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Antitumor Agents 293. 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

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Abstract

Novel dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB) analogs were designed and synthesized to improve their chemosensitizing action on KBvin (vincristine resistant nasopharyngeal carcinoma) cells, a multi-drug resistant cell line over-expressing P-glycoprotein (P-gp). Structure-activity relationship analysis showed that aromatic and bulky aliphatic side chains at the 2,2'-positions effectively and significantly sensitized P-gp overexpressing multidrug resistant (MDR) cells to anticancer drugs, such as paclitaxel (TAX), vincristine (VCR), and doxorubicin (DOX). DDB derivatives **16** and **23** showed 5–10 times more effective reversal ability than verapamil (VRP) for TAX and VCR. Analog **6** also exhibited five times greater chemosensitizing effect against DOX than VRP. Importantly, no cytotoxicity was observed by the active DDB analogs against both non-MDR and MDR cells, suggesting that DDB analogs serve as the novel lead compounds for the development of chemosensitizers to overcome MDR phenotype. The mechanism of action studies demonstrated that effective inhibition of P-glycoprotein by DDB analogs dramatically elevated cellular concentration of anticancer drugs.

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

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Supporting Information Available: Cytotoxicity of DDB analogs (Table S1); IC₅₀ ± standard deviation (SD) for reversal effects with paclitaxel (TAX), vincristine (VCR), and doxorubicin (DOX) in KBvin (Table S2); Standardized P values of compounds **6**, **23**, and VRP (Table S3); Effect of compounds **6** and **23** on P-gp function in KBvin cells (Table S4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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 An emerging understanding of cancer resistance results from cancer stem cell-like features.⁴ However, over-expression of drug efflux transporters, such as P-glycoprotein (P-gp) and MDR-associated protein (MRP), is 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 (CsA), 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. Although several third-generation P-gp inhibitors, including tariquidar, are now in phase II cancer clinical trials,⁹ their clinical efficacies are not yet clear. 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 1), the most abundant dibenzocyclooctadiene lignan from *Schisandra chinensis*, was found to inhibit P-gp/MDR1 and MRP1/ABCC1.^{10,11} Structurally similar lignans, schisandrin A, schisandrol A, 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/ABCC1.¹² Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB, **1**, Figure 1), which was discovered as a synthetic intermediate derivative of schisandrin C,¹³ shares the biphenyl partial structure of dibenzocyclooctadiene lignans. DDB (**1**) exhibited multidrug resistant reversal ability in MDR breast carcinoma MCF-7/Adr cells, KBv200, and intrinsic MDR human hepatocarcinoma Bel₇₄₀₂ cells via inhibition of P-gp and enhancement of apoptosis.¹⁴ However, a very high concentration (50 μ M) was required for effective reversal action. Co-treatment of **1** with VCR using nude mice with KBv200 xenografts also enhanced antitumor activity at doses of 300 and 500 mg/kg.¹⁴ DDB (**1**) has been used to treat chronic viral hepatitis B patients in China for more than 20 years, as well as in Korea and Egypt for more than 10 years, without any significant adverse effects.^{15,16} This important fact indicates that DDB analogs could be highly attractive MDR reversal agents with significant clinical potential due to their proven low toxicity. In addition, pharmacokinetic issues where chemosensitizers would interfere with the clearance of anticancer drugs often impede further development of an effective chemosensitizer. DDB was found not to alter the clearance of DOX by the evidence that plasma AUC₀₋₂₄ of DOX alone and DOX plus DDB were similar in ICR mice bearing S180 sarcoma model.¹⁴ In 2006, Zhu *et al* reported that an asymmetric analog of DDB, bicyclol (Figure 1), also exhibited a chemosensitizing effect in two established MDR cancer cell lines, Vin^RKB and Adr^RMCF-7.^{17,18} 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.

Design and Syntheses

Based on previous literature, DDB had effects on inhibition of P-gp and activation of cellular caspase-3 leading to DOX-induced apoptosis of innate MDR cell line Bel₇₄₀₂ and acquired MDR MCF-7/Adr.¹⁴ Since DDB acts against multi-targets, which is a common

feature of natural products or natural product-derived agents, ligand-based modification of DDB was applied. Bicyclol bears a primary alcohol at the C-2' position.¹⁷ Therefore, we selected the 2,2'-bis-hydroxymethyl biphenyl **2** as a base scaffold to design various esters **3–28** (Scheme 1) in order to define substituent effects at the C2 and C2' positions. The ester groups (R in Scheme 1) were selected by considering size, hydrophobicity, and electron density. The diverse set included aliphatic acyclic (Group-I), cyclic (Group-II), unsaturated aliphatic groups (Group-III), polar groups (Group-IV), and aromatic groups (Group-V), which could be transformed into water-soluble salts, if necessary. Esterifications of **2** were performed by treatment with the related acid (RCO₂H), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) to produce **3**, **7–12**, **15–17**, and **22**, as well as by treatment with the related acid chloride (RCOCl) and Et₃N to give **4–6**, **13–14**, **19–21**, and **23–28** (Scheme 1). Halogenated DDB analogs with various functional groups were also designed to explore their effects (Scheme 2) since 3,3'-dibromo-DDB has showed good activity in anti-HIV research.¹⁹ 3,3'-Dibromo-DDB (**29**) was previously synthesized.¹⁹ Iodination of **1** was achieved by treatment with silver trifluoroacetate and iodine to provide **30**.²⁰ The diester groups of **29** were reduced with diisobutylaluminum hydride (DIBAL) to afford bis-hydroxymethyl biphenyl **31** in good yield. The esters **32** and **33** were synthesized by esterification of **31** with butyric and benzoic acids, respectively, in the presence of EDCI and DMAP. Hydrolysis of dimethyl ester **29** under basic conditions resulted in dicarboxylic acid **34**. Reflux of **34** in Ac₂O gave anhydrous analog **35**, followed by asymmetric cleavage of carbonyl anhydrous bridge with NaBH₄ resulted in di-Br bicyclol analog **36**.²¹ All synthesized analogs were prepared as the racemic mixtures.

Results and Discussion

Evaluation of cytotoxicity and preliminary MDR reversal activity screening

All synthesized compounds were 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). Verapamil, the first generation chemosensitizer precluded from clinical use due to toxicity, showed some cytotoxicity especially to KB (IC₅₀ ~50 μM) and KBvin cells (IC₅₀ ~80 μM). Although compounds **28** and **32** were slightly cytotoxic, most of the DDB-derived compounds did not exhibit significant cytotoxicity (IC₅₀ > 100 μM), which implied low toxicity of these analogs (Suppl. Table S1).

For evaluating chemosensitizing activity, KB and KBvin cells were co-treated with test compounds at 10 μM and the anticancer drug paclitaxel (TAX) (Figure 2). As shown in Figure 2-B, DDB (**1**) and diol **2** did not exhibit MDR reversal effect at a concentration of 10 μM in KBvin cells. 3,3'-Dihalogenated DDB analogs with different 2,2'-functional groups did not show potent activity, except pentanoate **32** and benzoate **33**, but **32** was slightly cytotoxic to KBvin cells, which may result from bromo substitution. While a 2,2'-methyl ester group [-CH₂OC(O)R] appeared to be critical for reversal ability activity, 3,3'-halogenation reduced the potency (compare **33** with **19**).

Many of the analogs with aliphatic ester substituents (Group-I and -II in Figure 2) did show potent activity; however, acetate **3** (R = methyl) and dodecanoate **12** (R = undecanyl) were inactive, and 2-methylhexanoate **11** (R = 2-methylbutyl) was only moderately active. The screening results suggested 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) **5** and pivalate (R = *tert*-butyl) **6** displayed the most significant survival rate (13–14%) among all saturated Group-I

and-II compounds, and the unsaturated 3-methylbut-2-enoate (prenyl-like substituent) **16** in the Group-III compounds further increased the effect, showing a 5% survival rate. Among the Group-IV compounds with polar ester substituents, succinate **18** (R = CH₂CH₂COOH) 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. Especially, benzoate **19** and *p*-methoxybenzoate **21** exhibited less than 1% survival rate. From these findings, we speculated that decrease in electron density on the aromatic ring affected chemoreversal activity. Quinoline derivative **28** exhibited cytotoxicity against both KB and KBvin cells.

From these results, the Group I compounds (**4–11**), except for **3** and **12**, the Group-II and -III (**13–17**), and the most active Group V compounds (**19–23**) were selected for further investigation. The remaining compounds (**1–3**, **12**, **18**, **24–27**, **29–32**, and **35–36**), which generally had weaker reversal ability, as well as compounds as well as inherent cytotoxicity (**28**) were eliminated from further testing.

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

A quantitative evaluation of the reversal ability of DDB analogs **4–11**, **13–17**, and **19–23** 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, partly accounting for their resistance (Table 1). The IC₅₀ value of anticancer drugs in the presence of test compounds at 10 μM concentration was calculated and fold reversal was determined by dividing the IC₅₀ of anticancer drug alone by the IC₅₀ of anticancer drugs plus DDB analog. For chemoreversal ability against TAX resistance, compounds **9**, **15**, **16**, **19**, and **23** were 3–10 times more potent than the positive control VRP. Especially, **16**, with a prenyl-like ester substituent, and trimethoxybenzoate **23** effectively reversed the sensitivity of TAX in KBvin cells by 326- and 222-fold, respectively. Most of the tested analogs, including VRP, showed greater reversal against VCR resistance compared with TAX resistance. The reversal effects of compounds **6**, **10**, **13–16**, **22**, and **23** against VCR were significantly better than that of VRP. Especially, **16** and **23** showed 560-fold reversal effect, which was 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 **15** and **16** were generally more potent than saturated group I and II compounds. Compounds **15** and **16** displayed greater chemoreversal ability than **7** and **9**, 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 (**23**) > 3,4-di-OMe (**22**) > H (**19**) > 4-Me (**20**) ≈ 4-OMe (**21**). Different SAR correlations between TAX and VCR were found in the saturated aliphatic group. Cyclic aliphatic side chains (Group II, **13–14**) were more effective than non-cyclic aliphatic side chains (Group I, **4–11**) in the case of VCR resistance, while this difference was not present for TAX resistance. Within the tested aliphatic acyclic Group I compounds (**4–11**), 2-methylbutyrate (R = *iso*-butyl) **9** was most potent for TAX, while pivalate (R = *tert*-butyl) **6** and petanoate (R = *n*-butyl) **10** were most potent for VCR. In addition, **10** exhibited greater activity than the related unsaturated analog **17** against VCR resistance. All of the tested compounds were less effective at reversing DOX resistance than against TAX and VCR. This phenomenon might be correlated with the difference of efflux pump on the cell membrane with that on the nuclear membrane, because both TAX and VCR act by binding tubulin, while DOX interacts with DNA by intercalation.²² However, compounds **6**, **8**, **11–14**, and **20–23** still showed greater reversal effects than VRP for DOX chemosensitivity in MDR cells. Especially, pivalate **6**, with a bulky and short aliphatic ester chain, reversed the activity most

effectively, exhibiting medium more sensitivity than VRP. SAR against DOX was slightly different from that against VCR. Compounds **6**, **8**, **11**, **13**, and **14** with a branched substituent at the ester α -position showed significant MDR reversal activity. Benzoates with an electron donating group, such as *para*-methyl (**20**) and -methoxy (**21**), had little effect. Although an additional methoxy group at the *meta*-position increased the ability, no difference was found between trimethoxy **23** and dimethoxy **22**. The effects of substituent on phenyl ring resulted in the following order of potency: 3,4-di-OMe (**22**) \approx 3,4,5-tri-OMe (**23**) > H (**19**) \approx 4-Me (**20**) \approx 4-OMe (**21**). In conclusion, analogs **16** and **23** showed significant TAX and VCR cytotoxic reversal ability, and analog **6** exhibited the most potent DOX cytotoxic reversal ability.

Based on the above results, compounds **6** and **23** were selected for further evaluation of chemosensitizing efficacy. The dose-response proliferation inhibitory effects of TAX, VCR, and DOX at 10 μ M were analyzed against KB and KBvin cells (Figure 3). In the absence of DDB compound or VRP, KBvin cells were resistant to all three anticancer drugs, resulting in IC₅₀ values over 1000 nM. When 10 μ M of compound **6** or **23** or VRP was added, the sensitivity of KBvin cells to each anticancer drug was dramatically increased to at least the same levels of KB cells. The chemosensitizing efficacy of **6** or **23** was either similar or better than VRP. KBvin became even more sensitive to VCR than KB, when KBvin cells were co-treated with 10 μ M **23** and VCR. These results demonstrated that 10 μ M of **6** and **23** effectively chemosensitized MDR cells.

Dose-response effect of compounds **6** and **23** on sensitization of KBvin to TAX

To evaluate the reversal activity of **6** and **23** 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 4). As we expected, compounds **6** and **23** exhibited reversal activity in a dose dependent manner. Although the median effective concentration (EC₅₀) value of **6** (2.81 μ M) was similar to that of VRP (2.71 μ M), compound **23** (1.87 μ M) exhibited a lower EC₅₀ than VRP (P values are given in suppl. Table S3). These results demonstrate that **23** could be more effective than VRP in chemosensitizing the MDR cells to TAX.

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 **6** and **23** on P-gp function in KBvin cells using calcein-AM as a fluorogenic P-gp substrate was investigated (Figure 5, suppl. Table S4). Dose-dependent intracellular accumulation of calcein was observed in the presence of compounds. Although **6** was slightly less potent than VRP, **23** was up to two-fold more potent than VRP, especially at concentrations around the EC₅₀ value (1.87 μ M) of **23**. Therefore, these results clearly indicated that DDB analogs, especially **23**, are effective P-gp inhibitors.

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 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 (Table 1). 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.

Hydrophobicity evaluation of active DDB analoges

P-gp contains a large drug-binding pocket with a volume of around 6000Å.²³ 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 (hydrogen bond acceptor), and one or two protonatable nitrogens.^{24, 25} Because the upper half of the presumptive drug-binding pocket is composed of mainly hydrophobic and aromatic residues,²³ it is possible that hydrophobicity of DDB analogs could influence their MDR reversal activity. Table 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 **6**, **16**, and **23**, which were significantly active as described above, were between 4.8 and 6.2, which is close to that of VRP. This fact implied that hydrophobicity is an important parameter in P-gp inhibition.

Conclusions

Multidrug resistance is still a serious barrier to successful cancer chemotherapy. Among the possible reasons for multidrug resistance, reducing intracellular concentration of anticancer drug caused by the drug efflux pumps, such as P-gp, is a major cause of chemoresistance. 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 on clinically used anticancer drugs, such as TAX, VCR, and DOX. We succeeded to improve chemoreversal action by introducing a 2,2'-methylene group to DDB, and SAR studies were summarized in Figure 7. Insertion of halogen onto the 3-position reduced the ability. In the 2-position of the ester side chain, bulky groups, such as pivalate, 2-methylbutanoate, cyclic aliphatic, and trimethoxyphenyl, tended to enhance the reversal ability of DDB analogs. Among all tested compounds, DDB derivatives **16** and **23** were 5–10 times more effective than VRP for TAX and VCR reversal ability. Analog **6** also showed five-fold greater chemosensitizing effect against DOX than VRP. Importantly, active DDB analogs displayed no cytotoxicity against tumor cells established from different tissues, suggesting that our novel DDB analogs are significant lead compounds for further clinical development to overcome the MDR phenotype. Intracellular accumulation studies using Calcein-AM and DOX in KBvin cells clearly demonstrated that DDB analogs interfere with P-gp, drug efflux pump.

To conclude, newly synthesized DDB analogs were identified as P-gp inhibitors with new scaffold for non-toxic chemosensitizer drug development.

Experimental section

General

¹H NMR (400 MHz) spectra were measured on a Varian Inova spectrometer with TMS as the internal standard. Mass spectra were measured on a Shimadzu LCMS-IT-TOF. All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60 F254 plate, 20 × 20, Merck). 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 3, 7–12, 15–17, and 22—To a solution of **2** in CH₂Cl₂, 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 (3): Yield 85%; colorless oil; ¹H NMR (CDCl₃) 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 C₂₂H₂₂NaO₁₀ (M+Na)⁺ 469.1111, found 469.1116.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) dibutyrate (7): Yield 97%; colorless oil; ¹H NMR (CDCl₃) 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 (sex, *J* = 7.2 Hz, 4H), 0.90 (t, *J* = 7.2 Hz, 6H); HRMS calcd for C₂₆H₃₀NaO₁₀ (M+Na)⁺ 525.1737, found 525.1759.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(2-methylbutanoate) (8): Yield 83%; colorless oil; ¹H NMR (CDCl₃) 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 C₂₈H₃₄NaO₁₀ (M + Na)⁺ 553.2050, found 553.2063.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(3-methylbutanoate) (9): Yield 91%; colorless oil; ¹H NMR (CDCl₃) 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 C₂₈H₃₄NaO₁₀ (M + H)⁺ 531.2225, found.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) dipentanoate (10): Yield 99%; colorless oil; ¹H NMR (CDCl₃) 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 C₂₈H₃₄NaO₁₀ (M + Na)⁺ 553.2050, found 553.2067.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(2-methylpentanoate) (11): Yield 85%; colorless oil; ¹H NMR (CDCl₃) 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 C₃₀H₃₈NaO₁₀ (M + Na)⁺ 609.2676, found 609.2688.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) didodecanoate (12): Yield 88%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{42}\text{H}_{62}\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 749.4247, found 749.4225.

(2E,2'E)-(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(but-2-enoate) (15): Yield 8%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{26}\text{H}_{26}\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 521.1424, found 521.1449.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(3-methylbut-2-enoate) (16): Yield 51%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{28}\text{H}_{30}\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 549.1737, found 549.1761.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(hexa-2,4-dienoate) (17): Yield 55%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{30}\text{H}_{30}\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 573.1737, found 573.1746.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(3,4-dimethoxybenzoate) (22): Yield 90%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{36}\text{H}_{34}\text{NaO}_{14}$ ($\text{M} + \text{Na}$) $^+$ 713.1846, found 713.1839.

General procedure for compound 4–6, 13–14, 19–21, and 23–28—To a flask with anhydrous dichloromethane, compound **2** 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 was warmed up to room temperature gradually 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 (4): Yield 99%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{24}\text{H}_{27}\text{O}_{10}$ ($\text{M} + \text{H}$) $^+$ 497.1424, found 497.1432.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(2-methylpropanoate) (5): Yield 83%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{26}\text{H}_{30}\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 525.1737, found 525.1754.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(2,2-dimethylpropanoate) (6): Yield 82%; colorless oil; $^1\text{H NMR}$ (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 (13): Yield 99 %; colorless oil; ¹H NMR (CDCl₃) 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 (14): Yield 99%; colorless oil; ¹H NMR (CDCl₃) 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 (19): Yield 59%; colorless amorphous; ¹H NMR (CDCl₃) 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) (20): Yield 34%; colorless amorphous; ¹H NMR (CDCl₃) 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) (21): Yield 30%; colorless amorphous; ¹H NMR (CDCl₃) 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) (23): Yield 98%; colorless amorphous; ¹H NMR (CDCl₃) 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) (24): Yield 52%; colorless amorphous; ¹H NMR (CDCl₃) 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) (25): Yield 38%; colorless amorphous; ¹H NMR (CDCl₃) 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) (26): Yield 85%; colorless amorphous; $^1\text{H NMR}$ (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 $\text{C}_{34}\text{H}_{26}\text{NaO}_{14}$ ($\text{M} + \text{Na}$) $^+$ 681.1220, found 681.1188.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis[6-(trifluoromethyl)nicotinate] (27): Yield 61%; colorless oil; $^1\text{H NMR}$ (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 $\text{C}_{32}\text{H}_{22}\text{F}_6\text{N}_2\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 731.1076, found 731.1076.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) bis(quinoline-2-carboxylate) (28): Yield 97%; orange needles; mp: 129–130 °C; $^1\text{H NMR}$ (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 $\text{C}_{38}\text{H}_{29}\text{N}_2\text{O}_{10}$ ($\text{M} + \text{H}$) $^+$ 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) (18): To a solution of **2** (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 whole 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; $^1\text{H NMR}$ (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 $\text{C}_{26}\text{H}_{26}\text{NaO}_{14}$ ($\text{M} + \text{Na}$) $^+$ 585.1220, found 585.1228.

Dimethyl 3,3'-diiodo-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (30): Silver trifluoroacetate (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 with this condition. Yield 7%; colorless amorphous; $^1\text{H NMR}$ (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 $\text{C}_{20}\text{H}_{16}\text{I}_2\text{NaO}_{10}$ ($\text{M} + \text{Na}$) $^+$ 692.8731, found 692.8704.

3,3'-Dibromo-4,4'-dimethoxy-5,6,5',6'-bis(methylenedioxy)biphenyl-2,2'-dimethanol (31): 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; $^1\text{H NMR}$ (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 $\text{C}_{18}\text{H}_{16}\text{Br}_2\text{NaO}_8$ ($\text{M} + \text{Na}$) $^+$ 542.9089, found 542.9091.

(3,3'-Dibromo-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) dibutyrate (32): Compound **31** (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 CH₂Cl₂ overnight. The reaction mixture was subjected to preparative TLC to give the desired compound, which was recrystallized from CH₂Cl₂-hexane. Yield 86 %; colorless prisms; mp: 109–110 °C; ¹H NMR (CDCl₃) 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 C₂₈H₃₂Br₂NaO₁₀ (M + Na)⁺ 682.9926, found 682.9916.

(4,4'-Dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-diyl)bis(methylene) dibenzoate (33): The same procedure as for compound **32**, but benzoic acid was used instead of butyric acid. Yield 74 %; colorless prisms; mp: 127–128 °C; ¹H NMR (CDCl₃) 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 C₃₂H₂₄Br₂NaO₁₀ (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 (36): To a stirred solution containing anhydrous THF (5 mL) and **35** (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 CH₂Cl₂ (with less than 10 % MeOH), and the organic layer was dried over sodium sulfate. Flash chromatography (CH₂Cl₂—MeOH) was used to purify the desired compound. Yield 88 %; amorphous solid; ¹H 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 C₁₈H₁₄Br₂NaO₉ (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 were obtained from Lineberger Comprehensive 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 supplemented with 25 mM HEPES, 2 mM L-glutamine (Mediatech), 10 % heat inactivated fetal bovine serum (Hyclone), 100 IU penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Mediatech). KBvin cells were maintained in the culture medium containing 100 nM VCR. Cells were maintained at 37 °C in a humidifier with 5 % CO₂ atmosphere.

Cytotoxicity analysis (SRB assay)

Cytotoxicity was determined by the sulforhodamine B (SRB) colorimetric assay. Cells (3–5 × 10³ cells/well) were seeded in 96-well plates filled with culture medium containing various concentrations of samples for 72 h. At the end of the exposure period, the supernatant was removed and cells were washed with 100 μL fresh culture medium. The proliferated cells were fixed with 50% trichloroacetic acid for 30 min, and stained with 0.04% SRB (Sigma Chemical Co.) for 30 min. Protein-bound SRB dye was dissolved from stained cells in 10 mM Tris base, and absorbance was measured 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 chemosensitive cells were incubated with test compound in the presence of 100 nM TAX, which did not affect the cell growth. Multidrug resistant KBvin and parental KB cells were seeded at $5-7 \times 10^3$ cells/well into 96-well plates and incubated with 10 μ M test compound with 100 nM TAX for 72 h, and cell density was determined by a SRB assay. To assess the reversal activity of MDR by candidate compounds, it was evaluated by comparing IC₅₀ values of anticancer drugs (vincristine, paclitaxel, and doxorubicin) in the absence or presence of 10 μ M of test compound. IC₅₀ values were calculated by log-linear interpolation of data points. Verapamil, as a known P-gp inhibitor/modulator, was used as a positive control in all experiments. The reversal fold values, as potency parameter of test compounds, were calculated as: Reversal fold = IC₅₀ (anticancer drug alone) / IC₅₀ (anticancer drug + test compound). All experiments were performed at least three times.

Measurement of Intracellular Accumulation of Calcein or Doxorubicin

KBvin cells (5×10^3 cell/well) were seeded in 96-well plates and pretreated with samples for 1 h before Calcein-AM or doxorubicin was added to make final concentrations of 1 μ M Calcein-AM and 10 μ M doxorubicin. After 10 min for Calcein-AM or 3 h for DOX, the medium was removed by aspiration, and the cells were washed with ice-cold PBS followed by lysing with PBS containing 1% sodium dodecyl sulfate (SDS). The mean fluorescence intensity of Calcein or DOX was measured at Ex: 494 nm/Em: 517 nm or Ex: 488 nm/Em: 580 nm, respectively, by a fluorescence microplate reader (Plate Chameleon Multilabel Detection Platform, Hidex Oy, Turku, Finland) with MikroWin software. VRP or CsA was used as a positive control of P-gp modulator. All data for intracellular accumulation of DOX were calculated as the fluorescence intensity, and were represented as fold changes by treatment with compound compare with vehicle (DMSO) treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

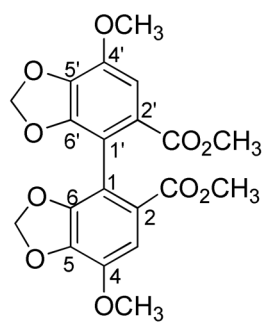
DDB	dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate
MDR	multi-drug resistance
P-gp	P-glycoprotein
MRP	multidrug resistance-associate protein
TAX	paclitaxel
VCR	vincristine
DOX	doxorubicin
VRP	verapamil
EDCI	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride

DMAP	4-dimethylaminopyridine
DIBAL	diisobutylaluminium hydride

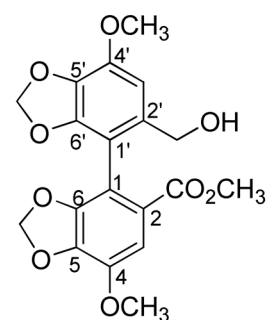
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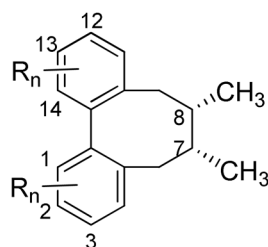
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1: DDB



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-dimethylenedioxy

Dibenzocyclooctadiene lignan

Figure 1.
Structures of DDB, Bicyclol and Dibenzocyclooctadiene Lignans

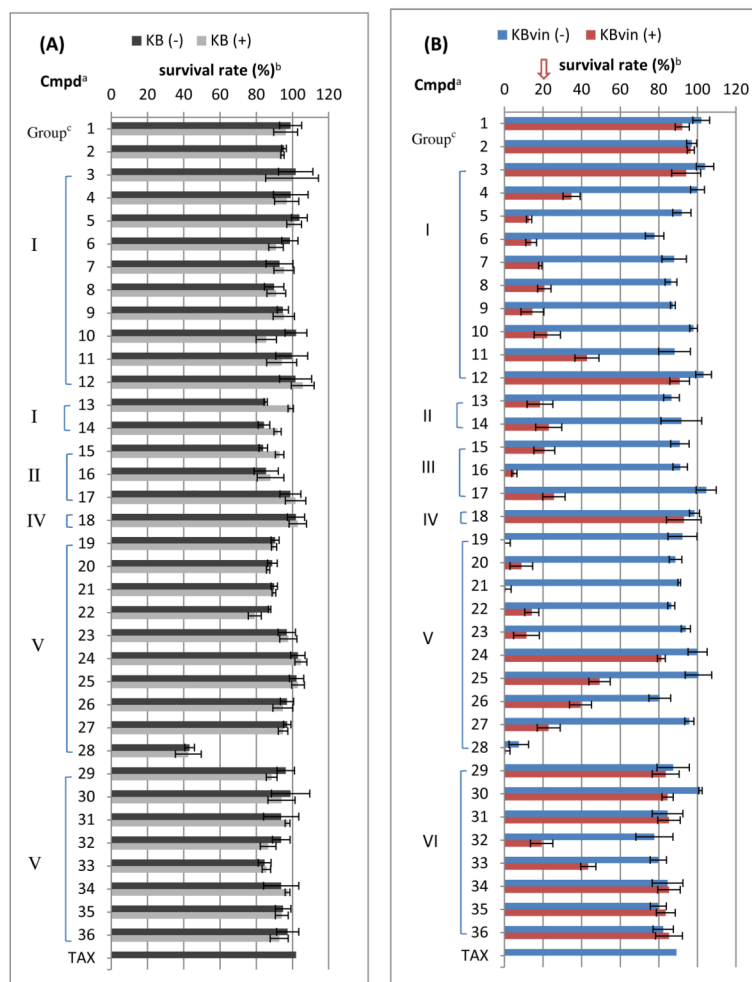
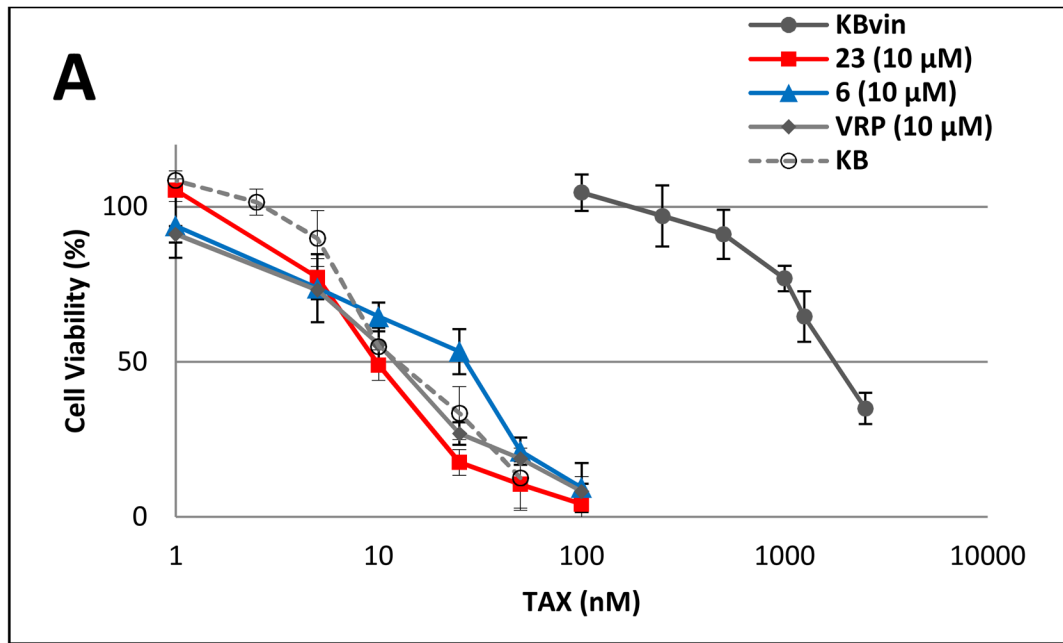


Figure 2. Screening of Reversal Abilities against KB (A) and KBvin (B)

Note: ^a Concentration of compounds: 10 μ M, ^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. ^cGroup (I) saturated acyclic alkyl; (II) cyclic alkyl ; (III) unsaturated; (IV) polar; (V): aromatic; (VI): halogenated.



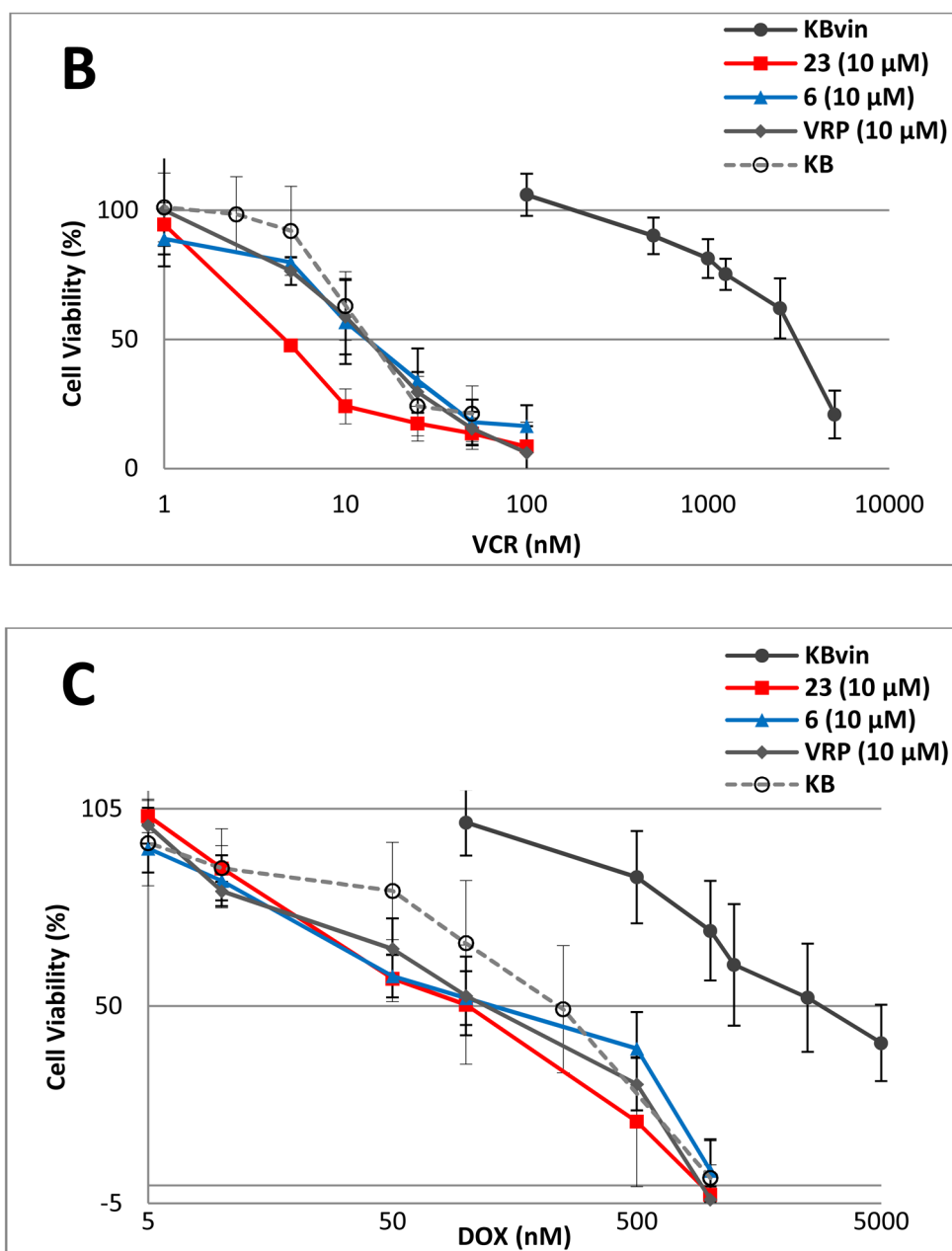


Figure 3. Reversal of chemoresensitivity of KBvin by 6 or 23

Chemoresistant KBvin cells were incubated with various concentrations of anticancer drugs TAX (A), VCR (B), or DOX (C) in the presence of test compounds, as indicated, for 72 h to evaluate the effect on chemosensitization. KB cells (○) were sensitive to anticancer drugs, while KBvin (●) were resistant in the absence of test compounds. Chemosensitization of KBvin cells was observed when the cells were co-treated with 10 μ M of 6 (▲), 23 (■), or VRP (◆).

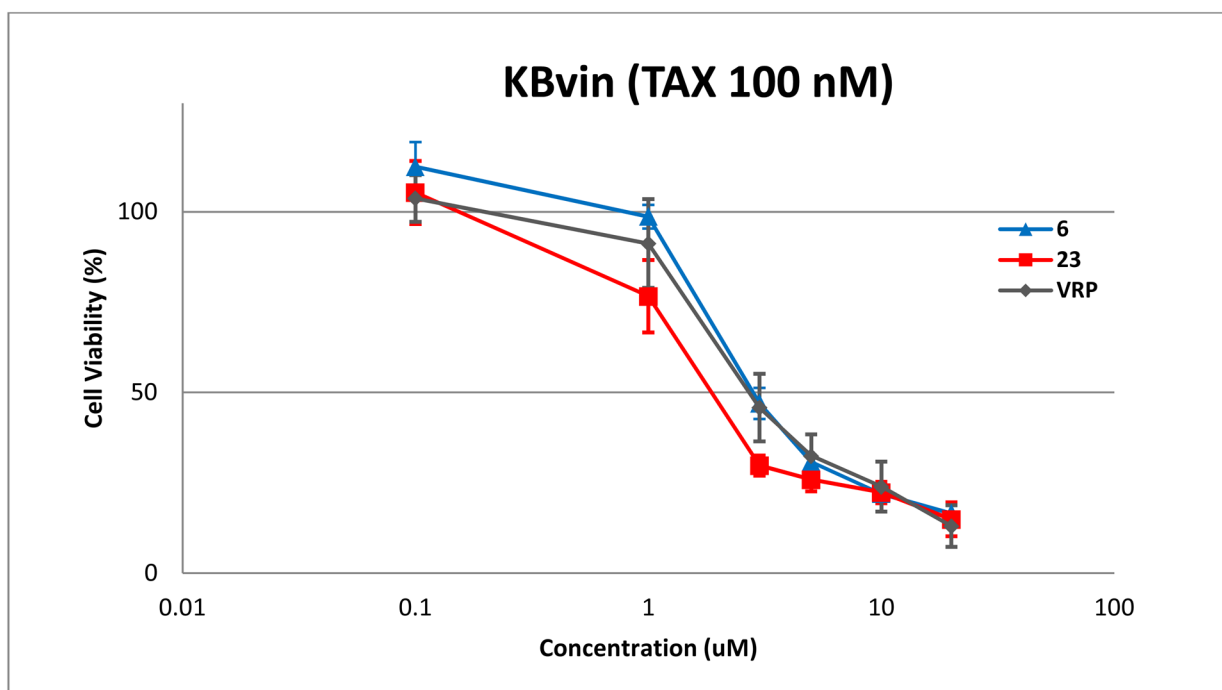


Figure 4. Dose response effect of compounds 6 and 23 on sensitization of KBvin to TAX
Multidrug resistant KBvin cells were treated with various concentrations of compounds **6** or **23** in the presence of 100 nM TAX, an absolutely non-toxic concentration for KBvin. Data are expressed as mean \pm SE of three independent experiments. Calculated median effective concentration (EC_{50}) of compounds **6**, **23**, or VRP was 2.81 μ M, 1.87 μ M or 2.71 μ M, respectively.

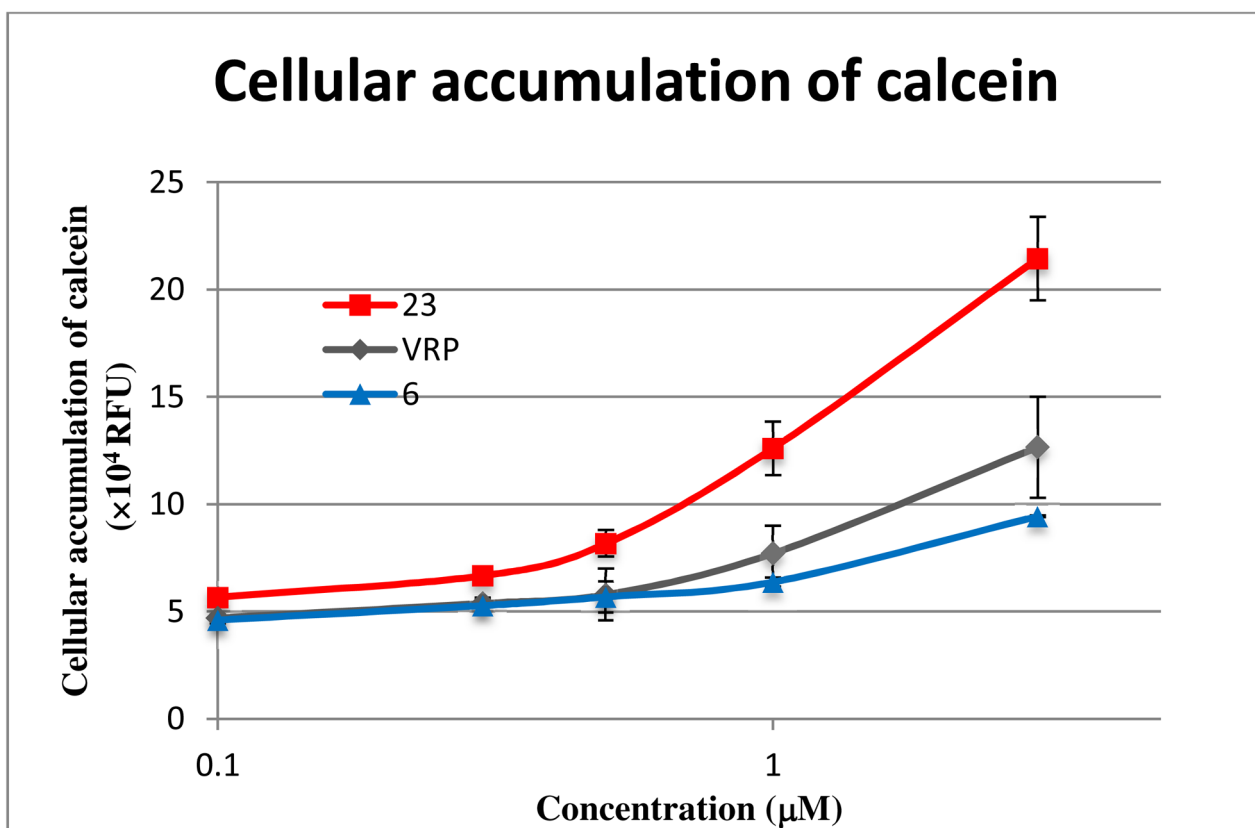


Figure 5. Effect of compounds on P-gp function in KBvin cells

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. Data with mean \pm SD of three independent experiments are shown in suppl. Table S2.

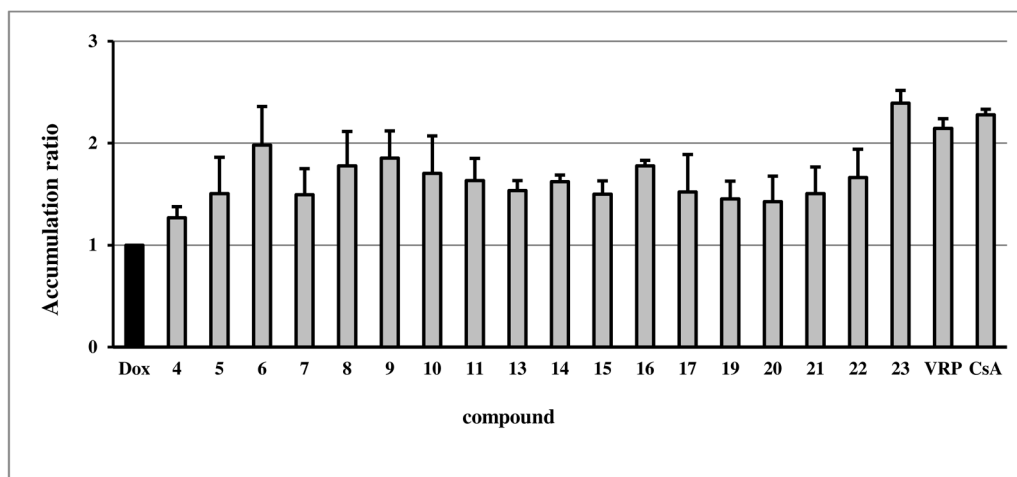


Figure 6. Recovered DOX Accumulation in DDB Analog-treated Drug Resistant KBvin Cells
KBvin cells were incubated in DOX medium (final conc. 10 μM) for 3 h in the presence of 10 μM DDB analogs, and then cellular accumulation of DOX was measured as intrinsic fluorescence intensity of DOX. Fluorescence intensity of DOX was expressed as the ratio of effect of compound to negative control (Dox). Intracellular accumulation of DOX was clearly observed in the presence of DDB-derived compounds. Data represent as mean \pm SD, $n=3$. P-gp inhibitor VRP (10 μM) or cyclosporine (CsA) (5 μM) was used as a positive control.

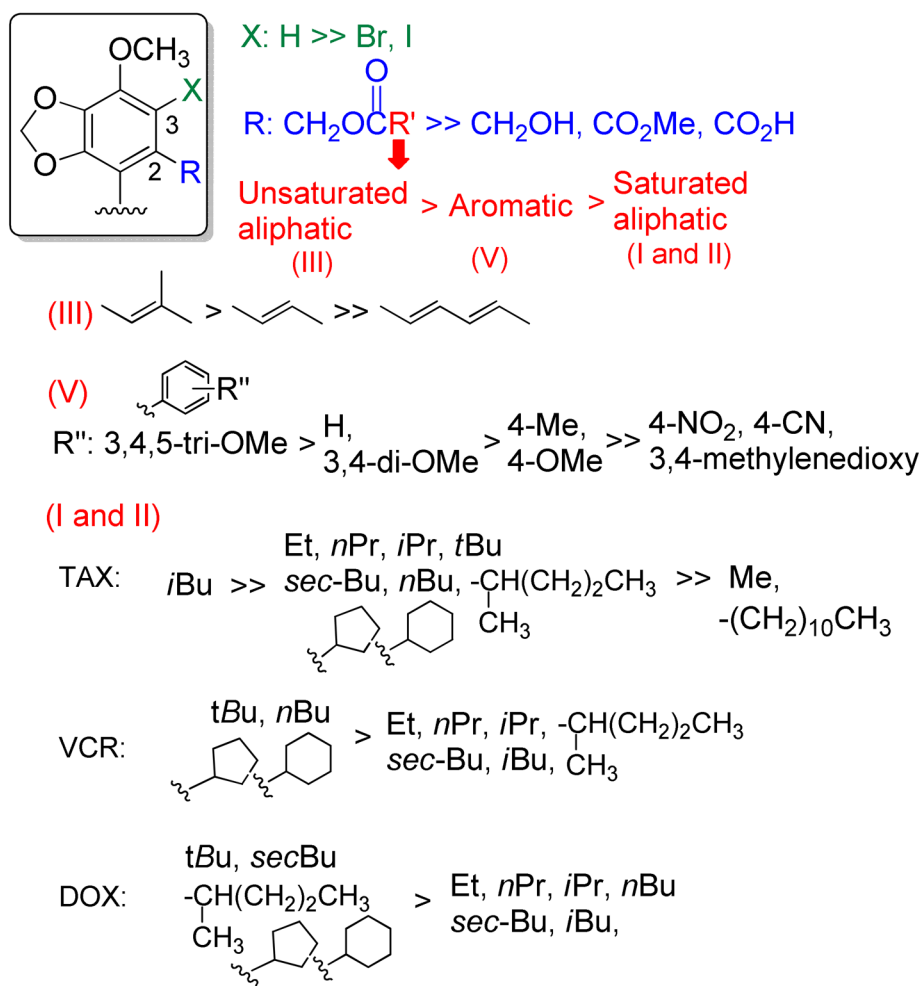
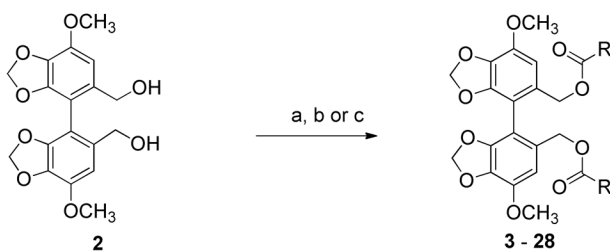
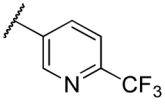
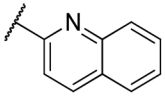


Figure 7.
Study of Structure-Activity Relationship

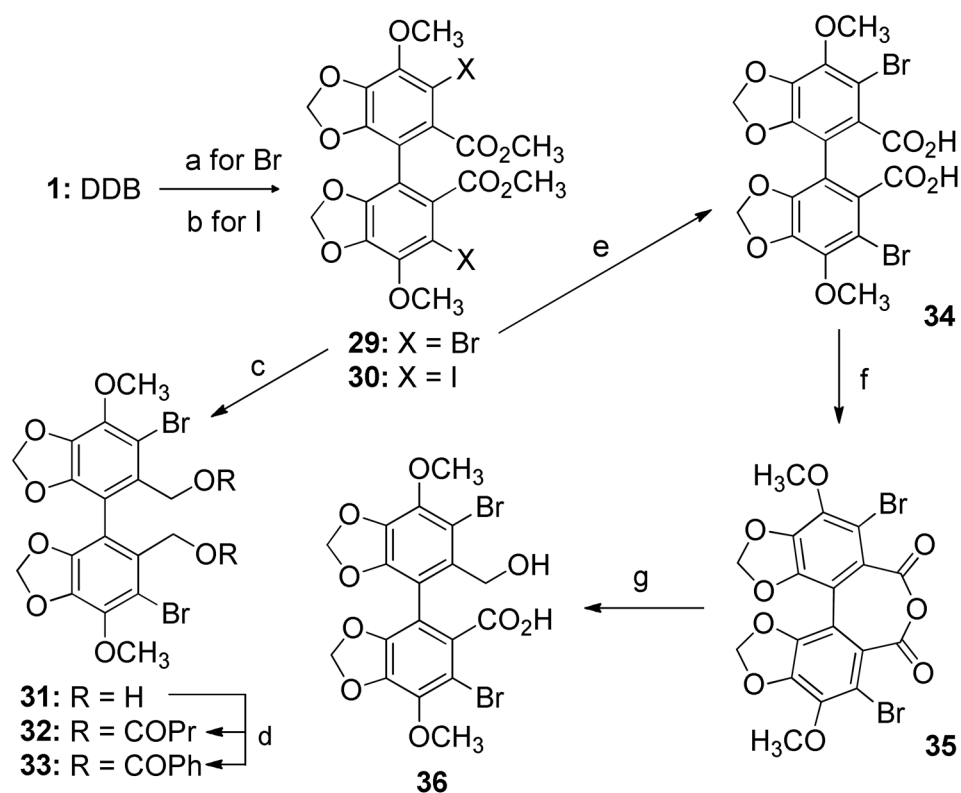


Reagents and conditions: (a) RCO_2H , EDCI, DMAP, for **3**, **7-12**, **15-17**, and **22**
 (b) RCOCl , Et_3N for **4-6**, **13-14**, **19-21**, and **23-28**
 (c) acid anhydride, DMAP for **18**

Cmpd	R		R		R	
	Aliphatic Acyclic Group (I)		Aliphatic Cyclic Group (II)		Aromatic Group (V)	
3	Me		13		19	R' = H
4	Et		14		20	R' = 4-Me
5	iPr				21	R' = 4-OMe
6	tBu			Unsaturated Group (III)	22	R' = 3,4-diOMe
7	Pr		15		23	R' = 3,4,5- triOMe
8	sec-Bu		16		24	R' = 4-NO ₂
9	iBu		17		25	R' = 4-CN

10	Bu			26	R' = 3,4-methylenedioxy
11	-CH(CH ₃)CH ₂ CH ₂ CH ₃		Polar Group (IV)	27	
12	-(CH ₂) ₁₀ CH ₃	18	-CH ₂ CH ₂ CO ₂ H	28	

Scheme 1. Syntheses of Diester Derivatives 3–28



Scheme 2. Syntheses of Dihalogenated DDB Analogues

Reagent and conditions: (a) Br_2 , CHCl_3 , 0°C , (b) $\text{CF}_3\text{CO}_2\text{Ag}$, I_2 , (c) DIBAL, CH_2Cl_2 , (d) EDCI, DMAP, CH_2Cl_2 , $\text{CH}_3(\text{CH}_2)_2\text{CO}_2\text{H}$ for 32, and PhCO_2H for 33, (e) KOH, EtOH, reflux, (f) Ac_2O , reflux, (g) NaBH_4 , THF, MeOH

Table 1

Reversal Effects with Paclitaxel (TAX), Vincristin (VCR), and Doxorubicin (DOX) in KBvin

Group	Cmpd ^a	IC ₅₀ of TAX (nM) ^b	Fold ^c	IC ₅₀ of VCR (nM)	Fold	IC ₅₀ of DOX (nM)	Fold
	TAX	976.8	-	2520.7	-	1942.3	-
I	4	82.93	12	80.96	31	507.5	4
	5	46.52	21	41.42	61	284.0	7
	6	26.15	37	7.22	349	42.6	46
	7	30.50	32	50.81	50	510.9	4
	8	37.06	26	35.92	70	93.9	21
	9	8.72	112	31.75	79	339.7	6
	10	40.74	24	15.65	161	221.5	9
II	11	39.11	25	47.31	53	61.9	31
	13	48.65	20	8.14	310	86.3	23
	14	39.89	24	10.55	239	65.8	30
	15	9.55	102	10.98	230	547.8	4
III	16	2.99	326	4.50	560	291.5	7
	17	47.45	21	46.53	54	573.9	3
	19	7.65	167	20.21	101	157.7	9
V	20	46.82	27	39.61	51	130.8	11
	21	47.23	27	40.53	50	131.4	11
	22	14.25	89	10.10	202	68.80	21
	23	4.41	222	4.45	566	84.2	23
	VRP	31.87	31	23.04	109	219	9

^aConcentration of compound: 10 μM.^bSD was shown in Supporting Information.^cThe reversal fold values were calculated as: Reversal fold = IC₅₀ (anticancer drug alone) / IC₅₀ (anticancer drug + test compound).

Table 2

cLogP Values of Synthesized DDB-derived Compounds

Cmpd	cLog P ^a	Cmpd	cLog P
1	4.27	19	6.93
2	1.26	20	7.93
3	2.98	21	7.28
4	4.03	22	6.69
5	4.65	23	5.93
6	5.45	24	6.42
7	5.09	25	5.80
8	5.71	26	7.31
9	5.89	27	6.11
10	6.15	28	6.21
11	6.77	29	4.68
12	N/A	30	5.02
13	5.92	31	2.60
14	7.04	32	7.49
15	5.24	33	8.27
16	6.04	34	1.96
17	6.51	35	5.34
18	2.40	36	2.28
		VRP	4.58

^a cLogP was calculated by ChemDraw Ultra Version 12.0