-2 and N-3 oligomers at the 3' -end from 50 pM to 1000 nM were added into human blank plasma and dose-response curves were constructed. Additionally, different concentrations of 5' N-2 and mismatch (Table 2.1) oligomers ranging from 50 pM to 1000 nM in human plasma were also evaluated for cross reactivity. All dose-response curves were fitted to the Sigmoid Emax model. The maximal response produced by each compound (Emax) and the concentration that produced 50% of the maximal response (EC50) was obtained by nonlinear regression analysis using WinNonLin (version 3.1, Pharsight Corporation, Mountain View, CA). The cross reactivity was calculated as EC50 of parent compound divided by EC50 of each metabolite or analog (10).

2.2.5 Cross-validation with the HPLC-UV method

Selected samples from the previous clinical phase I study (12) were analyzed by both the ELISA assay and the established HPLC-UV method (n=45). Since the previous HPLC-UV method had a higher LOQ (0.5 µg/ml, or 82 nM), only samples with levels above the LOQ were selected (11).

2.2.6 Data Analyses

The Mean and standard deviations (SD) were computed for all variables using standard methods. Relationships between continuous variables, wherever applicable, were performed using standard linear correlation and linear regression, as appropriate. When needed, log transforms were used to insure normal distributions and linearity.
Differences were considered statistically significant when $p \leq 0.05$. All graphs are plotted as mean ± standard deviation (SD). For cross validation with the HPLC-UV method, Pearson correlation was obtained using S-plus software (12). Equivalence test was performed with the Wilcoxon signed-rank test (13).

The following equations were used for the calculation of $T_m$ given sequence composition and ionic strength:

$$T_m = \frac{\Delta H^o}{\Delta S^o + R \log(C)}$$  \hspace{1cm} [Equation 2.1]

$$\frac{1}{T_m(Na^+)} = \frac{1}{T_m(1M_{salt})} + (4.29 f(GC content) - 3.95) \times 10^{-5} \log_2(Na^+) + 9.40 \times 10^{-6} \log_2(Na^+)$$  \hspace{1cm} [Equation 2.2]

In equation 2.1, the changes in standard enthalpy ($\Delta H^o$) and entropy ($\Delta S^o$) associated with duplex formation are calculated from nearest-neighbor thermodynamic parameters. $R$ is the ideal gas constant (1.987 cal.K$^{-1}$.mole$^{-1}$), and $C_t$ is the molar concentration of oligonucleotide. $T_m$ values at 1 M Na$^+$ ion buffers can be calculated by equation 2.1. Then the estimated $T_m$ was scaled to salt concentration corrected $T_m$ using equation 2.2 (14) where $f(GC content)$ is the fraction of GC base pair in the oligonucleotide of interest.

2.3 Results

2.3.1 Optimization of the hybridization ELISA assay

As shown in Figure 2.2, the hybridization ELISA involves multiple steps including two hybridization, one ligation and one enzyme cutting steps in the assay. Four
key parameters listed below were identified as the major factors influencing sensitivity, assay linearity and accuracy.

**Hybridization temperature.** Using equation 2.2, Tm of G3139 to its complementary strand varies with concentrations ranging from 61 to 68°C within the calibration ranges. Since the hybridization temperature should be set 18 – 30°C below Tm of G3139 which is 60°C, three temperatures (37, 42 and 50°C) were evaluated with different concentrations of G3139 for the highest signal intensity and best linearity (Figure 2.3). High temperature or high stringent hybridization condition has benefit of reducing species formed by mismatched ODN (for example, 1 or 2 mismatch ODNs). However, it also compromises the binding of the perfectly matched ODN (i.e. G3139) to the template therefore yielding a lower recovery and sensitivity. As shown in Figure 2.3, net fluorescence increased with increasing temperature from 37°C to 42°C, but decreased after higher temperature was applied. Linearity was only altered slightly at different temperature (data not shown). Since there is no mismatch present in our biological samples, maximal discrimination between G3139 and 2 base mismatch was not evaluated at a more stringent hybridization temperature (50°C) to avoid a potential decrease in sensitivity at high temperature. The observed cross reactivity of mismatch ODN (Table 2.2) is probably reflective of cross-hybridization due to the hybridization temperature we used.

**Capture and probe ODN concentration.** Different concentrations of capture ODN ranging from 0.2 to 100 nM were evaluated for the highest signal intensity when G3139 concentration was fixed at 5 nM and 50 nM of probe was used. As concentration of capture ODN was increased from 0.2 to 10 nM, there was a sharp increase of
fluorescence intensity. Thereafter, the fluorescence intensity appears to increase slowly until a plateau was reached at capture ODN concentration of 200 nM. Therefore, capture ODN concentration of 200 nM was chosen for optimal assay sensitivity without compromised linearity. We also optimized concentration of probe ODN by varying its concentration with a fixed G3139 concentration. Higher intensity was observed at higher probe ODN concentration as well as higher concentration of capture ODN (Figure 2.4B). At a fixed concentration of capture ODN and G3139, fluorescence intensity increased with an increase in probe ODN concentration and a plateau was achieved at probe concentration of 100 nM (Figure 2.4). Further increase of probe concentration did not increase the signal intensity.

**S1 nuclease concentration.** Since capture ODN was in excess amount of analyte, they will bind to probe ODN following the second hybridization/ligation step and is still capable of generating background signals. In order to remove the duplex of unligated probe bound to capture ODN (9mer plus 27 mer) on the microtiter plate wall, we used S1 nuclease which is single strand DNA specific endonuclease to cut all capture ODN, which did not bind to any G3139. Therefore, the effect of S1 nuclease concentration on signal intensity, linearity and accuracy was investigated with 15, 30 and 60 units nuclease per sample as described in Material and Methods. The results are shown in Figure 2.5. As shown, no significant loss of signal intensity at various concentration of G3139 was observed. S1 nuclease cuts excess amount of probe ODN bound to capture ODN but seems not to cut those duplex containing analytes. S1 nuclease reduced the background fluorescence from 3900 to 250, when higher S1 nuclease concentration was used without
compromised linearity (Table 2.3). A S1 nuclease concentration of 30 units per sample was therefore chosen for biological samples.

Effect of detergent on linearity. During development of the hybridization ELSIA assay, it was found that calibration curve in human and mouse plasma was not linear from 25 to 2500 pM. At high concentration range, signal tends to be lower than expected. For better regression analysis, log/log transformation was needed to ensure linearity. It is actually quite common that ELISA based assay needs four-parameter logistic regression analysis in which maximal and minimal signal/response ($E_{\text{max}}$ and $E_{\text{min}}$), $EC_{50}$ and slope of the curve (n) are used to describe the calibration curve. We hypothesized that some plasma or cellular protein (possibly albumin) might interfere with the hybridization of G3139 with capture ODNs at the first hybridization step, therefore causing non-linearity of the calibration curve. At higher concentration, it appears signal intensity was saturated and linearity could only be attained below 2500 pM. It was found that addition of 0.25 % Triton X-100 (final concentration, w/v) disrupt the nonspecific interaction between analyte and plasma protein and improved recovery, linearity and assay precision (Figure 2.6). For cell lysate, triton X-100 (1% final concentration) was required to lyse cells and calibration curve was found to be linear in concentration range from 25 to 4000 pM (Figure 2.7B).

2.3.2 Validation of the ELISA-based assay of G3139 in plasma and cell extracts.

The ELISA-based assay of G3139 was validated in human plasma and cell lysates. Standard curves in human or cell lysate ranging from 25 to 4000 pM were found to be linear and shown in Figure 2.7A and 2.7B. A limit of detection (LOD, defined as 10
times signal to background noise, (15)) of 25 pM and a limit of quantification (LOQ) of 50 pM equivalent to 0.15 and 0.3 ng/ml, respectively, were found. Serial dilution of a quality control (QC) sample at 500 nM with human plasma yielded a linear curve at concentration range from 25 to 2500 pM indistinguishable from a calibration curve at the same concentration range. The recovery from dilution was found to be 93±19 % (SD, n=6). These results indicated that the linear concentration range of the assay could be extended from 2500 pM up to 500 nM (a 200 fold increase in dynamic range) with acceptable precision and accuracy.

The within-day and between-day accuracy and precision of the assay in human plasma were then evaluated at 50 pM, 100 pM, 500 pM and 2000 pM. The mean within-day coefficients of variation (CVs) of the assay were found to be 13%, 6%, 6%, 3% (all n=6), respectively (Table 2.4). The corresponding accuracy values were 73, 103, 107 and 82%, respectively, based on the nominal concentrations. The between-day CVs of the assay in human plasma were found to be 10%, 5% and 10% for 50, 500 and 2000 pM, respectively, with the corresponding accuracy values of 94.2, 112, and 94.7%. In cell lysates, the mean within-day CVs at 50, 100, 500, and 2000 pM were 7, 5, 7, and 3%, with corresponding accuracy values of 109, 90, 103, and 93% (Table 2.5). The between-day CVs of the assay at 50, 500 and 2000 pM were found to be 12%, 6% and 7%, respectively, with the corresponding accuracy values of 96.7, 106.8 and 101.8%. Similar validation results were found in mouse plasma. The between-day CVs of the assay were found to be 12%, 5% and 8% for the 50, 500 and 2000 pM samples, respectively, with the corresponding accuracy values of 92, 110 and 97%. The mean within-day precision
were 13, 8, and 10%, with corresponding accuracy values of 110.6, 111, and 109% (Table 2.6).

**Specificity and cross-reactivity.** Insignificant background was found in human plasma and cell lysate. The specificity of the assay was assessed by measuring the fluorescence signal generated by 4 putative G3139 metabolites. Various concentrations ranging from 50 pM to 1000 nM of the 3'-end N-1, N-2, and N-3 ODNs and of the 5'-end N-2 ODN were spiked into human blank plasma and dose-response curves were constructed as shown in Figure 2.8. The fluorescence signal measured from blank plasma was found to be negligible. The maximal response (fluorescence intensity) produced by each compound (E<sub>max</sub>) and the concentration that produced 50% of the maximal response (E<sub>50</sub>) were determined by nonlinear regression analysis using WinNonlin (Figure 3). The cross-reactivity (C.R.) values as calculated as the ratio of E<sub>50</sub> of the parent compound to E<sub>50</sub> of each metabolite are shown in Table 2.1. The C.R. values of 3' N-1 and 3' N-2 was estimated to be:

\[
\text{C.R. } 3'\text{N-1} = \frac{E_{50} \text{ of } G3139}{E_{50} \text{ of } 3'\text{N-1}} = \frac{1.73}{27.58} \times 100\% = 6.3\% \quad \text{[Eq. 2.3]}
\]

\[
\text{C.R. } 3'\text{N-2} = \frac{E_{50} \text{ of } G3139}{E_{50} \text{ of } 3'\text{N-2}} = \frac{1.73}{51.0} \times 100\% = 3.4\% \quad \text{[Eq. 2.4]}
\]

Thus, the 3'-end N-1 metabolite at 5 nM gave a fluorescence signal <10% of that of G3139 at the same concentration. The 3'-end N-3 metabolite gave extremely low fluorescence so that 50% maximum G3139 fluorescence response could not be measured. Overall, the cross-reactivity of 3' N-3 was considered to be negligible (<0.04%). (Table 2.1) In contrast, the cross reactivity of the 5'-end N-2 was estimated to be 41% (Figure

68
2.9). This was not surprising, as the assay was initially set up only to discriminate G3139 from its 3’-end metabolites, which are more likely to be present in vivo (see Chapter 5). There was essentially no fluorescence signal when reverse control ODN was used (Table 2.1). Another control ODN that contains two mismatch nucleotides exhibited a cross-reactivity of 26% (Figure 2.9). However, this is a concern, since it is unlikely that there exist mismatch PS ODN in vivo.

2.3.3 Cross-validation with the HPLC-UV method for patients samples

Plasma G3139 levels are currently measured by an anion exchange HPLC/UV method (11, 16). In order to apply this method in clinical studies, cross-validation of our novel ELISA-based assay with this published method is necessary. Thus, we measured levels of G3139 in a number of plasma samples (n=45) obtained from acute leukemia patients treated with G3139 in our clinical protocol (OSU 9977), and compared the results with the results previously obtained using the HPLC/UV method (11). The results presented as a correlation curve between the ELISA and HPLC/UV methods are shown in Figure 2.10. The agreement of the results obtained with these two different methodologies was excellent with Pearson’s correlation coefficient of 0.968 (p <0.001). To test whether two methods are equivalent within 30 % difference, ratios of data from two methods were calculated and the Wilcoxon signed-rank test was performed. The result indicated that the ratio of HPLC to ELISA values were within 30% difference from unity at 98% confidence level. However, of note the main advantage of the new assay was its levels of sensitivity with at least three orders of magnitude higher than the HPLC/UV method.
2.4 Discussion

In order to obtain better kinetic information of disposition of G3139, a specific, sensitive and nonradioactive method is needed for quantification of G3139 in biological matrices. To date, none of the methods available for G3139 were either specific or adequately sensitive for the measuring intracellular levels of the drug. Thus, we developed and validated a hybridization ELISA assay for G3139 and used it to measure plasma, tissue and the intracellular drug in samples obtained from in vivo treated blood and BM MNCs (see Chapters 3, 4, 7). The hybridization ELISA assay depicted as in Figure 2.1 is based on the Watson-Crick base-pairing rules and anti-Dig detection system. The hybridization ELISA assay consists of the following steps: (1) hybridization binding of the analyte to a 3'-biotinylated capture ODN (first ODN) to form a double-strand complex with a 5' overhang; (2) attachment of a duplex containing the analyte to the 96-well plate by biotin-avidin binding; (3) Digoxigenin (Dig) labeling of the duplex containing the analyte by introducing a secondary ODN (probe ODN); (4) removal of the excess amount of probe ODN bound to template ODN by S1 nuclease; (5) binding of Dig-labeled product with anti-Dig-AP conjugate, and (6) fluorometric determination by Attophos® substrate.

There are many variables in the assay, which must be optimized. Four key parameters were identified as the major factors governing sensitivity, assay linearity and accuracy. Selection of the optimum hybridization temperature is very important to the selective assay of the parent drug. Hybridization between G3139 and capture ODN depends on the ability of two denatured oligomers to re-anneal with 100%
complementary binding between two ODNs. Hybridization temperature used in this assay essentially depends on the melting temperature (Tm) of each duplex in the assay. Tm values are primarily affected by ionic strength, concentration of probe and capture ODNs, length and sequence of ODN applied, and destabilizing solvents (for example, formamide). For the first hybridization step, hybridization temperature is very critical for selectivity and specificity of the assay. The hybridization temperature at the second hybridization step was not optimized since Tm of probe ODN is only 19.7°C. To maximize stability of probe-capture ODN duplex, incubation at 18°C for overnight was used to ensure complete hybridization and ligation. Capture concentration is the second critical variable for assay sensitivity and must be optimized. Two hundred nM of capture ODN was selected as the final condition. Apparently, a higher capture ODN concentration makes the cutting step by S1 nuclease extremely difficult and may also cause more binding with potential metabolites. Since Dig-labeled probe ODN is difficult to purify and associated with high cost, a concentration of 50 nM for probe ODN was selected in the final condition to preserve quantity of probe ODN. This combination of optimal capture and probe ODNs concentration offers good linearity and accuracy from 25 to 4000 pM. The third important factor is the concentration of S1 nuclease. Since capture ODN was used in excess, they will also bind to probe ODN following the second hybridization/ligation step and be able to generate background signal. In order to remove the duplex of unligated probe bound to capture ODN (9mer plus 27mer) on the microtitre plate wall, several washing procedure could be applied including high stringency wash (high temperature and low salt), denaturation, or destabilizing solvent formamide. Our results suggested destabilizing solvent such as formamide or washing temperature at
50°C reduce the binding of G3139 to capture ODN, therefore, yielding low recovery of analyte (data not shown). Another approach to remove unligated probe ODN while leaving the intact ligated analyte-probe duplex in the heterogeneous ELISA assay was to employ ddH₂O washing in which was applied to (7). To our surprise, however, we found that the unligated probe ODN could not be completely removed by this simple washing step. Apparently, binding affinity between probe ODN and capture ODN is strong enough which was not removable by ddH₂O washing. Of note, the residual probe bound to capture ODN not only limited the assay sensitivity but also compromised the linearity in the calibration curve. To further improve the assay sensitivity and linearity, S1 nuclease treatment was performed after second hybridization step (Assay Step 4). S1 nuclease is an endonuclease specific for cleavage of single strand DNA or the nick and gap in double strand DNA. We found that addition of S1 nuclease with 1 to 2 hr incubation at room temperature or 37°C, following the washing step after second hybridization step not only helped to reduce the background fluorescence but also enhanced the linearity of the assay. Detergent is known as effective additive to dissociate the nonspecific interaction between analyte and plasma protein thus may contribute to the improved linearity. Thus, detergent concentration was tested for its effect on the assay linearity. We found triton X-100 at low concentration appears to be effective in improving recovery, linearity and assay precision. Although this method utilized the similar approaches as that published for another antisense drug (7), the current method has substantial differences with respect to assay optimization as described above and appeared to be more robust.
We demonstrated that this assay is highly sensitive and specific and capable of differentiating from putative 3’-end metabolites. The design of the capture ODN was based on the sequence of G3139 in a way that its 3’ terminal sequence is complementary to the G3139 and the 5' terminal overhang complementary to sequence of the probe ODN. The probe ODN had a predetermined random sequence with no similar match from BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). The length and sequence of probe ODN were not critical for assay specificity or sensitivity and, therefore, it is likely that probe ODNs with different designs might work equally well, as long as the sequence of the capture template is complementary to the probe ODN at the 5' terminal sequence. In fact, the assay was design to detect only the 3'-end intact sequence of G3139, as truncated sequences of the drug could prevent ligation reaction with probe ODN and generation of a fluorescence signal. This design was purposely chosen to offer single nucleotide resolution toward the 3’-end metabolites, as base removal from the 3'-end by distinct exonucleases may be a major metabolic pathway of antisense oligonucleotides (17, 18). In contrast, the assay was not selective toward 5' metabolites; however, 5'-end metabolism has not been considered a major pathway (19, 20). Preliminary metabolism data in our laboratory (see Chapter 5) supported this contention. The signal interference from 5'-end metabolites could, however, be markedly reduced using a reverse sequence of capture and probe ODNs. N-3 and N-4 metabolites from the 5’-end of G3139 were not evaluated but we would expect a lower cross reactivity than 5’ N-2.
The applicability of the hybridization ELISA assay was demonstrated by pharmacokinetic analysis of G3139 in the mouse following *i.v.* administration. The assay enabled us to follow plasma concentration for significantly longer period of time *in vivo*, therefore, allowing a better characterization of pharmacokinetics of G3139. More importantly, this novel, highly sensitive and specific hybridization ELISA assay permitted for the first time the assessment of intracellular levels of the Bcl-2 antisense G3139 (see Chapters 3 and 4).
<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide Sequence (From 5’ to 3’)</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3139</td>
<td>TCT CCC AGC GTG CGC CAT</td>
<td>100</td>
</tr>
<tr>
<td>3’-N-1</td>
<td>TCT CCC AGC GTG CGC CA</td>
<td>6.3</td>
</tr>
<tr>
<td>3’-N-2</td>
<td>TCT CCC AGC GTG CGC C</td>
<td>3.4</td>
</tr>
<tr>
<td>3’-N-3</td>
<td>TCT CCC AGC GTG CGC</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>5’-N-2</td>
<td>TCC CAG CGT GCG CCA T</td>
<td>41</td>
</tr>
<tr>
<td>Reverse control</td>
<td>TAC CGC GTG CGA CCC TCT</td>
<td>0</td>
</tr>
<tr>
<td>Mismatch control</td>
<td>TCT CCC AGC ATG TGC CAT</td>
<td>26</td>
</tr>
</tbody>
</table>

**Table 2.1:** Oligonucleotide sequence of putative metabolites of G3139 and control ODN used in the cross reactivity studies.
<table>
<thead>
<tr>
<th>Name</th>
<th>NaCL Concentration</th>
<th>Oligonucleotide concentration</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3139</td>
<td>400 mM</td>
<td>10 nM</td>
<td>69</td>
</tr>
<tr>
<td>G3139</td>
<td>400 mM</td>
<td>100 pM</td>
<td>61</td>
</tr>
<tr>
<td>Capture ODN (27mer)</td>
<td>66 mM</td>
<td>100 nM</td>
<td>67.5</td>
</tr>
<tr>
<td>Probe ODN (9mer)</td>
<td>66 mM</td>
<td>100 nM</td>
<td>19.7</td>
</tr>
<tr>
<td>Ligation product (G3139 + probe ODN)</td>
<td>0.01 mM</td>
<td>10 nM</td>
<td>65</td>
</tr>
<tr>
<td>Mismatch control</td>
<td>400 mM</td>
<td>10 nM</td>
<td>63.7</td>
</tr>
</tbody>
</table>

**Table 2.2:** Optimization of hybridization temperature: oligonucleotide and probes used in the study and their corresponding Tm values
Concentration of S1 nuclease (units/sample)$^a$ | Calibration curve$^b$ | Background signal$^c$ |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Log/log transformation</td>
<td>3900 ± 195</td>
</tr>
</tbody>
</table>
| 15 | Slope = 10.91  
Intercept =725 | 1300 ± 60 |
| 30 | Slope = 10.78  
Intercept = 860 | 705 ± 25 |
| 60 | Slope = 10.14  
Intercept = -122 | 250 ± 16 |

$^a$1 unit of S1 nuclease hydrolyzes 1 µg of denatured DNA to acid-soluble material in 1 min at 37°C  
$^b$Quantification was shown to be linear from 25 to 4000 pM when S1 nuclease was used. Logarithmic transformation on both axis is needed without S1 nuclease.  
$^c$Two hundred nM of capture ODN and 50 nM of probe ODN were used without addition of G3139.

**Table 2.3**: Effect of S1 Nuclease concentration on linearity and background. The concentrations of capture ODN and probe ODN were 200 nM and 50 nM, respectively.
### Table 2.4: Within-run and between-run precision and accuracy of the ELISA assay of G3139 in human plasma.

<table>
<thead>
<tr>
<th>Nominal Concentration (pM)</th>
<th>Within-run precision and accuracy in plasma (n=6)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(^a) (pM) (±SD)</td>
<td>Precision(^b) (%CV)</td>
<td>Accuracy(^c) (% Nominal)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>36.7 (±4.8)</td>
<td>13</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>103.1 (±6.1)</td>
<td>6</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>532.8 (±31.1)</td>
<td>6</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>1646 (±44.4)</td>
<td>3</td>
<td>82</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nominal Concentration (pM)</th>
<th>Between-run validation in human plasma (n= 5 days)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured Mean(^a) (pM) (SD)</td>
<td>Precision(^b) (%CV)</td>
<td>Accuracy(^c) (% Nominal)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>47.1 (4.5)</td>
<td>10</td>
<td>94.2</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>562 (29.5)</td>
<td>5</td>
<td>112.0</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>1894 (196)</td>
<td>10</td>
<td>94.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Concentrations calculated from the linear least squares regression curve (n=6).
\(^b\)Expressed as coefficient of variation (%CV) (n=6).
\(^c\)Expressed as \[\left(\frac{\text{mean observed concentration}}{\text{nominal concentration}}\right) \times 100\].
<table>
<thead>
<tr>
<th>Nominal Concentration (pM)</th>
<th>Within-run precision and accuracy in cell lysate (n=6)</th>
<th>Between-run validation (n= 5 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(^a) (pM) (±SD)</td>
<td>Precision(^b) (%CV)</td>
</tr>
<tr>
<td>50</td>
<td>54.6 (±4.1)</td>
<td>7</td>
</tr>
<tr>
<td>100</td>
<td>90.0 (±4.2)</td>
<td>5</td>
</tr>
<tr>
<td>500</td>
<td>515.8 (±37.0)</td>
<td>7</td>
</tr>
<tr>
<td>2000</td>
<td>1865 (±52.4)</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations calculated from the linear least squares regression curve (n=6).

\(^b\) Expressed as coefficient of variation (%CV) (n=6).

\(^c\) Expressed as [(mean observed concentration / nominal concentration) \times 100].

**Table 2.5**: Within-run and between-run precision and accuracy of the ELISA assay of G3139 in K562 cell lysate
<table>
<thead>
<tr>
<th>Nominal Concentration (pM)</th>
<th>Measured Mean(^a) (pM) (±SD)</th>
<th>Precision(^b) (%CV)</th>
<th>Accuracy(^c) (% Nominal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>55.3 (±7.3)</td>
<td>13</td>
<td>110.6</td>
</tr>
<tr>
<td>500</td>
<td>554.3 (±45.7)</td>
<td>8</td>
<td>111</td>
</tr>
<tr>
<td>2000</td>
<td>2187 (±212)</td>
<td>10</td>
<td>109</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nominal Concentration (pM)</th>
<th>Measured Mean(^a) (pM) (±SD)</th>
<th>Precision(^b) (%CV)</th>
<th>Accuracy(^c) (% Nominal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>45.8 (±5.6)</td>
<td>12</td>
<td>92</td>
</tr>
<tr>
<td>500</td>
<td>550 (±29.5)</td>
<td>5</td>
<td>110</td>
</tr>
<tr>
<td>2000</td>
<td>1940 (±156)</td>
<td>8</td>
<td>97</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations calculated from the linear least squares regression curve.

\(^b\) Expressed as coefficient of variation (%CV).

\(^c\) Expressed as [(mean observed concentration / nominal concentration) \times 100].

Table 2.6: Validation data of the ELISA assay in mouse plasma
Figure 2.1: The principle of fluorogenic ELISA assay to measure G3139 concentrations in plasma and cell lysate.
Figure 2.2: The working procedure of fluorogenic ELISA assay to measure G3139 concentrations in plasma and cell lysate.
**Figure 2.3:** Effect of hybridization temperature on the hybridization signal. Different temperatures (37, 42 and 50 °C) was performed in the first hybridization step and the second hybridization temperature was at 18 °C.
Figure 2.4: Effect of capture and probe ODNs concentration on signal intensity. A) concentration of capture ODNs was changed from 0.2- 200 nM (n=3 each concentration), while the G3139 and probe ODN concentrations were fixed at 5 nM and 50 nM, respectively; B) the G3139 concentration was fixed at 5 nM. Concentrations of capture ODNs were evaluated at 5, 10, 50 and 200 nM (n=3).
Figure 2.5: Effect of S1 Nuclease concentrations on fluorescence signal intensity. Different concentrations of S1 nuclease were used to evaluate the effect of S1 nuclease concentration on linearity and background signals. The capture ODN was used at 200 nM and probe ODN at 50 nM.
Figure 2.6: Effect of detergent on assay linearity and calibration ranges. Average values for triplicates were shown. Error bar: standard deviation.
**Figure 2.7:** Representative standard curves of G3139 in human plasma (A) and cell lysate (B). The mean fluorescence signals were plotted against G3139 concentrations (pM). Each concentration was run in duplicates and the average was used for linear regression analysis with weight of 1/y.
Figure 2.8: Concentration-response curves of G3139 and its possible 3’-metabolites. Each concentration represents the average of triplicates.
**Figure 2.9:** Concentration-response curves of G3139, 5’ N-2 and 2 nucleotide mismatch. Each concentration represents the average of triplicates.
Figure 2.10: The correlation curve of G3139 between HPLC-UV and ELISA methods. Vertical axis represents the data from the ELISA-based assay and the X-axis those from the HPLC-UV assay. $Y = 0.39 + 0.7255X$. 
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CHAPTER 3

CELLULAR UPTAKE AND PHARMACOLOGY IN
LEUKEMIA CELL LINES AND LEUKEMIA BLASTS

3.1 Introduction

The use of antisense strategies in cancer has been supported by the potential of these ODN compounds to down-regulate oncogenic proteins that drive malignant transformation. The currently available phosphorothioate antisense compounds present a sulfur in place of an oxygen in the phosphodiester bond between two subsequent nucleotides, and have better characteristics such as nuclease resistance and ability to activate RNase H (1-3) that make them more suitable for in vivo administration for the treatment of neoplastic diseases (4-7).

Before ODNs can reach their molecular targets, several physical barriers must be overcome since most ODNs passing membrane are sequestered into endocytic compartments. Due to inefficient cellular uptake, cationic lipids are usually required to enhance cellular uptake and pharmacological effect. However, G3139 without a delivery agent is currently being investigated in several phase I to III clinical trials for both solid tumors and hematologic malignancies (5, 8-10). Despite encouraging clinical results (11),
many questions for this complex molecule remain: (1) the lack of correlations between plasma drug levels, Bcl-2 down-regulation and disease response, (2) how the drug was internalized in malignant cells \textit{in vivo} and what is the stoichiometry between G3139 and its target Bcl-2 RNA in cancer cells, (3) what is the threshold values of drug concentrations for Bcl-2 downregulation, and (4) how the intracellular trafficking relate to down-regulation of the intended target. In order to address these questions, we developed a novel, highly sensitive two step hybridization ELISA assay for quantification of G3139 in various biological matrices as described in Chapter 2. This assay allowed us to follow drug decay in plasma over much longer period of time than previously reported methods, distinguish the parent compound from its chain-shortened metabolites and, more importantly, quantify the intracellular drug concentrations in samples obtained from treated patients and cell lines. Herein, for the first time we reported that robust levels of G3139 were achievable in blood and BM mononuclear cells (MNCs) from patients with AML treated with the Bcl-2 antisense and these might relate to levels of Bcl-2 down-regulation.

High Bcl-2 levels in AML and other hematological cancers are associated with chemoresistance to cytotoxic drugs (Chapter 1). As overexpression of Bcl-2 in AML prevent leukemia cells from apoptosis and contribute to chemoresistance, suppression of Bcl-2 has been show to chemosensitize cancer cells to subsequent cytotoxic drugs (12, 13). In this study we used G3139 to down-regulate Bcl-2 protein expression in human acute myeloid leukemia (AML) cells \textit{in vitro}. The pretreatment with G3139 enhanced cytotoxicity and apoptosis rate induced by a subsequent treatment with Histone
deacetylase inhibitors (HDACi), such as SAHA or MS 275 in K562, NB4 and Kasumi-1 cells.

3.2 Materials and Methods

3.2.1 Cell lines and leukemia blasts

Two AML cell lines, K562 and Kasumi-1, obtained from the American Type Culture Collection (ATCC) (Manassas, VA) were used in the study. All cell lines were cultured in RPMI 1640 supplemented with antibiotics and 10% fetal bovine serum (Invitrogen, Rockville, MD). Cultures were maintained at 37°C in a humidified environment with 5% CO2. Viability and cell counts were determined using trypan blue dye exclusion and expressed as the mean ± SD of triplicate samples. Procurement of human bone marrow leukemia blasts from AML patients was consented under an IRB-approved protocol at The Ohio State University Hospitals. Leukemia blasts obtained from AML patients were cultured in IMDM medium containing 20% FBS, penicillin (50U/ml), streptomycin (50 U/ml), Interleukin-3 (20ng/ml), 50 U/ml of granulocyte–macrophage colony-stimulating factor (GM-CSF) and stem cell factor (50 ng/ml) (Invitrogen, Rockville, MD). Mononuclear cells (MNC) were separated immediately after procurement of bone marrow samples. BM diluted with RPMI1640 medium supplemented with 10% FBS (1:1) was centrifuged at 250 g for 30 min on Ficoll (Gibco, CA). Mononuclear cells were obtained from the middle layer and washed twice with PBS, cyropreserved in liquid nitrogen.
Approximately $10 \times 10^6$ of cyropreserved peripheral blood mononuclear cells (MNCs) from patient BM and blood samples collected before and after initiation of G3139 on the OSU 0164 protocol (11) were used. Bone marrow mononuclear cells were obtained by leukapheresis from AML patients.

3.2.2 Cellular uptake of G3139 in cell lines and in leukemia blasts in vitro and in vivo

K562 cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine (GIBCO, CA) and 10% heat-inactivated fetal bovine serum (GIBCO, CA). Approximately $2 \times 10^6$ of cyropreserved MNCs from patient BM and blood samples collected before and after initiation of G3139 on the OSU 9977 protocol (11) were used. Following centrifugation, the cell pellet was incubated with 200 µL, 0.1 µM phosphorothioate 28mer polycytidine (PS-dC28) for 2 min on ice and washed with PBS to remove membrane-bound ODN (14). Following addition of 200 µL lysis buffer (10 mM Tris-HCl, pH=8.0, 0.5 mM EDTA, 1% Triton X-100) and incubation on ice for 10 min, the cells were lysed by vortexing and sonication. The homogenate was then centrifuged at 10,000 g, and the supernatant was transferred to a new tube for the ELISA and protein assays (BioRad protein assay kit, Hercules, CA). The intracellular levels of G3139 were measured using the ELISA assay as described in Chapter 2. The intracellular concentration of G3139 was expressed as pmole per mg cellular total protein.

3.2.3 Transfection of cells with cationic liposomes

All transfection was performed in Opti-MEM medium (Invitrogen, CA). Stock solutions of Oligofectamine® reagent (Invitrogen, CA) and G3139 were prepared using
Opti-ME as the diluent. The appropriate amount of G3139 was diluted in 100 µL Opti-MEM to result in final concentrations of G3139 of 100 nM, 200 nM, 330 nM, 1.0 µM, 3.3 and 10 µM. For 100, 200, and 330 nM of G3139, 1.8, 3.6 and 6 µL of oligofectamine reagent was used. For 1.0, 3.3 and 10 µM of G3139, 20 µL of oligofectamine was used. To avoid cytotoxicity due to Oligofectamine®, its final concentration was kept below 20 µL/mL in all transfection experiments. These solutions were incubated at room temperature for 10-20 min to allow lipid-ODN complex to form. Then, 200 µL of each of these complex solutions was overlaid on the cells seeded at a density of 2x10⁶ cells per well in 0.8 mL medium on a 6 well plates for 4-5 hr. Another cationic liposome consisting of DDAB (Dimethyldioctadecylammonium bromide) and DOPE (L-α-dioleyl phosphatidylethanolamine) (a generous gift by Dr. Robert Lee, The Ohio State University) was also used and it was prepared as previously reported (15). The mean particle size of the cationic liposomes was determined to be 94 ± 48 (SD) nm. Since each DDAB molecule carries one positive charge, while one G3139 molecule possesses 17 negative charges, a preliminary cellular uptake study was first carried out to optimize the charge ratio of cationic lipid to G3139 to achieve the highest uptake value. The optimal ratio was found to be 1.43 on K562 cells, when 0.33 µM G3139 was used with various amounts of cationic liposomes. Therefore, 8, 24 and 60 µM of DDAB/DOPE was mixed with 0.33, 1 and 3.3 µM of G3139, and the DDAB/G3139 complexes were prepared in the similar fashion as in the case of Oligofectamine®. Following 4-5 hr incubation with Oligofectamine® or DDAB, 3 mL of medium containing 10% FBS was added to each
well and the content was gently mixed. Then the mixture was incubated for another 20 hr before cell lysis for total RNA isolation and G3139 quantification.

3.2.4 Quantification of Bcl-2 mRNA levels

Quantification of Bcl-2 RNA was performed by Real Time RT-PCR as previously reported (16). Briefly, total cellular RNA was extracted by commercially available RNA extraction kit (Qiagen RNeasy Mini kits, CA). The RNA concentration was determined using spectrophotometry (PerkinElmer, Boston, MA). For complementary DNA (cDNA) synthesis, total RNA (2 µg) from each sample was mixed with 1.5 µL of 20 µM random hexamer primer (Perkin Elmer, Boston, MA) and heated to 70°C for 2 min followed by cooling on ice for 5 min. 17 µL of master mixture containing M-Murine leukemia virus reverse transcriptase (Invitrogen, CA), 5X reaction buffer (Invitrogen, CA), 100 mM DTT, 10 mM of each dNTP and RNAsin (Promega, Madison, WI) was added into each sample. The samples were then incubated in a Thermal Cycler (Applied Biosystem, Foster City, CA) at 40°C for 60 min then at 94°C for 5 min. Following cDNA synthesis, real time PCR was performed on a ABI Prism 7700 Sequence Detection System (Applied Biosystem, Foster City, CA). Sequences of the fluorescence probes and primers were designed using the Primer Express program (Applied Biosystems, Foster City, CA): Bcl-2, forward primer CCCTGTGGATGACTGAGTACCTG; reverse primer CCAGCCTCCGTATCCTGGA; probe ACCGGCACCTGCACACCTGGA. Each cDNA samples were used as a template in a PCR amplification reaction containing (1) a set of primers and a carboxyfluorescein (FAM)/TAMRA (FAM: 5-Carboxyfluorescein, TAMRA: 5-Carboxytetramethylrhodamine) dual-labeled probe for the Bcl-2 transcripts,
and (2) primers and a VIC-labeled probe for a housekeeping gene 18S. The probe is labeled with a reporter dye (FAM) at 5’ end and a quencher dye at 3’ end (TAMRA). For each reaction the critical value $C_T$ for the target transcript was determined. Bcl-2 and 18S calibration standards were synthesized by cloning Bcl-2 or 18S cDNA using the TA cloning kit (Invitrogen, CA). Diluted standards were amplified to create separate calibration curves ($C_T$ versus log copy number) for the Bcl-2 or 18S. The amounts (copy numbers) of Bcl-2 and housekeeping transcripts (18S) in each sample were calculated against the calibration curves constructed at the same time as unknown samples. The result of the Real Time RT-PCR assay for each sample was reported as a ratio of Bcl-2 copies to $10^6$ 18S copies.

3.2.5 Flow cytometric analysis for study of cellular uptake and drug-induced apoptosis

K562 cells ($0.5 \times 10^6$) were exposed to 0.3 or 0.5 μM of FITC-G3139 (Calbiochem, San Diego, CA) in the presence or absence of delivery vehicle (DDAB or oligofectamine) at 37°C for 24 hrs. Then the cells were harvested, washed three times with cold PBS/1%FBS, and analyzed by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data was analyzed and displayed with CellQuest software (Becton-Dickinson, San Diego, CA). Histograms were drawn to compare difference in mean fluorescence intensity (MFI) of FITC positive cell population between treatments.

Apoptosis studies on cell lines (NB4, K562 and Kasumi-1) were performed following 24 and 48 hr treatment with G3139. Vehicle treated samples were used as control. Approximately $1 \times 10^6$ cells were harvested, washed, and assayed for apoptosis.
via dual staining with Annexin V-FITC and propidium iodide (PI) (BD Pharmingen, San Diego, CA). Cells were washed in cold PBS and resuspended in Annexin V binding buffer with calcium, stained with 2 µL of Annexin V-FITC, and 1.5 µL PI (50 µg/mL). Following a 10 min incubation at ambient temperature with protection from light, samples were analyzed by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, Franklin lakes, NJ). Results were interpreted as follows: Annexin V(positive)/PI (negative) cell population were considered apoptotic cells, Annexin V(positive)/PI (positive) events were considered necrotic cells, and Annexin V(negative)/PI(negative) events were considered viable cells. Values labeled in graphs represent the mean ± SD of triplicate samples.

3.2.6 Confocal microscopy analysis

The above cells incubation experiment was repeated. To the harvested cell pellet was added 200 µL 0.1 µM phosphorothioate 28 mer polycytidine (PS-dC28) and incubated for 2 min on ice (14). Following PBS wash, the harvested cells were fixed with 3.7% paraformaldehyde in PBS at room temperature for 10 min. The cells were then transferred onto slides by centrifugation (200 g, 5 minutes) with a Shandon Cytospin3 (Thermo Shandon, Pittsburgh, PA) and stained with 600 nM 4'-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR) for 5 min. The slides were mounted and examined with a Zeiss 510 META Laser Scanning Confocal microscope (Carl Zeiss Inc., Germany). Images were captured with a CCD camera and processed in Zeiss LSM Image Browser. Z-position scanning was performed by scanning incrementally along the z-axis of the cells at a space distance of 0.25 µm. The bottom of
the cells was set at 0 µm and the top was set at 11.55 µm. A total 46 optical sections were obtained and only 20 sections were shown.

3.2.7 Cytotoxicity studies by MTT assay

AML cells (K562, NB4 and Kasumi-1) growing in the exponential phase were diluted with fresh RPMI 1640 medium with 10% FBS to a final concentration of 2-5 × 10⁴ cells/ml. Cell suspensions (100 µl) were dispensed into each well of a 96-well tissue culture plate. The wells on the four outer sides of the plate contained medium alone to avoid evaporation issue. For single treatment with G3139 alone, solutions (20 µl) with serial concentrations of G3139 in presence or absence of cationic lipids were added to the individual wells simultaneously. For sequential drug exposure of G3139 followed by MS275 or SAHA, 20 µl of solutions of fixed concentrations of G3139 was added to each well, and the plates were incubated for 24 hrs. Then, the solutions (20 µl) of serial concentrations of MS275 or SAHA were added, and the plates were incubated continuously for additional 48 hrs. The cell growth was determined by the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) method, which is more appropriate for suspensions cells. Briefly, 10 µL of MTT reagent (Promega, Madison, WI) was added into each well and plates were incubated for an additional 4 h at 37°C before adding stop/solubilization solution. After completely solubilizing the formazan generated by viable cells overnight, the absorbance was read at 570 nm on a microplate reader Germini XS (Molecular devices, CA) plate reader with a dual wavelength filter 570/690 nm. Six replicates were performed at each drug concentration level. Data were
plotted and values calculated using GraphPad software (Version 4.0, San Diego, CA, USA). Chemosensitization ratio was calculated as the IC$_{50}$ of SAHA or MS275 without G3139 divided by the IC$_{50}$ of SAHA or MS275 with G3139.

3.2.8 Statistical analysis

The results were represented as the mean± standard deviation (SD) of 3 to 4 determinations. Means and standard deviations were computed for all variables using standard methods. Relationships between continuous variables, wherever applicable, were performed using standard linear correlation and linear regression, as appropriate. Two-sided multiple comparison method was performed to compare group mean difference with family wise error at 0.01 (17). Differences were considered statistically significant when p < 0.05.

3.3 Results

3.3.1 Cellular uptake of G3139 without or with delivery agents

Intracellular drug levels using our ELISA assay were quantified in K562 cell lysate following incubation with G3139 alone or in the presence of cationic lipid vehicle. Exposure to 0.33 to 10 $\mu$M G3139 without delivery vehicle for 24 hrs resulted in a dose-dependent intracellular drug concentration in the range of 2.04 to 11.4 pmole/mg protein (Figure 3.1). The uptake in K562 cells was estimated to be approximately 0.2-0.6% of the total exposed drug. This is the first chemical measurement showing intracellular levels of G3139, when cells were exposed to the free drug. In contrast, when G3139 was complexed with cationic lipids such as Oligofectamine® and DDAB/DOPE, cellular
uptakes of the antisense by K562 cells were found to increase by about 25 and 50 folds, respectively (Figure 3.1). Notably, at a concentration as high as 10 µM of noncomplexed G3139, the cellular uptake of the free drug was even lower than that measured when the cells were exposed to lower concentrations (i.e. 0.33, 1, 3.3 µM) of the antisense complexed with DDAB or Oligofectamine® (p<0.01). This difference may be due to the intrinsic difference in the uptake behaviors between noncomplex G3139 and G3139 complexed with cationic lipids generally considered for antisense ODN (18, 19), as the former is charged and the latter is essentially neutral (18, 19). Alternatively, the lower uptake of G3139 in cells may be due to the high binding of G3139 with proteins in FBS in the cell culture media, which may retard the uptake.

3.3.2 Intracellular distribution of G3139

To further validate our results, and to confirm that we in fact measured the internalized G3139, rather than cell membrane bound ODNs, we used a fluorescein labeled G3139 administered alone or complexed with Oligofectamine® to K562 cells and Kasumi-1 cells. Using flow cytometry, it appeared that both cell lines accumulated G3139 without liposomal vehicle 24 hr after incubation with a mean fluorescence intensity of 34 and 15 (FL-1 height) in K562 and Kasumi-1 cells, respectively (Figure 3.2 and 3.2B). However, the mean fluorescence in cells treated with ODN and cationic lipids was approximately 5 and 2-fold greater than that in K562 and Kasumi-1 cells treated with ODN treatment alone, respectively, which supports and validates the results obtained with our ELISA-based assay.
However, flow cytometry does not provide information about intracellular distribution. We further examined K562 cells by fluorescence microscopy after incubation with G3139 alone or in the presence of cationic lipids. DAPI was used for nuclear staining (blue). Following internalization, G3139 administered alone appeared to be sequestered into endosome/lysosomal compartments as evidenced by the faint, punctuate, cytoplasmic distribution of fluorescein-labeled G3139 (Figure 3.3B 1-3). In contrast, G3139 in the presence of cationic lipids resulted in a diffuse and bright cytoplasmic distribution as well as accumulation of large amounts of ODN in the nucleus after 24 hours incubation (Figure 3.3C 1-3) observed in more than 80% of cells. The z-scanning imaging of cells (Figure 3.4) with or without cationic lipids indicated that the fluorescence in Figures 3.3A and 3.3B was mainly from internalized FITC-G3139 and cytoplasmic membrane bound antisense (section 0, 0.61,10.94 and 11.55 µm in Figure 3.4) did not significantly contribute to it due to the washing steps applied before fixation and treatment with PS-dC28, which efficiently removed the membrane-bound drug.

As flow cytometry and IC determination do not provide information about intracellular distribution, we also examined differential drug distribution of different G3139 formulations in K562 cells by subcellular fractionation. Cell uptake for noncomplexed G3139 was found to be quite low (Figure 3.5), and approximately 60% to 80% of the internalized full length G3139 was found in the cytoplasmic fraction with a nucleus/cytoplasm drug ratio of 0.33 ± 0.053. In contrast, G3139 complexed with Oligofectamine® not only resulted in higher intracellular levels but also in a 7-fold higher nucleus/cytoplasm drug ratio (i.e. 0.33 ± 0.053 versus 2.5 ± 0.017) (Figure 3.5). To exclude the possibility of cytoplasm to nucleus contamination, the LDH content in
cytoplasm, nuclei wash fraction and nuclear fraction was measured and LDH in nuclei fraction and nuclei wash fraction was found to be <10% of that in cytoplasmic fraction, suggesting minimal cytoplasm to nucleus contamination.

3.3.3 Correlation of G3139 intracellular levels with Bcl-2 down regulation in AML leukemia cells in vitro

In order to determine whether intracellular levels of G3139 correlate with down-regulation of its target Bcl-2, K562 cells were again exposed to various concentrations of G3139 alone or in combination with Oligofectamine for 24 hrs. As shown in Figure 3.6A, at G3139 concentrations between 0.1–10 µM in the presence of cationic lipids, the down-regulation of Bcl-2 mRNA as measured by real-time RT-PCR occurred efficiently. Nonlinear regression analysis of the dose-response curve showed that the G3139 concentration that produced 50% of Bcl-2 down-regulation (IC50) was approximately 0.29 µM, and maximum Bcl-2 down-regulation (79% decrease relative to the control group) was observed at 10 µM relative to the control group (Figure 3.6B). The IC50 of 0.29 µM corresponds to an intracellular G3139 concentration of 37 pmole/mg protein, which was not achievable even at the highest concentration of G3139 (10 µM) when applied alone (Figure 3.1). Further, exposure to 3.3 µM G3139 without lipids failed to result in any significant suppression of Bcl-2 RNA (93% compared to control group). It is worth noting that exposure to 3.3 µM mismatch control (G4126) complexed with Oligofectamine failed to show any Bcl-2 down-regulation (102% of untreated) (data not shown), supporting a sequence-specific activity of the Bcl-2 antisense (Figure 3.6C).
3.3.4 Intracellular levels of G3139 in AML leukemia cells in vivo and in vitro

To study the spontaneous cellular uptake in human tumor cells in vitro, fresh leukemia blasts obtained from patient with AML were procured and incubated with either free drug or G3139 complexed with Oligofectamine®. Figures 3.6 A and B show the results obtained from two AML patients. In each patient, higher cellular uptakes of free drug were observed at all concentrations evaluated in comparison to those in K562 cells, suggesting different cellular uptake characteristics between cell line and tumor cells in vitro. Intracellular levels of G3139 in leukemia blasts were found to be 2 to 7 folds higher than that in K562 cells. Cellular uptake appeared to reach a plateau at extracellular concentration ≥ 3.3 µM (Figure 3.7).

To probe drug uptake in vivo, we measured levels of G3139 in BM and blood MNCs collected following 72 (Day 3) and 120 (Day 5) hrs of G3139 CIVI in AML patients treated on the protocol OSU 9977 (Table 3.1 and 3.2). In BM MNCs, G3139 levels ranging from 3.4 to 40.6 pmole/mg protein and in blood MNCs the intracellular levels ranging from 0.47 to 19.4 pmole/mg protein were found. Intracellular drug levels in blood MNC measured at 120 hrs were significantly higher than those measured at 72 hrs, despite unchanged or even decreased plasma levels, suggesting a slower intracellular clearance of the drug over time. Of note, intracellular levels of G3139 measured following 120 hrs of G3139 CIVI were found to be higher in BM MNCs than in blood MNCs, suggesting a site-specific preferential uptake of the drug (Tables 3.1 and 3.2).

Since only few pairs of plasma and viable cell samples were available, a statistical analysis between plasma and intracellular drug concentrations could not be made at this time. However, a direct correlation between drug plasma concentrations and cell uptakes
did not appear to occur, as higher concentrations of G3139 were achieved in patients in whom lower plasma concentrations of the antisense were measured.

3.3.5  G3139 enhances the cell killing effect of MS275 and SAHA

We hypothesize that pretreatment with Bcl-2 anti-sense could synergistically enhance histone deacetylase inhibitors (HDACi) MS275 or SAHA induced cytotoxicity and apoptosis in acute myeloid leukemia cells. Drug combination studies were performed with G3139 plus MS275 in three cell lines (K562, NB4 and Kasumi-1) or SAHA in NB4 cells, which express highest the highest Bcl-2 and CD34+. G3139 in the absence of cationic lipid was non-cytotoxic in all cell lines tested (Table 3.3). However, G3139 complexed with cationic lipids resulted in decreased cell viability in Kasumi-1 and NB4 but not K562 as evidenced by IC$_{50}$ of 35 and 39 µM for Kasumi-1 and NB4, respectively. Treatment with G3139 for 24 hr (Figure 3.8) or 48 hr (Figure 3.9) alone did not result in marked apoptosis. However, pretreatment with G3139 enhances MS275 induced reduction of cell proliferation in K562 cell lines with 5 to 6 fold increase in sensitivity at three G3139 concentrations tested. In Kasumi-1 cells, a 1.36 to 3.8-fold decrement in the IC$_{50}$. The sensitivity of NB4 to SAHA was increased 9.6-fold at the G3139 concentration of 0.3 and 1 µM (Figure 3.10 and Table 3.3). Potentiation of SAHA induced cell killing effect by G3139 was also confirmed by apoptosis analysis. The results are depicted in Figure 3.9B and Table 3.4. Treatment of NB4 cells with 0.33 µM G3139 then followed with SAHA 1 µM resulted in increased apoptotic and late apoptotic (necrotic) cells (17.1 and 31%) compared to that treated with 1 µM of SAHA alone (15 and 6.3%). Further, a dramatic decrease in viable cell population was observed, 51 vs 78.6 for combination and
SAHA single group (Table 3.4). Similar enhancement of SAHA induced apoptosis was observed at other combinations of 0.33 μM G3139 and SAHA although 1 μM of G3139 resulted in increase of percentage of early and late apoptosis cell population in less extent, suggesting the optimal concentration of G3139 was less than 1 μM.

3.4 Discussion

One of the limitations in assessing the clinical activity of G3139 or other antisense therapeutics has been the inability to obtain information on the fate of the drug following in vivo administration. Specifically, it is unknown whether detectable intracellular concentrations can be achieved and how these related to the drug plasma levels and if any stoichiometric relationship between the intracellular levels of the antisense and baseline Bcl-2 levels needs to be satisfied to attain a clinical significant down-regulation of the target. To date, none of the assay methods available for G3139 were either specific nor sensitive enough for the analysis intracellular levels of the drug (9, 11, 20-24). In order to overcome these problems and quantify Bcl-2 antisense in different biological matrices, we developed and validated a sensitive and specific ELISA assay for G3139 (Chapter 2) and used it to measure the intracellular levels of the drug in cell extracts from in vivo treated blood and BM MNCs, and to probe the PK/PD relationship in vitro.

The high sensitivity of our assay allowed us to measure intracellular concentrations of G3139. This is a critical point, since assessment of intracellular drug levels could provide us with currently unavailable information on cell uptake and the fate of the antisense, once it has been internalized following in vivo administration. To date,
the process through which ODN cellular uptake occurs remains to be elucidated, although adsorptive and fluid phase endocytosis appear to be involved (25-27). Once internalized, ODNs were sequestered in the endosomal-lysosomal compartment, and only a small proportion of ODNs escaped from the degradation vesicles and reached the intended targets either in the cytosol or nucleus. However, pharmacological activity of antisense could be limited if insufficient concentrations are attained. Our data showed low uptake and no antisense activity in K562 cells exposed to 3.3 μM G3139 in the absence of cationic lipids. Wu-pong et al. (28) found less than 2% of added oligonucleotide is internalized by HL-60 cells. This is also consistent with the general belief that the excess amounts of antisense drugs without cationic lipids are needed to achieve the desired target down-regulation (29, 30). In contrast, by using cationic lipids (Oligofectamine® and DDAB/DOPE) as delivery vehicles, marked concentration-dependent intracellular G3139 levels were observed. The increase in intracellular availability was 10 to 25 fold using Oligofectamine® and 20 to 50 fold by DDAB/DOPE. Using FITC-labeled G3139, we showed only a 5-fold increase of cellular uptake of ODN by cationic lipids. The discrepancy may be related to the pH dependent fluorescence intensity of FITC labeled G3139 (31), a small alteration in uptake behavior of labeled G3139, or difference in methodology. Cationic liposome formulations such as (N-(2,3-dioleyloxy)propyl trimethylammonium chloride) (DOTMA) were found to increase the potency of an antisense ODN targeted to human intercellular adhesion molecule-1 by 1000 fold, when compared with free ODN on various cell lines (32). Cationic lipids not only tremendously enhanced the rate and amount of G3139 uptake into K562 cells, but might also alter the intracellular distribution of G3139. It is widely accepted that cationic liposomes deliver
ODN into cells through an endocytotic pathway (33). Zelphati et al. (34) reported that cationic lipids were physically separated from ODN in subcellular levels. This mechanism is in agreement with our observations including sequence- and concentration-dependent down-regulation of Bcl-2 RNA and nuclear accumulation with diffuse cytoplasmic distribution pattern in the presence of cationic lipids.

Cationic lipids not only enhanced the rate and amount of G3139 uptake into K562 cells, but might also alter the intracellular distribution of G3139, as previously reported. It is widely accepted that cationic liposomes deliver ODN into cells through an endocytotic pathway (33), followed by dissociation between the ODNs and the cationic lipids (34). Here, we showed that the presence of cationic lipids enhanced nuclear accumulation and concentration dependent down-regulation of Bcl-2 RNA of the antisense in K562 cells. Our results for the intracellular localization of G3139 in K562 cells are consistent with the previous finding, in which more than 70% of the radiolabeled phosphorothioate oligonucleotides were found to be associated with the cytoplasmic fraction and various nucleus/cytoplasm ratio ranging from 0.146 to 0.34 was found for different sequences (35). Since the cytoplasmic fraction obtained with hypotonic lysis comprised membranes, cytosol, endosome/lysosome except the nuclei, the level of G3139 in cytoplasmic fraction might still be somewhat overestimated. Nevertheless, the distinct accumulation of G3139 in the nuclei by cationic liposome suggests that intranuclear content of G3139 may correlate with our observed concentration-dependent Bcl-2 down-regulation, since RNase H is enriched in the nuclei (36).
Interestingly, while the *in vitro* antisense activity required the use of cationic liposomes, G3139 in aqueous saline solution demonstrated pharmacological activity *in vivo* as shown by sequence-specific antisense target down-regulation. Thus, it appears that cationic liposomes are not required to achieve adequate intracellular levels of antisense *in vivo*. In fact, for the first time, we showed that a significant cellular uptake of G3139 occurs in MNC from patients’ BM and blood MNC samples collected following 72 to 120 hrs of CIVI of the antisense. Of 8 patients evaluated, 6 patients had intracellular noncomplexed drug concentrations >5 pmol/mg proteins, which correlated with efficient down-regulation of Bcl-2 mRNA. Despite G3139 being administered as an aqueous solution, the intracellular levels of the antisense in these 6 patients were higher than those observed in cell lines exposed to extracellular concentrations comparable to patients’ plasma levels. Consistent with previous reports that G3139 is extensively distributed in different animal tissues, with highest distribution in kidneys followed by liver, spleen, lung, heart and BM (21, 37), our findings showed a significant amount of antisense ODN following *iv* administration accumulated in BM. To our surprise, there was no correlation between plasma C_{ss} of G3139 and intracellular levels of the drug or Bcl-2 down-regulation. Further, despite the fact that G3139 was infused in patients in aqueous solution without any delivery vehicle, the cellular uptakes of G3139 in BM or blood samples’ were significantly higher than those observed in leukemia cell lines treated *in vitro* in the absence of cationic lipids. These results suggest that additional unidentified factors or conditions *in vivo* might be responsible for an efficient internalization of the antisense into MNCs. Future studies to recognize such factors or conditions are important
to optimize G3139 uptake *in vivo*, as Bcl-2 down-regulation was attained only in those patients with intracellular concentration of >5.0 pmole/mg protein.

**G3139 in combination with HDACi.** HDACi, such as SAHA and MS275, are a novel class of chemotherapeutic agent initially identified by their ability to reverse the malignant phenotype of transformed cells. SAHA has been shown to activate differentiation programs, inhibit the cell cycle, and induce apoptosis in a wide range of tumor-derived cell lines and to block angiogenesis and stimulate the immune system *in vivo* (38). Although the apoptosis induced by SAHA or MS275 is not fully understood, overexpression of Bcl-2 protein could protect against MS-275 or SAHA-induced apoptosis (39). It is possible SAHA targets the extrinsic apoptosis pathway and produce cellular stress on mitochondria. It becomes conceivable that Bcl-2 might act as an antagonist on apoptosis thus combinations of Bcl-2 antisense with SAHA or MS275 seems a reasonable strategy to target apoptosis in a complementary mechanistic fashion (40). In fact, our results suggest that SAHA interacts synergistically with G3139 to block cell proliferation and induce apoptosis. All leukemia cell lines were resistant to G3139 treatment alone, indicating other mechanism could overcome down-regulation of Bcl-2 by G3139 and maintain the cell proliferation. However, low dose G3139 chemosensitize these cells in response to SAHA or MS 275 induced cell-killing effect, which occurred in both low and high Bcl-2 expressing cells. Further study of the mechanism of synergy between SAHA and G3139 is warranted based on our promising results.
In conclusion, we have found for the first time evidence that measurable intracellular levels of Bcl-2 antisense G3139 are achievable *in vivo* in AML patients, when a non-complexed form of the drug was given, and that Bcl-2 down-regulation is likely to depend on the achievable intracellular concentration rather than plasma concentrations. Downregulation of Bcl-2 by G3139 sensitized leukemia cells to following treatment with SAHA or MS275 with higher apoptosis rate and more pronounced cell death.
Table 3.1: Plasma and intracellular concentrations (IC) of G3139 and corresponding Bcl-2 changes in BM cells at 120 hr (day 5) of the G3139 CIVI in acute leukemia patients enrolled on protocol OSU 9977.
<table>
<thead>
<tr>
<th>UPN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G3139 IC (pmole/mg protein)</th>
<th>G3139 Plasma concentration (µM)</th>
<th>Time point (hr)</th>
</tr>
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<tbody>
<tr>
<td>18</td>
<td>8.25</td>
<td>1.16</td>
<td>72</td>
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<td>18</td>
<td>19.44</td>
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<td>1.16</td>
<td>120</td>
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</table>

<sup>a</sup>UPN = unique patient number

**Table 3.2:** G3139 Concentrations in plasma and in blood MNCs at 72 hr (day 3) and 120 hr (day 5) following G3139 CIVI in acute leukemia patients enrolled on protocol OSU 9977.
<table>
<thead>
<tr>
<th>Treatment</th>
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<th>Chemosensitization ratio</th>
<th>Kasumi-1 cells</th>
<th>Chemosensitization ratio</th>
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</thead>
<tbody>
<tr>
<td>G3139 alone (without cationic lipids)</td>
<td>&gt;100 µM</td>
<td></td>
<td>&gt; 100 µM</td>
<td></td>
</tr>
<tr>
<td>G3139 alone (with cationic lipids)</td>
<td>&gt; 100µM</td>
<td></td>
<td>35 ± 0.42</td>
<td></td>
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<td>MS275 alone</td>
<td>25 ±0.19</td>
<td></td>
<td>19.0 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>G3139 0.33 µM + MS275</td>
<td>4.0±0.29</td>
<td>6.25</td>
<td>9.6 ± 0.11</td>
<td>1.98</td>
</tr>
<tr>
<td>G3139 1 µM + MS 275</td>
<td>4.2±0.27</td>
<td>5.9</td>
<td>14.0 ± 0.12</td>
<td>1.36</td>
</tr>
<tr>
<td>G3139 3.3 µM + MS 275</td>
<td>3.6± 0.12</td>
<td>6.94</td>
<td>5.0 ± 0.09</td>
<td>3.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NB4 cells</th>
<th>Chemosensitization ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAHA alone</td>
<td>12.71 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>G3139 alone (with cationic lipids)</td>
<td>39 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>G3139 0.33 µM +SAHA</td>
<td>1.32 ± 0.05</td>
<td>9.6</td>
</tr>
<tr>
<td>G3139 1 µM +SAHA</td>
<td>5.97 ± 0.09</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table 3.3:** Chemosensitization effect of G3139 for MS275 and SAHA in leukemia cell lines. The IC$_{50}$ (µM) data are presented as the mean of four replicates ± SD. Chemosensitization ratio was calculated as the IC$_{50}$ of SAHA or MS275 without G3139 divided by the IC$_{50}$ of combination with G3139.
Table 3.4: Potentiation of SAHA–induced apoptosis by pretreatment with G3139 in NB4 cells at 24 hr. The apoptosis was determined by Annexin V/PI double staining. Annexin V(positive)/PI (negative) cell population were considered apoptotic cells, Annexin V(positive)/PI (positive) events were considered necrotic cells, and Annexin V(negative)/PI(negative) events were considered viable cells. Values were presented in percentage of whole cell population.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells</th>
<th>Necrotic cells</th>
<th>Viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>1.68</td>
<td>1.77</td>
<td>96.45</td>
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<tr>
<td>G3139 0.33 µM</td>
<td>1.90</td>
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<td>G3139 1 µM</td>
<td>3.48</td>
<td>0.36</td>
<td>96.0</td>
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<tr>
<td>SAHA 0.1 µM</td>
<td>3.84</td>
<td>3.49</td>
<td>92.7</td>
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<td>SAHA 0.3 µM</td>
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<td>3.37</td>
<td>91.8</td>
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<td>91.2</td>
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<td>SAHA 1 µM</td>
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<td>78.6</td>
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<td>SAHA 3 µM</td>
<td>27.0</td>
<td>15.6</td>
<td>57.3</td>
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<tr>
<td>SAHA 10 µM</td>
<td>37.6</td>
<td>20.8</td>
<td>41.2</td>
</tr>
<tr>
<td>G3139 0.3 µM + 0.3 µM SAHA</td>
<td>7.8</td>
<td>14.02</td>
<td>78.0</td>
</tr>
<tr>
<td>G3139 0.3 µM + 0.6 µM SAHA</td>
<td>12.67</td>
<td>13.42</td>
<td>73.6</td>
</tr>
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<td>G3139 0.3 µM + 1 µM SAHA</td>
<td>30.89</td>
<td>17.7</td>
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<td>5.0</td>
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<td>85.8</td>
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<td>13.3</td>
<td>10.0</td>
<td>76.1</td>
</tr>
<tr>
<td>G3139 1 µM + 1 µM SAHA</td>
<td>30.3</td>
<td>13.76</td>
<td>55.65</td>
</tr>
</tbody>
</table>
Figure 3.1: Comparison of Intracellular uptake of G3139 antisense in the absence or presence of liposomal vehicle in K562 leukemic cells. Bars represented intracellular G3139 concentration following incubation with G3139 alone or in the presence of cationic lipid vehicle. The error bar is the standard deviation of three replicates. The uptake in K562 cells was estimated to be approximately 0.2-0.6% of the total exposed drug. Note that when complexed with ligofectamine® and DDAB/DOPE, G3139 delivery is enhanced by 25 and 50 fold, respectively.
Figure 3.2: Flow cytometry analysis of K562 and Kasumi-1 cells after incubation with FITC-labeled G3139 in the absence or presence of cationic lipids. (A) in K562 cells; (B) Kasumi-1 cells. Histograms were drawn to compare difference in fluorescence intensity between treatments. Control: autofluorescence intensity of untreated K562 cells or Kasumi-1; a: 0.5 µM FITC-G3139 without lipid. Both cell lines accumulated G3139 without liposomal vehicle 24 hr after incubation; b: 0.3 µM G3139 complexed with cationic lipid (6 µL Oligofectamine); c: 0.5 µM G3139 complexed with cationic lipid (6 µL Oligofectamine). The mean fluorescence in cells treated with ODN and cationic lipids was approximately 5 and 2 fold greater than that in K562 (A) and Kasumi-1 cells (B) treated with ODN alone.
**Figure 3.3:** Flow cytometric and microscopic analysis of K562 cells after incubation with FITC-labeled G3139 in the absence or presence of cationic lipids. (A) Histograms of fluorescence intensity in K562 cells. Histograms were drawn to compare difference in fluorescence intensity between treatments. Y-axis is cell counts and x-axis represents the logarithm of fluorescence intensity (FL-1 height). Legends: Control, autofluorescence intensity of untreated K562 cells; a, 0.5 µM FITC-G3139 without lipid; b, 0.3 µM G3139 complexed with cationic lipid (6 µL Oligofectamine®); c, 0.5 µM G3139 complexed with cationic lipid (6 µL Oligofectamine®). (B) and (C) are confocal fluorescence microscopy of cellular distribution of FITC-G3139 in K562 cells without (B) or with cationic lipids (C). Cells were transferred onto slides by Cytospin then stained with nuclear DAPI stain and processed for confocal microscopic analysis. B-3 and C-3 show superimposed images from FITC-G3139 (green, B-1 and C-1) and DAPI (blue, B-2 and C-2): (B) 0.33 µM G3139 without cationic lipid; (C) 0.33 µM G3139 with cationic lipid. Scale bar: 10 µm
Figure 3.3
**Figure 3.4:** Representative optical sections displaying internalization and intracellular distribution of FITC-labeled G3139. The optical sections (total 46) were scanned incrementally along the z-axis of the cells at a space distance of 0.25 µm. Only 20 sections were shown and section distance is given in the upper left corner in µm. The sections 0, 0.61, 10.94 and 11.55 µm are representative of membrane bound ODNs. Lack of Fluorescence suggests cytoplasmic membrane bound antisense did not significantly contribute to the images in Figure 3.3.
Figure 3.5: Comparison of G3139 levels in subcellular fractions between free G3139 and G3139 complexed with oligofectamine at 37°C after 24 hr incubation in K562 cells. Subcellular levels of G3139 are expressed as pmole per 1×10⁶ cells. Open bars, total cell lysate; diagonally hatched bars, cytoplasmic fraction; cross-hatched bars, nuclear fraction. Values represent the Mean ± SD (n=3 per group). Cell uptake for noncomplexed G3139 was low with approximately 60% to 80% of the internalized G3139 found in the cytoplasmic fraction. In contrast, G3139 complexed with Oligofectamine® not only resulted in higher intracellular levels but also a 7-fold higher nucleus/cytoplasm drug ratio.

**Figure 3.6:** Correlation of Intracellular G3139 concentration with Bcl-2 downregulation on K562 cells. (A) shows Bcl-2 mRNA as measured by Real Time RT-PCR (left Y axis, ▲) and intracellular G3139 levels as quantified by ELISA (right Y axis, ●). Intracellular levels of G3139 were normalized to total cellular protein quantified by BioRad protein assay. (B) Inhibition of Bcl-2 mRNA by G3139 on K562 cells. Quantification of Bcl-2 mRNA was performed by Real Time RT-PCR. Bcl-2 mRNA levels were normalized to 18S mRNA levels and presented as a percentage of lipid-control cells. The Bcl-2 mRNA was down-regulated by G3139 in a dose-dependent fashion with IC\textsubscript{50} = 0.29 μM. (C) The Bcl-2 mRNA was down-regulated by G3139 in a dose-dependent fashion whereas little or no change in Bcl-2 was detected in cells treated with reverse control (RC) oligonucleotides (3.3 and 10 μM) complexed with oligofectamine, mismatch (MM) antisense (3.3 and 10 μM) complexed with oligofectamine (20 μL/ml in Opti-MEM) and free drug (3.3 μM).
Figure 3.6
Figure 3.7: Comparison of cellular uptake of G3139 in leukemia blasts obtained from patient A (A), patient B (B) and K562 cells (A and B). Intracellular concentration of G3139 is represented as pmole/mg protein. G3139 uptake was examined in five concentrations (0.1, 0.33, 1, 3.3 and 5 µM) except in patient’s oligofectamine group where only four concentrations were tested. Different cellular uptake characteristics between cell lines and tumor cells obtained from untreated patients in vitro were observed. Intracellular levels of G3139 in leukemia blasts obtained from patients were found to be 2 to 7-folds higher than that in K562 cells. Cellular uptake appeared to reach a plateau at extracellular concentration $\geq 3.3$ µM.
Figure 3.8: G3139 induced apoptosis rate in NB4 cells at 24 hrs. A) lipid control, 24 hr; B) G3139 0.5 µM, 24 hr; C) G3139 1 µM, 24 hr. Treatment with G3139 alone for 24 hr or 48 hr did not result in a marked increase in apoptosis. The apoptosis rate was 1.85 and 3.48% at 0.5 and 1 µM of G3139, respectively.
Figure 3.9: G3139 induced apoptosis rate in NB4 cells at 48 hrs. A) lipid control, 48 hr; B) G3139 0.5 µM, 48 hr; C) G3139 1 µM, 48 hr.
Figure 3.10: G3139 enhances the cytotoxicity and apoptosis rate induced by SAHA in NB4 cells. A) Cell viability was determined by the MTT assay to detect metabolic activity. Each sample at each concentration was run in quadruplicate and was normalized to cells from control groups (n=4). Cells were pretreated with G3139 (0.33 or 1 µM) for 24 hr prior to SAHA treatment. B) Representative plot of cells treated with 0.3 µM of G3139 and 1 µM of SAHA for 24 hr. Apoptosis rate was determined by Annexin V/PI double staining. In (A), the sensitivity of NB4 to SAHA was increased when cells were pretreated with G3139. In (B), potentiation of SAHA induced cell killing effect by G3139 was also confirmed by apoptosis analysis.


CHAPTER 4

CLINICAL ACTIVITY, PHARMACOKINETICS AND METABOLISM OF G3139:
VALIDATION OF BCL-2 ANTISENSE STRATEGY IN ACUTE MYELOID LEUKEMIA

4.1 Introduction

Acute leukemia is a heterogeneous disease characterized by maturation arrest and uncontrolled proliferation of various hematopoietic precursor cells. Among a number of clinical and biological factors shown to predict poor prognosis, cytogenetic aberrations, age, hematologic disorders and prior chemotherapy are the most important ones (1). Despite many advances made in the last two decades in the management of acute leukemia, only 20 to 30% of adult patients are cured of their disease following intensive chemotherapy (2). Further, although cure can be achieved with allogeneic stem cell transplantation (SCT) in patients who fail initial treatment, many are not candidates for these aggressive strategies, underscoring the need for novel therapeutic approaches that could improve the current clinical results.
Most treatment failures in patients with acute leukemia are related to development of chemoresistance in malignant cells (3). Defects in apoptosis pathways contribute significantly to inducing resistance to a variety of chemotherapeutic agents. Bcl-2 is a potent inhibitor of caspase- and non-caspase-mediated apoptosis. Overexpression of this protein has been shown to result in resistance to a variety of apoptosis-inducing signals including radiation, steroids, and chemotherapy (4). In recent clinical studies, abnormal expression of Bcl-2 was proven to be predictive of poor response to treatment and adverse clinical outcome in patients with a variety of hematologic malignancies, including AML and non-Hodgkin’s lymphoma (5, 6). Based on these data, we have hypothesized that downregulation of Bcl-2 could ultimately induce a lower apoptotic threshold and restore chemosensitivity in chemoresistant leukemic cells.

To validate this strategy, we initiated two phase I studies of G3139 in acute leukemia. In the first phase I study, we combined G3139 with escalated doses of the Fludarabine, cytarabine or ARA-C and G-CSF combination (FLAG) in patients with refractory or relapsed acute leukemia. The primary objective of this trial was to determine the safety and tolerability of G3139 administered in combination with an intensive chemotherapy regimen, and to validate that the target Bcl-2 mRNA was down-regulated in the majority of the treated patients. Based on the encouraging results from the first study, the second phase I study was initiated to assess the feasibility of G3139 with a more standard remission induction regimen (Daunorubicin and Cytarabine) in patients ≥ 60 years of age with untreated AML. The primary goals of the second study was to provide safety data on the use of G3139 and cytarabine (Ara-C) in combination with
anthracyclines (daunorubicin) and to correlate the response with pharmacokinetics, baseline mRNA and protein of Bcl-2.

4.2 Patients and Methods

4.2.1 Drugs and dosage form

G3139 was supplied in glass vials as a concentrated sterile solution (30 mg/ml) that was further diluted with sterile normal saline (0.9% sodium chloride, USP). Cytarabine is commercially available as a sterile powder in vials of 100, 500, 1000, and 2000 mg or vials containing 100 mg and 1000 mg of parenteral solution in 0.68% NaCl. Powder formulation was reconstituted with sterile water for injection containing no preservatives. Daunorubicin is commercially available in 20 mg vials for injection which are stable at room temperature. Fludarabine is supplied as lyophilized powder and prepared in sterile water for injection.

4.2.2 Study design of two phase I studies

The first phase I dose escalation study included patients with refractory or relapsed acute myeloid (AML) or lymphoblastic (ALL) leukemia. Informed consent, approved by The Ohio State University Institutional Review Board (IRB), was obtained from all patients before entry into the study. G3139 was delivered as a continuous intravenous infusion (CIVI) on days 1 to 10 through a separate line at a dose of 4 mg/kg/d for the first four cohorts of patients, and at a dose of 7 mg/kg/d for the fifth cohort of patients. Both fludarabine (dose level 1: 15 mg/m² i.v. over 0.5 hour) and
cytarabine (dose level 1: 1,000 mg/m² i.v. over 4 hours) were given on days 6 to 10. Planned dose escalation was in approximately 25% increments to achieve the full dose of FLAG, which was administered to the fourth and fifth cohorts of patients (Figure 4.1 and Table 4.1). G-CSF was started at day 5 at a dose of 5 µg/kg/d in all cohorts until the absolute neutrophil count (ANC) was >3,000/µl for two consecutive days or >10,000/µl for one day. Adverse events were graded according to the NCI Common Toxicity Criteria (CTC version 2.0) (http://ctep.info.nih.gov). Either grade 3 or 4 non-hematologic systemic toxicity was considered Dose Limiting Toxicity (DLT) if directly related to G3139. Complete remission (CR) was defined as absence of leukemic blasts in BM and blood, BM cellularity of 20%, evidence of all three cell lineage maturation, neutrophil count ≥1,500/µL and platelet count of ≥100,000/µL (7). Incomplete remission (IR) was defined as the absence of leukemic blasts, but failure to achieve normal hematopoiesis, according to the criteria specified for CR.

The second phase I study included patients ≥ 60 years of age with either de novo primary or secondary AML. Two dose levels of daunorubicin 45 mg/m²/day (level 1) and 60 mg/m²/day (level 2) with same dosing of G3139 and cytarabine were studied. G3139 was delivered as CIVI on days 1 to 10 through a separate line at a dose of 7 mg/kg/day. Daunorubicin was given on days 4 to 6 as i.v. bolus administration. Cytarabine was given on days 4 to 10 at 100 mg/m² by CIVI through a central line, days 4-10 (73-240 hours), yielding a total dose 700 mg/m².
4.2.3 Quantification of G3139 levels in plasma, BM mononuclear cells and peripheral blood mononuclear cells (PBMC)

Initially, an anion exchange high-performance liquid chromatography (HPLC) method was used to assess plasma concentration of G3139 in the first phase I study (Protocol OSU9977). A 39mer ODN (Integrated DNA Technologies, IA) was used as an internal standard. Patient plasma samples were thawed at room temperature and thoroughly mixed by vortex. A 2.5 µg internal standard in 50 µl of 25 mM Tris buffer (pH=8.5) was added to each of a set of 16 mm×125 mm glass tubes, followed by an addition of 0.5 ml of a patient plasma sample or control plasma spiked with G3139 standards. Two ml of chloroform was used to precipitate the plasma proteins and remove the lipids. Following centrifugation at 1500×g for 15 min at 6°C, 75 µl of the supernatant was injected for HPLC analysis, which was conducted on a BioRad HPLC system (Hercules, CA). Separation was achieved on a Pharmacia HiTrap Q 1 ml strong anion exchange column (Piscataway, NJ). The mobile phase A was composed of 25 mM Tris buffer and 30% dimethyl formamide (pH=8.5) in water, and the mobile phase B consisted of 1 M sodium bromide in mobile phase A. A linear gradient was used with the solvent composition of: 20% B, 100% B, 100% B, 20% B and 20% B at times 0, 16, 20, 25 and 30 min, respectively. Using a flow-rate of 1 ml/min, the run time for each analysis was 30 min. ODNs were detected by UV at 267 nm. Chromatography was performed at room temperature. Prior to the analysis of the samples, HPLC assay validation was performed. Linearity was observed over the concentration range of 0.5 to 50 µg/ml in 0.5 ml plasma, with the linear regression coefficients ($r^2$) of >0.99 achieved routinely. The within-day coefficients of variation (CV) were 8.3, 3.9 and 4.3% at 1, 5, and 10 µg/ml level,
respectively. The between-day CVs were 11.2, 15.7 and 6.0 at 1, 10, and 25 µg/ml level, respectively. The limit of quantification was 0.5 µg/ml plasma. A calibration curve with a range of 0.5 to 25 µg/ml was performed with each analysis.

In the second phase I study, G3139 concentrations in patient plasma, BM mononuclear cells, and PBMC were measured by the validated two-step hybridization ELISA method as described in Chapter 2. Calibration curves were constructed in duplicates and quality control samples were run with each batch of samples. Separation of CD 34 positive cells from BM MNC was performed by MACS™ CD34 microbeads and selection columns (Miltenyi Biotec, Auburn, CA), as directed by the manufacturer’s instructions and as previously described (8). Post-selection cells were collected as CD 34 negative cells. Similar procedures described above were also used to quantify IC of G3139 in blood and BM MNCs collected from patients with AML.

4.2.4 Quantification of Bcl-2 protein and other apoptotic related proteins by immunoblotting analysis

Quantification of Bcl-2 protein was performed by immunoblotting as previously reported in Chapter 3. Expression of other Bcl-2 family protein, i.e. Bax, Mcl-1 and Bcl-XL were also be assessed by immunoblotting on the same nitrocellulose membrane after stripping with different monoclonal anti-human antibody.
4.2.5 Quantification of Bcl-2 mRNA and protein

Quantification of Bcl-2 mRNA was performed by real-time RT-PCR as previously reported in Chapter 3. The amounts (copy numbers) of Bcl-2 and transcripts (cABL, human oncogene overexpressed in AML) in each sample were calculated against the calibration curves constructed at the same time as unknown samples. The result of the real-time RT-PCR assay for each sample was reported as a ratio of Bcl-2 copies to $10^6$ cABL copies.

The quantitative Bc1-2 ELISA assay was performed using a commercially available ELISA kit from Oncogene (Boston, MA) as described in Chapter 3. The Bc1-2 concentration was determined in each cell sample in duplicate as Bcl-2 units/mL by comparing the absorbance obtained from each sample with that obtained from the standard curves. Bcl-2 levels in patient samples were normalized to total protein in the lysate and expressed as units/mg protein.

4.2.6 Blood sampling

Blood samples (6 ml each) were collected as described in the following protocols: in protocol OSU 9977, blood samples were collected before G3139 treatment, at 4, 24, 48, 120, 125.25, 240 hr from the beginning of G3139 infusion, and 0.25, 0.5, 1, 2, 3, 4 hr after the discontinuation of drug infusion. In protocol OSU 0164, blood samples were collected before G3139 treatment, at 24, 72 hr from the beginning of G3139 infusion, immediately before discontinuation of G3139 infusion, and 0.5, 1, 2, 4, 6, 8 hr after the discontinuation of drug infusion. Plasma samples were frozen immediately and stored in $–80 \, ^\circ C$ until analysis.
4.2.7 Pharmacokinetic Analysis

The pharmacokinetics of G3139 was analyzed by non-compartmental method or compartmental method and program WinNonLin (version 3.0, PharSight Corp, Mountain View, CA). The maximal plasma concentration of G3139 (Cmax) was determined by visual inspection of the plasma concentration-time profile and the terminal half-life was calculated by

$$t_{1/2} = \frac{0.693}{k_{el}}$$

where $k_{el}$ was the terminal elimination rate constant calculated by linear least-squares regression of the last three to four time points in the log concentration time profile. The area under the plasma concentration-time curve (AUC$_{0\text{-}t_{\text{last}}}$) from time 0 to the last sampling time ($t_{\text{last}}$) was calculated by linear trapezoidal rule. Residual AUC after $t_{\text{last}}$ (AUC$_{\text{tlast}\text{-}\infty}$) was calculated as the concentration ($C_{\text{last}}$) at last sampling time divided by $k_{el}$. Total AUC (AUC$_{0\text{-}\infty}$) was calculated as the sum of AUC$_{0\text{-}t_{\text{last}}}$ and AUC$_{\text{tlast}\text{-}\infty}$. Total body clearance (CL) of G3139 was calculated as the dose divided the AUC$_{0\text{-}\infty}$. Steady state plasma concentration ($C_{ss}$) was obtained by averaging the measured plasma concentration of G3139 at 24, 72 and 240 hr during the infusion. Plasma concentration-time profile was also fitted to a two-compartment open infusion model. Distribution ($\alpha$), elimination ($\beta$) rate constants and half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$, respectively) were determined by fitting to an appropriate model using nonlinear least squares regression analysis. AUC$_{0\text{-}\infty}$, CL, $\alpha$, $\beta$, and $V_1$ (volume of central compartment) were also estimated from model fitting.
4.2.8 Statistical Analysis

The estimated pharmacokinetic parameters of G3139 were analyzed using descriptive statistics. The differences within group or between two groups were examined by nonparametric test (Wilcoxon Mann-Whitney test) or analysis of variance (ANOVA), as appropriate, with the $\alpha$ value set at $p<0.05$ (SPSS, Version 12.0, Chicago, IL). Multiple linear regression was used to examine the correlation between pretreatment covariates (BSA, creatinine clearance, BUN, etc.) and pharmacokinetic parameters.

4.3 Results

4.3.1 Clinical pharmacokinetics and correlative results of G3139 in combination with FLAG (protocol OSU 9977)

In the phase I combination study (protocol OSU 9977), a total of twenty patients with the median age 56 years and a male/female ratio of 7/13 was given 4 or 7 mg/kg/day G3139 as CIVI (see Table 4.1 and Figure 4.1 for dosing regimen). The median time to relapse from the initial treatment for relapsed patients was seven months (range 3 to 21 months). Eighteen of the 20 patients developed pancytopenia following treatment with G3139 and FLAG. Hematologic toxicities were similar to those expected with FLAG alone. Median time for neutrophil recovery from start of chemotherapy (i.e., day 6) was 23 days (range 8 to 38 days); median time for platelet recovery ($\geq$ 50,000) was 39 days (range 21 to 56 days). Common adverse effects included fever, nausea, emesis, hypocalcemia, hypophosphatemia and fluid retention. They were manageable and not dose limiting.
Plasma pharmacokinetic profiles of 11 patients with a total of 12 CIVI courses at 4 mg/kg G3139, and of 6 patients with a total of 7 CIVI courses at 7 mg/kg G3139 were monitored. The composite plots of plasma concentration-time profiles for patients receiving G3139 at 4 mg/kg and 7 mg/kg are shown in Figures 4.3A and 4.3B, respectively. Steady-state plasma concentrations (Css) were achieved rapidly and remained so until the end of CIVI. Following the end of infusion, G3139 plasma concentrations declined mono-exponentially and became non detectable (<0.5 µg/ml) within 2 hrs. Css were less variable at 4 mg/kg than at 7 mg/kg (Figure 4.3). The composite plots for the mean plasma concentration-time profiles for these two doses of G3139 are shown in Figure 4.4. Using a one compartmental infusion model, the resultant relevant pharmacokinetic parameters were estimated and are shown in Table 4.3. The mean Css level for the 4 mg/kg dose was 3.19 ± 1.29 µg/ml (range 1.59-5.69 µg/ml), which is significantly lower than the Css of 5.47 ±2.16 µg/ml (range 2.67-8.38 µg/ml) for the 7 mg/kg dose (p=0.023). When normalized to dose, the Css values were 0.78 ± 0.33 and 0.78 ± 0.30 µg/ml for the 4 and 7 mg/kg doses, respectively. These results indicated that the Css levels were proportional to dose, and the pharmacokinetics were linear for the two doses (i.e., 4 and 7 mg/kg) administered. The linearity in pharmacokinetics was also reflected in dose-dependent differences in area under the curve (AUC) values (p<0.05) and similar total clearance (4.36 ±1.85 L/hr at 4 mg/kg and 3.89 ± 1.48 L/hr at 7 mg/kg, p > 0.5). The mean elimination constants for these two doses were 1.10±0.55 and 1.32 ± 0.55 hr⁻¹, respectively, yielding the mean t½ values of 0.63 hr (range 0.36-1.8 hr) and 0.52 hr (range 0.33-1.13 hrs) for 4 and 7 mg/kg doses, respectively (Table 4.3).
While clinical response was only a secondary endpoint of this study, complete responses (CR) were noted in 7 (35%) of the patients. Another two patients with AML achieved an incomplete remission (IR), but failed to recover normal neutrophil and/or platelet counts. Responses were seen at all dose levels. Response duration for two of these patients persisted for >12 months before relapse occurred (UPNs 3 and 4 with 18.8 and 12.7 months, respectively).

To fully evaluate the efficacy of G3139 in down-regulating its intended target, Bcl-2 mRNA levels were measured in bone marrow (BM) samples collected before treatment and at day 5 into G3139 infusion, prior to FLAG initiation. The analysis was restricted to these two time-points to avoid Bcl-2 variations due to the subsequent chemotherapy administration (Fig. 4.5). The relative differences in the pretreatment (set as 100%) and day 5 levels of Bcl-2 transcripts were measured in 12 patients’ specimen available for analysis (Table 4.4). Of the 12 patients, 9 (75%) showed down-regulation of Bcl-2 mRNA following G3139 administration (range 6.5 to 75.7% decrease in Bcl-2 transcripts). In contrast, in 3 patients (UPNs 2, 9 and 20) the Bcl-2 transcript increased following antisense treatment (21.1-38.4 % increase). In 5 of the 12 patients, additional BM specimen was available for Bcl-2 protein analysis by immunoblotting (Figure 4.5 and Table 4.4). In these patients, there was concordance in 4 of 5 (80%) serial samples relative to change in Bcl-2 mRNA and protein level.
4.3.2 Clinical pharmacokinetics of G3139 in combination with cytarabine and daunorubicin (protocol OSU0164)

Plasma pharmacokinetic profiles of 27 patients at 7 mg/kg G3139 dose were monitored. The composite plots of plasma concentration-time profiles for patients receiving G3139 during and post-infusion are shown in Figures 4.6A and 4.6B, respectively. Steady-state plasma concentrations (C_{ss}) were achieved within 24 hr and remained so until the end of CIVI (Figure 4.7A). A summary of pharmacokinetic parameters for all patients (n=27) in this trial is shown in Table 4.5. C_{ss} values of G3139 varied between 2-14.2 µg/mL. Following the end of infusion, G3139 plasma concentrations declined bi-exponentially and were detectable for up to 8 hrs monitored. The G3139 plasma-time curves were fitted to a two-compartment infusion model, and the relevant pharmacokinetic parameters were obtained (Figure 4.7 and Table 4.5). As shown, G1339 gave harmonic mean t_{1/2}^{\alpha} of 0.4 hr (range 0.1 to 1.1 hr) and t_{1/2}^{\beta} of 4.3 (range 1.8 –17 hr). These data differed from results in protocol OSU 9977 study which only showed a mono-exponential decline. The difference is probably due to differences in assay sensitivity. The mean total clearance value of the current study was 7.1 ± 3.7 L/hr or 1.50 ± 0.76 mg/min/kg which was similar to the previous reported study (protocol OSU 9977). There were no statistically significant differences in pharmacokinetic parameters (C_{ss}, AUC, CL and terminal t_{1/2}) between two daunorubicin dose levels (Table 4.5), indicating no significant drug interactions in the disposition of G3139 when given with daunorubicin and cytarabine. Multiple comparisons with and without chemotherapy suggest that there is no major pharmacokinetic interaction between G3139 and chemotherapeutic agents (Figure 4.8). Additionally, there was no significant difference in
C_{ss}, CL, AUC between CR and non-respond (NR) patients (Table 4.6), suggesting that the exposure of G3139 at 7mg/kg/d was adequate and difference in response may not relate to plasma pharmacokinetics.

Pharmacokinetic results suggested that G3139 at 7 mg/kg/d achieved adequate drug exposures (C_{ss}>2 \mu g/mL). G3139 was primarily metabolized to 3’-end deletion oligomers in plasma following CIVI of G3139 (see Chapter 5). Three major metabolites, i.e. 3’ N-1, N-2 and N-3, were quantified using ESI LC/Mass Spectrometry method and amounts of each metabolite were summarized in Table 4.8. Although appreciable amount of metabolites were observed at all time points, the predominant species in plasma was G3139 ranging from 50 to 71 % of the total measurable oligonucleotide pool (Table 4.7). The percentage of G3139 in plasma does not change before and after initiation of chemotherapy in samples available for analysis.

4.4 Discussion

Herein, we describe the clinical activity, safety and pharmacokinetic evaluation of G3139 in combination with intensive chemotherapy for the treatment of acute leukemia. Our study pursued this Bcl-2 antisense strategy to test the safety of this approach and to assess modulation of the target Bcl-2 in patients with acute leukemia who failed previous therapies. To test the feasibility of this approach and to determine whether the actual target was altered \textit{in vivo}, we performed two phase I, biologic correlative studies that combined G3139 with the FLAG salvage chemotherapy in patients with refractory/relapse acute leukemia and with cytarabine and daunorubicin in elderly acute leukemia. These studies demonstrated that the Bcl-2 antisense G3139 could be
administered safely with either FLAG chemotherapy in previously treat acute leukemia or cytarabine/daunorubicin in elderly untreated AML patients. Target Bcl-2 mRNA decrements were observed in 59% of the evaluated patients. While the primary objective of these studies was neither to validate modulation of the target Bcl-2 or therapeutic efficacy, the encouraging results of these studies provide justification for future large scale investigation of G3139 in AML.

**G3139 in combination with FLAG (Protocol OSU 9977)**

No dose limiting toxicity was observed in each of the cohorts examined in the OSU 9977 study. Furthermore, Konopleva et al. recently reported that low doses of Bcl-2 antisense *in vitro* recruits acute myeloid leukemic cells into the S phase and induce cell proliferation (9). While two patients had rapidly increasing blast counts after G3139 CIVI, this is unlikely related to G3139 as both of these individuals had rapidly proliferating disease prior to beginning therapy. A linear relation was noticed between the dose of the antisense and the AUC andCss, respectively. Therefore, 7 mg/kg/day was chosen as the safe dosing regimen in our OSU 0164 trial. Most common G3139-related toxicities were typical of those reported with other phosphorothioate oligonucleotides and are likely due to the phosphorothioate backbone, for example, fever and prolongation of clotting time (increased activated partial thromboplastin times, APTT) (10, 11). Nevertheless, it should be noted that among six patients treated at dose of 7 mg/kg G3139 in protocol OSU 9977 trial, UPN 7 developed methicillin-resistant *Staphylococcus aureus* (MRSA) sepsis complicated by multi-organ failure, and died at day 80 of the
second treatment course. Prior to G3139 and FLAG, she had received high-dose chemotherapy with autologous stem cell transplantation (SCT) as part of the consolidation treatment for AML. Although her multi-organ failure syndrome clearly occurred following a gram-positive sepsis, with prior splenectomy being a likely aggravating factor for this overwhelming infection, it should be noted that her G3139 AUC and Css were the highest among all patients studied. In previous animal and clinical studies, no unusual toxicity was reported in normal organs following Bcl-2 downregulation (12, 13). Nevertheless, the PK results in UPN 7 raises the question whether the high G3139 plasma concentration could have contributed to the severity of the multi-organ failure, and suggests a cautious use of this compound in patients who had previously received myeloablative doses of chemotherapy.

In our study, the overall response rate was 45%. However, this could be contributed from the anti-leukemic activity of the FLAG program, since response to FLAG alone has been reported to be between 30 and 80% depending upon the characteristics of the treated patients (14, 15). In a recent multi-center Phase II trial, a CR rate of 81% for AML patients with late relapse (> 6 months) and of 30% for patients with early relapse (< 6 months) was reported (16). In the latter group of patients, age negatively impacted on the probability of achieving remission with only 11% of the patients aged > 60 years attaining CR. Comparison of these clinical results with our current trial is obviously not possible. It is interesting, however, that in our study 5 patients achieved disease response despite not receiving the full dose of FLAG. Of the 5, 2 had primary refractory disease and one early relapse (UPN 5). Further, six of our responders had previously failed treatment with high dose Ara-C or autologous SCT.
Two patients had prolonged remission (>12 months) without any maintenance treatment following G3139 and FLAG.

**G3139 combined with cytarabine/daunorubicin (Protocol OSU 0164)**

It has recently been reported that Bcl-2 down-regulation significantly increases ischemic damage in rat myocardium (17). Therefore, it is important to carefully monitor our patient population for any potential, unexpected cardiac toxicity. Despite the high prevalence of coronary artery disease in patients aged >60 years, we observed only two cardiac events during induction and none during consolidation therapy. Importantly, no evidence of anthracycline-induced cardiomyopathy following G3139/cytarabine/daunorubicin induction was observed upon further follow-up, including patients who underwent a second induction. Finally, cerebellar toxicity, a well-known side effect of high dose Ara-C, was observed during only 2 of the 22 administered consolidation courses. Based on these results, we concluded that G3139 can be safely administered with an intensive multi-course induction and consolidation treatment program.

Of the 29 treated patients, approximately 50% achieved CR. As disease response was a secondary endpoint of this phase I trial, it not possible to confirm whether the addition of G3139 indeed enhanced the antitumor effect of the chemotherapy. Nevertheless, as a remission rate of approximately 40% has been shown in other studies of older AML patients treated with similar induction regimens, our results appear to be at least comparable with previously reported outcomes (18). Furthermore, with a median
follow-up of more than 1 year for patients alive and in CR, 50% of the CR patients remain in remission suggesting that durable response are achievable with this regimen. Of course, larger, randomized trials are necessary to fully address the clinical value of G3139 in the treatment of AML.

Our study also provides a detailed pharmacokinetic report of G3139 administered by CIVI in this elderly AML patient population. The plasma pharmacokinetics of G3139 was best described by a two-compartment \( iv \) infusion model with \( t_{1/2\beta} \) of 4.3 hr. The \( t_{1/2\beta} \) values for the current study with \( i.v. \) infusion were significantly shorter than the mean \( t_{1/2} \) of 7.46 ± 4.32 hours reported by Waters et al., using subcutaneous infusion (13). The longer \( t_{1/2} \) reported by these authors may be due to the sustained release effect of G3139 from the subcutaneous infusion site. Our results are closer to those reported by Morris et al., who used a \( i.v. \) infusion for 14 or 21 days (19). Pharmacokinetic parameters did not appear to be affected by the concomitant administration of the chemotherapy, and G3139 AUC and \( C_{ss} \) failed to correlate with toxicity or disease response. In contrast with our previous report, however, we determined that plasma concentrations of G3139 declined bi-exponentially and remained detectable up to 8 hours from the termination of the infusion. This resulted in a calculated terminal half-life at least three times longer than that previously reported by our laboratory (20). It is likely that such discrepancy relates to the much higher sensitivity of the novel ELISA-based assay utilized in the current study for quantification of the antisense levels. In fact, this methodology is at least three orders of magnitude more sensitive than the previously used HPLC assay, which allows us to
detect lower drug levels and follow the plasma decay of the antisense over a longer period of time.

We measured IC values of G3139 in bone marrow samples collected from previously untreated AML pts aged $\geq 60$ yrs enrolled on the phase I study in protocol OSU 0164. Robust levels of G3139 were detected in mononuclear cells obtained from BM. G3139 was preferentially taken up by CD 34 positive cells as oppose to CD 34 negative cells, suggesting tissue specific accumulation (see Chapter 7 section 7.3.4). A trend in higher median IC of G3139 was observed in CR pts (17.0 pmole/mg protein; range 1.5-30.0) as compared to NR pts (4.4 pmole/mg protein; range 0.33-28.0). Notably, no differences were observed in the G3139 plasma pharmacokinetics between the CR pts and NR pts in Css, AUC and CL, suggesting tumor/tissue site pharmacokinetics cannot be extrapolated from plasma pharmacokinetics.

Based upon the results from these two clinical studies, we conclude that the combination of G3139 with intensive chemotherapy is feasible in AML. The biologic data derived from this study suggest that G3139 is active against its target Bcl-2 mRNA. These data provide support and experimental platform for ongoing phase III studies (CALGB 10201) of G3139 with chemotherapy and to definitively assess the influence, safety and therapeutic role of the Bcl-2 antisense strategy in elderly acute leukemia patients.
Table 4.1: Dose levels and disease response of G3139 in combination with FLAG (protocol OSU9977)

<table>
<thead>
<tr>
<th>Dose Levels</th>
<th>G3139 mg/kg/d (d 1-10)</th>
<th>Fludarabine mg/m²/d (d 6-10)</th>
<th>cytarabine grams/m²/d (d 6-10)</th>
<th>G-CSF µg/kg/d (d 5-10)</th>
<th>Patients Treated (n)</th>
<th>CR (n)*</th>
<th>IR(n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>4</td>
<td>15</td>
<td>1.0</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Level 2</td>
<td>4</td>
<td>20</td>
<td>1.5</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Level 3</td>
<td>4</td>
<td>25</td>
<td>2.0</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Level 4</td>
<td>4</td>
<td>30</td>
<td>2.0</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Level 5</td>
<td>7</td>
<td>30</td>
<td>2.0</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

*CR, complete response; *IR, incomplete response
Table 4.2: Dose levels of G3139 in combination with cytarabine and daunorubicin (Protocol OSU0164)

<table>
<thead>
<tr>
<th>Dose Levels</th>
<th>G3139 mg/kg/day (d 1-10)</th>
<th>Daunorubicin mg/m²/d (d 4-6)</th>
<th>Cytarabine mg/m²/d (d 4-10)</th>
<th>Patients Treated (n)</th>
<th>CR (n)#</th>
<th>IR (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>7</td>
<td>45</td>
<td>100</td>
<td>9</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Level 2</td>
<td>7</td>
<td>60</td>
<td>100</td>
<td>20</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

#CR, complete response; *IR, incomplete response
Table 4.3: Relevant pharmacokinetic parameters\(^a\) for G3139 given at 4 and 7 mg/kg CIVI (Protocol OSU 9977)

<table>
<thead>
<tr>
<th>Patient UPN</th>
<th>No. of courses</th>
<th>(\text{AUC}_{0-\infty})(^b) (µg·hr/ml)</th>
<th>(\text{Css}) (µg/ml)(^c)</th>
<th>CL (l/hr)(^d)</th>
<th>(K_{el}) (hr(^{-1}))(^e)</th>
<th>(t_{1/2}) (hr)(^f)</th>
<th>(V) (l)(^g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg/kg</td>
<td>Average± SD</td>
<td>12</td>
<td>856±361</td>
<td>3.19±1.29</td>
<td>4.35±1.85</td>
<td>1.10±0.55</td>
<td>0.63±0.33 (0.36-1.80)</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 mg/kg</td>
<td>Average± SD</td>
<td>6</td>
<td>1546±659</td>
<td>5.47±2.16</td>
<td>3.89±1.48</td>
<td>1.32±0.56</td>
<td>0.52±0.23 (0.33-1.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total(^h)</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.20±1.71</td>
</tr>
</tbody>
</table>

\(^a\) all parameters indicated was mean± SD; \(^b\) \(\text{AUC}_{0-\infty}\), area under the plasma concentration-time curve calculated by linear trapezoid rule with actual data plus extrapolation; \(^c\) \(\text{Css}\), plasma steady-state concentration; \(^d\) CL, the total body clearance; \(^e\) \(K_{el}\), elimination rate constant; \(^f\) \(t_{1/2}\), plasma elimination phase half-life with harmonic mean half-life and its standard deviation; \(^g\) \(V\), Volume of distribution calculated by model-fitting; \(^h\) since all pharmacokinetic parameters are linear with dose, the appropriate mean values were computed for all patients.
<table>
<thead>
<tr>
<th>UPN</th>
<th>Response to G3139+FLA G</th>
<th>Changes (%) in Bcl-2 mRNA level by day 5 of G3139 CIVI</th>
<th>Changes (%) in Bcl-2 protein level by day 5 of G3139 CIVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CR^a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>IR^b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>IR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>NR^c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a CR, complete remission  
^b IR, incomplete remission (see text for definition)  
^c NR, no response  
^d (-36.7), percentage of change at day 5 of G3139 CIVI compared to pretreatment  
^e NA, not available

**Table 4.4**: Disease response and changes in Bcl-2 mRNA and proteins at day 5 of the G3139 CIVI (Protocol OSU 9977)
### Table 4.5: Relevant pharmacokinetic parameters of G3139 given at 7 mg/kg CIVI.

<table>
<thead>
<tr>
<th>Parameters / Units</th>
<th>Mean ± SD(^a) (n=27)</th>
<th>Dose of Daunorubicin(^b) (mg/m(^2)/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-compartment analysis</td>
<td>Two compartment iv infusion model</td>
</tr>
<tr>
<td>(C_{ss}^c), µg/ml (µM)</td>
<td>4.48 ± 3.70 (0.74 ± 0.50)</td>
<td>4.22 ± 2.90 (0.70 ± 0.50)</td>
</tr>
<tr>
<td>AUC(_{0-\infty}^d) (µg·hr/mL)</td>
<td>976 ± 696</td>
<td>1013 ± 692</td>
</tr>
<tr>
<td>CL(^e) (L/hr)</td>
<td>7.77 ± 4.62</td>
<td>7.10 ± 3.70</td>
</tr>
<tr>
<td>(t_{1/2,\lambda}^f) (hr)</td>
<td>2.6 (1.74-3.55)</td>
<td>-</td>
</tr>
<tr>
<td>MRT(^g) (hr)</td>
<td>11.8 ± 8.7</td>
<td>-</td>
</tr>
<tr>
<td>(t_{1/2\alpha}^h) (hr)</td>
<td>-</td>
<td>0.4 (0.1-1.1)</td>
</tr>
<tr>
<td>(V_1^i) (L)</td>
<td>-</td>
<td>4.3 (1.8 – 17)</td>
</tr>
</tbody>
</table>

\(^a\) all pharmacokinetic parameters indicated was mean ± SD except half-life; \(^b\) only non-compartment analysis was reported; \(^c\) \(C_{ss}\): plasma steady-state concentration; \(^d\) AUC\(_{0-\infty}\): area under the plasma concentration-time curve calculated by linear trapezoid rule with actual data plus extrapolated data; \(^e\) CL: the total body clearance; \(^f\) \(t_{1/2,\lambda}\): plasma terminal phase half-life with harmonic mean and ranges; \(^g\) MRT: mean residence time; \(^h\) and \(^i\): distribution (\(\alpha\)) and elimination (\(\beta\)) half-life \((t_{1/2\alpha} \text{ and } t_{1/2\beta})\), respectively) were reported as harmonic mean with ranges. \(^i\) volume of central compartment.
<table>
<thead>
<tr>
<th>Parameters / units</th>
<th>Clinical response</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Complete Response</td>
</tr>
<tr>
<td>No. of patients</td>
<td>13</td>
</tr>
<tr>
<td>CL(^a) (L/hr)</td>
<td>8.59 ± 3.41</td>
</tr>
<tr>
<td>C(_{\text{max}}) , µg/ml (µM)</td>
<td>3.63 ± 1.39</td>
</tr>
<tr>
<td>C(_{\text{ss}}) (^b) , µg/ml (µM)</td>
<td>3.90 ± 3.88</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (^c) (µg·hr/ml)</td>
<td>796 ± 303</td>
</tr>
</tbody>
</table>

\(^a\)CL: the total body clearance; \(^b\)C\(_{\text{ss}}\): plasma steady-state concentration; \(^c\)AUC\(_{0-\infty}\): area under the plasma concentration-time curve calculated by linear trapezoid rule with actual data plus extrapolated data.

Table 4.6: Comparison of pharmacokinetic parameters of G3139 between CR and NR patients
Table 4.7: Summary of G3139 major metabolite concentrations in human plasma at steady state

<table>
<thead>
<tr>
<th>Patients</th>
<th>Time point (hr)</th>
<th>3’N-1 (µg/ml)</th>
<th>3’N-2 (µg/ml)</th>
<th>3’N-3 (µg/ml)</th>
<th>G3139 (µg/ml)</th>
<th>N-1% of G3139</th>
<th>N-2% of G3139</th>
<th>N-3% of G3139</th>
<th>% G3139 in total ODNs pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>1.26</td>
<td>0.86</td>
<td>0.14</td>
<td>2.90</td>
<td>44%</td>
<td>30%</td>
<td>5%</td>
<td>56</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>1.80</td>
<td>0.67</td>
<td>0.16</td>
<td>3.60</td>
<td>50%</td>
<td>19%</td>
<td>4%</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>1.58</td>
<td>0.68</td>
<td>0.25</td>
<td>3.50</td>
<td>45%</td>
<td>19%</td>
<td>7%</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>2.00</td>
<td>0.80</td>
<td>0.14</td>
<td>4.60</td>
<td>43%</td>
<td>17%</td>
<td>3%</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>1.40</td>
<td>0.60</td>
<td>0.25</td>
<td>3.20</td>
<td>44%</td>
<td>19%</td>
<td>8%</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>2.60</td>
<td>1.00</td>
<td>0.32</td>
<td>5.50</td>
<td>47%</td>
<td>18%</td>
<td>6%</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>2.40</td>
<td>1.20</td>
<td>0.20</td>
<td>7.60</td>
<td>32%</td>
<td>16%</td>
<td>3%</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>2.20</td>
<td>0.80</td>
<td>0.40</td>
<td>7.70</td>
<td>29%</td>
<td>10%</td>
<td>5%</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>240</td>
<td>2.30</td>
<td>0.80</td>
<td>0.18</td>
<td>7.00</td>
<td>46%</td>
<td>16%</td>
<td>4%</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>1.50</td>
<td>0.70</td>
<td>0.50</td>
<td>5.40</td>
<td>28%</td>
<td>13%</td>
<td>9%</td>
<td>66</td>
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<td>5</td>
<td>72</td>
<td>1.90</td>
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<td>33%</td>
<td>16%</td>
<td>14%</td>
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<td>240</td>
<td>1.90</td>
<td>0.90</td>
<td>0.80</td>
<td>5.60</td>
<td>33%</td>
<td>16%</td>
<td>14%</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>1.40</td>
<td>0.73</td>
<td>0.50</td>
<td>4.90</td>
<td>29%</td>
<td>15%</td>
<td>10%</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>1.20</td>
<td>0.50</td>
<td>0.31</td>
<td>4.10</td>
<td>29%</td>
<td>12%</td>
<td>8%</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>1.20</td>
<td>0.50</td>
<td>0.30</td>
<td>4.10</td>
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<td>12%</td>
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Figure 4.1: Schematic representation of the treatment with G3139 in combination with FLAG utilized in the first phase I study. See Table 4.1 for dose levels and disease response.
**Figure 4.2**: Schematic representation of the treatment with G3139 in combination with cytarabine and daunorubicin utilized in the second phase I study. See Table 4.2 for dose levels and disease response.
Figure 4.3: A. Semi-logarithmic plasma concentration vs. time profiles during and post infusion of 4 mg/kg G3139; B. Semi-logarithmic plasma concentration vs. time profiles during and post infusion of 7 mg/kg G3139. The anion exchange HPLC method was used to assess plasma concentration of G3139 in protocol OSU 9977 study.
Figure 4.4: A. Mean plasma concentration vs. time profiles during and post infusion of both 4 mg/kg and 7 mg/kg G3139 doses. B. Post-infusion plasma concentration vs. time profiles at both 4 and 7 mg/kg G3139. The anion exchange HPLC method was used to assess plasma concentration of G3139 in protocol OSU 9977 study.
Figure 4.5: Bcl-2 protein levels as measured by immunoblotting (UPN 1). Bcl-2 levels pretreatment and at days 5 (prior to FLAG), 11 (completion of G3139 and FLAG treatment) and 28 (time of neutrophil and platelet count recovery) are shown for this patient who achieved a complete response to G3139 and FLAG. Inset: immunoblotting of Bcl-2 and β-actin levels is shown. Lanes 1-4 represent Bcl-2 and β-actin levels at day 0, 5, 11, and 28.
Figure 4.6: A. Semi-logarithmic plasma concentration vs. time profiles during and post infusion of 7 mg/kg G3139; B. Post-infusion plasma concentration-time profiles of G3139 in acute leukemia patients. The hybridization ELISA assay (Chapter 2) was used to assess plasma concentration of G3139 in protocol OSU 0164 study.
Figure 4.7: A) Mean Plasma concentration vs time profiles during and post infusion of 7 mg/kg/day G3139. The hybridization ELISA assay (Chapter 2) was used to assess plasma concentration of G3139 in protocol OSU 0164 study. Each dot represents the time-point at which plasma was collected for pharmacokinetic analysis. B) Post-infusion pharmacokinetic profile of G3139 (240-248 hr); C) A representative plasma concentration-time profile of G3139 after 10 day infusion of 7 mg/kg G3139. A fit of the data to a two-compartment infusion model is illustrated by the solid line. All solid lines were fitted curve.
**Figure 4.8:** Plasma concentration of G3139 by time. Daunorubicin and Ara-C began at 72 hr. Bars indicated the mean plasma concentration of G3139 with standard deviation at 24 hr (empty bar), 72 hr (upswing slash) and 240 hr (downswing slash).
REFERENCE FOR CHAPTER 4


CHAPTER 5

ELECTROSPRAY IONIZATION MASS SPECTROMETRY COUPLED WITH ION-PAIR REVERSE-PHASE HPLC CHROMATOGRAPHY FOR IN VIVO METABOLISM AND QUANTIFICATION OF G3139

5.1 Introduction

With the advent of electrospray ionization (ESI) techniques, liquid chromatography-mass spectrometry (LC-MS) has become a major qualitative and quantitative analytical tool for small organic molecules (1-3), peptides and proteins (4-6). For DNA, RNA, and oligonucleotides, this method has been less successful until relatively recently (7-9). This is probably due to the lower sensitivity and several analytical problems associated with this type of molecules, such as 1) inadequate chromatographic separation in either reversed phase or anion exchange chromatography, 2) lack of suitable ion-pairing reagents for both MS sensitivity and HPLC chromatography, and 3) extensive adduction with ubiquitous cations, such as sodium or potassium ions, in biological samples. While synthetic oligonucleotides have become increasingly important for the use for diagnostic and therapeutic purposes, characterization of these compounds, monitoring of purity, and more importantly quantification of these compounds in biological matrices, and elucidation of metabolic
products in preclinical and clinical evaluation are of paramount importance. In order to meet this demand a number of strategies have now been developed with respect to solvent additives and modifiers (8-10), higher performance analytical sorbents and columns (particle size and new materials) (11, 12), and improved instrumental parameters (flow rates and others) and manipulations (post column) (13, 14) to improve resolution and sensitivity.

It has been shown that phosphorothioate oligonucleotides are metabolized primarily by exonucleases. In plasma, the removal of bases from the 3'-end of the oligonucleotide is the major pathway (15, 16). Both 5' and 3' exonuclease excision may occur in liver and kidneys (17). On the other hand, endonuclease mediated degradation of phosphorothioate oligonucleotides is a minor pathway and generally not observed (17). The metabolism of PS ODNs also shows stereoselectivity (18). Due to the stereogenicity of the phosphorus atom, internucleotide phosphate groups are P-prochiral in natural oligos. Replacement of one of two nonbridging oxygens creates a new chiral center designated as Rp or Sp isomers (Figure 5.1). Sp and Rp isomers display different sensitivity to nucleases. The Rp isomer is less nuclease stable than the Sp isomer as demonstrated in an in vitro study using rat liver homogenates, in which rat liver nucleases preferentially digest phosphorothioate in the Rp and Rp/Sp racemic configuration. Rp isomer was metabolized at a much faster rate than the mixture, while oligonucleotides modified by the chirally pure Sp nucleotide were barely metabolized (19). Metabolites with higher molecular parent drug were also observed. It was suggested that these metabolites might be Phase II conjugate or addition of a ribonucleotide or a phosphorothioate deoxyribonucleotide to the parent compounds (17).
Identification of metabolic pathways for a drug can provide useful information about toxicity, pharmacological effect, and pharmacokinetics. Although pharmacokinetics of G3139 has been reasonably well studied previously (21, 22), and more recently by an ultra-sensitive non-LC-MS hybridization method (23, 24), little is known about its metabolic fate, nor was there LC-MS quantitative method for G3139 and its metabolites. Previous published results regarding disposition of G3139 were based on radioactivity measurement, which did not separate parent drug from the metabolites, therefore generating little information concerning disposition of G3139 and its metabolites (25, 26). In fact, to date there was only one publication using LC-MS for quantification of antisense drugs in biological samples (27). To overcome these problems, we first develop an ESI LC-MS method coupled to ion-pair reversed phase HPLC (IR-RP-HPLC) chromatography for separation and characterization of G3139 and three major metabolites in the mouse, rat and human. Further, we developed and validate a sensitive ESI LC-MS/MS N-in-one quantification method of G3139 and its metabolites in plasma, allowing characterization of G3139 and metabolite kinetics in human and in the rat (For metabolite kinetics of G3139 see Chapter 6).

5.2 Materials and Methods

5.2.1 Drugs and Chemicals

G3139 was supplied by the National Cancer Institute (Bethesda, MD) and used without further purification. The internal standard (IS), a 25mer phosphorothioate oligonucleotide, and other putative 3’-N-1, N-2, and N-3 of G3139 (here-to-fore G3139
is omitted) metabolites (Table 5.1) were obtained from Integrated DNA Technologies (Coralville, IA) and used without further purification. HPLC-grade methanol, triethylamine (TEA, 99.5%), triethylammonium bicarbonate (TEAB) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.8%) were purchased from Aldrich (Milwaukee, WI, USA). A Milli-Q system (Millipore, Bedford, MA, USA) used to prepare deionized water for HPLC. The purity and identity of each ODN was verified by HPLC-UV-Mass spectrometry (Finnigan LCQ, San Jose, CA). Blank human plasma was obtained from Red Cross (Columbus, OH). Drug-free rat plasma was purchased from Harlan Bioproducts for Science, Inc (Indianapolis, IN, USA).

5.2.2 Instrumentation

The LC/MS system used consisted of a Finnigan (ThermoFinnigan, San Jose, CA) LCQ ion trap mass spectrometer coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD) and SPD-M10A PDA detector (Shimadzu, Columbia, MD). The HPLC system was equipped with two LC-10AD pumps, a SIL-10AD autoinjector (Shimadzu, Columbia, MD).

5.2.3 HPLC chromatographic and mass spectrometric conditions

An Xterra MS18 2.5 µm (average pore diameter 120 Å) 50x2.1 mm stainless steel column (Waters Corp., Milford, MA) coupled to a MSC18 2.1x10 mm guard column (Waters Corp., Milford, MA) was used to separate G3139 and its metabolites. The HFIP-TEA buffer was prepared as a stock solution of 200 mM, adjusted to pH 8.35 with TEA. To prepare 500 mL of HFIP-TEA buffer (pH 8.35), 10.5 mL of HFIP was added into 485
ml water and 2 ml TEA was added with constant stirring for 1 hr. The pH was then titrated to 8.35 with TEA and the final volume was adjusted to 500 mL. This stock solution was diluted to 100 mM with water (50:50, v/v) for the solvent A (100 mM HFIP/8.6 mM TEA, pH 8.35) and with methanol for the mobile phase B consisted of 100 mM HFIP and 8.6 mM TEAB (pH 8.3) in methanol (50:50,v/v). Columns were kept at 50°C in a column heater (Keystone, Woburn, MA) throughout the analysis. The components were eluted with a gradient mode at a flow rate of 0.20 mL/min. Gradient was run from 25 to 50% mobile phase B in 30 min, returned to 25% A in 2 min, and equilibrated at 25% A for 8 min before next sample run. The LC eluant was introduced into the electrospray ion source without splitting.

All experiments were carried out using the LCQ ion trap mass spectrometer with an ESI source operated in the negative ion mode. The electrospray high voltage was 2.0–2.2 kV. The temperature of the heated capillary was set at 210°C. The mass spectrometer was operating with a background helium pressure of 1.75 x 10-3 torr, a typical electrospray needle voltage of 4.5 kV, a sheath gas flow of 80 (arbitrary unit), and an auxiliary nitrogen gas flow of 30 (arbitrary unit). Triple play mode was chosen for identification of major metabolites of G3139. Data dependent scan was performed as follows. Full mass scan was in the range of 600 to 2000 Da, zoom scan was based on the most intense peak from the full scan mass spectrum, and data dependent tandem MS/MS was derived from the most intense peak. The MS/MS mass spectrum of metabolites of G3139 was acquired with 25-30% of the normalized collision energy with isolation width of 3.0 Da. The mass spectrometer was tuned to its optimum sensitivity of charge states from [M-3H]3- to [M-7H]7- by infusion of either G3139 or 3’ N-1 through N-3. All
operations were controlled by Finnigan Xcaliber software in a Windows NT 4.0 system. Raw spectra were processed with Xcalibur (Version 1.2, Finnigan, CA). Molecular mass information was obtained by automatic deconvolution of the raw spectrum using the BioMass program (Xcalibur 2.1). The PDA was operated to give the spectra of 200 to 600 nm. All operations were controlled by EZstart 7.2 software in a Windows NT 4.0 system.

5.2.4 Sample preparation

Plasma samples were thawed and centrifuged at 1000g for 5 min. Solid phase extraction (SPE) was used for isolation of G3139 and metabolites from plasma. Rat and human plasma samples spiked with appropriate amounts of pure compounds were used to construct calibration curves for G3139 and three major metabolites. Samples (0.2 mL) were then spiked with 20 µL of the IS at 400 µg/mL in water. Thereafter, the samples and standards were mixed with 0.8 mL of 0.1 M TEAB buffer and extracted on an Oasis HLB cartridge packed with 30 mg material (Waters, Corp., Milford, MA). The extraction tubes were conditioned first with 1 ml of acetonitrile followed by 1 ml of 0.1 M TEAB (pH 8.5). Then the samples mixed with 1 ml of 0.1 M TEAB were loaded onto each of the columns. The proteins and salts were removed by sequential washes with 2 ml of TEAB, 1 ml of 10% acetonitrile in 0.1 M TEAB and 1 ml of water by gravity flow. The breakthrough was examined by UV measurement and found to be negligible. Then the ODNs were eluted with 0.5 ml of 50 % acetonitrile and the eluant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 100 µL of mobile phase A and 40 µL aliquot was analyzed by LC-MS.
Tissue samples were pulverized while still frozen, and an aliquot was weighed directly into a tube. Then, it was homogenized in 10:1 PBS with a tissue homogenizer (VIRTIS, Gardiner, NY). To the supernatant was added 2 mg/ml of proteinase K in buffer containing of Tris-HCl (pH, 8.0), 10 mM EDTA to digest tissue components (Sigma, WI). Samples were then incubated overnight at 37°C. The supernatant was extracted with phenol-chloroform-isoamylalcohol (25:24:1, pH, 8.0, Ambion, TX) to remove proteins and lipids. G3139 related species remain in the aqueous phase, while proteins and lipids stay in the organic phase. The phenol-chloroform layer was back-extracted with 2 ml of distilled H₂O, and the aqueous phases were pooled. Samples were then evaporated to dryness, resuspended in 3 ml of TEAB buffer, and then processed identically to plasma and urine samples. An aliquot of 40 µL was injected in the LC/MS system for analysis.

5.2.5 Identification of major metabolites of G3139 in vivo

For HPLC analysis, the ion-pair elution described above was used with optimization for each run for maximum peak separation. Data dependent scan was performed to obtain MS and tandem (MS/MS) spectra of the most intensive peak. Deconvolution was performed using Xcalibur Biomass to obtain the molecular weights. Data of the MS/MS spectra of metabolites were exported from the Xcalibur Software (Finnigan, CA) and sequenced by an external computer program, Simple Oligonucleotide Sequencer (SOS) (Version 1.1) [39]. Comparison of the measured and predicted spectra was performed with the same program.
5.2.6  N-in-one quantification of G3139 and major metabolites in rat and human plasma

Multiple reaction monitoring (MRM) mode was employed for monitoring ion transitions for four analytes. ESI LC/mass spectra G3139 and three major metabolites were first obtained. The [M-3H]^3- ions of G3139 and three major metabolites were isolated and activated for 30 ms to produce daughter ions with optimized normalized collision energy of 15% for G3139 and three metabolites, 18% for internal standard (25mer). The ion transitions (SRM) monitored were m/z 1893.7^3-→1842.8^3- for G3139, 1786.6^3-→1736.3^3- for 3’N-1, 1676.7^3-→1626.2^3- for 3’N-2, 1575.7^3-→1524.6^3- for 3’N-3, and 1985.4^4+→1947.5^4+ for the IS. An automatic gain control was set to ensure the high sensitivity, but avoiding the space charge effects. The mass spectrometer was tuned to its optimum sensitivity by flow infusion of G3139 at 20 μg/mL in 35% mobile phase B. All operations were controlled by Finnigan Navigator 1.2 software on a Window NT 4.0 system.

5.2.7  Assay validation

Plasma samples for the standard curves were prepared by spiking 0.2 mL of rat plasma and urine, and human plasma each with various amounts of G3139, three metabolites and a constant amount of the internal standard. The linearity was evaluated in the concentration range of 0.1-10 μg/mL. The within-day precision values were determined in five replicates at concentrations of 0.25, 1 and 5 μg/mL for each analyte. The between-day precision was determined across these three concentrations at five different days. The accuracy of the assay was determined by comparing the nominal
concentrations with the calculated values. The specificity of the assay was established by monitoring MRM for G3139 and each metabolite in blank plasma. The recovery of each analyte was estimated by comparing the peak area of the extract analyte to those of the unextracted at concentrations of 0.5 and 5 µg/mL.

5.2.8 *In vivo* metabolism of G3139 in rats and mice

Female Sprague Dawley rats weighing ~300 g were purchased from Harlan (Indianapolis, IN). Female CD2F1 mice were obtained from Harlan (Indianapolis, Indiana). The average body weight was 22 ± 2 g (mean ± SD). All animals were adapted to a 12-h light/dark cycle under controlled room temperature and humidity conditions. Food and water were given *ad libitum*. The animal facility is accredited by the American Association for Laboratory Animal Care. The study was performed with a protocol adhered to the “Principles of Laboratory Animal Care” by NIH and approved by The Ohio State University Vivaria. G3139 in sterile saline was given as an i.v. bolus dose of 5 mg/kg to mice and 20 mg/kg to rat. Animals were housed individually in metabolism cages that allowed for separate collection of urine and feces, and restricted to food only in the first 24 hr after dosing. Urine samples were collected 24 hr prior to dosing and at interval of 0-4, 4-8, 8-12 and 12- 24 hours after dosing. The total volume of urine was measured by weight difference and frozen at -80°C until analyzed. At the end of urine collection, animals were euthanized with 50 mg/kg Ketamine/Xylazine (Sigma, St. Louis, MO) and tissue samples were removed through thoracic opening. The plasma was obtained by centrifugation at 500 g at 4°C for 10 min and stored at -80°C until analysis. For mice, blood was collected by cardiac puncture under CO₂ anesthesia, transferred to
tubes containing 100 µg dry EDTA and centrifuged to separate plasma. Plasma and tissue samples were frozen and stored at -80°C until analysis.

5.3 Results

5.3.1 Identification of major metabolites of G3139 in vivo

The initial strategy involved characterization of the mass spectral fragmentation data of G3139. Figure 5.2 shows the ESI LC/mass spectrum of G3139, which indicated a base [M-3H]⁻³ at m/z 1893.7 and several ions at different charge states from –4 to –8 at lower and variable intensities (Table 5.2). Upon deconvolution, these multiple charged ions yielded the correct [MH]⁻ (m/z 5684.4) and associated isotope ions with ±0.009% mass accuracy, when compared to the calculated (m/z 5684.9) (Figure 5.2). Additionally, low levels of sodium and potassium adduct ions were detected. Upon collision induced dissociation (CID) of [M-3H]⁻³, the MS² spectrum gave fragment ions (Figure 5.2), consistent with w and a-B series based on the literature information on the base sequence of oligonucleotides in ion trap mass spectrometry (29, 30). The oligonucleotide fragmentation nomenclature, as shown in Figure 5.3, followed the widely accepted one proposed by McLuckey et al. (31). The formation of the most abundant fragment ion at m/z 1842.8 was readily accounted for by the loss of neutral guanine base from the –3 charge state molecular ion (m/z 1893.7) (Table 5.3). The procedure for sequencing of G3139 by SOS program is shown in Figure 5.4. Briefly, CID spectrum of G3139 was exported to a text file in Xcalibur and then loaded into the SOS program. From the isotopic spacing, the charge state of each ion was identified. The most diagnostic ions arise from the w series that are used to determine the 3' → 5' sequence and the a-B ions
that are used to determine the $5' \rightarrow 3'$ sequence. First, each $w$ ion was chosen based on the charge states and abundance rule to build the $w$ ions ladder. Then the $a-B$ ions ladder was established from $5'$ based on ion abundance rule and the charge states. Mass ladders were independently built from both terminals by searching for the best ion candidates at multiple charge states. Combined $w$ and $a-B$ ion series were finally aligned to generate a sequence candidate. If the mass of the candidate was not consistent with the molecular mass, it was discarded and the above procedure was repeated until a matching sequence was found. The CID spectrum of G3139 is shown in Figure 5.5. Using this strategy, assignments and mass/charge for the fragment ions observed in the CID spectrum of G3139 were made and the data are listed in Table 5.3 and depicted in Figure 5.5.

By comparing the plasma extract obtained from pretreatment (data not shown) with that obtained from the plasma sample in leukemia patient 120 hrs following i.v. infusion of G3139 at 7 mg/kg (32), six new peaks, denoted as M1, M2, M3, M4, M5 and M6 were found (Figure 5.6A). The parent compound G3139 was eluted after its potential metabolites with a retention time of 15.2 min (Figure 5.6A). The total ion current at the peak corresponding to M1 from 13.6 to 14.1 min was summed to generate the mass spectrum (Figure 5.6B), which shows several multiple charged ions. Following deconvolution, the molecular mass was found to be 5363.0 Da (Table 5.1). The most likely base composition for this mass was d(C$_8$T$_3$A$_2$G$_4$), a loss of dT from the 3’end from G3139 d(C$_8$T$_4$A$_2$G$_4$). Other possibilities of modified base or oxidative product were not supported by the mass computation. This component could be produced by either the 3’ or 5’ single nucleotide deletion from G3139. To distinguish these two possibilities, MS$^2$ spectrum of 3’ N-1 was obtained using the most abundant ion [M–3H]$^{-3}$ at m/z 1786.7
and compared with the MS$^2$ spectrum for M1 (Figure 5.7). Specifically, the ions from the w series and the a-B ions were examined and compared. Eleven w ions and 8 a-B ions generated from the MS$^2$ of M1 were identified and assigned (Table 5.4). The fragment ions as identified in MS$^2$ of 3’ N-1 were essentially the same, for example, w$^2$ (m/z 652.9), w$^3$ (m/z 956.9), and w$^4$ (m/z 803), corresponding to sequences of 3’-CA, 3’-CCA and 3’-GCCA, respectively. Similarly, the a-B fragment ions in MS$^2$ of 3’ N-1 are consistent with that of M1: a$^3$ (m/z 723), a$^4$ (m/z 1042), a$^15$ (m/z 1531), as corresponding to sequences of 5’-TCT, TCT, and TCTCCAGCGTGCGC, respectively.

MS$^2$ mass spectrum of 5’ N-1 (Figure 5.8) has distinct pattern of a-B ion and w ion series from that of 3’ N-1. For example, the fragment ions as identified in MS$^2$ of 5’ N-1 were w$^2$ (m/z 665.6), w$^3$ (m/z 970.9), and w$^4$ (m/z 1275.5), corresponding to sequences of 3’-AT, 3’-CAT and 3’-CCAT, respectively. Similarly, the fragment ions identified as the a-B ions were a$^4$ (m/z 1026.9), a$^6$ (m/z 1661), and a$^{15}$ (m/z 1628.9), as corresponding to sequences of 5’-CTCC, 5’-CTCCCA, and 5’-CTCCAGCGTGCGCC, respectively. The MS$^2$ mass spectrum of M1 was essentially identical to that of 3’N-1 but not to that of 5’N-1.

The second major peak (M2) in the plasma extracts shows several multiple charged ions with different intensities (Figure 5.6C), and upon deconvolution, had a mass at m/z 5035.1 (Table 5.1). This mass shows a decrease of 649.3 Da from the parent compound, corresponding to a loss of two phosphorothioate nucleotides from the 3’ end (-AT), but not from 5’end (-TC). Indeed, the mass accuracy of M2 was 0.016% that excludes any other possible mass assignment. Thus, M2 was assigned as the metabolite
derived from removal of 2 nucleotides from the 3’end or 3’N-2 of G3139 (Table 5.1).
The sequence of M2 was further verified by the SOS program (data not shown). MS\(^2\) ions
characteristic of M2 are of w ion series, for instance, w\(_2\)-1 (m/z 627), w\(_3\)-1 (m/z 917.9), w\(_5\)-2
(m/z 809.1), and w\(_7\)-2 (m/z 143.0) corresponding to 3’-CC, 3’-GCC, 3’-GCGCC, and 3’-
GTGCGCC, respectively. Fragment ions from the a-B ions were essentially the same as
those from M1, suggesting M2 shares the same sequence with M1 from the 5’ terminal
up to 15 nucleotides (data not shown).

Similarly, mass spectra of other major peaks are shown in Figure 5.6. Ion
envelopes were observed for all metabolites, and in most cases [M–3H]\(^{-3}\) to [M–6]\(^{-6}\) were
identifiable. Again, upon deconvolution molecular ions of these major components were
obtained, and M3, M4, and M5 were unambiguously assigned as 3’N-3, N-4 and N-5
with mass accuracy of 0.038%, 0.023% and 0.017%, respectively (Table 5.1). One
metabolite with the longest retention time (17.1 min) was not identified by ESI/MS/MS
(Figure 5.6 G). This component did not seem to relate to an oligonucleotide based on the
most abundant ion (m/z 1911.3), assuming that the ion was singly charged. Thus, based
on this result, metabolism of G3139 involves probably mainly 3’-exonuclease consistent
with metabolism of other antisense oligonucleotides (20, 29, 33).

5.3.2 Metabolism in organ tissues

The next step was to compare the metabolism of G3139 in different organ tissues
in different animal species. First, SD rats were dosed with i.v. G3139 of 20 mg/kg and
plasma samples were taken at 5 min for metabolite identification. Similarly, 3’ N-1 to 3’
N-5 were also identified in the 5 min plasma sample obtained from the rat given i.v. bolus
of G3139 at 20 mg/kg and the results are summarized in Figure 5.9 and Table 5.1. Similar pattern of metabolites was also observed in the 0-4 hr urinary extract taken from a rat given i.v. bolus of G3139 at 20 mg/kg. Selected ion monitoring (SIM) was performed and data for 3’ N-1 up to 3’ N-4 as well as G3139 were presented (Figure 5.10).

When the ion-pair reversed-phase LC-MS chromatogram of the kidney extract obtained from mice treated with G3139 at 5 mg/kg i.v. was compared with the same obtained from a control animal (Figure 5.11), five new peaks (Figure 5.12) denoted as M1, M2, M3, M4 and M5 were found. The parent compound G3139 was eluted after its metabolites with a retention time of 19.13 min. The identification of each major peak in the kidney extract was straightforward with deconvolution procedure except 3’N-1 (mass spectra of metabolites 3’N-2 to N-5 are shown in Figure 5.13). The principle described as above and procedure depicted in Figure 5.4 were applied to the assignment of 3’ N-1 in the kidney extract (data not shown). Further, we performed selected ion monitoring (SIM) by second HPLC injection of the kidney extract (Figure 5.14). The following ions were used to generate selected ion chromatograms for M1, M2, M3, M4, M5, and G3139 respectively, 1786.5, 1677, 1575, 1473, 1358.6, and 1983 with charge states of –3 for all species. Baseline separation was clearly demonstrated and resulting SIM confirm our previous finding that major metabolic pathway for G3139 was probably hydrolysis via 3’ exonuclease. Interestingly, two metabolites were seen at retention times of 17.48 and 18.70 min, which coeluted with 3’ N-1 and G3139, respectively (Figure 5.15). Calculation of mass for the peak 0.5 min before 3’ N-1 revealed a molecular weight of 5348.3 which was 16.0 Da different from mass of 3’N-1. This metabolite might be
generated by metabolic oxidation of sulfur at a single linkage. Similarly, there was a peak 0.4 min earlier than G3139 (MWT, 5684.9), which reveals mass of 5668.6, suggesting it is oxidation product of G3139 (Figure 5.15 and Table 5.5).

5.3.3 N-in-one quantification of G3139 and major metabolites in rat and human plasma, and rat urine

Using 100 mM HFIP combined with 8.6 mM TEA (pH 8.3) as the mobile phase with a gradient elution on a 2.5 µm C18 reverse phase column (Xterra MS C18) at 50°C, G3139, its 3’N-1, N-2, and N-3 were better than baseline resolved with UV detection (Figure 5.16A), and with LC-MS, they were nearly baseline separated (Figure 5.16B). The slight decrease in resolution of the LC-MS was probably due to the peak broadening frequently encountered in the online serial connection (LC/UV/MS). However, because of the MS resolution in the subsequent SIM or SRM assay, the small overlap in total ion did not present a problem for the assay.

Under the ESI LC/MS condition, G3139 gave a number of ions having different charge states, [M-8H]⁻⁸ (m/z 709.5), [M-7H]⁻⁷ (m/z 811.1), [M-6H]⁻⁶ (m/z 946.4), [M-5H]⁻⁵ (m/z 1135.7), [M-4H]⁻⁴ (m/z 1419.3) and [M-3H]⁻³ (m/z 1893.7, base ion) (Figure 5.2A). Therefore, the –3 charge state ion at m/z 1893.7 was selected for CID experiment which generated four major daughter ions at m/z 1856, 1848, 1842.8, 1797.5 and 1747.4 (Figure 5.17A). The parent/product ion pair at m/z 1893.7 and 1842.8 was chosen in the selected reaction monitoring (SRM) mode for quantification of G3139. Similarly, 3’ N-1 generated the triply charged ion as the most abundant peak, [M-3H]⁻³ (m/z 1786.6) in the full scan mode (Figure 5.6B) and the major daughter ion under CID at m/z 1736.3
(Figure 5.7). The parent/product ion pair at m/z 1786.6 and 1736.3 was thus selected in the SRM mode for quantification of 3’ N-1. Similarly, ion pairs at m/z 1676.7/1626.2 (Figures 5.6C and 5.17B) and at m/z 1575.6/1524.6 (Figures 5.6D and 5.17C) were used in the SRM mode for quantification of 3’ N-2 and 3’ N-3, respectively (Table 5.6). For the internal standard the 25mer (MW 7945) shows a parent/product ion pair at m/z 1985 and 1947.5 (Figure 5.17D) and it was selected in the SRM mode for the internal standard measurement.

5.3.4 Assay validations

Having identified the G3139 metabolites and establishment for the conditions for the separation and monitoring of the parent compound and its metabolites and the internal standard, we then used this method for their quantification in human plasma and rat plasma. Representative MRM chromatograms of human plasma spiked with pure G3139, 3’ N-1, N-2, N-3 each at concentration of 1 µg/mL and I.S. of 20 µg/mL are shown in Figure 5.18. As shown, G3139, three major metabolites and the internal standard were baseline separated with no interference found from plasma at the same retention time (Figure 5.19) under the SRM condition, thus further establishing the specificity of the assay.

The limit of quantification (LOQ) was set at 17.6 nM (100 ng/mL) in rat plasma and urine, and human plasma, on the basis of a signal-to-noise level above 3:1 (Figure 5.20). The recovery at 88 nM (0.5 µg/mL) (n=3) was 34, 30, 32, 54% for G3139, 3’ N-1, 3’ N-2, and 3’ N-3, respectively. The recovery (n=3) was 43, 58, 48, and 64% for G3139, 3’ N-1, 3’ N-2, and 3’ N-3, respectively, at 880 nM (5 µg/mL). The assay was linear from
17.6 nM (100 ng/mL) to 1760 nM (10 µg/mL), using 0.2 mL rat plasma (Figure 5.21) and human plasma (Figures 5.22). The within-day precision, expressed as % C.V. is shown in Table 5.7. As shown, the values ranged from 2.0 to 17.6 in rat plasma (n=5) and 2.0 to 22% in human plasma with majority of the precision values falling below 15%. The accuracy values ranged from 89-112%. The between-day CVs were 2 to 23% for 0.25, 1 and 5 µg/mL with majority <15%. The accuracy values of the assay varied in the range from 82 to 126% with majority of the values between 89 to 115% (Table 5.8).

The method was also utilized to quantify G3139 and three metabolites in rat urine. A representative MRM chromatogram of blank rat urine spiked with internal standard is shown in Figure 5.23. There were no endogenous peaks that interfered with the quantification of G3139 and three metabolites thus establishing the specificity of the assay. The limit of quantitation (LOQ) for G3139 was found to be 17.6 nM (100 ng/mL) in rat urine. The recovery of the method was approximately 70% (n=3). Quantification was shown to be linear for G3139, 3’ N-1, 3’ N-2 and 3’ N-3 from 17.6 to 1760 nM (0.1 to 10 µg/mL) (Figure 5.24).

5.4 Discussion

Adduct ions reduction. Characterization and quantitative analysis of ODNs oligonucleotides by ESI MS and MALDI has been rather difficult due to extensive adduction with ubiquitous cations such as Na\(^+\) and K\(^+\) (11). The formation of ion adducts causes dispersion of total ion among multiple charge states, thus reducing the ion intensity. With PS ODNs, this problem was compounded by the peak broadening effect due to the existence of multiple diastereomers imparting, when an oxygen atom in ODN
is replaced with a sulfur (34-36). Consequently, to date there was only one publication using LC/MS for quantification of antisense drugs in biological samples (27). To address to ion adducts formation, Greig and Griffey (8) showed that organic bases such as TEA and piperidine reduce the signals from bound Na⁺ most effectively, although they also decrease the ion signal intensity. Imidazole provides modest suppression of cation adducts with improved MS sensitivity. A 1:1 co-addition of imidazole and TEA/piperidine produces high ion abundance and good suppression of cation-adducts for PS ODNs. However, several other studies have shown the improved MS sensitivity using TEA as additive (7, 37). Muddiman et al. found that imidazole/piperidine/acetic acid in 80% acetonitrile reduced the charge states (shifting to a higher mass-to-charge ratio) with substantial suppression of cation formation (10). The use of solvents like isopropanol-water adjusted to pH 9.5 or acetonitrile-200 mM diisopropylamine adjusted to pH 7 have also been shown to yield good MS sensitivity and accuracy (38).

**Post-column manipulation.** Another strategy for reduction of cation adduction is the post-column addition of sheath liquid, such as methanol, acetonitrile, and iso-propanol, which also shifts the charge state distribution of multiply charged ions (13). Deguchi et al. proposed a method to combine acetonitrile/TEAA based mobile phase with 50-100 mM imidazole as an organic modifier in the post-column mode for enhanced MS sensitivity of ODNs (14).

**Sample preparation.** In complex biological matrices, salts, small organic and inorganic molecules, and other protein and non-protein macromolecules further contribute to the difficulty in the analysis of ODNs and PS ODNs because of matrix effects. Thus, sample preparation such as desalting and extraction of antisense molecules
is still required for the success of their analysis. However, general precipitation method for ODNs with ammonium acetate or acetonitrile results in low recovery. Isolation of analytes by solid phase extraction with proper sorbent and eluants remains a useful and attractive approach. We have found that bulk interference matrices could be effectively removed by solid phase extraction with sequential elution with TEAB buffer, TEAB-acetonitrile, and water. The analytes were eluted with 50% acetonitrile with reasonable recovery.

**Column Selection.** Oligonucleotides are usually separated by either anion-exchange or ion-pair-RP HPLC (34, 39). We and others have previously used HPLC-UV method with a strong anion exchange column with short length for the analysis of G3139 in plasma samples (22, 40). Although G3139 was separated from plasma matrix materials, resolution of G3139 from metabolites remained to be demonstrated. The use of a longer column will improve the resolution and would probably allow resolution with the metabolites. However, the high cost of the column makes the method unattractive and more importantly, the high salt content in the mobile phase renders the method impractical for the MS detection. On the other hand, the use of a reversed-phase column in combination with mobile phase with ion-pairing property is an attractive alternative. After a number of attempts, we have chosen a C18 reversed-phase column packed with a 2.5 μm fully porous C18 sorbent (Xterra MS C18). This column was specifically designed to tolerate high pH mobile phase so that the mobile phase at pH 8.3 did not seem to reduce the column lifetime. We also used an elevated temperature (50°C) to enhance the mass transfer and reduce the backpressure due to the fine particle of the column we used.
**Mobile phase.** Triethylammonium acetate buffer (TEAA) in acetonitrile is a commonly used buffer system for reversed phase ion pair separation of oligonucleotides. However, for MS analysis TEAA at concentrations of 50-100 mM has been shown to cause ion suppression due to its low volatility (9). Apffel et al. suggested that HFIP/TEA could substitute TEAA in this buffer system and TEA could serve as an efficient ion pair reagent for the negatively charged phosphorothioate oligonucleotides. As the solvent is electrosprayed, HFIP, being a highly volatile weak acid (b.p. 57°C), separates from the surface of oligonucleotide-TEA (b.p. 89°C) complex in the source, and the pH at the droplet surface rises toward 10 (9). At this pH in gas phase, TEA dissociates from the oligonucleotide, which results in ionization of the ODNs. A buffer system with 16.3 mM TEA/400 mM HFIP (pH 7.0) in methanol was recommended for optimal LC-MS analysis of oligonucleotides (9). However, our results have shown that such combination of TEA/HFIP did not result in satisfactory performance on ESI/MS. With 400 mM HFIP (pH 7.0) buffer, the most abundant charge state ion was \([M-7H]^-\) for G3139 (m/z 811.1) with minimal \([M-3H]^-\) ion (m/z 1893.7). Increasing pH values of buffer by reducing concentration of HFIP to 100 mM (pH 8.3) reduced the charge states of G3139 and increased the abundance of \([M-3H]^-\) ions (Figure 5.2A) with other higher charge state ions <30% relative abundance of \([M-3H]^-\) ion. This result suggests that IP-RP-HPLC coupled with ESI is essential for characterization of metabolites of G3139 in complex biological samples, since direct infusion into ESI/MS could not provide comparable sensitivity and mass accuracy due to cation adduct formation. The TEA serving as efficient ion pair reagent could displace cations adhered to the oligonucleotide phosphate backbone, thus resulting in superior deconvolution spectrum for G3139 with mass
accuracy of 0.009 % or 90 ppm (Figure 5.2B). It has been suggested that the TEA concentration rather than the concentration of HFIP plays a major role in the chromatography performance (41). We found 100 mM that HFIP combined with 8.6 mM TEA (pH 8.3) appears to offer the best ion pair efficiency and MS performance. Similar finding was also reported by Gilar and coworkers (41).

**Mass spectrometer.** Characterization of structures of metabolites relies on high accuracy in molecular mass measurements. It has been previously reported that ESI ion trap mass spectrometry provided mass accuracy ranging from 0.2 to 1.2 Da for small oligonucleotides and ranging from 0.1 to 6.4 Da for PCR products (20000-26000 Da) (42, 43). Using a LCQ ion trap instrument, we have found that the mass accuracy was ± 0.009%. Deconvolution readily yielded the unambiguous structural verification. Using this instrument, we were able to positively identify most metabolites of G3139 in biological samples following a single injection, with better than 300 ppm mass accuracy. Identification of majority of metabolites was straightforward by comparing ion envelope generated with theoretical one and deconvolution calculation. However, since both 3’ and 5’-terminals of G3139 are deoxyribo-thymine (dT), 3’ N-1 and 5’N-1 from M1 have exactly the same ion envelop and molecular mass upon deconvolution measurement, and both cannot be readily differentiated. Fortunately, algorithm for interpretation of CID mass spectra of oligonucleotides based on mass spectral fragmentation data has been developed for unknown nucleotides (28, 31, 44). This strategy was initially developed by McLuckey and co-workers (31) and the software was recently reported by McClosky based on fragmentation chemistry of oligonucleotides under ESI/MS/MS (28). We have used this interactive Simple Oligonucleotide Sequencer (SOS) (28) to successfully
elucidate and confirm the sequencing of M1 from MS/MS spectra. The extensive information obtained by data dependent scan provided a new strategy for characterization of metabolites of G3139. Our results indicate that the major metabolic pathway for G3139 is hydrolysis of the parent drug probably by 3’ exonuclease existing in various tissues including plasma. Further, mouse, rat and human exhibited very similar plasma metabolism (data not shown for mouse). In kidneys, 3’ deletion metabolites seem to be predominant species with small amounts of oxidative metabolites of 3’ N-1 and G3139. G3139 was always the predominant species in plasma as well as in kidney. Using the similar approach, we have also detected these three metabolites in rat urine, but the levels were only measurable for 8 hrs.

As stated in Chapter 1, one of our goals was to develop a sensitive and specific bioanalytical method for determining not only the parent drug but also all major metabolites in a single sample injection. Using this optimized HPLC/MS condition, a novel quantification method was developed to simultaneously quantify parent drug as well as major metabolites as “N in one” fashion. Synthetic G3139 and three major metabolites were used to construct calibration curves and the assay was found to be linear over ranges of 17.6 nM (100 ng/mL) to 1760 nM (10 µg/mL). In comparison to LC-UV, the LC-MS method clearly was more sensitive and specific. Although the novel hybridization ELISA assay (see Chapter 2) previously developed in our laboratory provided higher sensitivity, allowing measurement of intracellular drug levels (23, 24), it was not capable of determining various metabolites levels in biological fluids. Other previous pharmacokinetics and metabolism studies of oligonucleotides based primarily
on radiolabel method were also incapable of differentiating parent drug from metabolites. We have developed and validated a novel HPLC/MS/MS method to simultaneously quantify parent drug and metabolites in plasma. The between-run and within-run precision and accuracy of this ESI HPLC-MS/MS method in rat and human plasma are acceptable with LOQ of 17.6, 18.6, 20 and 21 nM (0.1 µg/mL) for G3139, 3’N-1, 3’N-2 and 3’N-3, respectively, in rat or human plasma.

In conclusion, a novel ion-pair reverse-phase ESI HPLC-MS method has been developed for identification and quantification of major metabolites of G3139 in vivo. TEA/HFIP is a useful additive in the method for the analysis of PS ODNs. Metabolism of G3139 in vivo is probably primarily mediated by 3’ exonuclease in mouse, rat and human.
<table>
<thead>
<tr>
<th>Identity</th>
<th>Proposed sequence (from 5’ to 3’)</th>
<th>Calculated MWT (Da)</th>
<th>Observed MWT (Da)</th>
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<tr>
<td></td>
<td></td>
<td>Human</td>
<td>Rat</td>
</tr>
<tr>
<td>G3139</td>
<td>TCTCCAGCGTGC GCCAT</td>
<td>5684.9</td>
<td>+ 5684.3</td>
</tr>
<tr>
<td>M1 3’ N-1</td>
<td>TCTCCAGCGTGC GCA</td>
<td>5364.3</td>
<td>+ 5364.0</td>
</tr>
<tr>
<td>M2 3’ N-2</td>
<td>TCTCCAGCGTGC GCC</td>
<td>5035.1</td>
<td>+ 5034.0</td>
</tr>
<tr>
<td>M3 3’ N-3</td>
<td>TCTCCAGCGTGC GC</td>
<td>4729.8</td>
<td>+ 4728.2</td>
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<tr>
<td>M4 3’ N-4</td>
<td>TCTCCAGCGTGC G</td>
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<td>+ 4425.0</td>
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<tr>
<td>M5 3’ N-5</td>
<td>TCTCCAGCGTGC</td>
<td>4079.3</td>
<td>+ 4078.6</td>
</tr>
<tr>
<td>M6</td>
<td>Unknown metabolite</td>
<td>+</td>
<td>Not oligonucleotide related</td>
</tr>
<tr>
<td>Internal standard</td>
<td>TCTCCAGCGTGC GCC CAT CAGCAT</td>
<td>7944</td>
<td>N/A</td>
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**Table 5.1:** Structures and Molecular weight of G3139 and its metabolites identified in rat and human plasma.
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<th>Metabolites</th>
<th>Calculated MWT (Da)</th>
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<th>-4 charge</th>
<th>-5 charge</th>
<th>-6 charge</th>
<th>-7 charge</th>
<th>-8 charge</th>
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<td>5684.9</td>
<td>1893.3</td>
<td>1420</td>
<td>1135.7</td>
<td>946.4</td>
<td>810.9</td>
<td>709.6</td>
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<td>3’ N-1</td>
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<td>1786.5</td>
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<td>1071.7</td>
<td>893.1</td>
<td>765.2</td>
<td>669.5</td>
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<td>1575.3</td>
<td>1181.3</td>
<td>944.8</td>
<td>787.4</td>
<td>675</td>
<td>590.2</td>
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<td>4424.6</td>
<td>1473.2</td>
<td>1105</td>
<td>883</td>
<td>736</td>
<td>631</td>
<td>552.1</td>
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<tr>
<td>3’ N-5</td>
<td>4079.3</td>
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<td>678</td>
<td>582</td>
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<td>3’ N-6</td>
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<td>1786.5</td>
<td>1339.8</td>
<td>1071.9</td>
<td>893.1</td>
<td>765.2</td>
<td>669.5</td>
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<td>5’ N-2</td>
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<td>1010.6</td>
<td>842</td>
<td>721.7</td>
<td>631.4</td>
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**Table 5.2:** Theoretical ion envelop of G3139 and possible metabolites
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<th>m/z</th>
<th>Ion assignment</th>
<th>Sequence (from 5’ to 3’)</th>
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</thead>
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<tr>
<td>971</td>
<td>W_3^+</td>
<td>CAT</td>
</tr>
<tr>
<td>1276</td>
<td>W_4^+</td>
<td>CCAT</td>
</tr>
<tr>
<td>1621.1</td>
<td>W_5^+</td>
<td>GCCAT</td>
</tr>
<tr>
<td>1926.2</td>
<td>W_6^+</td>
<td>CGCCAT</td>
</tr>
<tr>
<td>1455.1</td>
<td>W_9^-</td>
<td>GTGCGCCCAT</td>
</tr>
<tr>
<td>1792.6</td>
<td>W_11^-</td>
<td>GCGTGCGCCAT</td>
</tr>
<tr>
<td>1957</td>
<td>W_12^-</td>
<td>AGCGTGCGCCAT</td>
</tr>
<tr>
<td>1615.5</td>
<td>W_15^-</td>
<td>CCCAGCGTGCGCCAT</td>
</tr>
<tr>
<td>1717.7</td>
<td>W_16^-</td>
<td>TCCCAGCGTGCGCCAT</td>
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<td>1819</td>
<td>W_17^-</td>
<td>CTCCCAGCGTGCGCCAT</td>
</tr>
<tr>
<td>1042.8</td>
<td>a_4-B_4^-</td>
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</tr>
<tr>
<td>1347</td>
<td>a_5-B_5^-</td>
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</tr>
<tr>
<td>1652</td>
<td>a_6-B_6^-</td>
<td>TCTCCC</td>
</tr>
<tr>
<td>1959</td>
<td>a_7-B_7^-</td>
<td>TCTCCCA</td>
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<td>1142</td>
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<tr>
<td>1634</td>
<td>a_16-B_16^-</td>
<td>TCTCCAGCGTGCGCC</td>
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<td>1301.8</td>
<td>a_17-B_17^-</td>
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<tr>
<td>1842.8</td>
<td>(M-3H-G)^-3</td>
<td>TCTCCAGCGTGCGCCAT</td>
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**Table 5.3:** Assignment of fragment ions obtained from collision-induced dissociation of [M-3H]^3 of G3139 at m/z 1893.7 in human plasma
<table>
<thead>
<tr>
<th>m/z</th>
<th>Ion assignment</th>
<th>Sequence (from 5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>652.9</td>
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<td>CA</td>
</tr>
<tr>
<td>956.9</td>
<td>$W_3$&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>CCA</td>
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<td>803</td>
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<td>GCCA</td>
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<tr>
<td>956</td>
<td>$W_5$&lt;sup&gt;-2&lt;/sup&gt;</td>
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<td>1150.1</td>
<td>$W_7$&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>TGCGCCA</td>
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<td>1461.2</td>
<td>$W_9$&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>CGTGCGCCA</td>
</tr>
<tr>
<td>1634</td>
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<td>GCGTGCGCCA</td>
</tr>
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<td>1347.3</td>
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<td>1959.6</td>
<td>a&lt;sub&gt;7&lt;/sub&gt;-B&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;-1&lt;/sup&gt;</td>
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<td>TCTCCAGCGTGCGCC</td>
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<tr>
<td>1736.3</td>
<td>(M-3H-G)&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>TCTCCAGCGTGCGCCA</td>
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**Table 5.4**: Assignment of fragment ions obtained from collision-induced dissociation of [M-3H]<sup>3</sup> of M1 at m/z 1786.8 in human plasma
Table 5.5: Structures and molecular weights of metabolites observed in mouse kidneys after a single dose of 5 mg/kg

<table>
<thead>
<tr>
<th>Identity</th>
<th>Sequence (from 5’ to 3’)</th>
<th>Calculated MWT (Da)</th>
<th>Observed MWT (Da)</th>
<th>Difference in mass (Da)</th>
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<tr>
<td>M1</td>
<td>TCT CCC AGC GTG CGC CA</td>
<td>5364.3</td>
<td>5363.0</td>
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<td>M2</td>
<td>TCT CCC AGC GTG CGC C</td>
<td>5035.1</td>
<td>5034.3</td>
<td>0.8</td>
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<td>M3</td>
<td>TCT CCC AGC GTG CGC</td>
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<td>4728.0</td>
<td>1.8</td>
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<td>M4</td>
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</tr>
<tr>
<td>M7</td>
<td>TCT CCC AGC GTG CGC CAT+ OH⁺</td>
<td>5668.6</td>
<td>5671.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>
### Table 5.6: N-in-one quantification of G3139 and three major metabolites in rat and human plasma using multiple reaction monitoring (MRM)

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM</th>
<th>Charge state of selected precursor ions and daughter ions</th>
<th>Normalized collision energy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3139</td>
<td>1893.7 → 1842.8</td>
<td>-3</td>
<td>15</td>
</tr>
<tr>
<td>3’N-1</td>
<td>1786.6 → 1736.3</td>
<td>-3</td>
<td>15</td>
</tr>
<tr>
<td>3’N-2</td>
<td>1676.7 → 1626.2</td>
<td>-3</td>
<td>15</td>
</tr>
<tr>
<td>3’N-3</td>
<td>1575.7 → 1524.6</td>
<td>-3</td>
<td>15</td>
</tr>
<tr>
<td>Internal standard</td>
<td>1985.4 → 1947.5</td>
<td>-4</td>
<td>18</td>
</tr>
<tr>
<td>I.D. →</td>
<td>G3139</td>
<td>N-1</td>
<td>N-2</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>0.24</td>
<td>0.25</td>
</tr>
<tr>
<td>%CV</td>
<td>13.0</td>
<td>13.9</td>
<td>15.1</td>
</tr>
<tr>
<td>Accuracy&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Mean</td>
<td>1.04</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>%CV</td>
<td>12.2</td>
<td>17.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Accuracy&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mean</td>
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<td>4.72</td>
<td>4.71</td>
</tr>
<tr>
<td>%CV</td>
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<td>3.5</td>
<td>6.2</td>
</tr>
<tr>
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<td>94</td>
<td>94</td>
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<tr>
<td>Mean</td>
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<td>11.0</td>
<td>10.6</td>
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<tr>
<td>%CV</td>
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<td>5.7</td>
<td>5.9</td>
</tr>
<tr>
<td>Accuracy&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98</td>
<td>110</td>
<td>106</td>
</tr>
</tbody>
</table>

<sup>a</sup> For G3139 only
NE: not evaluated. <sup>a</sup>. Expressed as [(mean observed concentration/nominal concentration) × 100]

**Table 5.7:** Within-day assay validation characteristics of G3139 and three metabolites in rat and human plasma by IR-RP-HPLC coupled with ESI/MS
## Table 5.8: Between-day assay validation characteristics of G3139 and three metabolites in rat and human plasma by IR-RP-HPLC coupled with ESI/MS

<table>
<thead>
<tr>
<th>I.D. →</th>
<th>µg/mL (nM*) (n=5)</th>
<th>Rat plasma</th>
<th>Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G319</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-1</td>
<td>N-2</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>%CV</td>
<td></td>
<td>23.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Accuracy</td>
<td></td>
<td>85</td>
<td>87</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>%CV</td>
<td></td>
<td>13.3</td>
<td>9.2</td>
</tr>
<tr>
<td>Accuracy</td>
<td></td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.78</td>
<td>4.83</td>
</tr>
<tr>
<td>%CV</td>
<td></td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>Accuracy</td>
<td></td>
<td>96</td>
<td>97</td>
</tr>
</tbody>
</table>

* For G3139 only
Figure 5.1: First generation backbone modification in antisense ODNs: replacement of PO linkages by PS linkage introduces a chiral center at each phosphate linkage.
Figure 5.2: ESI LC-MS mass spectrum (A) and corresponding deconvoluted mass spectrum (B) of G3139. The charge states (–3 to –8) of the multiply charged ions are indicated above each ion in (A). Isotopic ions are indicated in (B).
Figure 5.3: Nomenclature of fragment ions generated from CID spectra of oligonucleotides (adapted from ref 44).
Figure 5.4: Procedures for de novo sequencing of unknown metabolites of G3139 using SOS program.
Figure 5.5: CID spectrum of triply charged molecular ion of G3139. Upper panel: fragmentation schema of G3139 according to McLuckey et al (Ref# 31). Lower panel: the most abundant ion was isolated and subjected to collision-induced dissociation by applying 35% of normalized collision energy. Complete ion assignments are listed in Table 5.3. The ion assignment an-B\(^{-1}\) is equivalent to an-Bn\(^{-1}\) (Ref# 44).
Figure 5.6: A representative TIC of G3139 and major metabolites obtained from plasma extract of a patient treated with G3139 (A) and the corresponding mass spectra of 3’ N-1 metabolite at retention time (RT) 13.90 min (B), of 3’ N-2 metabolite at RT 12.04 min (C), of 3’N-3 metabolite at RT 10.7 min (D).
Figure 5.6 (continued): the corresponding mass spectra of 3’ N-4 metabolite at retention time (RT) 9.41 min (E), of 3’ N-5 metabolite at RT 7.7 min (F), of unknown metabolite elute after G3139 (G).
Figure 5.7: Mass spectrum and CID spectrum of triply charged molecular ion of metabolite M1 in human plasma. Upper panel: Full mass spectrum of M1. Ions $[\text{M}–3\text{H}]^3$ to $[\text{M}–8\text{H}]^8$ were observed. Inset: deconvoluted spectrum of M1. Lower panel: CID spectrum of $[\text{M}-3\text{H}]^3$ of M1. The most abundant ion was isolated and subjected to collision induced dissociation by applying 35% of normalized collision energy. Complete ion assignments are listed in Table 5.4.
Figure 5.8: Mass spectrum and CID spectrum of triply charged molecular ion of 5’N-1. Upper panel: Full mass spectrum of M1. Ions \([M-3H]^{-3}\) to \([M-8H]^{-8}\) were observed. Lower panel: CID spectrum of \([M-3H]^{-3}\) of M1. The most abundant ion was isolated and subjected to collision induced dissociation by applying 35 % of normalized collision energy.
Figure 5.9: A representative TIC of G3139 and major metabolites in 5 min plasma extract obtained from a rat given 20 mg/kg of G3139 (A) and the corresponding mass spectra of 3’ N-1 metabolite at RT=13.5 min (B), of 3’ N-2 metabolite at RT 11.5 min (C), of 3’N-3 metabolite at RT 10.4 min (D).
Figure 5.10: TIC and Selected ion chromatograms of G3139 and its major metabolites in rat urine. Upper panel: TIC of G3139 and major metabolites in rat urine. Lower panel: ion current from the following m/z was monitored: G3139: 1983; 3’ N-1: 1786.5; 3’N-2: 1677; 3’N-3: 1575; 3’N-4: 1473. Isolation width was 3.0 Da.
**Figure 5.11**: Ion-pair RP-HPLC/MS chromatograms of control mouse kidneys. (A) HPLC chromatogram detected at 260 nm; (B) TIC MS chromatograms of kidney extract.
Figure 5.12: Representative ion-pair RP-HPLC/MS chromatograms of G3139 and its major metabolites in mouse kidneys. (A) HPLC chromatogram of G3139 and its major metabolites detected at 260 nm; (B) TIC MS chromatograms of corresponding ODNs.
Figure 5.13: Mass spectra of 3’N-2 (A), N-3 metabolite (B), N-4 (C) and N-5 (D) in mouse kidneys. Label above each major ions were charge states of each ion. Ions of [M–3H]⁻³ to [M–7H]⁻⁷ were observed.
Figure 5.13: Continued

C

D

Relative Abundance

m/z

Relative Abundance

m/z

(M-3H)^-3

(M-4H)^-4

(M-5H)^-5

(M-6H)^-6

883.74

737.37

1473.43

1399.37

1105.05

1160.12

1819.28

1194.79

1812.71

1941.79

600 800 1000 1200 1400 1600 1800 2000

600 800 1000 1200 1400 1600 1800 2000

651.31

651.31

994.98

1357.07

1590.32

1359.01

1018.57

1399.37

1590.32

1941.79

1812.71

1560.12

1810.28

1810.28

1812.71

1819.28
Figure 5.14: Extracted ion chromatograms of G3139 and its major metabolites in mouse kidneys. Ion current from the following m/z values monitored were: G3139, 1983; 3’N-1, 1786.5; 3’N-2, 1677; 3’N-3, 1575; 3’N-4: 1473; 3’N-5: 1358.6. The charge state for all species was [M-3H]⁻³. The isolation width was 3.0 Da.
**Figure 5.15**: Full scan mass spectra of an oxidation product of G3139 (A) and 3’N-1 (B) in mouse kidneys.
Figure 5.16: Representative ion-pair RP-HPLC-UV (A) and LC-MS total ion (TIC) chromatograms (B) of G3139, 3’ N-1, 3’ N-2 and 3’ N-3 of G3139. UV was set at 260 nm.
Figure 5.17: CID spectra of the triply charged molecular ions of G3139 (A), of 3’N-2 (B), of 3’N-3 (C) and of the internal standard (D). Ions at m/z 1676.6, m/z 1575.7 and m/z 1985.4 represent their parent [M-3H]⁻³ ions, respectively. CID spectra of triply charged ions of 3’N-1 are presented in Figure 5.7.
Figure 5.18: A representative TIC in rat plasma spiked with 1 µg/mL each of G3139 and three major metabolites and 20 µg/mL internal standard (A) and its corresponding MRM chromatograms (B).
Figure 5.19: MRM chromatograms in drug-free human (A) and rat (B) plasma spiked with the internal standard (IS). The location of G3139 and metabolites are indicated by the arrows and the spikes are amplified electronic noise.
Figure 5.20: Representative LC/ESI MRM chromatograms in extracts from human (A) and rat (B) plasma spiked with 0.1 µg/mL each of G3139 and three major metabolites and 20 µg/mL IS.
Figure 5.21: N-in-one calibration curves of G3139 (A), 3’ N-1 (B), 3’ N-2 (C), and 3’N-3 (D) metabolites in rat plasma.
Figure 5.22: N-in-one calibration curves of G3139 (A), 3’ N-1 (B), 3’ N-2 (C), and 3’N-3 (D) metabolites in human plasma.
Figure 5.23: Representative LC/UV coupled with ESI/MS/MS chromatograms in blank rat urine. (A) LC/UV chromatogram of drug-free rat urine extract spiked with internal standard. The location of G3139 and metabolites are indicated by the arrows and the spikes are amplified electronic noise. (B) MRM chromatograms in blank rat urine extract.
Figure 5.24: N-in-one Calibration curves of G3139 (A), 3’N-1 (B), 3’N-2 (C), and 3’N-3 (D) metabolites in rat urine.


CHAPTER 6

PHARMACOKINETICS OF G3139 IN THE MOUSE AND RAT, AND TISSUE DISPOSITION STUDY IN THE MOUSE

6.1 Introduction

Pharmacokinetic studies of phosphorothioate have been conducted in various species, including mice (1), rats (2) and monkeys (3), as well as in humans (4). In general, phosphorothioate oligonucleotides distribute rapidly into organ tissue with initial disappearance half-life less then 0.5 hr. Plasma concentration time profile exhibits multi-exponential decay with a terminal elimination half-life of 5 to 70 hr, depending on the bioanalytical methods used (5-8). Longer plasma terminal elimination phase was found using radiolabeled method but it probably did not truly represent the half-life of the parent drug since the method cannot separate the parent drugs from its metabolites. After repeated dosing at 20 mg/kg in mice and monkeys (3), nonlinear kinetics were observed as evidenced by greater than proportional increase in AUC and decrease in clearance. It is critical to evaluate tissue clearance since plasma clearance is not a determinant of overall disposition. Studies have shown that tissue clearance of antisense drugs is slow. Tissue exposure was dose-dependent but not linear relationship. Urinary excretion seems not playing important role in plasma clearance. It is also species independent in rat, dog,
monkey and human (9, 10). However, only limited data are available for G3139 regarding disposition and elimination in animals. Previous published results were based on radioactivity measurements, which generated little information on parent and metabolite disposition of G3139 (11, 12). In addition, similarity and difference in G3139 pharmacokinetics among different species was not addressed in previous studies. The objective of this study was to compare the differences of G3139 in the mouse and rat, and to fully characterize the tissue disposition in the mouse. The understanding of disposition property of G3139 among species may provide a powerful tool to scale up pharmacokinetic parameters to humans, thus many questions unanswered might be solved by an animal model. We also proposed a metabolite kinetic model to obtain the relationship between parent and metabolites and used this model to predict steady state concentration of parent drug and metabolites under infusion administration. Finally, preliminary pharmacokinetics of targeted liposome formulation of G3139 was reported.

6.2 Materials and Methods

6.2.1 Drugs and Chemicals

G3139 was supplied by the National Cancer Institute (Bethesda, MD) and used without further purification. The internal standard, 25mer, (the sequence is 5’-TCTCCC AGCGTGCGCCTCACAGCATA-3’, was obtained from Integrated DNA Technologies (Coralville, IA) and used without further purification. HPLC-grade methanol, triethylamine (TEA, 99.5%) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.8%) were purchased from Aldrich (Milwaukee, WI, USA). A Milli-Q system (Millipore, Bedford, MA, USA) used to prepare deionized water for HPLC. Other phosphorothioate ODNs
including 3’ N-1, N-2, and N-3 were purchased from Integrated DNA technologies (Coralville, Iowa). The purity and sequence of each ODN was examined by capillary gel electrophoresis (CGE) and was found to be consistent with the expected elution sequences of N-1, N-2 and N-3 oligomers relative to G3139. The purity and identity of each ODN was also verified by HPLC-UV-Mass spectrometry (Finnigan LCQ, San Jose, CA). Blank human plasma was obtained from the American Red Cross (Columbus, OH). Egg-PC (egg phosphatidylcholine) and PEG-DSPE2000 (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000) were purchased from Avanti Polar Lipids (Alabaster, AL). DC-Chol (3b-[N-(N’,N’-dimethylaminoethane-carbamol]cholesterol) was synthesized as reported previously (13). Folate-PEG-DSPE was synthesized as reported previously (14).

6.2.2 Animal studies

**Plasma PK and tissue disposition in the mouse.** A total of 60 CD1F2 mice (Harlan, Indianapolis, Indiana) with an average body weight of 22 ± 5 g (SD) were used in this study. The study was performed with a protocol adhered to the “Principles of Laboratory Animal Care” by NIH and approved by The Ohio State University Vivaria. G3139 in sterile saline was injected into the tail vein at a single bolus dose of 5 mg/kg. About 0.2-0.3 ml of blood samples each was collected at selected time points of 5, 15, 30 min, 1 hr, 2 hr, 6,10, 24, 48 and 72 hr post dose (6 animals/time point). The animals were euthanized in CO₂ atmosphere and blood samples were removed by cardiac puncture through thoracic opening. The plasma was obtained by centrifugation at 500 g at 4°C for 10 min and stored at -80°C until analysis. The following tissue samples were removed
through thoracic opening: Liver, kidneys, spleen, heart, lung and bone marrow. In a separate experiment, 4 mice were housed together in a metabolism cage that allowed for separate collection of urine and feces. Urine samples were collected 12 hr prior to dosing as blank and at interval of 0-24, 24-72 hours after dosing.

**Plasma pharmacokinetics and urinary excretion in the rat.** Female Sprague Dawley rats weighing approximately 340-380 g were purchased from Harlan (Indianapolis, IN). Five rats were implanted with catheters in the right jugular vein and serial blood samples (0.2 mL each) were taken at the following time points: 5, 10, 15, 30, 60 min and 2, 4, 6, 10 and 24 hr after dosing. The plasma was obtained by centrifugation at 5000 g at 4°C for 5 min and stored at -80°C until analysis. Plasma samples were frozen and stored at -80°C until analyzed. In a separate study, G3139 in sterile saline was injected into the tail vein at a single bolus dose of 20 mg/kg. Three rats were housed individually in metabolism cages that allowed for separate collection of urine and feces, and restricted to food only in the first 24 hr after dosing. Urine samples were collected 24 hr prior to dosing and at interval of 0-4, 4-8, 8-12 and 12-24 hours after dosing. The total volume of urine was measured by weight difference and frozen at -80°C until analysis.

**Pharmacokinetics of Liposomal G3139 in the mouse** a total of 16 CD1F2 mice (Harlan, Indianapolis, Indiana) with an average body weight of 22 ± 3 g (SD) were used in this study. Liposomal G3139 in sterile saline was injected into the tail vein at a single bolus dose of 5 mg/kg. About 0.5 ml of blood samples per time point was collected at selected time points of 5, 30 min, 1 hr, 2 hr, 4, 6, 24, 48 and 72 hr post dose (2 mice/ time point). The animals were euthanized in CO₂ atmosphere and blood samples were
removed by cardiac puncture through thoracic opening. The plasma was obtained by centrifugation at 500 g at 4°C for 10 min and stored at -80°C until analysis.

6.2.3 Plasma, urine and tissue sample extraction

Plasma and urine samples were thawed and centrifuged at 1000g for 5 min. Solid phase extraction (SPE) was used for isolation of G3139 and metabolites from plasma/urine as described in Chapter 5. Rat plasma or urine samples spiked with appropriate amounts of pure compounds were used to construct calibration curves for G3139 and three major metabolites. Plasma sample 0.2 mL was then spiked with 20 µL of the internal standard at 400 µg/mL in water. Thereafter, the samples and standards were mixed with 0.8 mL of 0.1 M TEAB buffer and extracted with Oasis HLB cartridge packed with 30 mg sorbent. The subsequent sample preparation procedure for plasma/urine samples analysis was essentially the same as that described in Chapter 5. G3139 levels in urine samples collected in mice were quantified by the ELISA assay as described in Chapter 2. Tissue samples were extracted as described in Chapter 5. Briefly, approximately 50 mg tissue samples were homogenized in 10:1 PBS with a tissue homogenizer (VIRTIS, Gardiner, NY). The homogenate was centrifuged at 3000 g for 5 min, and supernatant was separated. To the supernatant was added 2 mg/ml of proteinase K in buffer containing of Tris-HCl (pH, 8.0), 10 mM EDTA to digest tissue components (Sigma, WI). Samples were then incubated for 2 hrs at 37°C. A 0.5 ml aliquot of the supernatant was transferred into a new tube and extracted with 2 ml phenol-chloroform-isoamylalcohol (25:24:1, pH, 8.0, Ambion, TX) to remove proteins and lipids. The phenol-chloroform layer was back-extracted with 0.5 ml of distilled H2O, and the
aqueous phases were pooled. Samples were then subjected to assay by the ELISA method as described in Chapter 2.

6.2.4 N-in-one quantification of G3139 and major metabolites in the rat

N-in-one quantification of G3139 and major metabolites in rat plasma was performed with a novel ion-pair reverse phase (IR-RP) LC-MS/MS assay as described in Chapter 5. Selected reaction monitoring (SRM) mode was chosen for monitoring the daughter ions. The area ratio of each analyte from SRM chromatogram to that of internal standard (25mer) was obtained and plotted against the nominal concentration to generate the calibration curves. Eight points calibration curves were prepared for G3139, 3’ N-1, 3’ N-2 and 3’ N-3 using weighed least-squares linear regression analysis and used for calculation of unknown samples.

6.2.5 in vitro plasma protein binding of G3139

Plasma protein binding study of G3139 was carried out in rat plasma and human plasma. G3139 was incubated in rat or human plasma at 37°C for 20 min. A 300 µl aliquot was and placed onto an Amicon Centrifree micropartition device (molecular weight cut off of 30,000 Daltons, Millipore Corp., Bedford, MA). Centrifugation was carried out at 1500 rpm for 15 min at 37°C to obtain 100-300 µL of ultrafiltrate. The G3139 levels in the ultrafiltrate were measured by the validated ELISA method (see Chapter 2) after appropriate dilution by sample dilution buffer containing 0.2% Triton X-100. The percentage of unbound drug at each time point was obtained by dividing the
G3139 concentrations in the filtrate by those in the corresponding plasma samples and the percentage of bound drug was calculated by the following Equation 6.1:

\[
\text{Bound}\% = 1 - \frac{[\text{free G3139}]}{[\text{plasma G3139}]} \times 100\%
\]

[Equation 6.1]

Evaluation of non-specific absorption on filter was performed first and 0.2-0.3 % binding was found, which was taken into consideration in the final calculation of fraction bound.

6.2.6 Preparation of liposomal G3139 formulation

The liposome-entrapped G3139 was kindly provided by Sharon Chiu and Dr. Robert Lee, College of Pharmacy, The Ohio State University. Briefly, the non-targeted liposomal formulation was composed of egg-phosphatidylcholine (PC), dimethylaminoethane carbamoyl cholesterol (DC-Chol), and polyethylene glycol-distearoylphophatidylehtanolamine (PEG-DSPE) 2000 at molar ratio of 25:65:10. For folate-targeted liposomal formulations, 0.5 mol % of folate-PEG-DSPE was incorporated as part of the lipid composition. Lipid stock solutions were prepared in 100% ethanol and oligonucleotides were dissolved in 20mM citrate buffer (pH 4) before use. After mixing lipid components at predetermined ratios, the lipids were slowly added to oligonucleotide-containing citrate buffer (pH 4) under rapid mixing. The mixture was then passed 5 times through 200 nm polycarbonate filters at room temperature. The liposomes were dialyzed against 20 mM citrate buffer (pH4) for one hour to remove excess ethanol and then further dialyzed against HEPES buffered saline (HBS, 20 mM HEPES, 145 mM NaCl, pH 7.5) for 24 hours to remove citrate buffer, neutralize the
surface charge of DC-Chol, and, more importantly, release oligonucleotides that were associated on the surface of liposomes. Samples were stored at 4°C for future use. Encapsulation efficiencies were calculated based on the ratio of the initial and final oligonucleotide concentration, multiplied by 100. Encapsulation efficiencies were found to be approximately 70% for both non-targeted and folate-targeted formulations. The mean diameter and particle size distribution were determined by photon correlation spectroscopy using a NICOMP Particle Sizer (Model 370, CA). All liposomes have particle size distribution around 100 nm.

6.2.7 Metabolite kinetic model for parent and three major metabolites

To fully understand metabolite kinetics, a five-compartment model was proposed and depicted in Figure 6.11. The model is described by the following differential equations based on mass balance:

\[
\frac{dC_1}{dt} = \left( In - CLm \times C_1 - CL_{d} \times C_1 + CL_{d} \times C_2 \right) / V_1 \quad \text{[Equation 6.2]}
\]

\[
\frac{dC_2}{dt} = \left( CL_{d} \times C_1 - CL_{d} \times C_2 \right) / V_2 \quad \text{[Equation 6.3]}
\]

\[
\frac{dC_3}{dt} = \left( CLm \times C_1 - CLm_1 \times C_3 \right) / V_3 \quad \text{[Equation 6.4]}
\]

\[
\frac{dC_4}{dt} = \left( CLm_1 \times C_3 - CLm_2 \times C_4 \right) / V_4 \quad \text{[Equation 6.5]}
\]

\[
\frac{dC_5}{dt} = \left( CLm_2 \times C_4 - CLm_3 \times C_5 \right) / V_5 \quad \text{[Equation 6.6]}
\]

where:
In= input function of parent drug. In this case it is i.v. bolus

\[ C_1 = \text{G3139 concentration in the central compartment} \]

\[ C_2 = \text{G3139 concentration in the tissue compartment} \]

\[ C_3 = 3' \text{N-1 concentration in plasma} \]

\[ C_4 = 3' \text{N-2 concentration in plasma} \]

\[ C_5 = 3' \text{N-3 concentration in plasma} \]

\[ V_1 = \text{volume of the central compartment of G3139} \]

\[ V_2 = \text{volume of the tissue compartment of G3139} \]

\[ V_3 = \text{volume of } 3' \text{N-1 compartment} \]

\[ V_4 = \text{volume of } 3' \text{N-2 compartment} \]

\[ V_5 = \text{volume of } 3' \text{N-3 compartment} \]

\[ \text{In= input function of parent drug. In this case it is i.v. bolus} \]

\[ \text{CL}_m = \text{parent drug elimination clearance (metabolite 3’N-1 formation clearance)} \]

\[ \text{CL}_d = \text{parent drug distribution clearance} \]

\[ \text{CL}_{m1} = 3’ \text{N-1 elimination clearance} \]

\[ \text{CL}_{m2} = 3’ \text{N-2 elimination clearance} \]

\[ \text{CL}_{m3} = 3’ \text{N-3 elimination clearance} \]

\[ \text{V}_3 = \text{volume of } 3' \text{N-1 compartment} \]

The model is parameterized as clearance and rate constants are defined as secondary parameters:

\[ k_{12} = \frac{\text{CL}_d}{V_1} \]

\[ k_{13} = \frac{\text{CL}_m}{V_1} \]

\[ k_{21} = \frac{\text{CL}_d}{V_2} \]

\[ k_{34} = \frac{\text{CL}_{m1}}{V_3} \]

\[ k_{45} = \frac{\text{CL}_{m2}}{V_4} \]

\[ k_{50} = \frac{\text{CL}_{m3}}{V_5} \]

The nonlinear regression was performed with differential equations implemented in WinNonlin program. The computer program for the model is listed in Appendix C.
The proposed model was fitted to the geometric mean plasma concentration-time profiles of G3139 and three metabolites simultaneously. Distribution of residuals, F-test and Akaike information criterion (AIC) were used as statistical means to justify the model selection and some of model assumption. Following the model selection, each metabolite kinetic profile in the rat was individually fitted with this model. A weighting factor of $1/y^2$ or $1/y$ ($y$ is the observed concentration) and maximum likelihood algorithm (15) were compared and the best fitting justified the one used for the final estimation.

6.2.8 Data Analysis

The plasma concentration-time data were analyzed by WinNonlin (version 4.0, Pharsight Corporation, Mountain View, CA) via an appropriate pharmacokinetic model and relevant pharmacokinetic parameters of G3139 were obtained. For metabolites, noncompartmental analysis was used. Total body clearance, mean residence time (MRT), steady-state volume of distribution (Vss), and apparent renal clearance were calculated as follows:

$$CL_T = \frac{Dose}{AUC_{\infty}} \quad [\text{Equation 6.7}]$$

$$MRT = \frac{AUMC}{AUC} \quad [\text{Equation 6.8}]$$

$$V_{ss} = MRT \times CL_T \quad [\text{Equation 6.9}]$$

$$Cl_R = \frac{X_u(t)|_{t_2}^{t_1}}{AUC_{t_2}^{t_1}} = \frac{X_{u,0-\infty}}{AUC_{0-\infty}} \quad [\text{Equation 6.10}]$$
where AUC and AUMC are the area under the concentration-time curve and area under the first moment curve. \(Xu(t)\) is the amount of drug or metabolites excreted in urine within a period of \(t_1 - t_2\). \(AUC_{t_1}^{t_2}\) is the area under the plasma drug (or metabolite) curve of same period. The mean value and standard deviation of each parameter in each study group were calculated using Microsoft Excel.

6.2.9 Allometric scaling of G3139

The pharmacokinetics of G3139 was investigated in four species, mouse, rat, monkey, and human by compartmental analysis. The pharmacokinetic data in monkey was kindly provided by Dr. Zuyu Guo, of Sanofi-Aventis (Bridgewater, NJ). Allometric scaling was applied to \(V_{ss}/F\) and \(Cl\). Body weight model was employed:

\[
CL = a \times BW^b
\]  
\[
V_{ss} = c \times BW^d
\]

where \(a\) and \(c\) are coefficients and \(b\) and \(d\) are exponential terms in the allometric scaling equation. Prediction of human total body clearance was based on a 70 kg human.

6.3 Results

6.3.1 Plasma pharmacokinetics and tissue distribution of G3139 in CD2F1 mice

The mean plasma concentration-time profile of G3139 in mice given 5 mg/kg of G3139 showed a tri-exponential decline (Figure 6.1 and Table 6.1). The concentration-time data were therefore fitted to a three-compartment i.v. bolus model (Figure 6.2). The relevant pharmacokinetic parameters as estimated by WinNonlin computer software are
summarized in Table 6.2. The initial half-lives \( t_{1/2\alpha} \) was 10.2 min, the intermediate half-life \( t_{1/2\beta} \) was 2.7 hrs and the terminal half-life \( t_{1/2\gamma} \) was 17.2 hrs. The area under the concentration-time curve \( (\text{AUC}_{0-\infty}) \) was 14.7 \( \mu \text{g*hr/ml} \) (2.43 \( \mu \text{M} \)). The total body clearance \( (\text{CL}) \) value was 5.67 ml/min/kg. The steady state volumes of distribution \( (V_{ss}) \) was 2.85 mL/g, which was approximately 4-fold greater than the total body water of the mouse (14.5 ml for 20 g mouse or 0.73 ml/g) (16). Figure 6.1 shows a computer fitted curve of concentration-time profile of G3139 PK in mice. The percentage of the administered dose remaining in the blood after 24 hr was 10%.

Tissue distribution of G3139 in mouse was investigated at three time points: 1, 6 and 24 hr after dosing. The kidneys accumulated the highest concentration of G3139, 20.8 \( \pm \) 4.57 \( \mu \text{g/mL} \) at 1 hr, 29.8 \( \pm \) 4.79 \( \mu \text{g/mL} \) at 6 hr and 13.62 \( \pm \) 2.23 \( \mu \text{g/mL} \) at 24 hr after i.v. bolus of G3139 of 5 mg/kg (Table 6.3 and Figure 6.3). The liver attained the second highest concentration of G3139 as 16.8 \( \pm \) 1.79 \( \mu \text{g/mL} \) at 1 hr, 17.5 \( \pm \) 2.76 \( \mu \text{g/mL} \) at 6 hr and 9.93 \( \pm \) 1.10 \( \mu \text{g/mL} \) at 24 hr after iv bolus at 5 mg/kg G3139. Other organ tissues attained lower concentrations of G3139. However, tissue to plasma AUC ratios at 24 hr in all organs except heart exceeds 1 (Table 6.4), suggesting tissue distribution of G3139 is extensive and is a major elimination pathway of drug in circulation. Since liver has the highest mass in the mouse, liver was considered the major organ of disposition of G3139 rather than kidneys, although kidneys have a significant recovery of G3139 (Figure 6.4). It was worth noting that significant concentrations of G3139 (2.94 \( \pm \) 1.28 \( \mu \text{g/mL} \)) was also observed in bone marrow, which is a drug effect compartment for AML. That concentration decreased to 0.42 \( \pm \) 0.26 \( \mu \text{g/mL} \) 24 hr following drug
treatment. Twenty four hr cumulative urine was collected and quantified by the ELISA assay for parent drug (see Chapter 2 for the ELISA method). Dose recovered as G3139 in urine was found to be 1.65 ± 0.5 % of total administered dose. Total dose recovery as parent drug from all organ tissues including plasma was 45.4 %, 46.4% and 26.3 % at 1 hr, 6 hr and 24 hr, respectively (Fig. 6.4).

6.3.2 Plasma pharmacokinetics of liposomal G3139 in mice

The colloidal stability of liposomal G3139 formulation was evaluated by measuring the particle size change of both formulations up to 28 days. No significant particle size change was observed during this period for both formulations (Figure 6.5). A preliminary pharmacokinetic study was performed with G3139 encapsulated in a novel liposome formulation. As in Figure 6.6, plasma concentration-time profiles of G3139 in saline and the non-targeted PEGlyated liposomal formulation following single bolus administration at 5mg/kg dose have distinct characteristics. Total body clearance of G3139 was significantly prolonged (almost 10 fold decrease in clearance) for the liposome formulation as compared with that of free G3139 (Table 6.2 and Table 6.5). G3139 in the liposome formulation exhibited a bi-exponential decay over 72 hrs with t1/2α of 12.6 min and t1/2β of 576 min (Figure 6.6 and Table 6.5). The steady-state volume of distribution (Vss) was greatly decreased to about 13 fold as compared with G3139 in saline. Observed Vss of 8.5 ml is greater than blood volume of mouse but less than the total body water of the mouse, suggesting that the formulated G3139 was confined primarily in blood and well perfused organs such as liver and kidneys with less extent of distribution to other organ tissues compared to G3139 in saline. The plasma G3139
concentrations decreased slowly to approximately 0.38 µg/mL at 48 hrs after dosing. This concentration was approximately 10 fold higher than that in saline formulations (Table 6.1).

6.3.3 Plasma pharmacokinetics of G3139 and three major metabolites in rat

A novel ion-pair reverse-phase ESI HPLC-MS method has been developed for quantification of major metabolites of G3139 in vivo (Chapter 5). This method was then utilized for the quantification of G3139 and three major metabolites in the rat given a single bolus of G3139 at 20 mg/kg. Representative ESI LC-MS/MS chromatograms of G3139 and three metabolites in rat plasma at 5, 15, 30, 60 min and 2, 4, 6 hr are shown in Figures 6.7 A through G, respectively. The plasma concentration-time profiles of parent drug and three metabolites are shown in Figure 6.8 and Table 6.6. As shown, plasma G3139 pharmacokinetics follow a bi-exponential decay following its single bolus administration at 20 mg/kg in rat. The plasma concentration-time data of the parent drug was fitted to a two compartment open model with bolus input and first order elimination from the central compartment (Figure 6.9). Two representative semi-logarithmic plots are shown in Figure 6.10. Table 6.7 summarizes the relevant pharmacokinetic parameters of G3139 as estimated with this model. As shown, the mean total body clearance of G3139 was 3.74±1.70 (SD, n=5) ml/min/kg and the harmonic mean half-lives were 13 (ranges, 5-34 min) and 68.5 (ranges, 49-101 min) min for α and β phases, respectively. The estimated Vss was 217 ml/kg and V₁ was 103 ml/kg, which is greater than the total blood volume but less than total body water of the rat (16), suggesting that G3139 distributed extensively in well-perfused organs such as liver and kidneys.
After 6 hr post dose, plasma G3139 and metabolites levels were below the LOQs of the assay. Levels of 3’ N-1 reached the $C_{\text{max}}$ of 28.9 ± 5.04 µg/mL at about 11 min, and the other two metabolites also achieved their $C_{\text{max}}$ values of 13.76 and 12.0 µg/mL, respectively (Figure 6.8 and Table 6.5). Following their peak levels, plasma levels of the metabolites declined essentially in parallel to that of G3139. The relevant pharmacokinetic parameters of 3’ N-1, N-2 and N-3 are summarized in Table 6.8. Using noncompartmental analysis, the apparent harmonic mean terminal half-lives were 63.6, 68.6 and 69.8 min for N-1, N-2 and N-3, respectively, suggesting formation-limited behaviors of the metabolites. Mean %AUCm/AUCp were 25, 11.7 and 9.88 % for N-1, N-2 and N-3, respectively, indicating that the relative amounts of the metabolites, the 3’N-1 being the major metabolite. Together, the metabolite levels represent nearly 50% of the total drug in circulation. Estimated apparent renal clearance values of metabolite were 0.09, 0.28 and 0.227 ml/min/kg for N-1, N-2 and N-3, respectively, indicating a low urinary excretion (GFR of rat is 5.2 ml/min/kg, (16)).

6.3.4 Plasma protein binding

G3139 was highly protein bound in human and rat plasma (Table 6.9). The fractions bound of G3139 were found to be from 99.01 to 95.91 in human plasma and from 96.1 to 97.3 in rat plasma over a concentration range of 2 to 100 µg/mL.

6.3.5 Urinary excretion of G3139 and major metabolites in the rat

Following i.v. dose of G3139 at 20 mg/kg to the rat, it appears urinary excretion of G3139 was found to be only a minor pathway for elimination of G3139 in rat (Table 6.10). The parent drug was found to be the most abundant species in urine with 161 ± 12
(SD) µg excreted within the first 4 hr and 4.87 ± 1.76 (SD) µg from 4 to 8 hrs. There was no detectable amount of G3139 after 8 hr. Smaller amounts of 3’ N-1, 3’ N-2 and 3’ N-3 were detected within 4 hrs after dosing with 58.7 ± 4.87 (SD), 84.5 ± 31.3 (SD), and 46.6 ± 6 (SD) excreted, respectively (Table 6.10). No measurable metabolites were found after 8 hr. The percent of administered dose recovered as the parent drug was 2.78 % in 24 hr and 1.0, 1.4 and 0.82 % as 3’ N-1, 3’ N-2 and 3’ N-3 metabolite, respectively. The percent of administered dose recovered as oligonucleotides (G3139 + 3’N-1 + N-2 + N-3) within 24 hr was only 6 % in rat urine.

6.3.6 Metabolic kinetic model of G3139 in the rat

A metabolite kinetic model for G3139 (Model 3) was proposed as shown in Figure 6.11. G3139 follows a two-compartment open model. G3139 undergoes a first-order elimination or metabolite formation (K_{13} and CL_m) from the central compartment. Sequential metabolite formation was also proposed for 3’ N-2 and 3’ N-3 with first order rate constants of k_{34}, k_{45}. 3’ N-3 could be metabolized further to other metabolites or eliminated through other mechanism. Owing to the lack of data for metabolite given as an iv bolus, it was assumed that the entire amount of G3139 is available for metabolism to 3’ N-1 (fm=1 and CL_r=0). This assumption is reasonable, since urinary recovery of G3139 is low (<3%). Other assumptions of this model include: 1) Only sequential metabolism exists for each of compound in the model, i.e. there is insignificant urinary excretion of 3’ N-1, N-2 and N-3. This is justifiable, since urinary excretion of each metabolite was below 1% of total dose; 2) All metabolites distribute in a well-perfused compartment, since different volume terms for metabolites (V_3, V_4 and V_5) are not
identifiable individually but can be estimated globally ($V_3=V_4=V_5$). Thus a single volume term simplifies the nonlinear regression and accuracy of final estimates of each parameter; 3) Other metabolism does not significantly contribute to the elimination of parent drug. This has been verified in Chapter 5; 4) Only free fraction of each compound is subject to 3’-exonuclease, therefore enzymatic degradation occurs in tissues. During the model selection, it was found that addition of two peripheral compartments to 3’ N-1, N-2 as proposed in Model 4 (Figure 6.12) did not improve significantly to the final fitting and parameter estimations. Therefore the final model was proposed as shown in Model 3 (Figure 6.11).

Representative semi-logarithmic plots of the fitted G3139, 3’ N-1, N-2 and N-3 concentration-time profiles are shown in Figure 6.13. Relevant pharmacokinetic parameters of Model 3 as estimated by maximal likelihood estimation, are summarized in Table 6.11. The inter-compartment clearance values were 3.60 (SD=1.77), 3.94 (SD=1.10), 16.34 (SD=5.24), 37.42 (SD=13.10), 39.10 (SD=13.10) for parent drug distribution CL, metabolite formation CL, 3’ N-2 formation CL, 3’ N-3 formation CL and elimination CL of 3’ N-3, respectively (Table 6.11). The estimated clearance terms indicated the most rapid kinetic step was 3’ N-3 elimination from body and the slowest rate step or rate-limiting step was metabolite formation which was close to tissue distribution of parent drugs. Metabolite formation ($k_{13}V_1$) contributes approximately half of total clearance of parent drug with the half of drug going to the deep tissue compartment. The estimated volume of distribution of 3’ N-1 compartment was 62.4 ml/kg which was smaller than that of central compartment of parent drug (96 ml/kg). In
contrast, most small molecular weight drugs generate metabolites with higher volume of distribution since metabolites may be more polar than parent drugs and thus distribute more extensive in the body (17). Deep tissue compartment ($V_2$) has a larger volume than central compartment ($V_1$) for parent drug. The first order rate constants show the following rankings from highest to lowest: $k_{50} > k_{45} > k_{34} > k_{13} = k_{12} = k_{21}$ ($k_{13}$, $k_{12}$ and $k_{21}$ are essentially the same). This also confirms our observation that the rate-limiting step is the formation of 3’ N-1.

6.3.7 Simulation of plasma pharmacokinetics in rats

Model 3 (Figure 6.11) was used for the simulation using WinNonlin program. A 6 hr infusion at 20 mg/kg was given to rats and resulting pharmacokinetics of G3139 in central and deep tissue compartment was simulated using the final estimates of parameters in model 3 (Table 6.11). As shown in Figure 6.14, 6 hr infusion of 20 mg/kg resulted in a steady state concentration ($C_{ss}$) of G3139=13.8 µg/mL, $C_{ss}$ of 3’N-1= 3.3 µg/mL, $C_{ss}$ of N-2=1.46 µg/mL, and $C_{ss}$ of 3’N-3=1.21 µg/mL. Therefore, the percentage of metabolites relative to parent drug was 24.1%, 10.6% and 8.8% for 3’ N-1, N-2 and N-3, respectively. The G3139 levels in deep tissue compartment increased slowly and eliminated slower than that in the central compartment.

6.3.8 Allometric scaling of G3139

The pharmacokinetics of G3139 were investigated after i.v. administration in mice, rats, and cynomolgus monkeys. The allometric scaling results are shown in Figure 6.15. The correlation coefficient for this simple allometric model was 0.997, suggesting
good correlation of CL and BW. Based on this model, a CL value of 84 ml/min or 1.2 ml/min/kg in human was predicted, using a 70 kg human bodyweight. The predicted value is only 6% of hepatic blood flow in human and is rather close to observed value (observed CL=110 ± 61 ml/min). Other allometric scaling models incorporating brain weight, maximum life span might provide better predication and precision. Further investigation is warranted.

6.4 Discussion

As necessary steps for drug development, pharmacokinetic and toxicological studies of phosphorothioate oligonucleotides are critical for its evaluation. Since pharmacokinetics of G3139 in different species have not been fully characterized, herein we studied the stability, tissue disposition and excretion, and metabolite kinetics of G3139 in mouse and rat. Following i.v. administration in mice, we have found that G3139 was rapidly cleared from the circulation with a relatively high plasma clearance (5.67 ml/min/kg), which is consistent with results reported for G3139 and many other oligonucleotides in the mouse (11, 18). Compared to humans, plasma clearance of antisense ODN in the mouse is generally faster and ranged from 5.50 ~ 7.32 ml/min/kg (18). The terminal elimination half-life ($t_{1/2}$) was found to be 17 hrs, suggesting G3139 was slowly eliminated from blood in mouse during a period of 72 hrs. The low concentration of G3139 at 72 hr (0.01 µg/ml or 1.75 nM) is not measurable by conventional methods such as HPLC or CGE/UV (19, 20). The plasma clearance of G3139 was not largely affected by the terminal phase (24 hr to 72 hr) because the first 24 hr AUC$_{0-24}$ accounts for about 90% of total AUC$_{0-\infty}$. 
Following administration of G3139 to the mouse, there was a wide distribution of parent drug in various organ tissues despite its high plasma protein binding. This suggests that the binding affinity of G3139 to tissues is greater than to plasma proteins (21). The tissue drug uptake, especially liver and kidneys, appears to be the predominant factor in plasma clearance of G3139, although metabolism by nuclease in circulation may also play a role. The tissue distribution data presented have an important implication for the evaluation of G3139 in vivo in leukemia patients. The ability of G3139 to rapidly achieve substantial concentration in the bone marrow and remain relatively high at 24 hrs after i.v. dosing implies that G3139 has good access to tumor cells and might maintain relatively long duration of exposure if the drug is given as constant infusion.

We found that in 24 hr urine, 2.78% of the administered dose was recovered as the intact drugs in rats and 1.65% in mice. Other known metabolites only accounts for 3.22% of total dose in urine in the rats. These results differed from a previous study using radiolabeled G3139, which found 33% of total radioactivity recovered in the urine in mice (11). The discrepancy may be explained by different methodologies used. In that study, the $^{35}$S was incorporated into G3139 at the 5' end at 17-18 internucleotide linkages. This presented two potential issues. First, single base deletion from the 5'-end by 5'-exonuclease would liberate deoxythymidine with $^{35}$S label that is likely to be excreted in urine due to its low molecular weight. Thus, the amount of radioactivity recovered in urine could be quite high. But it is not real reflective of urinary excretion of parent drug. Second, multiple base deletion from the 3'-end by 3'-exonuclease would generate different 3' metabolites but the method in previous study was unable to differentiate the parent drug from those possible 3' end chain-shortened metabolites (11). Since we only
accounted for <5% of the administered dose as the known chain-shortened G3139 metabolites, we speculated that much of G3139 was metabolized to shorter oligonucleotides with sequential removal of phosphorothioate mononucleotides in both tissue and plasma. The fate of G3139 degradation products might follow similar pathway as endogenous nucleotides, nucleosides or bases and finally excreted through the renal pathway (2). Unfortunately, our LC/MS/MS method was not designed to detect those lower molecular weight metabolites. Similar inconsistent results about urinary recovery were also reported for other antisense oligonucleotides in animals. Some studies using radiotracer methods have shown that total excreted oligonucleotides were more than 30% of total dose (6, 7, 22, 23). In contrast, other studies using nonradioactive CGE/UV based methods found low urinary excretion (< 1%) (3, 24). Similarly, the discrepancy appears to come from the differences in methodologies applied rather than real differences in urinary excretion of oligonucleotides. For example, most radiotracer studies used either 5'-end or 3'-end labeled drugs, which could explain different urinary recovery data from the ones obtained from nonradioactive methods like CGE/UV. Furthermore, recovery data obtained from simple radioactivity counting without coupling to a separation method (i.e. reverse phase or anion exchange HPLC chromatography) was highly questionable.

In the pilot study with liposomal G3139 in comparison with G3139 in saline solution, the pegylated liposome formulation altered the pharmacokinetics of G3139 by prolonging circulation time. However, it is not certain that this formulation will enable deliver more drugs to tumor sites. Future studies on tumor-bearing mice with this encapsulated G3139 are warranted.
In the rat, the total clearance of G3139 was found to be 3.74 ml/min/kg. Assuming that the total clearance of G3139 is mainly hepatic in the rat (the hepatic blood flow in rat is 40 ml/min/kg), the hepatic extraction ratio of G3139 is less than 0.10. Therefore G3139 is a low hepatic extraction drug. This is not inconsistent with the tissue distribution results in mice, which showed relative high liver tissue uptake and the highest dose recovered in liver organ in mice. It was found that G3139 was quickly metabolized to 3’N-1 by 3’ exonuclease. Our current metabolism data show significantly high circulating chain-shortened metabolites and the kinetics of three profiles appear to be formation limited (25). It is possible that these metabolites of G3139 may contribute to the overall pharmacological activity of G3139. However, we do not have either pharmacological, toxicological effect, or protein binding property of these metabolites.

We are interested in estimating formation and elimination rate constant of metabolites. The proposed kinetic model (Model 3) provides reasonable estimates of inter-compartment clearance and kinetic rate constant. Elimination rate constant of 3’N-1 (k_{34}) was approximately 7-fold higher than the formation rate constant (k_{13}), suggesting formation limit kinetics. Elimination clearance of 3’N-3 was close to hepatic blood flow in rat (40 ml/min/kg), indicative further metabolism or degradation happened since urinary excretion of this metabolite was below 1% of total dose. Although tissue distribution contributes significantly to the disposition of G3139, the driving force of elimination of G3139 seems to be sequential metabolism by exonuclease after rapid distribution to the liver, which has been shown to be the major degradation organ for antisense drugs (26). One of the model assumptions was metabolite formation rate constants (k_{13}, k_{34}, k_{45}) are first order. It has been reported that phosphorothioate
oligonucleotides consist of racemic mixtures of Rp and Sp phosphorothioate diastereoisomers (see Chapter 5) that exhibit very different 3'-end exonuclease stability (27). Rp isomer has been found to be faster cleaved than Sp one. Thus, each metabolite formation rate constant might not be first order. They could be mixture of two kinetic steps, i.e. one rapid kinetic step due to Rp isomer and one slow step via Sp cleavage. Due to the complexity of probability of Rp or Sp at each linkage, we did not attempt to model stereoselective metabolism in this study.

Our allometric result shows a linear relationship between body weight and clearance in ml/min with slope of 0.796 (Figure 6.15). The predicted and observed clearance values of humans agreed reasonable well. It was shown that the exponential value of 0.75 appeared to be the central tendency for the CL of most compounds among 115 drugs, except for those drugs whose elimination was mainly via kidney (28). The associated 99% confidence interval for most compounds was 0.70-0.84 which covered value of 0.75. However, whether our metabolite kinetic model proposed in the rat can be extrapolated to human patients remains to be verified and requires further effort.

In conclusion, the pharmacokinetics and tissue distribution of G3139 were characterized across species and have been shown to be similar in its disposition. The remarkable similarity of the pharmacokinetics across species may be attributed to the physicochemical property of G3139, such as anionic backbone, the hydrophilicity. Metabolite model has been proposed and reasonably predicted metabolite formation and elimination. The ultimate utility of this metabolite model would be extrapolation from animal to human and predict metabolites level after infusion dosing in different clinical studies.
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* conversion factor for G3139 is 1 µM = 5.7 µg/mL

Table 6.1: Plasma concentration-time data of G3139 (µg/mL) in CD1F2 mice (n=6) given as an i.v. bolus injection at 5 mg/kg.
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<sup>a</sup>Obtained from a three compartment model using mean (n=6) plasma concentration-time data.

<sup>b</sup>AUC$_{0-\infty}$: area under the plasma concentration-time curve; $\alpha$, $\beta$, and $\gamma$: the first, second and terminal elimination phase rate constants, respectively; <sup>d</sup>CL: the total body clearance; <sup>e</sup>MRT: mean residence time.

**Table 6.2:** Relevant pharmacokinetic parameters of G3139 in mice (n=6) given as an i.v. bolus injection at 5 mg/kg.
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<tbody>
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<td>Mean± SD (µg/g)</td>
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<td>Mean± SD (µg/g)</td>
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<tr>
<td>Kidneys</td>
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<td>24.1</td>
<td>22.0</td>
<td>18.7</td>
<td>25.9</td>
<td>13.0</td>
<td>20.8±4.5</td>
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<td>26.0</td>
<td>33.0</td>
<td>37.0</td>
<td>31.0</td>
<td>28.0</td>
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<td>13.2</td>
<td>15.6</td>
<td>16.2±3.2</td>
<td>18.1</td>
<td>16.0</td>
<td>14.9</td>
<td>21.0</td>
<td>15.0</td>
<td>19.0</td>
<td>17.3±2.4</td>
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<tr>
<td>Spleen</td>
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<td>4.1</td>
<td>2.3</td>
<td>2.6</td>
<td>4.1</td>
<td>2.4</td>
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<td>1.6</td>
<td>1.9</td>
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<tr>
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<td>1.7</td>
<td>2.1</td>
<td>2.0</td>
<td>1.4</td>
<td>1.7</td>
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<td>1.1</td>
<td>1.3</td>
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<tr>
<td>Bone marrow</td>
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<td>2.1</td>
<td>2.9±1.3</td>
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<td>1.6</td>
<td>1.5</td>
<td>1.3</td>
<td>1.0</td>
<td>1.5</td>
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<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Mean± SD (µg/g)</td>
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<td>Mean± SD (µg/g)</td>
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</tr>
<tr>
<td>Kidneys</td>
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<td>15.0</td>
<td>12.0</td>
<td>10.5</td>
<td>17.1</td>
<td>14.5</td>
<td>13.6±2.4</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>11.0</td>
<td>10.0</td>
<td>9.1</td>
<td>8.2</td>
<td>10.5</td>
<td>10.8</td>
<td>9.9±1.1</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>2.1</td>
<td>1.1</td>
<td>1.4</td>
<td>1.6</td>
<td>1.9</td>
<td>2.0</td>
<td>1.7±0.4</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Gut</td>
<td>0.4</td>
<td>0.8</td>
<td>0.9</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6±0.2</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.7</td>
<td>1.0</td>
<td>0.7</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9±0.2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.3</td>
<td>0.9</td>
<td>0.2</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>0.4±0.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Heart</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND: not detectable

**Table 6.3:** Tissue concentrations of G3139 (µg/g) in CD1F2 mice (n=6) given as an i.v. bolus injection at 5 mg/kg.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>AUC\textsubscript{0-24hr} (µg·hr/ml or gram)</th>
<th>Tissue to plasma ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>13.7</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>528</td>
<td>38.5</td>
</tr>
<tr>
<td>Liver</td>
<td>341.0</td>
<td>24.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>55.1</td>
<td>4.02</td>
</tr>
<tr>
<td>Gut</td>
<td>50.6</td>
<td>3.70</td>
</tr>
<tr>
<td>Lung</td>
<td>29.0</td>
<td>2.11</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>28.0</td>
<td>2.04</td>
</tr>
<tr>
<td>Heart</td>
<td>2.73</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Table 6.4:** AUC values of tissue distribution and tissue to plasma ratio of G3139 at 24 hr in mice given as an i.v. bolus injection at 5 mg/kg.
## Table 6.5

A, Plasma concentration-time data of G3139. B, Relevant pharmacokinetic parameters of G3139 encapsulated in PEGlyated liposome formulation in mice given as an i.v. bolus injection at 5 mg/kg.

### A

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Concentration of G3139 (µg/mL)</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>20.73</td>
<td>20.80</td>
<td>0.12</td>
</tr>
<tr>
<td>0.5</td>
<td>17.1</td>
<td>17.65</td>
<td>0.78</td>
</tr>
<tr>
<td>1</td>
<td>9.61</td>
<td>9.52</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>8.9</td>
<td>8.80</td>
<td>0.14</td>
</tr>
<tr>
<td>6</td>
<td>9.52</td>
<td>10.91</td>
<td>1.97</td>
</tr>
<tr>
<td>26</td>
<td>2.95</td>
<td>3.04</td>
<td>0.13</td>
</tr>
<tr>
<td>48</td>
<td>0.35</td>
<td>0.37</td>
<td>0.04</td>
</tr>
<tr>
<td>72</td>
<td>0.055</td>
<td>0.0615</td>
<td>0.009</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Parameters/units</th>
<th>Values from curve-fitting&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$, µg/ml (µM)</td>
<td>24.4 (4.07)</td>
</tr>
<tr>
<td>$C_{5\text{min}}$, µg/ml (µM)</td>
<td>20.8 (3.45)</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$, µg·hr/ml (µM·hr)</td>
<td>172 (28.67)</td>
</tr>
<tr>
<td>$t_{1/2\alpha}$, (min)</td>
<td>12.6</td>
</tr>
<tr>
<td>$t_{1/2\beta}$, (min)</td>
<td>576</td>
</tr>
<tr>
<td>$\text{CL}$, (ml/min/kg)</td>
<td>0.48</td>
</tr>
<tr>
<td>$V_{\text{ss}}$, (ml/g)</td>
<td>0.39</td>
</tr>
<tr>
<td>$V_{1}$, (ml/g)</td>
<td>0.21</td>
</tr>
<tr>
<td>$\text{MRT}$, (hr)</td>
<td>13.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained from a two compartment model using mean (n=2) plasma concentration-time data;<br>
<sup>b</sup> AUC<sub>0-∞</sub>, area under the plasma-time curve;<br>
<sup>c</sup> $\alpha$, $\beta$, the first, second elimination phase rate constants, respectively;<br>
<sup>d</sup> CL, the total body clearance;<br>
<sup>e</sup> $V_{\text{ss}}$: steady state volume of distribution;<br>
<sup>f</sup> $V_{1}$, volume of central compartment;<br>
<sup>g</sup> MRT, mean residence time.
Table 6.6: Plasma concentration-time data of G3139 and three major metabolites (µg/mL) in SD rats (n=5) given as an i.v. bolus injection at 20 mg/kg.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8.95</td>
<td>2.88</td>
<td>8.92</td>
<td>8.17</td>
<td>9.75</td>
<td>7.74</td>
<td>2.77</td>
</tr>
<tr>
<td>10</td>
<td>10.60</td>
<td>9.24</td>
<td>10.26</td>
<td>10.81</td>
<td>10.75</td>
<td>10.33</td>
<td>0.65</td>
</tr>
<tr>
<td>30</td>
<td>4.06</td>
<td>7.08</td>
<td>3.45</td>
<td>3.16</td>
<td>5.55</td>
<td>4.66</td>
<td>1.64</td>
</tr>
<tr>
<td>60</td>
<td>1.53</td>
<td>7.72</td>
<td>0.98</td>
<td>2.29</td>
<td>1.33</td>
<td>2.77</td>
<td>2.81</td>
</tr>
<tr>
<td>120</td>
<td>0.70</td>
<td>1.40</td>
<td>0.58</td>
<td>0.68</td>
<td>0.83</td>
<td>0.84</td>
<td>0.33</td>
</tr>
<tr>
<td>180</td>
<td>0.53</td>
<td>0.60</td>
<td>0.30</td>
<td>0.92</td>
<td>0.37</td>
<td>0.55</td>
<td>0.24</td>
</tr>
<tr>
<td>240</td>
<td>0.31</td>
<td>0.35</td>
<td>0.15</td>
<td>0.50</td>
<td>0.27</td>
<td>0.32</td>
<td>0.13</td>
</tr>
<tr>
<td>360</td>
<td>ND</td>
<td>0.10</td>
<td>0.10</td>
<td>NA</td>
<td>0.07</td>
<td>0.11</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*NA: not available; ND: not detectable.
<table>
<thead>
<tr>
<th>Parameters/units</th>
<th>Values from curve-fitting (^a) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_{\text{max}}), µg/mL (µM)</td>
<td>200.7 ± 40.3 (33.4 ± 6.7)</td>
</tr>
<tr>
<td>(C_{5\text{min}}), µg/mL (µM)</td>
<td>145 ± 22.8 (24.17 ± 3.8)</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty})^b (µg·min/ml) (µM*min)</td>
<td>6057 ± 2787 (1009 ± 464)</td>
</tr>
<tr>
<td>A, µg/mL (µM)</td>
<td>170.4 ± 40.9 (28.4 ± 6.8)</td>
</tr>
<tr>
<td>B, µg/mL (µM)</td>
<td>30.2 ± 12.4 (5.0 ± 2.1)</td>
</tr>
<tr>
<td>(\alpha)^c (min(^{-1}))</td>
<td>0.081 ± 0.048</td>
</tr>
<tr>
<td>(t_{1/2\alpha}) (min)</td>
<td>13 (range: 5.2-34.7)</td>
</tr>
<tr>
<td>(\beta)^c (min(^{-1}))</td>
<td>0.0108 ± 0.003</td>
</tr>
<tr>
<td>(t_{1/2\beta}) (min)</td>
<td>68.5 (range: 49-101)</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>3.74 ± 1.7</td>
</tr>
<tr>
<td>(V_{\text{ss}})^d (mL/kg)</td>
<td>217 ± 37</td>
</tr>
<tr>
<td>(V_1)^e (ml/kg)</td>
<td>103 ± 22</td>
</tr>
<tr>
<td>MRT(^f) (min)</td>
<td>63 (35-109)</td>
</tr>
<tr>
<td>(k_{10})^g (min(^{-1}))</td>
<td>0.042 ± 0.024</td>
</tr>
<tr>
<td>(k_{12})^h (min(^{-1}))</td>
<td>0.030 ± 0.023</td>
</tr>
<tr>
<td>(k_{21})^h (min(^{-1}))</td>
<td>0.021 ± 0.008</td>
</tr>
</tbody>
</table>

\(^a\) Two compartment iv bolus model. \(^b\) AUC\(_{0-\infty}\): area under the plasma concentration-time curve; \(^c\) \(\alpha\) and \(\beta\): the first, second terminal elimination phase rate constants; \(^d\) \(V_{\text{ss}}\): steady state volume of distribution; \(^e\) \(V_1\), volume of central compartment; \(^f\) MRT, mean residence time; \(^g\) \(k_{10}\): elimination rate constant; \(^h\) \(k_{12}\) and \(k_{21}\): distribution rate constant between central and peripheral compartment.

**Table 6.7**: Relevant pharmacokinetic parameters of G3139 in the rat given as an i.v. bolus injection at 20 mg/kg.
<table>
<thead>
<tr>
<th></th>
<th>3’ N-1</th>
<th>3’ N-2</th>
<th>3’ N-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µg·min/ml)</td>
<td>1537 ± 749</td>
<td>707 ± 524</td>
<td>557 ± 202</td>
</tr>
<tr>
<td>%AUCm/AUCp</td>
<td>25.0 ± 7.0</td>
<td>11.7 ± 3.8</td>
<td>9.88 ± 3.60</td>
</tr>
<tr>
<td>t_{1/2, λ} (min)</td>
<td>63.6 (43.8 – 176)</td>
<td>68.6 (42.7 – 139.7)</td>
<td>69.8 (60 – 83)</td>
</tr>
<tr>
<td>T_{max} (min)</td>
<td>11 ± 2.2</td>
<td>11.0 ± 2.2</td>
<td>11.1 ± 2.3</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>82.8 ± 49.5</td>
<td>56.7 ± 14.0</td>
<td>60.5 ± 10.3</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>28.94 ± 5.04</td>
<td>13.76 ± 3.65</td>
<td>12.07 ± 3.32</td>
</tr>
<tr>
<td>CL\textsubscript{R} (ml/min/kg) of 3’ N-1</td>
<td>0.09</td>
<td>0.28</td>
<td>0.227</td>
</tr>
</tbody>
</table>

*From noncompartment analysis. \textsuperscript{a}AUCm/AUCp: metabolite AUC divided by parent drug AUC. \textsuperscript{b}Apparent renal clearance (parent to metabolite conversion unknown).

**Table 6.8**: Summary of relevant pharmacokinetic parameters of G3139 metabolites estimated by noncompartmental analysis in the rat given as an i.v. bolus injection at 20 mg/kg.
<table>
<thead>
<tr>
<th>Concentration of G3139 (µg/mL)</th>
<th>Human plasma (%)</th>
<th>Rat plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>99.01 ± 0.38a</td>
<td>97.20 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>98.16 ± 0.19</td>
<td>97.30 ± 0.09</td>
</tr>
<tr>
<td>100</td>
<td>95.91 ± 0.46</td>
<td>96.10 ± 0.10</td>
</tr>
</tbody>
</table>

* Data represent mean ± standard deviation of the percentage of drug bound (n=6, two separate experiments)

**Table 6.9:** Plasma protein binding of G3139 in human and rat plasma
<table>
<thead>
<tr>
<th></th>
<th>G3139</th>
<th>3’N-1</th>
<th>3’N-2</th>
<th>3’N-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection</td>
<td>0-4 hrs</td>
<td>4-8 hrs</td>
<td>0-4 hrs</td>
<td>4-8 hrs</td>
</tr>
<tr>
<td>period</td>
<td>hrs</td>
<td>hrs</td>
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</tr>
<tr>
<td>Amount</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excreted</td>
<td>161±13</td>
<td>4.87±1.76</td>
<td>58.7±4.87</td>
<td>2.88±0.8</td>
</tr>
<tr>
<td>(µg) (Mean ± SD, n=3)</td>
<td>84.5±31.3</td>
<td>2.05±1.05</td>
<td>46.6±2.67</td>
<td>2.0±0.8</td>
</tr>
<tr>
<td>% of dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>excreted in 24 hr* (n=3)</td>
<td>2.78</td>
<td>1.0</td>
<td>1.4</td>
<td>0.82</td>
</tr>
</tbody>
</table>

*Urine was collected to 24 hr; however, no G3139 and metabolites were detected after 8 hr.

**Table 6.10:** Urinary excretion of G3139 and its major metabolites in the rat following its i.v. bolus dose at 20 mg/kg
<table>
<thead>
<tr>
<th>System Parameters/units</th>
<th>Values from curve-fitting&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{12}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.04 ± 0.029</td>
</tr>
<tr>
<td>$k_{21}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>$k_{13}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>$k_{34}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.27 ± 0.11</td>
</tr>
<tr>
<td>$k_{45}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.61 ± 0.25</td>
</tr>
<tr>
<td>$k_{50}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.65 ± 0.29</td>
</tr>
<tr>
<td>$V_1$ (ml/kg)</td>
<td>96.50 ± 22.31</td>
</tr>
<tr>
<td>$V_2$ (ml/kg)</td>
<td>135.70 ± 55.67</td>
</tr>
<tr>
<td>$V_3$ (ml/kg)</td>
<td>62.44 ± 9.60</td>
</tr>
<tr>
<td>CL of parent distribution (ml/min/kg)</td>
<td>3.60 ± 1.77</td>
</tr>
<tr>
<td>CL of parent elimination (ml/min/kg) or metabolite formation</td>
<td>3.94 ± 1.10</td>
</tr>
<tr>
<td>CL of N-1 elimination (ml/min/kg)</td>
<td>16.34 ± 5.24</td>
</tr>
<tr>
<td>CL of N-2 elimination (ml/min/kg)</td>
<td>37.42 ± 13.10</td>
</tr>
<tr>
<td>CL of N-3 elimination (ml/min/kg)</td>
<td>39.10 ± 13.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent Mean ± SD (n=5).

**Table 6.11**: Relevant pharmacokinetic parameters of G3139 and metabolites using the metabolite model 3 (Fig.6.18) in rat given as an i.v. bolus injection at 20 mg/kg.
Figure 6.1: Plasma concentration-time profile of G3139 following i.v. bolus administration of 5 mg/kg G3139 in mice (n=6). Each point represents Mean ± SD of six mice.
Figure 6.2: A classical three compartment open model (Model 1) with bolus input and first-order elimination from the central compartment where 1 = central compartment, 2 = shallow tissue compartment, 3 = deep tissue compartment, $k_{10}$ = first-order elimination rate constant, $k_{12}$, $k_{21}$, $k_{13}$, $k_{31}$ = first-order distribution rate constant.
Figure 6.3: Tissue distribution of G3139 following i.v. bolus administration of 5 mg/kg G3139 in CD2F1 mice.
Figure 6.4: Total dose recovery of G3139 following i.v. bolus administration of 5 mg/kg G3139 in CD2F1 mice based on three time points (1, 6 and 24 hr).
Figure 6.5: Stability of targeted and non-targeted liposomes in HEPES buffered saline. The colloidal stability was evaluated by measuring the particle size change of both formulations for 28 days.
Figure 6.6: Plasma concentration-time profiles of G3139 in saline and liposome formulations following i.v. bolus administration of 20 mg/kg G3139 in mice.
Figure 6.7: Representative ESI LC/MS/MS chromatograms of G3139 and three major metabolites in rat plasma following iv bolus administration of 20 mg/kg G3139 at 5 min (A), 15 min (B), 30 min (C), and 1 hr (D), 2hr (E), 4 hr (F) and 6 hr (G) after dosing. Internal standard is at 20.46 min.
Figure 6.7: Continued
Figure 6.7: Continued
Figure 6.8: Plasma concentration-time profiles of G3139 and its major metabolites following i.v. bolus administration of 20 mg/kg G3139 in the rat.
Figure 6.9: A classical two compartment open model with bolus input and first-order elimination from the central compartment, where 1 = central compartment, 2 = tissue compartment, $k_{10} =$ first-order elimination rate constant, $k_{12}, k_{21} =$ first-order distribution rate constant, $V_1 =$ volume of central compartment, $V_2 =$ volume of tissue compartment
Figure 6.10: Two representative fitted Logarithm plasma concentration-time profiles of G3139 following i.v. bolus administration of 20 mg/kg G3139 in the rat.
Figure 6.11: Model 3, sequential metabolism model of G3139. G3139 follows a two compartment model with zero-order input and first-order metabolite formation from the central compartment, where 1 = parent drug compartment, 2 = tissue compartment for G3139, Clr = renal clearance, k_{12}, k_{21} = first-order distribution rate constant, f_m = fraction of parent being converted to metabolite, k_{13} = N-1 formation rate constant, k_{34} = N-2 formation rate constant, k_{45} = N-3 formation rate constant, k_{50} = further N-3 elimination rate constant.
Figure 6.12: Model 4, Metabolite model of sequential metabolism of G3139: G3139 follows two compartment model with zero-order input and first-order metabolite formation from the central compartment where 1 = parent drug compartment, 2 = tissue compartment for G3139, Clr = renal clearance, $k_{12}$, $k_{21}$ = first-order distribution rate constant, $k_{13}$=N-1 formation rate constant, $k_{34}$= N-2 formation rate constant, $k_{45}$=N-3 formation rate constant, $k_{50}$=N-3 elimination rate constant. 6=tissue compartment for 3’N-1, 7 = tissue compartment for 3’ N-2.
Figure 6.13: Representative fitted semi-logarithmic plot (rat 1) of plasma concentration-time profile of G3139 (open circle), 3’ N-1 (diamond), 3’ N-2 (square), 3’N-3 (down triangle) following i.v. bolus administration of 20 mg/kg G3139 in rat.
Figure 6.14: Semi-logarithmic plots of the simulated plasma concentration-time profiles of G3139 and three major metabolites following infusion of G3139 at 20 mg/kg for 6 hrs.

Symbols: G3139 in central compartment (open circle), 3’ N-1 (diamond), 3’ N-2 (square), 3’N-3 (down triangle), G3139 in deep tissue compartment (up triangle).
Figure 6.15: Allometric relationship between CL and body weight across four species for G3139.

Equation: $\text{CL} = 2.80 \times (\text{BW})^{0.796}$
REFERENCE FOR CHAPTER 6


12. Lopes de Menezes, D. E., Hudon, N., McIntosh, N., and Mayer, L. D. Molecular and pharmacokinetic properties associated with the therapeutics of bel-2 antisense


CHAPTER 7

PHARMACOKINETICS/PHARMACODYNAMICS CORRELATION OF G3139

7.1 Introduction

Antisense oligonucleotides (ODNs) have emerged as a powerful agent for the experimental and therapeutic down-regulation of gene expression. As discussed in Chapter 1, antisense ODNs indirectly affect the Bcl-2 protein function by inhibiting mRNA in several ways. Among them, cleavage of mRNA by RNase H is the most important antisense mechanism. The hybridization between antisense oligonucleotides and its target mRNA activates intracellular ribonuclease H (RNase-H) that catalyzes mRNA degradation. For the first generation antisense ODNs, RNase H activation appears to be the most potent and principal mechanism. Previously, it was found that low cellular uptake of naked G3139 was associated with negligible antisense activity in K562 cells (Chapter 3). In contrast, using cationic lipids as delivery vehicles, marked concentration-dependent intracellular G3139 levels were observed with a more effective target down-regulation. Cationic lipids not only enhance the rate and amount of G3139 uptake into K562 cells, but might also alter the intracellular distribution of G3139 and make it more available to Bcl-2 mRNA. These findings suggest concentration of G3139 at target site might correlate with its pharmacological effect or pharmacodynamic biomarker(s). This
is critical since the biophase of G3139 is within the cytoplasm, or more specifically the nucleus. We and others also found plasma concentrations do not correlate with suppression of Bcl-2 mRNA/protein (Chapter 4 and (1)). The PK/PD relationship of G3139 following drug administration remains unknown despite its evaluation in many clinical studies. The lack of such information might be due to the complexity of this molecule and confounding factors commonly encountered in clinical studies, for example, disease progression, limited sampling time, concomitant medication, and quality of PD samples. Therefore, it is our interest to establish a preliminary mathematical model(s) for dynamic change of Bcl-2 mRNA/protein in well-controlled leukemia cells treated with G3139. Herein, we characterized the pharmacokinetics (PK) and pharmacodynamics (PD) of G3139 in leukemia cell line using transfection reagents. Because kinetics of drugs and dynamics of Bcl-2 mRNA/protein interact in a time-dependent manner, a nonlinear dynamic model or physiological based system may provide a powerful tool to fit kinetics/dynamics simultaneously, thereby enabling us the design of a better dosing regimen that is based on the results from PK/PD modeling. Depending on the mechanism of action, a PK/PD correlation strategy may involve the selection of an appropriate model. Effect compartment models are often used to describe the lag between time course of concentrations and the time course of the pharmacodynamic responses due to slow distribution to site of action, or slow target equilibration kinetics (2, 3). On the other hand, a number of drug responses are considered indirect, since drugs produce pharmacological effect via altering synthesis rate or degradation rates. The PK/PD correlation is collectively called indirect response
model. Four Indirect response models have been well described by Jusko WJ et al. (4, 5) and applied to numerous drugs (6, 7).

To further evaluate the PK/PD correlation in vivo, we initiated a phase I study to assess the feasibility of G3139 with a standard remission induction regimen (Daunorubicin and Cytarabine) in patients ≥ 60 years (see Chapter 4 for pharmacokinetics). The primary goal of this study was to provide safety data on the use of G3139 and cytarabine in combination with daunorubicin and to correlate the response to pharmacokinetics, Bcl-2 mRNA levels and BCl-2 protein levels. A more detailed pharmacodynamic study was described in this Chapter to further evaluate the therapeutic role of G3139 in acute leukemia.

7.2 Materials and Methods

7.2.1 Cell lines and leukemia blasts

Four AML cell lines, namely K562, ML-1, NB4 and Kasumi-1, obtained from ATCC (Manassa, VA) were used in the in vitro PK/PD study. All cell lines were cultured in RPMI 1640 supplemented with 90 µg/ml of gentamicin and cefotaxine as antibiotics and 10% fetal bovine serum (Invitrogen, Rockville, MD). Cultures were maintained at 37°C in a humidified environment with 5% CO₂. Viability and cell counts were determined in triplicates using trypan blue dye exclusion and expressed as the mean ± SD. Procurement of human bone marrow leukemia blasts from AML patients was consented under an IRB-approved protocol (Protocol OSU 0164) at The Ohio State
University Hospitals. Mononuclear cells were separated immediately after procurement of bone marrow samples. BM diluted with RPMI1640 medium supplemented with 10% FBS (1:1) was centrifuged at 250 g for 30 min on Ficoll (Gibco, CA). Mononuclear cells were obtained from the middle layer and washed twice with PBS, cryopreserved in liquid nitrogen.

7.2.2 Transfection of cells with cationic liposomes

All transfection studies were performed as described in Chapter 3.

7.2.3 Cellular uptake study in NB4 cells and sample process

NB4 cells (~10×10^6) were exposed to 1 µM of G3139 in the presence of Oligofectamine at 37°C for 72 hrs. For intracellular concentration determination, about 2x 10^6 of cells per time point was collected at pre-selected time points of 15 min, 30 min, 1 hr, 2 hr, 4, 6, 8, 12, 16, 24, 32, 48 and 60 hr following exposure. After harvest, cells were processed as described in Chapter 3 to remove membrane-bound ODNs, and analyzed by the ELISA assay. Approximately 3x10^6 of cells each, removed at pre-selected time points of 0, 8 hr, 12, 16, 24, 32, 48 and 60 hr, were homogenize in 300 µL of RLT buffer (Qiagen, CA) immediately after cell harvest and frozen in –80°C for RNA analysis. The rest of NB4 cells (approximately 5 x10^6 cells) were collected at pre-selected time points of 0, 12, 24, 32, 48, 60 and 72 hr. The cells were processed as described in Section 7.2.5.
7.2.4 Quantification of Bcl-2 mRNA levels

Quantification of Bcl-2 mRNA was performed by Real Time RT-PCR as previously reported (see Chapter 3). ABL gene was used as the internal control for mRNA quality and amplification. Quantification of Bcl-2 transcript was normalized to ABL. The result of the Real Time RT-PCR assay for each sample was reported as Bcl-2 copies per ABL copies.

7.2.5 Quantification of Bcl-2 protein by ELISA assay

The quantitative Bc1-2 ELISA assay was performed using a commercially available ELISA kit from Oncogene (Boston, MA). Briefly, after 48 and 72 hr of culture, control and treated NB4 cells were counted and washed twice with phosphate buffered saline (PBS) before re-suspension at a cell concentration of 5x10^6/ml in suspension buffer (50 mM Tris, 5 mM EDTA, 0.2 mM PMSF, 1 µg/mL pepstatin and 0.5 µg/mL leupeptin, pH 7.4). Antigen was extracted by incubation of cells with an antigen extraction agent on ice for 30 min. The cell lysate was suspended at 1:5 in PBS containing 1% bovine serum albumin (BSA). All cell lysate samples and standards were incubated 2 hr in a 96-well microtitre plate coated with anti human Bc1-2 monoclonal antibody at room temperature. After washing with PBS containing 0.05% Tween-20, the anti-FITC peroxidase conjugate (diluted 1:200) was added followed by incubation for 30 min at room temperature. After washing with washing buffer and deionized water, the substrate was added and enzymatic reaction was allowed 30 min to occur and then stopped by 1% 2.5 N sulfuric acid. The color intensity developed was measured using Germini XS (Molecular devices, CA) plate reader with a dual wavelength filter 450/590 nm. A
standard curve was constructed by plotting the mean absorbance values of two measurements versus the nominal Bc1-2 standard concentrations. The Bc1-2 concentration was determined in each cell sample in duplicate as Bcl-2 Units/mL by comparing the absorbance obtained from each sample with that obtained from the standard curves.

7.2.6 Immunofluorescence staining and quantification of Bcl-2 protein by flow cytometry

Ten µL of mouse anti-human FITC-conjugated anti-CD34 antibody (BD Clonetech, CA) was added and incubated for 30 min at 4°C in the dark. Cells were washed once in PBS, then fixed, permeablized with cytofix/cytoperm buffer (Becton Dickinson, CA) and stained with mouse anti–human Bcl-2-Phycoerythrin (PE)-conjugated monoclonal antibody (BD Pharmingen, San Diego, CA). Irrelevant isotype-control was used as negative controls along with each staining. Analysis of double staining CD34 and Bcl-2 were acquired on a FACScan flow cytometer (Becton Dickinson, CA). Data was analyzed and displayed with CellQuest software (Becton-Dickinson, San Diego, CA). Histograms were drawn to compare difference in mean fluorescence intensity (MFI) of PE positive cell population.

7.2.7 Estimation methods and description of computer software for PK/PD modeling

The harvested NB4 cells were first incubated with 200 µL 0.1 µM phosphorothioate 28mer polycytidine (PS-dC28) for 2 min on ice and washed with PBS to remove membrane-bound G3139 (8). The cells were lysed as described in Chapter 3.
Kinetic profile of intracellular G3139 (pmole/mg) following exposure of 1 μM G3139 was determined by the validated hybridization ELISA assay (Chapter 2). Kinetic and dynamic changes of drug, Bcl-2 mRNA and Bcl-2 protein were modeled simultaneously by nonlinear regression in software ADAPTII (Biomedical Simulations Resource, University of Southern California) (ADAPTII codes were listed in appendix A and B) and relevant parameters were calculated using either weighted least squares method (WLS) or maximum likelihood method (ML) (9). Model selection was guided using the Akaike information criterion (AIC), coefficient of variance (CV) of each parameter estimate, and overall goodness of fit plots. For maximum likelihood method, the following equation was used to model the variance of the normally distributed output error for G3139, Bcl-2 mRNA, and Bcl-2 protein:

\[
\sigma_i^2(t_j) = (\sigma_1 + \sigma_2 \times Y_i(t_j))^2
\]

[Equation 7.1]

where \( \sigma_i^2(t_j) \) is the variance for the ith point, \( Y_i(t_j) \) is the ith predicted value from the model, and \( \sigma_1, \sigma_2 \) are the variance parameters, which were either estimated or fixed.

### 7.3 Results

#### 7.3.1 In vitro Pharmacokinetic/Pharmacodynamic modeling of Bcl-2 antisense: indirect response model

Basal Bcl-2 expression levels in Kasumi-1, NB4, K562 and ML-1 cell lines were initially determined by a validated Bcl-2 ELISA method (Figure 7.1). As shown, Bcl-2 expression levels in Kasumi-1 and NB4 cell lines were similar (Figure 7.1) and the
average concentrations were 57 and 61.9 Unit/10^6 cells. The expression of Bcl-2 in ML-1 cell line was medium with 23.7 U/10^6 cells. K562 has the lowest Bcl-2 level of 3.7 U/10^6 cells among four cell lines. These results were consistent with those from flow cytometry analysis (Figure 7.2), which essentially gave similar relative order of expression of Bcl-2. Using double staining by flow cytometry, only NB4 and Kasumi-1 were found to be both CD34 and Bcl-2 positive while the other two cell lines were CD34 negative. Since most AML blasts are CD34 positive and Bcl-2 overexpressed, NB4 cell was chosen for the following PK/PD study.

Table 7.1 and Figure 7.3 shows the overall intracellular concentration-time profile of G3139 in NB4 cells, when cells were incubated with 1 µM of G3139 for 72 hrs. The intracellular concentrations of G3139 in NB4 cells following exposure to 1 µM G3139 showed a rapid rise during the first 8 hrs and a slow decline over 60 hrs. The concentration-time data were then fitted with a simple model with first order input and first order elimination. The estimated $k_a$ and $k_{el}$ were 0.26 and 0.067 hr^{-1}, respectively. The intracellular G3139 half-life was calculated to be 10.3 hr. The cellular Bcl-2 mRNA levels as determined by RT PCR method and Bcl-2 protein levels as determined the ELISA method in NB4 cells following G3139 treatment are also shown in Table 7.1. As shown, both the baseline line values (set as 100%) of Bcl-2 mRNA and Bcl-2 protein decreased with time upon G3139 treatment. The rates and extents (~40 and ~60% of control mRNA, respectively) of decrease for mRNA appeared to be more profound than the protein. Levels of mRNA also appeared to return to the basal level at 60 hrs, but the depression of the protein expression was not recovered until 72 hr. The perturbation of
these biomarkers appears to correlate with the cellular levels of G3139 in some fashion. Therefore, we attempted to employ an appropriate PK/PD model to describe and fit these data.

Mechanistically, G3139 specifically binds to its target (Bcl-2 mRNA) in cytoplasm and nucleus and form duplex which will then trigger the activity of RNase H. RNase H mediated catabolism of Bcl-2 mRNA provides an efficient perturbation of transcript besides intrinsic pathway. Since concentrations of G3139 relates to stimulation of RNase H activity, or more specifically, the degradation of Bcl-2 mRNA, an indirect response models was proposed as shown in Figure 7.4A, which can be mathematically described in the following:

\[
\frac{d}{dt}(mRNA) = k_{in} - k_{out} \times (1 + \frac{E_{max} \cdot [G3139]^n}{EC_{50}^n + [G3139]^n}) \times (mRNA)
\]

[Equation 7.2]

where Emax is the maximum simulation fraction and EC50 is the concentration required for half-maximal stimulation. The pharmacodynamic model is based on this scheme in which Bcl-2 mRNA is synthesized with a zero-order rate coefficient (K_{in}) and a first-order degradation rate coefficient (K_{out}). Pre-perturbation concentrations of RNA was assumed to exist in a steady state, in which the rate of synthesis equals to the rate of decay, i.e. there is no net change in mRNA levels. When G3139 was taken up by cells, it decreases mRNA level by inducing its degradation, thereby decreasing the pharmacological response (mRNA production) (4). An initial modeling of data without sigmoidal factor (n) resulted in poor fitting of data and parameter estimates. Thus,
sigmoidal factor (n) was included into the model to describe the steepness of the dynamic curves. However, it was found that model gave a n value of 3.94 with high CV of 53% (Table 7.2A). Different n values were tested and n=3 was found to provide the best fit. Therefore, in the final model, n was fixed to 3 and the final estimates are provided in Table 7.2B.

In contrast, treatment with G3139 is expected to decrease Bcl-2 protein levels by inhibiting its production; however, G3139 is not mechanistically involved in the degradation pathway of protein. Thus, an indirect response model with inhibition of production of the response (Bcl-2 protein) was proposed for PD model of Bcl-2 protein (Figure 7.4B) using the following equation:

\[
\frac{d(\text{Protein})}{dt} = k_{in} \times \left(1 - \frac{I_{\text{max}} \cdot [G3139]^n}{IC_{50}^n + [G3139]^n}\right) - k_{out} \times (\text{Protein})
\]

[Equation 7.3]

where \(I_{\text{max}}\) is the maximum fraction of inhibition and \(IC_{50}\) is the concentration required for half-maximal inhibition. We also assume that a steady state of protein exists at pre-perturbation state, where the rate of synthesis (zero-order) equals to the rate of decay (first-order degradation rate). Thus, the pharmacokinetic and pharmacodynamic data of G3139 were simultaneously fitted with this final model and fitted curves are shown in Figures 7.5 and 7.6, respectively, with the respective estimated parameters listed in Tables 7.2 and 7.3 for Bcl-2 mRNA and protein.

According to this model, cellular concentrations of G3139 followed exposure of G3139-oligofectamine complex displayed a rapid uptake and a monoexponential decline.
with a half-life of about 10 hr. The peak concentration achieved was nearly 100 pmole/mg of protein, and when compared to the exposure to free drug (see Chapter 3), the drug levels were significantly higher, indicating efficient delivery of G3139 via cationic lipid complex. The model also indicated that the maximum down-regulation of mRNA reached approximately 40% of the control at about 20 hr after drug exposure. On the other hand, the protein nadir decreased to about 60% of the control at about 36 hrs. The pharmacodynamic parameters were estimated according to fitting of inhibitory Emax model. The estimated EC50 for Bcl-2 mRNA was 39 pmole/mg protein and IC50 for Bcl-2 protein was 43.8 pmole/mg protein (Table 7.2B and 7.3). The estimated Emax and Imax values were 1.58 and 0.42, respectively. The estimated values of kout provide a rough estimate of pseudo-degradation rate constant of RNA or protein. The half-life of RNA degradation was approximately 8.7 hr and half-life of protein degradation was 14 hr. Therefore, perturbation of the mRNA dynamics appears to be rapid as is its recovery. In contrast, protein levels decreases slowly and returns to baseline over a longer period of time. These dynamic profiles are consistent with the proposed mechanistic sequence that Bcl-2 mRNA is the precursor of Bcl-2 protein.

7.3.2 Simulation based on indirect response models

The key kinetic determinants in Bcl-2 mRNA model are k_in and k_out and using our PK/PD model, perturbation in the PD profile can be simulated. Figure 7.7 shows the simulated pharmacodynamics of Bcl-2 mRNA by altering the mRNA turnover rate. The k_in varied from 5 to 100 % mRNA/hr while values of k_out were changed accordingly in order to keep the ratio of k_in/k_out equals to 100 (%mRNA). For fast turnover mRNA, for
example, most oncogene related mRNA, a decrease in mRNA nadir was observed with less duration of the action. However, changes in mRNA nadir under various conditions were not significant neither was the duration of pharmacological effect. The pharmacodynamic profiles of Bcl-2 mRNA after exposure to various levels of G3139 are shown in Figure 7.8. The characteristics of these response curves is the increase in exposure results in increased maximal response or down-regulation of Bcl-2 mRNA and the maximal response appears to be 40% of control similar to the values observed. After the maximal response is reached, further increases in dose from 1 to 10 µM only prolong the duration of response at RNA nadir, in this case, at 40% of control.

Simulations were also performed to predict the PK/PD profiles of G3139 after multiple exposures (Figure 7.9). Dose regimen was altered from one exposure per 12 hr to one every 48 hr. The pharmacokinetics of IC G3139 are shown in Figure 7.9A and pharmacodynamics of Bcl-2 mRNA and protein are shown in Figure 7.9B and 7.10, respectively. When the exposure interval was 48 hr, mRNA rebound to baseline level at 48 hr, since intracellular concentration of G3139 was degraded by cellular nucleases (see Chapter 1). More frequent exposure schedule could circumvent this transient down-regulation of RNA by increasing cumulative intracellular drugs as shown in Figure 7.9A. Based on the simulation results, it appears that the optimal exposure interval is 12 hr, since a stable down-regulation was achieved when drug was given at 12 hr interval. In contrast, protein levels return to steady state condition slowly, presumably due to a slower turnover rate than that of RNA (Figure 7.10). To achieve the stable decrement, the optimal exposure interval is either 24 or 36 hr. In the clinical studies of G3139, constant i.v. infusion dosing schema with different infusion duration was explored (10-
12). Simulation results provide a rational for these empirical dosing schedules and suggest once per day dosing might not achieve the desired reduction of Bcl-2 mRNA or protein.

7.3.3 Pharmacokinetic/Pharmacodynamic modeling of G3139: mechanistic-based kinetic model for gene expression

A two-compartment gene expression model was originally proposed by Hargorove et al. (13). Based on this model, a cellular mechanistic based PK/PD model was proposed for gene expression when antisense was introduced into cell (Figure 7.11). The model is described by five differential equations based on the mass balance law as follows:

\[
\frac{dx_1}{dt} = k_{s1} - k_{d1} \cdot x_1 - k_1 \cdot x_1 \cdot x_3 \\
\frac{dx_2}{dt} = k_{s2} \cdot x_1 - k_{d2} \cdot x_2
\]

[Eq. 7.4] [Eq. 7.5]
\[
\frac{dx_3}{dt} = k_1 \cdot x_2 - k_{d3} \cdot x_3 - k_1 \cdot x_1 \cdot x_3 \quad \text{[Eq. 7.6]}
\]
\[
\frac{dx_4}{dt} = k_1 \cdot x_1 \cdot x_3 - k_{d4} \cdot x_4 \quad \text{[Eq. 7.7]}
\]
\[
\frac{dx_5}{dt} = -k_a \cdot x_5 \quad \text{[Eq. 7.8]}
\]

where:

<table>
<thead>
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<tr>
<td>1</td>
<td>RNA compartment, RNA is normalized to baseline</td>
</tr>
<tr>
<td>2</td>
<td>Protein compartment</td>
</tr>
<tr>
<td>3</td>
<td>Antisense compartment</td>
</tr>
<tr>
<td>4</td>
<td>Antisense-RNA duplex compartment</td>
</tr>
<tr>
<td>5</td>
<td>dosing compartment (extracellular compartment)</td>
</tr>
</tbody>
</table>

- \( k_{s1} \): First order rate constant for transcription
- \( k_{s2} \): First order rate constant for translation
- \( k_{d1} \): First order rate constant for RNA degradation
- \( k_{d2} \): First order rate constant for protein degradation
- \( k_{d3} \): First order rate constant for RNase-H mediated catabolism of antisense-RNA duplex
- \( k_{d4} \): First order rate constant for degradation of G3139
- \( k_1 \): Second order on-rate constant of antisense-RNA hybridization
- \( k_a \): First order rate constant for drug uptake from extracellular compartment
In this model, we assume that:

(1) G3139 only binds to cytoplasmic mRNA and the binding to cellular protein is negligible, (2) RNase H is a major antisense mechanism for pharmacological effect of G3139, (3) G3139 binds to mRNA and activate RNase H, thus the degradation of Bel-2 mRNA, (4) the binding of G3139 with mRNA is irreversible, i.e. there is no off kinetic from drug-RNA duplex, (5) before drug administration, there is a steady state condition for mRNA and protein, (6) intact mRNA is capable of translation to protein.

The pharmacokinetic and pharmacodynamic data of G3139 following single exposure of 1 µM was again fitted with this model and the results were depicted in Figure 7.12. The characteristics of mRNA response curve are a biphasic kinetics consisting of a rapid decline and a slowly return to pretreatment levels. Reversibility of mRNA was observed by 60-70 hrs. In contrast, protein response curve shows almost equal rate of reduction and increase over 72 hrs with maximum down-regulation observed around 36 hr. A concomitant downregulation of mRNA and protein in same level was not observed. The PK/PD model proposed did not satisfactorily describe mRNA response curve while protein response curve appeared to be reasonably modeled. The estimated parameters are listed in Table 7.4. Majority of parameter estimates associate with large variance, suggesting the model might be over-parameterized.

A simulation was performed in ADAPTII to predict the dynamics of RNA and protein after multiple exposures using the parameters obtained from the model estimates. As shown in Figure 7.13, when the exposure interval was 48 hr, mRNA profile returns to 70% of control at 48 hr. More frequent dosing schedules diminish this effect and the optimal dosing schedule appears to be every 24 hr. Similar changes in protein was also
observed when dosing interval was altered. Interestingly, based on this model, the optimal dosing interval for mRNA is consistent with that for protein (24 hr) while the indirect response model predicted different optimal dosing intervals for RNA and protein.

7.3.4 Pharmacodynamics of a phase I (Protocol OSU 0164) study of G3139

Intracellular concentration of G3139 was measured before and into 72 hrs of G3139 treatment. The median IC was 5.6 pmole/mg proteins (range, 0.33-29) (n=20). IC of G3139 in responders appears to be higher than that in non-responding patients (Figure 7.14). The median IC of G3139 in CR and NR patients was 17.01 and 4.41, respectively. Nonparametric statistical analysis (Wilcoxon sign test) showed that the difference between medians has p value of 0.06. IC of G3139 appears to be higher in bone marrow (BM) CD34-positive blasts than in the remaining CD34-negative cells, suggesting site-preferable uptake behavior (Figure 7.15).

A sensitive and reproducible real-time RT-PCR quantitation method was developed to determine Bcl-2 transcripts and internal positive control cABL in patient’s samples. A representative amplification plot is depicted Fig. 7.16 using Bcl-2 standard cDNA obtained from plasmid DNA. A calibration curve was constructed for C_{T} values against log copy number of Bcl-2 cDNA (Fig. 7.16). A slope greater than –2.9 and close to –3.0 is needed for subsequent quantification. Correlation coefficient of >0.99 was observed. The amplification plot and calibration curve for ABL are depicted in Figure 7.17. Using this validated real-time RT-PCR method, reduction of Bcl-2 mRNA was detected in 14 of 22 pts (Figure 7.18). The levels of Bcl-2 mRNA following G3139 CIVI was decreased in CR patients but increased in NR patients (Table 7.5). Since only limited
samples were available for analysis, the sign test was performed to compare differences between pre and post-treatments and their differences were classified as either positive, negative, or tied. Bcl-2 mRNA was decreased in CR patients with p=0.039 (n=12) and it was increased in NR patients (p=0.11), indicating down-regulation of Bcl-2 protein in CR patients but not in NR patients. A parametric test comparing percentage of changes between pre and post-treatment to 0 also gave similar significance level (data not shown). The difference in downregulation of Bcl-2 mRNA between CR and NR patients was statistically significant (p=0.002) as shown in Table 7.5. However, there is no correlation between IC of G139 and Bcl-2 mRNA.

Seven of the 15 patients showed down-regulation of ratio of Bcl-2/Bax as determined by immunoblotting (Fig. 7.19). Since ELISA assay is more sensitive and quantitative as opposed to standard methods like immunoblotting or flow cytometry, we performed quantification of Bcl-2 protein by a commercially available ELISA kit. Nine of 16 patients showed down-regulation of Bcl-2 protein (Fig. 7.20). In the responding population (n=9), Bcl-2 levels were decreased from 63.5 to 31 units/mg protein (median values) with p=0.004 (sign test). However, there was no significant changes in Bcl-2 levels in the NR population (n=6) (Median is approximately 50 unit/mg, Table 7.5). Similarly, the difference in down-regulation of Bcl-2 protein between two populations was statistically significant with p=0.013 by Wilcoxon Mann-Whitney. Although a significant correlation was not observed between Bcl-2 down-regulation and IC of G3139 due to the small sample size (p=0.10), it appears that a threshold for IC of G3139 might exist, since only those patients who achieved ≥ 5 pmole/mg had reduced Bcl-2 protein (Fig. 7.21). In 10 paired evaluable PBMC specimens, Bcl-2 decrement was observed in 5
of 6 CR patients while only 2 NR patients had decreased Bcl-2 levels. However, there was no statistical significance due to small sample size (Fig. 7.22).

Other Bcl-2 family genes were also evaluated using real-time RT-PCR and results are summarized in Table 7.6. Notably, Bcl-Xl increments occurred in 6 of 10 patients in the NR population while it only occurred in 3 of 13 CR patients, underscoring the possibility that other Bcl-2 associated genes might involve in the tumor response to G3139 therapy. The median change in Bcl-Xl in CR and NR group was 53.6 % and 163 %, respectively. Nonparametric test showed a difference in Bcl-Xl changes between these two groups with p=0.0506 (Figure 7.23A). Microarray data corroborated the results of real-time RT-PCR (Figure 7.23B). The difference in Mcl-1 was not statistically significant (Table 7.6).

7.4 Discussion

PK/PD modeling has contributed much to our understanding of drug concentrations and pharmacological effects of HIV protease inhibitors (14), corticosteroids (15-17), monoclonal antibody (18). We hypothesize that PK/PD modeling could be applied to antisense therapeutics and enhance our current understanding of pharmacology of this agent. Similarly to traditional small molecular drugs, mRNA can be considered to be drug receptors and antisense ODNs are then ligands that bind to mRNA. In such a case, the Watson-Crick base-pairing rule constitutes the ligand-receptor binding. The duplex of antisense-RNA triggers activity of RNase H which executes the downstream biological effects. The reduction of mRNA further results in a decrease of corresponding protein thus the final pharmacological effect. Since cells contain >100 fold
more proteins than RNA copy number (13), this strategy is practically attractive and potent. However, there was a lack of the information regarding to correlation of drug exposure with pharmacological effect (PK/PD correlation).

To further understand the dynamic perturbation of Bcl-2 mRNA and protein by G3139, we proposed an indirect response PK/PD model to fit the experimental data. The proposed model successfully fitted the data and provides useful parameters. The PD models used here for mRNA and proteins are rather simplistic representation of complex transcription and translation processes within the cytoplasm and nucleus (19). For example, based on the estimated $k_{out}$, the half-life of Bcl-2 mRNA was approximately 10.5 hr which is about two fold higher than that obtained from experiment which ranges from 3- 6 hr (20). Nevertheless, the value is still considered reasonable. Based on the model, apparent half-life of Bcl-2 protein was approximately 14 hr. Half-life of Bcl-2 protein depends on cell lines or cell origins. It was 20 hr in HL-60 cell line but is 38 hr in HL-60/adriamycin chemoresistant cell line (21). It is known that the dysregulation of Bcl-2 in leukemia might contribute prolongation of Bcl-2 protein or Bcl-2 RNA (21). This simple PK/PD model has formed a new concept for antisense action in leukemic cells expressing high level of Bcl-2 protein and provides a useful tool for further improvement of the PK/PD model incorporating more pertinent information. It is also realized that different dose levels are needed to fully characterize the underline PK/PD relationship of G1339 and its target Bcl-2. Nevertheless, results obtained from the simulation are consistent with current knowledge of antisense ODNs. For example, based on the simulation results, a frequent dose administration is needed to maintain the inhibitory effect of G3139 on the expression of both transcript and protein. The optimal
exposure interval is 12 or 24 hr and this is in line with the current clinical protocol using a continuous i.v. infusion. A common practice in the antisense field is the cells are treated with antisense ODNs once per day for consecutive 3 days to achieve maximum target downregulation. Our simulation results provide the rational and useful guide for the routine transfection experiments.

Although the indirect response models proposed above provided reasonable estimates of RNA and protein dynamics, it did not depict the underline physiological interaction between RNA and antisense, i.e. Watson-Crick base pairing and subsequent RNase H cleavage. An integrated mechanistic PK/PD model for both RNA and protein was proposed to depict the dynamic alteration of Bcl-2 at RNA and protein level following administration of G3139. Compared to the indirect response model, this model provides a more mechanistic representation of intracellular antisense-RNA-protein dynamics. We have not incorporated the effect of intracellular compartmentalization of G3139, protein binding of the drug, and other degradation process of duplex. Despite its simplicity, the current model might already be over-parameterized, since the variance for the parameter estimates is fairly large. Nevertheless, \( k_{s1} \) and \( k_{s2} \) were estimated to be 6.3% mRNA/hr and 7.0% protein/hr, which are similar to the estimation of \( k_{m} \) for both protein (5.0% protein/hr) and mRNA (6.6% mRNA/hr) obtained from the indirect response model. The model also provides reasonable estimates for another two key kinetic parameters, \( k_{d1} \) and \( k_{d2} \). It has been shown that the reduction of mRNA did not necessarily result in marked reduction of corresponding protein (22). This can be explained by the simulation study using the second model. Antisense ODNs will have little effect on those proteins with \( t_{1/2} > 48 \) hr, for example, \( \beta \)-Actin (60 hr) (23), GAPDH
(24) and many other housekeeping protein (13). This suggests that measurement of target protein is as important as that of target mRNA to demonstrate antisense effect, since concomitant down-regulation of mRNA and protein is usually not observed. The following factors need to be considered to optimize the pharmacological effect of antisense drugs: (1) pharmacokinetics of the drug in plasma, (2) cellular uptake profile, (3) pharmacokinetics of the drug intracellularly and compartmentalization of the drug, (4) stability of target mRNA and protein, (5) the binding affinity between ODNs and mRNA, and (6) the best dosing schedule.

The biological activity of the G3139 was evaluated in a phase I study. The Bcl-2 mRNA levels decreased in 14 of 22 pts (63.6%) patients as tested by Real Time RT-PCR. Although only limited samples (n=22) were available for analysis, a preliminary correlation was observed between disease response and the extent of Bcl-2 mRNA down-regulation. The difference in reduction of Bcl-2 mRNA between CR and NR patients was statistically significant with p <0.01. In fact, Bcl-2 mRNA was increased post treatment in NR patients as opposes to median decrement of 38% in CR patients. In an attempt to quantify the Bcl-2 protein level in bone marrow tumor cells, expression of bcl-2 was measured using ELSIA method and this result was found to be consistent with western blotting. However, measuring Bcl-2 in tumor cells using ELISA method appears to be more reliable in comparison to other standard methods as proven in previous publications (25, 26). Further, G3139-induced apoptosis might trigger degradation of some cellular proteins by caspase including the internal control we used (GAPDH). This ultimately makes quantification of BCI-2 by western blotting extremely difficult. Despite the longer half-life of Bcl-2 protein in acute leukemia (27, 28), 12 of 18 (67%) evaluable patients
achieved reduction of Bcl-2 protein. A similar correlation was observed between disease response and the extent of Bcl-2 protein downregulation, i.e. 9 of 9 responders (IR and CR) achieved reduction of Bcl-2, but only half NR patients had decrement of Bcl-2 post treatment with G3139. No linear correlation (n=21) was observed, however, between IC of G3139 and levels of Bcl-2 downregulation, underscoring that the complexity of this compound, the activity of which is likely to depend on several factors including intracellular drug trafficking, compartmentalization, endogenous levels of endonucleases and regulation of target expression in response to pharmacologic modulation. Nevertheless, using this sensitive assay in future studies, we expect to measure G3139 levels in different organelles or compartments upon cell subfractionation and identify specific patterns of drug distribution that may be predictive of both biological and clinical responses.

Circulating mononuclear cells (PBMC) offer an alternative way to evaluate pharmacological effect of drugs and have been widely used as a surrogate pharmacodynamic marker. However, they are biologically and pharmacologically different from tumor cells in bone marrow. It is not clear whether PBMC data we obtained are truly representative of target gene or protein status in comparison to BM results. A definitely correlation would facilitate future clinical studies, since PBMC samples are easier to attain than BM samples. The questions remain to be answered in future studies are: (1) whether Bcl-2 is an easy-accessible biomarker and (2) how procurement of tumor samples and analysis should be processed. For instance, 8 of 29 RNA samples and 11 of 29 protein samples in OSU0164 were not suitable for sample
analysis due to poor sample preparation or process procedure at different clinical study sites. In this regard, tissue handling and storage condition must be examined to validate the measurements without significant degradation of either nucleic acids or proteins. The assay for this biomarker must be reproducible and sensitive with calibration curve run simultaneously. Our experience already provide useful procedure and assay platform for how to handle and quantify Bcl-2 related biomarkers in the future clinical studies.

Another controversial aspect of Bcl-2 being a valid biomarker is that its expression in malignant cells are highly variable and its dynamics differ in different cell subpopulation (29-31). Selective survival of tumor cells with higher Bcl-2 expression also might happen during induction remission; therefore Bcl-2 levels do not necessarily reflect the chemosensitivity of tumor cells. Third, induction of apoptosis by cryopreservation and/or cell thawing might change Bcl-2 levels regardless therapy, thus introducing another variable to the final data analysis.
Table 7.1: Intracellular G3139 concentration-time data and corresponding Bcl-2 mRNA, protein levels (normalized to % of control) (n=3) following 1 µM exposure in NB4 cells.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Estimates</th>
<th>CV (%)</th>
<th>Confidence interval (95%)</th>
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</thead>
<tbody>
<tr>
<td>$k_{cl}$</td>
<td>hr$^{-1}$</td>
<td>0.063</td>
<td>5.2</td>
<td>[0.059, 0.076]</td>
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<tr>
<td>$V$</td>
<td>mg protein</td>
<td>6.5</td>
<td>22.8</td>
<td>[3.55, 9.51]</td>
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<tr>
<td>$k_a$</td>
<td>hr$^{-1}$</td>
<td>0.22</td>
<td>10.0</td>
<td>[0.16, 0.25]</td>
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<tr>
<td>$k_{in}$</td>
<td>% mRNA/hr</td>
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<td>[3.55, 9.51]</td>
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<tr>
<td>$k_{out}$</td>
<td>hr$^{-1}$</td>
<td>0.079</td>
<td>23.0</td>
<td>[0.035, 0.095]</td>
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<tr>
<td>$EC_{50}$</td>
<td>pmole/mg protein</td>
<td>37.9</td>
<td>21.2</td>
<td>[21.85, 53.94]</td>
</tr>
<tr>
<td>$E_{max}$</td>
<td></td>
<td>1.5</td>
<td>18.0</td>
<td>[0.96, 2.04]</td>
</tr>
<tr>
<td>Sigmoidal slope (n)</td>
<td></td>
<td>3.94</td>
<td>53</td>
<td>[-0.19, 8.07]</td>
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</tbody>
</table>

**Table 7.2:** Parameters for the pharmacokinetic/pharmacodynamic model of Bcl-2 antisense: indirect response model for Bcl-2 mRNA. The Maximum likelihood method was employed as estimation method. (A) Sigmoidal slope (n) was estimated and it was found the estimate was associated with large CV and CI included 0. (B) The sigmoidal slope (n) was fixed to 3 and it was found the parameter estimates were similar to those obtained without fixing sigmoidal slope.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Estimates</th>
<th>CV (%)</th>
<th>Confidence interval (95%)</th>
</tr>
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<tr>
<td>( k_{el} )</td>
<td>hr(^{-1})</td>
<td>0.067</td>
<td>2.0</td>
<td>[0.065, 0.070]</td>
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<tr>
<td>( V )</td>
<td>mg protein</td>
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<td>1.1</td>
<td>[6.60, 6.90]</td>
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<tr>
<td>( k_a )</td>
<td>hr(^{-1})</td>
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<td>( k_{in} )</td>
<td>% mRNA/hr</td>
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<td>22.0</td>
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<tr>
<td>( k_{out} )</td>
<td>hr(^{-1})</td>
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<td>22.0</td>
<td>[0.036, 0.094]</td>
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<tr>
<td>( EC_{50} )</td>
<td>pmole/mg protein</td>
<td>39.0</td>
<td>29</td>
<td>[16.15, 61.97]</td>
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<tr>
<td>( E_{max} )</td>
<td></td>
<td>1.58</td>
<td>24</td>
<td>[0.82, 2.36]</td>
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<td>Sigmoidal slope (n)</td>
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<td>3 (Fix)</td>
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<td>$k_{cl}$</td>
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<tr>
<td>$V$</td>
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<td>Imax</td>
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Sigmoidal slope (n) 3 (fixed)

Table 7.3: Parameters for the pharmacokinetic/pharmacodynamic model of Bcl-2 antisense: indirect response model for Bcl-2 protein. The Maximum likelihood method was employed as estimation method. Sigmoidal slope (n) was fixed to 3.
Table 7.4: Parameters for the pharmacokinetic/pharmacodynamic model of Bcl-2 antisense: gene expression model for Bcl-2 RNA and protein. The Maximum likelihood method was employed as the estimation method.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Estimates</th>
<th>CV (%)</th>
</tr>
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<tr>
<td>$k_{s1}$</td>
<td>% mRNA/hr</td>
<td>6.3</td>
<td>120</td>
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<tr>
<td>$k_{s2}$</td>
<td>% protein/hr</td>
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<td>$k_{d1}$</td>
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<td>$k_{d3}$</td>
<td>hr$^{-1}$</td>
<td>0.0672</td>
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<td>$k_{d4}$</td>
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<td>$k_a$</td>
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<td>0.2682</td>
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R-square: G3139: 0.980

Bcl-2 mRNA: 0.810

Bcl-2 protein: 0.957
<table>
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<tr>
<th>PD markers</th>
<th>Occasions</th>
<th>Median</th>
<th>25th and 75th percentile</th>
<th>P values $^a$</th>
<th>P values $^d$</th>
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<td>CR pt: Bcl-2 mRNA/cABL</td>
<td>Pretreatment (n=12)</td>
<td>85325 $^b$</td>
<td>61812, 120507.5</td>
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<td></td>
<td>Post-treatment (n=12)</td>
<td>52575</td>
<td>22157, 99012.5</td>
<td></td>
<td>0.002</td>
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<td>NR pt: Bcl-2 mRNA/cABL</td>
<td>Pretreatment (n=10)</td>
<td>32100</td>
<td>26590, 73877.5</td>
<td>0.109</td>
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<td>Post-treatment</td>
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<td>32080, 108635</td>
<td></td>
<td></td>
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<td>Responders: Bcl-2 protein</td>
<td>Pretreatment (n=9)</td>
<td>63.49$^c$</td>
<td>23.26, 86.92</td>
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<td>(negative 9/9)</td>
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<td>12.03, 72.04</td>
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<td>Pretreatment (n=6)</td>
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<td>Post-treatment</td>
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<td>8.38, 179.30</td>
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$^a$ sign test was performed to compare differences between the two variables and the differences of posttreatment from pretreatment were classified as either positive, negative, or tied. Negative difference indicates downregulation of Bcl-2 by treatment.

$^b$ Bcl-2 RNA levels were normalized to cABL copy numbers.

$^c$ Bcl-2 protein (unit/mg protein).

$^d$ % changes between CR and NR using Mann-Whitney test with two tailed.

**Table 7.5**: Summary of PD results using non-parametric statistical analysis
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>response</th>
<th>sample type</th>
<th>Bcl-XL mRNA (% of control)</th>
<th>MCI-1 mRNA (% of control)</th>
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<tr>
<td>SC</td>
<td>IR</td>
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<tr>
<td>EB</td>
<td>NR</td>
<td>BM</td>
<td>207.37</td>
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<tr>
<td>EB</td>
<td>NR</td>
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<td>157.87</td>
<td>100</td>
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<tr>
<td>WR</td>
<td>NR</td>
<td>BM</td>
<td>9.73</td>
<td>16</td>
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<tr>
<td>CG</td>
<td>CR</td>
<td>BM</td>
<td>33.23</td>
<td>39</td>
</tr>
<tr>
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<td>CR</td>
<td>CD34</td>
<td>70.86</td>
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<tr>
<td>SW</td>
<td>NR</td>
<td>BM</td>
<td>84.81</td>
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</tr>
<tr>
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<td>79</td>
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<td>BM</td>
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<td>BM</td>
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<td>BM</td>
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<td>BM</td>
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<td>BM</td>
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<td>CR</td>
<td>BM</td>
<td>27.77</td>
<td>52</td>
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<td>CR</td>
<td>BM</td>
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<td>CR</td>
<td>BM</td>
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<td>BM</td>
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<td>BM</td>
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<td>0.14</td>
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**Table 7.6**: Summary of Bcl-2 related transcript changes after treatment with G3139
**Figure 7.1:** Baseline levels of Bcl-2 protein in different leukemic cell lines. Bcl-2 protein levels were quantified by a validated ELISA method (n=3).
Figure 7.2: Baseline levels of Bcl-2 protein in four different leukemic cell lines, K562, NB4, Kasumi-1, and ML-1. Intracellular Bcl-2 level was determined by flow cytometry analysis. Histograms were shown to compare difference in phycoerythrin (PE) positive cells.
Figure 7.3: Intracellular concentration-time profile of G3139 in NB4 cells following exposure to G3139 at 1 μM. Symbols represent observed concentrations. Error bars represents standard deviation (n=3).
Figure 7.4: Scheme of Pharmacokinetic/Pharmacodynamic relationship of G3139 in vitro. A) the PD model was an indirect response model with removal of the response variable (Bcl-2 mRNA) stimulated by concentration of G3139. B) the PD model was an indirect response model with inhibition of production of the response (Bcl-2 protein).
Figure 7.5: Pharmacokinetic/Pharmacodynamic relationship of G3139 following exposure of 1 μM in vitro. A) A semi-logarithmic plot of the fitted curve and real data (n=3) of intracellular G3139 concentration-time profile. B) PD model was an indirect response model with removal of the response variable (Bcl-2 mRNA) stimulated by concentration of G3139 intracellularly.
Figure 7.6: Pharmacokinetic/Pharmacodynamic relationship of G3139 following exposure of 1 µM in vitro. A) A semi-logarithmic plot of the fitted curve and real data (n=3) of intracellular G3139 concentration-time profile. B) PD model was an indirect response model with inhibition of production of the response (Bcl-2 protein).
Figure 7.7: Dynamic changes of Bcl-2 mRNA as a function of the turnover rates of mRNA. Level of Bcl-2 mRNA was calculated as a function of the turnover rate based on the proposed PD model shown in Figure 7.4. Simulation was performed by altering the values of $k_{in}$ and $k_{out}$ but keeping the ratio of $k_{in}/k_{out}$ the same, assuming mRNA levels remaining at the steady state concentrations.
Figure 7.8: Dynamic changes of Bcl-2 mRNA as a function of exposure. Levels of Bcl-2 mRNA were calculated as a function of exposure based on the proposed PD model shown in Figure 7.4. Simulation was performed by altering exposures from 0.33 to 10 µM.
Figure 7.9: Pharmacokinetic/pharmacodynamic relationship of G3139. (A) Kinetics of G3139 following different exposure intervals ranging from 12 to 48 hr. (B) Dynamic change of Bcl-2 mRNA as a function of exposure interval. Level of Bcl-2 mRNA was calculated based on the proposed PD model (Figure 7.4). Simulation was performed by altering exposure interval from 12hr to 48 hr.
Figure 7.10: Dynamic change of Bcl-2 protein as a function of dosing interval. Level of Bcl-2 protein was calculated based on the proposed PD model (Figure 7.4). Simulation was performed by altering dosing interval from 36 hr to 72 hr.
Figure 7.11: Scheme of mechanistic Pharmacokinetic/Pharmacodynamic relationship of G3139 in vitro. A1: Bcl-2 mRNA; A2: Bcl-2 protein; A3: G3139 intracellular concentration; A4: duplex of G3139 to Bcl-2 mRNA; A5: dosing compartment. The corresponding rate constants are designated as $k_{s1}$, $k_{d1}$, $k_{d2}$, $k_{d3}$, $k_{d4}$, $k_1$. 

---

**DNA**  
$K_{s1}$  
$K_a$  
**RNA**  
$K_{d1}$  
**Antisense**  
$K_{d3}$  
$K_1$  
**duplex**  
$K_{d4}$  
**Protein**  
$K_{d2}$  
**Amino-acyl tRNAs**  
$K_{s2}$  
**Degradation by RNase H**  
$A_{d2} + T_{d2}$  
**Extracellular**  
**Intracellular**
**Figure 7.12:** Pharmacokinetic/Pharmacodynamic relationship using the gene perturbation model following exposure of 1 µM. A) plot of the fitted curve and real data (n=3) of intracellular G3139 concentration-time profile. B) Plot of the fitted curve and real data of Bcl-2 mRNA-time profile. (C) Plot of the fitted curve and real data of Bcl-2 protein-time profile.
Figure 7.12: Continued

C

![Graph showing Bcl-2 mRNA (% of control) over time (hr). The graph compares observed and predicted protein levels.](image-url)
Figure 7.13: Dynamic change of Bcl-2 mRNA (A) and protein (B) as a function of dosing interval. Level of Bcl-2 mRNA/protein was calculated based on the proposed PD model (Figure 7.11). Simulation was performed by altering dosing interval from 24 hr to 48 hr.
Figure 7.14: The box plot of intracellular G3139 against responders/non-responders (CR/NR). Median IC of G3139 in CR pts (n=11) was higher than that in non-responders (n=8) with p=0.06. Dot in NR patients represents an outlier in this group.
Figure 7.15: Preferential uptake of G3139 by proliferating cells: Unmanipulated BM MNC (empty bars), CD34 positive (slash bars) and CD34 negative (brick bars) were obtained from AML patients following 3 days G3139 CIVI and intracellular concentration (IC) of G3139 were measured in duplicate by the hybridization ELISA assay. In 4 of 5 patients, ICs of G3139 were higher in CD34-positive blasts than in the remaining CD34-negative cells and BM samples, suggesting site-preferable uptake behavior.
Figure 7.16: A) Representative amplification plots of Bcl-2 standard cDNA. B) A calibration curve of Bcl-2. The Ct values were plotted against log quantity of Bcl-2 cDNA (copy number). A good correlation was observed and the calibration curve was used to calculate the absolute quantity of Bcl-2 transcript in unknown samples.
Figure 7.17: A) Representative amplification plot of cABL standard cDNA. B) calibration curve of cABL. The Ct values were plotted against log quantity of ABL cDNA (copy number). The calibration curve was used to calculate the absolute quantity of ABL transcript in unknown samples.
Figure 7.18: Vertical Scatter plots of Bcl-2 mRNA as function of response. Bcl-2 mRNA levels were quantified by realtime RT-PCR and normalized to cABL copy numbers. Mann-Whitney test showed percentage of Bcl-2 down-regulation was significantly higher in CR than that in nonresponders (p=0.002).
Figure 7.19: A) Bcl-2/Bax protein quantification by immunoblotting. Bcl-2 levels in Patient KF before treatment and at day 4 prior to chemotherapy are shown. B) Bcl-2 levels in patient WC before treatment and at day 4 prior to chemotherapy are shown. Bcl-2 protein was decreased in these two patients.
Figure 7.20: Vertical Scatter plots of Bcl-2 protein levels as function of response. Bcl-2 levels were quantified by ELISA and expressed as units/mg protein. Wilcoxon Mann-Whitney test showed percentage of Bcl-2 downregulation was significantly higher in responders than that in nonresponders (p<0.05).
Figure 7.21: Correlation of Bcl-2 protein levels (% of baseline) with IC of G3139 (pmole/mg protein). Triangle represents NR patients and open circle are CR patients. Although a significant correlation was not observed between Bcl-2 down-regulation and IC of G3139 due to the small sample size (p=0.10), it appears that a threshold for IC of G3139 might exist, since only those patients who achieved ≥ 5 pmole/mg had reduced Bcl-2 protein.
Figure 7.22: Vertical Scatter plots of Bcl-2 protein in mononuclear cells (PBMC) as function of response. Bcl-2 levels were quantified by ELISA and expressed as units/mg protein. PBMC offer an alternative way to evaluate pharmacological effect of drugs and have been widely used as a surrogate pharmacodynamic marker. In 10 paired evaluable PBMC specimens, Bcl-2 decrement was observed in 5 of 6 CR patients while only 2 NR patients had decreased Bcl-2 levels. However, there was no statistical significance due to small sample size.
Figure 7.23: Vertical Scatter plots of Bcl-Xl mRNA in bone marrow as function of response. (A) Bcl-Xl levels were quantified by real time RT-PCR and normalized to cABL. (B) Bcl-Xl levels were measured by custom microarray. Bcl-Xl increments occurred in 6 of 10 patients in the NR population while it only occurred in 3 of 13 CR patients. The median change in Bcl-Xl in CR and NR group was 53.6 % and 163 %, respectively. Nonparametric test showed a difference in Bcl-Xl changes between these two groups (p=0.050, "Mann-Whitney test). Microarray data corroborated the results of real-time RT-PCR.
REFERENCE FOR CHAPTER 7


CHAPTER 8

CONCLUSIONS AND FUTURE PERSPECTIVES

A novel, nonradioactive, two-step hybridization ELISA method has been developed and validated for quantification of G3139 in a variety of matrixes. Its utility has been demonstrated in the disposition studies of G3139 in animal and clinical trails. With the design of appropriate capture and probe oligonucleotide sequences, and optimization of experimental condition, this assay has been successfully applied in assessment of plasma and intracellular levels of G3139 in animals and patients. This assay might be generally applicable in quantification of antisense compounds in a variety of biological matrices.

Cellular uptake and distribution of G3139 was studied in leukemic cells K562. When exposed to 0.33-10 \( \mu \)M of G3139, K562 cells exhibited intracellular concentrations (ICs) of G3139 in the range of 2.1-11.4 pmole/mg protein, which was only 0.2-0.6% of the total exposed drug. As a result, no significant suppression of Bcl-2 mRNA was observed with free drug. In contrast, a 10-25 fold increase of the ICs was observed when G3139 was delivered with cationic lipids. Down-regulation of Bcl-2 mRNA occurred efficiently and correlated with intracellular concentration of G3139 with IC\(_{50}\) of 37 pmole/mg protein. Robust ICs of G3139 were achieved \textit{in vivo} in bone marrow (BM) and peripheral blood mononuclear cells (PBMC) from AML patients treated with G3139.
CIVI. Two *in vitro* PK/PD models were developed to describe the dynamics of Bcl-2 mRNA/protein as well as kinetics of G3139 in AML cells. However, a single exposure was performed in this PK/PD effort. Other exposure such as 0.33, 3.3 and 10 µM need to be done to fully characterize the underline relationship between ICs and Bcl-2 reduction. More interestingly, similar experiments need to be performed in tumor cells obtained from leukemia patients *in vitro*. In this regard, transfection is probably not needed since we have shown that tumor cells obtained from patients took up G3139 more efficiently than cell lines (Chapter 3). The PK/PD results obtained from this study might be directly extrapolated to clinical study. On the other hand, the mechanistic based PK/PD model proposed was rather complex and prior information from literature or experimental measurement is needed for future investigation.

The combination strategy for G3139 with HDAi such as SAHA or MS275 appear to be promising based on our preliminary results. Our results suggest that SAHA interacts synergistically with G3139 to block cell proliferation and induce apoptosis. Low dose G3139 combined with low dose SAHA warrants further mechanistic studies and clinical investigation in AML. Some important questions need to be addressed in the future investigation are: (1) what are the major biological determinants of apoptosis induced by G3139 and SAHA or MS275? (2) what are the downstream events after induced apoptosis or cell differentiation? (3) whether chemosensitization effect is Bcl-2 dependent or independent. Specifically, we will use small interfering RNA targeting to human Bcl-2 (Chapter 1) at appropriate concentrations to produce specific and stable knockdown of Bcl-2 protein in Kasumi-1 and NB4 cells. The cells will be subject to
treatment of SAHA or MS275. Downstream apoptotic events, such as caspase 3, 8 activities and cleavage of poly(ADP-ribose) polymerase (PARP) will be measured and compared between different treatment groups.

Plasma pharmacokinetics of G3139 in treated AML patients was modeled as a two-compartment open infusion model with first-order elimination from the central compartment. Since most pharmacokinetic parameter estimates are associated with large variations, the sources of variability of pharmacokinetic parameters need to be identified in the future investigation. A population pharmacokinetic model will be proposed to identify important clinical characteristics, which might influence the exposure of G3139 in AML patients. The population pharmacokinetic model will then be used to simulate drug exposure based on those significant clinical covariates in the ongoing phase III study of G3139 in AML patients and provide the pharmacokinetics input for the PK/PD modeling (see below).

Correlation between the Bcl-2 mRNA/protein down-regulation and disease response was established (Chapter 7). Although a correlation between ICs of G3139 and Bcl-2 downregulation was not observed, it appears that a threshold for IC of G3139 existed, since only patients who achieved $\geq 5$ pmole/mg had reduced Bcl-2 protein. However, whether there is threshold value for IC of G3139 in AML can only be answered in a larger clinical study. It is widely recognized that Bcl-2 is not an easy-accessible biomarker and quantitative measurement could vary with conditions for tumor procurement. Our results derived from two phase I studies provided useful procedures
and assay platform for the handling and quantification of Bcl-2 and related biomarkers, that may be useful for ongoing phase III studies of G3139. While efforts have been made to quantify IC of G3139, the Bcl-2 mRNA/protein levels in previous two phase I studies, there was little information available to fully describe the PK/PD relationships in treated AML patients. In the ongoing phase III study, we propose to collect multiple biological specimens including bone marrow and PBMC at different time points during and post administration of G3139. The expression levels of Bcl-2 mRNA and protein will be quantified and the indirect response PK/PD model will then be used to explore the relationship between drug exposure and pharmacological response(s) (Bcl-2 down-regulation). A population PK/PD model will be developed using NONMEM (Version 5) and S plus (Version 6.2). More interestingly, the clinical outcomes for other prospective trials can be simulated from an exposure-response model based on this phase III study.

Several chain-shortened metabolites of G3139 were identified in various species including mice, rats and humans, indicating similarity in G3139 metabolism across species. It is likely that metabolism of G3139 in vivo is primarily through cleavages mediated by 3’ exonuclease. The pharmacological importance of these metabolites has yet to be evaluated in future study and the exact nature of the metabolism needs to be ascertained. Further, other metabolism mediated by 5’-exonuclease and endonuclease might exist in organ tissues and remains to be answered. A higher dose and longer duration of administration might be necessary to detect these metabolites.
In summary, this dissertation investigated cellular uptake, PK/PD correlation, disposition and metabolism of Bcl-2 antisense G3139. The results generated from these studies have addressed a couple of major issues in the field of antisense therapeutics. However, future studies as proposed above are needed to better define the therapeutic role of this novel drug in AML.


Hsu JC (1996) Multiple comparisons with a control, in Multiple Comparisons: Theory and Methods pp 43-78, CRC Pr LLC.


induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells. *Leukemia* 18:1207-1214.


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APPENDIX A:

COMPUTER PROGRAMS USED FOR FITTING OF

*IN VITRO* PK/PD MODEL OF BCL-2 MRNA IN ADAPTII
Subroutine DIFFEQ(T,X,XP)
Implicit None
Include 'globals.inc'
Include 'model.inc'
Real*8 T,X(MaxNDE),XP(MaxNDE)
C--------------------------------------------------------------------------------------------C
C 1. Enter Differential Equations Below  {e.g.  XP(1) = -P(1)*X(1) }  
C----c-----------------------------------------------------------------C
XP(1) = -P(6)*X(1)
XP(2) = P(6)*X(1)/P(2)-P(1)*X(2)
XP(3) = P(3)- (P(3)/IC(3))* (1 + P(5)*X(2)**P(7)/(P(4)**P(7)+X(2)**P(7)) )*X(3)
C######################################################################
Subroutine AMAT(A)
Implicit None
Include 'globals.inc'
Include 'model.inc'
Integer I,J
Real*8 A(MaxNDE,MaxNDE)
DO I=1,Ndeqs
  Do J=1,Ndeqs
    A(I,J)=0.0D0
  End Do
End Do
C----------------------------------------------------------------------C
C 2. Enter non zero elements of state matrix  {e.g.  A(1,1) = -P(1) }  C
C----c-----------------------------------------------------------------C
Return
End
C######################################################################
Subroutine OUTPUT(Y,T,X)
Implicit None
Include 'globals.inc'

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Include 'model.inc'
Real*8 Y(MaxNOE),T,X(MaxNDE)
C--------------------------------------------------------------------------------
C 3. Enter Output Equations Below {e.g. Y(1) = X(1)/P(2) } C
C--------------------------------------------------------------------------------
Y(1) = X(2)
Y(2) = X(3)
C--------------------------------------------------------------------------------
C--------------------------------------------------------------------------------
C######################################################################
Subroutine SYMBOL
Implicit None
Include 'globals.inc'
Include 'model.inc'
C----------------------------------------------------------------------
C 4. Enter as Indicated C
C--------------------------------------------------------------------------------
NDEqs   =  3  ! Enter # of Diff. Eqs.
NSParam =  7  ! Enter # of System Parameters.
NVparam =  4  ! Enter # of Variance Parameters.
NSecPar =  2  ! Enter # of Secondary Parameters.
NSecOut =  0   ! Enter # of Secondary Outputs (not used).
Ieqsol  =  1  ! Model type: 1 - DIFFEQ, 2 - AMAT, 3 - OUTPUT only.
Descr   = 'Bcl2.FOR: PK- 1 comp., PD- IndirectResponseM stimi.'
C----------------------------------------------------------------------
C 4. Enter Symbol for Each System Parameter (eg. Psym(1)='Kel') C
C--------------------------------------------------------------------------------
Psym(1) = 'Kel'
Psym(2) = 'V'
Psym(3) = 'Kin'
Psym(4) = 'EC50'
Psym(5) = 'Emax'
Psym(6) = 'ka'
Psym(7) = 'hill slope'

C--------------------------------------------------------------------------------
C 4. Enter Symbol for Each Variance Parameter {eg: PVsym(1)='Sigma'} C
C--------------------------------------------------------------------------------
PVsym(1) = 'SDinter1'
PVsym(2) = 'SDslope1'
PVsym(3) = 'SDinter2'
PVsym(4) = 'SDslope2'

C--------------------------------------------------------------------------------
C 4. Enter Symbol for Each Secondary Parameter {eg: PSsym(1)='CLt'} C
C--------------------------------------------------------------------------------

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PSsym(1) = 'CL'
PSsym(2) = 'Kout'

C******************************************************************************
C******************************************************************************
Return
End
C******************************************************************************
C******************************************************************************
Subroutine VARMOD(V,T,X,Y)
  Implicit None
  Include 'globals.inc'
  Include 'model.inc'
  Real*8 V(MaxNOE),T,X(MaxNDE),Y(MaxNOE)
C******************************************************************************
C 5.    Enter Variance Model Equations Below                               C
C         {e.g. V(1) = (PV(1) + PV(2)*Y(1)**2 }                        C
C----c-----------------------------------------------------------------C
  V(1) = (PV(1) + PV(2)*Y(1)**2
  V(2) = (PV(3) + PV(4)*Y(2)**2
C******************************************************************************
C******************************************************************************
Return
End
C******************************************************************************
C******************************************************************************
Subroutine PRIOR(Pmean,Pcov,ICmean,ICcov)
  Implicit None
  Include 'globals.inc'
  Include 'model.inc'
  Integer I,J
  Real*8 Pmean(MaxNSP+MaxNDE), ICmean(MaxNDE)
  Real*8 Pcov(MaxNSP+MaxNDE,MaxNSP+MaxNDE), ICcov(MaxNDE,MaxNDE)
APPENDIX B:

COMPUTER PROGRAMS USED FOR FITTING OF

IN VITRO PK/PD MODEL OF BCL-2 PROTEIN IN ADAPTII
Subroutine DIFFEQ(T,X,XP)
Implicit None
Include 'globals.inc'
Include 'model.inc'
Real*8 T,X(MaxNDE),XP(MaxNDE)

C----------------------------------------------------------------------------------------C
C 1. Enter Differential Equations Below  {e.g. XP(1) = -P(1)*X(1) }        C
C----------------------------------------------------------------------------------------C
XP(1) = -P(5)*X(1)
XP(2) = P(5)*X(1)/P(2)-P(1)*X(2)
XP(3) = P(3)*(1-P(7)*X(2)**P(6)/(P(4)**P(6)+X(2)**P(6)))-P(3)/IC(3)*X(3)

C-------------------------------------------------------------------------------------------C
C    Return
End
C######################################################################C
Subroutine AMAT(A)
Implicit None
Include 'globals.inc'
Include 'model.inc'
Integer I,J
Real*8 A(MaxNDE,MaxNDE)

DO I=1,Ndeqs
  Do J=1,Ndeqs
    A(I,J)=0.0D0
  End Do
End Do

C----------------------------------------------------------------------C
C 2. Enter non zero elements of state matrix  {e.g. A(1,1) = -P(1) }  C
C----------------------------------------------------------------------C

Subroutine OUTPUT(Y,T,X)
Implicit None

Include 'globals.inc'
Include 'model.inc'

Real*8 Y(MaxNOE),T,X(MaxNDE)

C
C 3. Enter Output Equations Below \{e.g. Y(1) = X(1)/P(2) \}      C
C
Y(1) = X(2)
Y(2) = X(3)
C
Return
End

C

Subroutine SYMBOL
Implicit None

Include 'globals.inc'
Include 'model.inc'

C
C 4. Enter as Indicated                                      C
C
NDEqs   =  3  ! Enter # of Diff. Eqs.
NSParam =  7  ! Enter # of System Parameters.
NVparam =  4  ! Enter # of Variance Parameters.
NSecPar =  2  ! Enter # of Secondary Parameters.
NSecOut =  0  ! Enter # of Secondary Outputs (not used).
Ieqsol  =  1  ! Model type: 1 - DIFFEQ, 2 - AMAT, 3 - OUTPUT only.
Descr   = 'Bcl2.FOR: PK- 1 comp., PD- IndirectResponseM stimi.'
C
C 4. Enter Symbol for Each System Parameter (eg. Psym(1)='Kel') C
C
Psym(1) = 'Kel'
Psym(2) = 'V'
Psym(3) = 'Kin'
Psym(4) = 'IC50_protein'
Psym(5) = 'ka'
Psym(6) = 'hill slope_protein'
Psym(7) = 'Imax'

C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
CC
C----------------------------------------------------------------------C
C 4. Enter Symbol for Each Variance Parameter {eg: PVsym(1)='Sigma'}  C
C----c-----------------------------------------------------------------C
PVsym(1) = 'SDinter1'
PVsym(2) = 'SDslope1'
PVsym(3) = 'SDinter2'
PVsym(4) = 'SDslope2'
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
CC
C----------------------------------------------------------------------C
C 4. Enter Symbol for Each Secondary Parameter {eg: PSsym(1)='CLt'}    C
C----c-----------------------------------------------------------------C
PSsym(1) = 'CL'
PSsym(2) = 'Kout'
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
Return
End
C######################################################################C
Subroutine VARMOD(V,T,X,Y)
Implicit None
Include 'globals.inc'
Include 'model.inc'

Real*8 V(MaxNOE),T,X(MaxNDE),Y(MaxNOE)
C
C----------------------------------------------------------------------C
C 5. Enter Variance Model Equations Below                              C
C {e.g. V(1) = (PV(1) + PV(2)*Y(1))**2 }                              C
C----c-----------------------------------------------------------------C
V(1) = (PV(1) + PV(2)*Y(1))**2
V(2) = (PV(3) + PV(4)*Y(2))**2
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
Return
End
C######################################################################C
Subroutine PRIOR(Pmean,Pcov,ICmean,ICcov)
Implicit None
Include 'globals.inc'
Include 'model.inc'
Integer I,J
Real*8 Pmean(MaxNSP+MaxNDE), ICmean(MaxNDE)
Real*8 Pcov(MaxNSP+MaxNDE,MaxNSP+MaxNDE), ICcov(MaxNDE,MaxNDE)
C
C-----------------------------------------------------------------------C
C 6. Enter Nonzero Elements of Prior Mean Vector
C     { e.g. Pmean(2) = 10.0 }                                       C
C-----------------------------------------------------------------------C
C-----------------------------------------------------------------------C
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
C 6. Enter Nonzero Elements of Covariance Matrix (Lower Triang.)
C     { e.g. Pcov(2,1) = 0.25 }                                      C
C-----------------------------------------------------------------------C
C-----------------------------------------------------------------------C
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
C----------------------------------------------------------------------C
Return
End
C########################################################################

Subroutine SPARAM(PS,P,IC)
Implicit None

Include 'globals.inc'
Real*8 PS(MaxNSECP), P(MaxNSP+MaxNDE), IC(MaxNDE)
C
C-----------------------------------------------------------------------C
C 7. Enter Equations Defining Secondary Parameters
C     { e.g. PS(1) = P(1)*P(2) }                                    C
C-----------------------------------------------------------------------C
PS(1) = P(1)*P(2)
IF(IC(3).ne.0.0) PS(2) = P(3)/IC(3)
C-----------------------------------------------------------------------C
APPENDIX C:

COMPUTER PROGRAM USED FOR FITTING OF G3139 AND THREE METABOLITES IN WINNONLIN SOFTWARE
remark  ************************************************************************************
remark  Developer: DAVID DAI
remark  G3139 metabolite model to fit three met and G3139
remark  Model Date:  08-22-2004
remark  Model Version:  2.0
remark  ************************************************************************************
remark
remark - define model-specific commands
COMMANDS
 NFUNCTIONS 4
 NDERIVATIVES 5
 NPARAMETERS 8
 NCON 1
 P NAMES 'V1', 'V2', 'CLD', 'CLM', 'CLM1', 'CLM2', 'CLM3', 'V3'
 NSECONDARY 6
 S NAMES 'K12', 'K21', 'K13', 'K34', 'K45', 'K50',
 NFUN 5
END
remark - define temporary variables
TEMPORARY
  T=X
  DOSE=CON(1)
  V1=P(1)
  V2=P(2)
  CLD=P(3)
  CLM=P(4)
  CLM1=P(5)
  CLM2=P(6)
  CLM3=P(7)
  V3=P(8)
END
remark - define differential equations starting values
START
  Z(1) = DOSE/V1
  Z(2) = 0
  Z(3) = 0
  Z(4) = 0
  Z(5) = 0
END
remark - define differential equations
DIFFERENTIAL
  DZ(1) = -CLM*Z(1)/V1-CLD*Z(1)/V1+CLD*Z(2)/V1
  DZ(2) = CLD*Z(1)/V2-CLD*Z(2)/V2
  DZ(3) = CLM*Z(1)/V3-CLM1*Z(3)/V3
  DZ(4) = CLM1*Z(3)/V3-CLM2*Z(4)/V3
  DZ(5) = CLM2*Z(4)/V3-CLM3*Z(5)/V3
END
remark - define algebraic functions
FUNCTION 1
F = Z(1)
END
FUNCTION 2
F = Z(3)
END
FUNCTION 3
F = Z(4)
END
FUNCTION 4
F = Z(5)
END
FUNCTION 5
F = Z(2)
END
remark - define any secondary parameters
SECONDARY
K12 = CLD/V1
K21 = CLD/V2
K13 = CLM/V1
K34 = CLM1/V3
K45 = CLM2/V3
K50 = CLM3/V3
END
EOM
Appendix D:

DATA RELEVANT TO CHAPTER 6
Figure A.1: fitted Logarithm plasma concentration-time profile of G3139 following i.v. bolus administration of 20 mg/kg G3139 in rat.
Figure A.2: Fitted semi-logarithmic plot of plasma concentration-time profile of G3139 (open circle), 3’ N-1 (diamond), 3’ N-2 (square), 3’N-3 (down triangle) following iv bolus administration of 20 mg/kg G3139 in rats.
Figure A.2 (continue)

Rat 3

![Graph showing concentration over time for Rat 3]

Rat 4

![Graph showing concentration over time for Rat 4]

Continued
Figure A.2 (continue)

**Rat 5**

![Graph showing the concentration over time for Rat 5 with various observed and predicted lines for different conditions.](image-url)