Novel Antimitotic Compounds with Potent *In Vitro* and *In Vivo* Antitumor Effects: the Use of Pharmacokinetics, Metabolism, Efficacy, and Toxicity Studies

Dissertation

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

By

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ABSTRACT

We identified novel indole derivatives inhibiting human cancer cell growth with nanomolar IC₅₀ values. Thus, we studied the mechanism of anticancer effects, pharmacokinetics, in vitro hepatic metabolism, in vivo efficacy, and/or toxicity of lead compounds I-13 and I-19. They induced a down-regulation and/or an inactivation by hyper-phosphorylation of Bcl-2. They arrested cells in the G₂M phase and inhibited tubulin polymerization by binding to the colchicine-binding site.

The pharmacokinetics of the lead compound, I-19, in ICR mice was determined after i.v., i.p., and p.o. administrations. I-19 was slowly cleared and highly distributed in mice. I.p. administration of I-19 was absorbed quickly with 65-94% bioavailability. In the in vitro metabolic stability study using mouse, rat, dog, monkey, and human liver microsomes, I-19 was metabolized mainly via phase I pathway and the metabolite with a reduced ketone was the predominant form by human liver microsomes.

In the PC-3 xenograft models, I-13 (10 mg/kg, q2d, i.p.) and I-19 (10 mg/kg, q2w, i.p.) inhibited 58% and 68% tumor growth, respectively. Furthermore, since multidrug resistant (MDR) cell lines that over-expressed ATP-binding cassette (ABC) transporters such as P-glycoprotein were not resistant to indole compounds, in vivo efficacy of I-19 was examined in xenografts using MES-SA/DX5 cells over-expressing P-glycoprotein. I-19 (10 mg/kg, q2d, i.p.) was effective with 76% tumor growth inhibition in xenograft models using MES-SA or MES-SA/DX5 cells. In vitro studies of NGF-dependent neurite outgrowth in PC12 cells and in vivo
studies of mouse behavior showed that I-19 was less neurotoxic than vinblastine and vincristine, 
tubulin destabilizers with known neurotoxicity.

In conclusion, indole compounds inhibit tubulin polymerization via the colchicine 
binding site and circumvent P-glycoprotein-mediated MDR, suggesting that they may be new 
antimitotic agents for the treatment of patients with drug resistant cancer.
ACKNOWLEDGMENTS

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Finally, I would like to thank my mom, brothers, aunt, and my Happy Virus Friends, Choco, Sticky, and DJ YOU for their endless encouragement and enduring support.
VITA

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Publication


Fields of Study

Major Field: Pharmacy
Pharmaceutics; In Vitro and In Vivo Anticancer Drug Discovery
TABLE OF CONTENTS

Abstract ............................................................................................................................................... ii
Acknowledgments ........................................................................................................................... iv
Vita................................................................................................................................................... v
Table of Contents ............................................................................................................................ vii
List of Tables .................................................................................................................................... xii
List of Figures .................................................................................................................................... xiii
List of Abbreviations ....................................................................................................................... xvi
List of Cell lines by Diseases .......................................................................................................... xix

CHAPTER 1: Introduction; Preclinical and Clinical Discovery and Development of Antimitotic Agents Binding to the Colchicine Binding Domain .................................................. 1
1.1. Introduction ............................................................................................................................... 1
1.2. Antitumor Action of Tubulin-Binding Agents ......................................................................... 5
1.3. Antivascular Action of Tubulin-Binding Agents ................................................................... 6
1.4. Toxicity ..................................................................................................................................... 6
1.5. Resistance .................................................................................................................................. 7
1.6. Colchicine Domain-Binding Agents ......................................................................................... 7
   1.6.1. Colchicine Derivatives with Methoxyphenyl Group ......................................................... 8
   1.6.2. Steroid Derivatives ........................................................................................................ 22
   1.6.3. Others ............................................................................................................................ 23
1.7. Vinca and Taxane Domain-Binding Agents in Clinical Use .................................................. 30
   1.7.1. Vinca Alkaloids .............................................................................................................. 30
   1.7.2. Taxanes .......................................................................................................................... 30
   1.7.3. Ixabepilone .................................................................................................................... 31
1.8. Conclusion ................................................................................................................................. 33
CHAPTER 2: Novel Indole Structural Pharmacophores with Anticancer Activity ........35
2.1. Introduction............................................................................................................................. 35
  2.1.1 Indole Compounds in Cancer Treatment........................................................................ 35
  2.1.2 Cytotoxicity, In Vitro Screen Processes for Novel Anticancer Agents ......................... 37
  2.1.3 Apoptosis......................................................................................................................... 37
  2.1.4 Apoptosis and Cancer................................................. .................................................. 38
  2.1.5 Cell Cycle Regulation in Cancer.................................................................................... 38
2.2. Materials and Methods..................................................................................................... 41
  2.2.1 Chemicals and Materials .......................................................................................... 41
  2.2.2 Cell Culture .................................................................................................................. 41
  2.2.3 Growth Inhibition Assay............................................................................................ 41
  2.2.4 Determination of DNA Fragmentation by ELISA ......................................................... 42
  2.2.5 Cell Cycle Analysis.................................................................................................... 42
  2.2.6 In Vitro Tubulin Depolymerization Assay ................................................................. 42
2.3. Results..................................................................................................................................... 43
  2.3.1 The Effect of Indoles on Cell Proliferation ................................................................. 43
  2.3.2 Apoptotic Effect Induced by Indoles............................................................................ 46
  2.3.3 Indoles Induce G2M Phase Arrest ............................................................................ 49
  2.3.4 The Effect of Indoles on Microtubule Polymerization................................................. 49
2.4. Discussion......................................................................................................................... 54
2.5. Acknowledgements.......................................................................................................... 55

CHAPTER 3: A Novel Bis-Indole Destabilizes Microtubules and Displays Potent In Vitro and In Vivo Antitumor Activity in Prostate Cancer ........................................................................56
3.1. Introduction......................................................................................................................... 56
3.2. Materials and Methods................................................................................................. 59
  3.2.1 Chemicals and Animals.......................................................................................... 59
  3.2.2 Cell Culture ................................................................................................................ 59
  3.2.3 Growth Inhibition Assay............................................................................................ 60
  3.2.4 Determination of DNA Fragmentation by ELISA .................................................. 60
3.2.5 Cell Cycle Analysis........................................................................................................ 60
3.2.6 Western Blot Analysis .................................................................................................... 61
3.2.7 In Vitro Tubulin Depolymerization Assay ................................................................. 61
3.2.8 Indirect Immunofluorescence Microscopy ................................................................ 61
3.2.9 In Vitro Binding Assay by Spin Column ..................................................................... 62
3.2.10 In Vivo Antitumor Efficacy Study .............................................................................. 62
3.2.11 Statistical Analysis ..................................................................................................... 62

3.3. Results ................................................................................................................................ 63
3.3.1 The Effect of Indoles on Cell Proliferation ................................................................. 63
3.3.2 The Effect of I-13 on Growth of Cells that Over-express P-glycoprotein, MRPs, and
BCRP ........................................................................................................................................ 63
3.3.3 Apoptotic Effect Induced by I-13 ................................................................................ 64
3.3.4 Effect of I-13 in Cell Cycle Distribution ..................................................................... 68
3.3.5 The Effect of I-13 on Microtubule Polymerization ..................................................... 68
3.3.6 In Vitro Competition of Binding of Vinblastine or Podophyllotoxin to Tubulin by I-13
.................................................................................................................................................. 71
3.3.7 In Vitro Pharmacology of I-13 .................................................................................. 76

3.4. Discussion .......................................................................................................................... 80
3.5. Acknowledgements .......................................................................................................... 82

CHAPTER 4:  I-19 Displays a Potent In Vitro and In Vivo Antitumor Activity with Less
Neurotoxicity ............................................................................................................................. 83
4.1. Introduction ....................................................................................................................... 83
4.2. Materials and Methods .................................................................................................... 85
  4.2.1 Chemicals and Animals ............................................................................................... 85
  4.2.2 Cell Culture .................................................................................................................. 85
  4.2.3 Growth Inhibition Assay ............................................................................................. 86
  4.2.4 Determination of DNA Fragmentation by ELISA .................................................... 86
  4.2.5 Cell Cycle Analysis ..................................................................................................... 87
  4.2.6 Western Blot Analysis ................................................................................................. 87
  4.2.7 In Vitro Tubulin Polymerization Assay ..................................................................... 87
  4.2.8 In Vitro Binding Assay by Spin Column ..................................................................... 87
4.2.9 Indirect Immunofluorescence Microscopy ................................................................. 88
4.2.10 In Vivo Antitumor Efficacy Study ........................................................................... 88
4.2.11 In Vitro Neurite Outgrowth .................................................................................. 89
4.2.12 Rotarod Performance ............................................................................................. 89
4.2.13 Statistical Analysis ............................................................................................... 89
4.3. Results .......................................................................................................................... 90
  4.3.1 The Effect of I-19 on Cell Proliferation ................................................................. 90
  4.3.2 Apoptotic Effect Induced by I-19 ........................................................................... 92
  4.3.3 Effect of I-19 in Cell Cycle Distribution ............................................................... 92
  4.3.4 The Effect of I-19 on Microtubule Polymerization ................................................ 98
  4.3.5 Efficacy of I-19 in Tumor Xenograft Models ......................................................... 105
  4.3.6 Neurotoxicity Studies of I-19 ............................................................................... 109
4.4. Discussion .................................................................................................................... 112
4.5. Acknowledgements ...................................................................................................... 114

CHAPTER 5: Biotransformation of a Novel Antimitotic Agent, I-19, by Mouse, Rat, Dog, Monkey, and Human Liver Microsomes and In Vivo Pharmacokinetics in Mice ............... 115
5.1. Introduction .................................................................................................................... 115
5.2. Materials and Methods .............................................................................................. 117
  5.2.1 Chemicals, Microsome, and Animals .................................................................. 117
  5.2.2 Pharmacokinetic Studies of I-19 in Mice ............................................................. 117
  5.2.3 Sample Preparation for Mass Spectrometric Analysis of Mouse Plasma ............ 118
  5.2.4 Pharmacokinetic Data Analysis .......................................................................... 118
  5.2.5 In Vitro Metabolism Studies of I-19 ..................................................................... 119
  5.2.6 LC-MS/MS Analyses ......................................................................................... 119
5.3. Results ......................................................................................................................... 120
  5.3.1 Pharmacokinetics of I-19 in Mice ....................................................................... 120
  5.3.2 Metabolic Stability of I-19 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes ............................................................................................................. 121
  5.3.3 Identification of Metabolites in Human Liver Microsomal Incubation................. 126
  5.3.4 Metabolites of I-19 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes ................................................................. 129
5.4. Discussion .................................................................................................................... 133
LIST OF TABLES

Table 1.1. Microtubule destabilizers targeting colchicine domain in clinical development..... 26
Table 1.2. Microtubule destabilizers targeting colchicine domain in preclinical development 28
Table 1.3. Microtubule-targeted drugs approved for clinical uses in US ................................. 32
Table 2.1. Structures and cell growth inhibition of indole derivatives in prostate cancer cell
lines ........................................................................................................................................... 44
Table 2.2. Structures and cell growth inhibition of indole derivatives in prostate cancer cell
lines ........................................................................................................................................... 45
Table 2.3. IC₅₀ values of different cancer cell lines treated with lead compounds .................. 46
Table 3.1. IC₅₀ values of different cancer cell lines treated with I-13 by the SRB assay .......... 65
Table 3.2. Effect of I-13 and other agents on the growth of cells expressing ABC MDR
transporters ................................................................................................................................. 65
Table 4.1. Anticancer efficacy of I-19 in different cancer cell lines and MDR cell lines with
different resistance phenotypes ................................................................................................. 91
Table 4.2. IC₅₀ values of I-19 in PC-12 cell line ........................................................................... 109
Table 5.1. Time points for sample collection for pharmacokinetic studies in mice ................. 118
Table 5.2. Pharmacokinetics of I-19 in male ICR mice after i.v. administration ...................... 122
Table 5.3. Pharmacokinetics of I-19 in male ICR mice after i.p. administration ...................... 123
Table 5.4. Pharmacokinetics of I-19 in male ICR mice after p.o. administration .................... 124
Table 5.5. Half life of I-19 in mouse, rat, dog, monkey, and human liver microsomes .......... 125
Table 5.6. Summary of the MS2 metabolite profile generated following incubation of I-19
with liver microsomes of mouse, rat, dog, monkey, and human ........................................... 131
LIST OF FIGURES

Figure 1.1. Microtubule polymerization ................................................................. 3
Figure 1.2. Binding sites for antimitotic drugs ....................................................... 4
Figure 1.3. Colchicine derivatives: methoxyphenyl fused to cycloheptane rings ... 9
Figure 1.4. Colchicine derivatives: methoxyphenyl as a substituent of ethylene ... 13
Figure 1.5. Colchicine derivatives: methoxyphenyl as a substituent of various 5-membered rings ......................................................................................................................... 16
Figure 1.6. Colchicine derivatives: methoxyphenyl with ketone linkage .............. 17
Figure 1.7. Colchicine derivatives: methoxyphenyl with sulfonamide linkage ...... 21
Figure 1.8. Colchicine derivatives: methoxyphenyl with aniline linkage .............. 21
Figure 1.9. Colchicine derivatives: methoxyphenyl linked to chroman ring ......... 21
Figure 1.10. Steroid derivatives targeting colchicine domain .............................. 25
Figure 1.11. Other tubulin destabilizers targeting colchicine domain .................. 25
Figure 2.1. Natural source- or synthetic indoles .................................................. 36
Figure 2.2. Anticancer agents targeting cell cycle regulation .............................. 40
Figure 2.3. Cytoplasmic histone-associated DNA fragments by lead compounds in PC-3 cells .......................................................... 47
Figure 2.4. EC₅₀ values of lead compounds to induce cytoplasmic histone-associated DNA fragments in PC-3 cells .......................................................... 48
Figure 2.5. Dose-response curves of compounds in cellular arrest in the G₂M phase .... 50
Figure 2.6. EC₅₀ values of compounds in cellular arrest in the G₂M phase ........... 51
Figure 2.7. Dose-response of tubulin depolymerization by indoles ....................... 52
Figure 2.8. SAR of the tubulin antagonists ............................................................. 54
Figure 3.1. Chemical structure of I-13 ................................................................... 58
Figure 3.2. Cytoplasmic histone-associated DNA fragments by I-13 in LNCaP and PC-3 cells .......................................................................................................................... 66
Figure 3.3. Changes in the protein expression of PARP and Bcl-2 by I-13 ............. 67
Figure 5.4. Mean plasma concentration–time profiles following p.o. doses of I-19 in ICR male mice
Figure 5.5. Fragmentation pattern of I-19 in MS2 mode of m/z 388.2
Figure 5.6. Chromatograms of I-19 incubated with human liver microsomes in precursor ion of m/z 192 and neutral loss of 167 u scan modes
Figure 5.7. TIC chromatograms of extracted ions of I-19 parent and metabolites in human liver microsomes using product ion scan mode and their fragmentation patterns
Figure 5.8. TIC chromatograms of extracted ions of I-19 metabolites in mouse, rat, dog, monkey, and human liver microsomes using EPI scan (MS2) mode
Figure 5.9. The proposed metabolic schemes of I-19 in the mouse, rat, dog, monkey, and human liver microsomes
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>amu</td>
<td>Atomic mass unit</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-promoting complex/cyclosome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>Area under the plasma concentration-time curve from time zero to infinity</td>
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<tr>
<td>AUMC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>Area under the first moment curve extrapolated to infinity</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>BCRP</td>
<td>Breast cancer resistance protein</td>
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<tr>
<td>cGMP-PDE</td>
<td>Cyclic guanosine 3',5'-monophosphate phosphodiesterases</td>
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<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DLT</td>
<td>Dose-limiting toxicities</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPM</td>
<td>Disintegrations per minute</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
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<td>Fluorescence-activated cell sorting</td>
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<td>Guanosine-5'-triphosphate</td>
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<td>Hsp</td>
<td>Heat shock proteins</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>VDA</td>
<td>Vascular Disrupting Agent(s)</td>
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<td>$V_{dss}$</td>
<td>Apparent volume of distribution at equilibrium</td>
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<td>XTT</td>
<td>(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)</td>
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</tr>
<tr>
<td>MES-SA</td>
<td>Human uterine sarcoma</td>
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CHAPTER 1: INTRODUCTION; PRECLINICAL AND CLINICAL DISCOVERY AND DEVELOPMENT OF ANTIMITOTIC AGENTS BINDING TO THE COLCHICINE BINDING DOMAIN

1.1. Introduction

Microtubules are critically important components of the cytoskeleton. Microtubules are made up of αβ-tubulin heterodimers which assemble into linear protofilaments, which then bundle to form a hollow tube (Figure 1.1). These microtubules can grow and shrink by the addition at the (+) end or removal of αβ-tubulin heterodimers from the (-) ends of microtubules. The intrinsic dynamic equilibrium between microtubules and αβ-tubulin heterodimers play an essential role in the functional diversity of microtubules. Microtubules are involved in a variety of cellular processes such as cell division, maintenance of cell shape, cell signaling, cell migration, and cellular transport (Jordan and Wilson 2004).

Since microtubules form the mitotic spindle, which dictates the proper segregation of chromosomes during mitosis, antimitotic drugs are known as “spindle poisons”. Antimitotic agents interact with either the αβ-tubulin heterodimers or the polymerized microtubules and thereby interfere with the dynamic equilibrium (Figure 1.1). Interference of the polymerization by antimitotic drugs induces the dissolution of microtubules and impairs cellular functions dependent on microtubules (Dumontet and Sikic 1999). Cancer cells are relatively sensitive to these drugs compared to normal cells, due to the fact that cancer cells divide more frequently than normal cells and therefore pass more frequently through a stage of vulnerability to mitotic poisons.
The drugs targeting tubulin are commonly classified into two major categories: the microtubule-stabilizing agents and the microtubule-destabilizing drugs. The microtubule-destabilizing drugs inhibit microtubule polymerization and include several compounds such as the vinca alkaloids (vinblastine, vincristine, vinorelbine, vindesine, and vinflunine), cryptophycins, halichondrins, estramustine, colchicine, and combretastatins. The vinca alkaloids are effectively used to treat a wide variety of human cancers including hematological malignancies, breast, non-small-cell lung and testicular carcinoma (Kruczynski and Hill 2001; Duflos, Kruczynski et al. 2002). Colchicine is used clinically for gout, but is not used for cancer due to toxicity in clinical trials. The microtubule-stabilizing agents block microtubule depolymerization and include paclitaxel, docetaxel, the epothilones, discodermolide, eleutherobins, sarcodictyins, laulimalide, rhazinalam, and certain steroids and polyisoprenyl benzophenones. Paclitaxel is widely used clinically for ovarian, breast, and non-small-cell lung carcinomas and Kaposi’s sarcoma (MedlinePlus Drug Information: Paclitaxel Injection 2008). Docetaxel, a taxane analog, was the first approved chemotherapy for the treatment of advanced prostate cancer by the US Food and Drug Administration (FDA) in 2004. However, the tubulin inhibitors in human chemotherapy have limitations such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance by MDR transporters and tubulin mutations. In order to combat these issues, there are active efforts to develop the next-generation of antimitotic agents.

In this chapter, novel compounds inducing tubulin depolymerization by binding to the colchicine binding site were introduced (Figure 1.2). The mechanism, toxicity, as well as resistance of microtubule inhibitors are discussed and we summarize preclinical and clinical achievements of tubulin depolymerizers binding to the colchicine binding sites. Since there is an active ongoing effort to develop new agents in this class, this review will be helpful as a foundation to development and design of novel tubulin inhibitors targeting colchicine site.
Figure 1.1. Microtubule polymerization. The αβ-tubulin heterodimers form a short microtubule nucleus. Each microtubule is elongated at both ends and makes a cylinder form with a so-called plus (+) end and a minus (-) end. The (+) end is composed of β-tubulin facing the solvent, and the (-) end with α-tubulin faces the solvent (Jordan and Wilson 2004).
Figure 1.2. Binding sites for antimitotic drugs. The vinca alkaloids bind in vinblastine domain (V) and suppress microtubule dynamics. The vinblastine site is at the surface exposed at the plus end of a microtubule. The colchicine domain (C) is at the intra-dimer interface and, Colchicine forms complexes with tubulin dimers and copolymerizes into the microtubule lattice, suppressing microtubule dynamics. Most microtubule stabilizers are known to bind in the taxane site (T) facing the lumen of the microtubule like the colchicine site. Nucleotides are indicated at the exchangeable (E) and nonexchangeable (N) sites (Downing 2000; Jordan and Wilson 2004).
1.2. Antitumor Action of Tubulin-Binding Agents

The antitumor effects of any tubulin-binding agent are dependent on several sequential cellular events. First, the drug binds to tubulin. In doing so, the drug disrupts the microtubule network and arrests cells in mitosis. Ultimately, the cell cycle arrest induces apoptotic signaling pathways.

Three main binding domains have been identified; vinca domain, taxane domain, and colchicine domain (Figure 1.2). Vinca alkaloids bind to tubulin with high affinity at the microtubule surface that is exposed at (+) ends and adjacent to the exchangeable Guanosine-5'-triphosphate (GTP)-binding site (Jordan 2002). The vinca alkaloids can also bind with considerably lower affinity to tubulin sites located along the sides of the microtubule cylinder (Jordan 2002). The taxane domain is in the middle of the β monomer in the inner face of the microtubule wall (Jordan 2002). The colchicine site is located at the intra-dimer interface toward the lumen of the microtubule, similar to the taxane domain (Downing 2000). In addition to these three binding sites, some tubulin-binding agents such as laulimalide are known to bind distinct sites (Pryor, O’Brate et al. 2002).

Immunofluorescence studies showed that microtubule destabilizers induce depolymerization of the microtubule network resulting in short microtubule fragmentations or tubulin paracrystals (Jordan, Thrower et al. 1991). Stabilizers such as taxanes result in the stabilization of microtubules with an increase in the apparent density of microtubules. In addition, the formation of aneuploid or polyploid cells has also been reported (Stavrovskaya and Kopnin 1975; Verdoodt, Decordier et al. 1999). The disruption of the microtubule network by tubulin-binding agents is known to inhibit spindle microtubule dynamics, thus causing improper attachment of chromosomes which impairs the kinetochore tension and in turn the signal for chromosome segregation. As such, interference with spindle formation inhibits transition from metaphase to anaphase resulting in cell arrest at the G2M phase. The G2M arrest results in apoptosis, but the link between microtubule network disruption and apoptosis induction is not fully understood.
1.3. Antivascular Action of Tubulin-Binding Agents

In addition to their ability to inhibit tumor cell growth, tubulin-binding agents have generated considerable interest due to potential tumor-selective antivascular activity including antiangiogenic and vascular-disrupting properties (Schwartz 2009). Low molecular weight drugs with rapid tumor selective vascular disrupting properties are categorized as vascular disrupting agents (VDAs) and microtubule destabilizers are considered as the largest family of VDAs. Although the cellular and molecular mechanisms for these actions are not clearly defined, the disruption of endothelial adherent junctions is considered as one of the main mechanisms of VDAs. The fragile and immature nature of tumor blood vessels is hypothesized to be the reason for the selective effects of these agents on tumors (Tozer, Kanthou et al. 2005). Low concentrations (non-toxic doses) of VDAs showed inhibition of endothelial cell proliferation, migration, and cell-cell interaction in vitro, which is related to the therapeutic antiangiogenic actions. In addition, they promoted rapid collapse in tumor blood flow (Tozer, Prise et al. 2001; Tozer, Kanthou et al. 2005).

1.4. Toxicity

In spite of their clinical success, toxicity and resistance have limited the effectiveness of antimitotic agents. Since tubulin-binding agents affect normal cells as well as tumor cells, reversible myelosuppression is a commonly observed side effect. However, peripheral neurotoxicities are considered to be more problematic due to permanent nerve damage during the clinical application of antimitotic agents (Rowinsky, Eisenhauer et al. 1993). Chemotherapy-induced neuropathy is often the dose-limiting factor in cancer therapy. It evokes an array of clinical symptoms: numbness and tingling, mechanical allodynia, cold allodynia, and ongoing burning pain in patients (Windebank and Grisold 2008). Little is known about the mechanism by which antimitotic agents affect neuronal tissue, in spite of a great number of in vitro and in vivo studies. Under normal circumstances, antimitotic agents rarely cross the blood-brain barrier, but do have better access to the peripheral nervous system (Greig, Soncrant et al. 1990; Ziske, Schottker et al. 2002). Thus, peripheral neurotoxicity tends to be produced rather than central neurotoxicity. Peripheral neuropathy is caused by morphologic or functional abnormalities in
peripheral nerves. Animal studies show swollen axons, changes in the density of microtubules within axons and Schwann cells, and abnormalities in the myelin sheath and internodes (Topp, Tanner et al. 2000; Flatters and Bennett 2006). Therefore, much research remains to be done to understand the role of tubulin in neurotoxicity and improve the safety of cancer treatment with tubulin-binding agents.

1.5. Resistance

Since resistance to tubulin-binding agents is one of the major problems in clinic, investigations into the mechanisms mediating multifactorial resistance and strategies to improve the efficacy by avoiding resistance are ongoing. Tubulin-binding agents enter cells by crossing the cell membrane. Thus, efflux transporters in cancer cell membranes like P-glycoprotein, MRP, and BCRP can induce drug resistance by pumping drugs out before they reach their cellular targets. Tubulin mutations, altered tubulin isotype expression, changes in microtubule regulatory proteins, and altered microtubule dynamics can also inhibit drug-target interaction and result in resistance (Verrills and Kavallaris 2005; Seve and Dumontet 2008; Perez 2009). In addition, alterations in the expression or function of antiapoptotic and signaling factors may also lead to resistance to these agents (Verrills and Kavallaris 2005). Observations from clinical samples obtained from drug resistant tumors as well as data obtained using in vitro drug-resistant cancer cell models have identified numerous possible reasons for resistance to antimitotic agents (Perez 2009).

1.6. Colchicine Domain-Binding Agents

Colchicine was the first drug found to bind to tubulin. Colchicine itself is not a useful clinical anticancer agent due to a fairly narrow range of effectiveness as a chemotherapy agent, but it has played a critical role as a tubulin depolymerizer (Lettre and Lettre 1966). All of the compounds described below inhibit in vitro cell growth of human cancer cell lines and induce G2M phase cell arrest in the nanomolar range. In addition, all of them inhibit tubulin polymerization or suppress microtubule dynamics by binding to or near the colchicine site. Some
of these drugs have entered clinical trials, but none of them are currently approved for clinical use.

### 1.6.1. Colchicine Derivatives with Methoxyphenyl Group

#### 1.6.1.1. Methoxyphenyl Fused to Cycloheptane Rings

A water-soluble phosphate prodrug of N-acetylcolchicinol, ZD6126 (ANG453) is a novel vascular targeting agent that selectively disrupts the cytoskeleton of endothelial cells in tumor. Antivascular activity of ZD6126 was examined in a murine tumor model (CaNT) (Davis, Dougherty et al. 2002). These studies showed a large reduction in vascular volume, induction of extensive necrosis in tumors, and a reduced tumor cell yield in a clonal excision assay. ZD6126 inhibits cell proliferation in HUVEC at micro molar concentrations, while its IC\textsubscript{50} value of ZD6126 in a human pancreatic cancer cell, L3.6pl, was 1 mM (Kleespies, Kohl et al. 2005). In the L3.6pl xenograft models, 150 mg/kg ZD6126 (single doses, i.p.) resulted in extensive tumor necrosis after 24 h, while 75 mg/kg ZD6126 (qd5, 2 days off × 4, i.p.) was effective in tumor growth inhibition (TGI 50%). ZD6126 inhibited liver and lymph node metastases in a model using heterotopic implantation of L3.6pl cells into the spleen. The frequency of peritoneal carcinosis was also significantly decreased. Decrease of tumor neovascularization by ZD6126 (75 mg/kg, qd × 10, i.p.) was observed in dorsal skinfold chamber analysis with implanted L3.6pl cells. In other solid tumor models, tumor growth delay was determined by the difference in time taken for the mean tumor volume to double in treated vs. control animals (Blakey, Westwood et al. 2002). ZD6126 (100 mg/kg, qd × 5, i.p.) resulted in increased growth delays in both Hras5 (the ras-transformed mouse 3T3 fibroblasts) and Calu-6 tumors. A single dose of ZD6126 (200 mg/kg) led to a significant growth delay in Calu-6 and Lovo tumors (Blakey, Westwood et al. 2002). Daily dosing with ZD6126 (100 mg/kg, qd5, 2 days off × 4, i.p.) caused a significant inhibition of tumor growth (95% TGI) in an orthotopic model of gastric cancer using TMK-1 cells (McCarty, Takeda et al. 2004). The effect of ZD6126 in lung metastases was examined in mouse models established via tail vein injection of PC14PE6 or NCI-H226 cells (Goto, Yano et al. 2002). ZD6126 (single doses) induced the hemorrhage and necrosis of already established lung metastases. ZD6126 induced apoptosis in the endothelial cells in the tumor, but not in the normal lung parenchyma. In tumor, the effect of ZD6126 was more selective to endothelial cells
than to tumor cells. Continuous administration of ZD6126 reduced tumor burden in the lung, but no change was observed in the number of metastatic nodules. A comprehensive series of neurotoxicity tests were examined; functional observation battery, measurement of muscle strength (forelimb and hind limb grip strength), nociception (tail flick test), locomotor activity, neuropathology, and whole nerve electrophysiology (Horner, Gould et al. 2004). Sub-acute (0 to 20 mg/kg, qd5, 2 days off ×1, i.v.) and chronic (0 to 10 mg/kg, qd5, 2 days off × 5-6, i.v.) toxicity studies of ZD6126 showed no evidence of neurotoxicity in rats. However, it is important to note that the doses at which the neurotoxicity of ZD6126 were evaluated were substantially lower than the doses at which antitumor activity in xenograft models was demonstrated. Phase I clinical trials identified pulmonary embolus, left ventricular ejection fraction decrease, fatigue, gastrointestinal effects and cardiac toxicities as dose-limiting toxicities (DLT) (Beerepoot, Radema et al. 2006; LoRusso, Gadgeel et al. 2008). Two Phase II clinical trials for metastatic cancer have been terminated or suspended.

Figure 1.3. Colchicine derivatives: methoxyphenyl fused to cycloheptane rings.
1.6.1.2. Methoxyphenyl as a Substituent of Ethylene Bridge

Combretastatins, natural product analogs of colchicine, were isolated from the South African willow tree *Combretum caffrum* (Hamel and Lin 1983). A number of these analogs have been investigated to date. The sodium-phosphate salt forms of combretastatin analogs such as CA4P (Combretastatin A-4 phosphate, CA4DP, Fosbretabulin, UNII-I5590ES2QZ, Zybrestat) and Oxi4503 (Combretastatin A-1 Phosphate) have been examined in preclinical and clinical studies against a wide range of cancers due to improved solubility (Dowlati, Robertson et al. 2002; Rustin, Galbraith et al. 2003; Stevenson, Rosen et al. 2003; Cooney, Radivoyevitch et al. 2004; Young and Chaplin 2004; Bilenker, Flaherty et al. 2005; Yeung, She et al. 2007; Koh, Blackledge et al. 2009; Meyer, Gaya et al. 2009). CA4P induced apoptosis via a mitochondria-dependent and a caspase-dependent manner, down-regulated VCAM-1 and accumulated reactive oxygen species (ROS) without incurring hematologic toxicity in acute myeloid leukemias (AMLs) (Petit, Karajannis et al. 2008). CA4P-induced cytotoxicity was also mediated by inhibition of the phosphoinositide-3 kinase/AKT pathway (Lin, Chiou et al. 2007). In animal xenograft studies, CA4P and combinations of CA4P with other agents were effective in a variety of tumors including medullary and anaplastic thyroid carcinoma, SW1222, LoVo, RIF-1, LS174T, Kaposi's sarcoma, CaNT, ES-2, TOV-21G, TOV-112D, Hey, SKOV-ip, Caki-1, HL-60, and MDA-MB-231 (Beauregard, Hill et al. 2001; Nelkin and Ball 2001; Pedley, Hill et al. 2001; Dziba, Marcinek et al. 2002; Hill, Chaplin et al. 2002; Li, Rojiani et al. 2002; El-Emir, Boxer et al. 2005; Salmon, Mladinich et al. 2006; Staflin, Jarnum et al. 2006; Petit, Karajannis et al. 2008; Siemann and Shi 2008; Zhao, Richer et al. 2008). CA4P inhibited gastric tumor metastasis (Lin, Chiou et al. 2007). In addition, tumor cell immunization with low-dose CA4P caused enhanced tumor growth inhibition and antitumor immune reactivity (Badn, Kalliomaki et al. 2006). Bowel ischemia, tumor pain, vagal syncope, motor neuropathy, reversible ataxia, cardiac ischemia, and dyspnea were the reported DLT in clinical trials of CA4P (Dowlati, Robertson et al. 2002; Rustin, Galbraith et al. 2003; Stevenson, Rosen et al. 2003). Phase II and III studies to assess the safety and efficacy of CA4P in combination with paclitaxel and carboplatin against anaplastic thyroid carcinoma are ongoing (Yeung, She et al. 2007). Phase II trials for the combination of other agents with CA4P against naïve non-small cell lung cancer and for single treatment of C4AP in Asian subjects with polypoidal choroidal vasculopathy are currently recruiting participants. Furthermore, the combination of CA4P and bevacizumab will be examined for recurrent high-grade gliomas in a Phase I trial.
Oxi4503 (Combretastatin A-1 Phosphate), an ortho-quinone prodrug, is a highly potent VDA. Oxi4503 was more potent at reducing the functional vascular volume in tumor tissue and inhibiting tumor growth than C4AP in a wide variety of xenograft models (Hill, Toze et al. 2002; Hua, Sheng et al. 2003; Salmon, Mladinich et al. 2006; Salmon and Siemann 2006; Malcontenti-Wilson, Chan et al. 2008; Dalal and Burchill 2009). Several preclinical studies showed that Oxi4503 selectively targets tumor blood vessels (Sheng, Hua et al. 2004; Chan, Malcontenti-Wilson et al. 2008). A recent study using Oxi4503 suggested G-CSF as a possible therapeutic target of re-growth of tumor following VDA treatment (Shaked, Tang et al. 2009). Although Oxi4503 induced significant tumor destruction, combination with cytotoxic and/or antiangiogenic agents was also suggested in order to achieve complete tumor regression and prolonged survival (Chan, Malcontenti-Wilson et al. 2007; Chan, Malcontenti-Wilson et al. 2008; Malcontenti-Wilson, Chan et al. 2008; Siemann and Shi 2008; Daenen, Shaked et al. 2009; Dalal and Burchill 2009). A Phase I dose-escalation study is ongoing to determine tolerability of Oxi4503 in patients with advanced solid tumors, and a Phase I/II clinical trial to examine the safety and toxicity of Oxi4503 in subjects with relapsed or refractory carcinomas with hepatic tumor burden is recruiting participants.

AVE8062 (AC7700, ombrabulin) is a prodrug, the serine of which is cleaved by aminopeptidases. AVE8062 reduced tumor tissue blood flow in a dose-dependent manner through an indirect effect on tumor vessels (Hori, Saito et al. 1999; Hori and Saito 2003). AVE8062 (10 mg/kg, q3d × 7, i.v.) resulted in inhibition of solid tumor growth (LY80 and Sato lung carcinoma) and lymph node metastases without signs of toxicity (Hori, Saito et al. 1999; Hori, Saito et al. 2002; Ohno, Kawano et al. 2002). In addition, AVE8062 showed a combination effect with cisplatin and docetaxel (Morinaga, Suga et al. 2003; Kim, Ravoori et al. 2007). Currently, several clinical trials to evaluate AVE8062 against solid tumors and sarcoma are recruiting participants. Transient myocardial ischemia, asymp hypotension, and transient neurological symptoms were reported as DLT in a phase I clinical trial.

Indanocine (Leoni, Hamel et al. 2000), a synthetic indanone, showed cell growth inhibition in MCF-7, MES-SA, MDA-MB-321, HL-60, CEM, KB-3-1, and MV522 which IC₅₀ values ranging from 7 to 85 nM. Nanomolar concentrations of indanocine induced apoptosis in MDR expressing cells. Indanocine-resistant cells showed a single point mutation in a region close to the putative colchicine-binding site and were cross-resistant to colchicine and vinblastine, but
not to paclitaxel (Hua, Genini et al. 2001). The expression of P-glycoprotein was not observed in those cells.

Sulindac sulfone derivatives including CP-248 (OSIP486823), CP-461 (OSI-461), and OSIP487703 were potent in human colon adrenocarcinoma or glioma cell growth inhibition at nanomolar concentrations (Yoon, Palazzo et al. 2002; Xiao, Deguchi et al. 2006). They target cyclic guanosine 3',5'-monophosphate phosphodiesterases (cGMP-PDE) and caused an increase in intracellular levels of cGMP, indicating that the cGMP-dependent enzyme protein kinase G was activated. Activated PKG triggered pathways to growth inhibition and apoptosis in cancer cells. CP-248 and CP-461 were also effective in prostate cancer cells (Lim, Piazza et al. 1999; Lim, Piazza et al. 2003). They caused a decrease in cellular levels of the prostate-specific antigen and androgen receptor proteins and inhibited the transcriptional activity of the promoter region of the AR gene in human prostate cancer cells (Lim, Piazza et al. 2003). A number of Phase I or Phase II trials for the safety and efficacy of CP-461 (p.o.) have been conducted in different kinds of cancer patients (Small, Halabi et al. 2003; Bayes, Rabasseda et al. 2004; O'Bryant, Lieu et al. 2009).

CC-5079 (Zhang, Wu et al. 2006) was cytotoxic in a variety of cancer cells including HT-29, HT-144, HCT 116, A549, NIH:OVCAR-3, PC-3, HCT-15, MCF7, and MES-SA with IC50 values from 4 to 50 nM. CC-5079 showed no resistance via P-glycoprotein. It caused elevation of MPM-2, cyclin B1, and p53 and induced phosphorylation of cdc25C and Bcl-2. In addition, tumor necrosis factor-α secretion was inhibited from lipopolysaccharide-stimulated human peripheral blood mononuclear cells. In vivo treatment with CC-5079 (25 mg/kg, qd × 5 and changed to q3d × 4, i.p.) showed 46% TGI in HCT-116 xenografts. In addition, the antiangiogenic effect of CC-5079 was examined in chorioallantoic membrane and rat aortic ring assays and CC-5079 stimulated mitogen-activated protein kinase (MAPK) phosphatase 1 expression in HUVEC and fibroblast (Vu, Miller et al. 2009).
Figure 1.4. Colchicine derivatives: methoxyphenyl as a substituent of ethylene.
1.6.1.3. Methoxyphenyl as a Substituent of Various 5-membered Rings

T115 (Arora, Wang et al. 2009) was cytotoxic in MCF7, BC-19, A2780, KB-3-1, PC-3, and P388S cell lines with low nanomolar IC\textsubscript{50} values (2.1-21 nM). In normal cells including GM 05659 and FBCL, however, IC\textsubscript{50} values were >10µM. It was also cytotoxic in cells over-expressing P-glycoprotein, multi-drug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP) at low nanomolar concentrations. T115 also inhibited tumor growth in mouse xenograft models bearing HT-29 and PC-3 xenografts with 50% TGI (90 mg/kg, q2d × 4, i.p.).

JG-03-14 (Arthur, Gupton et al. 2007; Mooberry, Weiderhold et al. 2007; Dalyot-Herman, Delgado-Lopez et al. 2009) showed potential antiangiogenic and vascular-disrupting activity. JG-03-14 inhibited endothelial cell migration and tube formation at non-cytotoxic concentrations and increased the permeability of the endothelial cell layer. JG-03-14 treatment resulted in abnormally elongated adherent junctions, reduced VE-cadherin phosphorylation, and disrupted VE-cadherin/b-catenin complexes. JG-03-14 was cytotoxic in MDA-MB-435, HeLa, PC-3, and DU-145 cells with nanomolar IC\textsubscript{50} values (36-80 nM). JG-03-14 was also potent in cells over-expressing P-glycoprotein. JG-03-14 induced phosphorylation of Bcl-2.

KRIBB3 (Shin, Lee et al. 2005; Shin, Yoon et al. 2008) was potent in cell growth inhibition in cancer cells including HCT 116, HCT-15, SW620, HCA-7, NCI-H23, DU-145, PC-3, SK-OV-3, and HeLa with IC\textsubscript{50} values ranging from 300 to 800 nM. Elevation of cyclin B1, inhibition of Mad2 with p55CDC (mammalian homologue of CDC20), and activation of APC/C (anaphase-promoting complex/cyclosome) suggests that the mitotic spindle checkpoint was activated by KRIBB3. KRIBB3 inhibited tumor cell migration and invasion by blocking protein kinase C-dependent phosphorylation of Hsp27. An in vivo efficacy study using doses of 50 mg/kg and 100 mg/kg of KRIBB3 (qd × 16, i.p.) inhibited the growth of subcutaneous HCT-116 xenografts by 50% and 70%, respectively.

A-105972, an oxasolidine analog, and its modified derivatives showed cytotoxic activity against a variety of cancer cell lines (Szczepankiewicz, Liu et al. 2001; Wu-Wong, Alder et al. 2001). A-105972, the lead compound, inhibited human cancer cell proliferation with low IC\textsubscript{50} values (3-158 nM) in A549, COLO 205, HCT-15, HL-60, HS 578T, HT-29, HT-1080, LNCaP, MCF-7, MDA-MB-468, NCI-H226, NCI-H460, and PC-3 cells. Evaluation by the NCI in a panel of 60 human cancer cell lines showed that A-105972 exhibited a significant growth inhibition efficacy in most cancer cell lines except OVCAR-4, OVCAR-5, and TK-10. The binding site of
A-105972 likely partially overlaps the colchicine-binding site, but is not identical. The potency of A-105972 was not affected by the level of P-glycoprotein, MRP, and LRP (lung resistance protein) and was also independent of the status of p53 protein. A-105972 also induced phosphorylation of Bcl-2. A-105972 (25 mg/kg, qd × 9, i.p.) increased the life span in mouse models inoculated with B16, P388, and Adriamycin-resistant P388.

A-204197 (Tahir, Han et al. 2001), another oxazolidine analog, was cytotoxic in MDR cells as well as H462 cells with IC_{50} values ranging from 36 to 48 nM. Cell cycle arrest in mitosis by A-204197 was evaluated examining the effects of A-204197 on phosphorylation of cell cycle signaling molecules. As a result, A-204197 induced accumulation of cyclin B1, active Cdc2 kinase and phosphorylation of Cdc25C, and MPM-2 epitopes in vitro.

A-289099, an oxazole analog, was also a very potent inhibitor of cancer cell growth regardless of P-glycoprotein expression (Li, Woods et al. 2002; Tahir, Nukkala et al. 2003). The S-isomer, A-289099, was found to be significantly more potent than the R-isomer. The range of IC_{50} values of S-isomer was from 6 to 13 nM in H460/T200, HCT-15, NCI-H460, A549, HCT-116, DLD-1, MDA-MB-231, and U-87-MG cell lines, which were comparable to combretastatin A4 and up to 74-fold more potent than the R-isomer. Overall, the two enantiomers had similar pharmacokinetic properties with oral bioavailabilities ranging from 7 to 44 % in mouse, rat, dog, and monkey. A-289099 showed approximately five times better oral bioavailability than A-105972 in rat. The bioavailability of A-289099 in monkey was 19%. Oral administration of A-289099 (50 mg/kg, qd × 12) showed potent efficacy (TGI 89%) in M5076 xenograft model.

NSC 676693 (Lisowski, Leonce et al. 2004), an analog of thienopyrrolizinones, was cytotoxic in L1210, B16, OVCAR3, A2780, DU-145, A549, HT-29, LS174T, and P388 cells with low IC_{50} values (15-887 nM). The IC_{50} of NSC 676693 in resistant KBA1 cells with MDR was 15 nM. In a mouse xenograft study in which introduced by i.p. injection of P388 cells, efficacy was examined via median survival time. The result is considered as significant when ratio T/C (%) is above 125%. T/C of NSC 676693 (25-400 mg/kg, once, i.p.) was from 108 to 121%. I.v. injection of NSC 676693 (25-400 mg/kg, once) was not effective with T/C values of 19-102% due to poor aqueous solubility.
Figure 1.5. Colchicine derivatives: methoxyphenyl as a substituent of various 5-membered rings.
1.6.1.4. Methoxyphenyl with Ketone Linkage

The indole-based agent, BpR0L075 (Kuo, Hsieh et al. 2004; Liou, Mahindroo et al. 2006), displayed potent cytotoxicity against a number of human cancer cells including KB, HONE-1, HT-29, DBTRG, MCF-7, MKN-45, Hep G2, and CEM with IC₅₀ values in the single-digit nanomolar range. However, the IC₅₀ value in fibroblast Detroit 551 cells was >1000 nM. This result showed the selectivity of BpR0L075 between normal and cancer cells. BpR0L075 was also cytotoxic in cells over-expressing P-glycoprotein and MRP. BpR0L075 induced Bcl-2 phosphorylation. In the cell cycle signaling pathway, cyclin B1, pCdc25C, and MPM2 were increased and phosphocdc2 was decreased by BpR0L075. To evaluate the in vivo efficacy of BpR0L075, mouse xenograft models bearing KB, MKN-45, and KB-VIN10 cells were used. In all xenograft models, 50 mg/kg BpR0L075 (qd5, 2 days off, i.v.) showed tumor growth inhibition (TGI 82%) without significant body weight loss. Additionally, the effects of BpR0L075 toward MDR xenograft using the KB-VIN10 cell line were notable.

![BpR0L075](image)

**Figure 1.6. Colchicine derivatives: methoxyphenyl with ketone linkage.**
1.6.1.5. Methoxyphenyl with Sulfonamide Linkage

ABT-751 (E 7010), a novel orally active antimitotic agent, is currently in Phase I and/or II clinical development. Previously *in vitro* cell growth experiments showed IC\textsubscript{50} values ranging from 0.6 to 4.6 mM in neuroblastoma cell lines including RD, TC-71, LD, Daoy, HOS, SK-N-AS, SK-N-DZ, and KCNR (Meany, Sackett et al. 2010). ABT-751 was effective in a variety of preclinical xenograft models including NSCLC, colon cancer, acute lymphocytic leukemia, neuroblastoma, osteosarcoma, Ewing sarcoma, rhabdomyosarcoma, medulloblastoma, and kidney cancer lines (Edelman 2006; Apostolidou, Swords et al. 2007; Morton, Favours et al. 2007). In addition, ABT-751 enhanced efficacy of cisplatin, 5-FU, and radiation in the Calu-6 NSCLC, HT-29 colon, and HCT-116 colon carcinoma xenograft models (Jorgensen, Tian et al. 2007). ABT-751 further showed a potent VDA by reducing tumor blood flow and no significant effects on normal vascular function in the intact rat at doses of 30 mg/kg (Segreti, Polakowski et al. 2004). Clinical trials (Phase I or II) have been completed against advanced lung, colorectal, renal cell, and recurrent breast cancer. Trials are currently ongoing in patients with refractory solid tumors, neuroblastoma that relapsed or not responded to previous treatment, and metastatic prostate cancer that did not respond to hormone therapy (Fox, Maris et al. 2010; Meany, Sackett et al. 2010; Michels, Ellard et al. 2010; Yee, Hagey et al. 2005; Fox, Maris et al. 2006; Hande, Hagey et al. 2006; Fox, Maris et al. 2008; Mauer, Cohen et al. 2008). Fatigue, constipation, and dehydration were the only treatment related, grade 3 adverse events. ABT-751 was not associated with myelosuppression (Mauer, Cohen et al. 2008).

An aminoindole derivative, J30 (Chang, Hsieh et al. 2006; Liou, Hsu et al. 2007) inhibited cancer cell proliferation at nanomolar concentrations (IC\textsubscript{50} values; 16-23 nM) in KB, HONE-1, MKN-45, A-498, Hep3B, NIH-H460, HT-29, DBTRG, and TSGH cells. J30 showed cytotoxic efficacy against resistant sublines expressing P-glycoprotein or MRP. J30 increased levels of MPM-2 and cyclin B1, induced phosphorylation of Cdc25C and dephosphorylation of Cdc2. J30 (20 mg/kg, qd5, 2 days off × 2, p.o.) showed tumor growth suppression (41-49% TGI) in xenograft models using KB-VIN10 and KB and MKN-45 cells.
1.6.1.6. Methoxyphenyl with Aniline Linkage

MPC-6827 (Azixa) (Kasibhatla, Baichwal et al. 2007) was cytotoxic at low nanomolar concentrations in MCF-7, MX-1, and MB-MDA-435, HT-29, MIA PaCa-2, OVCAR-3, and B16 cancer cells. MPC-6827 induced cell death via the intrinsic apoptotic pathway and avoided resistance mediated by P-glycoprotein, MRP-1, and BCRP. In a number of in vivo xenograft models, MPC-6827 was effective at inhibiting tumor growth. In OVCAR-3 xenografts, MPC-6827 (5 mg/kg, q3d × 4, i.v. and 7.5 mg/kg, q1w × 3, i.v.) showed 88% TGI and 95% TGI, respectively. MPC-6827 (5 mg/kg, q1w × 3, i.v.) was more effective in MX-1 (96% TGI) than in MCF-7 (53% TGI) xenograft. In the xenograft model bearing B16-F1, MPC-6827 (q1w × 2, i.v.) resulted in 67% TGI. Administrations of MPC-6827 (5 mg/kg, q1w × 7, i.v.) for seven weeks were effective in MDA-MB-435 xenograft (71% TGI). Phase I trials of MPC-6827 have been completed in patients with refractory brain metastases and refractory solid tumors. Two Phase I clinical trials are currently ongoing to evaluate effects of MPC-6827 alone or in combination with other anticancer agents with MPC-6827 against recurrent glioblastoma multiforme and metastatic melanoma. A Phase II trial is currently recruiting participants for recurrent glioblastoma multiforme.

MPI-0441138 (EP128265) (Sirisoma, Kasibhatla et al. 2008) was also cytotoxic in cells over-expressing P-glycoprotein as well as in T47D, HCT-116, and P388 cancer cells with single nanomolar IC$_{50}$ values. MPI-0441138 (10 mg/kg, q1w × 2, i.v.) inhibited tumor growth to 95% and 66% in MX-1 and PC-3 xenograft models, respectively. 2.5mg/kg MPI-0441138 (qd5, 2 days off × 2, i.v.) showed 90% TGI in the MX-1 model. The maximally tolerated dose (MTD) of MPI-0441138 was 25 mg/kg (q1w, i.v.). MPI-0441138 penetrated through the blood-brain barrier (BBB) significantly.

The oral alternative of MPC-6827, MPI-0443803 was reported in an AACR 2009 poster presentation. MPI-0443803 was soluble in 5% dextrose:water and a half life of (60 mg/kg, p.o., not fasted) approximately 10 h in male Nu/+ mice. MPI-0443803 crossed the blood brain barrier and distributed rapidly into the CNS with exposure in the brain approximately 7 times higher than in plasma after oral or i.v. dosing. In the xenograft model bearing B16-F0 tumors, MPI-0443803 (50 mg/kg, qd × 5, p.o. and 200 mg/kg, q1w × 2, p.o.) inhibited tumor growth significantly.
1.6.1.7. Methoxyphenyl Linked to Chroman Ring

EPC2407 (Crinobulin, MX-116407) is a novel VDA and apoptosis inducer for the treatment of patients with advanced solid tumors and lymphomas (Gourdeau, Leblond et al. 2004; Cai 2007). In T47D, DLD-1, and NCI-H1299 cells, IC$_{50}$ values were 33-170 nM and EPC2407 induced phosphorylation of Bcl-2. It was also potent in cells over-expressing P-glycoprotein. In addition, EPC2407 showed vascular disruption activity at 11-42 nM. In nude mice, MTD (single i.v. dose) was 25 mg/kg with body weight loss (<20%). EPC2407 (45 mg/kg, qd5, 2 days off × 3, i.v.) resulted in 57% TGI in MDA-MB-435 xenograft. EPC2407 (45 mg/kg, qd5, 2 days off × 6, i.v.) showed approximately 86% TGI in Calu-6 xenograft. In MX-1 xenograft model, tumor regression was observed by EPC2407 (45 mg/kg, qd5, 2 days off × 2, i.v.) with 93% TGI. EPC2407 caused no thrombocytopenia, leukopenia, or neutropenia at any dose studied and no renal or hepatic dysfunction in preclinical studies. A Phase I trial has been completed to evaluate the safety, pharmacokinetics, and pharmacodynamic effects of EPC2407 for advanced cancer in 2009.
Figure 1.7. Colchicine derivatives: methoxyphenyl with sulfonamide linkage.

Figure 1.8. Colchicine derivatives: methoxyphenyl with aniline linkage.

Figure 1.9. Colchicine derivatives: methoxyphenyl linked to chroman ring.
1.6.2. Steroid Derivatives

2-ME (2-Methoxyestradiol, 2-ME2, 2-MEO, 2-Methoxyestradiol-17beta, NSC 659853, Panzem), an estrogen metabolite, is an antimitotic and antiangiogenic drug candidate in Phase I and II clinical trials for the treatment of a variety of tumor types (Lakhani, Sarkar et al. 2003; Dahut, Lakhani et al. 2006; James, Murry et al. 2007). Capsule and nanocrystal colloidal dispersion formulations of 2-ME also have been evaluated in Phase I and II clinical trials (Sweeney, Liu et al. 2005; Rajkumar, Richardson et al. 2007; Matei, Schilder et al. 2009; Tevaarwerk, Holen et al. 2009). DLT included fatigue, hypophosphatemia, increased alanine aminotransferase, and muscle weakness (Tevaarwerk, Holen et al. 2009). In vitro, 2-ME inhibits proliferation of many cancer and endothelial cell lines in the nanomolar to low micromolar ranges. I.p. or oral administration of 2-ME was efficacious for many types of xenografts including myeloma, head and neck, breast, doxorubicin-resistant breast, and cervical tumors with lack of toxicity (Brueggemeier, Bhat et al. 2001; Dingli, Timm et al. 2002; Tinley, Leal et al. 2003; Ricker, Chen et al. 2004; Li, Da et al. 2005; Sutherland, Schuliga et al. 2005; Azab, Salama et al. 2008). In addition, 2-ME is known to have therapeutic potential in rheumatoid arthritis based on its antiangiogenic, antiinflammatory, and antiosteoclastic properties. In preclinical models, 2-ME significantly delayed the onset and reduced the development of clinical and radiographic type II collagen-induced arthritis (Josefsson and Tarkowski 1997). 2-ME inhibited the expression of synovial VEGF and fibroblast growth factor-2 with parallel reduction of synovial blood vessels (Brahn, Banquerigo et al. 2008). An investigational new drug trial for 2-ME in rheumatoid arthritis has been accepted by FDA and a Phase I clinical trial of 2-ME in health volunteers was completed in 2008.

ENMD-1198 (Tinley, Leal et al. 2003; LaVallee, Burke et al. 2008), an analog of 2-ME, was cytotoxic in a number of human cancer cells including HUVEC, MDA-MB-231, LLC, U-87-MG, PC-3, and HT-29 with nanomolar IC\textsubscript{50} values (130-440 nM). ENMD-1198 does not exhibit sensitivity to MDR mechanisms in preclinical studies. In LLC metastasis model, ENMD-1198 (200 mg/kg, p.o.) treatment groups survived longer than vehicle-control group. In addition, in the mouse xenograft model bearing MDA-MB-231 cells, ENMD-1198 (200 mg/kg, p.o.) resulted in 77\% TGI. Recently a Phase I clinical trial of ENMD-1198 was completed to evaluate safety, tolerability, pharmacokinetics, and clinical benefit in advanced cancer patients (Zhou, Gustafson et al.). Oral treatment was well tolerated in the clinical study.
1.6.3. Others

IRC-083927 (Liberatore, Coulomb et al. 2008) is a compound without a phenyl methoxy group developed by Ipsen Inc. It was very potent against a number of human cancer cells including MIA PaCa-2, MDA-MB-231, DU-145, NCI-H69, and A549 with low IC₅₀ values (7-15 nM). It was also cytotoxic in MDR cells over-expressing P-glycoprotein and MRP as well as cells resistant to epothilone B or paclitaxel (A549.EpoB40 and A549. EpoB480). A549.EpoB40 and A549. EpoB480 cells are known to have mutated α- and/or β-tubulins. In addition, antiangiogenic effects in endothelial cell and HUVEC cell morphology were demonstrated with IRC-083927.

IRC-083927 (5 mg/kg, qd5, 2 days off × 4, p.o.) inhibited 60% tumor growth in C-33A xenograft model. In MEA-MB-231 xenograft studies, IRC-083927 (5 mg/kg, qd5, 2 days off × 4, p.o.) was effective as a higher dose of ABT-751 (100 mg/kg, qd5, 2 days off × 4, p.o.) resulting in 80% TGI.

A vascular targeting agent, NPI-2358 (KPU-2, Plinabulin) (Nicholson, Lloyd et al. 2006; Yamazaki, Kohno et al. 2008) is a halimide derivative from a marine microbial source. NPI-2358 showed potent in vitro activity with IC₅₀ values of 5 to 20 nM in several human cancer cell lines. NPI-2358 was active in MDR cell lines including taxol-resistant cell lines. NPI-2358 increased HUVEC monolayer permeability in a dose-dependent manner, which is an in-vitro model of tumor vascular collapse. In a xenograft model established using HT-29 and MDA-MB-231 cells, NPI-2358 (7.5 mg/kg, q3d × 5, i.p.) inhibited tumor growth by 37% and 53%, respectively. NPI-2358 entered a Phase I clinical trial to examine the safety, pharmacokinetics, and pharmacodynamics in patients with refractory solid tumors or lymphoma. A Phase II clinical trial is currently recruiting participants to evaluate NPI-2358 combined with docetaxel in patients with advanced non-small cell lung cancer.

XRP44X (Wasylyk, Zheng et al. 2008) is a potent in inhibitor of cell growth in vitro. The range of IC₅₀ values was 2 to 11 nM in PC-3, A549, SK-MEL-5, HeLa, NCI-H460, HCT-116, NCI-H1299, MDA-MB-231, SW480, HUVEC, NIH 3T3, HCT-116, and NIH3T3–Ki-Ras cells. XRP44X showed similar effects as combretastatin-A4 on inhibition of FGF-2 Ras-Net signaling as well as on the cytoskeleton. In addition, XRP44X inhibited aortic microvessel sprouting.

CYT997 (Burns, Fantino et al. 2009; Burns, Harte et al. 2009; Monaghan, Khong et al. 2009) showed vascular disrupting activity as well as tumor growth inhibition. In vitro cancer cell growth studies showed that CYT997 was cytotoxic in DU-145, PC-3, A549, Ramos, KHOS/NP,
A-375, HCT-15, HT-1376, BT-20, A-431, PA-1, U-937, HepG2, TF-1, Baf3/TelJAK2, and K-562 cells with nanomolar IC₅₀ values (9-93 nM). CYP997 induced phosphorylation of Bcl-2, activated caspases-3, and increased the level of cyclin B1. The vascular disrupting activity was measured by effects on the permeability of *in vitro* HUVEC monolayer and by effects on *in vivo* tumor blood flow. Oral bioavailability of CYT997 was 78%. Thus, *in vivo* tumor growth inhibition of CYT-997 (p.o.) was observed in PC-3 and 4T1 xenograft models. In the PC-3 model, CYP997 (25-30 mg/kg, qid) resulted in approximately 70% TGI. In 4T1 xenograft, three different regimens of CYT997 (30 mg/kg, qd; 70 mg/kg q1w, 80 mg/kg, q2w) showed approximately 60% TGI. Since CYT997 was effective against multiple myeloma (Monaghan, Khong et al. 2009), a Phase I clinical trial to evaluate safety and activity of CYT997 against multiple myeloma is ongoing. Furthermore, Phase I and II trials for the efficacy study of CYT997 in combination with carboplatin in glioblastoma are currently recruiting participants. In a Phase I trial in patients with advanced cancer, CYT997 was well tolerated (poster, ASCO 2008).

CHM-1 (Hsu, Yang et al. 2009) was identified as a potent and selective antitumor agent in human hepatocellular carcinoma. CHM-1 induced growth inhibition of HA22T, Hep3B, and HepG2 cells with IC₅₀ values of approximately 750 nM. CHM-1 treatment resulted in cell cycle arrest at G₂/M phase by activating cdc2/cyclin B1 complex activity. CHM-1 did not modulate the caspase cascade, but induced the translocation of apoptosis-inducing factor from mitochondria to the nucleus. In the HA22T xenograft, CHM-1 (10 mg/kg, q2w × 4, i.p.) showed approximately 70% TGI. For *in vivo* antitumor activity assay, HA22T cells were injected i.p. into male severe combined immunodeficient mice on day 0 and CHM-1 (10 mg/kg, q2w × 4, i.p.) was injected on day 1. CHM-1 prolonged the lifespan; T/C (life span) value was 167%.

A novel benzimidazole carbamate, MN-029 is a VDA. MN-029 (100 mg/kg, i.p.) showed a significant suppression of functional vessel and secondary tumor cell necrosis (approximately 90%) in KHT sarcomas (Shi and Siemann 2005; Traynor, Gordon et al. 2009). In clinical studies, the most common side effects of MN-029 were nausea, vomiting, hypotension, fatigue and diarrhea. The DLT was acute coronary ischemia possibly due to coronary vasospasm in a Phase I clinical trial in patients with advanced solid tumors (poster, ASCO 2006).
Figure 1.10. Steroid derivatives targeting colchicine domain.

Figure 1.11. Other tubulin destabilizers targeting colchicine domain.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Phase</th>
<th>Status in clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZD-6126</td>
<td>Astra Zeneca</td>
<td>I, II</td>
<td>Two Phase II clinical trials for metastatic renal cell and colorectal cancer have been terminated and suspended, respectively.</td>
</tr>
<tr>
<td>CA4P</td>
<td>Oxigene</td>
<td>I, II, III</td>
<td>Completed Phase I/II trials of CA4P only or combined with other drugs or radiation for advanced solid tumors, advanced anaplastic thyroid cancer Currently in a Phase I trial for advanced solid tumorsCurrently in Phase II and III trials to assess the safety and efficacy of CA4P in combination with paclitaxel and carboplatin against anaplastic thyroid carcinomaPhase II trials for the combination of other agents with CA4P against naïve non-small cell lung cancer and for single treatment of CA4P in Asian subjects with polypoidal choroidal vasculopathy are currently recruiting participants for a Phase II trial.The combination of CA4P and bevacizumab will be examined for recurrent high-grade gliomas in a Phase I trial.</td>
</tr>
<tr>
<td>Oxi4503</td>
<td>Oxigene</td>
<td>I, II</td>
<td>Currently in Phase I trials for advanced solid tumors Currently in Phase I/II clinical trial for relapsed or refractory carcinomas with hepatic tumor burden is recruiting participants</td>
</tr>
<tr>
<td>AVE8062</td>
<td>Sanofi-Aventis</td>
<td>I, II, III</td>
<td>Currently six clinical trials are recruiting participants for sarcoma and solid tumors.</td>
</tr>
<tr>
<td></td>
<td>icals</td>
<td></td>
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</tr>
<tr>
<td>ABT-751</td>
<td>Abbott</td>
<td>I, II</td>
<td>Completed trials against advanced lung cancer, colorectal cancer, renal cell cancer, and recurrent breast cancer after taxane therapy. Terminated trials for advanced lung cancer, relapsed pediatric acute lymphoblastic leukemia, and refractory hematologic malignancies. Currently in Phase I and/or II trials for refractory solid tumors, neuroblastoma that has relapsed or not responded to previous treatment, and metastatic prostate cancer that did not respond to hormone therapy.</td>
</tr>
<tr>
<td>MPC-6827</td>
<td>Myraid</td>
<td>I, II</td>
<td>Completed Phase I trials for refractory brain metastases and refractory solid tumors. Currently in two Phase I clinical trials against recurrent glioblastoma multiforme and metastatic melanoma. A phase II trial is currently recruiting participants for recurrent glioblastoma multiforme.</td>
</tr>
<tr>
<td>EPC2407</td>
<td>EpiCept</td>
<td>I</td>
<td>Completed a trial for advanced cancer (September 2009).</td>
</tr>
<tr>
<td>Drug</td>
<td>Company</td>
<td>Phase</td>
<td>Status in clinical trials</td>
</tr>
<tr>
<td>-----------</td>
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<tr>
<td>2-ME</td>
<td>EntreMed</td>
<td>I, II</td>
<td>Completed Phase II and I trials for multiple myeloma and advanced solid tumors, respectively.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Completed Phase II trials of 2-ME NCD for glioblastoma, metastatic breast, ovarian, hormone-refractory prostate and renal cell cancer carcinoid cancer, advanced cancer (No more after 2009 Dec).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Completed Phase I/II trials of 2-ME Capsule for multiple myeloma, advanced solid, prostate, and metastatic breast cancer.</td>
</tr>
<tr>
<td>ENMD-1198</td>
<td>EntreMed</td>
<td>I</td>
<td>Completed a Phase I trial for advanced cancer.</td>
</tr>
<tr>
<td>NPI-2358</td>
<td>Nereus Pharmaceuticals</td>
<td>I, II</td>
<td>Currently in a Phase I trial for refractory solid tumors or lymphoma.</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>A Phase I/II clinical trial is currently recruiting participants to evaluate NPI-2358 combined with docetaxel for advanced NSCLC.</td>
</tr>
<tr>
<td>CYT997</td>
<td>Cytopia Research Pty Ltd</td>
<td>I, II</td>
<td>In a Phase I trial in patients with advanced cancer, CYT997 was well tolerated (just in poster).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Currently in a Phase I trial for multiple myeloma.</td>
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<tr>
<td></td>
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<td></td>
<td>Phase I and II trials for the efficacy study of CYT997 in combination with carboplatin in glioblastoma are currently recruiting participants.</td>
</tr>
<tr>
<td>MN-029</td>
<td>MediciNova</td>
<td>I</td>
<td>Completed Phase I trials for solid tumors (only in company homepage).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Currently limited the development of MN-029 for the treatment of solid tumors to those activities necessary to maximize MN-029’s value while pursuing a variety of initiatives to monetize this product candidate.</td>
</tr>
</tbody>
</table>
Table 1.2. Microtubule destabilizers targeting colchicine domain in preclinical development.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Selectivity</th>
<th>In vitro studies</th>
<th>In vivo efficacy models</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indanocine</td>
<td>P-glycoprotein, MRP, the glutathione transferase π isoform</td>
<td>Activation of caspase-3.</td>
<td>-</td>
</tr>
<tr>
<td>CP-248 and OSIP487703</td>
<td>-</td>
<td>↑ cGMP and activate PKG. ↓ PSA and AR. Inhibits the transcriptional activity of the promoter region of the AR gene (Lim, Piazza et al. 1999; Lim, Piazza et al. 2003).</td>
<td>-</td>
</tr>
<tr>
<td>T115</td>
<td>P-glycoprotein, MRP, and BCRP</td>
<td>-</td>
<td>HT-29 and PC-3 xenografts (i.p.)</td>
</tr>
<tr>
<td>JG-03-14</td>
<td>P-glycoprotein</td>
<td>↓ endothelial cell migration and tube formation. ↑ the permeability of an endothelial cell layer. Abnormally elongated adherent junctions and reduced VE-cadherin phosphorylation, and disrupted the VE-cadherin/b-catenin complex. ↑ pBcl-2</td>
<td>-</td>
</tr>
<tr>
<td>KRIBB3</td>
<td>-</td>
<td>↑ cyclinB1, APC/C. ↓ Mad2, p55CDC. Inhibits tumor cell migration and invasion by blocking pHsp27.</td>
<td>HCT-116 xenograft (i.p.)</td>
</tr>
<tr>
<td>A-105972</td>
<td>P-glycoprotein, MRP, and LRP</td>
<td>Independent of p53. ↑ pBcl-2.</td>
<td>B16, P388, and adriamycin-resistant P388 xenografts (i.p.), Fp.o. in rat &lt; 10%</td>
</tr>
<tr>
<td>A-289099</td>
<td>P-glycoprotein</td>
<td>S-isomer is more potent than R-isomer. Both isomers showed similar PK profile (7-44 % Fp.o. in mouse, rat, dog, and monkey.</td>
<td>M5076 xenograft (p.o.)</td>
</tr>
<tr>
<td>Drug</td>
<td>Selectivity</td>
<td>In vitro studies</td>
<td>In vivo efficacy models</td>
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<tr>
<td>-------------</td>
<td>------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>NSC 676693</td>
<td>-</td>
<td></td>
<td>P388 xenograft (i.p., i.v.) Poor aqueous solubility</td>
</tr>
<tr>
<td>BpR0L075</td>
<td>P-glycoprotein and MRP</td>
<td>↑ pBcl-2, MPM-2, pCdc25C, and cyclinB1. ↓ pCdc2.</td>
<td>KB, MKN-45, and KB-VIN10 xenografts (i.v.)</td>
</tr>
<tr>
<td>MPI-0441138</td>
<td>P-glycoprotein</td>
<td>Caspase-3 activation. PARP cleavage.</td>
<td>MX-1 and PC-3 xenografts (i.v.) High BBB penetration</td>
</tr>
<tr>
<td>MPI-0443803</td>
<td>-</td>
<td></td>
<td>B16-F0 xenograft (p.o.) Soluble in 5% dextrose:water High BBB penetration</td>
</tr>
<tr>
<td>IRC-083927</td>
<td>P-glycoprotein and cells resistant to epoB or PTX</td>
<td>Inhibits Angiogenesis in vitro. ↓ endothelial cell proliferation, migration, and vessel formation.</td>
<td>C-33A xenograft (p.o.) MEA-MB-231 (p.o.)</td>
</tr>
<tr>
<td>XRP44X</td>
<td>-</td>
<td>↓ FGF-2 Ras-Net signaling</td>
<td>↓ aortic microvessel sprouting</td>
</tr>
<tr>
<td>CHM-1</td>
<td>-</td>
<td>No modulation of caspase cascade. Induces the translocation of AIF from mitochondria to nucleus. ↑ MPM-2 and cyclinB1. ↑ Cdc2 kinase activity.</td>
<td>HA22T xenograft (i.p.)</td>
</tr>
</tbody>
</table>

(continued)
1.7. Vinca and Taxane Domain-Binding Agents in Clinical Use

The information is obtained from the official label of each drug.

1.7.1. Vinca Alkaloids

Vinca alkaloids, natural compounds isolated from the Madagascar periwinkle plant *Catharanthus roseus*, have been used in clinical chemotherapy since 1960s. These agents disrupt the mitotic machinery by binding to the vinblastine domain in tubulin. Vinblastine (Velban®) is active in Hodgkin's disease, lymphomas, and breast cancer. Vincristine (Oncovin®) is mainly used to treat acute leukemia, lymphomas, and neuroblastoma. Vinorelbine (Navelbine®) was approved for the treatment of advanced non-small cell lung cancer in 1994. However, they all have side effects such as immunosuppression and neuropathy. Peripheral neuropathy is the primary DLT of vincristine. Vinorelbine is known to have less neurotoxicity than other vinca alkaloids. In addition, similarity of the chemical structures of these agents is known to result in cross-resistance. MDR via efflux transporters and tubulin mutations is also considered to be a major cause of failure to cure advanced malignancies when using the vinca alkaloids.

1.7.2. Taxanes

Three taxanes, paclitaxel (Taxol®), docetaxel (Taxotere®), and cabazitaxel (Jevtana®), are approved for clinical use. They stabilize tubulin by binding to the taxane-binding domain. Paclitaxel isolated from the bark of the Pacific Yew tree first won FDA approval in 1992 for second-line treatment of ovarian cancer. Later, paclitaxel was approved for second-line breast cancer therapy and for the treatment of AIDS-related Kaposi’s sarcoma. Since paclitaxel is highly lipophilic and insoluble in water, it is dissolved in Cremophor EL and ethanol and it is given as a chemotherapy infusion. However, due to the toxicity of Cremophore EL, nanoparticle albumin-bound paclitaxel (Abraxane®) was being developed as an alternative. Abraxane was approved for the treatment of breast cancer in 2005. Extensive trials have been done to find a way to avoid toxicity by altering its administration.
Docetaxel was developed due to the limited resources of Pacific Yew tree. In addition, extraction of paclitaxel from the tree was a complicated and expensive process. The demand for paclitaxel led to the production of a partially-synthetic derivative of paclitaxel, docetaxel, using renewable resources as a starting material. The US FDA approved docetaxel in 2004 for prostate, breast, gastric, head and neck, and lung cancer. Side effects of docetaxel are reported to be more severe but more short-lived than paclitaxel. MDR remains a challenge for successful treatment using taxanes.

Recently a novel semi-synthetic taxane, cabazitaxel is also approved in US FDA (June 2010). Cabazitaxel is active in docetaxel-sensitive tumors. Cabazitaxel also demonstrated antitumor activity in tumor models insensitive to chemotherapy including docetaxel. Therefore, the combination therapy with cabazitaxel and prednisone is used in hormone-refractory metastatic prostate cancer previously treated with a docetaxel-containing treatment regimen. However, severe neutropenia, hypersensitivity, GI symptom, and renal failure have been reported as adverse reactions in clinical studies. Cabazitaxel is contraindicated in patients with history of severe hypersensitivity reactions to contraindicate or to drugs formulated with polysorbate 80. In addition, cabazitaxel can cause fetal harm in a pregnant woman.

1.7.3. Ixabepilone

Monotherapy with ixabepilone (Ixempra® Kit, BMS-247550), a semi-synthetic analog of epothilone B, and combination therapy with ixabepilone and capecitabine were approved in 2007 for breast cancer in patients after failure of an anthracycline and a taxane. Ixabepilone inhibits the dynamic instability of αβ-II and αβ-III microtubules by binding to the taxane domain directly. Ixabepilone is active against MDR cells via over-expression of ABC transporters and βIII tubulin isotype or tubulin mutations. Antiangiogenic activity of ixabepilone has also been reported. However, several adverse reactions of ixabepilone such as peripheral sensory neuropathy and hematologic abnormalities including neutropenia, leukopenia, anemia, and thrombocytopenia are known as major DLT. In addition, since it is also formulated with Cremophor EL, hypersensitivity due to Cremophor EL or its derivatives is common.
<table>
<thead>
<tr>
<th>Binding site</th>
<th>Drug</th>
<th>Administration</th>
<th>Therapeutic uses</th>
<th>Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vinblastine domain</strong></td>
<td>Vinblastine (Velban)</td>
<td>i.v. infusion</td>
<td>Hodgkin’s disease, Bladder and Testicular germ cell cancer, Kaposi’s sarcoma, Neuroblastoma</td>
<td>Hematologic abnormality, GI disturbances, cellulitis, phlebitis, Neuropathy, Drug resistance</td>
</tr>
<tr>
<td><strong>Vincristine (Oncovin)</strong></td>
<td>i.v. infusion</td>
<td>Pediatric leukemia, Lymphomas, Neuroblastoma, Rhabdomyosarcoma, Lung cancer</td>
<td></td>
<td>Hematologic abnormality, Neuropathy, Drug resistance</td>
</tr>
<tr>
<td><strong>Vinorelbine (Navelbine)</strong></td>
<td>i.v. infusion</td>
<td>Lung and breast cancer</td>
<td></td>
<td>Hematologic abnormality</td>
</tr>
<tr>
<td><strong>Taxane domain</strong></td>
<td>Paclitaxel (Taxol)</td>
<td>i.v. infusion</td>
<td>Ovarian, breast, lung tumors, Kaposi’s sarcoma</td>
<td>Hypersensitivity (Cremophor EL), Neuropathy, Hematologic abnormality, Cardiac toxicity, Drug resistance</td>
</tr>
<tr>
<td><strong>Docetaxel (Taxotere)</strong></td>
<td>i.v. infusion</td>
<td>Prostate, head and neck, breast, gastric, and lung cancer</td>
<td></td>
<td>Hypersensitivity, Fluid Retention, Hematologic abnormality, Neuropathy, Drug resistance</td>
</tr>
<tr>
<td><strong>Cabazitaxel (Jevtana)</strong></td>
<td>i.v. infusion</td>
<td>Prostate cancer</td>
<td></td>
<td>Hematologic abnormality, Hypersensitivity, GI disturbances, Renal failure, Neuropathy, Hepatic impairment, Fetal harm to pregnant women</td>
</tr>
<tr>
<td><strong>Ixabepilone (Ixempra)</strong></td>
<td>i.v. infusion</td>
<td>Breast cancer</td>
<td></td>
<td>Hypersensitivity (Cremophor EL), Neuropathy, Hematologic abnormality</td>
</tr>
</tbody>
</table>
1.8. Conclusion

Tubulin binding agents have been considered to be one of the most important targets in chemotherapy for decades. Recently, extensive efforts have begun to develop antimitotic agents that target the colchicine domain. Therefore, in this chapter we introduced and summarized a number of tubulin inhibitors that bind the colchicine domain.

Considerable evidence from in vitro studies suggests that agents binding the colchicine domain of tubulin have a common mechanism of action. They inhibit microtubule dynamics, block the cell cycle at the metaphase/anaphase transition, and thus induce apoptosis. For the listed compounds here, it indicates that suppression of microtubule dynamics correlates well with induction of mitotic arrest. However, the ability to destabilize microtubules is not simply dependent on the binding affinity to tubulin protein and furthermore, the in vitro potency of action does not always represent the in vivo efficacy.

The molecular and cellular events underlying the apoptosis induced by antimitotic agents are not clearly elucidated yet. Therefore, to date, various in vitro apoptosis studies have been conducted to figure out the apoptosis mechanisms of tubulin inhibitors. Several studies showed that the antimitotic effects of tubulin inhibitors probably have a more direct impact on mitochondrial function (Kraus, Samuel et al. 2003; Esteve, Carre et al. 2007).

In clinical trials, the tubulin destabilizers are generally investigated as stand-alone anticancer drugs or in combination therapy for various malignancies including advanced solid tumors, leukemia, lymphoma, etc. Since most of these agents are relatively small molecules that avoid MDR via ABC transporters, their benefit in drug resistant tumors has been extensively studied. Applications in glioblastoma have also been considered due to the high BBB penetrating property of some agents such as MPC-6827, MPI-0441138, and MPI-0443803.

Some of these colchicine binding agents showed very potent vascular disruption effects as well as antitumor effects. In addition, these compounds have much simpler structures compared to most antimitotic agents that are derived from natural products. Thus, one of the most obvious advantages of the newer colchicine-binding agents is their amenability to synthesis and modification. Furthermore, there is no orally available antimitotic agent approved for clinical cancer treatment yet. Thus, a number of clinical trials of orally available colchicine-binding compounds suggest a higher probability of developing promising oral antimitotic agents to improve patient response and reduce toxicity.
In conclusion, this chapter introduced a great variety of antimitotic agents with potent effects in vivo as well as in vitro that target the colchicine-binding site. Considering the diversity and similarity of the structures of these agents, the clinical and preclinical effects and limitations of them could help to develop novel pharmacophores of antimitotic agents to improve efficacy with less toxicity or resistance.

1.9. Scope and Objectives of Dissertation

The mitotic spindle is an important target for cancer chemotherapy. Antimitotic agents suppress microtubule function by stabilization or destabilization of microtubules. Tubulin inhibitors induce cellular arrest in the mitotic phase of the cell cycle and result in apoptotic cell death. They interact physically with tubulin by binding to one of the three known binding sites: colchicine-, vinblastine-, or paclitaxel-binding site. Over the past decades, development of novel agents binding to the colchicine site has gained extensive interest because of their benefits in drug resistance, VDA, relatively simple synthetic processes, and/or oral availability compared to other tubulin inhibitors. A number of preclinical and clinical studies are ongoing in this field, but none of them are currently approved for clinical use. Therefore, the discovery of new antimitotic agents targeting the colchicine domain with an improved safety profile and a wider therapeutic range avoiding drug resistance could be meaningful for the cancer treatment.

During drug screening efforts, we identified a series of novel indoles that exhibit potent anticancer activities in vitro. We employed a rational experimental design to test the hypothesis that treatment of cancer in animal models with novel indoles would exert advantages in MDR and neurotoxicity. The specific aims of this project were to: determine the anticancer effects of new pharmacophores targeting tubulin (Chapter 2), determine in vitro mechanism of anticancer effects on tubulin, in vivo efficacy, and toxicity of lead compounds, I-13 (Chapter 3) and I-19 (Chapter 4), and examine in vivo pharmacokinetics in mice and in vitro biotransformation by animal liver microsomes of I-19 (Chapter 5). A summary of our findings and analysis of future perspectives for indoles in cancer treatment is presented in Chapter 6.
CHAPTER 2: NOVEL INDOLE STRUCTURAL PHARMACOPHORES WITH ANTICANCER ACTIVITY

2.1. Introduction

The discovery and development of novel chemotherapeutic agents is of key importance to cancer patients. Empirical approaches by extracts of natural organisms and rational approaches considering target structure or process could be considered for new pharmacophores to alter or inhibit cancer cell growth. In this study, several indole derivatives with anticancer effect were investigated. All of the test compounds were synthesized in Dr. Miller’s lab, University of Tennessee Health Science Center.

2.1.1 Indole Compounds in Cancer Treatment

A wide diversity of cytotoxic indole compounds have been reported from marine or vegetable origins such as vinca alkaloids, indole-3-carbinol, 3, 3’-diindolylmethane, and hemiasterlin (Figure 2.1) (da Rocha, Lopes et al. 2001; Rawat, Joshi et al. 2006). However, the structural complexity of many natural-derived products presents a formidable challenge for organic medicinal chemists to develop feasible and high yield syntheses, therefore limiting the use of conventional, broad-scale screening approaches to characterize structure and activity relationships (SAR), identify active compounds and proceed to preclinical and clinical evaluation. Thus, several novel indole tubulin inhibitors with simple structures have been reported such as BPR0L075, D-24851, T115, Tryprostatin A, A-289099, and J30 (Figure 2.1) (Usui, Kondoh et al.
1998; Mahboobi, Pongratz et al. 2001; Kuo, Hsieh et al. 2004; Liou, Chang et al. 2004; Liou, Wu et al. 2007; Mahboobi, Sellmer et al. 2007).

Vincristine \((R_1 = \text{CHO}, R_2 = \text{OCH}_3, R_3 = \text{COCH}_3)\)

Vinblastine \((R_1 = \text{CH}_3, R_2 = \text{OCH}_3, R_3 = \text{COCH}_3)\)

Vindesine \((R_1 = \text{CH}_3, R_2 = \text{NH}_2, R_3 = \text{H})\)

Vinorelbine \((R=\text{H})\)

Vinflunine \((R=\text{F})\)

Figure 2.1. Natural source- or synthetic indoles.
2.1.2 Cytotoxicity, In Vitro Screen Processes for Novel Anticancer Agents

Short term in vitro assay systems are used for screening potential candidates. These systems require the preparation of suspensions of tumor cells, which are then exposed to serial concentrations of cytotoxic agents. Cytotoxicity can be assessed according to several different criteria so that the effects of different compounds on each tumor cell line can be compared. Cell viability and cell growth inhibition are generally used because exposure to anticancer agents decreases the number of viable tumor cells by direct cell killing or by simply decreasing the rate of cellular proliferation. For cellular growth and viability, three alternative assays, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT), sulforhodamine B (SRB) assays, have been extensively investigated (Alley, Scudiero et al. 1988; Rubinstein, Shoemaker et al. 1990; Skehan, Storeng et al. 1990). MTT and XTT are metabolic assays in which the cellular reduction of a colorless tetrazolium salt (MTT or XTT) yields a colored formazan product in proportion to viable cell number. The formazans can be measured conveniently in an automated colorimeter (Alley, Scudiero et al. 1988). SRB is a bright pink anionic dye that in diluted acetic acid binds electrostatically to the basic amino acid residues of proteins in Trichloroacid-fixed cells (Skehan, Storeng et al. 1990). In a large-scale screening application, SRB assay was recommended by the National Cancer Institute (NCI) because of the best stain intensity, signal-to-noise ratio, and linearity with cell number (Rubinstein, Shoemaker et al. 1990).

2.1.3 Apoptosis

Cytotoxicity is the cell-killing property regardless the mechanisms of death. There are two different types of cell death: necrosis and apoptosis. Necrosis is defined as accidental cell death and can be caused by external events such as hypoxia, chemical exposure, radiation injury, and many others. It is often associated with inflammation. On the other hand, apoptosis described as “programmed cell death” is a highly regulated process. Apoptosis is critical to maintain tissue function, homeostasis, and development in multicellular organisms. Furthermore, apoptosis functions to eliminate damaged or unwanted cells. It is generally accepted that subsequent
induction of apoptosis by anticancer drugs may be the primary mechanism of cancer cell death (Hannun 1997).

The apoptotic cascade can be initiated via two major pathways by intrinsic and extrinsic inducers. The intrinsic pathway is triggered from within the cell such as DNA damage, leading to mitochondrial depolarization and release of cytochrome C. The extrinsic pathway is initiated by pro-apoptotic ligands binding to receptors at the cell membrane such as Fas and TNF-α receptors. Activation of caspases followed cleavage of apoptosis-related proteins and the “flipping” of phosphatidylserine occur from the inner side of the cell membrane to the outer side. It is the first evidence of morphological changes, which later lead to cell shrinkage and membrane blebbing. Then, DNA fragmentation occurs before disintegration of the cell (Hengartner 2000).

### 2.1.4 Apoptosis and Cancer

During cancer development, various imbalances result in increased activity of the cell cycle, decreased activity of differentiation pathways, decreased DNA repair, and/or decreased cell death. Defects in the processes controlling apoptosis create abnormal environment allowing excessive expansion of neoplastic cells through several ways. Genetic instability and gene mutations promote resistance of neoplastic cells to immune-based cell death and disobedance of cell cycle checkpoints allows mutated cancer cell growth. Cancer cells can also survive independently of growth factor/hormone, nutrients, or oxygen. Furthermore, during metastasis, anchorage-independent cell survival can be facilitated. These various abnormal environments can also cause resistance to anticancer therapy in clinic (Reed 1999). Thus, during cancer drug development, extensive research has been conducted in order to understand the molecular mechanisms of apoptosis and how resistant forms of cancer evade apoptotic events.

### 2.1.5 Cell Cycle Regulation in Cancer

Apoptosis and proliferation are closely related with tissue homeostasis being dependent on the balance between cell proliferation and cell death. Dividing cells pass through several well-controlled phases: G₀, G₁, S, G₂, and M phases. In the G₁ phase, cells commit to enter the cell-cycle and prepare components required for duplicating their DNA during S phase. After S phase,
cells enter the G₂ phase which consists of preparing adequate components for mitosis in M phase. During the M phase, chromatids and daughter cells separate through prophase, metaphase, anaphase, and telophase. After cytokinesis, the cells can enter G₁ again or G₀ which is a quiescent phase. Entry into certain phases of the cell-cycle is carefully regulated by cell-cycle checkpoints in G₁, G₂, and M phases.

In cancer, alterations in the control of cell division result in unrestrained cell proliferation (Malumbres and Barbacid 2001). Thus, there are various anticancer agents targeting cell cycle pathway in human cancer (Figure 2.2). For example, antimetabolites such as methotrexate and 5-fluorouracil stop cells from making the building blocks of DNA and stop cell division at the G₁-S phase (Johnson, Young et al. 1999). Alkylating agents, antitumor antibiotics, and platinum compounds work on S phase by stopping DNA synthesis (Tercero and Diffley 2001; Tu, Melendy et al. 2004; Temmink, Hoebe et al. 2007). DNA-damaging drugs such as topoisomerase inhibitors activate the DNA damage checkpoint involving the function of the tumor suppressor p53 and result in cell cycle arrest in the G₂ phase (Xiao, Chen et al. 2003). In addition, antimitotic agents such as taxanes and vinca alkaloids arrest cells in the G₂M phase because they inhibit microtubules which are essential for mitosis (DiPaola 2002). Therefore, we investigated the cell cycle distribution after treatment with indoles to elucidate the mechanism of action for anticancer activity.
Figure 2.2. Anticancer agents targeting cell cycle regulation
2.2. Materials and Methods

2.2.1 Chemicals and Materials

All compounds were synthesized and characterized in the College of Pharmacy at the University of Tennessee Health Science Center. The purity of the synthesized compound was confirmed by NMR, elemental analysis and mass spectrometry. Cell Death Detection ELISA (anti-histone ELISA) was purchased from Roche Applied Science (Indianapolis, IN). Bovine brain tubulin protein was purchased from Cytoskeleton, Inc (Denver, CO). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2.2 Cell Culture

All human cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS). All cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide. RPMI 1640 media, trypsin-Ethylenediaminetetraacetic acid (EDTA) and L-glutamine were purchased from Mediatech (Herndon, VA). FBS was purchased from Invitrogen (Carlsbad, CA).

2.2.3 Growth Inhibition Assay

The cytotoxic or antiproliferative activity of test compounds was investigated in cancer cell lines using SRB assay. Cultured cells were plated into 96-well plates and incubated with medium containing different concentrations of the test compounds for 96 h. Cells were stained with SRB solution, and the optical density was determined at 540 nm on a microplate reader (Dynex Technologies, Chantilly, VA). The concentration that inhibited cell growth by 50% relative to the untreated control (IC$_{50}$) was determined by nonlinear least squares regression using WinNonlin software (Pharsight Corporation, Cary, NC). WinNonlin was provided by a Pharsight Academic License to The Ohio State University.
2.2.4 Determination of DNA Fragmentation by ELISA

Apoptosis was measured by quantitation of cytoplasmic histone-associated DNA fragments using a cell death detection ELISA kit. PC-3 cells were seeded in 6-well plates and exposed to indoles for 24 h at different concentrations. The quantitation of DNA fragmentation was performed according to the manufacturer’s instructions. Results are expressed as the enrichment factor (i.e., the ratio of the optical density in treated cells to the optical density in control cells).

2.2.5 Cell Cycle Analysis

Cell cycle distribution was determined by propidium iodide staining. Treated cells were washed with phosphate buffer solution (PBS) and fixed with 70% ice-cold ethanol overnight. Fixed cells were then stained with propidium iodide (20 μg/mL) in the presence of RNase A (300 μg/mL) at 37°C for 30 min. Cell cycle distribution was analyzed by fluorescence-activated cell sorting (FACS) analysis core services at The Ohio State University.

2.2.6 In Vitro Tubulin Depolymerization Assay

Tubulin polymerization kits (Cytoskeleton, Denver, CO) were used to study tubulin depolymerization. The reaction contained 50 μL of 4 mg/mL tubulin in PEM buffer (80 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), pH 7.0, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 2 mM MgCl₂, 1 mM GTP, 10% glycerol). The procedure was done according to the manufacturer’s protocol. Tubulin polymerization was monitored in a UV spectrophotometer for 40 minutes at 340 nm at 37°C.
2.3. Results

2.3.1 The Effect of Indoles on Cell Proliferation

To explore the chemosensitivity of indoles in solid tumor cell lines, cells were seeded in 96-well plates and incubated with different concentrations of compounds for 96 h. Cell survival was quantitated by the SRB assay. The concentration that inhibited cell growth by 50% relative to the vehicle control (IC\textsubscript{50}) was determined by nonlinear least squares regression using WinNonlin. In this cell-based screening assay, we found 6 compounds with potent cytotoxicity in several human prostate cancer cell lines with nanomolar IC\textsubscript{50} values (Table 2.1-2.2). Those 6 compounds were also potent in TSU-Pr1, HT-29, and MCF-7 cells (Table 2.3).

Compared to I-13, reduction of the methanone linkage to an alcohol (I-14) significantly reduced activity, with IC\textsubscript{50} values over 100-fold higher for I-14 in LNCaP and PC-3 cells (Table 2.1). Complete reduction to a methylene linker, I-12 almost completely abolished activity (Table 2.1). N-substituted compounds (I-9, I-10, and I-11) were also devoid of activity, while partial intermediates without the R\textsubscript{3} ring (I-6 and I-7) also demonstrated substantially reduced activity in these cancer cell lines (Table 2.1).

I-19 with a 3,4,5-trimethoxyphenyl group (I-19) in R\textsubscript{3} increased activity twofold in LNCaP, PC-3, DU145, and PPC-1 cells compared to I-13 (Table 2.1). Compared to I-19, reduction of the methanone linkage to an alcohol (I-47) significantly reduced activity, with IC\textsubscript{50} values over 100-fold higher for I-47 in the cells studied (Table 2.1). N-substituted compounds (I-17 and I-18) showed lower activity (Table 2.1). Replacement of the A-ring indolyl group with 5-fluoroindole (I-37) was as potent as I-19 (Table 2.1). Removal of a single meta-methoxy group, the para methoxy group, or both a meta and para methoxy group doubled the observed IC\textsubscript{50} values (I-35, I-34, and I-33, respectively) (Table 2.1). Unexpectedly, removal of both of the meta-methoxy groups (I-36) resulted in a 100-fold increase in IC\textsubscript{50} in the tested cancer cell lines (Table 2.1).

Compared to the most active compounds in Tables 2.1 and 2.2, I-13 and I-19, respectively, replacement of the A-ring indolyl group with 3,4,5-trimethoxyphenyl (I-20, I-21, I-22, I-23, and I-24) significantly reduced activity, with IC\textsubscript{50} values over 100-fold higher in all cell lines studied regardless of the linker and C-ring (Table 2.2).
Table 2.1. Structures and cell growth inhibition of indole derivatives in prostate cancer cell lines

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>X</th>
<th>R₃</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LNCaP</td>
</tr>
<tr>
<td>I-6</td>
<td>H</td>
<td>SO₂ph CO</td>
<td>H</td>
<td>17.7</td>
</tr>
<tr>
<td>I-7</td>
<td>H</td>
<td>H CHOH</td>
<td>H</td>
<td>23.8</td>
</tr>
<tr>
<td>I-9</td>
<td>H</td>
<td>SO₂ph CHOH</td>
<td>2-(1-(phenyl sulfonfonyl)indolyl)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>I-10</td>
<td>H</td>
<td>SO₂ph CH₂</td>
<td>2-(1-(phenyl sulfonfonyl)indolyl)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>I-11</td>
<td>H</td>
<td>SO₂ph CO</td>
<td>2-(1-(phenyl sulfonfonyl)indolyl)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>I-12</td>
<td>H</td>
<td>H CH₂ 2-indolyl</td>
<td></td>
<td>&gt;50</td>
</tr>
<tr>
<td>I-13</td>
<td>H</td>
<td>H CO</td>
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</tr>
<tr>
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<td>H</td>
<td>H CHOH</td>
<td>2-indolyl</td>
<td>5.6</td>
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<td>SO₂ph CHOH</td>
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<td>10.1</td>
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<tr>
<td>I-18</td>
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<td>SO₂ph CO</td>
<td>3,4,5,-Trimethoxyphenyl</td>
<td>7.8</td>
</tr>
<tr>
<td>I-19</td>
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<td>H CO</td>
<td>3,4,5-Trimethoxyphenyl</td>
<td>0.031</td>
</tr>
<tr>
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<td>H CO</td>
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<td>0.032</td>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>I-36</td>
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<td>4-Methoxyphenyl</td>
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Table 2.2. Structures and cell growth inhibition of indole derivatives in prostate cancer cell lines

<table>
<thead>
<tr>
<th>X</th>
<th>R₁</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LNCaP</td>
</tr>
<tr>
<td>I-20</td>
<td>CHOH</td>
<td>H</td>
</tr>
<tr>
<td>I-21</td>
<td>CO</td>
<td>3,4,5,-Trimethoxyphenyl</td>
</tr>
<tr>
<td>I-22</td>
<td>CO</td>
<td>2-(1-(phenylsulfonyl)indolyl)</td>
</tr>
<tr>
<td>I-23</td>
<td>CHOH</td>
<td>2-(1-(phenylsulfonyl)indolyl)</td>
</tr>
<tr>
<td>I-24</td>
<td>CO</td>
<td>2-indolyl</td>
</tr>
</tbody>
</table>
2.3.2 Apoptotic Effect Induced by Indoles

To determine apoptosis of cancer cells induced by compounds, we examined DNA fragmentation by using a cell death detection ELISA kit. Compounds were incubated with PC-3 cells for 24 h in six-well plates (n=2-3 per condition). The lead compounds increased the enrichment factor (EF, absorbance in treated cells/absorbance in control cells) in a concentration-dependent manner (Figure 2.3). The concentration that induced histone-complexed DNA fragments halfway between the baseline and maximum (EC$_{50}$) were determined by nonlinear least squares regression using WinNonlin. I-19 and I-37 were the most potent at inducing DNA fragmentation with EC$_{50}$ values less than 100 nM (Figure 2.4).

| Table 2.3. IC$_{50}$ values of different cancer cell lines treated with lead compounds |
|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|
| Mean ± SD, n=3-4                    |     |     |     |     |     |     |     |
|                                    | LNCaP | PC-3 | DU-145 | PPC-1 | TSU-Pr1 | HT-29 | MCF-7 |
| Vinblastine                         | 3.4 ± 0.9 | 1.4 ± 0.3 | 2.6 ± 1.0 | 1.1 ± 0.4 | 1.6 ± 0.1 | 2.4 ± 0.2 | 1.4 ± 0.4 |
| Docetaxel                           | 4.7 ± 1.3 | 6.3 ± 0.4 | 5.2 ± 1.0 | 2.7 ± 1.0 | 2.6 ± 0.9 | 4.1 ± 0.2 | 3.8 ± 0.8 |
| I-13                                | 44.2 ± 2.4 | 81.1 ± 9.6 | 138.1 ± 12.6 | 67.3 ± 1.1 | 33.8 ± 1.8 | 61.6 ± 0.8 | 161.5 ± 10.2 |
| I-19                                | 30.9 ± 3.5 | 28.2 ± 2.0 | 22.8 ± 4.1 | 18.7 ± 1.5 | 17.6 ± 2.1 | 15.1 ± 1.3 | 39.2 ± 2.9 |
| I-33                                | 47.3 ± 8.5 | 72.0 ± 13.0 | 128.9 ± 8.8 | 43.4 ± 5.8 | 42.4 ± 2.5 | 100.1 ± 5.5 | 43.2 ± 7.5 |
| I-34                                | 81.5 ± 12.6 | 74.9 ± 16.1 | 133.8 ± 9.3 | 68.3 ± 8.1 | 64.6 ± 4.0 | 91.7 ± 7.8 | 53.5 ± 3.0 |
| I-35                                | 51.0 ± 4.9 | 69.0 ± 12.3 | 131.4 ± 12.2 | 40.3 ± 3.3 | 43.9 ± 1.2 | 119 ± 13.9 | 43.3 ± 1.9 |
| I-37                                | 32.4 ± 5.3 | 31.4 ± 4.0 | 28.3 ± 5.1 | 27.6 ± 4.3 | ND | ND | 39.3 ± 15.5 |

ND Not determined
Figure 2.3. Cytoplasmic histone-associated DNA fragments by lead compounds in PC-3 cells. The extent of apoptosis was determined using a commercially available anti-histone ELISA after 24 h treatment. Graphs show the concentrations of compounds (x-axis) versus EF (absorbance in treated cells/absorbance in control cells) of each sample (y-axis). Vinblastine was used as a positive control. Mean ± SD, n=2-3.
Figure 2.4. EC₅₀ values of lead compounds that induced cytoplasmic histone-associated DNA fragments in PC-3 cells. Mean ± SD, n=2-3.
2.3.3 Indoles Induce G₂M Phase Arrest

As an initial step to understand the mechanism of action of indoles, we determined the effect of indoles on the cell cycle. PC-3 cells were significantly arrested in the G₂M phase in a concentration-dependent manner, a pattern that is commonly observed with taxanes and the vinca alkaloids. When the percentages of cells in the G₂M phase were plotted against different concentrations of the compounds (0 to 1.5 μM), vinblastine, docetaxel, I-13, I-19, I-33, I-34, I-35, and I-37 arrested the cell cycle with EC₅₀ values of 7, 14, 282, 34, 180, 235, 263, and 38 nM respectively (Figure 2.5-2.6). The effect of these drugs arresting cells in the G₂M phase is closely related to their IC₅₀ values of cytotoxicity in PC-3 cells (Table 2.3), suggesting that this is a direct link to their mechanism of action.

2.3.4 The Effect of Indoles on Microtubule Polymerization

Vinblastine and indoles were compared for their ability to inhibit tubulin polymerization at different concentrations of the compounds. Microtubule polymerization was measured by absorbance over time since assembled microtubules cause turbidity in the assay solution (Figure 2.7). These data indicate that indoles mimic the effects of vinca alkaloids on tubulin polymerization to reduce polymerization and destabilize microtubule formation as is evident by the decrease in absorbance with increasing concentration. Whereas the IC₅₀ value of vinblastine was 283 nM, I-13, I-19, I-33, I-34, and I-35 suppressed tubulin polymerization in concentration dependent manner with IC₅₀ values of 554, 381, 898, 765, and 879 nM, respectively (Figure 2.7).
Figure 2.5. Dose-response curves of compounds in cellular arrest in the G2M phase.
PC-3 cells were treated with different concentrations (0 to 1.5 μM) of compounds for 24 h, and DNA content of the cells was analyzed by FACS. The percentage of cells in the G2M phase of cell cycle division was quantified. Dose-response curves for indoles are shown.
Figure 2.6. EC$_{50}$ values of compounds in cellular arrest in the G$_2$M phase.
The percentage of cells in the G$_2$M phase of the cell division cycle was quantified and EC$_{50}$ values were compared, since they arrested cells in the G$_2$M phase in a concentration-dependent manner.
Figure 2.7. Dose-Response of tubulin depolymerization by indoles.

The microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. Indoles depolymerized microtubules in a concentration-dependent manner. IC_{50} values for tubulin inhibition were calculated (vehicle control value set at 100%). Mean ± SD, n=2.

<table>
<thead>
<tr>
<th></th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine</td>
<td>283 ± 18</td>
</tr>
<tr>
<td>I-13</td>
<td>554 ± 50</td>
</tr>
<tr>
<td>I-19</td>
<td>381 ± 68</td>
</tr>
<tr>
<td>I-33</td>
<td>898 ± 84</td>
</tr>
<tr>
<td>I-34</td>
<td>765 ± 113</td>
</tr>
<tr>
<td>I-35</td>
<td>879 ± 185</td>
</tr>
</tbody>
</table>
A-ring, indole is required. 5-fluorindole didn't reduce the cytotoxic effect.

Methanone linkage is required. Reduction abolishes activity.

C-ring
Indole is very active.
3,4,5-trimethoxy is the best.
3-methoxy, 3,4-dimethoxy, and 3,5-dimethoxy are less active compared to 3,4,5-trimethoxy.
4-methoxy significantly reduces activity.

Figure 2.8. SAR of the tubulin antagonists.
2.4. Discussion

Indole derivatives have been a major interest in anticancer drug development for decades. Indole-3-carbinol and diindolylmethane demonstrated apoptotic effects in human breast and prostate cancer cell lines in vitro (Fares, Ge et al. 1998; Ge, Fares et al. 1999). Furthermore, a great number of novel indole compounds have been investigated for antimitotic effects such as BpR0L075, D-24851, aroylindoles, A-289099, tryprostatin A, T115, J30, and 2-acyl-1H-indole-4,7-diones (Usui, Kondoh et al. 1998; Bacher, Nickel et al. 2001; Mahboobi, Pongratz et al. 2001; Beckers, Reissmann et al. 2002; Li, Woods et al. 2002; Kuo, Hsieh et al. 2004; Mahboobi, Sellmer et al. 2005; Liou, Hsu et al. 2007; Liou, Wu et al. 2007; Liou, Wu et al. 2008; Arora, Wang et al. 2009). During drug screening efforts, we identified a series of novel indoles that exhibit potent anticancer activities.

To evaluate the role of the indole substitution pattern, we synthesized various derivatives of the indole and examined their effects in the inhibition of cell proliferation by SRB assay in prostate cancer cell lines including LNCaP, PC-3, DU-145, and PPC-1 (Table 2.1-2.2). In conclusion, as shown in Figure 2.8, 2-indolyl, 5-indolyl or 5-fluoro-2-indolyl ring for the A-ring showed very potent activity. In addition, electron donor linkages were required between A-ring and B-ring. A number of modifications to the C-ring did not affect activity (indole, 3,4,5-trimethoxy, 3,4-dimethoxy, and 3,5-dimethoxy). The SAR was summarized in Figure 2.8.

We identified 6 potent compounds (I-13, I-19, I-33, I-34, I-35, and I-37) with nanomolar IC50 values of cell growth inhibition in human cancer cell lines. In further studies, they induced apoptosis, arrested PC-3 cells in the G2M phase of the cell cycle, and destabilized tubulin polymerization in a dose-dependent manner. The effect of these drugs arresting cells in the G2M phase and inhibiting tubulin polymerization are closely related to their IC50 values of cytotoxicity in human cancer cell lines (Table 2.3), suggesting that this is a direct link to their mechanism of action. In chapters 3 and 4, further in vivo as well as in vitro studies to examine the antitumor activities of I-13 and I-19 will be described in detail, respectively.
2.5. Acknowledgements

I would like to thank Dr. James T. Dalton and Dr. Jun Yang for his guidance and assistance for this project. The studies in table 2.1 was conducted with Jun Yang. I would like to thank Dr. Duane D. Miller’s group for synthesis of compounds.
3.1. Introduction

Tubulin inhibitors are a major group of antitumor agents. Microtubules are critically important for the formation of the mitotic spindle, which dictates the proper segregation of chromosomes during mitosis. It is generally accepted that the antiproliferative properties of microtubule-interacting drugs arise largely from their interactions with tubulin resulting in disruption of microtubule dynamics (Dumontet and Sikic 1999). These drugs are commonly classified into two major categories: the microtubule-destabilizing agents (i.e., the vinca alkaloids and colchicines) and the microtubule-stabilizing drugs (i.e., taxanes). The vinca alkaloids, targeting the vinblastine-binding site, are effectively used to treat a wide variety of human cancers including hematological malignancies, breast, non-small-cell lung, and testicular carcinoma. Colchicine, which binds at a different site on β-tubulin, is used clinically for gout, but not cancer. Taxanes are also widely used in ovarian, breast, and non-small-cell lung carcinomas and bind to distinct binding sites on tubulin other than the vinblastine and colchicine sites. Docetaxel, a taxane analog, was the first chemotherapeutic agent specifically approved for the treatment of advanced prostate cancer by the US Food and Drug Administration in 2004 (Tannock, de Wit et al. 2004). The clinical success of targeting tubulin for anticancer drugs has led to the search for new agents that inhibit tubulin activity with an improved safety profile. Tubulin inhibitors in human chemotherapy have severe limitations such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug

High levels of intrinsic MDR transporters and their over-expression induced by long-term exposure to chemotherapy has been recently suggested as one of the reasons for the low chemotherapeutic responses in primary cell cultures from prostate cancer patients (Sanchez, Mendoza et al. 2009). P-glycoprotein is a member of large family of ABC including MRP and BCRP, that are responsible for drug efflux and MDR. Cancer cells expressing P-glycoprotein are resistant to a number of structurally unrelated agents including vinca alkaloids, taxanes, colchicines, epipodophyllotoxins, and anthracyclines with overlapping but not identical substrate specificity (Leonard, Fojo et al. 2003). To date, no clinical solutions exist to overcome MDR caused by P-glycoprotein expression, but many research groups are actively pursuing different therapies to diminish this dilemma. In vivo and in vitro studies using some anticancer drugs that are not substrates of P-glycoprotein and therefore are not susceptible to being extruded from tumor cells have been reported. Epothilones, a new class of microtubule stabilizers, and 2-arylimidoles, orally active small molecule tubulin inhibitors, are recent examples of such agents (Giannakakou, Sackett et al. 1997; Chou, Zhang et al. 1998; Chou, O'Connor et al. 2001; Lee, Borzilleri et al. 2001; Beckers, Reissmann et al. 2002; Lin, Catley et al. 2005). Ixabepilone, an epothilone analog, is currently in phase II clinical trials for hormone-resistant metastatic prostate cancer, but thus far no results have been reported as to the success of the trial in http://clinicaltrials.gov.

The structural complexity of the vinca alkaloids, taxanes, and natural and marine-derived products (Hamel 1992) that inhibit tubulin presents a formidable challenge to organic medicinal chemists to develop feasible and high yield syntheses. Therefore, this limits the use of conventional, broad-scale screening approaches to characterize structure-activity relationships, identify active compounds, and proceed to preclinical and clinical evaluation.

During our studies to identify new pharmacophores for prostate cancer, we identified a novel class of indoles that act as potent anticancer agents. Indoles that inhibit tubulin action but circumvent MDR represent a novel chemical approach to this dilemma. We examined the sensitivity of a variety of human cancer cell lines to our lead, (3-(1H-indol-2-yl)phenyl)(1H-indol-2-yl)methanone (hereafter referred to as I-13; Figure 3.1), and explored underlying mechanisms to explain the ability of I-13 to induce apoptosis. We further compared the anticancer activity and toxicity of I-13 to vinblastine and docetaxel in a human prostate cancer
PC-3 xenograft model and provide compelling evidence that I-13 is the first member of a new class of tubulin inhibitors that circumvent ABC transporter-mediated drug resistance with an improved safety profile compared to existing agents and other tubulin inhibitors in development.

Figure 3.1. Chemical structure of I-13.
3.2. Materials and Methods

3.2.1 Chemicals and Animals

Monoclonal antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bovine brain tubulin protein was purchased from Cytoskeleton, Inc (Denver, CO). [\(^3\)H]vinblastine and [\(^3\)H]podophyllotoxin were purchased from Moravek, Inc (Brea, CA). Sephadex G25 column and Cell Death Detection ELISA (anti-histone ELISA) were purchased from Roche Applied Science (Indianapolis, IN).

All other chemicals were purchased from Sigma (St. Louis, MO).

Four- to 5-week-old male ICR mice and male nu/nu nude mice were purchased from Harlan Biosciences (Indianapolis, IN). All animal protocols were approved by the Animal Care and Use Committee at The Ohio State University or the University of Tennessee Health Science Center.

3.2.2 Cell Culture

All human cancer cell lines except K562/DOX, HEK293-pcDNA3.1, HEK293-MRP1, HEK293-MRP2, HEK293-pcDNA3-10 and HEK293-482R2 were obtained from American Type Culture Collection (Manassas, VA). K562/DOX, HEK293-pcDNA3-10 and HEK293-482R2 were kindly provided by Dr. Duxin Sun (College of Pharmacy, The Ohio State University). The plasmids for MRP1 and MRP2 were kindly provided by Dr. Susan P. C. Cole (Department of Pharmacology & Toxicology, Queen’s University) (Letourneau, Slot et al. 2007). K562/DOX was obtained by in vitro selection of K562 in progressively increasing doses of doxorubicin (Tsuruo, Iida-Saito et al. 1986; Fang, Zhang et al. 2006). HEK293-MRP1 and HEK293-MRP2 were transfected with the plasmid encoding the full-length MRP1 and MRP2 genes, respectively, according to the calcium phosphate transfection method (Chen and Okayama 1987). HEK293-pcDNA3.1 was transfected with empty pcDNA 3.1(−) vector. HEK293-MRP1, HEK293-MRP2, and HEK293-pcDNA3.1 cells were selected with 200 µg/mL G418 (Geneticin) for 4 weeks after transfection. HEK293-pcDNA3-10 and HEK293-482R2 were HEK293 cells transfected with empty pcDNA3 vector and pcDNA3 vector containing full-length BCRP, respectively (Robey,
Honjo et al. 2003). Cells were maintained in RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10% fetal bovine serum. All cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide. RPMI-1640 media, trypsin–EDTA and L-glutamine were purchased from Mediatech (Herndon, VA). FBS was purchased from Invitrogen (Carlsbad, CA).

3.2.3 Growth Inhibition Assay

The cytotoxic or antiproliferative activity of test compounds was investigated in cancer cell lines using the SRB assay. The MTT assay was also used for the leukemia cell lines. Cultured cells were plated into 96-well plates and incubated with medium containing different concentrations of the test compounds for 4 days. Cells were stained with SRB or MTT solution. The optical density was determined at 540 nm on a microplate reader (Dynex Technologies, Chantilly, VA). The concentration that inhibited cell growth by 50% relative to the untreated control (IC50) was determined by nonlinear least squares regression using WinNonlin software (Pharsight Corporation, Cary, NC). WinNonlin was provided by a Pharsight Academic License to The Ohio State University.

3.2.4 Determination of DNA Fragmentation by ELISA

Apoptosis was measured by quantitation of cytoplasmic histone-associated DNA fragments using the cell death detection ELISA kit. LNCaP and PC-3 cells were seeded in six-well plates and exposed to I-13 for 24 h at final concentrations of 100 and 200 nM (approximating the IC90 values in these cell lines), respectively. The quantitation of DNA fragments was performed according to the manufacturer’s instructions.

3.2.5 Cell Cycle Analysis

Treated cells were washed with PBS and fixed with 70% ice-cold ethanol overnight. Fixed cells were then stained with propidium iodide (20 µg/mL) in the presence of RNase A (300
μg/mL) at 37°C for 30 min. Cell cycle distribution was analyzed by FACS analysis core services at The Ohio State University.

3.2.6 Western Blot Analysis

Treated cells were lysed in cold lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na2 EDTA, 1% Triton–X 100, and freshly added Na3VO4 (2 mM), NaF (20 mM) and complete protease inhibitor cocktail]. For each sample, an aliquot (20–40 µg) of total protein was loaded and run on a SDS-PAGE gel. Protein was transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), blocked with 5% non-fat milk, incubated with primary antibody overnight at 4°C, and then incubated with secondary antibody at room temperature for 1 h. The ECL™ (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used for detection.

3.2.7 In Vitro Tubulin Depolymerization Assay

BK004 kits (Cytoskeleton, Denver, CO) were used to study tubulin depolymerization. The reaction contained 50 µL of 4 mg/mL tubulin in PEM buffer (80 mM PIPES, pH 7.0, 0.5 mM EGTA, 2 mM MgCl2, 1 mM GTP, 10% glycerol). The procedure was done according to the manufacturer’s protocol. Tubulin polymerization was monitored in a UV spectrophotometer for 40 min at 340 nm at 37°C.

3.2.8 Indirect Immunofluorescence Microscopy

PC-3 cells were plated on poly-D-lysine coated glass cover slips in six-well plates (2.5×10⁴ cells/well) the day before treatment. After cells were incubated with cytotoxic agents at 37°C for 6 h, cells were fixed on cover slips with 4% formaldehyde in PBS for 10 min and permeabilized by 2% Triton-X in PBS for 5 min. They were blocked with 3% bovine serum albumin in TBST for 30 min and incubated with anti-α-tubulin-FITC antibody (Sigma, St. Louis, MO) used at 1:100 dilution at 4°C overnight. They were mounted on slides and observed by a
Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, Thornwood, NY). Images were acquired with a Zeiss Axiocam HRc, using Zeiss AxioVision.

### 3.2.9 In Vitro Binding Assay by Spin Column

A spin column binding assay was used to determine whether I-13 competes for either the vinblastine- or colchicine-binding sites (Bacher, Nickel et al. 2001). Tubulin (0.1 mg/mL) was incubated either with 3 μM vinblastine containing [³H]vinblastine (4×10⁴ dpm/nmol) in the presence of different concentrations of vincristine or I-13 in PEM buffer or with 3 μM podophyllotoxin containing [³H]podophyllotoxin (4×10⁴ dpm/nmol) in the presence of different concentrations of colchicine or I-13 in PEM buffer in a total volume of 140 μL at 37°C for 1 h. Samples (120 μL) were then loaded onto size-exclusion Sephadex G25 columns that were pre-washed with 3 mL of PEM buffer. The columns were then placed into empty tubes and centrifuged at 200g for 1 min and radioactivity in the flow-through was analyzed by scintillation counting.

### 3.2.10 In Vivo Antitumor Efficacy Study

PC-3 cells (2.5×10⁶ cells/site) were injected subcutaneously into the flanks of male nu/nu mice. Tumor size was measured using calipers every 3 days and calculated as \( V = \pi/6 \times (\text{length}) \times (\text{width})^2 \) (Dethlefsen, Prewitt et al. 1968). When tumors reached a volume of approximately 150 mm³, drug treatment was initiated. The control group was treated with vehicle (10% DMSO in PEG300) only. During the treatment, tumor size and animal body weight were measured twice per week. Relative tumor growths (%) were calculated considering the mean tumor volumes at day 0 as 100%.

### 3.2.11 Statistical Analysis

The results (mean values ± SD) were subjected to statistical analysis by single-factor ANOVA (Analysis of variance). The level of significance was set at \( P < 0.05 \).
3.3. Results

3.3.1 The Effect of Indoles on Cell Proliferation

Compound I-13 demonstrated a potent growth inhibitory effect in several human cancer cell lines of differing tissue origin, including prostate cancer cell lines DU-145, LNCaP, PC-3, and PPC-1, bladder cancer TSU-Pr1 cells, breast adenocarcinoma MCF7 cells, and the colorectal carcinoma HT29 cell line (Table 3.1). Vinblastine and docetaxel confirmed the assay validity by exhibiting high potency in cancer cell lines with IC$_{50}$ values ranging from 1.05 to 6.3 nM. IC$_{50}$ values of Colchicine were from 10 to 20 nM in DU-145, LNCaP, PC-3, and PPC-1 (Lu, Li et al. 2009).

3.3.2 The Effect of I-13 on Growth of Cells that Over-express P-glycoprotein, MRPs, and BCRP

We next determined whether cells that over-express P-glycoprotein, MRP1, MRP2, and BCRP were resistant to I-13. To examine the P-glycoprotein-induced drug resistance, the parental K562 leukemia cell line and its doxorubicin-resistant sub-line (K562/DOX) that over-expresses P-glycoprotein were co-incubated with different concentrations of I-13, doxorubicin, docetaxel, and vinblastine. It is well established that doxorubicin, docetaxel, and vinblastine are substrates for P-glycoprotein (Schinkel and Jonker 2003). The IC$_{50}$ values for doxorubicin, vinblastine, and docetaxel were increased 32-, 191-, and 203-fold, respectively, in the K562/DOX cell line as compared to parental K562 cells, while I-13 demonstrated equal potent anticancer activity in the two cell lines (Table 3.2).

Studies were performed using MDR-expressing HEK293-MRP1 and HEK293-MRP2 cells treated with I-13 to determine if I-13 is a substrate for MRP1 and MRP2. Vinblastine, doxorubicin, SN-38, and docetaxel were substantially less effective in HEK293-MRP1 and HEK293-MRP2 (Table 3.2). IC$_{50}$ values for vinblastine, doxorubicin, SN-38, and docetaxel were 4.9-, 3.7-, 2.1- and 7.2-fold greater in cells with high MRP1 expression and 3.7-, 2.2-, 1.5-, and 7.6-fold greater in cells over-expressing MRP2 than in HEK293-pcDNA3.1 empty vector cells. I-
13, however, demonstrated similar IC$_{50}$ values in HEK293-pcDNA3.1 and HEK293-MRP1 and HEK293-MRP2 cells.

For the BCRP transporter, HEK293 cells transfected with pcDNA3 empty vector and HEK293-482R2 transfected with the vector including BCRP gene were used. Upon treatment with SN-38 and doxorubicin, significant differences between the IC$_{50}$ values in HEK293-pcDNA3-10 and HEK293-482R2 cell line were seen (Table 3.2). IC$_{50}$ values for SN-38 and doxorubicin were 33- and 4-fold greater, respectively, in cells with high BCRP expression as compared to nonexpressing cells. I-13, however, retained similar potency in HEK293-482R2 cells, as well as K562/DOX, HEK293-MRP1, and HEK293-MRP2 cell lines as compared to the control cell lines. These data indicate that I-13 is not a substrate for P-glycoprotein, MRP1, MRP2, and BCRP, while other chemotherapeutic agents that target tubulin (vinblastine, docetaxel, and paclitaxel) or other cellular mechanisms (SN-38 and doxorubicin) are substrates for these ABC transporters.

3.3.3 Apoptotic Effect Induced by I-13

In the apoptosis study using the cell death detection ELISA kit, I-13 increased the enrichment factor (absorbance in treated cells/absorbance in control cells) 4.5- and 4-fold in LNCaP and PC-3 cells, respectively ($P < 0.05$; Figure 3.2). The extent of apoptosis was greater in LNCaP as compared to PC-3 cells, which is consistent with the lower IC$_{50}$ value of I-13 in LNCaP (44 nM) versus PC-3 cells (81 nM). These studies provide persuasive evidence that I-13 potently induces apoptosis in prostate cancer cell lines, with potency similar to but slightly less than paclitaxel.

To further elucidate the apoptotic mechanism of I-13, we examined its effects on PARP cleavage, a marker of apoptotic cell death, and the expression of the antiapoptotic protein Bcl-2 in PC-3 cells after 24-, 48-, and 72-h treatments (Figure 3.3). Lysates of PC-3 showed no cleavage of PARP and a high expression of Bcl-2 before drug treatment. PARP cleavage was observed after 48-h exposure with I-13 (200 nM; approximate IC$_{90}$ value in PC-3 cell growth inhibition study). Bcl-2 expression decreased in a time-dependent manner after treatment with I-13 (200 nM), suggesting that I-13-induced apoptosis is at least partially mediated by down-regulation of Bcl-2.
Table 3.1. IC₅₀ values of different cancer cell lines treated with I-13 by the SRB assay

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC₅₀ (nM)</th>
<th>Vinblastine</th>
<th>Docetaxel</th>
<th>I-13</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>3.43 ± 0.9</td>
<td>4.66 ± 1.3</td>
<td>44.2 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>1.43 ± 0.3</td>
<td>6.29 ± 0.4</td>
<td>81.1 ± 9.6</td>
<td></td>
</tr>
<tr>
<td>DU-145</td>
<td>2.60 ± 1.0</td>
<td>5.19 ± 1.0</td>
<td>138.1 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>PPC-1</td>
<td>1.05 ± 0.4</td>
<td>2.67 ± 1.0</td>
<td>67.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>1.63 ± 0.1</td>
<td>2.64 ± 0.9</td>
<td>33.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>2.42 ± 0.21</td>
<td>4.13 ± 0.2</td>
<td>61.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.35 ± 0.41</td>
<td>3.77 ± 0.8</td>
<td>161.5 ± 10.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Effect of I-13 and other agents on the growth of cells expressing ABC MDR transporters

<table>
<thead>
<tr>
<th>Resistance phenotype and cell line</th>
<th>I-13 (nM)</th>
<th>Doxorubicin (nM)</th>
<th>Vinblastine (nM)</th>
<th>SN-38 (nM)</th>
<th>Docetaxel (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>63.6 ± 2</td>
<td>27.1 ± 6</td>
<td>1.4 ± 0.4</td>
<td>ND</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>K562/Dox</td>
<td>78.2 ± 3 (1.2)</td>
<td>859 ± 27 (31.7)</td>
<td>268.1 ± 48 (191)</td>
<td>ND</td>
<td>548.5 ± 76 (203)</td>
</tr>
<tr>
<td>MRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293-pcDNA3.1</td>
<td>60.2 ± 7</td>
<td>80.6 ± 3</td>
<td>5.0 ± 1</td>
<td>8.4 ± 1</td>
<td>4.4 ± 2</td>
</tr>
<tr>
<td>HEK293-MRP1</td>
<td>89.9 ± 13 (1.5)</td>
<td>298.1 ± 16 (3.7)</td>
<td>24.3 ± 2 (4.9)</td>
<td>17.7 ± 1 (2.1)</td>
<td>31.3 ± 5 (7.2)</td>
</tr>
<tr>
<td>HEK293-MRP2</td>
<td>90.4 ± 2 (1.5)</td>
<td>177.6 ± 11 (2.2)</td>
<td>18.1 ± 4 (3.7)</td>
<td>12.4 ± 1 (1.5)</td>
<td>33.1 ± 4 (7.6)</td>
</tr>
<tr>
<td>BCRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293-pcDNA3-10</td>
<td>91.6 ± 10</td>
<td>ND</td>
<td>ND</td>
<td>2.9 ± 0.3</td>
<td>4.04 ± 0.8</td>
</tr>
<tr>
<td>HEK293-482R2</td>
<td>105 ± 4 (1.15)</td>
<td>ND</td>
<td>ND</td>
<td>94 ± 20 (32.5)</td>
<td>15.7 ± 2 (3.89)</td>
</tr>
</tbody>
</table>

NOTE: P-glycoprotein, MRP1, MRP2, BCRP were over-expressed in K562/DOX, HEK293-MRP1, HEK293-MRP2 and HEK293-482R2. The resistance factor (in parentheses) was calculated as the ratio of IC₅₀ values for the resistant cell sub-line to that of the parental cell line. All experiments were performed at least in three replicates. ND: not determined
Figure 3.2 Cytoplasmic histone-associated DNA fragments by I-13 in LNCaP and PC-3 cells. Cells were incubated with I-13 at a concentration of IC_{90} to each cell line for 24 h. The extent of apoptosis was determined using a commercially available anti-histone ELISA.

* $P < 0.05.$
Figure 3.3. Changes in the protein expression of PARP and Bcl-2 by I-13. PARP cleavage and decreased expression of Bcl-2 were observed in PC-3 cells treated with 200 nM of Vinblastine and I-13 for 24-, 48- and 72-h by Western blot analysis.
### 3.3.4 Effect of I-13 in Cell Cycle Distribution

As an initial step to understand the mechanism of action of I-13, we determined the effect of I-13 on the cell cycle. The percentage of PC-3 cells in the G2M phase increased (Figure 3.4) from 13% (control) to 24, 46, and 73% at I-13 concentrations of 100, 300, and 500 nM, respectively, indicating that PC-3 cells were significantly arrested in the G2M phase in a concentration-dependent manner, a pattern that is commonly observed with taxanes and vinca alkaloids. When the percentages of cells in the G2M phase were plotted against different concentrations of the compounds (0 to 1 μM), vinblastine, docetaxel, and I-13 arrested the cell cycle with EC50 values of 7, 14, and 282 nM, respectively (Figure 3.5). The effect of these drug arresting cells in the G2M phase is closely related to their IC50 values of cytotoxicity in PC-3 cells (1, 6, and 81 nM, respectively; Table 3.1), suggesting that this is a direct link to their mechanism of action.

### 3.3.5 The Effect of I-13 on Microtubule Polymerization

Vinblastine and I-13 were compared for their ability to inhibit tubulin polymerization at different concentrations of the compounds. Microtubule polymerization was measured by absorbance over time since assembled microtubules cause turbidity in the assay solution (Figure 3.6). These data indicate that I-13 mimics the effects of vinca alkaloids on tubulin polymerization to reduce polymerization and destabilize microtubule formation as is evident by the decrease in absorbance with increasing concentration. I-13 suppressed tubulin polymerization in a concentration-dependent manner with an IC50 of 554 nM, whereas the IC50 of vinblastine was 283 nM (Figure 3.7). In order to visualize the microtubule changes in the cells, immunofluorescence microscopy was used. Vinblastine inhibited microtubule polymerization with the appearance of short microtubule fragmentations in the cytoplasm. In contrast, treatment with docetaxel resulted in stabilization of microtubule with an increase in the density of microtubules. Treatment with I-13 induced similar changes to microtubules as compared to vinblastine (Figure 3.8).
Figure 3.4. The cellular arrest in the G2/M phase by I-13.

PC-3 cells were treated with different concentrations of compounds for 24 h and DNA content of the cells was analyzed by FACS. Selected FACS histograms are shown.
Figure 3.5. Dose-response curve of I-13 in cellular arrest in the G2M phase.

PC-3 cells were treated with different concentrations (0 to 1.5 μM) of I-13, vinblastine, or docetaxel for 24 h, and DNA content of the cells was analyzed by FACS. The percentage of cells in the G2M phase of the cell cycle division was quantified. Dose-response curves are shown.
3.3.6 *In Vitro* Competition of Binding of Vinblastine or Podophyllotoxin to Tubulin by I-13

Antimitotic agents are known to bind at different binding sites in the tubulin protein. Since microtubule polymerization inhibitors usually bind to the vinblastine- or colchicine-binding sites, we assessed the binding of I-13 to tubulin with $[^3]$Hvinblastine and $[^3]$Hpodophyllotoxin (binds to the colchicine site) in a competition binding assay using vincristine and colchicine as positive control inhibitors. When depolymerized tubulin was incubated with radiolabeled vinblastine in the presence of different concentrations of the unlabeled vincristine, a known competitor of vinblastine binding to tubulin, the amount of protein-bound $[^3]$Hvinblastine found in the flow-through was strongly reduced (Figure 3.9). Thus, vincristine competes for the binding of vinblastine to tubulin. However, when different concentrations of I-13 instead of vincristine were used, no effect on binding of $[^3]$Hvinblastine to tubulin was observed (Figure 3.9). When tubulin was incubated with $[^3]$Hpodophyllotoxin in the presence of different concentrations of unlabeled colchicine, the amount of $[^3]$Hpodophyllotoxin in the flow through the column was reduced (Figure 3.9). Thus, podophyllotoxin competes for the binding of colchicine to tubulin. Increasing concentrations of I-13 also decreased $[^3]$Hpodophyllotoxin binding to tubulin, indicating that I-13 binds to the colchicine-binding site to induce disassembly of microtubules (Figure 3.9).
Figure 3.6. Effect of I-13 in *in vitro* tubulin polymerization.

The microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. Representative experiment. Control (○); I-13, 0.1 µM (○); I-13, 1 µM (△); I-13, 5 µM (△); I-13, 10 µM (■). Mean ± SD, n=2.
Figure 3.7. Dose-response curve of tubulin depolymerization by I-13.
The microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. I-13 depolymerized microtubule in a concentration-dependent manner. IC_{50} values for tubulin inhibition were calculated (non-treated control considered as 100%). Mean ± SD, n=2.

<table>
<thead>
<tr>
<th></th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine</td>
<td>283 ± 18</td>
</tr>
<tr>
<td>I-13</td>
<td>554 ± 50</td>
</tr>
</tbody>
</table>
Figure 3.8. *In vitro* cellular microtubule changes by I-13.

PC-3 cells were treated with 2 μM of antimitotic agents for 6 h. Fixed cells were reacted with anti-α-tubulin-FITC antibody and the cellular microtubules were observed by a Zeiss Axioplan 2 fluorescent microscope.
Figure 3.9. *In vitro* competition of I-13 for colchicine or vinblastine binding sites on tubulin. Spin column assay was used to investigate binding sites of I-13 on tubulin. [³H]vinblastine was incubated with tubulin in the presence of different concentrations of vincristine or I-13 (*upper panel*). [³H]podophyllotoxin was incubated with tubulin in the presence of different concentrations of colchicine or I-13 (*lower panel*). Tubulin-bound [³H]vinblastine and [³H]podophyllotoxin were plotted against the concentrations of the competitors. Mean ± SD, n=2.
3.3.7 *In Vitro* Pharmacology of I-13

The acute toxicity (as measured by changes in morbidity in 24 h) of vinblastine and I-13 was examined in ICR mice via i.v. injection to identify the MTD. The acute MTD of vinblastine was 25 mg/kg, whereas I-13 did not induce any severe acute toxicity at doses up to 500 mg/kg. For sub-chronic toxicity, doses were administered via i.p. injection (5 days/week) in ICR mice. The positive control group was divided into groups of five (n=10 per group) and received vinblastine doses of 25, 5, 2.5, 1, and 0.5 mg/kg. For I-13, mice were divided into 4 groups (n=10 per group) that received 250, 125, 50, and 25 mg/kg. There was no significant difference in morbidity in 2.5, 1, and 0.5 mg/kg vinblastine or in any I-13 treatment groups (Figure 3.10). These results suggest that I-13 was well tolerated with up to 4-week treatment.

We further examined the effects of I-13 (5 and 25 mg/kg, i.p., 2 days/week and 10 mg/kg, i.p., q2d) on *in vivo* tumor growth for 4 weeks in a PC-3 xenograft model. Vinblastine- (0.5 mg/kg) and docetaxel-treated (5 mg/kg) mice were included as a positive control. Tumor size in the vehicle control group increased seven- to eightfold over a period of 4 weeks. I-13 (25 mg/kg) and vinblastine (0.5 mg/kg) twice weekly treatment resulted in 40% and 50% T/C (treated over control tumor volume ratio × 100%), respectively (Figure 3.11). Also with this dosing regimen, 5 mg/kg docetaxel inhibited tumor growth with 21% T/C, but induced a greater than 15% body weight loss after 2 weeks. In addition, the survival rate of docetaxel treated mice was 70% after 3 weeks and less than 30% after 4 weeks, while I-13 and vinblastine treated groups had 100% survival. A second xenograft study with q2d dosing schedule resulted in comparable efficacy of the 10 mg/kg I-13 and 0.5 mg/kg vinblastine (Figure 3.12) with 42 and 38% T/C, respectively. Unfortunately, study with q2d dosing schedule resulted in comparable efficacy of the 10 mg/kg I-13 and 0.5 mg/kg vinblastine (Figure 3.12) with 42% and 38% T/C, respectively. Unfortunately, docetaxel (5 mg/kg) treatment following every other day dosing was too toxic. Since the vehicle-control group bearing tumor did not induce any body weight loss and morbidity, the low survival rate caused by docetaxel could be considered due to general toxicity not tumor burden. I-13 was also well tolerated with no signs of toxicity with an increased dosing schedule. Loss of body weight after treatment was < 15% of the initial body weight in all treatment groups receiving I-13 (Figure 3.11 and 3.12). Thus, I-13 exerts potent antitumoral efficacy toward PC-3 solid tumor xenografts without any general toxicity.
Figure 3.10. Sub-chronic toxicity of I-13.
Doses were administrated via i.p. injection (5 days/week × 4) in ICR mice. For the positive control drug, vinblastine, mice were divided into groups of five; 25 (the acute MTD), 5, 2.5, 1, and 0.5 mg/kg were injected. For I-13, mice were divided into 4 groups; 250, 125, 50, and 25 mg/kg n=10.
Figure 3.11. Efficacy of I-13 in PC-3 xenografts (2 days/week, i.p.).

PC-3 xenografts were treated with vehicle control, vinblastine (0.5 mg/kg, i.p.; 2 days/week), docetaxel (5 mg/kg, i.p.; 2 days/week), and I-13 (5 and 25 mg/kg, i.p.; 2 days/week). The relative tumor growths (%) (upper panel) and body weights (lower panel) were plotted against time. Mean ± SD, n=6-8.
PC-3 xenografts were treated with vehicle control, vinblastine (0.5 mg/kg i.p.; q2d) docetaxel (5 mg/kg, i.p.; q2d), and I-13 (10 mg/kg, i.p.; q2d). The relative tumor growths (%) and body weights were plotted against time. Mean ± SD, n=6-8.
3.4. Discussion

Indole derivatives have been a major interest in anticancer drug development for decades. Indole-3-carbinol and diindolylmethane demonstrated apoptotic effects in human breast and prostate cancer cell lines in vitro (Fares, Ge et al. 1998; Ge, Fares et al. 1999) and a great number of novel indole compounds have been investigated for antimitotic effects such as BpR0L075, D24851, aroylindoles, tryprostatin A, and 2-acyl-1H-indole-4,7-diones (Usui, Kondoh et al. 1998; Bacher, Nickel et al. 2001; Mahboobi, Pongratz et al. 2001; Beckers, Reissmann et al. 2002; Kuo, Hsieh et al. 2004; Mahboobi, Sellmer et al. 2005; Liou, Wu et al. 2007; Liou, Wu et al. 2008). During drug screening efforts, we identified a series of novel indoles that exhibit potent anticancer activities. These indoles were synthesized by Suzuki coupling pathways, oxidation, and de-protection and their activity evaluated against a variety of human cancer cell lines. The most active compound, I-13, (3-(1H-indol-2-yl)phenyl)(1H-indol-2-yl)methanone was further studied to understand its basic mechanism of action and its potential use in vivo.

Apoptosis was induced by I-13 at nanomolar concentrations in several prostate cancer cell lines including LNCaP, PPC-1, DU-145, and PC-3. Like other microtubule-destabilizing agents, I-13 arrested cancer cells at the G2M phase in a concentration-dependent manner by binding to the colchicine-binding site and preventing tubulin polymerization.

P-glycoprotein is thought to be one of the major causes of failure of cancer therapy in the clinic. As such, the search has turned toward the identification of cytotoxic compounds that are not substrates for P-glycoprotein. I-13 showed potent cytotoxic effects in cells expressing P-glycoprotein, as well as MRP1-, MRP2- and BCRP-positive cell lines (Table 3.2). These data suggest that I-13 is a poor substrate of P-glycoprotein, MRP1, MRP2, and BCRP. The ability to retain activity in MDR over-expressing cell lines strongly supports the potential use of I-13 in taxane- and vinca alkaloid-resistant cancer diseases.

We also investigated the involvement of I-13 in an important cell survival pathway. It is known that microtubule damage leads to cancer cell apoptosis through inactivation of Bcl-2, an important guardian of microtubule integrity (Haldar, Basu et al. 1997). In our Western blot study using PC-3 cells, vinblastine and I-13 reduced the level of Bcl-2 in a time-dependent manner (Figure 3.3). These agents, however, also induced cytotoxicity in DU-145 prostate carcinoma, which does not express Bcl-2. The IC50 value of I-13 in DU-145 cells was somewhat higher (138 nM) than the IC50 values in other prostate cancer cell lines (44–81 nM) (Table 3.1). These data
suggest that microtubule-damaging agents induce apoptosis in connection with Bcl-2 down-regulation, but that decreases in Bcl-2 are not the only anticancer mechanism of antimitotic drugs. Docetaxel, a microtubule stabilizer, has also been shown to be an effective inhibitor of tumor growth in DU-145 xenograft model, thus supporting a Bcl-2 independent mechanism for tubulin inhibitors (Kraus, Samuel et al. 2003).

The *in vivo* toxicity study in ICR mice showed no severe toxicity of I-13 at doses up to 250 mg/kg, the maximum dose tested (Figure 3.10). Since vinca alkaloids are widely used antimitotic agents in clinical chemotherapy, we used vinblastine as one of the positive controls during *in vivo* efficacy and toxicity studies. In addition, I-13 was compared to docetaxel, an antimitotic taxane analog, which is the first approved chemotherapy for the treatment of advanced prostate cancer. A 25-fold lower dose of I-13 (10 mg/kg) was shown to be as effective in the PC-3 xenograft model as 0.5 mg/kg vinblastine, a dose only five-fold lower than the sub-chronic MTD of vinblastine in the q2d regimen. Docetaxel showed severe body weight loss, as well as tumor growth inhibition. The body weight loss (>15%) in docetaxel group (5 mg/kg, i.p., 2 days/week) was observed with 33% T/C after 2-week treatment. Docetaxel was very efficacious (21% T/C) but less than 30% of the mice survived by the end of this study. I-13 (10 mg/kg, i.p., q2d) induced tumor growth inhibition (42% T/C) with no signs of general toxicity. Ixabepilone (BMS-247550), the most widely clinically investigated epothilone produced significant antitumor activities at doses between 6.6 and 15 mg/kg (q4d × 3, i.v.), but the MTD of ixabepilone was between 10 and 16 mg/kg (q4d × 3, i.v.) in several mice xenograft models (Lee, Borzilleri et al. 2001; Peterson, Tucker et al. 2005; Morris and Fornier 2008). Our data suggest that I-13 may also provide a wider therapeutic window between efficacy and toxicity than available agents or ixabepilone, perhaps due to its utilization of the colchicine binding site in tubulin. More definitive animal studies to examine the general safety and neurotoxicity of I-13 are on going in our laboratory.

Clinical use of indole alkaloids is currently restricted to vinblastine, vincristine and vinorelbine, all of which target the vinca alkaloid binding site in tubulin. Although a number of agents are undergoing early clinical trials, there are no therapies available for clinical use that bind the colchicine binding site and avoid MDR. The relatively large structural complexity of vinca alkaloids and taxanes has remained a challenge for industry to develop a feasible synthesis. In this study, we show that a simple molecule, I-13, elicits high anticancer activity by targeting the colchicine binding site in a broad spectrum of human cancer cells including ABC transporter
positive cell lines. As a whole, our pilot in vivo anticancer activity and MDR insensitivity of I-13 validates the development of structurally related antimitotic agents against prostate cancer and other paclitaxel, vinca alkaloid or MDR cancers.

3.5. Acknowledgements

I would like to thank Dr. Duane D. Miller’s group for synthesis of I-13, Dr. Yun Wang for her assistance in the transfection experiments, and Dr. James T. Dalton, Dr. Christina M. Barrett, and Dr. Jun Yang for their guidance, valuable discussions, and manuscript review. This chapter was originally published in Cancer Chemotherapy and Pharmacology 2010 Apr 11 [Epub ahead of print].
4.1. Introduction

Microtubules, which are comprised of α- and β-tubulin dimers, are one of the most useful subcellular targets in chemotherapy. It is generally accepted that the antiproliferative properties of tubulin inhibitors result from their interference with microtubule dynamics, since microtubules are critically important components in cell mitosis and cell signaling (Jordan and Wilson 2004). In animal cells, mitosis requires the formation of a unique structure, the mitotic spindle, which is essential for the maintenance of euploidy. The proper assembly and function of the mitotic spindle is necessary for adequate chromatid segregation (Beghin, Galmarini et al. 2007).

There are two major groups of antimitotic agents consisting of microtubule-stabilizing agents (i.e., taxanes) and microtubule-destabilizing drugs (i.e., vinca alkaloids and colchicines). They interact physically with tubulin by binding to one of the three main binding sites: colchicine-, vinblastine-, or paclitaxel-binding site (Islam and Iskander 2004). Tubulin binding agents alter the dynamic behaviors of microtubules and arrest mitotic cells in the M-phase of the cell cycle, thus leading to apoptotic cell death. Despite the clinical success of tubulin inhibitors, agents currently used for chemotherapy are known to have limitations such as peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance due to multidrug resistance (MDR) transporters and tubulin mutation (Hamel 1992; Dumontet and Sikic 1999; Verrills and Kavallaris 2005; Malik and Stillman 2008). These limitations have led to the search for new agents that inhibit tubulin activity and circumvent these limitations. (3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone (hereafter referred to as I-19, Figure 4.1) was identified during studies to discover new pharmacophores for treatment
of cancer. We examined the tubulin binding properties as well as the \textit{in vitro} and \textit{in vivo} efficacy and toxicity of I-19.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{chemical_structure.png}
\caption{Chemical structure of I-19.}
\end{figure}
4.2. Materials and Methods

4.2.1 Chemicals and Animals

Monoclonal antibodies to phospho-Bcl-2 (pBcl-2), cyclin B1, Cdc25C, phospho-Cdc25C (pCdc25C), Cdc2, phospho-Cdc2 (pCdc2), and horseradish peroxidase conjugated secondary antibodies were purchased from Millipore Corporation (Billerica, MA). Bovine brain tubulin protein was purchased from Cytoskeleton, Inc (Denver, CO). [\(^3\)H]Vinblastine and [\(^3\)H]podophyllotoxin were purchased from Moravek, Inc (Brea, California). Sephadex G25 column and Cell Death Detection ELISA (anti-histone ELISA) were purchased from Roche Applied Science (Indianapolis, IN). Murine 2.5S nerve growth factor was purchased from Promega (Madison, WI). All other chemicals were purchased from Sigma (St. Louis, MO).

Four to five week old male ICR mice and male nu/nu nude mice were purchased from Harlan Biosciences (Indianapolis, IN). All animal protocols were approved by the Animal Care and Use Committee at The Ohio State University or the University of Tennessee Health Science Center.

4.2.2 Cell Culture

All human cancer cell lines except K562/DOX, HEK293-MRP1, HEK293-MRP2, HEK293-pcDNA3-10, and HEK293-482R2 were obtained from American Type Culture Collection (Manassas, VA). K562/DOX, HEK293-pcDNA3-10, and HEK293-482R2 were kindly provided by Dr. Duxin Sun (College of Pharmacy, The Ohio State University). The plasmids for MRP1 and MRP2 were kindly provided by Dr. Susan P. C. Cole (Department of Pharmacology & Toxicology, Queen’s University) (Letourneau, Slot et al. 2007). MES-SA/DX5 and K562/DOX cell lines were obtained by in vitro selection of MES-SA and K562, respectively, in progressively increasing doses of doxorubicin (Harker and Sikic 1985; Tsuruo, Iida-Saito et al. 1986; Fang, Zhang et al. 2006). HEK293-pcDNA3-10 and HEK293-482R2 were HEK293 cells transfected with empty pcDNA3 vector and pcDNA3 vector containing full-length BCRP, respectively (Robey, Honjo et al. 2003). HEK293 cells stably expressing MRP1, MRP2, and pcDNA3.1(-) have been described before (Letourneau, Slot et al. 2007). MES-SA and MES-SA/DX5 were
maintained in McCoy's 5A Medium containing 2 mM L-glutamine supplemented with 10% FBS. PC12 was maintained in RPMI-1640 medium with 5% FBS and 10% heat-inactivated horse serum. All other cells were maintained in RPMI-1640 medium with 2 mM L-glutamine and 10% FBS. All cells were grown at 37 °C in a humidified atmosphere containing 5% carbon dioxide. McCoy's 5A Media, RPMI-1640 media, trypsin-EDTA and L-glutamine were purchased from Hyclone (Logan, UT). FBS and horse serum were purchased from Invitrogen (Carlsbad, CA) and American Type Culture Collection (Manassas, VA), respectively.

4.2.3 Growth Inhibition Assay

The cytotoxic or antiproliferative activity of test compounds was investigated in several cell lines using the SRB assay (Skehan, Storeng et al. 1990). MTT assay was also used for the leukemia cell lines (Alley, Scudiero et al. 1988). Cultured cells were plated into 96-well plates and incubated with medium containing different concentrations of the test compounds for 96 h. Cells were stained with SRB solution or MTT solution. The optical density was determined at 540 nm on a microplate reader (Dynex Technologies, Chantilly, VA). Plots of percent inhibition of cell growth versus drug concentration were constructed, and the concentration that inhibited cell growth by 50% relative to the untreated control (IC50) was determined by nonlinear least squares regression using WinNonlin software (Pharsight Corporation, Cary, NC). WinNonlin was provided by a Pharsight Academic License to The Ohio State University.

4.2.4 Determination of DNA Fragmentation by ELISA

Apoptosis was measured by quantitation of cytoplasmic histone-associated DNA fragments using the cell death detection ELISA kit. Cells were seeded in 6-well plates and exposed to I-19 for 24 h at different concentrations. The quantitation of DNA fragments was performed according to the manufacturer’s instructions. Results are expressed as the enrichment factor (i.e., the ratio of the optical density in treated cells to the optical density in control cells).
4.2.5 Cell Cycle Analysis

Cell cycle distribution was determined by propidium iodide staining. Treated cells were washed with PBS and fixed with 70% ice-cold ethanol overnight. Fixed cells were then stained with 20 μg/ml of PI in the presence of RNase A (300 μg/ml) at 37°C for 30 min. Cell cycle distribution was analyzed by FACS analysis core services at The Ohio State University.

4.2.6 Western Blot Analysis

Treated cells were lysed in cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton x-100, and freshly added Na₃VO₄ (2 mM), NaF (20 mM) and complete protease inhibitor cocktail). For each sample, an aliquot (20 to 40 μg) of total protein was loaded and run on a SDS-PAGE gel. Protein was transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), blocked with 5% non-fat milk, incubated with primary antibody overnight at 4°C, and then incubated with secondary antibody at room temperature for 1 h. ECL™ (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used for detection.

4.2.7 In Vitro Tubulin Polymerization Assay

Tubulin polymerization kits (Cytoskeleton, Denver, CO) were used to study tubulin depolymerization. The reaction contained 50 μL of 4 mg/mL tubulin in PEM buffer (80 mM PIPES, pH 7.0, 0.5 mM EGTA, 2 mM MgCl₂, 1 mM GTP, 10% glycerol). The procedure was done according to the manufacturer’s protocol. Tubulin polymerization was monitored in a UV spectrophotometer for 40 minutes at 340 nm at 37°C.

4.2.8 In Vitro Binding Assay by Spin Column

A spin column binding assay was done as reported previously (Bacher, Nickel et al. 2001) using bovine tubulin, size-exclusion Sephadex G25 columns, and ³H-labeled ligands. Briefly, tubulin (0.1 mg/mL) was incubated in PEM buffer either with 3 μM vinblastine
containing $[^3\text{H}]$vinblastine (4×10⁴ dpm/nmol) in the presence of different concentrations of vincristine or I-19 or with 3 μM podophyllotoxin containing $[^3\text{H}]$podophyllotoxin (4×10⁴ dpm/nmol) in the presence of different concentrations of colchicine or I-19 at 37 °C for 1 h. Samples were then loaded onto size-exclusion Sephadex G25 columns and centrifuged at 200 g for 1 min, and radioactivity in the flow-through was analyzed by scintillation counting.

4.2.9 Indirect Immunofluorescence Microscopy

PC-3 cells were plated on poly-D-lysine coated glass cover slips in 6-well plates (5×10⁴ cells/well) the day before treatment. After cells were incubated with cytotoxic agents at 37°C for 24 h, cells were fixed on the cover slips with 4% formaldehyde in PBS for 10 min and permeabilized by 2% Triton-X in PBS for 5 min at room temperature. Fixed cells were blocked with 3% bovine serum albumin in TBST for 30 min and incubated with anti-α-tubulin-FITC antibody (1:100 dilution) at 4°C overnight. Cover slips were mounted on slides using mounting medium followed by microscopic analysis with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, Thornwood, NY). Images were acquired with a Zeiss Axiocam HRc, using Zeiss AxioVision.

4.2.10 In Vivo Antitumor Efficacy Study

PC-3 cells (2.5×10⁶ cells/site), MES-SA cells (2×10⁶ cells/site), and MES-SA/DX5 (2×10⁶ cells/site) plus Matrigel (BD biosciences, San Jose, CA) were injected subcutaneously into flanks of male nu/nu mice. Tumor size was measured using calipers every 3-4 days and calculated as $V = \pi / 6 \times (\text{length}) \times (\text{width})^2$ (Dethlefsen, Prewitt et al. 1968). When tumors reached a volume of approximately 100-150 mm³, drug treatment was initiated. The control group was treated with vehicle (10% DMSO in PEG300). During the treatment, tumor size and body weights were measured every 2-4 days.
4.2.11 *In Vitro* Neurite Outgrowth

PC-12 cells were seeded in 6-well plates coated with poly-D-lysine (BD, Franklin Lakes, NJ) (10000 cells/well). After 6 h, cells were pretreated for 3 days by adding 100 ng/ml murine 2.5S β-NGF (Promega, Madison, WI) to induce neuronal differentiation and neurite outgrowth. Differentiated cells were then treated for 24 h with compounds at various concentrations in the presence of NGF (100 ng/ml). Vinblastine and docetaxel were used as positive controls. After compound treatments, approximately 100 cells in each well were randomly chosen and the numbers of cells with no neurites, short neurites (<2 × cell body), and long neurites (> 2 × cell body) were counted (n=3).

4.2.12 Rotarod Performance

The training and rotarod test was conducted as described previously (Karl, Pabst et al. 2003). Briefly, during the training phase for 2 consecutive days (3 trials/day), mice (10 mice/group) were trained to run in a rotarod apparatus without falling at a constant speed (12 rpm) for a maximum of 2 min. For the rotarod performance test, duration of each trial was less than 5 min. During this time, the rotation speed was constantly increased from 5 rpm to 40 rpm. Tests were performed for 2 weeks (2 days/week). The animals were tested in three trials per day with an intertrial interval of 30 min. The animal performance was recorded as the average elapsed time for the mice to fall off.

4.2.13 Statistical Analysis

The results (mean values ± SD) were subjected to statistical analysis by single-factor ANOVA. The level of significance was set at $P < 0.05$. 
4.3. Results

4.3.1 The Effect of I-19 on Cell Proliferation

To explore the effect of I-19 on cancer cell proliferation, we treated human cancer cell lines from prostate, colon, breast, and bladder with different concentrations of compounds using vinblastine and docetaxel as positive controls. Cell proliferation was measured by SRB assay. Proliferation of all cell lines was inhibited by I-19 in a concentration-dependent manner with IC\textsubscript{50} values ranging from 15.1 to 39.2 nM (Table 4.1). Vinblastine and docetaxel confirmed the assay validity by exhibiting high potency in cancer cell lines with IC\textsubscript{50} values ranging from 1.1 nM to 6.3 nM.

Since ABC transporters are thought to be one of the major causes of MDR of anticancer drugs (Van Brussel, Jan Van Steenbrugge et al. 2001; Verrills and Kavallaris 2005), the antitumoral efficacy of I-19 was compared with other anticancer drugs in a cytotoxicity assay using MDR cell lines with high levels of P-glycoprotein, MRP1, MRP2, or BCRP (Table 4.1) (Harker and Sikic 1985; Tsuruo, Iida-Saito et al. 1986; Robey, Honjo et al. 2003; Fang, Zhang et al. 2006; Letourneau, Slot et al. 2007). As judged from the resistance factor (RF, the ratio of the IC\textsubscript{50} values in cells over-expressing MDR transporters relative to the IC\textsubscript{50} values in their control cells), cells with P-glycoprotein were 13- to 203-fold resistant to vinblastine and docetaxel. RFs for vinblastine, SN-38, and docetaxel were 4.9, 2.1 and 7.2 in cells with high MRP1 expression and 3.7, 1.5, and 7.6 in cells over-expressing MRP2. Cells transfected with the vector including BCRP gene were also 33- fold resistant to SN-38. In contrast, the cytotoxicity efficacy of I-19 against cancer cells was unaltered by the MDR phenotypes.
Table 4.1. Anticancer efficacy of I-19 in different cancer cell lines and MDR cell lines with different resistance phenotypes

<table>
<thead>
<tr>
<th></th>
<th>I-19</th>
<th>Vinblastine</th>
<th>SN-38</th>
<th>Docetaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>30.9 ± 3.5</td>
<td>3.4 ± 0.9</td>
<td>ND</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>PC-3</td>
<td>28.2 ± 2.0</td>
<td>1.4 ± 0.3</td>
<td>ND</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>DU-145</td>
<td>22.8 ± 4.1</td>
<td>2.6 ± 1.0</td>
<td>ND</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>PPC-1</td>
<td>18.7 ± 1.5</td>
<td>1.1 ± 0.4</td>
<td>ND</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>17.6 ± 2.1</td>
<td>1.6 ± 0.1</td>
<td>ND</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>HT-29</td>
<td>15.1 ± 1.3</td>
<td>2.4 ± 0.2</td>
<td>ND</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>MCF-7</td>
<td>39.2 ± 2.9</td>
<td>1.4 ± 0.4</td>
<td>ND</td>
<td>3.8 ± 0.8</td>
</tr>
<tr>
<td>K562</td>
<td>23.6 ± 2.4</td>
<td>1.4 ± 0.4</td>
<td>ND</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>K562/Dox</td>
<td>18.0 ± 4.4 (0.8)</td>
<td>268 ± 48 (191)</td>
<td>ND</td>
<td>548 ± 76 (203)</td>
</tr>
<tr>
<td>MES-SA</td>
<td>31.5 ± 2.9</td>
<td>2.3 ± 0.8</td>
<td>ND</td>
<td>5.9 ± 1.1</td>
</tr>
<tr>
<td>MES-SA/DX5</td>
<td>32.3 ± 8.2 (1.0)</td>
<td>45.7 ± 5.3 (20)</td>
<td>ND</td>
<td>76.4 ± 8.7 (13)</td>
</tr>
<tr>
<td>HEK293-pcDNA3.1</td>
<td>23.8 ± 1.7</td>
<td>5.0 ± 1.1</td>
<td>8.4 ± 0.6</td>
<td>4.4 ± 1.7</td>
</tr>
<tr>
<td>HEK293-MRP1</td>
<td>30.4 ± 6.4 (1.3)</td>
<td>24.3 ± 2.0 (4.9)</td>
<td>17.7 ± 1.4 (2.1)</td>
<td>31.3 ± 5.1 (7.2)</td>
</tr>
<tr>
<td>HEK293-MRP2</td>
<td>31.5 ± 8.1 (1.3)</td>
<td>18.1 ± 4.2 (3.7)</td>
<td>12.4 ± 0.5 (1.5)</td>
<td>33.1 ± 4.1 (7.6)</td>
</tr>
<tr>
<td>HEK293-pcDNA3-10</td>
<td>13.5 ±1.6</td>
<td>ND</td>
<td>2.9 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>HEK293-482R2</td>
<td>13.6 ± 1.3 (1.0)</td>
<td>ND</td>
<td>94.3 ± 20 (32.5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: P-glycoprotein, MRP1, MRP2, BCRP were over-expressed in K562/DOX, MES-SA/DX5, HEK293-MRP1, HEK293-MRP2 and HEK293-482R2. The resistance factor (in parentheses) was calculated as the ratio of IC50 values for the resistant cell subline to that of the parental cell line. All experiments were performed at least in three replicates.

ND: not determined
4.3.2 Apoptotic Effect Induced by I-19

To determine whether I-19 induces apoptosis of cancer cells, we examined DNA fragmentation by using a cell death detection ELISA kit. Vinblastine was used as a positive control to confirm assay validity. Vinblastine and I-19 increased the enrichment factor (absorbance of treated cells/absorbance of vehicle control cells) in a concentration-dependent manner in PC-3 cells with EC\textsubscript{50} values of 8.1 and 42.7 nM, respectively (Figure 4.2). We also assessed whether I-19 induced the phosphorylation of Bcl-2, thus inactivating the protein. Following 24 h treatment in PC-3 cells, immunoblots of pBcl-2 showed that 5 nM vinblastine and 50 nM I-19 induced phosphorylation (Ser70) of Bcl-2 (Figure 4.3). As a whole, these studies provide persuasive evidence that I-19 potently induces apoptosis in PC-3 cells that is at least partially mediated by inactivation of Bcl-2.

4.3.3 Effect of I-19 in Cell Cycle Distribution.

The potent antiproliferative activity and apoptosis of I-19 on cancer cells prompted us to test its effects on the cell cycle. The percentage of PC-3 cells in the G2M phase increased (Figure 4.4) from 19 (control) to 24, 63, and 77% at I-19 concentrations of 10, 50, and 100 nM, respectively, indicating that PC-3 cells were significantly arrested in the G2M phase in a concentration-dependent manner, a pattern that is commonly observed with taxanes and vinca alkaloids. When the percentages of cells in the G2M phase were plotted against different concentrations of the compounds (0.1 nM to 1 \mu M), vinblastine, docetaxel, and I-19 arrested the cell cycle with EC\textsubscript{50} values of 7, 14, and 34 nM, respectively (Figure 4.5). The effect of these drugs arresting cells in the G2M phase is closely related to the IC\textsubscript{50} values of cytotoxicity in PC-3 cells (1, 6, and 28 nM, respectively; Table 4.1), suggesting that this is a direct link to the mechanism of action. Therefore, changes of expressed and phosphorylated levels of key mitotic regulators following I-19 treatment were evaluated. Cyclin B1 accumulation, Cdc25C phosphorylation, and Cdc2 dephosphorylation were observed at I-19 concentrations of higher than 50 nM (24 h treatment) in PC-3 cells (Figure 4.6). However, protein expression of pCdc25C (Ser 216) was decreased in PC-3 cells (Figure 4.6).
Figure 4.2. Cytoplasmic histone-associated DNA fragments by I-19 in PC-3 cells.

The extent of apoptosis (i.e., the enrichment factor) was determined using a commercially available anti-histone ELISA after 24 h treatment. Vinblastine was used as a positive control. Mean ± SD, n=3.
Figure 4.3. Bcl-2 phosphorylation by I-19.

Bcl-2 phosphorylation in PC-3 cells was analyzed by Western blot analysis after 24 h treatment of I-19 in different concentrations. Actin was used as a loading control.

Mean ± SD, n=3. * $P < 0.05$. 
Figure 4.4. The cellular arrest in the G2M phase by I-19.

PC-3 cells were treated with different concentrations of vinblastine and I-19 for 24 h and DNA content of the cells was analyzed by FACS. Vinblastine and I-19 arrested cells in the G2M phase.
Figure 4.5. Dose-response curve of I-19 in cellular arrest in the G₂M phase.

PC-3 cells were treated with different concentrations of docetaxel, vinblastine, and I-19 for 24 h. The percentage of cells in the G₂M phase of the cell cycle division was quantified and dose-response curves are shown.
Figure 4.6. Changes of protein expressions of G2M regulators by I-19.

Changes in expressed and phosphorylated status of G2M regulators including cyclin B1, Cdc25C, pCdc25C (Ser216), Cdc2, and pCdc2 (Tyr15) by I-19 (24 h treatment) were evaluated in PC-3 cells by Western blot analysis. Actin was used as a loading control. Mean ± SD, n=2-3. Significant differences from control: * $P < 0.05$; # $P < 0.07$. 
4.3.4 The Effect of I-19 on Microtubule Polymerization

Since I-19 caused cell arrest in the G2M phase, we investigated whether I-19 affects microtubule organization. In order to visualize the microtubule changes in the cells, immunofluorescence microscopy was used. As expected, vehicle-treated control cells demonstrated a variety of fluorescence patterns showing different phases of the cell cycle. On the other hand, vinblastine treated cells demonstrated the appearance of short microtubules due to fragments in the cytoplasm (Figure 4.7). In contrast, treatment with docetaxel resulted in stabilization of microtubules illustrated by an increase in the density of microtubules with brighter fluorescence in a time- and concentration-dependent manner (Figure 4.8). I-19 treatment showed fluorescence similar to vinblastine-induced microtubule changes (Figure 4.9). Therefore, vinblastine and I-19 were compared for their ability to inhibit tubulin polymerization in a cell free system using tubulin polymerization kits (Figure 4.10). As expected, vinblastine decreased the microtubule formation in a concentration-dependent manner with an IC50 of 283 nM, and I-19 mimicked the effects of vinca alkaloids with an IC50 of 381 nM (Figure 4.11). It is generally observed that antimitotic drugs interact with tubulin either at the colchicine-, vinblastine-, and paclitaxel-binding sites. Competition-binding studies using a spin column assay (Figure 4.12) showed that I-19 competitively inhibited [3H]podophyllotoxin binding to tubulin as colchicine did, whereas I-19 was not able to displace [3H]vinblastine. This suggests that I-19 directly interacts with tubulin by binding to the colchicine-binding domain.
Figure 4.7. *In vitro* cellular microtubule changes by vinblastine.

PC-3 cells were treated with vinblastine in different concentrations for 6, 12, or 24 h. Fixed cells were incubated with anti-α-tubulin-FITC antibody and the cellular microtubules were observed with a Zeiss Axioplan 2 fluorescent microscope.
Figure 4.8. *In vitro* cellular microtubule changes by docetaxel.

PC-3 cells were treated with docetaxel in different concentrations for 6, 12, or 24 h. Fixed cells were incubated with anti-α-tubulin-FITC antibody and the cellular microtubules were observed with a Zeiss Axioplan 2 fluorescent microscope.
Figure 4.9. *In vitro* cellular microtubule changes by I-19.

PC-3 cells were treated with I-19 in different concentrations for 6, 12, or 24 h. Fixed cells were incubated with anti-α-tubulin-FITC antibody and the cellular microtubules were observed with a Zeiss Axioplan 2 fluorescent microscope.
Figure 4.10. Effect of I-19 in *in vitro* tubulin polymerization.

The microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. Representative experiment. Control (■); I-19, 0.1 µM (●); I-19, 1 µM (○); I-19, 5 µM (▲); I-19, 10 µM (▲).
Figure 4.11. Dose-response curve of tubulin depolymerization by I-19.

The microtubule polymerization was monitored by measuring the turbidity at 340 nm in the absence or presence of drugs. I-19 depolymerized microtubule in a concentration-dependent manner. IC<sub>50</sub> values for tubulin inhibition were calculated (vehicle control considered as 100%). Mean ± SD, n=2.
Figure 4.12. *In vitro* competition of I-19 for colchicine or vinblastine binding sites on tubulin. Spin column assay was used to investigate binding sites of I-19 on tubulin. $[^3]$Hvinblastine was incubated with tubulin in the presence of different concentrations of vincristine or I-19 (*upper panel*). $[^3]$Hpodophyllotoxin was incubated with tubulin in the presence of different concentrations of colchicine or I-19 (*lower panel*). Tubulin-bound $[^3]$Hvinblastine and $[^3]$Hpodophyllotoxin were plotted against the concentrations of the competitors. Mean ± SD, n=2.
4.3.5 Efficacy of I-19 in Tumor Xenograft Models

We next investigated the ability of I-19 to inhibit growth in PC-3, MES-SA, and MES-SA/DX5 xenograft models after i.p. injection. Docetaxel (5 mg/kg) treated mice were included as a positive control. In twice weekly treatments against PC-3 xenograft models, doses of 5 and 10 mg/kg I-19 were well tolerated. Tumor growth was inhibited 68% and 82% for the 5 mg/kg and 10 mg/kg I-19, respectively. The 10 mg/kg I-19 group showed comparable efficacy to the 5 mg/kg docetaxel group in this regimen (Figure 4.13) without any general toxicity. In contrast, only 70% of the mice survived in the docetaxel treated group after 3 weeks with an end of study survival rate less than 30%. In the second xenograft study, the efficacy of I-19 against tumors with ABC transporter related resistance was tested using MES-SA/DX5 cells over-expressing P-glycoprotein (Harker and Sikic 1985). Doses of 5 mg/kg docetaxel and 0.5 mg/kg vinblastine showed tumor growth inhibition in MES-SA xenografts with TGIs of 54% and 48%, respectively (Figure 4.14). These control compounds, however, were not effective in MES-SA/DX5 xenografts with much lower TGIs of 9% (5 mg/kg docetaxel, q2d) and 24% (0.5 mg/kg vinblastine, q2d) (Figure 4.15). In contrast, I-19 inhibited tumor growth significantly in MES-SA/DX5 as well as in MES-SA xenografts with the q2d regimen (Figure 4.14 and 4.15). In the MES-SA/DX5 xenograft model, I-19 showed similar TGIs of 47% and 76% in the 5 and 10 mg/kg treatment groups, respectively, as compared to the MES-SA model with 47% and 74%. I-19 treatment did not induce any general toxicity or significant body weight loss in either the 5 or 10 mg/kg treatment groups.
Figure 4.13. Efficacy of I-19 in PC-3 xenografts (2 days/week, i.p.)

PC-3 xenografts were treated with vehicle control, docetaxel (5 mg/kg, i.p.; 2 days/week), and I-19 (5 and 10 mg/kg, i.p.; 2 days/week). The relative tumor growths (%) (upper panel) and survival rates (lower panel) were plotted against time. Mean ± SD, n=8.
Figure 4.14. Efficacy of I-19 in MES-SA xenografts (q2d, i.p.)

MES-SA xenografts were treated with vehicle control, vinblastine (0.5 mg/kg, i.p., q2d), docetaxel (5 mg/kg, i.p., q2d), and I-19 (5 and 10 mg/kg, i.p, q2d). The relative tumor growths (%) (upper panel) and body weights (lower panel) were plotted against time. Mean ± SD, n=8.
Figure 4.15. Efficacy of I-19 in MES-SA/DX5 xenografts (q2d, i.p.)
MES-SA/DX5 xenografts were treated with vehicle control, vinblastine (0.5 mg/kg, i.p., q2d),
docetaxel (5 mg/kg, i.p., q2d), and I-19 (5 and 10 mg/kg, i.p, q2d). The relative tumor growths
(%) (upper panel) and body weights (lower panel) were plotted against time. Mean ± SD, n=8.
4.3.6 Neurotoxicity Studies of I-19

NGF-dependent neurite outgrowth is commonly used as a *in vitro* model to study the neurotoxic effects of drugs (Geldof 1995; Geldof, Minneboo et al. 1998). NGF-dependent neurite outgrowth assay showed dose-dependent reduction of PC12 neurite extensions in the vinblastine and I-19 treatment groups. 88% of vehicle treated cells expressed neurite elongation (Figure 4.16). Twenty-one percent of cells expressed neurite elongation in 5 nM vinblastine treatment and only long neurites were observed in 1.5% of cells. I-19, however, resulted in higher percentages of neurite forming cells than vinblastine with 65% of cells showing neurite elongation with 10 nM I-19 treatment. Further, 50 nM I-19, a more toxic dose than IC₅₀ value, did not reduce neurite growth showing 60% of cells with neurites (Figure 4.16 and Table 4.2). To evaluate *in vivo* neurotoxicity, mouse performance on the accelerating rotarod was examined following exposure to vehicle, vinblastine, vincristine, or I-19. Vehicle-treated mice stayed on the rotating rod for 151 s at the end of 2 weeks, whereas 0.5 mg/kg vinblastine- and vincristine-treated mice showed significant reduction in their ability to stay on the rotating rod with mean times on the rod of 121 (P=0.0006) and 104 s (P=0.00006), respectively. I-19-treated mice (10 mg/kg) did not show impaired rotarod performance as compared to the vehicle control (P=0.6) (Figure 4.17).

<table>
<thead>
<tr>
<th>IC₅₀ values of I-19 in PC-12 cell line. Mean ± SD, n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC₅₀ (nM)</strong></td>
</tr>
<tr>
<td>Vinblastine</td>
</tr>
<tr>
<td>Vincristine</td>
</tr>
<tr>
<td>I-19</td>
</tr>
</tbody>
</table>
Figure 4.16. Effect of I-19 on \textit{in vitro} PC-12 neurite outgrowth.

Pretreated PC-12 cells with 100 ng/ml murine 2.5S β-NGF were treated with vehicle-control, vinblastine, and I-19 to various final concentrations in presence of 100 ng/ml NGF for 24 h. The numbers of cells with no neurite, short neurite (<2 × cell body), and long neurite (> 2 × cell body) were counted. Mean ± SD, n=3.
Figure 4.17. Effects of I-19 to the mouse performance on accelerating rotating rod.
ICR mice were dosed with vehicle control, vinblastine (0.5 mg/kg, i.p., q2d), vincristine (0.5 mg/kg, i.p., q2d), or I-19 (10 mg/kg, i.p., q2d) for 2 weeks. Mice were placed on an accelerating rotating rod and the performance on the rotarod was monitored 2 days/week. Mean ± SD, n=10.
4.4. Discussion

Microtubules are considered to be one of the major therapeutic targets of anticancer drugs to date. In spite of clinical success of tubulin inhibitors, some limitations remain, such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance conferred by MDR transporters and tubulin mutations (Hamel 1992; Dumontet and Sikic 1999; Verrills and Kavallaris 2005; Malik and Stillman 2008). The structural complexity of naturally-derived tubulin inhibitors such as vinca alkaloids, taxanes, maytansin, and halichondrin B analogs limits the use of conventional approaches to characterize structure-activity relationships and to isolate active compounds (Hamel 1992). Drug resistance is also one of the main problems in clinical chemotherapy. The expression of multidrug resistance proteins, such as P-glycoprotein and MRPs, and mutations of the target protein are known to result in drug resistance. In addition, peripheral neurotoxicity is considered to be a major side effect in the clinical application of antimitotic agents because they bind to beta-tubulin which is the primary component of the axonal microtubules (Windebank and Grisold 2008).

Here, we identified a structurally novel compound, I-19, (3-(1H-indol-2-yl)phenyl)(3,4,5-trimethoxyphenyl)methanone, which inhibits cell growth in a variety of human tumor cells and arrests cell cycle progression in the G2M phase. We, therefore, examined the influence of I-19 on changes of key mitotic regulators. I-19 treatment induced abnormal cyclin B1 accumulation and Cdc2 dephosphorylation in PC-3 cells. Phosphorylation of Cdc25C (Ser216) is known to induce nuclear export and reduction of activity of Cdc25C by allowing the binding of 14-3-3 to pCdc25C, while abundant phosphorylation of Cdc25C (Ser191, Ser198, etc) activates Cdc25C (Perdiguero and Nebreda 2004). Phosphorylation in Cdc25C (Ser216) decreased significantly after I-19 treatment, resulting in dephosphorylation of Cdc2 (Thr14 and Tyr15) that is required for activation of Cdc2/cyclin B1 and subsequent entry into mitosis (Hutchins and Clarke 2004). These data suggested that I-19 regulated the cell transition from G2 to mitosis by constitutive activation of Cdc2/cyclin B1 complex. Since I-19 caused cell arrest in the G2M phase, further in vitro studies were conducted to investigate whether I-19 affects microtubule organization. These studies showed that I-19 destabilizes microtubules in human tumor cells by binding to the colchicine-binding site. In the xenograft models using PC-3 cell line, 10 mg/kg I-19 (2 days/week, i.p.) showed comparable tumor growth inhibition to 5 mg/kg docetaxel (2 days/week, i.p.) without signs of toxicity. Furthermore, I-19 induced potent cell growth inhibition against
drug-resistant cell lines over-expressing ABC transporters and showed potent efficacy in the animal xenograft models with MES-SA/DX5 as well as MES-SA. In contrast, 0.5 mg/kg vinblastine and 5 mg/kg docetaxel were not effective in MES-SA/DX5 resistance model as compared to sensitive MES-SA. These *in vivo* results suggested I-19 could be very effective in tumors that have obtained resistance by over-expressing P-glycoprotein.

Mitotic spindle poisons leading to dynamic instability of microtubules induce tumor cell death. However, since microtubules fulfill important functions in interphase, resting, and differentiated cells for the maintenance of cytoskeletal functions and intracellular transport processes, microtubule inhibitors are known to exhibit unwanted side effects including peripheral neuropathies. The peripheral neurotoxicity might result from a disruption of microtubule mediated axonal flow. Microtubule inhibitors such as taxanes and vinca alkaloids are also known to induce myelosuppression and neutropenia due to the inhibition of the proliferation of non-transformed cells such as hematopoietic precursor cells (Canta, Chiorazzi et al. 2009; Schiff, Wen et al. 2009). A neurite outgrowth assay in PC12 cells has been proposed as an *in vitro* model to investigate chemotherapy-induced neuropathy (Geldof 1995; Geldof, Minneboo et al. 1998). In this neurite outgrowth study, I-19 caused less neurotoxic damage with higher percentages of neurite forming cells compared to vinblastine. Furthermore, since *in vivo* behavioral assays are also recommended to detect neuropathic pain, rotarod performance was examined. The rotarod is one of the widely used tests for neuromotor performance since 1968 (Karl, Pabst et al. 2003) (Jones and Roberts 1968). The status of the motor system of ICR mice was tested on the accelerating rotarod. Vinblastine (0.5 mg/kg, q2d, i.p.) and vincristine (0.5 mg/kg, q2d, i.p.) showed impaired performance from day 4 to day 14. I-19 (10 mg/kg, q2d, i.p.), in comparison, showed no loss of function, thus indicating that I-19 had no effect on motor performance. Although studies are needed to elucidate the effects of I-19 in the central as well as peripheral nervous system, our rotarod studies provide initial but compelling comparison of the peripheral neurotoxicity of I-19 to the vinca alkaloids, due to the limited access of vinca alkaloids to the central nervous system. Additional electrophysiologic and nerve biopsy studies would be useful to understand how I-19 affects neuronal cell bodies, Schwann cells, myelins, axons, and/or nerve roots (Bal-Price, Sunol et al, 2008). In addition, other animal behavior studies using hot plate and von Frey fiber could be considered (Karl, Pabst et al. 2003). However, it is important to note that the clinical neuropathy in humans is not always predictable by the results in animal or *in vitro*
models (Cata, Weng et al, 2006). Neuropathic pain in humans is usually spontaneous with individual variance making it very difficult to quantify or predict from animal models.

In conclusion, despite clinical success, a number of studies are ongoing in the field of tubulin-binding agents to improve the safety profile and overcome other limitations (e.g., drug resistance) of conventional agents. In this study, I-19 exhibited potent anticancer activity by targeting the colchicine-binding site in a broad spectrum of human cancer cells including those that express ABC transporters without any detrimental effects on animal motor performance. Our study indicates that I-19 has potential for use as a stand-alone anticancer drug or combination therapy for various malignancies including drug-resistant tumors.

4.5. Acknowledgements

I would like to thank Dr. Duane D. Miller’s group for synthesis of I-19, Dr. Yun Wang for her assistance in the transfection experiments, and Dr. James T. Dalton and Dr. Christina M. Barrett for their guidance, valuable discussions, and manuscript review.
CHAPTER 5: BIOTRANSFORMATION OF A NOVEL ANTIMITOTIC AGENT, I-19, BY MOUSE, RAT, DOG, MONKEY, AND HUMAN LIVER MICROSOMES AND IN VIVO PHARMACOKINETICS IN MICE

5.1. Introduction

In general, optimization of drug absorption, distribution, metabolism, excretion, and toxicity properties are critical in drug discovery. Following our discovery of a novel potent antimitotic agent, I-19, we began characterization of in vitro metabolism across several species as well as in vivo pharmacokinetics in mice. The results from those studies will be used to optimize dosing regimens of I-19 in various animal models. Furthermore, it will aid in the identification of metabolically liable sites for future structural modification.

In vitro hepatic metabolism studies provide a means to predict in vivo clearance (CL), half-life (T_{1/2}), and oral bioavailability (F_{p.o.}). Drug biotransformation is divided into two types of reactions; phase I (hydrolysis, oxidation, and reduction) and phase II (conjugation). The cytochrome P450 (CYP) enzyme superfamily plays a dominant role in phase I metabolism and phase II conjugation is mediated by uridine diphosphoglucuronosyl transferase (UGT), N-acetyltransferase, glutathione S-transferase, sulfotransferase and so on. Current FDA guidelines for human drug development specify in vitro system for metabolism studies such as microsomes, supersomes, cytosol, S9 fraction, cell lines, transgenic cell lines, and primary hepatocytes. Liver microsomes are vesicles derived from the hepatocyte endoplasmic reticulum, which contains CYP and UGT enzymes. Supersomes are the microsomes of human CYP- or UGT-transfected insect cells. The liver cytosolic fraction contains the soluble phase II enzymes such as NAT, GST and ST. The liver S9 fraction contains both microsomal and cytosolic fractions.
Pharmacokinetics is concerned with the changes in drug concentration over time resulting from absorption, distribution, metabolism, and elimination. Since it is necessary to maintain a certain concentration of drug at the site of action for a finite period time, pharmacokinetic studies provide the basis to determine the dosage regimen required for efficacy in vivo.

In this study, we examined the pharmacokinetics of I-19 in mice following i.p., i.v., and p.o. dosing. Additionally, the metabolic fate of I-19 was investigated in mouse, rat, dog, monkey, and human liver microsomes.

Figure 5.1. Chemical structure of I-19.
For better understanding putative structures of I-19 metabolites, we will refer to sections denoted in the figure as the A, B, and C rings.
5.2. Materials and Methods

5.2.1 Chemicals, Microsome, and Animals

I-19, I-13 (an internal standard), and I-47 (one of putative metabolites) were synthesized in Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee, Memphis, TN. Pooled liver microsomes from mice, rats, dogs, monkeys, and humans were purchased from Xenotech (Lenexa, KS). Nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system was purchased from BD (Franklin Lakes, NJ). All other chemicals were purchased from Sigma (St. Louis, MO). Male ICR mice (20-25 g) were purchased from Harlan Biosciences (Indianapolis, IN). All animal protocols were approved by the Animal Care and Use Committee at The Ohio State University or the University of Tennessee Health Science Center.

5.2.2 Pharmacokinetic Studies of I-19 in Mice

Male ICR mice (5-6 weeks, 20-25 g) were purchased from Harlan Biosciences (Indianapolis, IN). Thirty animals were weighed, ear tagged, randomized by body weight, and assigned into groups with five animals per group.

Three doses (1, 5, and 15 mg/kg) were administered via each route of administration (i.v., i.p., and p.o.). These doses were chosen based on safety studies with I-19. Dosing vehicles were composed of 10% DMSO in PEG300 and dosing solutions were prepared at an appropriate concentration to deliver the dose in a final volume of 0.05 ml. I.v. doses were administered via the tail vein. Oral doses were administered by gavage.

Blood samples (up to 600 μl each) were taken from the posterior vena cava using heparinized syringes under isoflurane anesthesia (Baxter Healthcare, Deerfield, IL) at different time points after dose administration (Table 5.1).

One animal was euthanized at each blood sampling. Blood samples were collected into 1.7 ml heparinized microcentrifuge tubes and placed on ice until plasma was separated by centrifugation at 800 g for 10 min at 4°C. Plasma samples were stored at -20°C prior to analysis.
Table 5.1. Time points for sample collection for pharmacokinetic studies in mice

<table>
<thead>
<tr>
<th>Administration route</th>
<th>Doses (mg/kg)</th>
<th>Sampling time points (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>1 and 5</td>
<td>3, 10, 30, 60, 120, 200, 360, 600, and 1200</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3, 5, 10, 30, 60, 180, 360, 600, and 1200</td>
</tr>
<tr>
<td>i.p.</td>
<td>1 and 5</td>
<td>5, 20, 60, 120, 200, 360, 600, 1200, and 1800</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>10, 20, 60, 120, 180, 360, 540, 960, 1440, and 1800</td>
</tr>
<tr>
<td>p.o.</td>
<td>1, 5, and 15</td>
<td>10, 30, 60, 180, 360, 480, 600, 1200, 1800 and 2400</td>
</tr>
</tbody>
</table>

5.2.3 Sample Preparation for Mass Spectrometric Analysis of Mouse Plasma

Plasma proteins were precipitated by the addition of acetonitrile (150 µl, containing the internal standard) to 100 µl of mouse plasma. Samples were vortexed and then centrifuged at 8000 rpm for 10 min. The supernatant was transferred to a clean vial for injection into the mass spectrometer for analysis.

5.2.4 Pharmacokinetic Data Analysis

Mean plasma concentration-time data were analyzed using noncompartmental methods (WinNonlin Version 5.2.1, Pharsight Corporation, Mountain View, CA). The area under the plasma concentration-time curve from time 0 to infinity ($AUC_{inf}$) was calculated by the trapezoidal rule with extrapolation to time infinity. The terminal $T_{1/2}$ was calculated as $\ln(2)/\lambda_z$, where $\lambda_z$ is the first order rate constant associated with the terminal (log-linear) portion of the curve. The plasma CL was calculated as $\text{Dose}/AUC_{inf}$. The maximum plasma concentration ($C_{max}$) and the time when it occurred ($T_{max}$) were obtained by visual inspection of the plasma concentration-time curve. The apparent volume of distribution at steady state ($V_{dss}$) was calculated by $\text{CL} \times \text{MRT}_{inf}$ where MRT is the mean residence time extrapolated to infinity calculated as $\text{AUMC}_{inf}/AUC_{inf}$, where AUMC$_{inf}$ is the area under the first moment curve extrapolated to infinity. The bioavailability (F) was calculated as $(\text{AUC}_{non-intravenous} \times \text{dose}_{i.v})/(\text{AUC}_{i.v} \times \text{dose}_{non-intravenous})$. 

118
5.2.5 In Vitro Metabolism Studies of I-19

For both phase I and phase I & II metabolism studies, the incubation mixture, in 65 mM potassium phosphate buffer (pH 7.4), consisted of 1 mg/ml liver microsomal proteins, 3 mM NADPH, and 0.5 µM test compound. For the phase I & II metabolism, 5 mM uridine diphosphate glucuronic acid (UDPGA), 5 mM D-Saccharolactone, 50 µg/ml Alamethicin, and 3 mM magnesium chloride were added to the phase I metabolism study mixture. The concentration of methanol (used for dissolving the substrate) was 1 % (v/v). Total volume of the incubation was 200 µl and the reaction mixtures were incubated at 37°C. For metabolite identification, the reaction mixture was incubated for 2 hr. To generate the stability curves for I-19 different incubations were stopped at 10, 20, 30, 60, and 90 minutes for analysis of I-19 remaining. All reactions were stopped by the addition of 200 µl ice-cold acetonitrile containing the internal standard to a 100 µl sample of the reaction mixture. Subsequently, the samples were then centrifuged at 3000 g for 5 min and supernatant was analyzed by LC-MS/MS.

5.2.6 LC-MS/MS Analyses

The LC-MS/MS analysis was performed on a 4000 Q trap triple quadrupole/linear ion trap mass spectrometer (Applied Bioscience, Foster City, CA) with a nanospray interface. The nanospray temperature was set as 500°C, curtain gas at 30 psi, ion spray energy at 5500 V, Nebulizer gas (gas 1) at 30 and Nano gas (gas 2) at 40 psi. Nitrogen was used as the collision gas. The collision energy was 45 eV. The protonated molecules were investigated. 5 to 10 µl of samples were injected into the HPLC system (Model 1100 Series Chemstation, Agilent Technology Co, Santa Clara, CA).

Samples for pharmacokinetic studies were separated with a Halo 2.1 x 50 mm C18 column (Wilmington, DE) within a runtime of 8 min. Gradient mode was used to achieve separation of the analytes using mixtures of mobile phase A (95% acetonitrile and 5% water with 0.1% folic acid) and mobile phase B (95% water and 5% acetonitrile with 0.1% folic acid) at a flow rate of 300 µL/min. Mobile phase A was used at 20% from 0 to 1 min followed by a linearly programmed gradient to 80% of mobile phase A within 1.5 min, and 80% of mobile phase A was maintained for 2 min before a quick ramp to 20% mobile phase A for 30 sec. 20% of mobile
phase A was continued for another 3 min towards the end of analysis. Multiple reaction monitoring (MRM) mode scanning m/z 388.2 → 192 was used to obtain the most sensitive signals for I-19.

Metabolite separation was performed on an Alltech Alltima HP 2.1 x 100 mm C$_{18}$ column (Deerfield, IL). The mobile phase was comprised of 0% of A for the first 1 min and increased to 100% A in a linear gradient over 7 min. 100% of mobile phase A was maintained for 2 min and it quickly returned to 0% for 30 sec. 100% mobile phase B was continued for 4 more min until the end of analysis. Q1 full (data was not shown), precursor ion, neutral loss, and enhanced product ion (EPI, MS2) scan modes, were utilized to identify metabolites of I-19.

5.3. Results

5.3.1 Pharmacokinetics of I-19 in Mice

Doses of 1, 5, and 15 mg/kg were administrated via i.v., i.p., and p.o. in order to elucidate the pharmacokinetic parameters of I-19 in ICR mice. Plasma concentrations of I-19 in mice declined in a biexponential manner after i.v. injection, with a terminal T$_{1/2}$ of 4-9 h. The V$_{dss}$ of I-19 was 3.8 to 9.8 l/kg following i.v. administration indicating high tissue binding. The high tissue binding of I-19 is likely a result of the hydrophobic nature of this compound. CL decreased for the 1, 5, and 15 mg/kg at 27, 21, and 19 ml/min/kg (Table 5.2 and Figure 5.2), which is 20-30% of hepatic blood flow (Davies and Morris 1993). MRT$_{inf}$ increased from 3 and 3.3 h at the 1 and 5 mg/kg doses, respectively, to 8.8 h following the 15 mg/kg dose in i.v. administration.

After i.p. administration, I-19 was absorbed quickly with T$_{max}$ values of 5-10 min. Systemic exposure following i.p. doses was 65-95% of the analogous i.v. dose (Table 5.3 and Figure 5.3). Plasma concentrations of I-19 peaked at about 30 min after p.o. administration with absolute bioavailability of 24, 13, and 8% in doses of 1, 5, and 15 mg/kg, respectively (Table 5.3 and Figure 5.3). A drug administered by i.p. injection is subject to the same potential hepatic first pass effects as oral administration. However, the i.p. bioavailability (65-95%) of I-19 greatly exceeded that of orally administered drug (8-24%), indicating that the oral bioavailability of I-19 is primarily limited by its poor stability in gastric or intestinal fluids or its incomplete absorption due to poor permeability and/or low aqueous solubility. The approximate terminal T$_{1/2}$ (mean T$_{1/2}$
=7 h) after i.v. administration was similar to that observed after i.p. and p.o. doses (mean \( T_{1/2} \approx 7-8 \) h) (Table 5.2, 5.3, and 5.4).

### 5.3.2 Metabolic Stability of I-19 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes

Metabolic stability was investigated by incubating I-19 with liver microsomes from mouse, rat, dog, monkey, and human. In order to evaluate the metabolism by phase II conjugation, UDPGA was introduced to the microsomal preparation. Half-life was determined with a 2 parameter single exponential decay curves using SigmaPlot Version 10.0 (Systat Software, Inc., Chicago, IL). Metabolic stability of I-19 in the presence and absence of UDPGA was not significantly different with the similar half-lives in phase I and phase I & II pathway (Table 5.5). This result suggests that the primary metabolic pathway of I-19 is regulated by phase I enzymes. I-19 was the most stable in rat liver microsomes with the longest \( T_{1/2} \) (41.9 min) and degraded quickly in monkey liver microsomes with the shortest \( T_{1/2} \) (15 min) in phase I metabolism. The metabolic stability of I-19 in human liver microsomes (23.6 min) appears to be similar to those of mouse and dog with half-lives of 36.7, and 29.4 min, respectively (Table 5.5).
Figure 5.2. Mean plasma concentration–time profiles following i.v. doses of I-19 in male ICR mice. Mean ± SD, n=3-4 in each time point.

Table 5.2. Pharmacokinetics of I-19 in male ICR mice after i.v. administration

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>1 mg/kg</th>
<th>5 mg/kg</th>
<th>15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>ml / min / kg</td>
<td>27</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Vdss</td>
<td>l / kg</td>
<td>5.3</td>
<td>3.8</td>
<td>9.8</td>
</tr>
<tr>
<td>T₁/₂</td>
<td>h</td>
<td>7</td>
<td>4.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Cₘₐₓ</td>
<td>µg / ml</td>
<td>0.55</td>
<td>2.86</td>
<td>7.53</td>
</tr>
<tr>
<td>MRT₉₀</td>
<td>h</td>
<td>3.3</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>AUC₉₀</td>
<td>min × µg / ml</td>
<td>37</td>
<td>237</td>
<td>805</td>
</tr>
</tbody>
</table>
Figure 5.3. Mean plasma concentration–time profiles following i.p. doses of I-19 in male ICR mice. Mean ± SD, n=3-4 in each time point.

| Table 5.3. Pharmacokinetics of I-19 in male ICR mice after i.p. administration |
|-------------------|----------------|----------------|----------------|
|                   | Unit           | 1 mg/kg        | 5 mg/kg        | 15 mg/kg       |
| $T_{1/2}$         | h              | 8              | 4.9            | 8.3            |
| $C_{max}$         | $\mu g / ml$  | 0.25           | 1.44           | 4.29           |
| $T_{max}$         | min            | 5              | 5              | 10             |
| $AUC_{inf}$       | min $\times$ $\mu g / ml$ | 24             | 225            | 755            |
| $F$               | %              | 65             | 95             | 94             |
Figure 5.4. Mean plasma concentration–time profiles following p.o. doses of I-19 in ICR male mice. Mean ± SD, n=3-4 in each time point.

Table 5.4. Pharmacokinetics of I-19 in male ICR mice after p.o. administration

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>1 mg/kg</th>
<th>5 mg/kg</th>
<th>15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2}$</td>
<td>h</td>
<td>8.7</td>
<td>7.7</td>
<td>7.9</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>µg / ml</td>
<td>0.03</td>
<td>0.10</td>
<td>0.26</td>
</tr>
<tr>
<td>$T_{max}$</td>
<td>min</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>$AUC_{inf}$</td>
<td>min × µg / ml</td>
<td>8.9</td>
<td>31</td>
<td>64</td>
</tr>
<tr>
<td>F</td>
<td>%</td>
<td>24</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>
Table 5.5. Half life of I-19 in mouse, rat, dog, monkey, and human liver microsomes. Mean ± SD, n=2-3

<table>
<thead>
<tr>
<th></th>
<th>Phase I</th>
<th>Phase I &amp; II</th>
<th>T ½ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>36.7 ± 3</td>
<td>39.0 ± 6</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>41.9 ± 1</td>
<td>53.7 ± 4</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>29.4 ± 4</td>
<td>32.7 ± 1</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>15.0 ± 2</td>
<td>10.5 ± 7</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>23.6 ± 3</td>
<td>29.5 ± 3</td>
<td></td>
</tr>
</tbody>
</table>
5.3.3 Identification of Metabolites in Human Liver Microsomal Incubation

To identify the metabolites of I-19 in human liver microsomes, I-19 was incubated with human liver microsomes in the presence of NADPH for 2 h. Samples were analyzed by LC-MS/MS. The MS2 spectrum of I-19 (m/z 388.2) results in two prominent fragment ions of m/z 192 and 220 (Figure 5.5). Thus, precursor ion of m/z 192 and neutral loss of 167 u scan modes were used to identify metabolites of I-19 and its MS2 fragments. Total ion current (TIC) chromatograms of the acetonitrile extracted I-19 in precursor ion and neutral loss scan modes are shown in the Figure 5.6. The results are described using the terms of A, B, and C rings of I-19 as shown in Figure 5.1. The precursor ion scan of m/z 192 was utilized to identify modifications in the ketone group and/or C-ring. The metabolites modified in A/B-ring and a ketone group comparing to I-19 could be expected to be shown through the neutral loss of 167 u.

In addition to the unchanged I-19, three metabolites (M1, M2, and M3) were detected after 2 hr incubation in human liver microsomes (Figure 5.6). In the precursor scan mode, M1 showed the [M + H]+ ion at m/z 374.7, which was 13.5 amu lower than that of the parent compound (388.2 amu), suggesting the loss of a CH3 group from the C-ring. M2 observed in neutral loss as well as precursor scan modes showed the [M + H]+ ion at m/z 390.6, which was 2.4 amu higher than that of the parent compound. These results suggest that the ketone group was reduced. M3 showed the [M + H]+ ion at m/z 404.7, which was 16.5 amu higher than that of the parent compound in the neutral loss scan mode, indicating the addition of an oxygen atom to the A or B ring of parent compound. The fragment ion masses of M1, M2, and M3 obtained in MS2 spectrum clearly indicated O-demethylation in C-ring, reduction of a ketone group between B and C ring, and hydroxylation in A or B ring (Figure 5.7). However, only structure M2 was verified due to the lack of reference standards for M1 and M3.
Figure 5.5. Fragmentation pattern of I-19 in MS2 mode of m/z 388.2.

Figure 5.6. Chromatograms of I-19 incubated with human liver microsomes in precursor ion of m/z 192 and neutral loss of 167 u scan modes. Chromatograms of I-19 samples in the precursor ion scan mode (A and B) and in the neutral loss scan mode (C and D) were shown.
Figure 5.7. TIC chromatograms of extracted ions of I-19 parent and metabolites in human liver microsomes using product ion scan mode and their fragmentation patterns.
5.3.4 Metabolites of I-19 in Mouse, Rat, Dog, Monkey, and Human Liver Microsomes

To compare the metabolic fate of I-19 in the different species, I-19 was incubated with mouse, rat, dog, and monkey liver microsomes under the same conditions applied to the human liver microsomes for 2 hr. TIC chromatogram of linear ion trap MS2 scan according to their m/z; 375, 390.5, and 404.5 were shown (Figure 5.8). In mouse, rat, dog, and monkey liver microsomes, 9 metabolites (M1-M9) were observed. Table 5.6 shows the retention time, [M + H]^+ and major fragment ions of I-19 and the metabolites in the liver microsomal incubations of the five species. In the MS2 of m/z 375 and 404.5, additional metabolites (M4, M8, and M9) were observed in different retention times compared to M1 and M3. M4 yielded same fragment ion at m/z 192 as M1. Both M8 and M9 generated the diagnostic fragment ion at m/z 208 as M3 did. In the MS2 of m/z 390.5, not only the metabolite with the reduced ketone group (M3) but additional metabolites (M5, M6, and M7) were also detected. The LC-MS/MS fragmentation pattern of M5, M6, and M7 yielded the diagnostic fragment ion at m/z 208 indicating that demethylation and hydroxylation occurred.

The relative amount of each metabolite was calculated from peak areas in the chromatogram of MS2 (Figure 5.8). The metabolism of I-19 in monkey and human liver microsomes showed the similar patterns with reduction of a ketone group and hydroxylation. In dog liver microsomes, hydroxylation was the primary metabolic pathway. Dog liver microsomes generate three different hydroxylated metabolites (M3, M8, and M9). M3 and/or M8 were also observed in the microsomes of other species. O-demethylation was observed to a lesser degree in dog, monkey, and human microsomes. Incubation of mouse and rat liver microsomes with I-19 reduced ketone, O-demethylationed, and hydroxylated metabolites in similar proportions. Monkey liver microsomes produced similar patterns of metabolism of I-19 as human liver microsomes in vitro. Figure 5.9 depicts the proposed metabolic pathways of I-19 in the liver microsomes of the five species.
Figure 5.8. TIC chromatograms of extracted ions of I-19 metabolites in mouse, rat, dog, monkey, and human liver microsomes using EPI scan (MS2) mode. The relative amount of each metabolite was analyzed based on its fragmentation pattern and was shown in right panel; O-demethylation (■), reduction of a ketone group (▧), and hydroxylation in A or B ring (□).
Table 5.6. Summary of the MS2 metabolite profile generated following incubation of I-19 with liver microsomes of mouse, rat, dog, monkey, and human

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>[M+H]$^+$</th>
<th>Fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-19</td>
<td>11.3-11.4</td>
<td>388</td>
<td>192</td>
</tr>
<tr>
<td>Demethylation in C ring</td>
<td>M1</td>
<td>10.6</td>
<td>375</td>
</tr>
<tr>
<td>Demethylation in C ring</td>
<td>M4</td>
<td>10.8</td>
<td>375</td>
</tr>
<tr>
<td>Reduction of ketone</td>
<td>M2</td>
<td>10.5</td>
<td>390.5</td>
</tr>
<tr>
<td>Demethylation in C ring and hydroxylation in A/B ring</td>
<td>M5</td>
<td>9.5</td>
<td>390.5</td>
</tr>
<tr>
<td>Demethylation in C ring</td>
<td>M6</td>
<td>9.7</td>
<td>390.5</td>
</tr>
<tr>
<td>Demethylation in C ring and hydroxylation in A/B ring</td>
<td>M7</td>
<td>11.4</td>
<td>390.5</td>
</tr>
<tr>
<td>Hydroxylation in A/B ring</td>
<td>M8</td>
<td>10</td>
<td>404.5</td>
</tr>
<tr>
<td>Hydroxylation in A/B ring</td>
<td>M3</td>
<td>10.2-10.3</td>
<td>404.5</td>
</tr>
<tr>
<td>Hydroxylation in A/B ring</td>
<td>M9</td>
<td>10.6</td>
<td>404.5</td>
</tr>
</tbody>
</table>
Figure 5.9. The proposed metabolic schemes of I-19 in the mouse, rat, dog, monkey, and human liver microsomes.
5.4. Discussion

In order to understand the pharmacokinetic profile of I-19, a lead antimitotic compound, predict pharmacologic doses and to optimize the novel pharmacophore in terms of metabolic stability, several in vivo and in vitro studies were conducted.

For the pharmacokinetic studies of I-19 in ICR mice, three doses (1, 5, and 15 mg/kg) of I-19 were administered via i.v., i.p., and p.o. Compounds were prepared in 10% DMSO in PEG300. Non-compartmental pharmacokinetic analysis after i.v. injection of I-19 showed high volume of distribution and low clearance in mice. The relatively long half-life of I-19 via i.v., i.p., and p.o. administration appears to be consistent with once-daily dosing. The oral bioavailability of I-19 was limited with 8-24% of bioavailability. Therefore, for the further efficacy studies in mice, i.p. injection could be appropriate to achieve enough exposure of I-19 in the animal model. Based on the anticancer potency of I-19 in the in vitro studies, at least 5 mg/kg dose should be considered.

In vitro metabolic stability in animal liver microsomes of five different species was examined in the presence of NADPH. I-19 was metabolized mainly via phase I pathway in mouse, rat, dog, monkey, and human liver microsomes. Since I-19 showed similar stability in mouse and dog compared to human, further efficacy studies in mice or dogs might be helpful to predict the effects of I-19 in human compared to two other species.

In vitro metabolite identification studies showed that ketone reduction, O-demethylation, and/or hydroxylation are likely the predominant metabolic pathways for I-19. The positions of hydroxylation and/or demethylation were different in the liver microsomes of the five species. Quantitative differences in metabolism were also observed in the liver microsomes of different species. As a result, ketone reduction was the most important metabolic pathway in human suggesting that further modification in the ketone linkage could improve metabolic stability of this structural pharmacophore in the human.

5.5. Acknowledgements

I would like to thank Dr. Duane D. Miller’s group for synthesis of compounds, Dr. James T. Dalton, Dr. Jeffrey D. Kearbey, and Chien-Ming Li for their valuable discussions and review.
CHAPTER 6: SUMMARY AND DISCUSSION

The objectives and conclusions of this dissertation are as follows:

1. To determine the anticancer effects of new pharmacophores targeting tubulin.

For decades, microtubules are considered to be one of the major targets for cancer chemotherapy. In preclinical and clinical studies, the colchicine binding agents showed a great potential as anticancer agents with several advantages in drug resistance, safety profile, simplified structure, and/or oral bioavailability which can potentially to increase convenience and flexibility in cancer treatment as compared to other antimitotic agents targeting vinca or taxane domain. However, there is no agent approved for clinical use targeting this domain in spite of extensive efforts to develop new agents in the pharmaceutical industries as well as academic fields. Therefore, we have developed novel indole compounds that may have more advantages with chemical stability, better bioavailability, and less peripheral neurotoxicity.

Since several potent antimitotic compounds with indoles have been reported, we made a variety of structural modifications to the indole ring. The cancer cell growth inhibition was examined by SRB assay as an initial step to screen compounds in four different prostate cancer cell lines including LNCaP, PC-3, DU-145, and PPC-1. As a result, three-ring system (A-, B-, and C-rings) linked with O- or N-linkage between B- and C-ring was cytotoxic. Reduction on the ketone linkage abolished the activity. Both 2-indolyl and 5-indolyl ring for A-ring showed potent activity. In addition, 5-fluoro modification on 2-indolyl ring did not affect the activity. In addition, a variety of modifications on C-ring (indole, 3,4,5-trimethoxy, 3,4-dimethoxy, and 3,5-dimethoxy) maintained the activity. Especially, 3,4,5-trimethoxy derivatives were most potent in cell growth inhibition.
Further *in vitro* studies showed the effects of lead compounds on apoptosis, cell cycle distribution, and tubulin destabilization. Apoptosis induced by I-13, I-19, I-33, I-34, I-35, and I-37 was confirmed by DNA fragmentation. They arrested PC-3 cell in the G2M phase after 24 h incubation. Indoles inhibited tubulin polymerization in a concentration-dependent manner. The effect of these drugs on tubulin depolymerization and arresting cells in the G2M phase are closely related to their IC50 values of cytotoxicity in human cancer cell lines.

2. To determine the *in vivo* and *in vitro* anticancer effects of I-13; *in vitro* mechanism of anticancer effect on tubulin, *in vivo* efficacy, and toxicity studies

The potent cytotoxic compound, I-13, was identified in cell-based screening using human cancer cell lines including LNCaP, PC-3, DU-145, PPC-1, MCF-7, Tsu-Pr1, and HT-29. I-13 treatment arrested PC-3 cells in the G2M phase and inhibited tubulin polymerization in a concentration-dependent manner. I-13 is synthetically accessible *via* a simple five-step mechanism (Ahn, Hwang et al. 2010). Here, *in vitro* and *in vivo* studies were examined to elucidate the anticancer effect of I-13.

Apoptosis induced by I-13 was evaluated by DNA fragmentation and PARP cleavage. I-13 also decreased the protein expression of Bcl-2. Interestingly, I-13 retained cytotoxic activity in MDR cells over-expressing ABC transporters. Since I-13 arrested cells in the G2M phase, further studies to evaluate anticancer mechanism was investigated in microtubules. Using indirect immunofluorescence microscopy with anti-α-tubulin-FITC antibody, similar cellular microtubule changes were observed in PC-3 cells after treatment with I-13 and vinblastine. *In vitro* studies showed that I-13 depolymerizes microtubules by binding to the colchicine binding site.

*In vivo* toxicity and efficacy of I-13 was examined in mice models. As a result, MTD of I-13 in ICR mice was over 250 mg/kg (qd5, 2 days off × 4, i.p.) and *in vivo*, I-13 treatment (10 mg/kg, q2d, i.p.) showed 58% tumor growth inhibition in mice bearing PC-3 tumor xenografts without significant loss of body weight.

In conclusion, I-13 inhibits tubulin polymerization targeting the colchicine binding site and induces tumor growth inhibition in *in vivo* PC-3 xenograft model. Since I-13 is sensitive against MDR cells mediated ABC transporters, we suggest that I-13 could be considered a lead compound for the development of chemotherapeutic strategies for drug-resistant malignancies. The studies of leukemic cells showed the most reproducible results on expression of ABC transporters in human tumors and P-glycoprotein is expressed in acute myelocytic leukemia cells
in approximately 30% of patients at diagnosis, but in over 50% at relapse (Han, Kahng et al. 2000). In addition, hyper-expression of P-glycoprotein in BBB is also known to impair drug access to the brain tumor cells. Therefore, we could further examine the effect of I-13 in brain cancer and/or leukemia in the future.

3. To determine the in vitro and in vivo anticancer effects of I-19; in vitro mechanism of anticancer effect in tubulin, in vivo efficacy, and toxicity studies

During the drug development, we identified a novel compound, I-19, which inhibits the in vitro growth of a number of human cancer cell lines with nanomolar IC50 values. I-19 arrested cells in the G2M phase and inhibited tubulin polymerization in vitro. We further studied about the in vitro cellular mechanisms of apoptosis and in vivo efficacy as well as toxicity of I-19.

I-19 regulated the cell transition from G2 to mitosis by constitutive activation of Cdc2/cyclin B1 complex and inhibited tubulin polymerization by targeting the colchicine domain in vitro. In addition, in vivo, I-19 treatment (10 mg/kg, q2w, i.p.) showed 68% tumor growth inhibition in mice bearing PC-3 tumor xenografts. Interestingly, multidrug resistant cell lines that over-expressed P-glycoprotein, MRP1, MRP2, and BCRP were not rendered resistant to I-19. In vivo efficacy study showed that I-19 (10 mg/kg, q2d, i.p.) was effective with 76% tumor growth inhibition in xenograft models using MES-SA uterine sarcoma cells or MES-SA/DX5 cells over-expressing P-glycoprotein. In contrast, in MES-SA/DX5 xenograft models, docetaxel and vinblastine were not effective. Furthermore, in vitro studies of NGF-dependent neurite outgrowth in PC12 cells and in vivo studies of mouse behavior showed that I-19 was less neurotoxic than vinblastine and vincristine, tubulin destabilizers with known neurotoxicity. In conclusion, I-19 inhibits tubulin polymerization via the colchicine binding site and significantly circumvents P-glycoprotein-mediated MDR, suggesting that it may be a new antimitotic agent for the treatment of patients with drug resistant cancer.

It remains to be determined what effect I-19 has on other resistance mechanisms of tubulin inhibitors. Over-expression of Aurora-A (oncogene) leads to resistance against paclitaxel (Anand, Penrhyn-Lowe et al. 2003). Survivin is also known to contribute to the resistance of paclitaxel treatment (Tirro, Consoli et al. 2006). The mutations to the binding sites in α- and/or β-tubulin and microtubule dynamics may play a role in the mechanism of resistance (Seve, Isaac et al. 2005; Perez 2009). In addition, the role of microtubule destabilizing proteins and microtubule-stabilizing proteins such as stathmin, MAP 4, γ-actin, and tau in the chemoresistance was
reported (Balachandran, Welsh et al. 2003; Martello, Verdier-Pinard et al. 2003). In future studies, we could examine these factors of resistance in cells undergoing selection by long-term treatment with I-19.

In addition, tubulin binding agents are often used with DNA-damaging agents such as cisplatin and anthracyclines (Kavallaris 2010; Nelkin and Ball 2001; Morinaga, Suga et al. 2003; Montero, Fossella et al. 2005). Therefore, we might be able to enhance tolerability as well as efficacy in cancer models by the combination therapy I-19 and other anticancer agents targeting DNA.

4. To examine in vivo pharmacokinetics in mice, and in vitro biotransformation by animal liver microsomes of a novel antimitotic indole, I-19

I-19 is a novel antimitotic compound with potent antitumor activity in various preclinical models. We determined the pharmacokinetics in ICR mice and in vitro hepatic metabolic fate of I-19.

Three different doses of I-19 (1, 5, and 15 mg/kg) were administrated via i.v., i.p., or p.o. in mice and in vivo pharmacokinetics was studied using non-compartment pharmacokinetic analysis. The studies demonstrate that I-19 was slowly cleared and highly distributed in mice when vehicle was PEG300 with 10% DMSO. I.p administration of I-19 was absorbed quickly with 65-94% bioavailability, while oral bioavailability was 8-24% of the analogous i.v. dose. This study suggests that i.p. administrations of I-19 in 10% DMSO in PEG300 could be appropriate to achieve high enough exposure of I-19 in the efficacy study using mice models. In the future, we may be able to obtain better oral derivatives of I-19 by understanding the reason for low oral bioavailability of I-19.

Metabolic stability of I-19 by mouse, rat, dog, monkey, and human liver microsomes was evaluated using LC-MS/MS. I-19 was metabolized mainly via phase I pathway in mouse, rat, dog, monkey, and human liver microsomes. I-19 showed similar stability in mouse, dog, and human liver microsomes. Therefore, further efficacy studies in mice or dogs could be more appropriate to predict the effects of I-19 in human rather than rat and monkey in terms of hepatic stability.

In vitro studies for metabolite identification showed that ketone reduction, O-demethylation, and/or hydroxylation are likely the major phase I metabolic pathways for I-19 in the mouse, rat, dog, monkey, and human. In particular, the metabolite with reduced ketone was
the predominant in the phase I metabolism by human liver microsomes. It suggests that further modification of the ketone linkage will be critical to improve the metabolic stability of related structural pharmacophores.
REFERENCES


