DDB AND BETULINIC ACID-DERIVATIVES AS POTENT CHEMOSENSITIZING, CHEMOPREVENTIVE, AND ANTI-HIV AGENTS

Hsin-Yi Hung

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Pharmacy.

Chapel Hill
2012

Approved by

Kuo-Hsiung Lee, PhD.

Chin-Ho Chen, Ph.D.

Jian Liu, Ph.D.

Kyoko Nakagawa-Goto, Ph.D.

Qisheng Zhang, Ph.D.

ABSTRACT

HSIN-YI HUNG: DDB and Betulinic Acid-derivatives as Potent Chemosensitizing, Chemopreventive, and Anti-HIV Agents
(Under the direction of Kenan Professor Kuo-Hsiung Lee)

The overall goals of this research are to design and synthesize novel DDB and betulinic acid derivatives to evaluate their chemosensitizing, chemopreventive, and anti-HIV activities. Structure-activity relationships (SAR) are established in order to discover novel chemical entities, and to explore the mechanism(s) of action.

Dimethyl diphenyl bicarboxylate (DDB) and its analog, bicyclol, were reported to have chemosensitizing activity in several multidrug resistant cancer cell lines. In a continuing study, an easy modification was accomplished by reduction to 2,2′-bismethylhydroxy DDB followed by esterification with various acids. This synthetic route was applied to develop a series of compounds and establish SAR. SAR studies on these compounds revealed that analogs with aromatic and bulky aliphatic side chains at the 2,2′-positions effectively and significantly sensitized MDR-1 over-expressed cells to three anticancer drugs, paclitaxel (TAX), vincristine (VCR), and doxorubicin (DOX). DDB derivatives **83** and **90** were five to ten times more effective than verapamil (VRP) for TAX and VCR reversal ability. Analog **73** also showed five times greater chemosensitizing effect than VRP against DOX. The mechanism of action studies showed that DDB analogs elevate cellular concentration of DOX via inhibition of P-glycoprotein.

Nineteen DDB analogs were evaluated in an *in vitro* short-term Epstein Barr virus early antigen (EBA-EA) activation assay. Three of the most potent compounds (**83**, **85** and **106**) were also tested for inhibitory effects on skin tumor promotion in an *in vivo* two-stage mouse-skin

carcinogenesis test. Compound **106** with a prenyl ether had the most significant cancer preventive activity *in vitro*, and it also exhibited a remarkable inhibitory effect on skin tumor promotion in an *in vivo* two-stage mouse-skin carcinogenesis test.

Importantly, active DDB analogs displayed no cytotoxicity either in chemoreversal or cancer prevention assays, suggesting that they are good leads for further development.

In chapter 5, another natural product, betulinic acid, was studied since our group has focused on modification of this molecule for years. Most researches were on the modifications of positions 3, 19 and 28. Modifications on ring A were largely under investigation. Therefore, thirteen new betulinic acid derivatives were designed, synthesized, and evaluated for anticancer, cancer prevention, or anti-HIV activity. In the cytotoxic activity evaluation, compounds **120**, **123**, **126**, and **129** exhibited GI_{50} < 10 μM, and compound **120** with a 3-methyl ester and 4, 23-vinyl group was the most potent. SAR study showed that the 3-methyl ester and 4, 23-vinyl group are important for potency. A C-3 carboxylic acid modification totally abolished the activity. A carboxylic acid was better than an *N*-heptane acetamide at C-28. In the cancer prevention assay, compound **121** showed the strongest inhibition in EBV-EA activation, and was more potent than curcumin, even at low concentrations. The SAR trends were similar to those for anticancer activity, except that a C-3 carboxylic acid was preferred. In the anti-HIV assay, only compound **119** with an ε-lactone A-ring and 28-*N* heptane acetamide showed weak activity, and was identified as an entry inhibitor. However, its activity was weaker than a known entry inhibitor, A43-D, with the same C-28 side chain.

ACKNOWLEDGEMENTS

First, my sincere gratitude and respect are extended to my mentor, Dr. Kuo-Hsiung Lee for his sage guidance, warm encouragement, and continuous support during my graduate studies and research.

I am especially grateful to the members of my doctoral committee: Dr. Chin-Ho Chen, Dr. Jian Liu, Dr. Kyoko Nakagawa-Goto, and Dr. Qisheng Zhang for their advice, criticism, and encouragement.

I am especially thankful to Dr. Kyoko Nakagawa-Goto for mentoring my experimental skills and generous sharing of her experiences in medicinal chemistry research either in the concept of developing a good paper or the direction of modification.

I am especially grateful to Dr. Kenneth F. Bastow, Dr. Emika Ohkoshi, and Chia-Ya Wang for the cytotoxicity assay and chemosensitizing assays, to Dr. Harukuni Tokuda, Dr. Akira Iida, and Dr. Nobutaka Suzuki for their contributions to *in vivo* EBA-EA inhibition and *in vivo* two-stage mouse skin carcinogenesis assays, and to Dr. Chin-Ho Chen for anti-HIV assay and interpretation of the results.

I am very grateful to Dr. Susan Morris-Natschke for her great help for the preparation of papers and this dissertation.

My thanks are also extended to all the faculty and staff members, students, and especially current or former colleagues of Dr. Lee's laboratory for their support and help. Besides, I also thank my roommate, Ying-Hsuan Lin, and all of my friends for their kindness and warmness friendship support. Finally, I thank my parents for their warmth support of my passion for researches.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF SYMBOLS AND ABBREVIATIONS

BA	Betulinic acid
DBDMH	1,3-dibromo-5,5-dimethylhydantoin
DDB	dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EBV-EA	Epstein Barr virus early antigen
EC_{50}	effective dose which causes 50% inhibition
FDA	Food and Drug Administration
1H NMR	proton nuclear magnetic resonance
MDR	Multi-drug resistance
MeOH	methanol
mp	melting point (°C)
MS	mass spectrum
TPA	12-*O*-tetradecanoyl phorbal 13-acetate
μM	micromolar concentration
WHO	World Health Organization

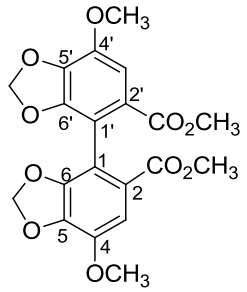
Chapter 1

Origin, Synthesis, and Bioactivities of DDB-related Compounds on Cancer Chemosensitization, Cancer Prevention, and Anti-HIV Activity

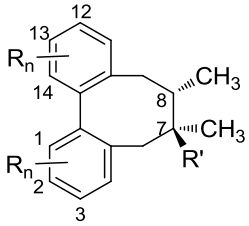
1.1 Origin and Synthesis of Dimethyl Diphenyl Bicarboxylate (DDB)

The Chinese herb *Schisandra chinesis* Baill (Schisandraceae), Wu Wei Zi, has been used as a bactericidal agent as well as to treat other reported indications, such as chronic cough and dyspnea, diarrhea, night sweats, wasting disorders, irritability, palpitations, and sleep disorders. Several dibenzocylcooctane ligans have been isolated from Wu Wei Zi, including schisandrin A, schisandrin B, schisandrin C, and gomisin A (Figure 1.1).[1] Schisandrin B and schisandrin C, the two most investigated constituents in *Schisandra chinesis*, account for the therapeutic effects in liver diseases.[2] The biphenyl unit of these lignans is critical for their pharmacological activities. Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB, **1**) was found as a synthetic intermediate derivative of schisandrin C (Figure 1.1). Different acronyms for DDB, including diphenyl dimethyl bicarboxylate, biphenyl dimethyl dicarboxylate (BDD)[3], and diphenyl dimethyl dicarboxylate (PMC), are used in the literature.[4]

Figure 1.1 Structures of DDB and Dibenzocylcooctadiene Lignans



1: DDB



Dibenzocyclooctadiene lignan

Schisandrin A:
R_n= 1,2,3,12,13,14-hexamethoxy, R'= H

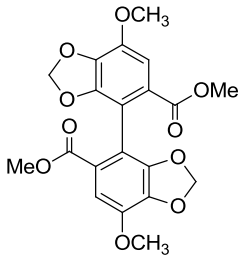
Schisandrin B (Gomisin N):
R_n= 1,2,3,14-tetramethoxy-12,13-methylenedioxy, R'= H

Schisandrin C:
R_n= 1,14-dimethoxy-2,3,12,13-dimethylendioxy, R'= H

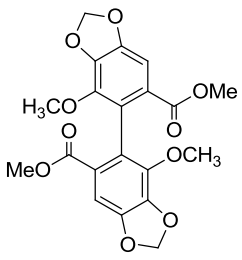
Gomisin A:
R_n= 1,2,3,14-tetramethoxy-12,13-methylenedioxy, R'= OH

DDB shares the biphenyl partial structure of dibenzocyclooctadiene lignans, but lacks the cyclooctadiene ring. The three regioisomers of DDB (α-, β- and γ-DDB) are shown in Figure 1.2. Because different structures can display different biological activities, DDB as a hepatoprotectant generally referred to α-DDB. The two regioisomers, β-DDB and γ-DDB, were found as by-products during the synthesis of α-DDB.

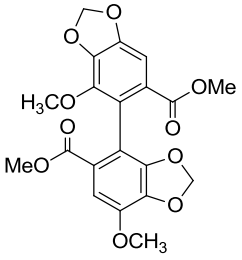
Figure 1.2 Structures of Regioisomers of DDB.



α-DDB



β-DDB

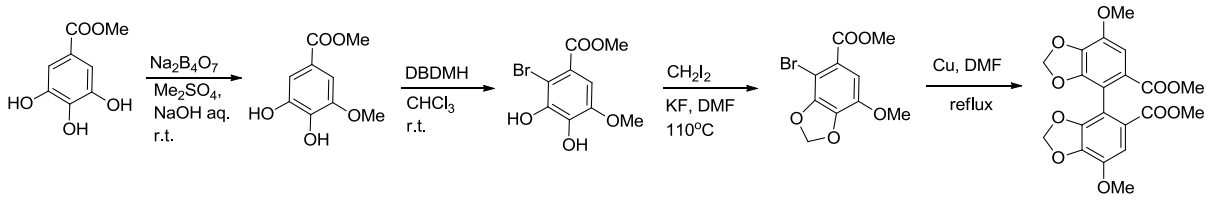


γ-DDB

α-DDB was synthesized regio-selectively from commercially available and inexpensive methyl gallate (Figure 1.3).[5, 6] Selective methylation with methyl sulfate through a borax-chelated transition state, followed by ortho-hydroxy-directed regioselective bromination with 1,3-dibromo-5,5-dimethylhydantoin (DBDMH) gave methyl 2-bromo-3,3-dihydroxy-5-methoxybenzoate. After

protection of the diol as a methylene acetal, homocoupling of the resulting phenylbromide under Ullmann conditions produced α-DDB in 25% overall yield.

Figure 1.3 Regio-selective Synthesis of α-DDB



1.2 DDB-related Compounds and Their Bioactivities

DDB is now used as an adjuvant hepatoprotectant in China and other Asian countries for the treatment of chronic viral hepatitis and other liver diseases.[7] DDB itself also exhibits many pharmacological effects as listed below.

(1) Hepatoprotectant: DDB in clinical research has been shown to lower levels of serum alanine aminotransferase (ALT), bilirubin, and α-fetoprotein in patients and to alleviate symptoms in patients with chronic viral hepatitis.[7] Serum aspartate aminotransferase (AST) and ALT are commonly used biomarkers for liver function. Elevated serum AST and ALT, resulting from these enzymes being released into blood circulation from the cytosol of hepatocytes, can be seen upon hepatocellular damage. In an *in vitro* study, DDB treatment affected synthesis and/or degradation of ALT in cultured hepatocytes, resulting in decrease of cellular ALT.[8, 9] However, in some clinical studies, no improvement was seen in the histological grade or stage of liver disease,[10] which implied that DDB can only decrease the level of liver enzymes, such as ALT. Therefore, DDB is not suitable for the routine treatment of chronic liver diseases, especially in anti-HBV therapy, because the ALT level is the only marker to monitor the anti-viral efficacy. Another study showed that DDB can reduce tamoxifen-induced liver toxicity by increasing the activity of anti-oxidant enzymes and decreasing glutathione level and lipid peroxides. Interestingly, the protective effect of DDB (treated

before tamoxifen intoxication) was less significant than its curative effect against tamoxifen-induced liver injury, which suggested that DDB might have a curative effect on liver damage.[11]

(2) Anti-HBV: Although DDB is usually prescribed as an adjuvant agent for viral hepatitis, a recent study showed that DDB may contribute to the anti-HBV effect by stimulating Jak/Stat signaling and inducing the expression of interferon alpha (IFN-α) stimulated genes, mostly 6-16 and ISG12.[12]

(3) Prevention of immunotoxicity: In two different studies, DDB restored cellular immune function suppressed by carbon tetrachloride (CCl_4) and ketoconazole, which likely leads to decreased numbers of natural killer cells and phagocytic activity in mice.[4, 13] In addition, DDB also reversed humoral immunosuppression caused by ethanol in mice.[14]

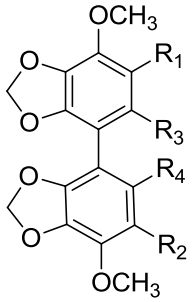
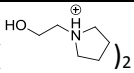
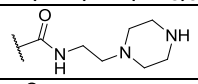
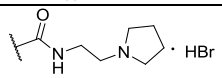
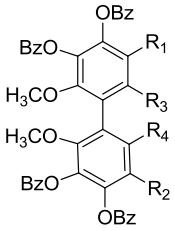
(4) Chemosensitization: DDB exhibited multidrug resistant (MDR) reversal ability in MDR breast carcinoma MCF-7/Adr cells, KBv200, and intrinsic MDR human hepatocarcinoma Bel_{7402} cells via inhibition of P-gp and enhancement of apoptosis. At doses of 300 and 500 mg/kg, co-treatment with DDB and vincristine (VCR) in nude mice bearing KBv200 xenografts also enhanced antitumor activity.[15]

(5) Chemoprevention: At doses of 1, 2, and 4 μmol/L, DDB exhibited a preventive effect on oncogenic transformation of WB-F344 (rat liver epithelial) cells induced by 3-methylcholanthrene and 12-*O*-tetradecanoyl phorbal 13-acetate (TPA). In a soft-agar colony formation assay to examine the ability of cells to grow unattached, colony numbers were reduced in transformed cells treated with DDB. Furthermore, DDB inhibited TPA-induced down-regulation of the gap junctional intercellular communication (GJIC), a process commonly seen in transformed cells. These findings suggested that DDB prevented oncogenic transformation of WB-F344 cells.[16]

Besides DDB itself, DDB derivatives also show various bioactivities as summarized below.

(1) Anti-HIV activity: Sixteen 3, 3′-dibromo-DDB derivatives were reported, and their structures and activities are summarized in Table 1.1. Among all 16 compounds, 3,3′-dibromo-DDB (**3**) was the most potent (EC_{50}= 0.23 μg/ml, Therapeutic Index > 480), and reverse transcriptase was the possible biological target. SAR study suggested that the types of substituents on the phenolic hydroxy groups were more important than the number of bromine(s) on the aromatic rings.[17]

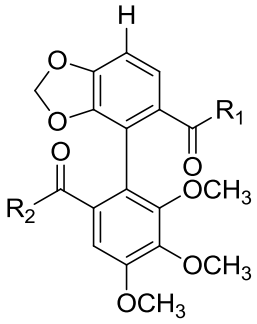
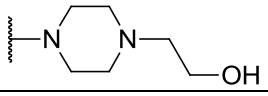
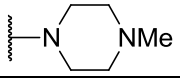
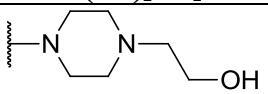
Table 1.1. Anti-HIV Activities of Brominated Biphenyl Derivatives in Acutely Infected H9 Lymphocytes

compd		R_1	R_2	R_3	R_4	EC_{50} (μg/ml)	TI[a]
1		H	H	$COOCH_3$	$COOCH_3$	5	>20
2		Br	H	$COOCH_3$	$COOCH_3$	0.52	>190
3			Br	$COOCH_3$	$COOCH_3$	0.23	>480
4				COOH	COOH	>100	1
5				$COO(CH_2)_5CH_3$	$COO(CH_2)_5CH_3$	2	>50
6				CO-O-CO		-	-
7				COO^-		17	>1.2 <5.9
8				COOH	$COOCH_2CH_3$	60	>1.7
9				COOH	$COO(CH_2)_2N^+(CH_3)_3Cl^-$	>100	1
10				COOH		30	>3.3
11				COOH		>100	1
12				COO^-Na^+	COO^-Na^+	>100	1
13				COO^-Na^+	$COOC_2H_5$	-	-
14		Br	H	$COOCH_3$	$COOCH_3$	>100	1
15		Br	Br	$COOCH_3$	$COOCH_3$	>100	1
16		Br	H	CH_2OH	CH_2OH	100	>1
17		Br	Br	CH_2OH	CH_2OH	>100	1

[a]TI (therapeutic index) is calculated by EC_{50}/IC_{50}. IC_{50} of all sixteen compounds was >100 μg/ml, except **7** (IC_{50} >20 μg/ml).

(2) Protective effect on CCl_4-induced liver injury: 2,2′-Disubstituted DDB analogs exhibited suppressive effects on CCl_4-induced liver injury. The 2,2′-disubstituted unsymmetric biphenyls were synthesized by intramolecular Ullman coupling. Their structures and *in vitro* suppressive activity on CCl_4-induced liver injury are summarized in Table 1.2. Compounds **18**, **21**, **28**, and **30** showed potent suppressive effects and were further evaluated *in vivo* in mice at the doses of 100, 30, and 10 mg/kg body weight. Among all tested compounds, compound **30** was the most potent and exhibited no acute toxicity.[18]

Table 1.2. Effect of 2,2′-Disubstituted Biphenyls on CCl_4-induced Liver Injury in Mice

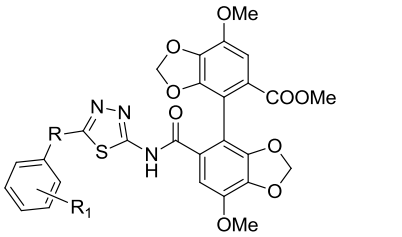
Compd		R_1	R_2	Suppressive activity[a]
1 (DDB)				+++
18		OEt	OEt	++++
19		-O(CH$_2$)$_3$O-		-
20		NHEt	NHEt	+++
21		NEt_2	NEt_2	++++
22		$NHC(Me)_2CH_2OH$	$NHC(Me)_2CH_2OH$	+++
23		NEt_2	OEt	++
24		NEt_2	O-(2-Me-Ph)	+++
25		$NHC(Me)_2CH_2OH$	O-(2-Me-Ph)	++
26		$NHCH_2CH_2OH$	O-(2-Me-Ph)	+
27		$NH(CH_2)_5CH_3$	O-(2-Me-Ph)	++
28			O-(2-Me-Ph)	++++
29			O-(2-Me-Ph)	+
30		O-(2-Me-Ph)	$NHC(Me)_2CH_2OH$	++++
31		O-(2-Me-Ph)		++

[a]Suppression (%) of the CCl_4-induced elevation of GPT in mice: – ≤ 20, 20 ≤ + ≤ 40, 40 < ++ ≤ 60, 60 < +++ ≤ 80, 80 > ++++.

(3) Anticancer activity: DDB derivatives bearing different 1,3,4-thiadiazole moiety (Figure 1.4) were investigated for cytotoxic activity against five human cancer cell lines: HepG2 (human hepatoma cells), KB (human oral epidermoid cancer cells), A549 (human lung carcinoma

cells), K562 (human chronic myeloid leukemia cells), and MCF-7 (human breast cancer cells). The two most active compounds contained a sulfone linkage and 3- or 4-substituted nitrobenzene ring, and exhibited IC_{50} values of 6.6 / 6.9 μM for HePG2, 19.2 / 18.9 μM for KB, 8.9 / 9.9 μM for A549, 7.1 / 6.4 μM for K562, and 12.6 / 11.8 μM for MCF-7, respectively.[19]

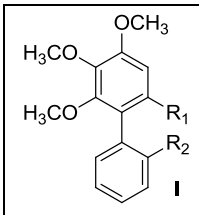
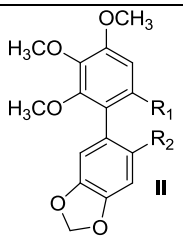
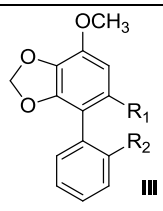
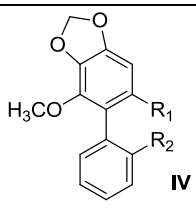
Figure 1.4. DDB derivatives with different 1,3,4-substituted thiadiazole moieties.



R = SCH_2 or SO_2CH_2
R_1 = H, 2-Cl, 3-Cl, 4-Cl, 3-Me, 4-Me, 3-OMe, 4-OMe, 2-NO_2, 3-NO_2, 4-NO_2

Other research studied a series of unsymmetric biphenyls for cytotoxic activity against DU145 (prostate cancer), A549 (lung cancer), KB (nasopharyngeal) and KBvin (KB multidrug resistant cell) cell lines. Table 1.3 lists the structures and cytotoxic activities of several potent compounds. Compounds **33**, **35**, and **36** showed potent activity within an IC_{50} range of 0.04 – 3.23 μM. SAR study showed that it is critical to have a bulky group on the 2,2′-position of the biphenyl skeleton.[20]

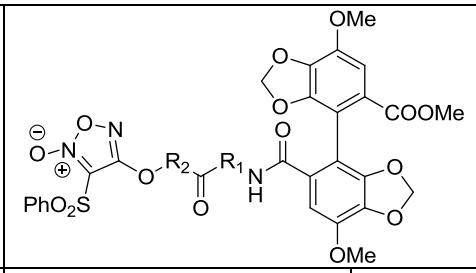
Table 1.3. Cytotoxic Asymmetric Biphenyls

 I	 II	 III	 IV	IC_{50} (μM)			
Compd	Type	R_1	R_2	DU145	A549	KB	KBvin
32	I	COOMe	CHO	5.25	7.76	12.0	4.97

33	I	$CH=C(Me)NO_2$	$CH=C(Me)NO_2$	NT	0.11	0.41	0.51
34	II	CHO	CHO	3.66	20.2	15.7	4.10
35	II	CH_2OCOPh	CH_2OCOPh	0.04	0.04	0.04	0.04
36	III	COOMe	CHO	0.31	1.70	3.23	0.86
37	IV	COOMe	CHO	2.23	19.6	12.8	1.88

(4) Chemosensitizing activity: Several novel furoxan-based nitric oxide (NO)-releasing DDB derivatives were synthesized and tested for anti-proliferative activity and chemoreversal activity in HepG2 and MCF-7/Adr multidrug resistant cell lines, respectively (Table 1.4). Increased intracellular doxorubicin concentrations were observed resulting from inhibition for P-gp-mediated drug efflux and downregulation of P-gp expression.[21]

Table 1.4. Furoxan-based Nitric Oxide-releasing DDB Derivatives and Their Anti-proliferative Activity against HepG2 and Their MDR Reversal Activity against MCF-7/Adr.

			IC_{50} (μM)		
Compd	R_1	R_2	HepG2[a]	MCF-7[b] (+ DOX[c])	MCF-7/Adr[b] (+ DOX[c])
38	$-O(CH_2)_2-$	$-(CH_2)_2-$	1.35	-	-
39	$-O(CH_2)_3-$	$-(CH_2)_2-$	1.14	-	-
40	$-O(CH_2)_4-$	$-(CH_2)_2-$	1.36	-	-
41	$-NH(CH_2)_2-$	$-(CH_2)_2-$	1.25	-	-
42	$-O(CH_2)_2O(CH_2)_2-$	$-(CH_2)_2-$	1.88	-	-
43	$-OCH(CH_3)(CH_2)_2-$	$-(CH_2)_2-$	1.15	-	-
44	$-OC(CH_3)_2(CH_2)_2-$	$-(CH_2)_2-$	1.36	-	-
45	$-O(CH_2)_3-$	$-CH_3CH-$	1.25	-	-
46	$-O(CH_2)_4-$	$-CH_3CH-$	1.21	0.224 (0.1 μM) 0.066 (1 μM) 0.026 (10 μM)	46.28 (0.1 μM) 23.17 (1 μM) 0.26 (10 μM)
47	$-O(CH_2)_2O(CH_2)_2-$	$-CH_3CH-$	1.49	0.183 (1 μM) 0.155 (10 μM)	9.98 (1 μM) 0.41 (10 μM)
DDB			>100	0.287 (0.1 μM) 0.271 (1 μM) 0.259 (10 μM)	32.29 (0.1 μM) 19.08 (1 μM) 16.15 (10 μM)
5-FU			15.92		

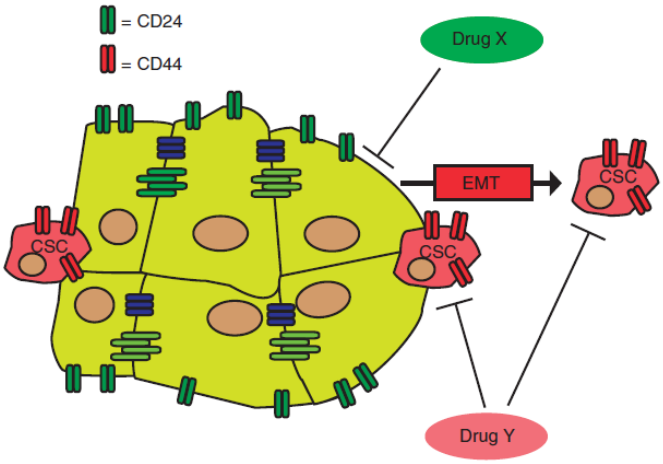
a. Anti-proliferative assay for HepG2 cell line. b. Chemosensitizing assay: MTT assay was applied to examine the cell viability of co-treatment of compounds and doxorubicin. C. DOX: doxorubicin

1.3 Cancer Resistance and Chemosensitization

1.3.1. Chemotherapeutic Resistance

Although immense effort has been put on cancer research, cancers still remain among the leading causes of death in the US. Chemotherapy, a critical therapeutic strategy for cancer treatment, fails to eliminate all tumor cells associated with cell-intrinsic processes or acquired drug resistance, the most common cause of tumor recurrence. Emerging evidence suggests that epithelial-mesenchymal transition (EMT)-type cells and cancer stem cells (CSCs) or initiating cells are more resistant to conventional chemotherapy and are linked to anticancer drug resistance.[22] During acquired drug resistance, the occurrence of reversible epigenetic changes imply different differentiation states of the tumor, reflecting EMT and the presence of stem cell-like chemo-refractory cells. A schematic strategy for eliminating stem cell-like cancer cells is shown in Figure 1.5. Because conventional anticancer drugs can only eliminate bulk differentiated cancer cells, a combination of selectively targeted CSC agents and current chemotherapeutic agents is preferred. Several drug targets have been identified, such as histone deacetylase and transforming growth factor beta 1 (TGF-β) pathways. Inhibitors of these pathways prevent cell differentiation or kill CSCs.[22]

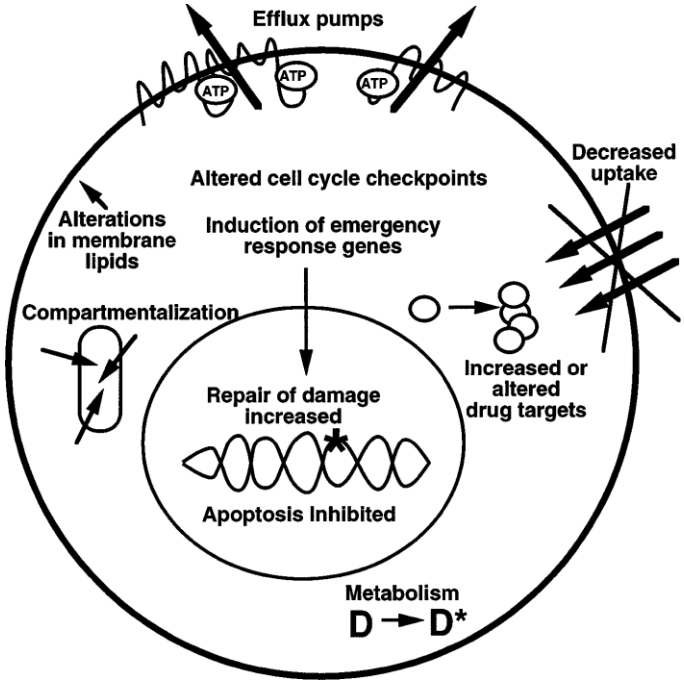
Figure 1.5. Schematic Strategy of Eliminating Stem Cell-like Features (CSC) Cancer Cells.[22] Drug X is a chemotherapeutic agent that can kill bulk differentiated tumor cells, while Drug Y either acts as a chemosensitizer to prevent tumor cells from undergoing EMT to become stem cell-like resistant cancer cells or directly and selectively kill CSCs.



Another issue of chemotherapeutic resistance is the tumor microenvironment, in which cancer cells are surrounded by various sets of non-transformed cells and a heterogenous stromal compartment.[23] The interaction between tumor cells and normal cells in this tumor microenvironment occurs via secreted and surface-bound proteins, and is critical for tumor progression. Firstly, the tumor cells can use the general stress-induced secretory machinery to survive and progress after administration of frontline chemotherapy. This finding is supported by the evidence that within 24 hours of treatment with doxorubicin in the thymus, interleukin 6 (IL-6), which conferred resistance to the drug, was released from endothelial cells, which can sense DNA damage and acutely activate a protective secretory program for both normal cells and tumor cells. Secondly, increased physical barriers to drug accessibility have evolved to affect cancer treatment by paracrine prosurvival signaling of factors such as IL-6. A strategy to eliminate the interference from the tumor microenvironment and improve the outcome of chemotherapy is to combine current chemotherapeutic agents with prosurvival signaling inhibitors, which may potentiate the killing effect of anticancer agents.[24]

In addition to the tumor microenvironment, cancer cells also adapt other mechanisms to deal with chemotherapeutic agents. Cellular mechanisms of multidrug resistance include decreased uptake of chemotherapeutic agents, such as expression of vacuolar ATPase (V-ATPase), or adaptation of cancer cells to affect the cytotoxic ability of chemotherapeutic agents, such as down-regulation of the drug target (e.g., topoisomerase II), over-expression of metabolizing enzymes (e.g., glutathione S-transferase π-1 or -2 (GST p-1 / p-2), heterdimerization and receptor cross-activation for decreasing activity of receptor kinase inhibitors, and microtubule dynamic changes. Over-expression of drug efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) is the primary cause leading to multidrug resistance.[25] A cartoon summarizing cellular mechanisms of cancer drug resistance is shown below (Figure 1.6).[26]

Figure 1.6. Schematic Representation of the Main Cellular Mechanisms of Chemoresistances.



1.3.2. Chemosensitization

Chemosensitization is one strategy to overcome chemotherapeutic resistance. The concept of chemosensitization is to administrate another agent to enhance the activity of current chemotherapeutic agents.[27] Great efforts have been put into developing chemosensitizing agents, categorized as apoptosis modulators and efflux pump inhibitors. A new research area in chemosensitization is the differentiation of CSCs into epithelial-like cancer cells. Several drug targets for differentiation of CSCs have been mentioned above. Over-expression of efflux pumps on the surface cell membrane, which results in resistance to different structural and functional anticancer drugs, accounts for most multidrug resistance. Drug efflux is mediated by ATP-dependent multidrug transporters including multidrug resistance protein (MDR, P-glycoprotein, P-gp), multidrug resistance associated protein (MRP1), lung resistance-related protein (LRP), and breast cancer resistance protein (BRCP). Two first generation MDR reversal agents, verapamil and cyclosporine A, are usually used

in MDR research as positive controls. However, they were precluded from clinical use due to their significant toxicity. Additional MDR modulators were discovered as second or third generation agents; however, unsatisfactory toxicity and pharmacokinetic issues still impeded their further development. Although several third generation P-gp inhibitors, including Tariquidar, are in phase III clinical trial for breast cancer, the existing MDR inhibitors are not yet in clinical use.

Natural products have been proven to be an important source for drug discovery. More than 60 % of the current anticancer drugs are from natural products. Several favorable features including multi-targeting properties, low cost, low toxicity, and immediate availability have led to increased interest in and discovery of natural products as anticancer drugs over the years.[28] One example is resveratrol, 3,4′,5-trihydroxy-trans-stilbene, first isolated from the roots of white hellebore (*Veratrum grandiflorum* O. Loes). Since the first article on its anticancer potential was published in 1997, resveratrol has been significantly investigated. Its anticancer activities are mediated through modulation of cell cycle progression, inflammation, proliferation, apoptosis, invasion, metastasis, and angiogenesis of tumor cells. Resveratrol also acts as a chemosensitizer by modulating apoptotic pathways, down-regulating drug transporters, and down-regulating proliferative proteins in NF-κB and STAT3 pathways.[29]

1.3.3 Chemosensitizing Potential of DDB[15] and Bicyclol[30]

Like resveratrol, DDB, a synthetic intermediate derivative of schisandrin C, also exhibited chemosensitizing effects on multidrug resistant cancer cell lines. Table 1.5 summarizes the chemoreversal effects of DDB on MCF-7 (breast cancer), MCF-7/Adr (multidrug-resistant breast cancer), Bel_{7402} (human hepatocellular carcinoma), KB (human nasopharyngeal carcinoma), and KBvin (vincristine-selected KB MDR subline) cell lines. At non-toxic concentrations of 12.5, 25, and 50 μM, DDB markedly enhanced the sensitivity of MCF-Adr and KBvin cells to vincristine (VCR) and doxorubicin (DOX) in a dose-dependent manner. The chemosensitizing ability was also seen in an innate MDR cell line, Bel_{7402}. Moreover, increased cellular doxorubicin concentration (2 to 2.9

fold) was found in DDB treated groups at the doses of 25 and 50 μM, respectively, but this increase was seen only in an MDR resistant subline such as MCF-Adr. When detecting the surface expression of P-gp by fluorescence, the fluorescence intensity of P-gp on the surface of MCF-Adr cells was much higher than that on sensitive MCF-7 cells, as expected. After MCF-7/Adr cells were exposed to 25 and 50 μM of DDB, the fluorescence intensity of surface P-gp decreased markedly, indicating that DDB inhibited P-gp levels in resistant cells. The expression level of mdr1, a gene encoding P-gp expression in MCF-7 cells, was examined by semi-quantitative RT-PCR. As expected, the mdr1 band could be observed clearly in resistant MCF-7/Adr cells, while no band was seen in the MCF-7 group. However, no decrease in mdr1 RNA levels was seen in MCF-7/Adr cells treated with DDB for 24 h.[15]

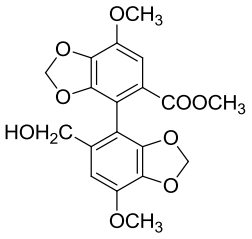
Table 1.5. Effect of DDB on the Sensitivities of MCF-7, MCF-7/Adr, Bel_{7402}, KB and KBvin to VCR, DOX and TAX. (VRP= verapamil)

Compd	Conc. (μM)	KB Nasopharyngeal Carcinoma		KBv200		MCF-7 Breast cancer		MCF-7/Adr	
					IC_{50} of VCR (nM)				
Control	0	28±23		3917±325		487±130		2125±290	
VPL	10	26±14	1.1	9±8	435.2	219±41	1.5	380±49	5.6
DDB	12.5	30±6	0.9	1690±213	2.3	283±198	1.2	1170±42	1.8
	25	24±12	1.2	1092±243	3.6	539±14	0.6	759±296	2.8
	50	30±19	0.9	287±90	13.6	470±6	1.0	459±21	4.6
					IC_{50} of DOX (nM)				
Control	0	30±9		1055±7		323±13		28750±7000	
VPL	10	26±6	1.2	73±29	14.5	333±25	1.0	1142±492	25.2
DDB	12.5	29±3	1.0	461±41	2.3	373±310	0.9	14967±851	1.9
	25	29±9	1.0	219±66	4.8	348±261	0.9	5720±891	5.0
	50	32±17	0.9	70±21	15.1	380±74	0.9	3757±1000	7.7
					IC_{50} of TAX (nM)				
Control	0	4.5±2.1		644±201		–		–	
VPL	10	1.0±0.2	4.5	13±0.7	48.8	–		–	
DDB	12.5	7.5±1.1	0.6	268±148	2.4	–		–	
	25	2.1±0.4	2.1	127±84	5.1	–		–	
	50	2.0±0.3	2.3	31±12	20.5	–		–	

In addition, KB and KBv200 xenografts were implanted in both flanks of nude mice to investigate the *in vivo* MDR reversal activity of DDB. Injection of VCR alone for 16 days inhibited

about 40 % of the growth of KB xenografts, but did not affect the growth of KBv200 xenografts, which is a sign of resistance. However, co-treatment of DDB with VCR at 300 and 500 mg/kg body weight markedly potentiated the antitumor effect of VCR on KBv200 xenografts, but had only a slight effect on KB xenografts.

A major challenge in developing clinical useful MDR reversal agents is that such agents would affect the clearance of chemotherapeutic agents. The pharmacokinetics of DDB with DOX were studied by measuring DOX concentration in plasma and tumor tissues of ICR mice bearing S180 sarcoma. Similar values were found for the area under curve for 24 hrs ($AUC_{0-24\,h}$) of DOX alone and DOX plus DDB in plasma, indicating that co-administration of DDB did not affect the pharmacokinetics of DOX. A higher Dox concentration in tumor tissues was also found in the DDB plus DOX group compared with the DOX alone group.



Bicyclol

Moreover, bicyclol, a second generation hepatoprotectant used in China, also showed chemosensitizing effects in Kbvin and MCF-7/ adr cell lines.[30] The fold of reversal for vincristine in the Kbvin cell line was about 20 and for paclitaxel in the Kbvin cell line was about 13 and in the MCF-7 cell line was about 11. DDB and bicylcol have the same structure, except that one methylcarboxylate ($COOCH_3$) group in DDB has been reduced to a hydroxymethylene group (CH_2OH) in bicyclol, which could be a starting point for the design of DDB derivatives.

To conclude, DDB possesses the features of low toxicity, easy synthesis, chemosensitizing effects *in vitro* as well as *in vivo,* and no alteration of DOX clearance, which suggests that DDB is a promising lead for further modification aimed at chemosensitizer development.

1.4 Cancer Prevention

1.4.1 Introduction of Cancer Prevention

Cancer is a complex disease. A major focus of carcinogenesis research has been the issue of latency. Cancer is a multistep process, which often involves delayed development. As early as the 1930's, researchers were already investigating whether cancer involves multiple steps, but it was not until the publication of Berenblum and Shubik in 1947 that a model distinguishing the steps in carcinogenesis was actually demonstrated.[31] This model described three different stages of cancer development: initiation, promotion, and progression. Initiation was an irreversible event that we now know involves permanent alteration in the DNA, whereas promotion was a reversible process that involved clonal expansion of initiated cells to form benign lesions. Another characteristic of initiation was that the effect appeared to be addictive, while promotion was not addictive. Progression would be the step that converts the benign lesions to malignancy.[32] The concept of chemoprevention is to block each step to prevent cells from progressing to the next step. Inhibition of the long and reversible promotion step has been widely studied, and is an effective approach to control cancer. Cancer chemoprevention is defined as the use of natural dietary compounds and/or synthetic substances that can delay, prevent, or even reverse the development of adenomas, as well as the progression from adenoma to carcinoma.[33] Strategies for discovering cancer preventive agents can be summarized briefly into three categories: quenching inflammation (such as with aspirin), curbing premalignant cells (such as with a combination of TRAIL and RAc), and preventing metastasis (such as with genistein).[34] Possible mechanisms of chemoprevention include cell cycle arrest and apoptosis or inhibition of signal transduction pathways including mitogen-activated protein kinases (MAPK), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and glycogen synthase kinase (GSK).[35] Various phytochemicals, such as carotenoids, green tea polyphenols, curcumin, glycyrrhizin, and related compounds, from herbs and medicinal plants have been identified to exhibit cancer preventive ability.[36] Besides these dietary phytochemicals, the US FDA has approved several drugs for cancer prevention, which is indicative of the emerging importance of this field (Table 1.6). Because the

pathogeneses of cancers are different, prevention has to be specific in order to gain FDA approval. For example, tamoxifen and raloxifen, which are selective estrogen receptor modulators, have been approved for women with high breast cancer risk, and use of these two drugs can result in a 50% risk reduction.[34]

Table 1.6. FDA-Approved Chemoprevention Drugs[34].

Drug	Brand name(s)	Cancer type	Year first approved	Target / mechanism	Original manufacturer
Tamoxifen	Nolvadex Istubal Valodex	Breast	1998	Selective estrogen receptor modulator (SERM)	AstraZeneca
Raloxifene	Evista	Breast	2007	SERM	Eli Lilly
HPV vaccine	Gardasil Cervarix	Cervix Vulva Vagina/Anus	2006	Elicit immune response to prevent infection by the most common cancer-causing types of HPV	Merck & Co GlaxoSmithKline
Porfimer sodium + photodynamic therapy (PDT) & omeprazole	Photofrin	Esophageal	2003	Lodges in precancerous cells and upon exposure to certain light produces an active form of oxygen that kills nearby cancer cells	Axcan
Fluorouracil	Efudex Fluoroplex Carac	Skin	1970	Interferes with DNA synthesis and leads to cell death	Valeant
Diclofenac sodium 3%	Solaraze	Skin	2000	Exact mechanism is unknown	PharmaDerm
5-aminolevulinic acid + PDT*	Levulan	Skin	1999	Solution kills precancerous cell when exposed to light	DUSA
Imiquimod	Aldara (5 %) Zyclara (3.75%)	Skin	2004	Enhances immune response and promotes apoptosis	Graceway Pharmaceuticals

1.4.2 Cancer Preventive Potential of DDB[16]

DDB was also tested for chemopreventive ability in three different experiments involving hepatocarcinoma. The first experiment was two stage chemical oncogenesis of WB-F344 (rat liver epithelial cells). WB-F344 cells were incubated in medium containing 3-methylcholanthrene (3-MC, initiator) or acetone (negative control) for 72 h, and then incubated in medium with TPA (a promoter) for 14 days after washing out of initiator. Finally, the cells were stained and scored for transformed colonies. The transformed cells were grown in a disorganized multilayer rather than a monolayer. At concentrations of 1, 2, 4 μmol/ml, DDB significantly inhibited transformation of WB-F344 cells in a dose dependent manner (inhibitory rates of 10%, 37%, and 47%, respectively). Next, a soft-agar colony formation assay was conducted. The cells underwent the procedure described above, but then were seeded in soft agar for 28 days instead of being stained. This assay is designed to evaluate a

cell's ability to grow unattached to a surface and, therefore, growth in suspended in agar is a sign of transformed cells. No colony formation was seen with untreated WB-F344 cells, whereas the cells initiated with 3-MC and promoted with TPA developed phenotypic colony formation. The cells treated with DDB also formed colonies, but the numbers of colonies were significantly decreased. The third experiment involved gap junctional intercellular communication. Gap junctions are comprised of proteinaceous, plasma membrane channels that link the interiors of adjacent cells and permit cells to directly exchange small (< 1,000 Daltons) molecules and ions. This exchange, termed gap junctional intercellular communication (GJIC), is involved in growth regulation, differentiation, and apoptosis. Growth controlling factors may pass between cells through the junctions. The loss of gap junctions or impairment of their permeability has been observed in many neoplastic cells and cells treated with growth promoting carcinogens and other agents. The loss of GJIC appears to be an important event in the conversion of a normal cell into a neoplastic one. In the experiment, cells were treated with DDB and then TPA. A fluorescent dye permeating gap junctional channel was used to detect GJIC. The GJIC of normal WB-F344 cells was good. After exposing the cells to TPA, over 85% inhibition of GJIC was detected. When the cells were pretreated with DDB, GJIC was recovered dose dependently. From the results of these three experiments, it can be concluded that DDB can prevent the malignant transformation of WB-F344 cells and restore GJIC. Therefore, DDB has a potential chemopreventive effect on hepatocarcinogenesis.

1.5 References

1. Hikino, H.; Kiso, Y.; Taguchi, H.; Ikeya, Y. Antihepatotoxic actions of lignoids from *Schizandra chinensis* fruits. *Planta Med.* **1984,** *50*, 213-218.

2. Sinclair, S. Chinese herbs: a clinical review of *Astragalus*, *Ligusticum*, and *Schizandrae*. *Altern. Med. Rev.* **1998,** *3*, 338-344.

3. Kim, C. K.; Cho, Y. J.; Gao, Z. G. Preparation and evaluation of biphenyl dimethyl dicarboxylate microemulsions for oral delivery. *J. Control Release* **2001,** *70*, 149-155.

4. Ahn, Y. K.; Kim, J. H. Preventive effects of diphenyl dimethyl dicarboxylate on the immunotoxicity of carbon tetrachloride in ICR mice. *J. Toxicol. Sci.* **1993,** *18*, 185-195.

5. Alam, A.; Takaguchi, Y.; Ito, H.; Yoshida, T.; Tsuboi, S. Multi-functionalization of gallic acid towards improved synthesis of [alpha]- and [beta]-DDB. *Tetrahedron* **2005,** *61*, 1909-1918.

6. Chang, J.; Chen, R.; Guo, R.; Dong, C.; Zhao, K. Synthesis, separation, and theoretical studies of chiral biphenyl lignans (α- and β-DDB). *Helvetica Chimica Acta* **2003,** *86*, 2239-2246.

7. Wang, C., Xu, You Qing. Diphenyl dimethyl bicarboxylate in the treatment of viral hepatitis, adjuvant or curative? *Gastroenterol. Res.* **2008,** *1*, 2-7.

8. Fu, T.; Liu, G. Protective effects of dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate on damages of isolated rat hepatocytes induced by carbon tetrachloride and D-galactosamine. *Biomed. Environ. Sci.* **1992,** *5*, 185-194.

9. Ip, S. P.; Yiu, H. Y.; Ko, K. M. Differential effect of schisandrin B and dimethyl diphenyl bicarboxylate (DDB) on hepatic mitochondrial glutathione redox status in carbon tetrachloride intoxicated mice. *Mol. Cell Biochem.* **2000,** *205*, 111-114.

10. Huber, R.; Hockenjos, B.; Blum, H. E. DDB treatment of patients with chronic hepatitis. *Hepatology* **2004,** *39*, 1732-1733.

11. El-Beshbishy, H. A. The effect of dimethyl dimethoxy biphenyl dicarboxylate (DDB) against tamoxifen-induced liver injury in rats: DDB use is curative or protective. *J. Biochem. Mol. Biol.* **2005,** *38*, 300-306.

12. Joo, S. S.; Won, T. J.; Kim, M. J.; Hwang, K. W.; Lee do, I. Interferon signal transduction of biphenyl dimethyl dicarboxylate/amantadine and anti-HBV activity in HepG2 *Arch Pharm. Res.* **2006,** *29*, 405-411.

13. Kim, J. H.; Kang, T. W. Effect of biphenyl dimethyl dicarboxylate on the cellular and nonspecific immunosuppressions by ketoconazole in mice. *Arch Pharm. Res.* **1999,** *22*, 255-261.

14. Kim, J. H.; Mun, Y. J.; Chun, H. J.; Jeon, K. S.; Kim, Y. O.; Woo, W. H. Effect of biphenyl dimethyl dicarboxylate on the humoral immunosuppression by ethanol. *Int. J. Immunopharmacol.* **2000,** *22*, 905-913.

15. Jin, J.; Sun, H.; Wei, H.; Liu, G. The anti-hepatitis drug DDB chemosensitizes multidrug resistant cancer cells in vitro and in vivo by inhibiting P-gp and enhancing apoptosis. *Invest. New Drugs* **2007,** *25*, 95-105.

16. Sun, H.; Liu, G. T. Chemopreventive effect of dimethyl dicarboxylate biphenyl on malignant transformation of WB-F344 rat liver epithelial cells. *Acta Pharmacol. Sin.* **2005,** *26*, 1339-1344.

17. Xie, L.; Xie, J. X.; Kashiwada, Y.; Cosentino, L. M.; Liu, S. H.; Pai, R. B.; Cheng, Y. C.; Lee, K. H. Anti-AIDS (acquired immune deficiency syndrome) agents. 17. New brominated hexahydroxybiphenyl derivatives as potent anti-HIV agents. *J. Med .Chem.* **1995,** *38*, 3003-3008.

18. Kondo, K.; Takahashi, M.; Ohmizu, H.; Matsumoto, M.; Taguchi, I.; Iwasaki, T. 2,2'-Disubstituted biphenyls: synthesis and suppressive effect against carbon tetrachloride-induced liver injury. *Chem. Pharm. Bull. (Tokyo)* **1994,** *42*, 62-66.

19. Kong, X. W.; Zhang, Y. H.; Wang, T.; Lai, Y. S.; Peng, S. X. Synthesis and cytotoxic evaluation of novel dimethyl [1,1'-biphenyl]-2,2'-dicarboxylates bearing 1,3,4-thiadiazole moieties. *Chem. Biodivers.* **2008,** *5*, 1743-1752.

20. Wu, G.; Guo, H. F.; Gao, K.; Liu, Y. N.; Bastow, K. F.; Morris-Natschke, S. L.; Lee, K. H.; Xie, L. Synthesis of unsymmetrical biphenyls as potent cytotoxic agents. *Bioorg. Med. Chem. Lett.* **2008,** *18*, 5272-5276.

21. Tang, X.; Gu, X.; Ai, H.; Wang, G.; Peng, H.; Lai, Y.; Zhang, Y. Synthesis and evaluation of nitric oxide-releasing DDB derivatives as potential Pgp-mediated MDR reversal agents in MCF-7/Adr cells. *Bioorg. Med. Chem. Lett.* **2012,** *22*, 801-805.

22. Singh, A.; Settleman, J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **2010,** *29*, 4741-4751.

23. Egeblad, M.; Nakasone, E. S.; Werb, Z. Tumors as organs: complex tissues that interface with the entire organism. *Dev. Cell* **2010,** *18*, 884-901.

24. Gilbert, L. A.; Hemann, M. T. Chemotherapeutic resistance: surviving stressful situations. *Cancer Res.* **2011,** *71*, 5062-5066.

25. Fodale, V.; Pierobon, M.; Liotta, L.; Petricoin, E. Mechanism of cell adaptation: when and how do cancer cells develop chemoresistance? *Cancer J.* **2011,** *17*, 89-95.

26. Gottesman, M. M. Mechanisms of cancer drug resistance. *Annu. Rev. Med.* **2002,** *53*, 615-627.

27. Coleman, C. N.; Bump, E. A.; Kramer, R. A. Chemical modifiers of cancer treatment. *J. Clin. Oncol.* **1988,** *6*, 709-733.

28. Newman, D. J.; Cragg, G. M.; Snader, K. M. Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* **2003,** *66*, 1022-1037.

29. Gupta, S. C.; Kannappan, R.; Reuter, S.; Kim, J. H.; Aggarwal, B. B. Chemosensitization of tumors by resveratrol. *Ann. N. Y. Acad. Sci.* **2011,** *1215*, 150-160.

30. Zhu, B.; Liu, G. T.; Zhao, Y. M.; Wu, R. S.; Strada, S. J. Chemosensitizing multiple drug resistance of human carcinoma by bicyclol involves attenuated p-glycoprotein, GST-P and Bcl-2. *Cancer Biol. Ther.* **2006,** *5*, 536-543.

31. Berenblum, I.; Shubik, P. A new, quantitative approach to the study of the stages of chemical cartinogenesis in the mouse's skin. *Br. J. Cancer* **1947,** *1*, 383-391.

32. Cohen, S. M.; Arnold, L. L. Chemical carcinogenesis. *Toxicol. Sci.* **2011,** *120 Suppl 1*, S76-92.

33. Pan, M. H.; Lai, C. S.; Wu, J. C.; Ho, C. T. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. *Mol. Nutr. Food Res.* **2011,** *55*, 32-45.

34. Gravitz, L. Chemoprevention: First line of defense. *Nature* **2011,** *471*, S5-7.

35. Neergheen, V. S.; Bahorun, T.; Taylor, E. W.; Jen, L. S.; Aruoma, O. I. Targeting specific cell signaling transduction pathways by dietary and medicinal phytochemicals in cancer chemoprevention. *Toxicology* **2010,** *278*, 229-241.

36. Nishino, H.; Tokuda, H.; Satomi, Y.; Masuda, M.; Onozuka, M.; Yamaguchi, S.; Takayasu, J.; Tsuruta, J.; Takemura, M.; Ii, T.; Ichiishi, E.; Kuchide, S.; Okuda, M.; Murakoshi, M. Cancer chemoprevention by phytochemicals and their related compounds. *Asian Pac. J. Cancer Prev.* **2000,** *1*, 49-55.

Chapter 2

Introduction of Betulinic Acid on the Researches of Anti-HIV Activity, Anticancer Activity, and Cancer Prevention

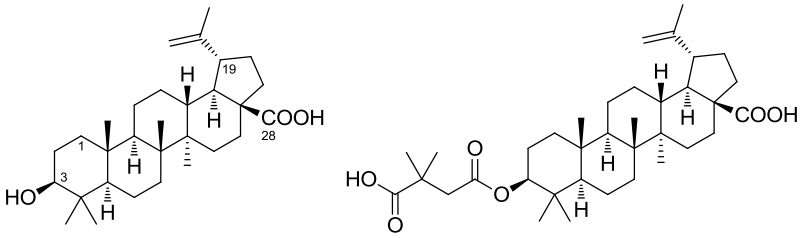
Natural products are promising source for new drug discovery. Many triterpenoids have been shown to have anti-HIV activity and anticancer activity.[1, 2] A huge breakthrough regarding triterpenoids in anti-HIV research occurred when betulinic acid was found to be potent against HIV in 1994, which led to the discovery and development of a new class of anti-HIV agents, namely, maturation inhibitors (MI).

2.1 Introduction of Betulinic Acid and Bevirimat

Betulinic acid (BA), 3β-hydroxy-lup-20(29)-en-28-oic acid, a lupane type pentacyclic triterpene, was isolated from *Betula papyrifera* (North American birch tree). Considerable amounts of BA (up to 2.5%) are available in the outer bark of many terrestrial plant species such as *Ancistrocladus heyneanus*, *Diospyros leucomelas*, *Syzygium formosanum*, *Tetracera Boliviana*, *Tryphyllum peltatum*, and *Ziziphus Vulgaris*.[3] Betulin, a structurally close compound and a major constituent of white-barked birch trees, can be easily converted to BA via Jone's oxidation followed sodium borohydride reduction to give 3α- and 3β-hydroxy products (3:95). Crystallization of the product mixture affords BA (3β).[4] BA exhibits many pharmacological activities, including antitumor, anti-HIV, antibacterial, antimalarial, analgesic, anti-inflammatory, and anthelmintic activities. [5]

Bevirimat (BVM), also known as PA-457, DSB, and MPC-4326, is 3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid, a derivative of BA. Bevirimat, now in clinical phase II trials, is the first member of the novel class of HIV maturation inhibitors. Except for protease inhibitors, most FDA-approved drugs target the early steps of the HIV-1 life cycle. However, bevirimat inhibits HIV-1 maturation, which occurs in the late steps of the HIV-1 life cycle, primarily after virus release from infected cells. Bevirimat inhibits HIV-1 maturation by preventing the cleavage of CA-SP1 (p25) gag into capsid (p24) and the gag small peptide 1 [SP1 (p2)], which leads to the formation of an immature p25 CA protein-SP1 rather than the expected p24 CA protein. This defective immature p25 CA protein-SP1 cannot condense to form the capsid core and results in non-infectious virion progeny. The presumed BVM binding site is located near the CA protein-SP1 cleavage site and was found to be highly associated with the surrounding gag tertiary structure.[6] The structures of betulinic acid and bevirimat are shown below.

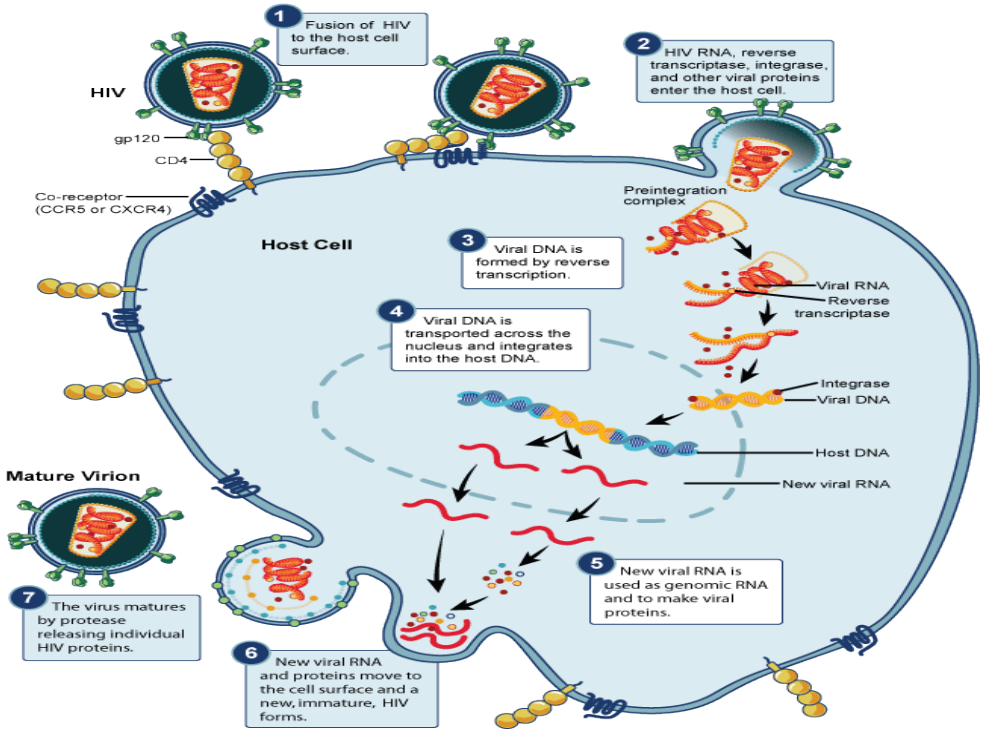
Figure 2.1. Structures of Betulinic Acid and Bevirimat.



2.2 HIV Life Cycle and Possible Drug Targets

An estimated 34 million people continue to live with acquired immunodeficiency syndrome (AIDS) at the end of 2010. Although the number of AIDS-related deaths worldwide has steadily decreased to an estimated 1.8 million in 2010 based on WHO reports, new drugs are still needed, particularly in less developed countries. Numerous studies have been done to investigate the life cycle of HIV in order to identify possible drug targets. The brief HIV life cycle and drug targets are shown below.

Figure 2.2. HIV Replication Cycle



(From: National institution of Allergy and Infectious Disease (NIAID))

HIV entry into host cells, a complicated multi-process associated with the envelope glycoprotein (Env), involves three steps. Initially, a conformational change caused by the binding of viral gp120 with cellular CD4 receptors allows co-receptor binding. Secondly, a further conformational change leading to exposure of the hydrophobic fusion domain of gp41 occurs subsequent to by the binding of gp120 with CCR5 and/or CXCR4 co-receptor. Finally, a fusion process is mediated by gp41. Once the HIV virion is fused together with the cell, the viral capsid is released into the target cell and undergoes uncoating to release the HIV RNA genome and various enzymes, including reverse transcriptase (RT), integrase (IN), and protease (PR), into cytoplasm. Viral cDNA is reverse transcribed by RT using viral genomic RNA as a template. The viral cDNA translocates to the host cell nucleus and incorporates into host DNA genome through viral integrase (IN).

Following HIV integration, provirus gene expression is tightly controlled via the interplay of viral and multiple cellular factors. After viral DNA is transcribed into viral messenger RNA (mRNA), mRNA is transported to the cytoplasm and translated into viral proteins by the cell's machinery. Once the Gag polyprotein precursors ($Pr55^{gag}$) and Gag-Pol polyprotein precursors ($Pr160^{Gag\text{-}Pol}$) are translated, they recruit two copies of viral RNA and assemble with accessory proteins to form the core virion near the inner face of the cell membrane. The modified cell membrane containing envelope glycoproteins (Env) then surrounds the core particle, and budding begins from the host cell. This process leads to the production of immature non-infectious particles.

During or right after budding, the Gag and Gag-Pol polyproteins are cleaved. This cleavage is catalyzed by the third product of the pol gene – protease (PR). PR recognizes and cleaves Gag and Gag-Pol polyproteins at several specific sites to produce viral structural proteins [matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, as well as three pol products RT, IN and PR itself], which leads to the formation of a mature infectious HIV virion that can infect another target cell.

Based on the knowledge of HIV life cycle, the potential strategies to inhibit HIV are fusion inhibition, RT inhibition, IN inhibition, PR inhibition, and maturation inhibition.

2.3 Progression of Betulinic Acid Modification on Anti-HIV research

After BA was first found to inhibit HIV-1_{IIIB} replication in 1994, it has been used as a pre-eminent molecular scaffold in the development of triterpene analogs as anti-HIV drugs.[7] Systematic structural modifications were explored on the A ring C-3 hydroxyl, C-19 isopropenyl group, and C-28 carboxyl moiety.[8] However, except for dihydrobetulinic acid (saturation of the C-19 isopropenyl to isopropyl group), modifications of C-19 reduced or even eliminated anti-HIV activity. The more successful SAR findings are discussed below.

2.3.1 C-3 position

Numerous side chains, including a simple acetyl group[7], different lengths of acid[9], and rigid acids,[10] have been connected at the C-3 position via an ester or amide linkage.[11] Among all side chains used, 2,2′-dimethylsuccinate is very effective and better than its regio-isomer, 3,3′-dimethylsuccinate. Only 2,2′-methylethylsuccinate resulted in greater activity active than 2,2′-dimethylsuccinate.[7, 9, 12]

2.3.2 C-28 position[13-16]

The explored side chains incorporated different chain lengths, amide / ester linkage, and bulky amine linkage. Among them, C-28 *N*-(aminooctanoyl)-4-amino-3-hydroxy-6-methylheptanoic acid linkage successfully elevated the activity; however, the mechanism of action of the compound was anti-entry rather than anti-maturation as with C-3 modified analogs including bevirimat.

2.3.3 Bi-functional Modification: C-3 and C-28[14-17]

Based on the modification studies at C-3 and C-28, Huang *et al* selected active C-3 side chains and C-28 side chains to produce bi-functional derivatives with activity up to 20-fold higher than that of bevirimat.[18] When the bi-functional derivatives were compared with the same C-28 side chain modified analogs without a C-3 side chain, the entry inhibition of the bi-functional molecules would be lower, implying that C-3 modification might hinder or reduce entry inhibition. However, the bi-functional derivatives were still more potent than C-28 modified parent compound in terms of total inhibition of virus.

Less research has been performed on A-ring modification for anti-HIV activity. Although an A-ring dimethylsuccinate side chain is critical for activity, the effects of other A-ring modifications remain unclear. In order to explore new BA-based scaffolds, A-ring opened derivatives were designed and synthesized, and six ring derivatives were also proposed.

2.4 Progression of Betulinic Acid Modification on Anticancer research

Another important field of BA research is anticancer activity. BA has been tested and proven to be active against several cancer cell lines (Table 2.1).[19]

Table 2.1 *In vitro* Cytotoxic Effect of Betulinic Acid on Human Cancer Cell Lines.

Cancer type	ED_{50} (μg/ml)	Cancer type	ED_{50} (μg/ml)	Cancer type	ED_{50} (μg/ml)
melanoma	1.1–4.8	glioblastoma	5–16	cervix carcinoma	1.8
neuroblastoma	2–10	head & neck cancer	8	lung carcinoma	1.5–4.2
medulloblastoma	3–15	ovarian carcinoma	1.8–4.5	leukemia	2–15

Induction of apoptosis via perturbation of the mitochondrial pathway accounts partly for BA's cytotoxicity (Figure 2.3). In a cell-free system, BA induced loss of mitochondrial membrane potential, while in intact cells, BA was shown to trigger cytochrome c in a capase-independent and permeability transition pore-dependent manner. Perturbance of mitochondrial permeability plays an important role in BA-induced apoptosis leading to caspase activation and apoptotic DNA fragmentation.[20, 21] Other mechanisms were also found for BA's activity, such as regulation of Bcl-2 family proteins, modulation of NF-κB activity, and regulation of angiogenesis. Moreover, BA was identified as potent activator of the chymotrypsin-like activity of the proteasome.[22] Interestingly, neuroblastoma cells resistant to doxorubicin-mediated apoptosis still responded to BA treatment, suggesting that BA may overcome some forms of drug resistance.[23]Moreover, BA acted collaboratively with different cytotoxic agents to suppress tumor growth, including ionic radiation[24], and chemotherapeutic agents.[25, 26] Thus, BA may be used as a chemosensitizing agent in combination regimens to enhance the efficacy of anticancer therapy.

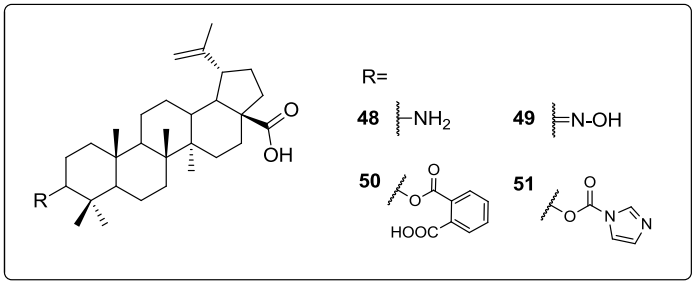
Four positions, the A-ring, C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid moieties, have served as the targets for most derivatization studies of BA for cytotoxic activity. Based on the

literature results for modification of the C-20 alkene, C-20 position does not appear to be a critical site for structural modification.

2.4.1 C-3 Position (Figure 2.3)

The 3β-hydroxy moiety represents a readily available position for chemical modifications, including acetylation, oxidation to a ketone, and formation of various nitrogen-containing analogs (amines, oximes, or carbamates). Oxidation to a ketone increased cytotoxic activity but decreased selectivity.[27] An amine (**48**) or oxime (**49**) at the C-3 position reduced activity or selectivity.[27] However, 3-*O*-glycosylated derivatives were 8- to 12-fold more potent than BA and showed higher selectivity between normal and cancer cells.[28] Esterification with phthalic acid (**50**) increased activity in some cell lines,[29] and with 1*H*-imidazole-1-carboxylic acid (**51**) significantly elevated cytotoxicity.[30] In summary, modification on the C-3 position resulted in altered potency or selectivity.

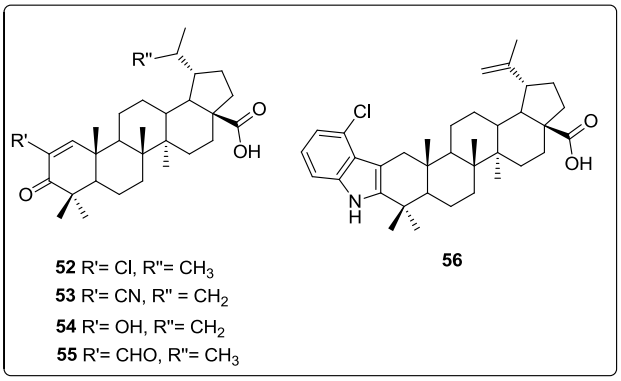
Figure 2.3 Selected C-3 Modified BA derivatives for Anticancer Activity.



2.3.2 Ring A Modification (Figure 2.4)

In recent studies, modifications at C-2, such as introduction of nitrile, hydroxy, or heterocyclic groups, led to increased cytotoxicity. Oxidation of the C-3 hydroxyl coupled with introduction of a C-2 hydroxyl (**54**) significantly elevated activity in many cancer cell lines.[31, 32] Extension of the ring system with an indole moiety (**56**) also led to better activity.[33]

Figure 2.4 Selected BA Derivatives on A-ring Modification.



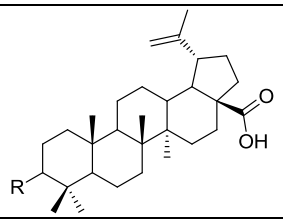
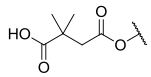
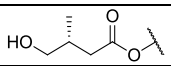
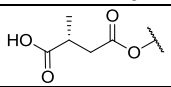
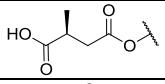
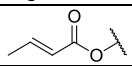
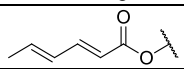
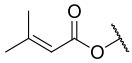
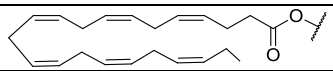
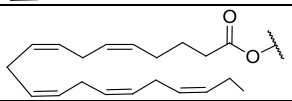
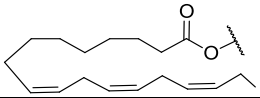
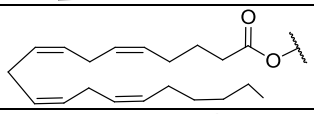
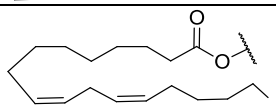
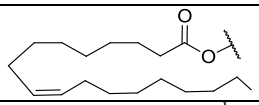
2.4.3 C-28 Position

Modification studies at C-28 showed that the C-28 carbonyl group was critical for cytotoxic activity.[8] Modification at C-28 was usually combined with modification of other position to achieve better activity. Several BA analogs with amino acid side chains at C-28 exhibited melanoma-specific cytotoxicity and improved water solubility.[34] In addition, introduction of polar groups, such as acid, amine, or hydroxyl groups, l at the C-28 position could elevate activity.[30] However, C-28 glucosidation did not improve activity.[35]

2.5 Progression of Betulinic Acid Modification on Cancer Prevention Research

BA can inhibit TPA-induced tumors *in vivo* and ovarian or melanoma xenograft in mice.[36-38] Importantly, BA did not exhibit any toxic effect in mice even at a concentration of 500 mg/kg, but effectively impeded tumor growth even at 5mg/kg.[39] Various analogs with fatty acid side chains at C-3 were synthesized and evaluated for inhibition of TPA-induced tumor activity (Table 2.2).[40] Compounds with short fatty acid chains, such as crotonic (**60**), sorbic (**61**) or prenyl (**62**), exhibited better cancer preventive activity than BA. In addition, chiral monomethylsuccinyl analogs of BA (**57**–**59**), as well as analogs of ceanothic acid, had comparable activity to BA.[40]

Table 2.2 BA Derivatives for Cancer Preventive Activity

		Percentage EBV-EA positive cells Compound concentration (mol ratio/TPA[a])				
Compd	R	1000	500	100	10	IC_{50}[b]
Betulinic acid	-OH	7.9	37.2	75.1	100	403
Bevirimat		6.1	36.1	74.2	96.5	396
57		0.0	29.3	70.1	93.1	303
58		0.0	31.5	71.0	94.2	308
59		0.0	22.8	71.2	94.7	309
60		0.0	26.2	66.7	91.6	299
61		0.0	25.7	65.7	90.2	295
62		0.0	23.5	62.5	88.7	285
63		17.0	53.8	83.2	100	546
64		15.3	50.4	80.1	100	523
65		10.9	43.3	77.8	97.0	445
66		12.3	46.1	79.0	100	482
67		7.3	37.9	75.9	94.8	390
68		4.8	33.7	72.4	92.5	344

[a] Concentration of TPA is 32 pmol/ml. [b] Concentration of IC_{50} also presents as mol ratio/TPA.

2.6 References

1. Singh, I. P.; Bodiwala, H. S. Recent advances in anti-HIV natural products. *Nat. Prod. Rep* .**2010,** *27*, 1781-1800.

2. Cassels, B.; Asencio, M. Anti-HIV activity of natural triterpenoids and hemisynthetic derivatives 2004–2009. *Phytochem. Rev.* **2011**,*10*, 545-564.

3. Kuo, R. Y.; Qian, K.; Morris-Natschke, S. L.; Lee, K. H. Plant-derived triterpenoids and analogues as antitumor and anti-HIV agents. *Nat. Prod. Rep.* **2009,** *26*, 1321-1344.

4. Kim, D. S. H. L.; Chen, Z.; Nguyen, v. T.; Pezzuto, J. M.; Qiu, S.; Lu, Z.-Z. A concise semi-synthetic approach to betulinic acid from betulin. *Synth. Commun.* **1997,** *27*, 1607-1612.

5. Yogeeswari, P.; Sriram, D. Betulinic acid and its derivatives: a review on their biological properties. *Curr. Med. Chem.* **2005,** *12*, 657-666.

6. Sakalian, M.; McMurtrey, C. P.; Deeg, F. J.; Maloy, C. W.; Li, F.; Wild, C. T.; Salzwedel, K. 3-*O*-(3',3'-Dimethysuccinyl) betulinic acid inhibits maturation of the human immunodeficiency virus type 1 Gag precursor assembled in vitro. *J. Virol.* **2006,** *80*, 5716-5722.

7. Fujioka, T.; Kashiwada, Y.; Kilkuskie, R. E.; Cosentino, L. M.; Ballas, L. M.; Jiang, J. B.; Janzen, W. P.; Chen, I. S.; Lee, K. H. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* **1994,** *57*, 243-247.

8. Cichewicz, R. H.; Kouzi, S. A. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Med. Res. Rev.* **2004,** *24*, 90-114.

9. Hashimoto, F.; Kashiwada, Y.; Cosentino, L. M.; Chen, C. H.; Garrett, P. E.; Lee, K. H. Anti-AIDS agents--XXVII. Synthesis and anti-HIV activity of betulinic acid and dihydrobetulinic acid derivatives. *Bioorg. Med. Chem.* **1997,** *5*, 2133-2143.

10. Qian, K.; Kuo, R. Y.; Chen, C. H.; Huang, L.; Morris-Natschke, S. L.; Lee, K. H. Anti-AIDS agents 81. Design, synthesis, and structure-activity relationship study of betulinic acid and moronic acid derivatives as potent HIV maturation inhibitors. *J. Med. Chem.* **2010,** *53*, 3133-3141.

11. Kashiwada, Y.; Chiyo, J.; Ikeshiro, Y.; Nagao, T.; Okabe, H.; Cosentino, L. M.; Fowke, K.; Morris-Natschke, S. L.; Lee, K. H. Synthesis and anti-HIV activity of 3-alkylamido-3-deoxy-betulinic acid derivatives. *Chem. Pharm. Bull. (Tokyo)* **2000,** *48*, 1387-1390.

12. Qian, K.; Nakagawa-Goto, K.; Yu, D.; Morris-Natschke, S. L.; Nitz, T. J.; Kilgore, N.; Allaway, G. P.; Lee, K. H. Anti-AIDS agents 73: structure-activity relationship study and asymmetric synthesis of 3-O-monomethylsuccinyl-betulinic acid derivatives. *Bioorg. Med. Chem. Lett.* **2007,** *17*, 6553-6557.

13. Soler, F.; Poujade, C.; Evers, M.; Carry, J. C.; Henin, Y.; Bousseau, A.; Huet, T.; Pauwels, R.; De Clercq, E.; Mayaux, J. F.; Le Pecq, J. B.; Dereu, N. Betulinic acid derivatives: a new class of specific inhibitors of human immunodeficiency virus type 1 entry. *J. Med. Chem.* **1996,** *39*, 1069-1083.

14. Sun, I. C.; Wang, H. K.; Kashiwada, Y.; Shen, J. K.; Cosentino, L. M.; Chen, C. H.; Yang, L. M.; Lee, K. H. Anti-AIDS agents. 34. Synthesis and structure-activity relationships of betulin derivatives as anti-HIV agents. *J. Med. Chem.* **1998,** *41*, 4648-4657.

15. Kashiwada, Y.; Chiyo, J.; Ikeshiro, Y.; Nagao, T.; Okabe, H.; Cosentino, L. M.; Fowke, K.; Lee, K. H. 3,28-Di-*O*-(dimethylsuccinyl)-betulin isomers as anti-HIV agents. *Bioorg. Med. Chem. Lett.* **2001,** *11*, 183-185.

16. Sun, I. C.; Chen, C. H.; Kashiwada, Y.; Wu, J. H.; Wang, H. K.; Lee, K. H. Anti-AIDS agents 49. Synthesis, anti-HIV, and anti-fusion activities of IC9564 analogues based on betulinic acid. *J. Med. Chem.* **2002,** *45*, 4271-4275.

17. Gerrish, D.; Kim, I. C.; Kumar, D. V.; Austin, H.; Garrus, J. E.; Baichwal, V.; Saunders, M.; McKinnon, R. S.; Anderson, M. B.; Carlson, R.; Arranz-Plaza, E.; Yager, K. M. Triterpene based compounds with potent anti-maturation activity against HIV-1. *Bioorg. Med. Chem. Lett.* **2008,** *18*, 6377-6380.

18. Huang, L.; Ho, P.; Lee, K. H.; Chen, C. H. Synthesis and anti-HIV activity of bi-functional betulinic acid derivatives. *Bioorg. Med. Chem.* **2006,** *14*, 2279-2289.

19. Fulda, S. Betulinic Acid for cancer treatment and prevention. *Int. J. Mol. Sci.* **2008,** *9*, 1096-1107.

20. Fulda, S.; Scaffidi, C.; Susin, S. A.; Krammer, P. H.; Kroemer, G.; Peter, M. E.; Debatin, K. M. Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. *J. Biol. Chem.* **1998,** *273*, 33942-33948.

21. Andre, N.; Carre, M.; Brasseur, G.; Pourroy, B.; Kovacic, H.; Briand, C.; Braguer, D. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *FEBS Lett.* **2002,** *532*, 256-260.

22. Huang, L.; Ho, P.; Chen, C. H. Activation and inhibition of the proteasome by betulinic acid and its derivatives. *FEBS Lett.* **2007,** *581*, 4955-4959.

23. Fulda, S.; Susin, S. A.; Kroemer, G.; Debatin, K. M. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. *Cancer Res.* **1998,** *58*, 4453-4460.

24. Selzer, E.; Pimentel, E.; Wacheck, V.; Schlegel, W.; Pehamberger, H.; Jansen, B.; Kodym, R. Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. *J. Invest. Dermatol.* **2000,** *114*, 935-940.

25. Fulda, S.; Debatin, K. M. Sensitization for anticancer drug-induced apoptosis by betulinic Acid. *Neoplasia* **2005,** *7*, 162-170.

26. Sawada, N.; Kataoka, K.; Kondo, K.; Arimochi, H.; Fujino, H.; Takahashi, Y.; Miyoshi, T.; Kuwahara, T.; Monden, Y.; Ohnishi, Y. Betulinic acid augments the inhibitory effects of

vincristine on growth and lung metastasis of B16F10 melanoma cells in mice. *Br. J. Cancer* **2004,** *90*, 1672-1678.

27. Kim, D. S.; Pezzuto, J. M.; Pisha, E. Synthesis of betulinic acid derivatives with activity against human melanoma. *Bioorg. Med. Chem. Lett.* **1998,** *8*, 1707-1712.

28. Gauthier, C.; Legault, J.; Lebrun, M.; Dufour, P.; Pichette, A. Glycosidation of lupane-type triterpenoids as potent in vitro cytotoxic agents. *Bioorg. Med. Chem.* **2006,** *14*, 6713-6725.

29. Kvasnica, M.; Sarek, J.; Klinotova, E.; Dzubak, P.; Hajduch, M. Synthesis of phthalates of betulinic acid and betulin with cytotoxic activity. *Bioorg. Med. Chem.* **2005,** *13*, 3447-3454.

30. Santos, R. C.; Salvador, J. A.; Marin, S.; Cascante, M. Novel semisynthetic derivatives of betulin and betulinic acid with cytotoxic activity. *Bioorg. Med. Chem.* **2009,** *17*, 6241-6250.

31. You, Y. J.; Kim, Y.; Nam, N. H.; Ahn, B. Z. Synthesis and cytotoxic activity of A-ring modified betulinic acid derivatives. *Bioorg. Med. Chem. Lett.* **2003,** *13*, 3137-3140.

32. Koohang, A.; Majewski, N. D.; Szotek, E. L.; Mar, A. A.; Eiznhamer, D. A.; Flavin, M. T.; Xu, Z. Q. Synthesis and cytotoxicity of 2-cyano-28-hydroxy-lup-1-en-3-ones. *Bioorg. Med. Chem. Lett.* **2009,** *19*, 2168-2171.

33. Kumar, V.; Rani, N.; Aggarwal, P.; Sanna, V. K.; Singh, A. T.; Jaggi, M.; Joshi, N.; Sharma, P. K.; Irchhaiya, R.; Burman, A. C. Synthesis and cytotoxic activity of heterocyclic ring-substituted betulinic acid derivatives. *Bioorg. Med. Chem. Lett.* **2008,** *18*, 5058-5062.

34. Jeong, H. J.; Chai, H. B.; Park, S. Y.; Kim, D. S. Preparation of amino acid conjugates of betulinic acid with activity against human melanoma. *Bioorg. Med. Chem. Lett.* **1999,** *9*, 1201-1204.

35. Chatterjee, P.; Pezzuto, J. M.; Kouzi, S. A. Glucosidation of betulinic acid by Cunninghamella species. *J. Nat. Prod.* **1999,** *62*, 761-763.

36. Zuco, V.; Supino, R.; Righetti, S. C.; Cleris, L.; Marchesi, E.; Gambacorti-Passerini, C.; Formelli, F. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Lett.* **2002,** *175*, 17-25.

37. Yasukawa, K.; Takido, M.; Matsumoto, T.; Takeuchi, M.; Nakagawa, S. Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis. *Oncology* **1991,** *48*, 72-76.

38. Yasukawa, K.; Yu, S. Y.; Yamanouchi, S.; Takido, M.; Akihisa, T.; Tamura, T. Some lupane-type triterpenes inhibit tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. *Phytomedicine* **1995,** *1*, 309-313.

39. Pisha, E.; Chai, H.; Lee, I. S.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W.; Fong, H. H.; Kinghorn, A. D.; Brown, D. M.; et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat. Med.* **1995,** *1*, 1046-1051.

40. Nakagawa-Goto, K.; Yamada, K.; Taniguchi, M.; Tokuda, H.; Lee, K. H. Cancer preventive agents 9. Betulinic acid derivatives as potent cancer chemopreventive agents. *Bioorg. Med. Chem. Lett.* **2009,** *19*, 3378-3381.

Chapter 3

Non-toxic Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB) Analogs Chemosensitize Multidrug Resistant Cancer Cells to Clinical Anticancer Drugs

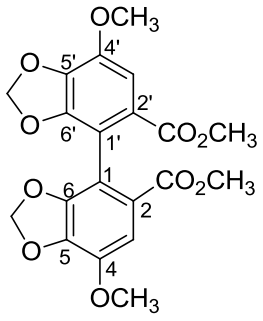
3.1 Introduction

Despite substantial biomedical research on cancer therapy, cancers still remain the leading cause of death. Among all factors resulting in the ultimate failure of cancer treatment, chemotherapy resistance is a significant player, and multidrug resistance (MDR), cross-resistance to different chemical drug classes, occurs in various cancer types. Cellular mechanisms of MDR include decreased uptake of chemotherapeutic agents, via expression of vacuolar ATPase (V-ATPase), or adaptation of cancer cells to the cytotoxic ability of chemotherapeutic agents, via down-regulation of topoisomerase II and over-expression of glutathione S-transferase-π.[1-3] Recent studies on cancer stem cell-like features are leading to new understanding of cancer resistance.[4] However, over-expression of drug efflux transporters, such as P-glycoprotein (P-gp) and MDR-associated protein (MRP), is still believe to be the primary cause leading to multidrug resistance.[5] In order to surmount MDR, great efforts have been put into developing clinically usable chemosensitizing agents, categorized as either apoptosis modulators[6, 7] or MDR modulators, also known as P-gp inhibitors.[8] Verapamil (VRP) and cyclosporine A, two first-generation chemosensitizers, were precluded from clinical use due to significant toxicity, but are used in experiments as positive controls. Second- and third-generation chemosensitizers were developed subsequently; however, unsatisfactory toxicity and pharmacokinetic complications still impeded drug candidate development. Thus, the discovery of safe and effective

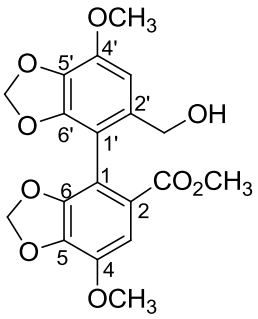
MDR modulators is still attractive and greatly needed to overcome the MDR issue in the field of cancer chemotherapy.

Schisandrin B (Figure 3.1), the most abundant dibenzocyclooctadiene lignan from *Schisandra chinensis,* was found to inhibit P-gp/MDR1 and MRP1/ABCC1.[9,10] Structurally similar lignans, schisandrin A, schisandrol A, and schisantherins A and B, also chemosensitized various anticancer drugs, including vincristine (VCR), daunorubicin, and etoposide, in human promyelocytic leukemia cell lines with over-expressed MRP1/ABCC1.[11] Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene dioxybiphenyl-2,2′-dicarboxylate (DDB, **1**, Figure 3.1), which was discovered as a synthetic intermediate derivative of schisandrin C,[12] shares the biphenyl partial structure of dibenzocyclooctadiene lignans. DDB (**1**) exhibited multidrug resistant reversal ability *in vitro* and *in vivo* and DDB's analog, bicyclol also shown chemoreversal activity mentioned in chapter 1.[13,14] However, a very high concentration (50 μM) was required for their effective reversal action. DDB's low toxic feature makes DDB analogs highly attractive MDR reversal agents. Although DDB and bicyclol have high potentials as MDR reversal agents and various DDB analogs have been prepared, their MDR reversal abilities and structure activity relationship (SAR) correlations have been not investigated. To explore more potent non-toxic MDR reversal analogs with lower effective dosing and to study SAR, we designed and synthesized additional DDB analogs. Herein, we report the chemosensitizing effects of newly synthesized DDB analogs.

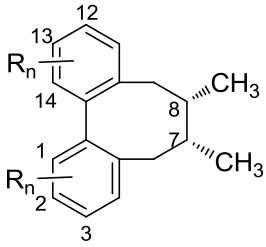
Figure 3.1 Structures of DDB, Bicyclol, and Dibenzocyclooctadiene Lignans



1: DDB



Bicyclol



Schisandrin A:
R_n= 1,2,3,12,13,14-hexamethoxy
Schisandrin B:
R_n= 1,2,3,14-tetramethoxy-12,13-methylenedioxy
Schisandrin C:
R_n= 1,14-dimethoxy-2,3,12,13-dimethylendioxy

Dibenzocyclooctadiene lignan

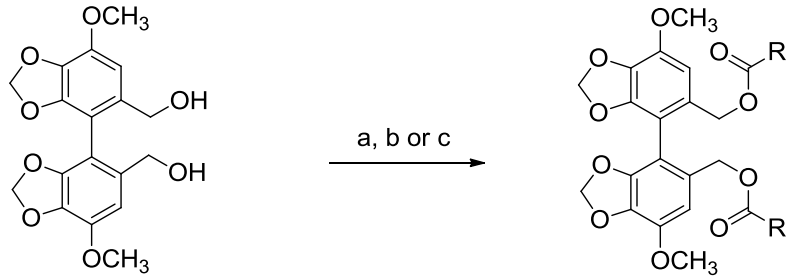
3.2 Design and Synthesis

The P-gp contains a large drug-binding pocket with a volume of around 6000Å^3.[15] The pocket includes predominantly hydrophobic and aromatic residues in its upper half and more polar and charged residues in its lower half. Thus, because of similarity, hydrophobic substrates would bind to the hydrophobic residues, and aromatic substrates would overlap with the π-orbitals of aromatic residues in the binding pocket. Further evaluation of the chemical structures of various known P-gp inhibitors identified common features, including high hydrophobicity, two or more aromatic rings, a methoxy group on the aromatic ring, and one or two protonatable nitrogens.[16] Among the above mentioned features, DDB already possesses two aromatic rings with a methoxy group on each. Because bicyclol bears a primary alcohol at the C-2′ position, we selected the 2,2′-bis-hydroxymethyl analog **69** as a base scaffold to design various esters **70**–**95** (Scheme 3.1) and define substituent effects at the C2 and C2′ positions. The ester groups (R in Scheme 3.1) were selected by considering size, hydrophobicity, and electron density, which might affect the binding affinity to P-gp. The

diverse set included acyclic, cyclic, unsaturated aliphatic groups, aromatic groups, and polar groups, which could be transformed into water-soluble salts, if necessary. Esterifications of **69** were performed by treatment with the appropriate acid (RCO_2H), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI), and 4-dimethylaminopyridine (DMAP) to produce **70**, **74**–**79**, **82**–**84**, and **89**, as well as by treatment with the related acid chloride (RCOCl) and Et_3N to give **71**–**73**, **80**–**81**, **86**–**88**, and **90**–**95** (Scheme 3.1).

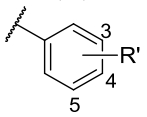
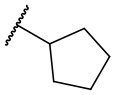
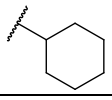
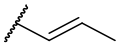
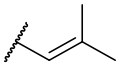
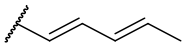
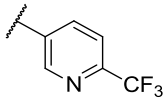
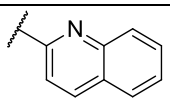
Halogenated DDB analogs were also designed to explore their effects (Scheme 3.2). 3,3′-Dibromo-DDB (**96**) was previously synthesized.[17] Iodination of **1** was achieved by treatment with silver trifluoacetate and iodine to provide **97**.[18] The diester groups of **96** were reduced with diisobutylaluminium hydride (DIBAL) to afford bis-hydroxymethyl biphenyl **98** in good yield. A bulky group or aromatic ring is a common feature of several MDR modulators as mentioned above. Therefore, the esters **99** and **100** were also synthesized by esterification of **98** with butyric and benzoic acids, respectively, in the presence of EDCI and DMAP. Hydrolysis of dimethyl ester **96** under basic conditions resulted in dicarboxylic acid **101**. Reflux of **101** in Ac_2O gave anhydrous analog **102**, followed by asymmetric cleavage of carbonyl anhydrous bridge with $NaBH_4$ resulted in di-Br bicyclol analog **103**.[19] All synthesized analogs were prepared as racemic mixtures.

Scheme 3.1. Syntheses of Diester Derivatives 70 - 95

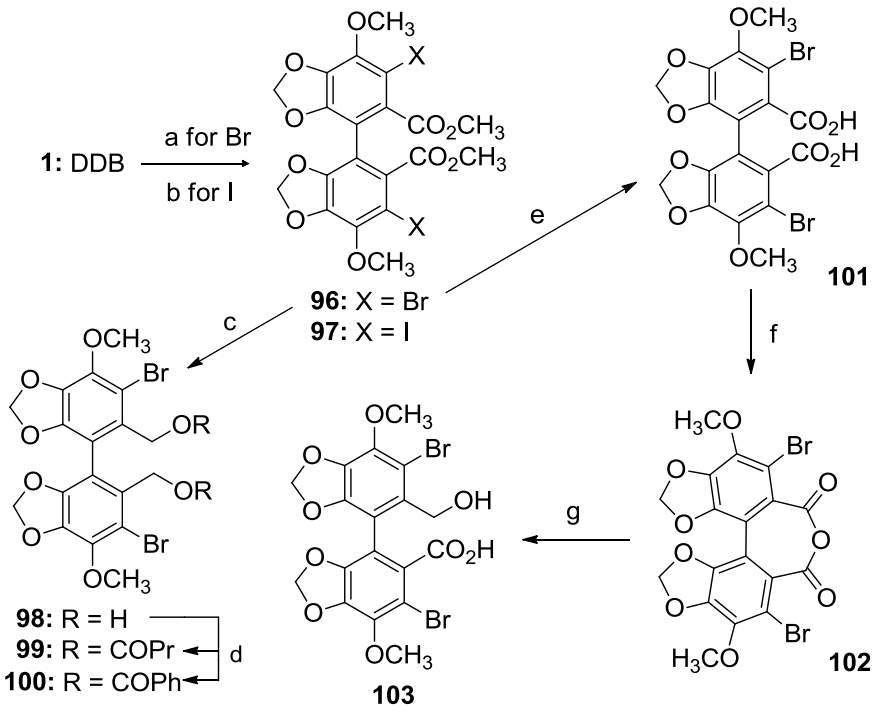


69 **70 - 95**

Reagents and conditions: (a) RCO_2H, EDCI, DMAP, for **70**, **74-79**, **82-84**, and **89**
(b) RCOCl, Et_3N for **71-73**, **80-81**, **86-88**, and **90-95**
(c) acid anhydride, DMAP for **85**

Cmpd	**R** **Aliphatic Acyclic Group (I)**	Cmpd	**R** **Aliphatic Cyclic Group (II)**	Cmpd	**R** **Aromatic Group (V)** 
70	Me	**80**		**86**	R' = H
71	Et	**81**		**87**	R' = 4-Me
72	*i*Pr			**88**	R' = 4-OMe
73	*t*Bu		**Unsaturated Group (III)**	**89**	R' = 3,4-diOMe
74	Pr	**82**		**90**	R' = 3,4,5-triOMe
75	*sec*-Bu	**83**		**91**	R' = NO_2
76	*i*Bu	**84**		**92**	R' = CN
77	Bu			**93**	R' = 3,4-methylenedioxy
78	-$CH(CH_3)CH_2CH_2CH_3$		**Polar Group (IV)**	**94**	
79	-$(CH_2)_{10}CH_3$	**85**	-$CH_2CH_2CO_2H$	**95**	

Scheme 3.2. Syntheses of Dihalogenated DDB Analogs



Reagent and conditions: (a) Br_2, $CHCl_3$, 0°C, (b) CF_3CO_2Ag, I_2, (c) DIBAL, CH_2Cl_2, (d) EDCI, DMAP, CH_2Cl_2, $CH_3(CH_2)_2CO_2H$ for **99**, and $PhCO_2H$ for **100,** (e) KOH, EtOH, reflux, (f) Ac_2O, reflux, (g) $NaBH_4$, THF, MeOH

3.3 Results and Discussion

Evaluation of cytotoxicity and preliminary MDR reversal activity screening.

All synthesized compounds were initially evaluated in a cytotoxic activity assay using four tumor cell lines, A549 (lung cancer), DU145 (prostate cancer), KB (epidermoid carcinoma of the nasopharynx) and a resistant sub-line, KBvin (over-expression of P-gp selected using increasing concentrations of vincristine). Although compounds **95** and **99** were slightly cytotoxic, the other compounds did not exhibit significant cytotoxicity (Table 3.1), which implied low toxicity of these analogs.

For evaluating chemosensitizing activity, KB and KBvin cells were co-treated with test compound at 10 μM and the anticancer drug paclitaxel (TAX), as well as with test compound alone at

the same concentration (Figure 3.2). As shown in Figure 3.2-B, DDB (**1**) and diol **69** did not exhibit an MDR reversal effect at a concentration of 10 μM in KBvin cells. However, many of the analogs with aliphatic ester substituents (I and II in Figure 3.2) did show potent activity, although acetate **70** (R = methyl) and dodecanoate **79** (R = undecanyl) were inactive, and 2-methylhexanoate **78** (R = 2-methybutyl) was only moderately active. The screening results implied a rough SAR; at least a two carbon linear ester chain (R = ethyl) was necessary for chemosensitizing activity, but a long chain (R > approximately five carbons), led to reduced or no activity. Isobutyrate (R = isopropyl) **72** and pivalate (R = *tert*-butyl) **73** resulted in the most significant chemosensitization (cell survival rate of 13–14%) among all saturated Group I and II compounds, and the unsaturated 3-methylbut-2-enoate (prenyl-like substituent) **83** in the Group III compounds further increased the effect, resulting in a cell survival rate of only 5%. Among the Group IV compounds with polar ester substituents, succinate **85** (R = CH_2CH_2COOH) was inactive, while compounds with phenyl or pyridinyl aromatic rings showed potent reversal ability, unless an electron-withdrawing group, such as nitro and cyano, was present on the aromatic ring. Notably, benzoate **86** and *p*-methoxybenzoate **88** led to less than 1% cell survival rate. From these findings, we speculated that a decrease in electron density on the aromatic ring affected the overlap of the compound with the π–orbitals of aromatic residues in the P-gp drug-binding pocket. Quinoline derivative **95** exhibited cytotoxicity against both KB and KBvin cells. 3,3′-Dihalogenated DDB analogs with different 2,2′-functional groups did not show potent reversal activity, except pentanoate **99** and benzoate **100**, but **99** was cytotoxic to KBvin cells (Table 3.1). While a 2,2′-methylester group [-$CH_2OC(O)R$] appeared to be critical for reversal ability, 3,3′-halogenation reduced the potency (compare **100** with **86**).

Because the presumptive drug-binding pocket is composed of mostly hydrophobic and aromatic residues,[15] it is possible that hydrophobicity of DDB analogs could influence their MDR reversal activity. Table 3.2 shows the Clog *P* values of synthesized compounds. Although a few exceptions were present, the chemosensitizing effects of compounds were moderately correlated with their Clog *P* values. Active compounds had Clog *P* values of 4–8; those with Clog *P* lower than 4 tended to lose

chemosensitizing activity. The Clog *P* values of **73**, **83**, and **90**, which as described below were significantly active, were between 4.8 and 6.2, which is close to that of VRP. This fact implied that hydrophobicity is an important parameter, although other factors could also affect potency.

From these results, compounds **71**–**78**, **80**–**84**, and **86**–**91** were selected for further investigation; compounds with weaker reversal ability (**1, 69**–**70**, **79**, **85**, **91**–**94**, **96**–**98**, and **102**–**103**) as well as inherent cytotoxicity (**95** and **99**) were eliminated from further testing**.**

Table 3.1. Cytotoxicity of DDB Analogs

Compd	GI_{50} (μM)[a]			
	A549	DU145	KB	KBvin
1, 69-72	>100	>100	>100	>100
73	98	>100	>100	>100
74-94	>100	>100	>100	>100
95	45	>100	88	71
96-98	>100	>100	>100	>100
99	13	19	24	19
100-103	>100	>100	>100	>100
TAX[b]	7.0 nM	2.9 nM	1.2 nM	1290.9 nM

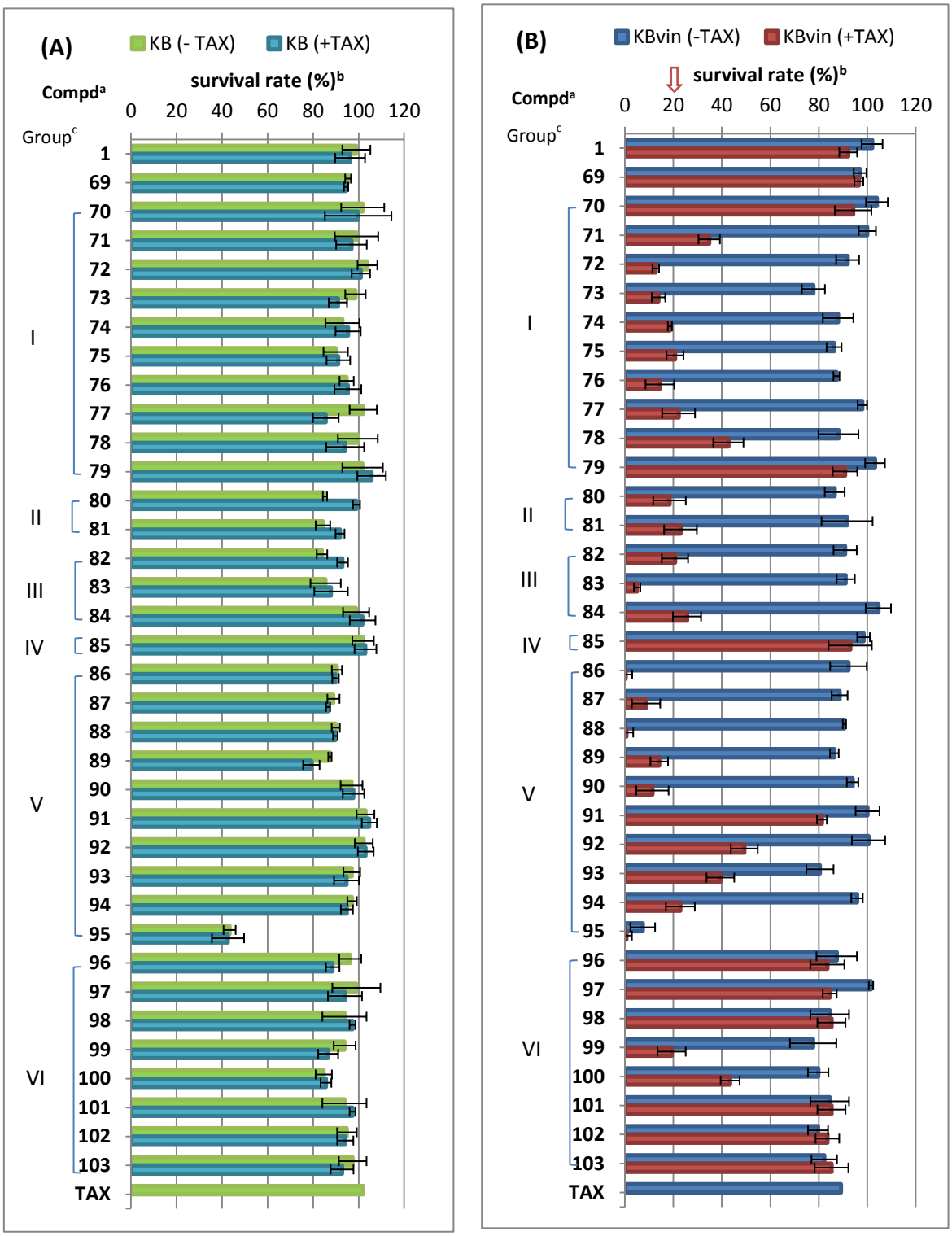
[a] Antiproliferative activity as GI_{50} values for each cell line, the concentration of compound that caused 50% reduction in absorbance at 562 nm relative to untreated cells using the sulforhodamine B assay. Human lung carcinoma (A549), prostate cancer (DU145), epidermoid carcinoma of the nasopharynx (KB), and MDR expressing P-glycoprotein (KB-VIN). All compounds, besides **95** and **89**, exhibited no cytotoxicity. [b]Paclitaxel

Table 3.2. CLogP Values of Synthesized Compounds

Compd	CLog P[a]	Compd	CLog P	Compd	CLog P	Compd	CLog P
1	4.27	**77**	6.15	**86**	6.93	**95**	6.21
69	1.26	**78**	6.77	**87**	7.93	**96**	4.68
70	2.98	**79**	N/A	**88**	7.28	**97**	5.02
71	4.03	**80**	5.92	**89**	6.69	**98**	2.60
72	4.65	**81**	7.04	**90**	5.93	**99**	7.49
73	5.45	**82**	5.24	**91**	6.42	**100**	8.27
74	5.09	**83**	6.04	**92**	5.80	**101**	1.96
75	5.71	**84**	6.51	**93**	7.31	**102**	5.34
76	5.89	**85**	2.40	**94**	6.11	**103**	2.28
						VRP	4.58

[a] CLogP was calculated by ChemDraw Ultra Version 12.0

Figure 3.2. Screening of Reversal Abilities against KB (A) and KBvin (B)



Note: [a] Concentration of compounds: 10 µM, [b] Survival rate (%) was measured by SRB method using KB and KBvin cells in the presence (+) or absence (-) of paclitaxel(TAX). Compounds with survival rates below 20 % were considered very potent and moved to further experiments. [c]Group (I) saturated acyclic alkyl; (II) cyclic alkyl ; (III) unsaturated; (IV) polar; (V): aromatic; (VI): halogenated.

Chemoreversal ability of DDB analogs with TAX, VCR, and doxorubicin (DOX).

A quantitative evaluation of the reversal ability of DDB analogs **71**–**78**, **80**–**84**, and **86**–**90** was performed using MDR KBvin cells with various concentrations of TAX, VCR, and DOX, which are clinically used and known as significant P-gp substrates (Table 3.3). The IC_{50} value of cancer drug in the presence of test compound at 10 μM concentration was calculated and fold reversal was determined by dividing the IC_{50} of anticancer drug alone by the IC_{50} of anticancer drug plus DDB analog. For chemoreversal ability against TAX resistance, compounds **76**, **82**, **83**, **86**, and **90** were 3–10 times more potent than the positive control VRP. Especially, **83**, with a prenyl-like ester substituent, and **90**, with a trimethoxybenzoate substituent, effectively reversed the sensitivity of TAX in KBvin cells by 326- and 222-fold, respectively. Most of the tested analogs, along with VRP, showed greater reversal against VCR resistance compared with TAX resistance. The reversal effects of compounds **73**, **77**, **80**–**83**, **89,** and **90** against VCR were significantly better than that of VRP. Notably, **83** and **90** showed a 560-fold reversal effect, 5.1 times greater than that of VRP. The following SAR correlations were proposed based on the chemosensitizing effects against TAX and VCR. In compounds with aliphatic esters, unsaturated Group III compounds **82** and **83** were generally more potent than saturated group I and II compounds. Compounds **82** and **83** displayed greater chemoreversal ability than **74** and **76**, which contain structurally related saturated groups. In the case of compounds with aromatic esters (Group V), an electron donating group, such as methyl and methoxy, at the *para*-position reduced the reversal ability, while additional methoxy groups at the *meta*-position enhanced the ability. The following rank order of potency was seen: 3,4,5-tri-OMe (**90**) > 3,4-di-OMe (**89**) > H (**86**) > 4-Me (**87**) ≈ 4-OMe (**88**). Different SAR correlations were found between TAX and VCR within the saturated aliphatic group. Cyclic aliphatic side chains (Group II, **80**–**81**) were more effective than non-cyclic aliphatic side chains (Group I, **71**–**78**) in the case of VCR resistance, while a difference was not present for TAX resistance. Within the tested aliphatic acyclic Group I compounds (**71**–**78**), 2-methylbutyrate (R = *iso*-butyl) **76** was most potent for TAX, while pivalate (R = *tert*-butyl) **73** and petanoate (R = *n*-butyl) **77** were most potent for VCR. In addition, **77**

exhibited greater activity than the related unsaturated analog **84** against VCR resistance. All of the tested compounds were less effective at reversing DOX resistance compared with TAX and VCR resistance. This phenomenon might be correlated with the location of the efflux pump, either on the cell membrane or on the nuclear membrane, because both TAX and VCR act by binding tubulin, while DOX interacts with DNA by intercalation.[20] However, compounds **73**, **75**, **78**–**81**, and **87**–**90** still showed greater reversal effects than VRP for DOX chemosensitivity in MDR cells. Pivalate **73**, with a bulky and short aliphatic ester chain, reversed the activity most effectively, exhibiting five-fold more sensitivity than VRP. SAR against DOX was slightly different than that against VCR. Compounds **73**, **75**, **78**, **80**, and **81** with a branched substituent at the ester α-position showed significant MDR reversal activity. Benzoates with an electron donating group, such as *para*-methyl (**87**) and –methoxy (**88**), had little effect. Although an additional methoxy group at the *meta*-position increased the ability, no difference was found between trimethoxy **90** and dimethoxy **89**. The effects of substituent on phenyl ring resulted in the following order of potency: 3,4-di-OMe (**89**)≈3,4,5-tri-OMe (**90**) > H (**86**) ≈4-Me (**87**) ≈4-OMe (**88**). In conclusion, analogs **83** and **90** showed the most significant TAX and VCR cytotoxic reversal ability, while analog **73** exhibited the most potent DOX cytotoxic reversal ability.

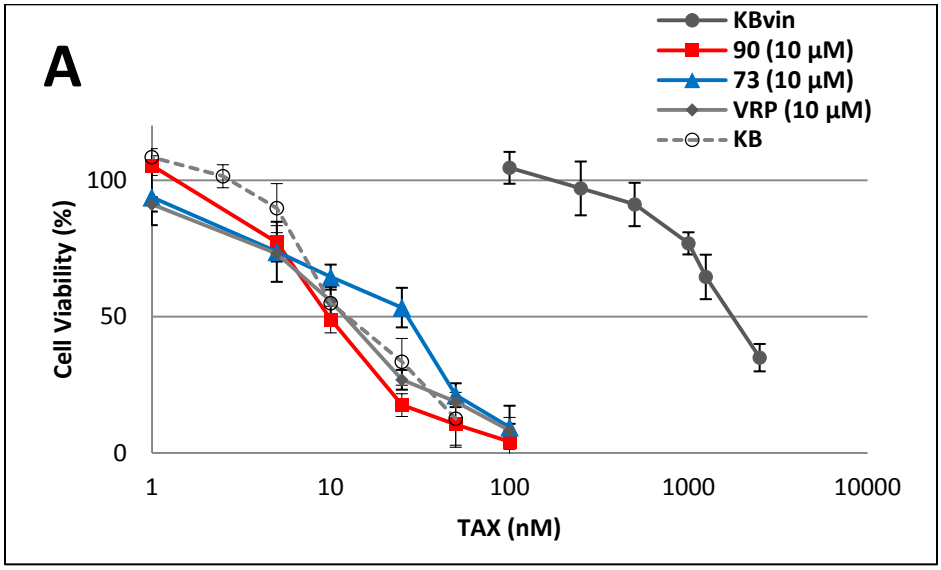
Based on the above results, compounds **73** and **90** were selected for a dose-response analysis of chemosensitization efficacy. Because their ability to induce full cancer drug sensitivity is unclear, dose-response curves of growth inhibition by TAX (A), VCR (B), and DOX (C) in the presence of **73**, **90**, or VRP were compared to parent cell drug sensitivity (Figure 3.3). After treatment with **73**, **90**, or VRP at 10 μM, the growth inhibition curves for the three anticancer drugs in KBvin cells shifted toward and were similar to those against the parental KB cell line. This result shows that **73** and **90** can completely inhibit cellular P-gp and thereby confer full sensitivity to the tested clinically relevant anticancer drugs.

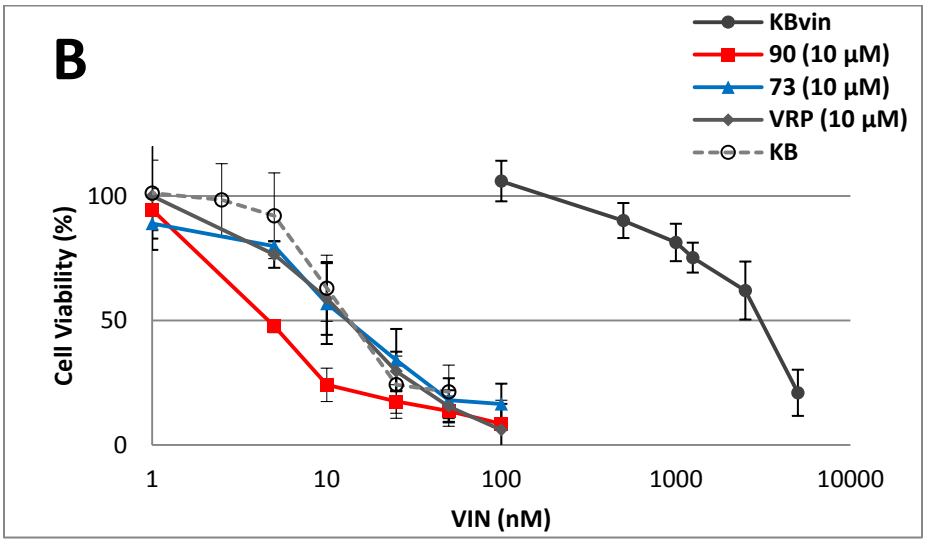
Table 3.3. Reversal Effects with Paclitaxel (TAX), Vincristine (VCR), and Doxorubicin (DOX) in KBvin

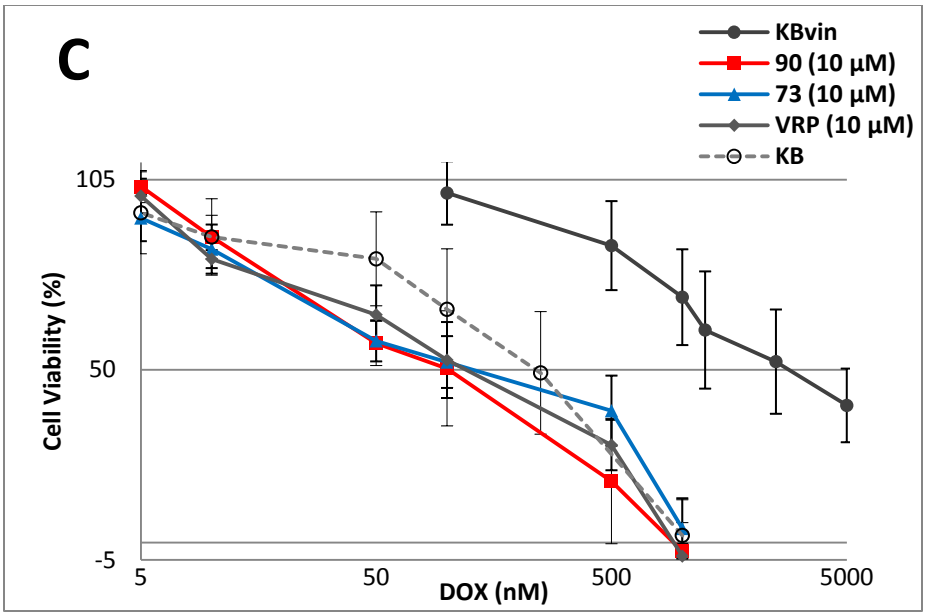
Group	Cmpd[a]	GI_{50} of TAX (nM)[b]	Fold[c]	GI_{50} of VCR (nM)	Fold	GI_{50} of DOX (nM)	Fold
	TAX	976.8	-	2520.7	-	1942.3	-
I	**71**	82.93	12	80.96	31	507.5	4
	72	46.52	21	41.42	61	284.0	7
	73	26.15	37	7.22	349	42.6	46
	74	30.50	32	50.81	50	510.9	4
	75	37.06	26	35.92	70	93.9	21
	76	8.72	112	31.75	79	339.7	6
	77	40.74	24	15.65	161	221.5	9
	78	39.11	25	47.31	53	61.9	31
II	**80**	48.65	20	8.14	310	86.3	23
	81	39.89	24	10.55	239	65.8	30
III	**82**	9.55	102	10.98	230	547.8	4
	83	2.99	326	4.50	560	291.5	7
	84	47.45	21	46.53	54	573.9	3
V	**86**	7.65	167	20.21	101	157.7	9
	87	46.82	27	39.61	51	130.8	11
	88	47.23	27	40.53	50	131.4	11
	89	14.25	89	10.10	202	68.80	21
	90	4.41	222	4.45	566	84.2	23
	VRP	31.87	31	23.04	109	219	9

[a] Concentration of compound: 10 μM, [b]SD was shown in Supporting Information, [c] The reversal fold values were calculated as: Reversal fold = IC_{50} (anticancer drug alone) / IC_{50} (anticancer drug + test compound).

Figure 3.3. Dose-response of growth inhibition of 73 and 90 together with Paclitaxel, Vincristine and Doxorubicin against KB and KBvin. KBvin (●) and KB (○) cells were treated with various concentrations of TAX (A), VCR (B), or DOX (C) alone or together with 10 μM of **73** (▲), **90** (■), or verapamil (◆). The incubation period was 72 hours before the cell viability was measured by SRB method as described in Materials and Methods. Compounds **73** and **90** were able to elevate the sensitivity of KBvin to the anticancer drug (TAX, VCR, and DOX) to a level similar to or lower than that of KB.



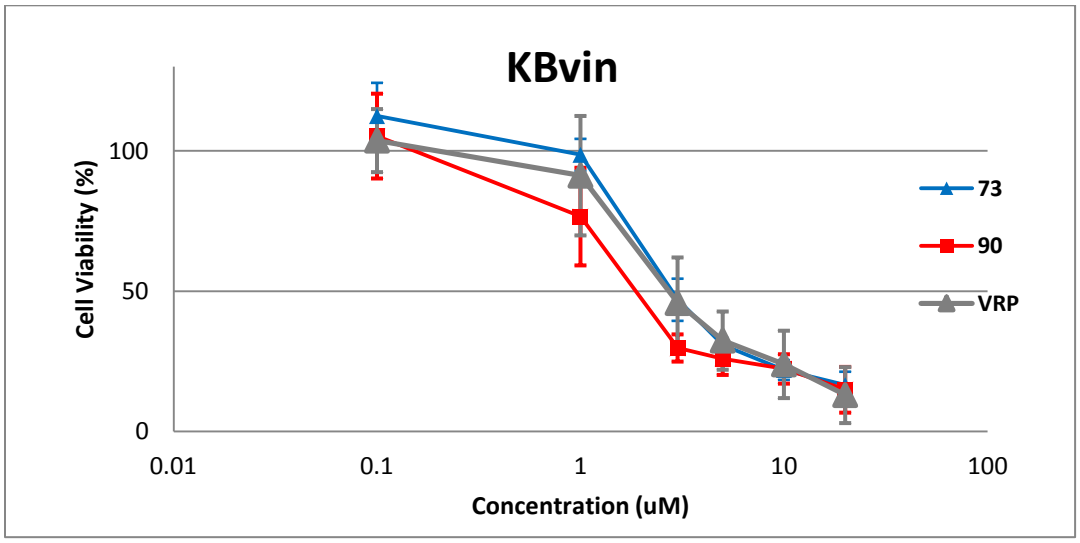




Dose-response effect of compounds 73 and 90 on sensitization of KBvin to TAX.

To evaluate the reversal activity of **73** and **90** in a dose-response manner, KBvin cells were cultured with non-toxic concentration of TAX (100 nM) in the presence of various concentrations of compounds (Figure 3.4). As we expected, compounds **73** and **90** exhibited reversal activity in a dose dependent manner. Although median effective concentration (EC_{50}) value of **73** (2.81 µM) was similar to that of VRP (2.71 µM), **90** (1.87 µM) was more potent than VRP. These results demonstrate that **90** is more effective than VRP in chemosensitizing the MDR cells to TAX.

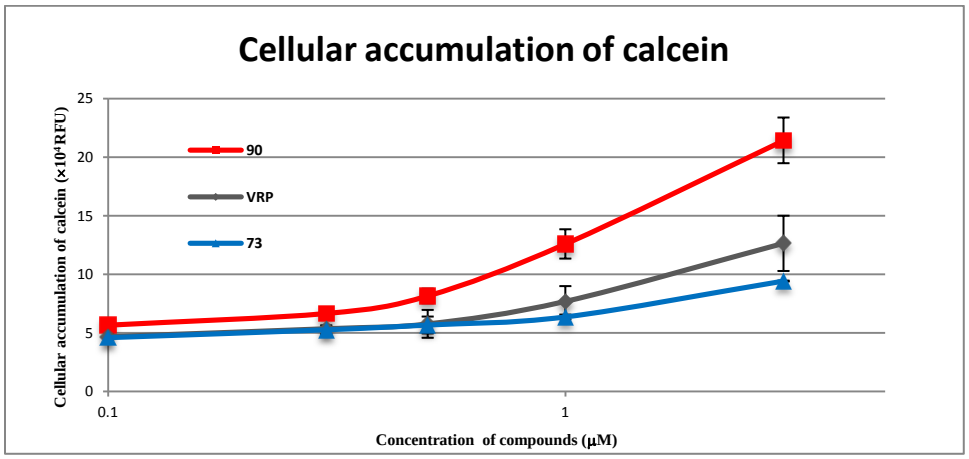
Figure 3.4. Dose-response effect of compounds 73 and 90 on sensitization of KBvin to TAX. Multidrug resistant KBvin cells were treated with various concentrations of compounds 73 or 90 in the presence of 100 nM TAX, an absolutely non-toxic concentration for KBvin. Data are expressed as mean ± SD of three independent experiments. Calculated median effective concentration (EC_{50}) of compounds **73**, **90**, or VRP was 2.81 µM, 1.87 µM or 2.71 µM, respectively.



The effect of DDB analogs on P-gp function in KBvin cells.

To confirm our hypothesis that DDB analogs inhibit efflux activity of P-gp resulting in elevated concentration of anticancer drugs in MDR cells, the effect of compounds **73** and **90** on P-gp function in KBvin cells using Calcein-AM as a fluorogenic P-gp substrate was investigated (Figure 3.5). Dose dependent intracellular accumulation of Calcein was observed in the presence of compounds. Although **73** was slightly less potent than VRP, **90** displayed two-fold more potent than VRP, especially at a concentration of EC_{50} value (1.87 µM) for TAX. Therefore, these results clearly indicated that DDB analogs, especially **90**, are effective P-gp inhibitors.

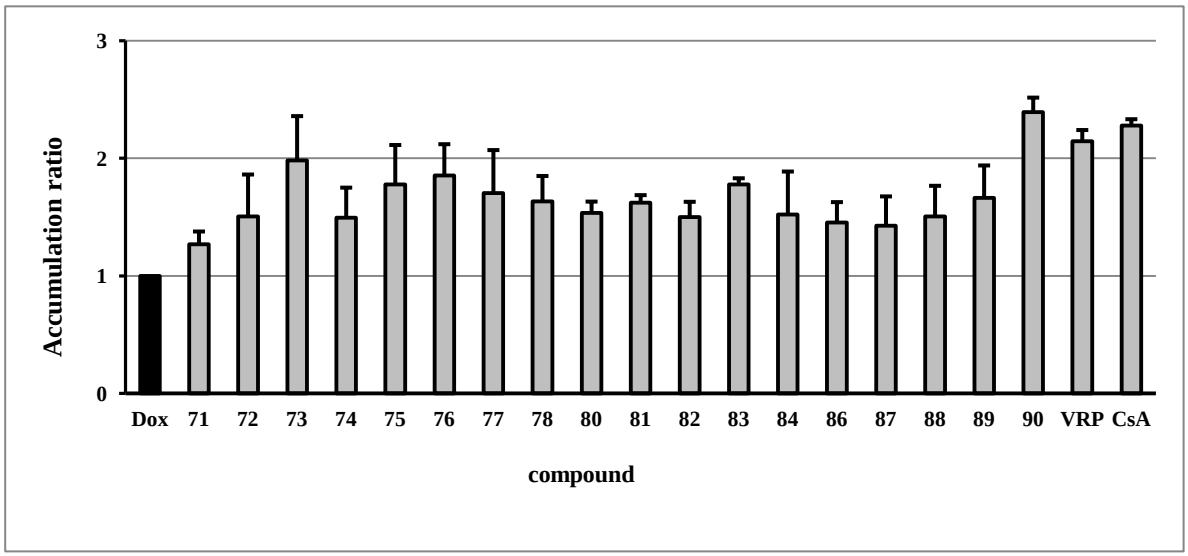
Figure 3.5. Effect of compounds on P-gp function in KBvin cells. KBvin cells were pre-treated with compounds followed by addition of calcein-AM. The cellular accumulation of calcein is represented by the relative fluorescent unit ($\times 10^4$ RFU). Cellular accumulation of calcein demonstrates inhibition of efflux activity of P-gp.



To demonstrate the effective efflux inhibition of anticancer drugs, direct measurement of cellular accumulation of DOX in KBvin cells was studied as the intensity of intrinsic fluorescence of DOX (Figure 3.6). KBvin cells were pre-treated with compounds followed by addition of DOX. Intracellular accumulation of DOX was measured as the fluorescence intensity and standardized as fold ratio. All DDB analogs induced DOX accumulation in KBvin cells at 1.2- to 2.4-fold. The cellular accumulation of DOX by DDB analogs was consistent with sensitization of KBvin cells to DOX. Thus, these data further support that DDB-derived chemosensitizers function as P-gp inhibitors resulting in cellular accumulation of anticancer drugs.

Further screening studies demonstrated that DDB analogs sensitized NIH3T3-MDR cells (murine fibroblast NIH3T3 cells with overexpressing human P-gp protein) to TAX and VCR (unpublished data). These results also support our conclusion that DDB analogs interfere with drug efflux function of P-gp.

Figure 3.6. Recovered Doxorubicin Accumulation in DDB Analog-treated Drug Resistant KBvin cells. Cells were incubated in DOX medium for 3 h in the presence of 10 μM DDB analogs to measure DOX accumulation. DDB analogs were able to recover DOX accumulation level to the same or greater extent than VRP. Data represent as mean ± SD, n= 3. VRP and cyclosporine (CsA) were used as positive controls.



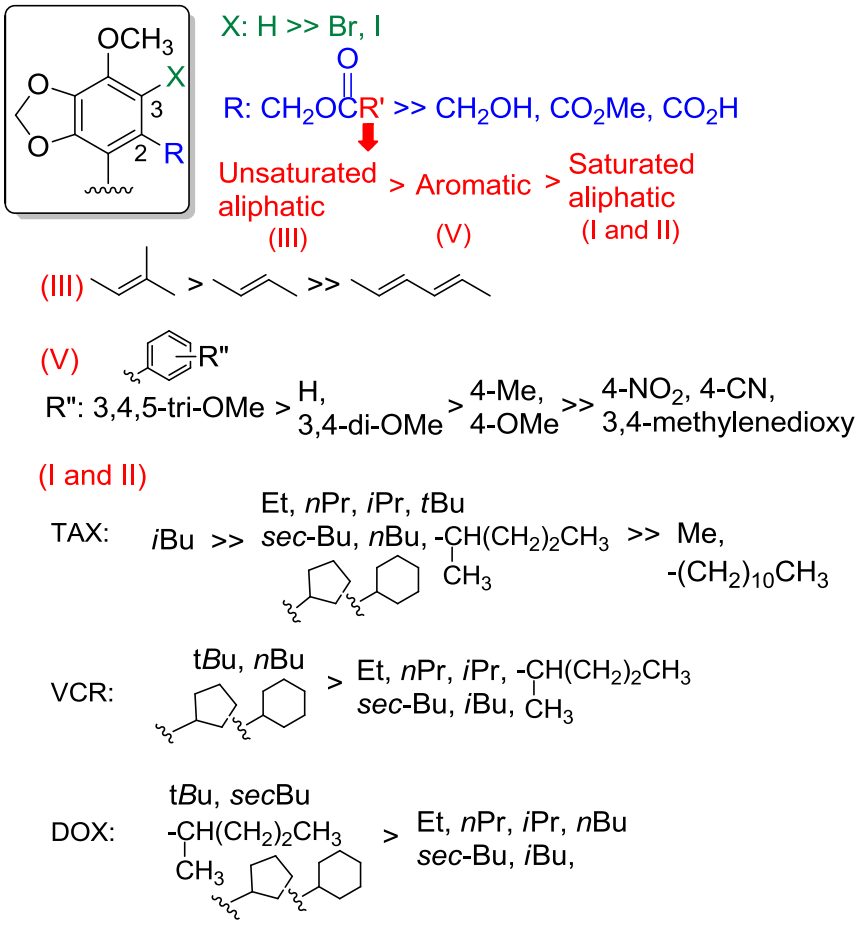
3.4 Conclusions

Multidrug resistance is still a serious barrier to successful cancer treatment. Among the possible reasons for multidrug resistance, the top cause is the reduction in the concentration of the anticancer drug caused by the P-gp and/or MRP drug efflux pump. Effective P-gp modulators are still unavailable for clinical use, due partly to the toxicity of the currently available compounds. We selected DDB, a clinically used hepatoprotective compound, as a lead, and 33 new DDB analogs were newly designed and synthesized. All synthesized analogs were evaluated for MDR chemosensitizing effects of the clinical cancer drug TAX, and selected compounds were also tested for effects on the drugs VCR and DOX. Most of the 2,2′-methylester DDB analogs exhibited improved chemoreversal action compared with the parent compound. The SAR findings are summarized in Figure 3.5. Insertion of halogen at the 3-position reduced the reversal ability, while. bulky groups, such as pivalate, 2-methylbutanoate, cyclic aliphatic, and trimethoxyphenyl, in the ester side chain at the 2-

position, tended to enhance the reversal ability of DDB analogs. Among all tested compounds, DDB derivatives **83** and **90** were 5–10 times more effective than VRP for enhancing TAX and VCR activity, and analog **73** was five times more effective than VRP for enhancing DOX activity. Importantly, active DDB analogs displayed no cytotoxicity at 10-fold higher concentration, suggesting that they are significant lead compounds for further clinical development to overcome multidrug resistance. DOX accumulation inside KBvin cells upon treatment with DDB analogs confirmed that the mechanism of reversal ability is due, at least partly, to drug efflux pump inhibition. Preliminary mechanism of action studies showed that the most promising DDB analogs impeded the efflux ability of resistant cancer cells and thereby facilitated accumulation of the cytotoxic drug DOX.

To conclude, DDB was identified as a new scaffold for chemosensitizer drug development, and newly synthesized non-toxic DDB analogs were discovered. Detailed mechanism of action studies are ongoing to completely elucidate the mechanism of chemoreversal by DDB analogs.

Figure 3.7. Study of Structure-Activity Relationship



3.5 Materials and Methods

Chemistry

General. ^{1}H NMR (400 MHz) spectra were measured on a Varian Inova spectrometer with TMS as the internal standard. Mass spectra were measured on a Shimazu LCMS-IT-TOF. All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60 F254 plate, 20 × 20, Merk). Melting points were recorded on a Fisher Johns melting apparatus without correction. Medium-pressure column chromatography was used in Biotage Flash and Isco companion systems with silica 40 μM columns from Grace Inc. All final compounds are >95 % pure based on HPLC. Anhydrous solvents were purchased from commercial suppliers.

General synthetic procedure for compounds 70, 74-79, 82-84, and 89

To a solution of **69** in CH_2Cl_2, an appropriate carboxylic acid (5 equiv mol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (5 equiv mol) and 4-(dimethylamino)pyridine (1 equiv mol) were added and stirred overnight. The reaction mixture was then applied directly to preparative TLC (hexane-EtOAc) without work-up.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) diacetate (**70**). Yield 85%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.69 (s, 2H), 5.97 (s, 2H), 5.95 (s, 2H), 4.85 (s, 4H), 3.95 (s, 6H), 1.99 (s, 6H); HRMS calcd for $C_{22}H_{22}NaO_{10}$ $(M+Na)^+$ 469.1111, found 469.1116.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dibutyrate (**74**). Yield 97%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 1H), 5.96 (d, *J* = 1.4 Hz, 2H), 5.95 (d, *J* = 1.4 Hz, 2H) 4.86 (s, 4H), 3.93 (s, 6H), 2.22 (t, *J* = 7.2, 4H), 1.59 (sex, *J* = 7.2 Hz, 4H), 0.90 (t, *J* = 7.2 Hz, 6H); HRMS calcd for $C_{26}H_{30}NaO_{10}$ $(M+ Na)^+$ 525.1737, found 525.1759.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(2-methylbutanoate) (**75**). Yield 83%; colorless oil ; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.97 (s, 2H), 5.95

(s, 2H), 4.91–4.82 (m, 4H), 3.93 (s, 6H), 2.32 (sex, *J* = 6 Hz, 2H), 1.67–1.56(m, 2H), 1.48–1.36 (m, 2H), 1.09 (d, *J* = 7.2 Hz, 3H), 1.07 (d, *J* = 7.2 Hz, 3H), 0.84 (dd, *J* = 7.4, 14.6 Hz, 6H); HRMS calcd for $C_{28}H_{34}NaO_{10}$ $(M + Na)^+$ 553.2050, found 553.2063.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(3-methylbutanoate) (**76**). Yield 91%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (s, 2H), 5.95 (s, 2H), 4.86 (s, 4H), 3.93 (s, 6H), 2.12 (d, *J* = 6.4 Hz, 4H), 2.03 (m, 2H), 0.90 (dd, *J* = 2.8, 6.6 Hz, 12H); HRMS calcd for $C_{28}H_{34}NaO_{10}$ $(M + Na)^+$ 553.2050, found 553.2063.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dipentanoate (**77**). Yield 99%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, *J* = 1.5 Hz, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.23 (t, *J* = 7.6 Hz, 4H), 1.54 (pent, *J* = 7.6 Hz, 4H), 1.29 (sex, *J* = 7.6 Hz, 4H), 0.88 (t, *J* = 7.6 Hz, 6H); HRMS calcd for $C_{28}H_{34}NaO_{10}$ $(M + Na)^+$ 553.2050, found 553.2067.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(2-methylpentanoate) (**78**). Yield 85%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.97 (s, 2H), 5.95 (s, 2H) 4.91–4.81 (m, 4H), 3.93 (s, 6H), 2.37 (dd, *J* = 7.2, 14 Hz, 2H), 1.64–1.13 (m, 8H), 1.09 (d, *J* = 7 Hz, 3H), 1.07 (d, *J* = 7 Hz, 3H), 0.86 (t, *J* = 6.8 Hz, 6H); HRMS calcd for $C_{30}H_{38}NaO_{10}$ $(M + Na)^+$ 609.2676, found 609.2688.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) didodecanoate (**79**). Yield 88%; colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.68 (s, 2H), 5.96 (d, *J* = 1.5 Hz, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.22 (t, *J*= Hz, 4H), 1.57–1.19 (m), 0.87 (t, *J* = 6.8 Hz, 6H); HRMS calcd for $C_{42}H_{62}NaO_{10}$ $(M + Na)^+$ 749.4247, found 749.4225.

(2*E*,2'*E*)-(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(but-2-enoate) (**82**). Yield 8%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.96–6.87 (m), 6.7 (s, 2H), 5.94 (s, 4H),

5.78 (d, *J*= 15.6 Hz, 2H), 4.94 (d, *J* = 12.4 Hz, 2H), 4.85 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H), 1.85 (d, *J* = 6.8 Hz, 6H); HRMS calcd for $C_{26}H_{26}NaO_{10}$ $(M + Na)^+$ 521.1424, found 521.1449.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(3-methylbut-2-enoate) (**83**). Yield 51%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.70 (s, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 5.93 (d, *J* = 1.5 Hz, 2H), 5.62 (s, 2H), 4.95 (d, *J* = 12.5 Hz, 2H), 4.82 (d, *J* = 12.5 Hz, 2H), 3.93 (s, 6H), 2.12 (s, 6H), 1.86 (s, 6H); HRMS calcd for $C_{28}H_{30}NaO_{10}$ $(M + Na)^+$ 549.1737, found 549.1761.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(hexa-2,4-dienoate) (**84**). Yield 55%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.19 (d, *J* = 9.8 Hz, 1H), 7.15 (d, *J* = 9.8 Hz, 1H), 6.70 (s, 2H), 6.18–6.08 (m, 4H), 5.93 (d, *J* = 1.5 Hz, 4H), 5.71 (s, 1H), 5.68 (s, 1H), 4.96 (d, *J* = 12.3 Hz, 4H), 4.87 (d, *J* = 12.3 Hz, 4H), 3.93 (s, 6H), 1.83 (d, *J*= 5.2 Hz, 6H); HRMS calcd for $C_{30}H_{30}NaO_{10}$ $(M + Na)^+$ 573.1737, found 573.1746.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(3,4-dimethoxybenzoate) (**89**). Yield 90%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.55 (dd, *J* = 2, 8.4 Hz, 2H), 7.43 (d, *J* = 2 Hz, 2H), 6.78 (d, *J* = 8.4 Hz, 2H), 6.76 (s, 2H), 5.91 (s, 2H), 5.81 (s, 2H), 5.14 (d, *J* = 12.4 Hz, 2H), 5.04 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H), 3.89 (s, 6H), 3.88 (s, 6H); HRMS calcd for $C_{36}H_{34}NaO_{14}$ $(M + Na)^+$ 713.1846, found 713.1839.

General procedure for compound 71-73, 80-81, 86-88, and 90-95

To a flask with anhydrous dichloromethane, compound **69** and triethylamine (5-10 equiv mol) were added first and then the appropriate acyl chloride (2.2 equiv mol) was added at 0 °C under nitrogen. The reaction mixture was warmed gradually to room temperature and stirred for 1 – 3 h. After the reaction was completed, water and saturated sodium carbonate solution were added and the reaction mixture was extracted with dichloromethane, dried over sodium sulfate, and concentrated. Further purification was done by combiflash (hexane-EtOAc gradient).

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dipropionate (**71**). Yield 99%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.95 (d, *J* = 6.5 Hz, 4H), 4.85 (s, 4H), 3.93 (s, 6H), 2.26 (q, *J* = 7.2 Hz, 4H), 1.08 (t, *J* = 7.6 Hz, 6H); HRMS calcd for $C_{24}H_{27}O_{10}$ $(M + H)^+$ 497.1424, found 497.1432.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(2-methylpropanoate) (**72**). Yield 83%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (dd, *J* = 9.2 Hz, 4H), 4.88 (d, *J* = 12.6 Hz, 2H), 4.84 (d, *J* = 12.6 Hz, 2H), 3.93 (s, 6H), 2.49 (sept, *J* = 7.2 Hz, 2H), 1.11 (t, *J* = 7.2 Hz, 12H); HRMS calcd for $C_{26}H_{30}NaO_{10}$ $(M + Na)^+$ 525.1737, found 525.1754.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(2,2-dimethylpropanoate) (**73**). Yield 82%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, *J* = 13.8 Hz, 4H), 4.91 (d, *J* = 12.8 Hz, 2H), 4.82 (d, *J* = 12.8 Hz, 2H), 3.93 (s, 6H), 1.15 (s, 18H); HRMS calcd for $C_{28}H_{34}NaO_{10}$ $(M + Na)^+$ 553.2050, found 553.2054.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dicyclopentanecarboxylate (**80**). Yield 99 %; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.95 (d, *J* = 7.9 Hz, 4H), 4.88 (d, *J* = 12.6 Hz, 2H), 4.84 (d, *J* = 12.6 Hz, 2H), 3.93 (s, 6H), 2.71–2.65 (m, 2H), 1.88–1.52 (m, 16H); HRMS calcd for $C_{30}H_{34}NaO_{10}$ $(M + Na)^+$ 577.2050, found 577.2052.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dicyclohexanecarboxylate (**81**). Yield 99%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.67 (s, 2H), 5.95 (d, *J* = 7.6 Hz, 4H), 4.87 (d, *J* = 12.6 Hz, 2H), 4.83 (d, *J*= 12.6 Hz, 2H), 3.93 (s, 6H), 2.27–2.20 (m, 2H), 1.95–1.13 (m, 20H); HRMS calcd for $C_{32}H_{38}NaO_{10}$ $(M + Na)^+$ 605.2363, found 605.2387.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dibenzoate (**86**). Yield 59%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.94–7.92 (m, 4H), 7.52–7.47 (m, 2H), 7.38–

7.34 (m, 4H), 6.76 (s, 2H), 5.90 (s, 2H), 5.79 (s, 2H), 5.15 (d, *J* = 12.3 Hz, 2H), 5.06 (d, *J* = 12.3 Hz, 2H), 3.93 (s, 6H); HRMS calcd for $C_{32}H_{26}NaO_{10}$ $(M + Na)^+$ 593.1424, found 593.1452.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(4-methylbenzoate) (**87**). Yield 34%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.81 (d, *J* = 8.0 Hz, 4H), 7.15 (d, *J* = 8.0 Hz, 4H), 6.75 (s, 2H), 5.91 (s, 2H), 5.81 (s, 2H), 5.12 (d, *J* = 12.5 Hz, 2H), 5.04 (d, *J* = 12.5 Hz, 2H), 3.92 (s, 6H), 2.35 (s, 6H) ; HRMS calcd for $C_{34}H_{30}NaO_{10}$ $(M + Na)^+$ 621.1737, found 621.1761.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(4-methoxybenzoate) (**88**). Yield 30%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.90–7.86 (m, 4H), 6.85–6.81 (m, 4H), 6.75 (s, 2H), 5.91 (s, 2H), 5.82 (s, 2H), 5.12 (d, *J* = 12.3 Hz, 2H), 5.03 (d, *J* = 12.3 Hz, 2H), 3.93 (s, 6H), 3.80 (s, 6H); HRMS calcd for $C_{34}H_{30}NaO_{12}$ $(M + Na)^+$ 653.1635, found 653.1615.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(2,3,4-trimethoxybenzoate) (**90**). Yield 98%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.18 (s, 4H), 6.73 (s, 2H), 5.90 (s, 2H), 5.77 (s, 2H), 5.16 (d, *J* = 12.3 Hz, 2H), 5.04 (d, *J* = 12.3 Hz, 2H), 3.92 (s, 6H), 3.87 (s, 6H), 3.86 (s, 12H); HRMS calcd for $C_{38}H_{39}O_{16}$ $(M + H)^+$ 773.2058, found 773.2050.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(4-nitrobenzoate) (**91**). Yield 52%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 8.22–8.19 (m, 4H), 8.09–8.06 (m, 4H), 6.75 (s, 2H), 5.92 (s, 2H), 5.85 (s, 2H), 5.18 (d, *J* = 12.4 Hz, 2H), 5.12 (d, *J* = 12.4 Hz, 2H), 3.94 (s, 6H); HRMS calcd for $C_{32}H_{25}N_2O_{14}$ $(M + H)^+$ 683.1125, found 683.1119.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(4-cyanobenzoate) (**92**). Yield 38%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 8.02–7.99 (m, 4H),

7.69–7.66 (m, 4H), 6.73 (s, 2H), 5.91 (s, 2H), 5.81 (s, 2H), 5.16 (d, *J* = 12.4 Hz, 2H), 5.08 (d, *J* = 12.4 Hz, 2H), 3.94 (s, 6H); HRMS calcd for $C_{34}H_{24}N_2NaO_{10}$ $(M + Na)^+$ 643.1329, found 643.1314.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(benzo[*d*][1,3]dioxole-5-carboxylate) (**93**). Yield 85%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.53 (m, 2H), 7.34 (s, 2H), 6.77 (s, 2H), 6.75 (s, 2H), 5.99 (s, 4H), 5.93 (s,2H), 5.88 (s, 2H), 5.10 (d, *J* = 12.4 Hz, 2H), 5.02 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H); HRMS calcd for $C_{34}H_{26}NaO_{14}$ $(M + Na)^+$ 681.1220, found 681.1188.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis[6-(trifluoromethyl)nicotinate] (**94**). Yield 61%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 9.15 (m, 2H), 8.38 (m, 2H), 7.71 (m, 2H), 6.74 (s, 2H), 5.93 (dd, *J*= 1.6, 8.8 Hz, 4H), 5.21 (d, *J*= 7.6 Hz, 4H), 3.93 (s, 3H); HRMS calcd for $C_{32}H_{22}F_6N_2NaO_{10}$ $(M + Na)^+$ 731.1076, found 731.1076.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) bis(quinoline-2-carboxylate) (**95**). Yield 97%; orange needles; mp: 129–130 °C; ^{1}H NMR ($CDCl_3$) δ 8.25 (m, 2H), 8.16 (m, 2H), 7.98 (m, 2H), 7.77–7.70 (m, 4H), 7.59–7.55 (m, 2H), 6.86 (s, 2H), 5.88 (dd, *J*= 1.6, 19.6 Hz, 4H), 5.32 (d, *J*= 2.4 Hz, 4H), 3.93 (s, 6H); HRMS calcd for $C_{38}H_{29}N_2O_{10}$ $(M + H)^+$ 673.1822, found 673.1798.

4,4'-[(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene)]bis(oxy)bis(4-oxobutanoic acid) (85). To a solution of **69** (26 mg, 0.072 mmol) in anhydrous THF (2.0 mL), succinic anhydride (9 mg, 0.090 mmol) and DMAP (5 mg, 0.040 mmol) was added. The mixture was refluxed overnight. After cooling to rt, the solution was acidified with 1N HCl aq. and partitioned with EtOAc. The organic phase was concentrated. The residue was purified by preparative TLC (CH_2Cl_2:MeOH:TFA =95:5:0.25). Yield 99%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.67 (s, 2H), 5.96 (d, *J* = 16.8 Hz, 4H), 4.94 (d, *J* = 12.6 Hz, 2H), 4.89 (d, *J* = 12.6 Hz, 2H), 3.93 (s, 6H), 2.59–2.54 (m, 8H); HRMS calcd for $C_{26}H_{26}NaO_{14}$ $(M + Na)^+$ 585.1220, found 585.1228.

Dimethyl 3,3'-diiodo-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (**97**). Silver trifluoroactate (89 mg, 0.4 mmol) was added to a solution containing **1** (42.1 mg, 0.1 mmol) and $CHCl_3$ (1 mL). Then iodine (102.2 mg, 0.4 mmol) was poured into the solution, and the reaction mixture was stirred overnight at room temperature. Further isolation was done by preparative TLC (hexane-EtOAc: 7:3). Mono- and di-iodo products were found under these conditions. Yield 7%; colorless amorphous solid; ^{1}HNMR ($CDCl_3$) δ 6.01 (d, *J* = 1.6 Hz, 2H), 5.99 (d, *J* = 1.2 Hz, 2H), 4.05 (s, 6H), 3.68 (s, 6H); HRMS calcd for $C_{20}H_{16}I_2NaO_{10}$ $(M + Na)^+$ 692.8731, found 692.8704.

3,3′-Dibromo-4,4′-dimethoxy-5,6,5′,6′-bis(methylenedioxy)biphenyl-2,2′-dimethanol (**98**). To a stirred solution containing **1** (95.2 mg, 0.165 mmol) and 1 mL anhydrous CH_2Cl_2 under nitrogen at around -20 °C (ice with brine), Diisobutylaluminum hydride (DIBAL) (0.83 mL, 0.83 mmol) was added dropwise. After one h, another portion of DIBAL (0.4 mL, 0.4 mmol was added and stirring continued until starting material disappeared. The reaction was quenched with MeOH (3 mL), 10% Rochelle salt solution (3 mL) was added, and the mixture stirred for 30 min. Water was added to the mixture, which was then extracted with EtOAc three times, dried over sodium sulfate, and concentrated. The compound has low solubility in various solvents (EtOAc, CH_2Cl_2, MeOH, acetone). A small portion was taken for further purification for bioassay. The remaining portion was used in the next reaction without further purification. Colorless amorphous solid; ^{1}H NMR ($CDCl_3$) δ 5.96 (s, 4H), 4.67 (d, *J* = 12.4 Hz, 2H), 4.26 (d, *J* = 12 Hz, 2H), 4.09 (s, 6H), 3.19 (bs, 2H); HRMS calcd for $C_{18}H_{16}Br_2NaO_8$ $(M + Na)^+$ 542.9089, found 542.9091.

(3,3′-Dibromo-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dibutyrate (**99**). Compound **98** (16.8 mg, 0.033 mmol), butyric acid (0.02 mL, 0.21 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (31.5 mg, 0.16 mmol), 4-(dimethylamino)pyridine (4.1 mg, 0.033 mmol) were mixed together in CH_2Cl_2 overnight. The reaction mixture was subjected to preparative TLC to give the desired compound, which was recrystallized from CH_2Cl_2 -hexane. Yield 86%; colorless prisms; mp: 109–110 °C; ^{1}H NMR ($CDCl_3$)

δ 5.94 (d, *J* = 1.2 Hz, 2H), 5.92 (d, *J* = 1.6 Hz, 2H), 4.97 (d, *J* = 1.2 Hz, 2H), 4.89 (d, *J* = 1.2 Hz, 2H), 4.06 (s, 6H), 2.18 (t, *J* = 7.6 Hz, 6H), 1.57 (sex, *J* = 7.6 Hz, 4H), 0.88 (t, *J* = 7.2 Hz, 6H); HRMS calcd for $C_{28}H_{32}Br_2NaO_{10}$ $(M + Na)^+$ 682.9926, found 682.9916.

(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)bis(methylene) dibenzoate (**100**). The same procedure as for compound **99**, but benzoic acid was used instead of butyric acid. Yield 74%; colorless prisms; mp: 127–128 °C; ^{1}H NMR ($CDCl_3$) δ 7.92 (d, *J* = 7.6 Hz, 4H), 7.46–7.52 (m, 2H), 7.36 (t, *J* = 7.6 Hz, 4H), 5.88 (s, 2H), 5.70 (s, 2H), 5.31 (d, *J* = 11.6 Hz, 2H), 5.12 (d, *J* = 11.6 Hz, 2H), 4.07 (s, 3H); HRMS calcd for $C_{32}H_{24}Br_2NaO_{10}$ $(M + Na)^+$ 750.9613, found 750.9614.

3,3′-Dibromo-4,4′-dimethoxy-5,6,5′,6′-bis(methylenedioxy)biphenyl-2-hydroxymethyl-2′-carboxylic acid (**103**). To a stirred solution containing anhydrous THF (5 mL) and **102** (69.4 mg, 0.13 mmol) under nitrogen, sodium borohydride (15 mg, 0.39 mmol), followed by MeOH (0.1 mL), was added at room temperature. After the reaction was completed, 2 N HCl solution was added to acidify the solution to pH= 2. The solution was extracted with CH_2Cl_2 (with less than 10% MeOH), and the organic layer was dried over sodium sulfate. Flash chromatography (CH_2Cl_2—MeOH) was used to purify the desired compound. Yield 88%; amorphous solid; ^{1}H NMR (DMSO, 400 MHz) δ 13.2 (bs), 6.04 (d, *J* = 34 Hz, 2H), 5.97 (d, *J* = 40 Hz, 2H), 4.55 (bs), 4.28 (dd, *J* = 11.2, 25.8 Hz, 4H), 3.99 (s, 6H), 3.95 (s, 6H); HRMS calcd for $C_{18}H_{14}Br_2NaO_9$ $(M + Na)^+$ 582.9029, found 582.9038.

Cell Culture

A549 (lung carcinoma), DU-145 (prostate cancer), K562 (chronic myelogenous leukemia) and KB (epidermoid carcinoma) cell lines (ATCC) were obtained from Lineberger Cancer Center (UNC-CH). KBvin (vincristine-resistant KB subline) was generously provided by Professor Y.-C. Cheng, Yale University, CT. Cells were cultured in RPMI 1640 medium with 25 mM HEPES and 2 mM L-glutamine (Mediatech), supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 100 IU penicillin, 100 µg streptomycin, and 0.25 µg/mL amphotericin B (Mediatech). KBvin cells were

maintained in media containing 100 nM vincristine and were cultured for 7–10 days without vincristine before experiments were performed. Cells were maintained at 37 °C in a humidied 5% CO_2 atmosphere. The cells were sub-cultured every 3–4 days.

Cytotoxicity Analysis (SRB assay)

Cytotoxicity was determined by the sulforhodamine B (SRB) colorimetric assay. Cells ($3\text{-}5 \times 10^3$ cells/well) were seeded in 96-well plates filled with culture medium containing various concentrations of samples for 3 days. At the end of the exposure period, the supernatant was removed and fresh culture medium (100 µL) was added to each well. The cells were fixed with 50% trichloroacetic acid solution for 30 min, and 0.04% SRB (Sigma Chemical Co.) was added to each well. After 30 min incubation, the plates were washed, and dye was dissolved in 10 mM unbuffered Tris base and read at 515 nm on Microplate Reader ELx800 (Bio-Tek instruments, Winooski, VT) with a Gen5 software.

MDR Reversal Activity

For screening of chemosensitizing ability of test compounds, MDR and parental sensitive cells were incubated with test compound in the presence and absence of paclitaxel at a fixed concentration that did not affect the cell growth. Vincristine-resistant KB (KBvin) and parental KB cells were seeded at $5 - 7 \times 10^3$ cells/well into 96-well plates and incubated with 10 µM test compound with and without paclitaxel at 100 nM or 1 nM, respectively. After 3 days incubation, cell density was determined with a SRB assay. To assess the reversal activity of MDR by candidate compounds, a comparison was made of the IC_{50} values of anticancer drug (vincristine, paclitaxel, and doxorubicin) in the presence and absence of 10 µM of each test compound. IC_{50} values were calculated by log–linear interpolation of data points. Verapamil, a known P-gp inhibitor/modulator, was used as the positive control in all of the experiments. The reversal fold values, as potency parameter of test compounds, were calculated

as: Reversal fold = IC_{50} (anticancer drug alone) / IC_{50} (anticancer drug + test compound). All experiments were performed at least three times.

Measurement of the Intracellular Accumulation of calcein-AM and Doxorubicin (Dox)

KBvin cells (5×10^3 cell/well) were seeded in 96-well plates and pre-incubated for 72 h in a 5% CO_2 incubator at 37 °C. Then, the cells were washed with the culture medium and pretreated with samples or vehicle 1 h before calcein or doxorubicin was added at a final concentration of 10 μM. After 3 h incubation, the medium was removed by aspiration and the cells were washed with ice-cold PBS, and lysed with 1% sodium dodecyl sulfate (SDS) in PBS. Calcein or doxorubicin-associated mean fluorescence intensity were measured at Ex: 494 nm/Em: 517 nm or Ex: 488 nm/Em: 580 nm respectively with a fluorescence microplate reader (Plate Chameleon Multilabel Detection Platform, Hidex Oy, Turku, Finland) with MikroWin software. Verapamil was used as the positive control. All data were calculated as the ratio of doxorubicin fluorescence with test compound divided by doxorubicin fluorescence without test compound after subtraction of the fluorescence of the control.

3.6 References

1. Perez-Sayans, M.; Somoza-Martin, J. M.; Barros-Angueira, F.; Diz, P. G.; Rey, J. M.; Garcia-Garcia, A. Multidrug resistance in oral squamous cell carcinoma: The role of vacuolar ATPases. *Cancer Lett.* **2010,** *295*, 135-143.

2. Zhou, T.; Shi, Q.; Bastow, K. F.; Lee, K. H. Antitumor agents 286. Design, synthesis, and structure-activity relationships of 3'R,4'R-disubstituted-2',2'-dimethyldihydropyrano[2,3-f]chromone (DSP) analogues as potent chemosensitizers to overcome multidrug resistance. *J. Med. Chem.* **2010,** 53, 8700-8708.

3. Fodale, V.; Pierobon, M.; Liotta, L.; Petricoin, E. Mechanism of cell adaptation: when and how do cancer cells develop chemoresistance? *Cancer J.* **2011,** *17*, 89-95.

4. Singh, A.; Settleman, J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **2010,** *29*, 4741-4751.

5. Torres-Romero, D.; Munoz-Martinez, F.; Jimenez, I. A.; Castanys, S.; Gamarro, F.; Bazzocchi, I. L. Novel dihydro-[small beta]-agarofuran sesquiterpenes as potent modulators of human P-glycoprotein dependent multidrug resistance. *Org.Biomol.Chem.* **2009,** *7*, 5166-5172.

6. Das, S. G.; Doshi, J. M.; Tian, D.; Addo, S. N.; Srinivasan, B.; Hermanson, D. L.; Xing, C. Structure-activity relationship and molecular mechanisms of ethyl 2-amino-4-(2-ethoxy-2-oxoethyl)-6-phenyl-4h-chromene-3-carboxylate (sha 14-1) and its analogues. *J .Med. Chem.* **2009**, *52*, 5937-5949.

7. Mor, G.; Montagna, M. K.; Alvero, A. B. Modulation of apoptosis to reverse chemoresistance. *Methods Mol. Biol.* **2008**, *414*, 1-12.

8. Zhang, P. Y.; Wong, I. L.; Yan, C. S.; Zhang, X. Y.; Jiang, T.; Chow, L. M.; Wan, S. B. Design and syntheses of permethyl ningalin B analogues: potent multidrug resistance (MDR) reversal agents of cancer cells. *J. Med. Chem.* **2010**, *53*, 5108-5120.

9. Sun, M.; Xu, X.; Lu, Q.; Pan, Q.; Hu, X. Schisandrin B: A dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1. *Cancer Lett.* **2007**, *246*, 300-307.

10. Qiangrong, P.; Wang, T.; Lu, Q.; Hu, X. Schisandrin B--A novel inhibitor of P-glycoprotein. *Biochem. Biophys. Res. Commun.* **2005**, *335*, 406-411.

11. Li, L.; Pan, Q.; Sun, M.; Lu, Q.; Hu, X. Dibenzocyclooctadiene lignans -- a class of novel inhibitors of multidrug resistance-associated protein 1. *Life Sci.* **2007**, *80*, 741-748.

12. Sun, H.; Liu, G. T. Chemopreventive effect of dimethyl dicarboxylate biphenyl on malignant transformation of WB-F344 rat liver epithelial cells. *Acta Pharmacol. Sin.* **2005**, *26*, 1339-1344.

13. Jin, J.; Sun, H.; Wei, H.; Liu, G. The anti-hepatitis drug DDB chemosensitizes multidrug resistant cancer cells in vitro and in vivo by inhibiting P-gp and enhancing apoptosis. *Invest. New Drugs* **2007**, *25*, 95-105.

14. Zhu, B.; Liu, G. T.; Zhao, Y. M.; Wu, R. S.; Strada, S. J. Chemosensitizing multiple drug resistance of human carcinoma by Bicyclol involves attenuated p-glycoprotein, GST-P and Bcl-2. *Cancer Biol. Ther*. **2006**, *5*, 536-543.

15. Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q.; Urbatsch, I. L.; Chang, G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **2009**, *323*, 1718-1722.

16. Pajeva, I. K.; Wiese, M. Pharmacophore model of drugs involved in P-glycoprotein multidrug resistance: explanation of structural variety (hypothesis). *J .Med. Chem.* **2002**, *45*, 5671-586.

17. Xie, L.; Xie, J. X.; Kashiwada, Y.; Cosentino, L. M.; Liu, S. H.; Pai, R. B.; Cheng, Y. C.; Lee, K. H. Anti-AIDS (acquired immune deficiency syndrome) agents. 17. New brominated hexahydroxybiphenyl derivatives as potent anti-HIV agents. *J. Med .Chem.* **1995,** *38*, 3003-3008.

18. Molander, G. A.; George, K. M.; Monovich, L. G. Total synthesis of (+)-isoschizandrin utilizing a samarium(II) iodide-promoted 8-endo ketyl-olefin cyclization. *J. Org. Chem.* 2003, *68*, 9533-9540.

19. Soai, K.; Yokoyama, S.; Mochida, K. Reduction of symmetric and mixed anhydrides of carboxylic acids by sodium borohydride with dropwise addition of methanol. *Synthesis* 1987, 1987, 647-648.

20. Calcabrini, A.; Meschini, S.; Stringaro, A.; Cianfriglia, M.; Arancia, G.; Molinari, A. Detection of P-glycoprotein in the nuclear envelope of multidrug resistant cells. *Histochem. J.* 2000, *32*, 599-606.

Chapter 4

Novel Analogs of Dimethyl Dicarboxylate Biphenyl as Potent Cancer Chemopreventive Agents

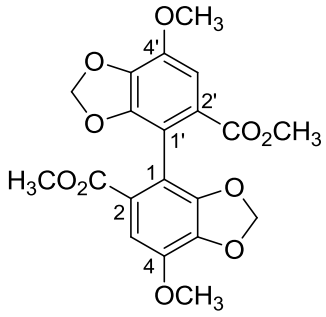
4.1 Introduction

Carcinogenesis is a complex process involving initiation, promotion, and progression steps. The promotion step is a long and reversible process and has been widely studied.[1] Inhibition of this step, which is known as cancer prevention, should be an effective approach to control cancer, and various phytochemicals, such as carotenoids, green tea polyphenols, curcumin, glycyrrhizin and its related compounds, from herbs and medicinal plants have been reported to exhibit cancer preventive ability.[2] However, despite its recognized effectiveness at blocking the long process of cancer development, relatively limited numbers of studies have been reported on cancer prevention. Therefore, more efforts aimed at the discovery and development of cancer preventive agents are needed. To evaluate cancer preventive ability, a short term *in vitro* assay can be applied for determining cancer preventive agents. Epstein-Barr virus (EBV) is known to be activated by tumor promoters to produce early antigens. Inhibition of Epstein Barr virus early antigen (EBV-EA) is used to evaluate antitumor promoting ability.[3]

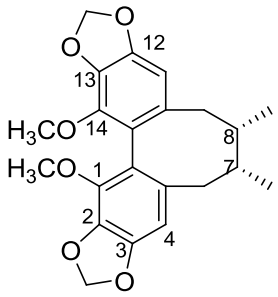
Dimethyl dicarboxylate biphenyl (DDB, **1**), a synthetic analog of schisandrin C (Figure 4.1) isolated from *Schizandrae chinensis*, is a hepatoprotective agent used to treat hepatitis B in China and to treat HBV and HCV in many Asian countries.[4, 5] In addition, DDB was shown to reverse multidrug resistant cancer cells, breast carcinoma MCF-7/Adr, KBv200, and Bel_{7402} *in vitro* and increase antitumor activity of vincristine to KBv200 xenografts *in vivo*.[5] Moreover, DDB also

prevented the oncogenic transformation of WB-F344 rat liver epithelial cells induced by 3-methylcholanthrene and 12-*O*-tetradecanoyl phorbal 13-acetate (TPA) at the doses of 1, 2, and 4 μmol/L. In a soft-agar colony formation assay, colony numbers were reduced in transformed cells treated with DDB. Furthermore, DDB could inhibit TPA-induced down-regulation of the gap junctional intercellular communication (GJIC).[4] These findings suggest that DDB has chemopreventive potential.

Figure 4.1. Structures of DDB and Schisandrin C



1: DDB



Schizandrin C

In our design of new DDB analogs, we found that known DDB analogs with different functional groups were previously synthesized and tested for cancer preventive ability.[6] In addition, while many 2,2′-carboxylate ester derivatives have been covered in various patents and papers, the modification of a 2,2′-bismethylene alcohol DDB intermediate (**69**) appears to be a new avenue of exploration. A prenylated side chain, which has been found to be effective in cancer chemoprevention studies of other compound classes, was a logical first choice.[7] In addition, short unsaturated fatty acid chains, which have resulted in good chemopreventive activity in betulinic acid derivatives, were included in our modification scheme.[8] Water-solubility is an important factor for drug discovery, because it is always associated with important pharmaceutical drug indices. Thus, a hydrophilic carboxylic acid moiety was incorporated into new DDB analogs by coupling the 2,2′-bismethylene alcohol DDB intermediate with succinic and glutaric anhydrides. Accordingly, in this study, we will

discuss the synthesis of new DDB analogs (Figure 4.2), structure-activity relationship findings, and EBV-EA inhibition ability. *In vivo* data of the most potent compounds are also described.

4.2 Results and Discussion

Chemistry.

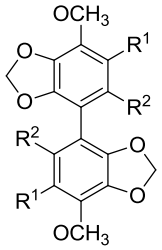
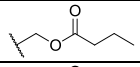
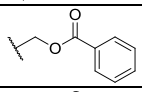
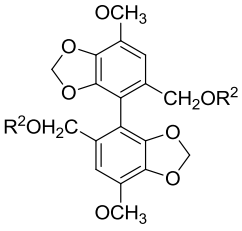
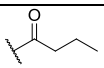
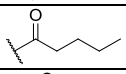
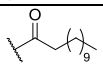
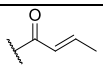
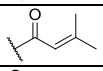
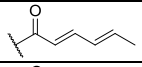
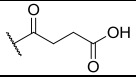
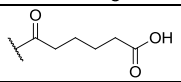
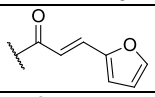
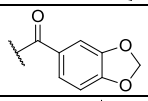
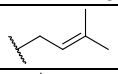
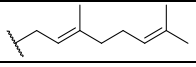
Compounds **101** were synthesized following literature methods.[6] Reduction of **1** and 3,3′-dibromo-DDB (**96**) with diisobutylaluminum hydride (DIBAL) resulted in the related 2,2′-methylene alcohols **69** and **98**, respectively. Diols **69** and **98** were then converted to various ester and ether analogs as shown in Figure 2. Esterifications of **69** and **98** were carried out in the presence of either excess carboxylic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), and 4-dimethylaminopyridine (DMAP) or acyl chloride and triethylamine at 0°C. A hydrophilic group was introduced by coupling with succinic anhydride or glutaric anhydride. Bisprenyl ether **106** and mono-geranyl ether **107** were obtained by Williamson ether synthesis of **69** with prenylbromide and geranylbromide, respectively, in the presence of sodium hydride. It should be noted that the bisgeranyl ether was not stable and decomposed easily after purification. Therefore, only the mono-geranyl ether **107** was obtained.

In vitro EBV-EA inhibition of DDB analogs.

All analogs were evaluated in a short-term *in vitro* EBV-EA inhibition assay to determine their cancer prevention potential, and the results are shown in Table 1. All tested compounds showed relatively potent inhibition of EBV activation. The analogs with unsaturated alkyl side chains and terminal carboxylic acids, such as **82-85**, **93**, **104-107**, significantly inhibited EBV-EA activation, showing 95.9–100.0% inhibition at the highest tested concentration, and showed greater inhibitory effects than the parent compound **1**. In particular, the most potent compound **106** displayed 100%

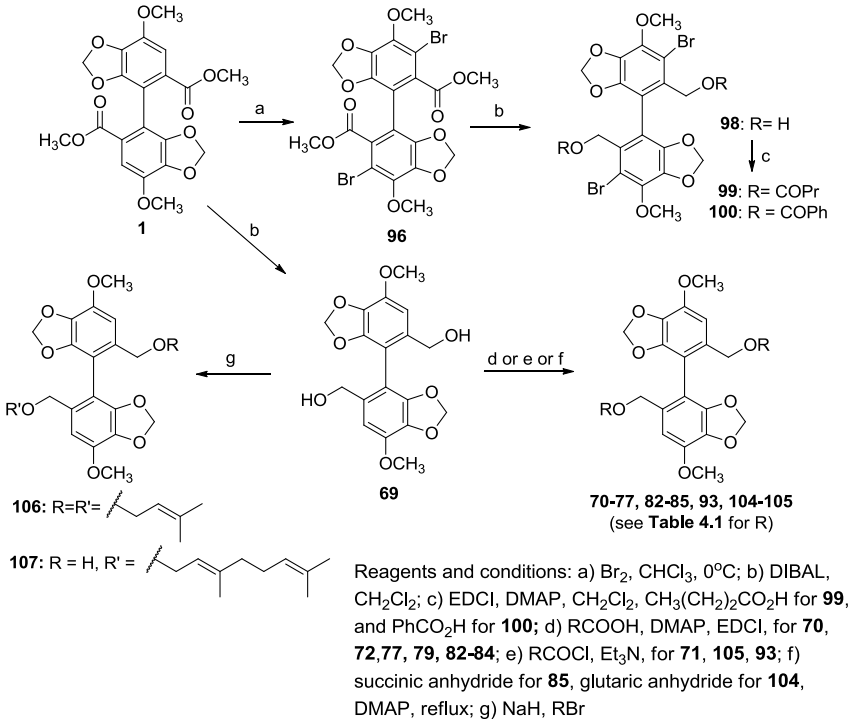
inhibition at 1×10^3 mol ratio/TPA, and 78.4%, 49.7% and 10.9% inhibition at 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively, with an IC_{50} value of 252 mol ratio/TPA. At the higher concentrations of 1×10^3 and 5×10^2 mol ratio/TPA, the inhibition values with **106** were comparable to those of curcumin, which is a known potent cancer preventive agent. Moreover, even at low concentrations, **106** inhibited EBV-EA activation and the inhibitory effects of **106** were notably greater than those of curcumin at 1×10^2 and 1×10 mol ratio/TPA. The analogs with prenyl-like unsaturated alkyl groups, such as **82-84**, **106**, and **107** exhibited relatively high activity. This finding is consistent with other reports that a prenyl-like group tends to enhance the inhibitory effect on EBA activation.[7] The presence of an aromatic ring on the C-2,2′ side chain, as found in **100**, **105**, and **93**, reduced the inhibitory effect on EBV-EA activation. The effect of bromide depended on the functional group at C-2 and -2′. With 2,2′-biscarbomethoxy substitution, the 3,3′-dibromo analog **96** showed lower potency than the parent compound **1**, while with 2,2′-bisbutyryloxymethyl substitution, the 3,3′-dibromo analog **99** showed higher potency than the related non-brominated compound **72**. The esters **70**–**72** and **77** with linear saturated fatty acids of varying lengths demonstrated almost equal potency, indicating that the length of the alkyl chain is not crucial for the activity; however, the activity decreased with a chain length of 12 carbons (analog **79**). Compounds **85** and **104** with terminal carboxylic acids on the 2,2′-ester groups exhibited better activity than the parent compound **1**. In a direct comparison, the succinate side chain (**85**) was better than glutarate side chain (**104**) in terms of potency.

Table 4.1. DDB Analogs and Their EBV-EA Inhibition Ability.

No.		R^1	R^2	Percentage of EBV-EA positive cells Concentration (mol ratio/TPA[b])				IC_{50}[d]
				1000	500	100	10	
1		H		6.3 (70)[c]	32.8	67.8	97.3	341
96		Br		14.6 (60)	40.2	76.0	100	403
98				8.4 (60)	36.0	71.2	100	358
101				7.9 (60)	35.8	68.2	98.6	349
99				7.9 (60)	35.8	68.2	98.6	349
100				11.5 (60)	38.6	75.5	100	389
70				13.1 (60)	37.0	71.3	100	390
71				12.0 (60)	35.9	70.0	100	381
72				11.2 (60)	37.0	72.0	100	380
77				10.3 (60)	37.4	71.7	100	379
79				15.6 (60)	41.0	76.9	100	426
82				3.1 (70)	27.0	54.9	93.4	278
83				1.7 (70)	23.5	51.6	90.3	260
84				2.9 (70)	25.1	53.7	91.7	269
85				2.1 (60)	24.8	52.6	91.5	265
104				4.1 (60)	28.6	57.4	96.6	287
105				9.5 (60)	36.0	71.0	100	372
93				8.9 (60)	35.7	71.5	100	369
106				0 (70)	21.6	50.3	89.1	252
107[a]				1.9 (70)	24.6	52.9	91.6	263
curcumin				0 (60)	21.1	80.1	100	379

Notes: [a] In this structure, only one R^2 is the moiety shown above; the other R^2 is a hydrogen group.
[b] TPA concentration is 32 pmol/mL.
[c] Values in parentheses are viability percentages of Raji cells.
[d] IC_{50} in mol/ratio TPA.

Figure 4.2. Syntheses of DDB analogs



Reagents and conditions: a) Br_2, $CHCl_3$, 0°C; b) DIBAL, CH_2Cl_2; c) EDCI, DMAP, CH_2Cl_2, $CH_3(CH_2)_2CO_2H$ for **99**, and $PhCO_2H$ for **100;** d) RCOOH, DMAP, EDCI, for **70**, **72**,**77, 79, 82-84**; e) RCOCl, Et_3N, for **71**, **105**, **93**; f) succinic anhydride for **85**, glutaric anhydride for **104**, DMAP, reflux; g) NaH, RBr

***In vivo* mouse skin carcinogenesis inhibition.**

The *in vitro* inhibitory effects determined in the EBV-EA assay generally have been found to correlate well with *in vivo* inhibitory effects on tumor promotion as reported in many studies.[9-13] Therefore, based on the *in vitro* data, only three of the most potent compounds (**83**, **85**, and **106**) were examined in a two-stage *in vivo* skin carcinogenesis test evaluating mouse skin papilloma induced by DMBA as an initiator and TPA as a promoter (Table 4.2). The compounds' activities were determined by both the percentage of papilloma-bearing mice (Figure 4.3A) and the average number of papillomas/mouse (Figure 4.3B), compared with the positive control. All three compounds delayed the appearance of the first tumor for two weeks compared with the positive control. In the positive control group, 6.6, 40, and 100% of the mice bore papillomas after 6, 8, and 11 weeks of promotion, respectively, and 6.3 papillomas were formed/mouse after 15 weeks. However, in the groups treated with compounds **83** and **106**, 0, 7, and 33% of the mice bore papillomas at weeks 6, 8, and 11,

respectively, and 4.4–5.1 papillomas/mouse were found with all three tested compounds, even after 15 weeks of promotion.

Table 4.2. *In vivo* inhibitory effects of **83**, **85**, and **106** on two-stage mouse carcinogenesis

Week	**Papilloma (%)**				**Papillomas/Mouse**			
	Positive control[a]	**83**[b]	**85**[b]	**106**[b]	Positive control[a]	**83**[b]	**85**[b]	**106**[b]
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	6.6	0	0	0	0.4	0	0	0
7	20.0	0	0	0	0.9	0	0	0
8	40.0	6.6	13.3	6.6	1.8	0.7	0.9	0.6
9	73.3	13.3	26.6	13.3	2.4	1.6	2.0	1.4
10	86.6	26.6	33.3	26.6	3.5	2.0	2.2	1.8
11	100	33.3	40.0	33.3	3.9	2.4	2.6	2.0
12	100	40.0	53.3	40.0	4.3	2.6	2.9	2.4
13	100	53.3	66.6	53.3	5.2	3.3	3.6	3.1
14	100	66.6	73.3	66.6	5.9	4.3	4.5	4.0
15	100	73.3	73.3	66.6	6.3	4.6	5.1	4.4

Note: [a] The positive control is DMBA (390 nmol) plus TPA (1.7 nmol) [b] The concentration of compound is 85 nmol.

Figure 4.3. Inhibitory effects of compounds 83, 85, and 106 on DMBA-TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly beginning 1 week after initiation. (A) Papilloma percentage in mice. (B) Average number of papillomas/mouse. (◆) Control TPA alone; (■) TPA + compound **83** (85 nmol); (×) TPA+ compound **85** (85 nmol); (▲) TPA + compound **106** (85 nmol). After 15 weeks of promotion, a significant difference in the number of papillomas/mouse between the treated groups and the control group was evident ($p < 0.05$). In Fig. 4.3A, the trace for compound **106** is superimposed with that for compound **83**.

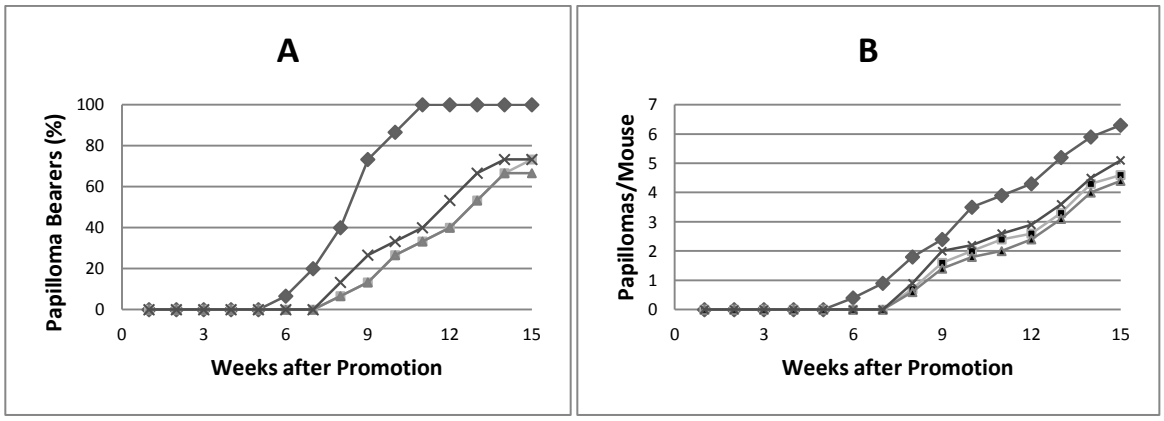
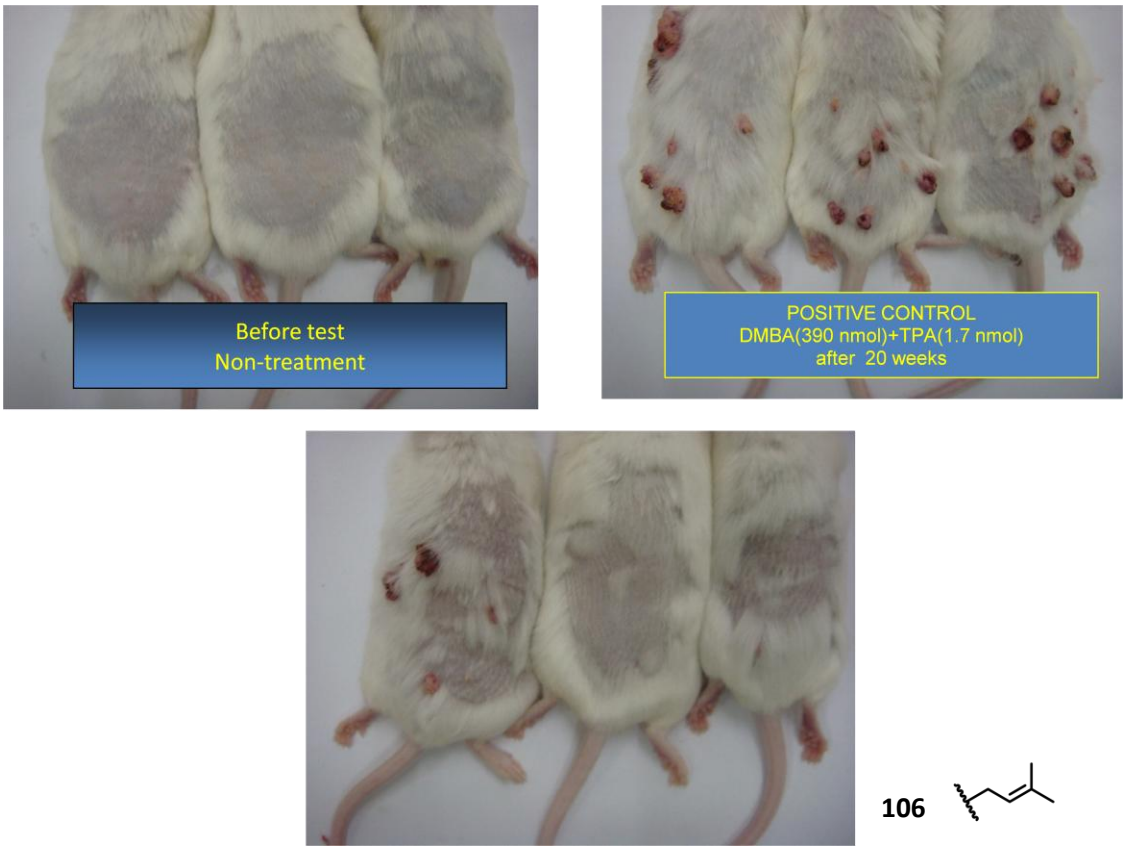


Figure 4.4. Pictures of Tested Rat of *In Vivo* Two Stage Mouse Skin Carcinogenesis Assay.



4.3 Conclusions

Several 2,2′-bismethyl ester and ether DDB analogs were designed and synthesized. All analogs showed potent EBV-EA inhibition *in vitro*. Among them, analogs **82**–**85**, **104**, **106**, and **107** with unsaturated side chains or terminal carboxylic acids significantly inhibited the EBV-EA activation. In particular, prenyl derivative **106** showed the highest inhibitory effects (100% , 78.4%, 49.7% and 10.9% inhibition at 1×10^3, 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively), which were greater than those of curcumin at the low concentrations. In an *in vivo* assay, DDB analogs **83**, **85**, and **106** also delayed the formation of mouse skin papillomas after initiation and promotion by a cancer promoting substance. DDB has been used clinically, which implies that DDB analogs have good probability to be further developed as potent cancer preventive agents for clinical use. Thus, DDB analog **106** could be a valuable candidate as a cancer preventive agent or as a lead for the development of new antitumor promoter drugs.

4.4 Material and Methods

Chemistry

^{1}H NMR (400 MHz) spectra were measured on a Varian Inova spectrometer with TMS as the internal standard. All chemical shifts are reported in ppm. Mass spectra were measured on a Shimazu LCMS-2010 (ESI-MS). All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60 F254 plate, 20 × 20, Merk). Melting points were recorded on a Fisher Johns melting apparatus without correction. Medium-pressure column chromatography was used in Biotage Flash and Isco companion systems with silica 40 μM columns from Grace Inc. All final compounds were > 95% purity based on HPLC. Anhydrous solvents were purchased from commercial suppliers.

General procedure for compounds **70**, **72**, **77**, **82**-**84**, and **99**-**100**

To a solution of **69** in dichloromethane, the appropriate carboxylic acid (5 eq. mole), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (5 eq. mole) and 4-(dimethylamino)pyridine (1 eq. mole) were added and stirred overnight. The reaction mixture was subjected to preparative TLC (hexane- ethyl acetate) without work-up.

Compound **99**: Yield, 86%; Colorless prisms; mp: 109-110°C; ^{1}H NMR ($CDCl_3$) δ 5.94 (d, *J* = 1.2 Hz, 2H), 5.92 (d, *J* = 1.6 Hz, 2H), 4.97 (d, *J* = 1.2 Hz, 2H), 4.89 (d, *J* = 1.2 Hz, 2H), 4.06 (s, 6H), 2.18 (t, *J* = 7.6 Hz, 6H), 1.57 (sext, *J* = 7.6 Hz, 4H), 0.88 (t, *J* = 7.2 Hz, 6H); ESI-MS *m/z*: 678 $[M+18(H_2O)]^+$.

Compound **100**: Yield, 75%; Colorless prisms; mp: 127-128°C; ^{1}H NMR ($CDCl_3$) δ 7.92 (d, *J* = 7.6 Hz, 4H), 7.46–7.52 (m, 2H), 7.36 (t, *J* = 7.6 Hz, 4H), 5.88 (s, 2H), 5.70 (s, 2H), 5.31 (d, *J* = 11.6 Hz, 2H), 5.12 (d, *J* = 11.6 Hz, 2H), 4.07 (s, 3H); ESI-MS *m/z*: 746 $[M+18(H_2O)]^+$.

Compound **70**: Yield, 85%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.69 (s, 2H), 5.97 (s, 2H), 5.95 (s, 2H), 4.85 (s, 4H), 3.95 (s, 6H), 1.99 (s, 6H); ESI-MS *m/z*: 464 $[M+18(H_2O)]^+$.

Compound **72**: Yield, 97%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 1H), 5.96 (d, *J* = 1.4 Hz, 2H), 5.95 (d, *J* = 1.4 Hz, 2H) 4.86 (s, 4H), 3.93 (s, 6H), 2.22 (t, *J* = 7.2 Hz, 4H), 1.59 (m, 4H), 0.90 (t, *J* = 7.2 Hz, 6H); ESI-MS *m/z*: 520 $[M+18(H_2O)]^+$.

Compound **77**: Yield, 99%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, *J* = 1.5 Hz, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.23 (t, *J* = 7.6 Hz, 4H), 1.54 (pent, *J* = 7.6 Hz, 4H), 1.34–1.25 (m, 4H), 0.88 (t, *J* = 7.6 Hz, 6H); ESI-MS *m/z*: 548 $[M+18(H_2O)]^+$.

Compound **79**: Yield, 88%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, *J* = 1.5 Hz, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.24–2.20 (m, 4H), 1.57–1.19 (m), 0.87 (t, *J* = 6.8 Hz, 6H); ESI-MS *m/z*: 744 $[M+18(H_2O)]^+$.

Compound **82**: Yield, 8%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.96–6.87 (m), 6.7 (s, 2H), 5.94 (s, 4H), 5.78 (d, *J*= 15.6 Hz, 2H), 4.94 (d, *J* = 12.4 Hz, 2H), 4.85 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H), 1.85 (d, *J* = 6.8 Hz, 6H); ESI-MS *m/z*: 544 $[M+46(HCOOH)]^+$.

Compound **83**: Yield, 51%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.70 (s, 2H), 5.94 (d, *J* = 1.5 Hz, 2H), 5.93 (d, *J* = 1.5 Hz, 2H), 5.62 (s, 2H), 4.95 (d, *J* = 12.5 Hz, 2H), 4.82 (d, *J* = 12.5 Hz, 2H), 3.93 (s, 6H), 2.12 (s, 6H), 1.86 (s, 6H); ESI-MS *m/z*: 568 $[M+42(CH_3CN+H)]^+$.

Compound **84**: Yield, 55%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.19 (d, *J* = 9.8 Hz, 1H), 7.15 (d, *J* = 9.8 Hz, 1H), 6.70 (s, 2H), 6.18–6.08 (m, 4H), 5.93 (d, *J* = 1.5 Hz, 4H), 5.71 (s, 1H), 5.68 (s, 1H), 4.96 (d, *J* = 12.3 Hz, 4H), 4.87 (d, *J* = 12.3 Hz, 4H), 3.93 (s, 6H), 1.83 (d, *J*= 5.2 Hz, 6H); ESI-MS *m/z*: 516 $[M-34]^+$.

General procedure for compounds **71**, **105**, and **93**

Compound **69** and triethylamine (5–10 eq. mole) were first added to anhydrous dichloromethane, and then the appropriate acyl chloride (2.2 eq. mole) was added at 0°C under nitrogen. The reaction was warmed gradually to room temperature and stirred for 1–3 h. After the reaction was completed, water and saturated sodium carbonate solution were added and the reaction mixture was extracted with dichloromethane, dried over sodium sulfate, and concentrated. Further purification was done by combiflash (hexane-ethyl acetate gradient).

Compound **71**: Yield, 99%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.95 (d, *J* = 6.5 Hz, 4H), 4.85 (s, 4H), 3.93 (s, 6H), 2.26 (q, *J* = 7.2 Hz, 4H), 1.08 (t, *J* = 7.6 Hz, 6H); ESI-MS *m/z*: 492 $[M+18(H_2O)]^+$. Compound **17**: Yield, 85%; Orange amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.43 (s, 2H), 7.34 (d, *J* = 15.6Hz, 2H), 6.73 (s, 2H), 6.58 (d, *J* = 1.2 Hz, 2H), 6.44 (m, 2H), 6.22 (d, *J* = 16 Hz, 2H), 5.95 (d, *J* = 2.4 Hz, 4H), 5.01 (d, *J* = 12.4 Hz, 2H), 4.95 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H); ESI-MS *m/z*: 620 $[M+18(H_2O)]^+$.

Compound **93**: Yield, 85%; Colorless amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.53 (m, 2H), 7.34 (s, 2H), 6.77 (s, 2H), 6.75 (s, 2H), 5.99 (s, 4H), 5.93 (s, 2H), 5.88 (s, 2H), 5.10 (d, *J* = 12.4 Hz, 2H), 5.02 (d, *J* = 12.4 Hz, 2H), 3.93 (s, 6H); ESI-MS *m/z*: 676 $[M+18(H_2O)]^+$.

General procedure for compounds **85** and **104**

Succinic anhydride (for **85**) or glutaric anhydride (for **104**) (1.2 eq. mole) and DMAP (5% w/w) was added to a flask containing **69** in anhydrous tetrahydrofuran. The solution was refluxed under nitrogen overnight. A solution of 1N hydrochloric acid was added to acidify the reaction mixture and ethyl acetate was used three times successively for extraction of the aqueous layer. Preparative TLC (dichloromethane–methanol) was applied to purify the desired compound from the combined aqueous extracts.

Compound **85**: Yield, 28%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.67 (s, 2H), 5.96 (d, *J* = 16.8 Hz, 4H), 4.94 (d, *J* = 12.6 Hz, 2H), 4.89 (d, *J* = 12.6 Hz, 2H), 3.93 (s, 6H), 2.59–2.54 (m, 8H); ESI-MS *m/z*: 580 $[M+18(H_2O)]^+$.

Compound **104**: Yield, 7%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, *J* = 13.6 Hz, 4H), 4.90 (d, *J* = 12.5 Hz, 2H), 4.84 (d, *J* = 12.5 Hz, 2H), 2.38–2.31 (m, 8H), 1.93–1.84 (m, 4H); ESI-MS *m/z*: 608 $[M+18(H_2O)]^+$.

General procedure for compounds **106** and **107**

Compound **69** in anhydrous tetrahydrofuran was added slowly to a flask with sodium hydride (5 eq. mole) in anhydrous tetrahydrofuran under nitrogen at 0°C. After 10 min, 3,3-dimethylallyl bromide (3 eq. mole for **106**) or geranyl bromide (3 eq. mole for **107**) was added. When the starting material disappeared, water was added to quench the reaction. The aqueous solution was partitioned with ethyl acetate. The organic layer was washed with sodium bicarbonate solution and then dried over sodium sulfate. Desired compounds were purified by preparative TLC with a hexane-ethyl acetate system.

Compound **106**: Yield, 43%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.78 (s, 2H), 5.92 (s, 4H), 5.23 (m, 2H), 4.23 (d, *J* = 12.1 Hz, 2H), 4.16 (d, *J* = 12.1 Hz, 2H), 3.94 (s, 6H), 3.82 (d, *J* = 6.9 Hz, 4H), 1.70 (s, 6H), 1.59 (s, 6H); ESI-MS *m/z*: 521 $[M+23(Na)]^+$.

Compound **107**: Yield, 47%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.76 (s, 1H), 6.69 (s, 1H), 5.69–5.93(m, 4H), 5.11–5.05 (m, 2H), 4.39–4.11 (m, 4H), 3.94 (s, 6H), 3.84 (m, 2H), 2.10–1.95 (m, 4H), 1.67 (s, 3H), 1.58 (s, 3H), 1.57 (s, 3H); ESI-MS *m/z*: 481 $[M-17(OH)]^+$.

***In vitro* EBV-EA activation experiment**

Raji cells (10^6 cells/mL) (with EBV latent, non-producing cells) were incubated at 37°C for 48 h in RPMI 1640 medium with 10% fetal calf serum (FCS) with *n*-butyric acid (4 mmol), TPA (32 pmol), and test compounds at various concentrations (10, 100, 500, 1000 mol ratio/TPA). Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by an indirect immunofluorescence technique (anti-human EBV-EA antibody followed by IgG-FITC tag). In each assay, at least 500 cells were counted and the number of stained cells (positive cells) was recorded. Each assay was repeated three times for one test compound. The EBV-EA-inhibiting activity of the test compound was estimated on the basis of the percentage of the number of positive cells compared with that of the control without the test compound. The viability of the cells was assayed by the TrypanBlue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60%.[14]

***In vivo* two-stage mouse skin carcinogenesis test**

A total of 30 female ICR mice (6 weeks old, purchased from SLC Co. Ltd., Shizouka, Japan) were used. Two groups, with each group consisting of 15 animals, housed at five/cage, were painted with 390 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone, 0.1 mL/mouse, on a shaved region of skin on the back. After 1 week, the mice were treated topically with 1.7 nmol of TPA in acetone

(0.1 mol) twice a week for 20 weeks. One hour prior to TPA treatment, the animals in group I were treated with acetone (0.1 mL) alone, serving as a promotion-positive control. The animals in group II were treated with the test compound (85 nmol) in acetone (0.1 mL). The incidence of papilloma was observed weekly for 15 weeks. The differences in the occurrence of mouse skin papillomas between the control and treatment groups were analyzed by means of the Student's *t*-test after 15 weeks of promotion.

4.5 References

1. Itoigawa, M.; Ito, C.; Ju-ichi, M.; Nobukuni, T.; Ichiishi, E.; Tokuda, H.; Nishino, H.; Furukawa, H. Cancer chemopreventive activity of flavanones on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. *Cancer Lett.* **2002**, *176*, 25-29.

2. Nishino, H.; Tokuda, H.; Satomi, Y.; Masuda, M.; Onozuka, M.; Yamaguchi, S.; Takayasu, J.; Tsuruta, J.; Takemura, M.; Ii, T.; Ichiishi, E.; Kuchide, S.; Okuda, M.; Murakoshi, M. Cancer chemoprevention by phytochemicals and their related compounds. *Asian Pac. J. Cancer Prev.* **2000**, *1*, 49-55.

3. Ito, Y.; Yanase, S.; Fujita, J.; Harayama, T.; Takashima, M.; Imanaka, H. A short-term in vitro assay for promoter substances using human lymphoblastoid cells latently infected with Epstein-Barr virus. *Cancer Lett.* **1981**, *13*, 29-37.

4. Sun, H.; Liu, G. T. Chemopreventive effect of dimethyl dicarboxylate biphenyl on malignant transformation of WB-F344 rat liver epithelial cells. *Acta Pharmacol. Sin.* **2005**, *26*, 1339-1344.

5. Jin, J.; Sun, H.; Wei, H.; Liu, G. The anti-hepatitis drug DDB chemosensitizes multidrug resistant cancer cells in vitro and in vivo by inhibiting P-gp and enhancing apoptosis. *Invest. New Drugs* **2007**, *25*, 95-105.

6. Xie, L.; Xie, J. X.; Kashiwada, Y.; Cosentino, L. M.; Liu, S. H.; Pai, R. B.; Cheng, Y. C.; Lee, K. H. Anti-AIDS (acquired immune deficiency syndrome) agents. 17. New brominated hexahydroxybiphenyl derivatives as potent anti-HIV agents. *J. Med. Chem.* **1995**, *38*, 3003-3008.

7. Tatsuzaki, J.; Nakagawa-Goto, K.; Tokuda, H.; Lee, K. H. Cancer preventive agents 10. Prenylated dehydrozingerone analogs as potent chemopreventive agents. *J. Asian Nat. Prod. Res.* **2010**, *12*, 227-232.

8. Nakagawa-Goto, K.; Yamada, K.; Taniguchi, M.; Tokuda, H.; Lee, K. H. Cancer preventive agents 9. Betulinic acid derivatives as potent cancer chemopreventive agents. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3378-3381.

9. Konoshima, T.; Takasaki, M.; Tatsumoto, T.; Kozuka, M.; Kasai, R.; Tanaka, O.; Nie, R. L.; Tokuda, H.; Nishino, H.; Iwashima, A. Inhibitory effects of cucurbitane triterpenoids on Epstein-Barr virus activation and two-stage carcinogenesis of skin tumors. *Biol. Pharm. Bull.* **1994**, *17*, 668-671.

10. Ishida, J.; Kozuka, M.; Wang, H.; Konoshima, T.; Tokuda, H.; Okuda, M.; Yang Mou, X.; Nishino, H.; Sakurai, N.; Lee, K. H.; Nagai, M. Antitumor-promoting effects of cyclic diarylheptanoids on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. *Cancer Lett.* **2000**, *159*, 135-140.

11. Ishida, J.; Kozuka, M.; Tokuda, H.; Nishino, H.; Nagumo, S.; Lee, K. H.; Nagai, M. Chemopreventive potential of cyclic diarylheptanoids. *Bioorg. Med. Chem.* **2002**, *10*, 3361-3365.

12. Sakurai, N.; Kozuka, M.; Tokuda, H.; Nobukuni, Y.; Takayasu, J.; Nishino, H.; Kusano, A.; Kusano, G.; Nagai, M.; Sakurai, Y.; Lee, K. H. Antitumor agents 220. Antitumor-promoting effects of cimigenol and related compounds on Epstein-Barr virus activation and two-stage mouse skin carcinogenesis. *Bioorg. Med. Chem.* **2003**, *11*, 1137-1140.

13. Wang, X.; Nakagawa-Goto, K.; Kozuka, M.; Tokuda, H.; Nishino, H.; Lee, K.-H. Cancer preventive agents. Part 6: Chemopreventive potential of furanocoumarins and related compounds. *Pharm. Biol.* **2006**, *44*, 116-120.

14. Iwase, Y.; Takemura, Y.; Ju-ichi, M.; Ito, C.; Furukawa, H.; Kawaii, S.; Yano, M.; Mou, X. Y.; Takayasu, J.; Tokuda, H.; Nishino, H. Inhibitory effect of flavonoids from citrus plants on Epstein-Barr virus activation and two-stage carcinogenesis of skin tumors. *Cancer Lett.* **2000**, *154*, 101-105.

Chapter 5

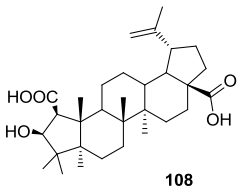
A-ring Modified Betulinic Acid Analogs as Novel Cancer Preventive, Anticancer and Anti-HIV Agents

5.1 Introduction

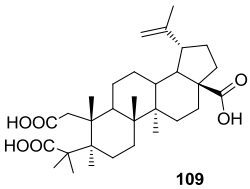
Triterpenoids are abundant in the plant kingdom, and many of them exhibit anti-HIV activity.[1, 2] A huge break-through occurred in this research field when studies showed that betulinic acid (BA) was potent against HIV in 1994, which led to the discovery and development of a new class of anti-HIV agents, maturation inhibitors (MI). Bevirimat, 3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid with EC_{50} < 0.35 nM and a selective index of 20,000, is now in phase II clinical trial as the first drug in the class of maturation inhibitors. [3, 4] MIs interfere with viral Gag precursor polyprotein and prevent conversion of p25 (CA-SP1) to functional p24 (CA), which leads to production of noninfectious immature HIV-1 particles. Importantly, MIs retain their high anti-HIV potency against different viral strains that are resistant to current antiviral therapies.[5]

Extensive studies have been reported on modification of BA, mainly on the C-3 and C-28 positions, to enhance anti-HIV activity (Figure 5.1). Analogs have included conformationally restricted 3-*O*-acyl BA,[6] 3-*O*-monosuccinyl BA,[6] and 3,28-disubstituted 28-piperidine BA derivatives.[7] In addition, a lupane-like triterpene with a five-membered ring A, 2β-carboxy-3β-hydroxyl-norlupA(1)-20(29)-en-28-oic acid (**108**), also showed anti-HIV activity with EC_{50} < 0.13 μM.[8] Moreover, a 2,3-seco-lupane triterpene, 16 β-hydroxy-2,3-seco-lup-20(29)-ene-2,3-dioic acid (**109**), exhibited significant anti-HIV protease activity, which suggested that a seco A-ring with two carboxylic acids might increase protease activity.[9] Meanwhile, lancifoic acid A (**110**), which

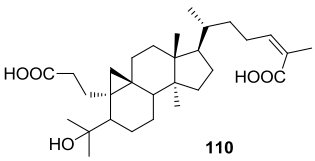
exhibited moderate anti-HIV activity (EC_{50} = 33.2 μM) contained a secocycloartane A ring.[10] Another anti-HIV (EC_{50} 5.1 μM) natural product, 3,4-seco-(24*Z*)-cycloart-4(28),24-diene-3,26-dioic acid 26-methyl ester (**111**), isolated from *Illicium verum,* also has a secoclycoartane A ring. [11]



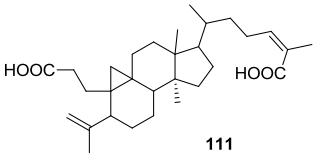
108



109

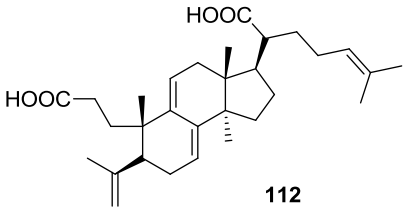


110

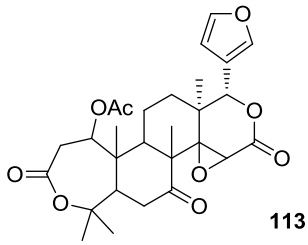


111

In addition to anti-HIV activity, a 3,4-seco lanostane triterpene (**112**) exhibited inhibitory effects in an EBV-EA activation assay in Raji cells.[12] Moreover, some limonoids with an A-ring lactone [ex. nomilin (**113**)] have shown various actions against neuroblastoma cancer cells (SH-SY5Y), including apoptosis induction, cancer cell cycle arrest, and aneuploidic effect.[13] 3,4-Seco ursolic acid derivatives were also obtained by first constructing and then opening a lactone ring. These derivatives induced cell cycle arrest and apoptosis in a human bladder cancer cell line (NTUB1).[14]



112



113

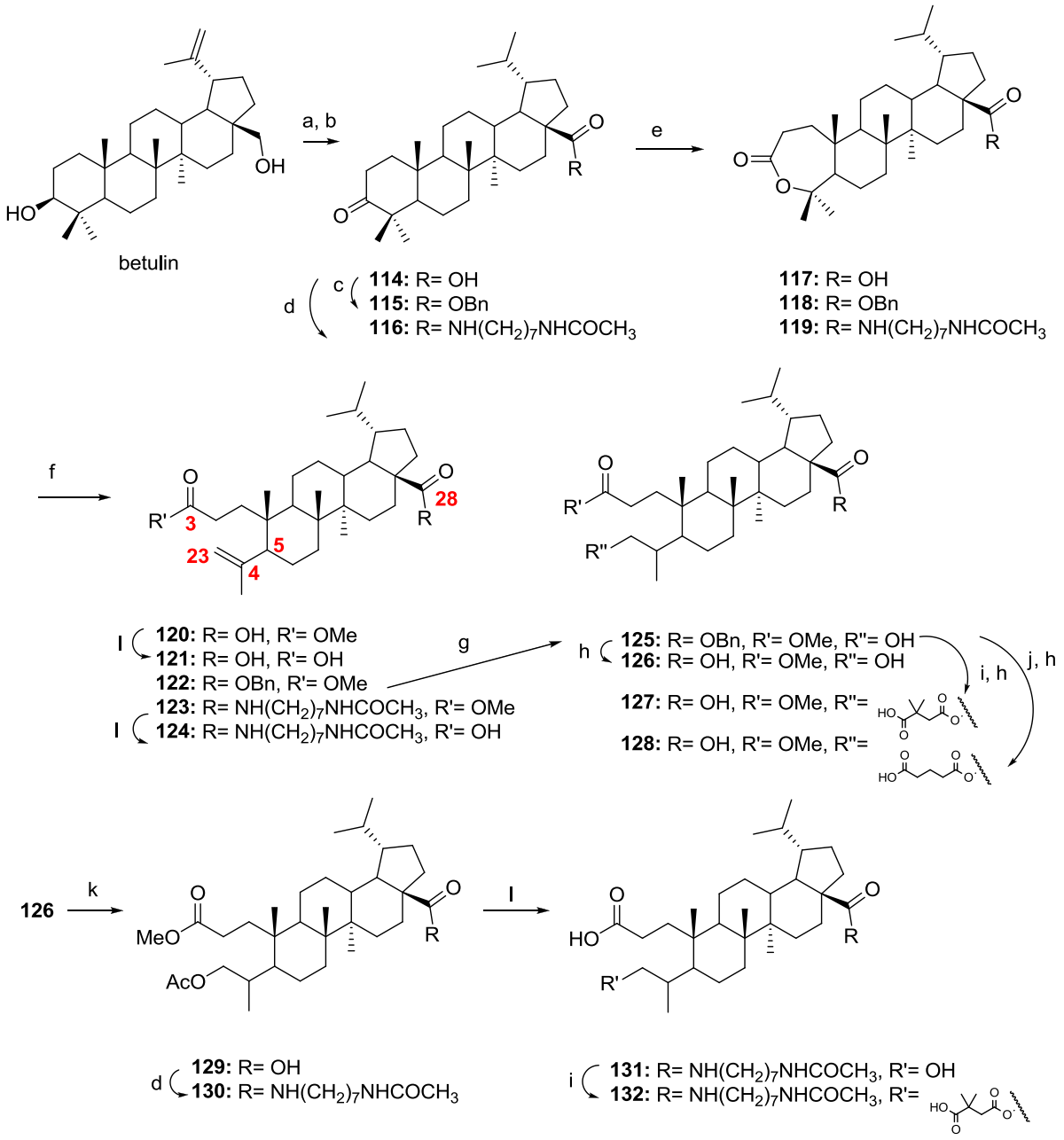
Based on these findings and studies, novel A-ring modified BA derivatives were designed, synthesized, and evaluated for anti-HIV, anticancer, and cancer preventive activities.

5.2 Design and Synthesis

3,4-Seco analogs, **120**–**132**, were designed and synthesized through oxepanone ring-A analogs, **117**–**119**, which could provide information on the effect of an expanded ring-A (Scheme 5.1). Based on previous study, an *N*-heptane acetamide side chain can enhance anti-HIV activity, either anti-maturation or anti-entry activity.[15] Therefore, this side chain was included in the analog design as shown in **123**, **124**, and **130**–**132**. A benzylic group was selected to serve as a protective group, as well as to assess the biological effect of aromatic substitution. Betulin, a commercial available

pentacyclic triterpene, was used as starting material. Jone's oxidation of betulin easily produced **114**, with a 3-ketone and 28-carboxylic acid. Compound **114** was converted separately to benzyl ester **115** and amide **116**, which has the same amide side chain found in **A43-D**, a prototypic HIV entry inhibitor. A Baeyer-Villiger reaction using 3-chloroperbenzoic acid (mCPBA) produced oxepanone ring A-analogs, **117**–**119**. Acid-catalytic lactone ring opening in methanol led to the related 4-methylene-3-methyl ester analogs **120**, **122**, and **123**, respectively. The prop-1-en-2-yl group generated at C-5 is similar to a prenyl group, which is critical in cancer prevention research.[16] From previous study, compounds **109**–**112** have an acetic or propanoic acid side chain, and they showed good protease inhibition. Therefore, the acids **121** and **124** were prepared by hydrolysis of the methyl ester groups in **120** and **123**. Hydroboration of the double bond in **122** gave a terminal hydroxy group in **125**. Esterification of this hydroxy group with 2,2-dimethylsuccinic or glutaric anhydride, followed by catalytic debenzylation, gave compounds **127** and **128**, respectively. From the studies leading to bevirimat, a dimethylsuccinate side chain can effectively increase anti-HIV activity. Glutaric acid was selected to compare the effect of chain length. Alternatively, catalytic debenzylation of **125** gave **126**, which was esterified using acetic anhydride to provide acetate **129**. Amidation of the free C-28 carboxylic acid gave the amide **130**. The acetyl group served as a protective group, as well as provided a polarity comparison with the corresponding hydroxy group, after the methyl ester and acetate on **130** were hydrolyzed to obtain **131**. The treatment of **131** with 2,2-dimethylsuccinic anhydride produced **132**.

Scheme 5.1 Synthetic Scheme of Dihydro-BA A-Ring Modified Derivatives.

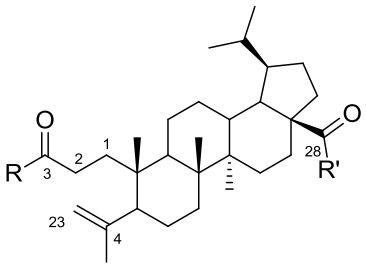


Reagents and Conditions: (a) Jone's oxidation, (b) Pd/C, H_2, AcOH, DCM-MeOH (c) BnBr, K_2CO_3, THF, 55 ^oC, (d) 1) $(COCl)_2$, $NH_2(CH_2)_7NH_2$, CH_2Cl_2, 2) Ac_2O, DMAP, pyridine, r.t., (e) mCPBA, CH_2Cl_2, (f) H_2SO_4, MeOH (g) 1) $BH_3{\cdot}SMe_2$, 2) NaOH, H_2O_2, (h) Pd/C, H_2, DCM-MeOH, (i) 2,2-dimethylsuccinic anhydride, DMAP, pyridine, 160^oC, (j) glutaric anhydride, DMAP, pyridine, (k) Ac_2O, DMAP, pyridine, r.t., (l) 4N NaOH/THF and MeOH

5.3 Results and Discussion

5.3.1 Results of Cytotoxicity Activity

Compounds **119**–**121**, **123**, **124**, **126**, **127**, and **129**–**132** were evaluated for cytotoxicity against four different human cancer cell lines, A549 (lung carcinoma), DU-145 (prostate cancer), KB (nasopharyngeal carcinoma), and KBvin (KB multidrug resistant subline). Compounds **120**, **123**, **126**, and **129** exhibited cytotoxicity (IC_{50}< 10 μM) against at least three four cell lines, (Table 5.1). Analog **120** with a 3-methyl ester, 4,23-vinyl group, and 28-carboxylic acid was the most potent. Compound **121** with a 3-carboxylic acid group rather than methyl ester was inactive, indicating that a more hydrophobic group may be preferred at this position. From the data for **120** and **123**, addition of a 28-*N*-heptane acetamide group in the latter compound decreased the activity. Comparison of **120**, **126**, and **129** resulted in the general rank order of potency, **120** ($C=CH_2$) > **129** ($CH-CH_2OAc$) > **126** ($CH-CH_2OH$). Interestingly, especially **126** was more cytotoxic against the multidrug resistant strain, KBvin (IC_{50} = 4.8 μM) than its parent strain KB (IC_{50} = 8.03 μM), suggesting a potential chemosensitizing activity. Further evaluation of this possible effect is ongoing. A brief schematic summary of the structure/activity results is shown below.



3-position	4-position	28-position
OMe >> OH	$=CH_2$ > CH_2OH, CH_2OAc	OH > $NH(CH_2)_7NHCOCH_3$

Table 5.1. Cytotoxicity of BA A-ring Opened Derivatives

	IC_{50} (μM)			
Compd	A549	DU-145	KB	KBvin
119	>10	>10	>10	9.01 ± 0.3
120	5.59 ± 0.3	4.46 ± 0.3	5.08 ± 0.3	4.55 ± 0.4
121	>10	>10	>10	>10
123	>10	6.35 ± 0.2	8.79 ± 0.06	9.48 ± 0.8
124	>10	>10	>10	>10
126	6.78 ± 0.5	7.16 ± 0.7	8.03 ± 0.5	4.86 ± 0.4
127	9.68 ± 0.5	>10	>10	>10
129	6.18 ± 0.03	5.29 ± 0.5	7.01 ± 0.5	5.02 ± 0.3
130	>10	8.38 ± 0.4	>10	>10
131	>10	>10	>10	>10
132	>10	>10	>10	>10

5.3.2 Results for Cancer Preventive Activity

Compounds **119**–**121**, **123**, **124**, **126**, **127**, **129**–**132** were evaluated for *in vitro* EBV-EA inhibition, and the results are shown in Table 5.2. From the results, an-*N*-heptane acetamide group reduced the activity compared with a carboxylic acid (**123** vs **120**, **124** vs **121**, **130** vs **129**) at C-28. The four most potent analogs (**120**, **121**, **126**, and **129**) all contained a C-28 carboxylic acid, and the most active compound **121**, with a 3-carboxylic acid, 4,23-vinyl group, and 28-carboxylic acid, displayed 100%, 91.6%, 65.1%, and 26.6% inhibition at 1×10^3 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively, with an IC_{50} value of 295 mol ratio/TPA. Even at the low concentration (1×10 mol ratio/TPA), **121** exhibited more inhibitory activity than curcurmin, a known cancer preventive agent. This result may because the prop-1-en-2-yl group at C-5 is similar to a prenyl group, which is

an effective modification in cancer prevention research. Compounds **120** and **121** with a carboxylic acid and methyl ester, respectively, at C-3showed similar potency. Regarding the C-4 position,comparison of **120**, **126**, and **129** resulted in the general rank order of potency, **120** ($C=CH_2$) > **126** ($CH\text{-}CH_2OH$) > **129** ($CH\text{-}CH_2OAc$). Overall, the main difference between the SAR for cancer prevention and anticancer activity was that a carboxylic acid was better than a methyl ester at C-3 for the former effect. Further *in vivo* study is ongoing.

Table 5.2 EBV-EA Inhibition Ability of Newly Synthesized Betulinic Acid Derivatives.

	Percentage EBV-EA positive cells Compound concentration (mol ratio/TPA[a])				
compd	1000	500	100	10	IC_{50}[c]
119	15.3±0.4 (60)[b]	61.4±1.4	83.2±2.1	100±0.3	523
120	0.0±0.5 (70)	29.1±1.5	66.9±2.5	94.2±0.4	311
121	0.0±0.4 (70)	26.6±1.5	65.1±2.4	91.6±0.5	295
123	7.1±0.6 (60)	54.4±1.6	77.2±2.5	100±0.3	479
124	6.7±0.5 (60)	50.2±1.5	76.3±2.4	100±0.2	460
126	0.0±0.4 (70)	31.6±1.3	68.1±2.3	96.3±0.4	323
127	3.7±0.5 (70)	36.4±1.6	71.3±2.5	100±0.4	386
129	0.0±0.5 (70)	33.8±1.5	68.8±2.3	98.2±0.5	335
130	7.8±0.6 (60)	56.0±1.6	79.4±2.3	100±0.4	490
131	6.6±0.5 (60)	52.1±1.4	77.5±2.5	100±0.4	471
132	11.3±0.4 (60)	57.2±1.6	81.3±2.3	100±0.2	501
BA	7.9±0.4 (60)	37.2±1.2	75.1±2.3	100±0.6	403
Bevirimat	6.1±0.3 (60)	36.1±1.3	74.2±2.2	96.5±0.9	396
curcurmin	0.0±0.2 (60)	22.8±1.2	81.7±2.5	100±0.5	341

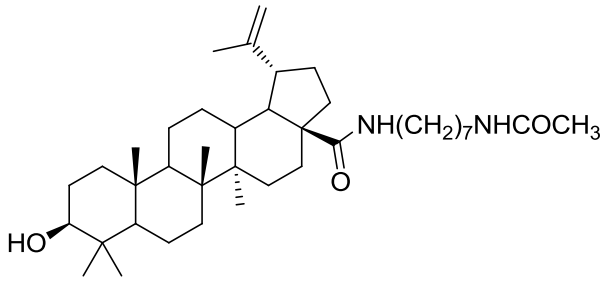
[a] TPA concentration is 32 pmol/mL.
[b] Values in parentheses are viability percentages of Raji cells.
[c] IC_{50} in mol/ratio TPA

5.3.3 Results of Anti-HIV Activity

All of the compounds were assayed for anti-HIV replication activity. All compounds, except for **127**, **128**, **131**, and **132**, exhibited some cytotoxicity against MT-4 cells at 4 μg/ml, and thus, cytotoxic and anti-HIV activities could not be differentiated. Only the oxepanone ring A-analog **119** retained some anti-HIV activity when the concentration was reduced below 4 μg/ml. However, it was less potent than bevirimat or A43-D, which has the same C-28 side chain as **119**. Further mechanistic study is ongoing, and the anti-viral activity against a DSB-resistant strain will also be tested.

Although several literature articles have indicated that 3,4-seco compounds exhibited HIV protease inhibition, they did not report inhibition of viral replication data, implying that these compounds are unlikely to inhibit viral replication. Accordingly, while our newly synthesized compounds may have protease inhibition activity (not tested), they cannot inhibit viral replication, which diminishes their value for further protease inhibition study.

Figure 5.1. Structure of A43-D.



5.4 Conclusions

In this study, 13 new BA derivatives were designed, synthesized, and evaluated for anticancer, cancer prevention, or anti-HIV activity. For cytotoxicity activity, compounds **120**, **123**, **126**, and **129** exhibited GI_{50} < 10 μM. Compound **120** with a 3-methyl ester, 4,23-vinyl group, and 28-carboxylic acid was the most potent. SAR study suggested a methyl ester at C-3, hydroxyl or acetate at C-23 would reduce the activity compared with a double bond between C-4 and C-23, and a carboxylic acid was more effective than an *N*-heptane acetamide at C-28. Notably, compound **126** was more toxic to MDR KBvin than the parental KB cell line, indicating a potential chemosensitizing activity. Further chemosensitizing assays are in progress. In the cancer prevention assay, the SAR trends were similar to those found with anticancer activity, except that a free carboxylic acid was preferred at C-3. For anti-HIV activity, only compound **119** with an ε-lactone A ring and 28-*N* heptane acetamide showed weak activity, and its mechanism of action is under study.

5.5 Materials and Methods

Chemistry

General. ^{1}H NMR (400 MHz) spectra were measured on a Varian Inova spectrometer with TMS as the internal standard. Mass spectra were measured on a Shimazu LCMS-IT-TOF. All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60 F254 plate, 20 × 20, Merk). Melting points were recorded on a Fisher Johns melting apparatus without correction. Medium-pressure column chromatography was used in Grace Companion systems with silica 40 μM columns from Grace Inc. All final compounds are >95% pure, based on HPLC. Anhydrous solvents were purchased from commercial suppliers.

General Procedure for Compounds 116 and 130

A mixture of **114** or **129** and oxalyl chloride (10 mol eq.) was stirred at 0 ^oC for 30 min and then dried under vacuum. The residue was redissolved in anhydrous CH_2Cl_2 and added dropwise to a solution containing 1,7-diaminoheptane (3 mol eq.) in anhydrous CH_2Cl_2 at r.t..After stirring overnight, the mixture was washed with water and the organic layer was concentrated. The crude product was purified by flash chromatography on a silica gel column eluted with CH_2Cl_2/MeOH. The amine intermediate in anhydrous pyridine was added to acetic anhydride (2 mol eq.) and DMAP (catalytic amount) and stirred overnight at room temperature. The reaction mixture was added CH_2Cl_2 and washed with 1N HCl and Sat. $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4, concentrated and purified by flash chromatography on a silica gel column eluted with CH_2Cl_2/MeOH to yield the corresponding product.

Compound 116. Yield 55%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 5.59-5.62 (2H, m, 2 × NH), 3.05-3.65 (4H, m, 2 × NH*CH*$_2$), 1.97 (3H, s, -NHCO*CH*$_3$), 1.07, 1.02, 0.96, 0.95, 0.93 (each 3H, s, 4 × CH_3), 0.85, 0.74 (each 3H, d, *J* = 6.8 Hz, H-29, 30); ESI-MS *m*/*z*: 611 $[M+H]^+$

Compound 130. Yield 46%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 5.68-5.62 (2H, m, 2 × NH), 4.17 (1H, dd, *J* = 3.2, 10.8 Hz, H-23), 3.69 (1H, t-like, H-23), 3.67 (3H, s, OMe), 3.29-3.12 (4H, m, 2 × NH*CH*$_2$), 2.03 (3H, s, -OCO*CH*$_3$), 1.97 (3H, s, -NHCO*CH*$_3$), 0.99 (3H, d, *J* = 6.8 Hz, CH_3), 0.93 (6H, s, 2 × CH_3), 0.86-0.84 (6H, 2 × CH_3), 0.74 (3H, d, *J* = 6.8 Hz, CH_3); ESI-MS *m*/*z*: 702 $[M+H]^+$

General Procedure for Compounds 117, 118 and 119

3-Chloroperoxybenzoic acid (77%, 5-10 mol eq.) was added to the flask containing compounds **114**, **115**, or **116** in $CHCl_3$ and the mixture was stirred overnight at room temperature. $NaHCO_3$ (aq.) solution was added to stop the reaction. The solution was partitioned three times with CH_2Cl_2, dried over Na_2SO_4, and concentrated. The crude product was purified by flash chromatography on a silica gel column eluted with hexane/EtOAc.

Compound 118. Yield 42%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.36-7.30 (5H, m. aromatic H), 5.13, 5.07 (2H, d, *J* = 12.4 Hz, benzylic H), 2.58-2.64 (1H, m, H-2), 2.45-2.51 (1H, m, H-2), 1.46, 1.38 (each 3H, s, H-23, 24), 1.04, 0.91. 0.78 (each 3H, s, 3 × CH_3), 0.84, 0.74 (each 3H, d, *J* = 6.8 Hz, H-29, 30); ESI-MS *m*/*z*: 563 $[M+H]^+$

Compound 119. Yield 52%; colorless amorphous ; ^{1}H NMR ($CDCl_3$) δ 5.58 (1H, t-like, -NH), 5.52 (1H, bs, -NH), 3.33-3.13 (4H, m, 2 × NH*CH*$_2$), 1.97 (3H, s, -NHCO*CH*$_3$), 1.46, 1.39 (each 3H, s, H-23,24), 1.06, 0.98, 0.94 (each 3H, s, 3 × CH_3), 0.85, 0.74 (each 3H, d, *J* = 6.8 Hz, H-29, 30); ESI-MS *m*/*z*: 627 $[M+H]^+$

General Procedure for Compounds 120, 122 and 123

To a solution containing **117, 118,** or **119** in methanol, several drop of conc. H_2SO_4 was added. The solution was stirred overnight and only one spot was obtained. Methanol was evaporated and the crude product was purified by flash chromatography on a silica gel column eluted with hexane/EtOAc

Compound 120. Yield 55%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 4.84, 4.64 (each 1H, s, H-23), 3.65 (3H, s, OMe), 1.71 (3H, s, H-24), 0.97 (6H, s, 2 × CH_3), 0.85 (3H, d, *J* = 6.8 Hz, CH_3), 0.83 (3H, s, CH_3), 0.76 (3H, d, *J* = 6.8 Hz, CH_3); ESI-MS *m*/*z*: 485 $[M-H]^-$

Compound 122. Yield 80%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 7.36-7.32 (5H, m, $CH_2C_6H_5$), 4.84 (1H, s, H-23), 4.63 (1H, s, H-23), 3.65 (3H, s, OMe), 1.71 (3H, s, H-24), 0.93 (3H, s, CH_3), 0.84 (3H, d, *J* = 6.8 Hz, CH_3), 0.80, 0.78 (each 3H, s, 2 × CH_3), 0.74 (3H, d, *J* = 6.8 Hz, CH_3)

Compound 123. Yield 62%; colorless amorphous ; ^{1}H NMR ($CDCl_3$) δ 5.58 (1H, t-like, -NH), 5.52 (1H, bs, -NH), 4.84 (1H, s, H-23), 4.64 (1H, s, H-23), 3.65 (3H, s, -OMe), 3.29-3.13 (4H, m, 2 × NHCH_2), 1.97 (3H, s, -NHCOCH_3), 1.72 (3H, s, H-24), 0.97, 0.96, 0.83 (each 3H, s, 3 × CH_3), 0.85, 0.74 (each 3H, d, *J* = 6.8 Hz, H-29, 30); ESI-MS *m*/*z*: 641 $[M+H]^+$

Synthesis of Compound 125

To a solution of **120** (1.4 g, 2.4 mmol) in anhydrous THF (20 ml) at 0 ^oC under argon, 2M of BH_3SMe_2 solution was added (6 ml, 12 mmol). After starting material disappeared, NaOH solution [1200 mg in EtOH/H_2O (65:35)] was added at 0 ^oC, and then H_2O_2 solution was added (30%wt, 1.5 ml). After one hour, water was added, the mixture was extracted three times with EtOAc, and the organic layer was dried over Na_2SO_4. The crude product was purified by Grace flash chromatography using silica gel column eluted with hexane/EtOAc.

Compound 125. Yield 61%; colorless amorphous ; ^{1}H NMR ($CDCl_3$) δ 3.76 (1H, dd, *J* = 3.2, 10.4 Hz, H-23), 3.66 (3H, s, OMe), 3.26 (1H, t-like, H-23), 1.02 (3H, d, *J* = 6.8 Hz, CH_3), 0.91 (3H, s, CH_3), 0.84 (3H, d, *J* = 6.8 Hz, CH_3), 0.74-0.73 (6H, 2 × CH_3); ESI-MS *m*/*z*: 595 $[M+H]^+$

General Procedure for De-protection of Benzylic Group

Compound **125** was dissolved in CH_2Cl_2/MeOH solution (minimal amount of CH_2Cl_2) and excess Pd/C was added. Pressurized hygrogen was applied for one hour. Pd/C was filtered and solvent was

evaporated. The crude product was purified by Grace flash chromatography using silica gel column eluted with hexane/EtOAc.

Compound 126. Yield 72%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 3.76 (1H, dd, *J* = 2.4, 10 Hz, H-23), 3.67 (3H, s, OMe), 3.25 (1H, t, *J* = 9.6 Hz, H-23), 1.03 (3H, d, *J* = 6.8 Hz, CH_3), 0.94, 0.92 (each 3H, s, 2 × CH_3), 0.86-0.84 (6H, 2 × CH_3), 0.75 (3H, d, *J* = 6.8 Hz, CH_3); ESI-MS *m*/*z*: 503 $[M-H]^-$

General Procedure for Compounds 127, 128 and 132

A mixture of **125** or **131**, 2,2-dimethylsuccinic anhydride (for **127**, **132**) or glutaric anhydride (for **128**) (5 mol eq.), and DMAP (1 mol eq.) in anhydrous pyridine was refluxed overnight. The mixture was concentrated to remove pyridine, 1N HCl solution was added, and the solutions extracted with EtOAc. The crude product was purified by Grace flash chromatography using silica gel column eluted with hexane/EtOAc (for **127**, **128**) or CH_2Cl_2/MeOH (for **132**) with minor amount of the dimethyl positional isomers for **127** and **132.** Compounds **127** and **128** were obtained following the de-protection procedure described above.

Compound 127. Yield 57%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 4.30 (1H, dd, J = 2.8, 8 Hz, H-23), 3.67 (3H, s, OMe), 3.61 (1H, t-like, J = 11.2, 10.4 Hz, H-23), 2.59 (2H, s, -CH_2COOCH_2), 1.30, 1.27 (each 3H, s, -C(CH_3)-COOH), 0.99 (3H, d, *J* = 6.8 Hz, CH_3), 0.94, 0.92 (each 3H, s, CH_3), 0.84 (3H, d, *J* = 7.2 Hz, CH_3), 0.74 (3H, d, *J* = 6.8 Hz, CH_3); ESI-MS *m*/*z*: 631 $[M-H]^-$

Compound 128. Yield 17%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 4.18 (1H, dd, J = 3.2, 10.8 Hz, H-23), 3.69 (1H, t-like, H-23), 3.64 (3H, s, OMe), 2.40-2.34 (4H, m, 2 × OCO$CH_2$$CH_2$), 0.96 (3H, d, *J* = 6.4 Hz, CH_3), 0.918, 0.911 (6H, s, 2 × CH_3), 0.85 (3H, s, CH_3), 0.82 (3H, d, *J* = 7.2 Hz, CH_3), 0.72 (3H, d, *J* = 6.4 Hz, CH_3); ESI-MS *m*/*z*: 617 $[M-H]^-$

Compound 132. Yield 35%; colorless oil; ^{1}H NMR ($CDCl_3$) δ 5.62-5.55 (2H, m, 2 × NH), 4.28 (1H, dd, *J* = 2.8, 7.6 Hz, H-23), 3.61 (1H, t-like, *J* = 10.4 Hz, H-23), 3.31-3.13 (4H, m, 2 × NHCH_2), 2.66, 2.65 (1H, s, -CH_2COOCH_2), 1.98 (3H, s, -NHCOCH_3), 1.29, 1.27 (each 3H, s, -C(CH_3)-COOH), 0.96

(3H, d, J = 6.8 Hz, CH_3), 0.94, 0.92 (3H, s, 2 × CH_3), 0.87-0.84 (6H, m, 2 × CH_3), 0.74 (3H, d, J = 6.8 Hz, CH_3); ESI-MS m/z: 772 $[M-H]^-$

Synthesis of Compound 129

A mixture of **126**, acetic anhydride (2 mol eq.), and DMAP (1 mol eq.) in anhydrous pyridine was stirred overnight at room temperature. The mixture was concentrated to remove pyridine, 1N HCl added, and the solution was extracted with EtOAc. The crude product was purified by Grace flash chromatography using silica gel column eluted with hexane/EtOAc. Compound **129** was obtained following the de-protection procedure mentioned above.

Compound 129. Yield 85%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 4.18 (1H, dd, J = 3.6, 10.4 Hz, H-23), 3.68 (1H, t-like, H-23), 3.67 (3H, s, OMe), 2.03 (3H, s, OCOCH_3), 0.99 (3H, d, J = 6.8 Hz), 0.94, 0.93 (each 3H, s, 2× CH_3), 0.87-0.84 (6H, 2× CH_3), 0.75 (3H, d, J = 6.8 Hz); ESI-MS m/z: 545 $[M-H]^-$

General Procedure for Compounds 121, 124 and 131

Compounds **120**, **123** or **130** was stirred in 4N NaOH THF solution for overnight. EtOAc was added and the solution was washed with water and 1N HCl solution. The organic layer was dried over Na_2SO_4 and the crude product was purified by flash chromatography on a silica gel column eluted with CH_2Cl_2/MeOH.

Compound 121. Yield 82%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 4.85, 4.65 (each 1H, s, H-23), 1.72 (3H, s, H-24), 0.98, 0.97 (each 3H, s, CH_3), 0.86-0.84 (6H, 2 × CH_3), 0.76 (3H, d, J = 6.8 Hz, CH_3); ESI-MS m/z: 471 $[M-H]^-$

Compound 124. Yield 51%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 5.58 (1H, t-like, NH), 5.51 (1H, bs, NH), 4.84 (1H, s, H-23), 4.65 (1H, s, H-23), 3.29-3.13 (4H, m, 2 × NHCH_2), 1.97 (3H, s, NHCOCH_3), 1.72 (3H, s, CH_3), 0.97, 0.96 (3H, s, 2 × CH_3), 0.85 (3H, d, J = 6.8 Hz, CH_3), 0.84 (3H, s, CH_3), 0.75 (3H, d, J = 6.8 Hz, CH_3); ESI-MS m/z: 625 $[M-H]^-$

Compound 131. Yield 58%; colorless amorphous; ^{1}H NMR ($CDCl_3$) δ 5.59-5.54 (2H, 2 × NH), 3.77 (1H, dd, *J* = 3.2, 10.4 Hz, H-23), 3.31-3.11 (5H, m, 2 × NHCH_2 and H-23), 1.97 (3H, s, NHCOCH_3), 1.03 (3H, d, *J* = 6.8 Hz, CH_3), 0.94-0.93 (6H, 2 × CH_3), 0.86-0.84 (6H, 2 × CH_3), 0.74 (3H, d, *J* = 6.8 Hz, CH_3); ESI-MS *m*/*z*: 643 $[M-H]^-$

Anti-HIV Assay

An anti-HIV-1 infectivity assay previously reported was used in the experiments. A 96-well microtiter plate was used to set up the HIV-1 NL4-3 replication assay. HIV-1 NL4-3 at a multiplicity of infection (MOI) of 0.01 was used to infect MT4 cells. Culture supernatants were collected on day 4 post infection for the p24 assay using an ELISA kit from ZeptoMetrix Corporation (Buffalo, New York).

5.6 References

1. Singh, I. P.; Bodiwala, H. S. Recent advances in anti-HIV natural products. *Nat. Prod. Rep.* **2010**, *27*, 1781-1800.

2. Cassels, B.; Asencio, M. Anti-HIV activity of natural triterpenoids and hemisynthetic derivatives 2004–2009. *Phytochem. Rev.* **2011**,*10*, 545-564.

3. Fujioka, T.; Kashiwada, Y.; Kilkuskie, R. E.; Cosentino, L. M.; Ballas, L. M.; Jiang, J. B.; Janzen, W. P.; Chen, I. S.; Lee, K. H. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* **1994,** *57*, 243-247.

4. Hashimoto, F.; Kashiwada, Y.; Cosentino, L. M.; Chen, C. H.; Garrett, P. E.; Lee, K. H. Anti-AIDS agents--XXVII. Synthesis and anti-HIV activity of betulinic acid and dihydrobetulinic acid derivatives. *Bioorg. Med. Chem.* **1997,** *5*, 2133-43.

5. Smith, P. F.; Ogundele, A.; Forrest, A.; Wilton, J.; Salzwedel, K.; Doto, J.; Allaway, G. P.; Martin, D. E. Phase I and II study of the safety, virologic effect, and pharmacokinetics/pharmacodynamics of single-dose 3-*O*-(3',3'-dimethylsuccinyl)betulinic acid (bevirimat) against human immunodeficiency virus infection. *Antimicrob. Agents Chemother.* **2007,** *51*, 3574-3581.

6. Qian, K.; Kuo, R. Y.; Chen, C. H.; Huang, L.; Morris-Natschke, S. L.; Lee, K. H. Anti-AIDS agents 81. Design, synthesis, and structure-activity relationship study of betulinic acid and moronic acid derivatives as potent HIV maturation inhibitors. *J. Med. Chem.* **2010**, *53*, 3133-3141.

7. Qian, K.; Yu, D.; Chen, C. H.; Huang, L.; Morris-Natschke, S. L.; Nitz, T. J.; Salzwedel, K.; Reddick, M.; Allaway, G. P.; Lee, K. H. Anti-AIDS agents. 78. Design, synthesis, metabolic stability assessment, and antiviral evaluation of novel betulinic acid derivatives as potent anti-human immunodeficiency virus (HIV) agents. *J. Med. Chem.* **2009,** *52*, 3248-3258.

8. Sun, M.; Xu, X.; Lu, Q.; Pan, Q.; Hu, X. Schisandrin B: A dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1. *Cancer Letters* **2007,** *246*, 300-307.

9. Wei, Y.; Ma, C. M.; Chen, D. Y.; Hattori, M. Anti-HIV-1 protease triterpenoids from *Stauntonia obovatifoliola* Hayata subsp. intermedia. *Phytochemistry* **2008,** *69*, 1875-1879.

10. Xiao, W. L.; Tian, R. R.; Pu, J. X.; Li, X.; Wu, L.; Lu, Y.; Li, S. H.; Li, R. T.; Zheng, Y. T.; Zheng, Q. T.; Sun, H. D. Triterpenoids from *Schisandra lancifolia* with anti-HIV-1 activity. *J. Nat. Prod.* **2006,** *69*, 277-9.

11. Song, W. Y.; Ma, Y. B.; Bai, X.; Zhang, X. M.; Gu, Q.; Zheng, Y. T.; Zhou, J.; Chen, J. J. Two new compounds and anti-HIV active constituents from *Illicium verum*. *Planta Med.* **2007,** *73*, 372-5.

12. Akihisa, T.; Nakamura, Y.; Tokuda, H.; Uchiyama, E.; Suzuki, T.; Kimura, Y.; Uchikura, K.; Nishino, H. Triterpene acids from Poria cocos and their anti-tumor-promoting effects. *J. Nat. Prod.* **2007,** *70*, 948-953.

13. Manners, G. D. Citrus limonoids: analysis, bioactivity, and biomedical prospects. *J. Agric. Food Chem.* **2007,** *55*, 8285-8294.

14. Tu, H. Y.; Huang, A. M.; Wei, B. L.; Gan, K. H.; Hour, T. C.; Yang, S. C.; Pu, Y. S.; Lin, C. N. Ursolic acid derivatives induce cell cycle arrest and apoptosis in NTUB1 cells associated with reactive oxygen species. *Bioorg. Med. Chem.* **2009,** *17*, 7265-7274.

15. Huang, L.; Ho, P.; Lee, K. H.; Chen, C. H. Synthesis and anti-HIV activity of bi-functional betulinic acid derivatives. *Bioorg. Med. Chem.* **2006,** *14*, 2279-2289.

16. Nakagawa-Goto, K.; Yamada, K.; Taniguchi, M.; Tokuda, H.; Lee, K. H. Cancer preventive agents 9. Betulinic acid derivatives as potent cancer chemopreventive agents. *Bioorg. Med .Chem. Lett.* **2009,** *19*, 3378-3381.

Chapter 6

Concluding Remarks and Perspectives for Future Directions of Research on DDB and BA Derivatives

6.1 DDB as a Promising Lead for Cancer Prevention and Chemosensitization

6.1.1 SAR Conclusions on DDB Analogs as Leads for Chemosensitization and Cancer Prevention

DDB, a clinically used hepatoprotectant, served as a lead to further design and synthesize new analogs for chemosensitizing and chemopreventive activities.

Thirty-three DDB analogs were newly synthesized and evaluated for chemoreversal action in comparison with the parent compound, DDB. Reduced activity was seen for compounds containing a 3-position halogen, such as bromide or iodide, while increased activity was seen for compounds bearing a 2,2′-position methyl ester side chain, such as pivalate, 2-methylbutanoate, cyclic aliphatic, and trimethoxyphenyl. Among all tested compounds, DDB derivatives **83** and **90** were 5–10 times more effective than VRP for TAX and VCR reversal ability. Analog **73** with a pivalate side chain also showed five-fold greater chemosensitizing effect than VRP for DOX.

Nineteen DDB analogs with 2,2′-bismethyl ester and ether substituents were designed and synthesized for chemopreventive activity. All analogs showed potent EBV-EA inhibition *in vitro* and significant inhibition of the EBV-EA activation was seen for analogs **82-85, 104**, **106**, and **107**, with unsaturated side chains or terminal carboxylic acids. Remarkably, the highest inhibitory effects (100%, 78.4%, 49.7% and 10.9% inhibition at 1×10^3, 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively) were seen with prenyl derivative **106**, which were greater than those of curcumin at the low concentrations. In an *in vivo* assay, DDB analogs **83**, **85**, and **106** significantly delayed the

formation of mouse skin papillomas and reduced the number of papillomas after initiation and promotion by a cancer promoting substance, TPA.

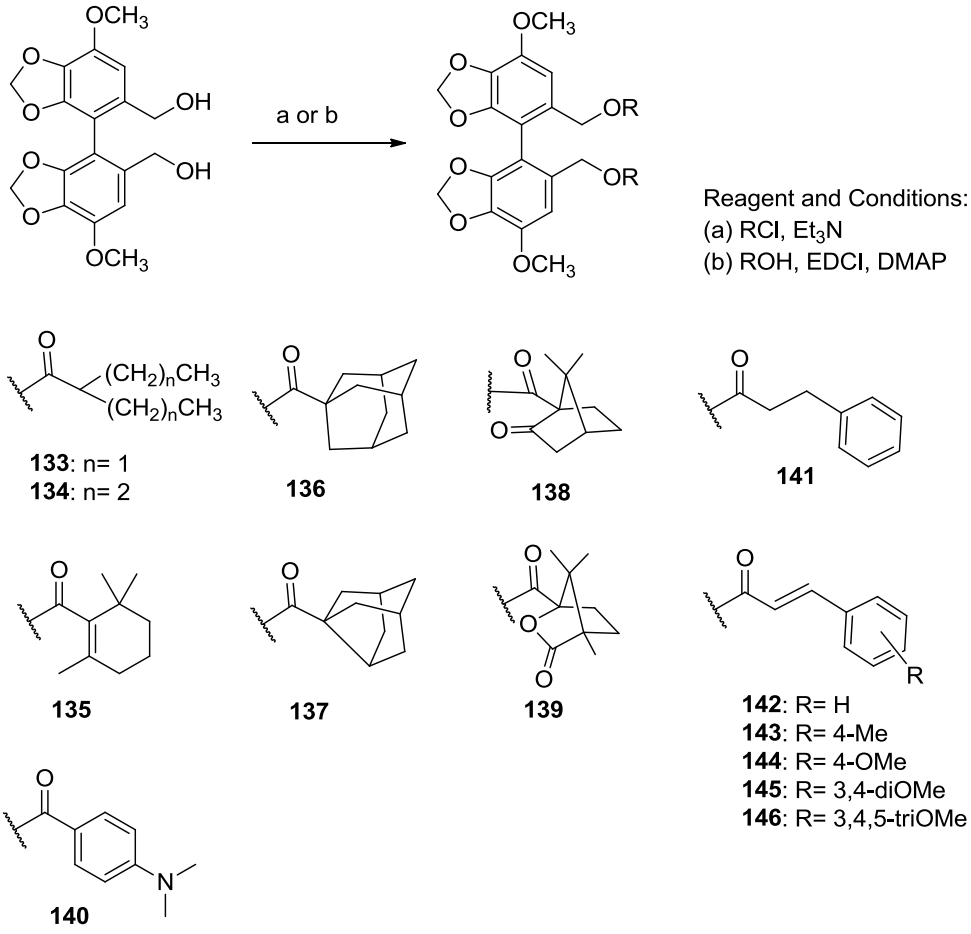
Importantly, all active DDB analogs displayed no cytotoxicity as chemosensitizers or anti-tumor promoters, suggesting that they have potential for long term use for further clinical development as chemosensitizing or chemopreventive agents.

6.1.2 Design, Synthesis of DDB Analogs as Novel Chemosensitizers

Based on the SAR study, 2,2′-methylene ester DDB analogs were effective for chemosensitization of certain anticancer drugs. Therefore, additional 2,2′-methylene ester DDB analogs are proposed to extend the current SAR. To quickly assess the effect of each direction of modification, the proposed analogs can be readily prepared using commercially available materials via the synthetic scheme shown below in Scheme 6.1. Compounds **133** and **134** are proposed in order to compare the effects of chain length and hydrophobicity compared with previously synthesized compounds **71**, **72**, **74**, and **75**. Compound **135** is proposed to evaluate the combined effects of a prenyl side chain and cyclohexane ring. Hydrophobicity is critical in designing P-gp inhibitors, and Clog P values from 4.8–6.5 are considered to be important. However, a wide spectrum of hydrophobicity should be studied to establish a clear cut off of suitable hydrophobicity. Accordingly, compounds **136** and **137** contain very bulky and hydrophobic ring systems. The design of compounds **138** and **139** is based on the chemosensitizing modification of dicamphanoyl-dihydropyranochrome (DCP) and dicamphanoyl-dihydropyranoxanthone (DCX) compounds (unpublished data). Commercially available camphanic and (1R,4S)-7,7-dimethyl-2-oxobicyclo[2.2.1]heptane-1-carbonyl groups are proposed. Nitrogen is a bioisostere substituent of the oxygen atom, so the simple and commercially available 4-(dimethylamino)benzoyl group in **140** should provide a rational comparison with compounds **87** and **88** for SAR study. Compounds **141**–**146** are designed based on a modification study of 3′R,4′R-disubstituted-2′,2′-dimethyldihydropyrano[2,3-*f*]chromone (DSP).[1] In

the study, an (E)-3-(4-methoxyphenyl)acryloyl substituent led to good activity, but di- and tri-methoxy substituents were not included. As found in Chapter 3, a DDB analog with trimethoxy substitution (**90**) showed quite potent activity. Therefore, mono-, di-, tri-methoxy substituents are proposed.

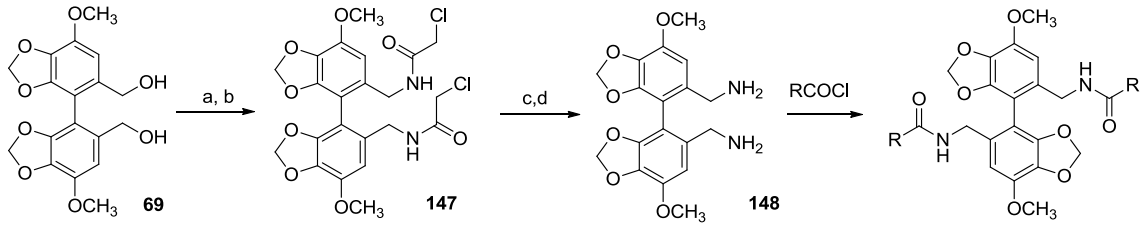
Scheme 6.1. Proposed Synthetic Pathway and Substitutions of 2,2′-Methylene Ester DDB Analogs.



As mentioned above, nitrogen often serves as a bioisostere for oxygen in drug design.[2] In addition, an amide linkage was effective in previous chemosensitizing research.[3] Thus, 2,2′-bis-methylene amide DDB analogs (in order to avoid stereochemistry issues) are proposed, and the synthetic pathway is also shown below (Scheme 6.2). 2,2′-Bishydroxymethyl biphenyl will be used as the

starting material. A Ritter reaction will be applied to avoid hazardous and explosive azide reagents (ex. NaN_3).[4, 5] Carbenium ion formation followed by electrophilic addition of chloroacetonitrile will lead to **147**. Subsequent cleavage of the chloroacetyl group with thiourea and heating will give the desired compound **148**. Various acyl chlorides, including previous active substitutions, can be used to prepare various analogs, which will be tested for chemosensitizing activity to compare the effect of ester and amide.

Scheme 6.2. Proposed Synthetic Pathway to 2,2′-Methylene amide DDB Analogs.



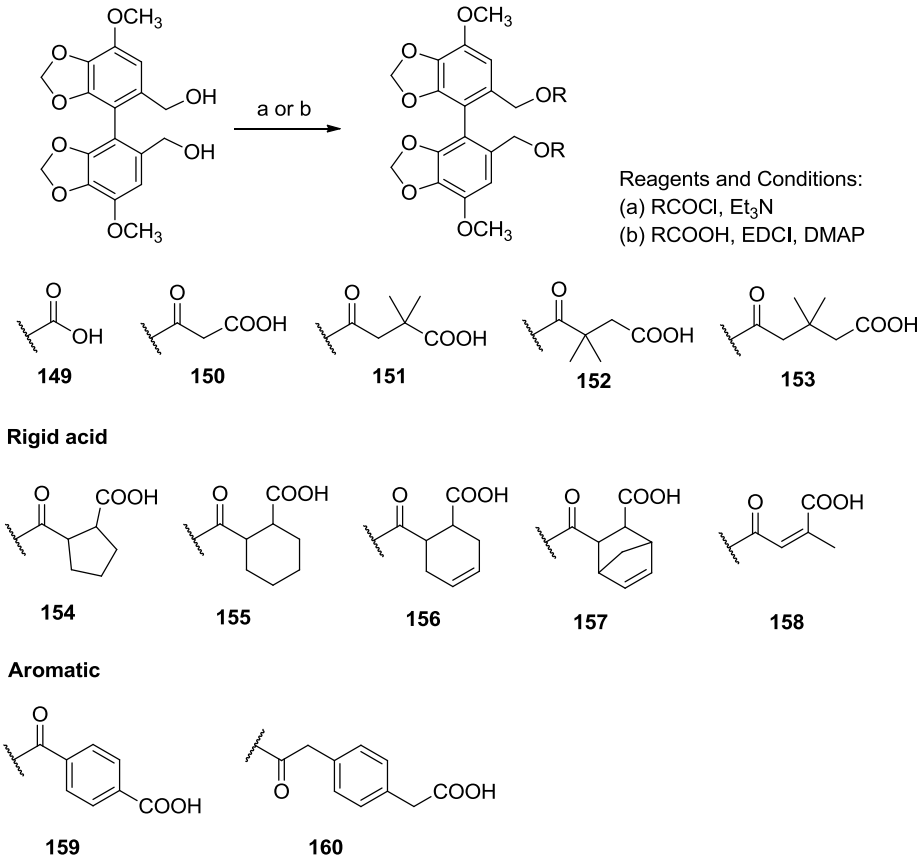
Reagent and conditions: (a) $ClCH_2CN$, $MsOH/Al_2O_3$, (b) heating, 60 °C, 5h or sonication, 60 °C, 2h, (c) thiourea, AcOH, (d) heating, 60 °C, 5h or sonication, 60 °C, 2h

6.1.3 Design and Synthesis of DDB Analogs as Novel Chemopreventive Agents

From the conclusions of Chapter 4, 2,2′-methylene ester DDB analogs are effective for chemopreventive activity, especially with the presence of an unsaturated side chain or terminal carboxylic acid. Based on this information, several 2,2′-methylene ester analogs have been designed (Scheme 6.3) and will be prepared by the same synthetic pathway described in Chapter 4. Although DDB analogs with a terminal carboxylic acid have been effective (see compounds **85** and **104**), the SAR has not been fully explored. Therefore, the acids selected will have different lengths or branching (**149**–**153**) or contain more rigid (**154**–**158**) or aromatic (**159**–**160**) systems, as well as be commercially available. The branched substituents in **151**, **152**, and **153** were also used in betulinic acid modification studies. Bulky and rigid acids (**154** and **155**) are proposed to study the effect of steric hindrance. Compounds **156**–**158** are proposed to evaluate the combined effect of an acid and unsaturated group, especially the presence of a carboxylic acid in a prenyl group (**158**). Two benzoate

substituents (**159**–**160**) were selected to compare the effect of insertion of an aromatic group. Analogs with an ether linkage will also be designed based on the results of corresponding ester linked compounds (see compounds **83** and **106**). In addition, amide analogs, again based on bioisostere design, can be prepared by the synthetic pathway shown above.

Scheme 6.3. Proposed DDB Analogs and Synthetic Pathway for Cancer Prevention.



6.2 A-ring Modification of Betulinic Acid Derivatives

6.2.1 SAR Conclusions with A-ring Opened Betulinic Acid Derivatives

Thirteen new 3,4-seco BA derivatives were designed, synthesized, and evaluated for cancer prevention, anticancer, and anti-HIV activity. In the cancer prevention study, analogs with a terminal (C-28) carboxylic acid, such as **120**, **121**, **126**, and **129**, significantly inhibited EBV-EA activation,

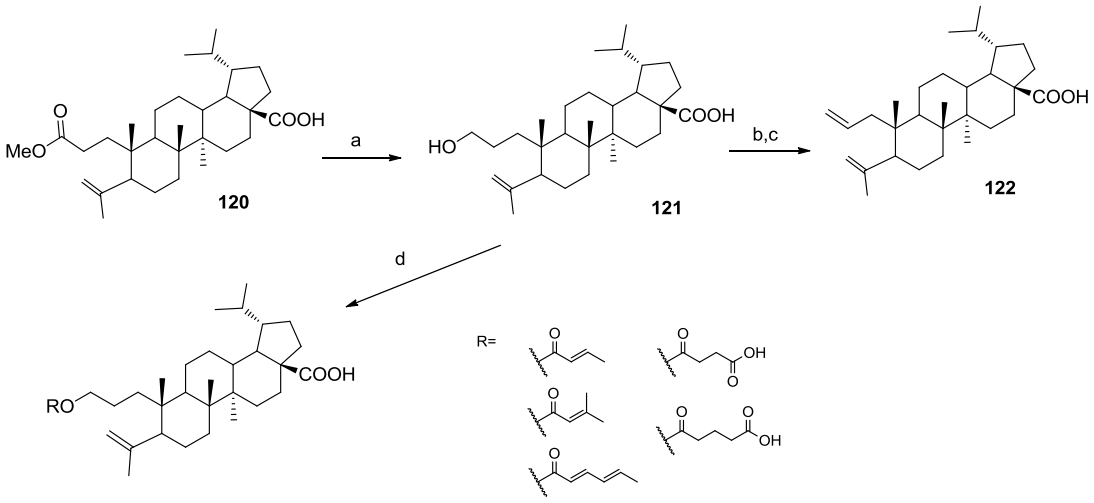
showing 100.0% inhibition at the highest tested concentration. The most active compound **121** with a 3-carboxylic acid and 4,23-vinyl group displayed 100% inhibition at 1×10^3 mol ratio/TPA, and 91.6%, 65.1% and 26.6% inhibition at 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively, with an IC_{50} value of 295 mol ratio/TPA, more potent than the known cancer preventive agent curcumin. In the cytotoxicity assays in A549, DU-145, K562 and KB cell lines, compounds **119**, **120**, **123**, **126**, and **129** exhibited $GI_{50} < 10$ μM. Compound **120** with a 3-methyl ester and 4,23-vinyl group was the most potent. Hydration of the 4,23 double bond to form a hydroxymethylene and subsequent esterification with acetic, 3,3′-dimethylsuccinic, or glutarie acid reduced activity. A C-28 carboxylic acid analog was more potent than the corresponding C-28 *N*-heptane acetamide. The chemosensitizing effects of the compounds at non-toxic concentrations are under investigation. Regarding anti-HIV activity, most of the compounds were toxic to MT-4 cells. However, compound **119** with an ε-lactone A-ring and 28-*N*-heptane acetamide showed activity and its mechanism of action is under study. A long side chain on C-28 and retained A-ring seem to be crucial for anti-HIV activity.

6.2.2 Future Research Directions with A ring Opened Betulinic Acid Derivatives

From the cancer prevention results in Chapter 5, A-ring opened BA analogs with a C-4,23 vinyl group were better than analogs with hydroxymethylene or acetoxymethylene groups, and a carboxylic acid was better than its methyl ester at C-3. In addition, SAR in Chapter 4 suggested that short fatty acid and acidic side chains were effective in DDB compounds. Based on these results, A-ring opened BA analogs will be prepared by the synthetic scheme shown in Scheme 6.4. The terminal methyl ester in starting material **120** can be selectively reduced to a hydroxymethylene group (**121**) without altering the C-28 carboxylic acid. The allyl group in **122** can be produced by treating **121** with trifluoromethanesulfonic anhydride (Tf_2O) followed by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The effects of the polarity and water solubility of the three compounds can then be compared. The hydroxy group found in A-ring opened **121** can be seen as analogous to the C-3 hydroxyl found in

betulinic acid (BA). Thus, it will be esterified with the active side chains found in Chapter 4 to assess whether the corresponding A-ring opened analogs will exhibit cancer preventive activity.

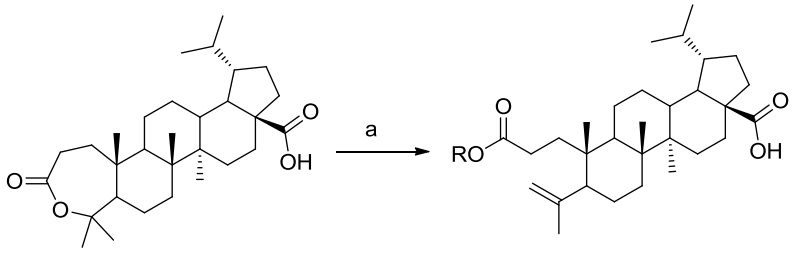
Scheme 6.4. Proposed A-ring Opened BA Derivatives for Cancer Prevention.



Reagent and conditions: (a) $LiBH_4$, (b) Tf_2O, THF, (c) DBU, heat, (d) RCl, DMAP, THF

Based on previous BA cytotoxicity results, several analogs are proposed and their synthetic scheme is shown below (Scheme 6.5). The main goal is to explore esters with different chain lengths or branching to establish complete SAR at the C-3 position. The oxepanone ring-A starting material can be produced from 3-oxo-BA using mCPBA. Different alcohols, such as ethanol and propanol, can be used as solvents as well as reagents to form esters with different chain lengths at C-3.

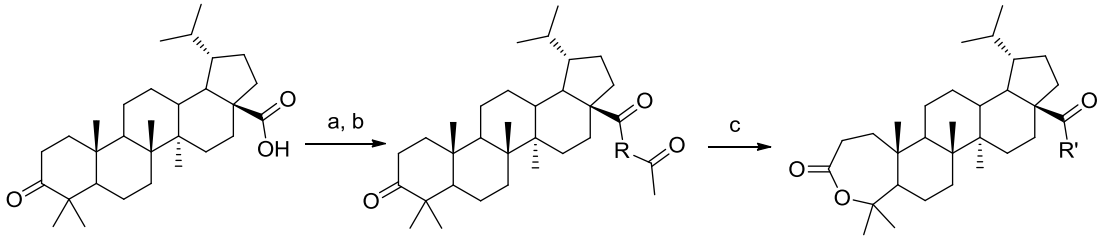
Scheme 6.5. Proposed New BA Derivatives for Cytotoxicity.



Reagents and conditions: (a) H_2SO_4, ROH
ROH= EtOH, PrOH, BuOH, *i*-PA, etc.

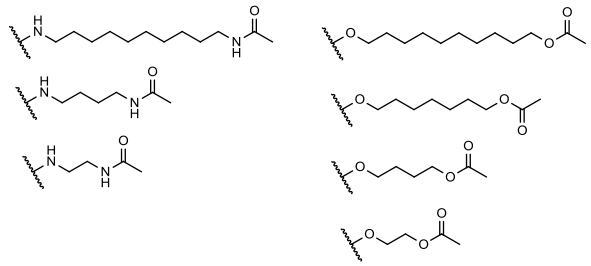
According to the SAR analysis in Chapter 5, a long chain on C-28 is essential for anti-HIV activity. Anti-entry assays are now ongoing, as prior BA derivatives with long chains at C-28 usually exhibited anti-entry activity. Further synthetic modifications are proposed, while the mechanism is being confirmed. Different chain lengths and different linkages will be incorporated through the synthetic pathway shown below (Scheme 6.6). Two, five, and ten carbon linking (R) groups are designed to optimize suitable chain length. As oxygen and nitrogen are bioisosteres, ester linked compounds with different chain lengths are also designed. In the synthetic scheme, the 3-oxo-28-carboxylic acid is used as starting material. The C-28 side chains can easily be introduced by acyl chloride formation, nucleophilic attack of amine or alcohol, and acetylation with acetic anhydride. Desired products with the expanded A-ring lactone can be achieved via a Baeyer-Villiger reaction using mCPBA.

Scheme 6.6. Proposed New BA Derivatives for Anti-HIV Activity and Their Synthetic Scheme.



Reagents and conditions: (a) $(CO_2)_2Cl_2$, RNH_2 or ROH, (b) Ac_2O, DMAP, (c) mCPBA

R'=



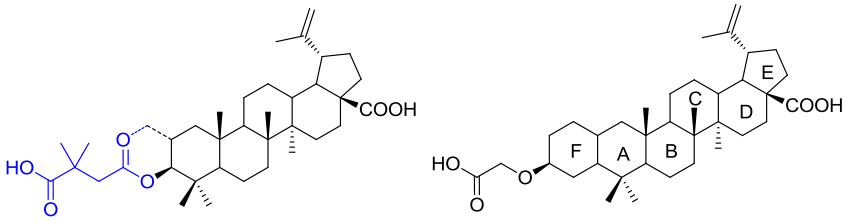
6.2.3 Successful Six Member Ring BA Derivatives

6.2.3.1 Introduction

The main target of the BA derivative bevirimat is the HIV maturation step. Inhibition of this step blocks the cleavage of precursor Gag protein to functional p24, resulting in non-infectious virus. However, the crystal structure of Gag protein is still unavailable, and the actual binding site of bevirimat is still unknown. Therefore, in order to explore new BA analogs for anti-maturation activity, ligand-based drug design will be applied.

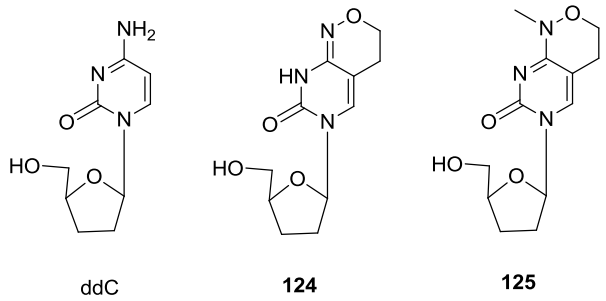
Common strategies for analog design are 1) bioisosteric replacement, 2) rigid analogs, 3) homologation of alkyl chain or alteration of chain branching, changes in ring size, and ring position isomers, 4) stereoisomers and geometric isomers, 5) fragment of a lead molecule, and 6) alteration in interatomic distance.[2] The advantage of making rigid analogs is to reduce three-dimensional movement of a side chain or to investigate the effect of freedom of a side chain. Based on this logic, the BA analog **123** was designed (Figure 6.1).

Figure 6.1. Proposed Rigid BA Analog.



Addition of a sixth ring (F-ring) joined to the A-ring will reduce the freedom of the prior DSB side chain. A recent example of successful ring construction in antiviral research convinced us of the validity of this approach. N-4-Hydroxy pyrimidine nucleosides are a recent novel class of anti-herpes agent. Two bicyclic derivatives (**124** and **125**) of ddC, a NRTI targeting HIV reverse transcriptase, are shown in Figure 6.2. These two compounds inhibited cultural herpes simplex (HSV) and varicella zoster virus (VZV) and had similar inhibitory potency toward VZV replication on VZV nuclease kinase with a 40-fold selectivity.[6]

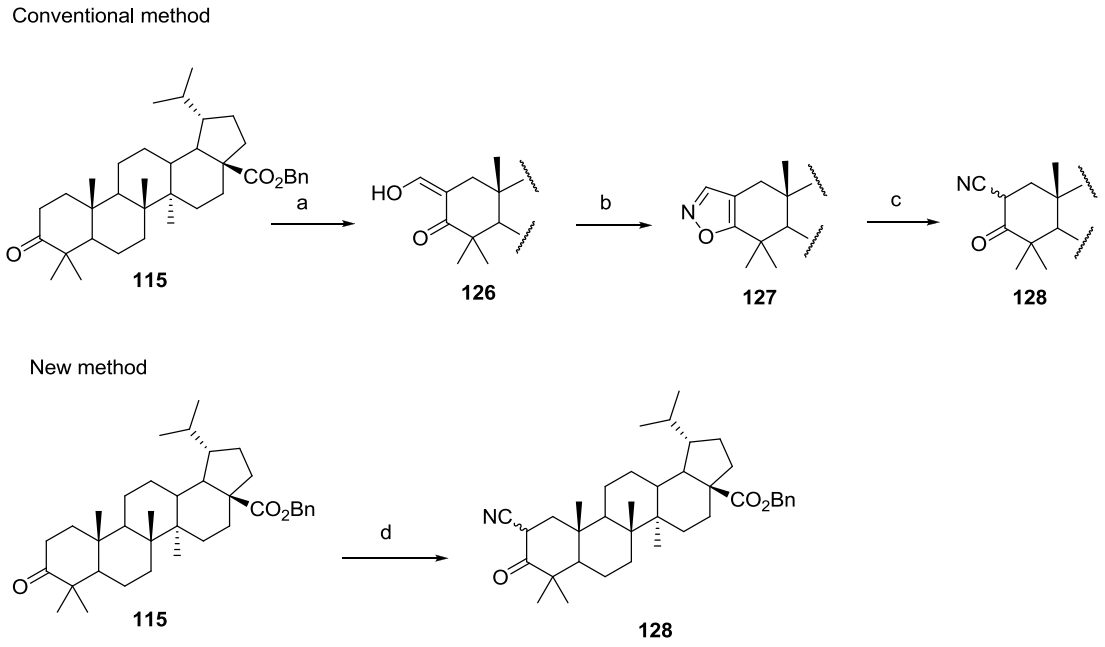
Figure 6.2 *N*-4-Hydroxy Pyrimidine Nucleoside and Related Bicyclic Analogs



6.3.2.2 Synthesis of the Six-Ring BA Derivative

Robinson annulation, which is Michael addition followed by ring cyclization, can be applied to construct the F-ring of a new BA derivative. Michael addition without an activator (Mukaiyama Michael reaction) was tried several times, but resulted in low yields with mostly starting material recovered and two isomers obtained. Therefore, a Michael addition with a nitrile group (β-keto nitrile) as an activator was applied. The β-keto nitrile group in **128** can be introduced by two methods (Figure 6.3). The conventional method is a three-step process: 1) hydroxymethylene ketone formation (**126**) using sodium hydride and ethyl formate, 2) isoxazole formation (**127**) using hydroxylamine hydrochloride and sodium acetate, and 3) β-keto nitrile group formation (**128**) using sodium methoxide. Low yields (20-40 %) were obtained and the three steps took three days to complete. An easier synthetic pathway to the β-keto nitrile (**128**) was found by nucleophilic attack of the enolate of **115**, formed with lithium diisopropylamide (LDA), on *p*-toluenesulfonyl cyanide (p-TsCN), which led to relative high yields (60-80 %) of **128** within a short time period (one hour).

Figure 6.3. Two Synthetic Methods for Forming β-Keto Nitrile BA Derivative (128).



Reagents and Conditions: (a) NaH, ethyl formate, dry ether, (b) $NH_2OH{\cdot}HCl$, CH_3COONa, $CHCl_3$/EtOH (c) NaOMe/MeOH, Et_2O, (d) LDA, p-TsCN, THF, -78°C

Michael addition on the β-keto nitrile BA (**128**) was achieved using methyl vinyl ketone (MVK) and triethylamine (Scheme 6.7). Only one product (**129**) was obtained in high yield. The product's stereochemistry is explained in Figure 6.4. The axial C-24 methyl group would disfavor an approach on MVK from the β-direction (and BA cannot easily flip into another configuration due to its pentacyclic structure). Therefore, the attack would come from the α-direction, and the nitrile group would end up in the β-position.[7]

Scheme 6.7 Synthetic Scheme for the F-ring BA Derivative.

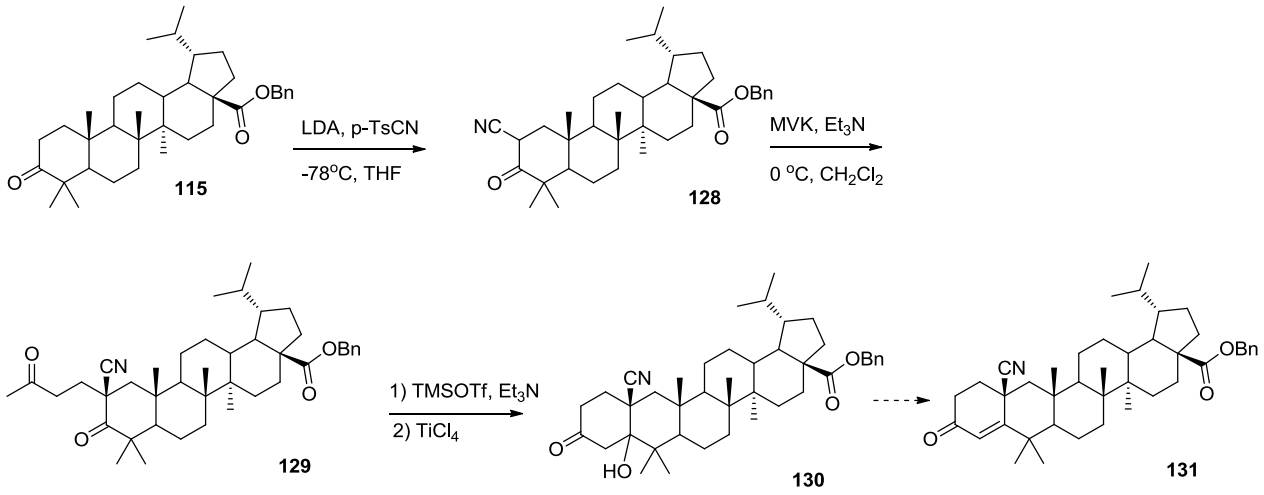
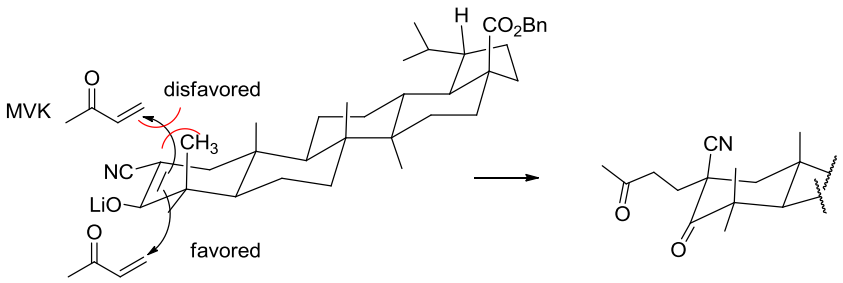


Figure 6.4. Explanation of Stereochemistry of C-2 Nitrile Group of 129.



Cyclization of the Michael addition product (**129**) was needed to complete the Robison annulation. Several basic conditions (Table 6.1) have been reported in the literature and were attempted here to obtain cyclic products. However, because of the huge steric effect on the A-ring, retro Michael reaction took place predominately to return to the starting cyano ketone (**128**). Only a small amount of cyclic product (**130**) was obtained with a bulky base or moderate basicity at low temperature (ex. 0.022 M NaOMe at 0 °C). Finally, a Mukaiyama aldol condensation [formation of the trimethylsilylenol ether of the C-3 ketone followed by cyclization with the Lewis acid titanium tetrachloride ($TiCl_4$)] was more effective and raised the yield of **130** up to 40–50%.[8] However, dehydration of the β-hydroxyketone was not achieved using trifluoroacetic anhydride and triethylamine. Other dehydration conditions will be needed to complete the synthesis of **131**.

Table 6.1. Basic Cyclization Conditions.

Base	Solvent	Temperature	Time	**128** (Retro Michael product)	**130**
0.022 M NaOMe	THF/MeOH (8:1)	0 °C	15 min	major	< 2%
0.022 M NaOMe	THF/MeOH (8:1)	0 °C	30 min	major	< 1%
0.022 M NaOMe	THF/MeOH (8:1)	r.t.	overnight	major	0
0.6 mmol NaOMe	Toluene	r.t.	30 min or overnight	major	0 (Unknown product appeared with double bond signal in NMR)
0.8 equiv. LDA[a]	THF	-78 °C	30 min	major	< 5 %
4N NaOH	THF/MeOH	r.t.	30 min or overnight	major	0
1M tBuOK	THF/MeOH	r.t.	30 min or overnight	major	0
1.1 eq. KHMDS[b]	THF	-78 ° C	30 min	major	< 5%

[a.]LDA: lithium diisopropylamide, [b.] KHMDS: Potassium bis(trimethylsilyl)amide

6.2.4 Future Research Direction of F-ring BA Derivative Modification

6.2.4.1 Optimize Cyclization Condition and Complete Dehydration of β-Hydroxyketone

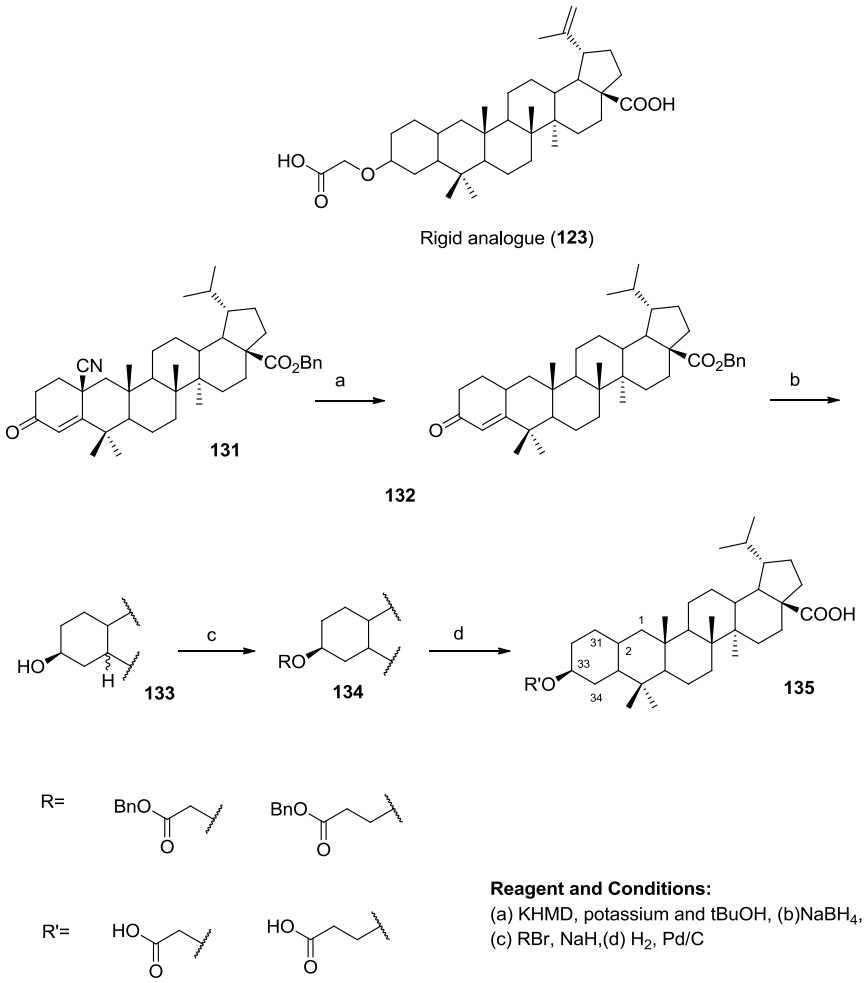
Since $TiCl_4$ (a Lewis acid) worked for the cyclization to form β-hydroxyketone (**130**), a series of Lewis acids such as BF_3, $SnCl_4$, and $ZrCl_4$ will be used to optimize the cyclization step. Another rational method is Knoevenagel condensation using pyrrolidine with pH adjusted by acetic acid.[9]

6.2.4.2 New F-ring BA derivatives

Based on previous rigid analog design, several new F-ring BA derivatives are proposed and their synthetic pathway is shown below (Scheme 6.8). Newly synthesized BA derivative **131** will serve as a starting material. Exposure to potassium and hexamethyl phosphoric triamide (KHMD) will eliminate the nitrile group at C-2 as well as the C-28 benzyl group.[10] 1,4-Reduction by sodium

borohydride should give mainly β-hydroxylation at C-33 (**133**). Etherification with various protected ω-bromoacids (e.g., benzyl 2-bromoacetate and 3-bromopropanoate) followed by deprotection of the benzylic group by hydrogenation will give the desired compounds (**135**).

Scheme 6.8. Proposed New F-ring BA Derivatives and Their Synthetic Route.



6.3 References

1. Zhou, T.; Shi, Q.; Bastow, K. F.; Lee, K. H. Antitumor agents 286. Design, synthesis, and structure-activity relationships of 3'R,4'R-disubstituted-2',2'-dimethyldihydropyrano[2,3-f]chromone (DSP) analogues as potent chemosensitizers to overcome multidrug resistance. *J. Med. Chem.* **2010,** *53*, 8700-8708.

2. Wolff, M. E. *Burger's Medicinal Chemistry and Drug Discovery*. 5th ed.; John Wiley & Sons, Inc: New York, 1994; Vol. 1, p 783-802.

3. Tang, X.; Gu, X.; Ai, H.; Wang, G.; Peng, H.; Lai, Y.; Zhang, Y. Synthesis and evaluation of nitric oxide-releasing DDB derivatives as potential Pgp-mediated MDR reversal agents in MCF-7/Adr cells. *Bioorg. Med. Chem. Lett.* **2012,** *22*, 801-805.

4. Liu, Y.-Q. L., Lin-Hai; Yang, Liu; Li, Hong-Yu. A novel, stereoselective and practical protocol for the synthesis of 4-beta-aminopodophyllotoxins. *Chemical Papers* **2010,** *64*, 533-536.

5. Jirgensons, A. K., V.; Kalvinsh, I. ; Gold, M. R. A practical synthesis of tert-alkylamines via Ritter reaction with chloroacetonitrile. *Synthesis* **2000,** *12*, 1709-1712.

6. Wolf, M. E. *Burger's Medicinal Chemistry and Drug Discovery*. John Wiley & Sons, Inc.: New York, 1994; Vol. 4, p 487-549.

7. Jansen, B. J. M.; Hendrikx, C. C. J.; Masalov, N.; Stork, G. A.; Meulemans, T. M.; Macaev, F. Z.; de Groot, A. Enantioselective synthesis of functionalised decalones by Robinson annulation of substituted cyclohexanones, derived from R-(-)-carvone. *Tetrahedron* **2000,** *56*, 2075-2094.

8. Bouhlel, E.; Hassine, B. B. One pot synthesis of alpha,beta-unsaturated ketones from the Mukaiyama Aldol condensation. *Synthetic. Commun.* **1992,** *22*, 2183-2186.

9. Nussbaumer, C. Stereochemistry of the Robinson anellation: studies on the mode of formation of the intermediate hydroxy ketones. *Helvetica Chimica Acta* **1990,** *73*, 1621-1636.

10. Rojas, G.; Wagener, K. B. Avoiding olefin isomerization during decyanation of alkylcyano α,ω-dienes: a deuterium labeling and structural study of mechanism. *J. Org. Chem.* **2008,** *73*, 4962-4970.