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Antitumor Agents. 293. Nontoxic Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB) Analogues Chemosensitize Multidrug-Resistant Cancer Cells to Clinical Anticancer Drugs

Hsin-Yi Hung,[†] Emika Ohkoshi,[†] Masuo Goto,[‡] Kenneth F. Bastow,[§] Kyoko Nakagawa-Goto,*[,†] and Kuo-Hsiung Lee*[,†,⊥]

[†]Natural Products Research Laboratories, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599-7568, United States

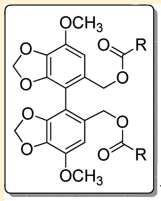
[‡]Cell and Developmental Biology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7090, United States

[§]Division of Chemical Biology & Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599-7568, United States

[⊥]Chinese Medicine Research and Development Center, China Medical University and Hospital, Taichung, Taiwan

Supporting Information

ABSTRACT: Novel dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB) analogues were designed and synthesized to improve their chemosensitizing action on KBvin (vincristine-resistant nasopharyngeal carcinoma) cells, a multidrug resistant cell line overexpressing 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. Analogue **6** also exhibited five times greater chemosensitizing effect against DOX than VRP. Importantly, no cytotoxicity was observed by the active DDB analogues against both non-MDR and MDR cells, suggesting that DDB analogues serve as novel lead compounds for the development of chemosensitizers to overcome the MDR phenotype. The mechanism of action studies demonstrated that effective inhibition of P-glycoprotein by DDB analogues dramatically elevated the cellular concentration of anticancer drugs.



Compounds	R	Chemoreversal Ability[a] with Paclitaxel	Vincristine	Doxorubicin
6	tBu	37	349	46
16	-CH=C(CH$_3$)$_2$	326	560	7
23		222	566	23
	Verapamil	31	109	9

[a]Chemoreversal abilities were shown as the reversal fold values [IC_{50} (anticancer drug alone) / IC_{50} (anticancer drug + test compound)].

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 overexpression of glutathione S-transferase-π.[1−3] An emerging understanding of cancer resistance results from cancer stem cell-like features.[4] However, overexpression 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 because of 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

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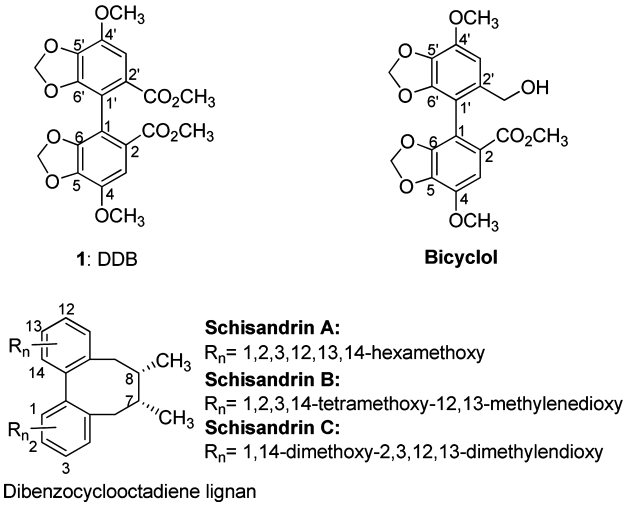

Figure 1. Structures of DDB, bicyclol, and dibenzocyclooctadiene lignans.

and B, also chemosensitized various anticancer drugs, including vincristine (VCR), daunorubicin, and etoposide, in human promyelocytic leukemia cell lines with overexpressed MRP1/ABCC1.[12] Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxy-biphenyl-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 Bel_{7402} cells via inhibition of P-gp and enhancement of apoptosis.[14] However, a very high concentration (50 μM) was required for effective reversal action. Cotreatment 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 analogues 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 AUC_{0-24} 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 analogue 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 analogues have been prepared, their MDR reversal abilities and structure–activity relationship (SAR) correlations have not been investigated. To explore more potent nontoxic MDR reversal analogues with lower effective dosing and to study SAR, we designed and synthesized additional DDB analogues. Herein, we report the chemosensitizing effects of newly synthesized DDB analogues.

DESIGN AND SYNTHESES

On the basis of 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 Bel_{7402} and acquired MDR MCF-7/Adr.[14] Since DDB acts against multitargets, 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′- bishydroxymethyl 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 (group III), polar (group IV), and aromatic (group V) groups, which could be transformed into water-soluble salts, if necessary. Esterifications of **2** were performed by treatment with the related acid (RCO_2H), *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 Et_3N to give **4**–**6**, **13**–**14**, **19**–**21**, and **23**–**28** (Scheme 1). Halogenated DDB analogues with various functional groups were also designed to explore their effects (Scheme 2) since 3,3′-dibromo-DDB has shown 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 trifluoacetate and iodine to provide **30**.[20] The diester groups of **29** were reduced with diisobutylaluminium 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 Ac_2O gave anhydrous analogue **35**, followed by asymmetric cleavage of the carbonyl anhydrous bridge with $NaBH_4$, resulting in di-Br bicyclol analogue **36**.[21] All synthesized analogues 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 subline, KBvin (overexpression 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 (IC_{50} ~50 μM) and KBvin cells (IC_{50} ~ 80 μM). Although compounds **28** and **32** were slightly cytotoxic, most of the DDB-derived compounds did not exhibit significant cytotoxicity (IC_{50} > 100 μM), which implied low toxicity of these analogues (Supporting Information Table S1).

For evaluating chemosensitizing activity, KB and KBvin cells were cotreated with test compounds at 10 μM and the anticancer drug paclitaxel (TAX) (Figure 2). As shown in Figure 2B, DDB (**1**) and diol **2** did not exhibit MDR reversal effect at a concentration of 10 μM in KBvin cells. 3,3′-Dihalogenated DDB analogues with different 2,2′-functional groups did not show potent activity, except for 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 [$CH_2OC(O)R$] appeared to be critical for reversal ability activity, 3,3′-halogenation reduced the potency (compare **33** with **19**).

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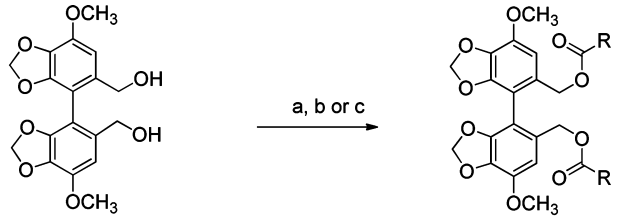
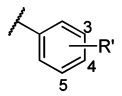
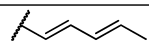
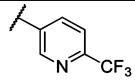
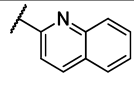
Scheme 1. Syntheses of Diester Derivatives 3−28[a]



2 **3 - 28**

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 **Aliphatic Acyclic Group (I)**	Cmpd	R **Aliphatic Cyclic Group (II)**	Cmpd	R **Aromatic Group (V)** 
3	Me	**13**		**19**	R' = H
4	Et	**14**		**20**	R' = 4-Me
5	*i*Pr			**21**	R' = 4-OMe
6	*t*Bu		**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	*i*Bu	**17**		**25**	R' = 4-CN
10	Bu			**26**	R' = 3,4-methylenedioxy
11	-$CH(CH_3)CH_2CH_2CH_3$		**Polar Group (IV)**	**27**	
12	-$(CH_2)_{10}CH_3$	**18**	-$CH_2CH_2CO_2H$	**28**	

[a]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**−**38**, (c) acid anhydride, DMAP for **18**.

Many of the analogues with aliphatic ester substituents (groups 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-methybutyl) 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.

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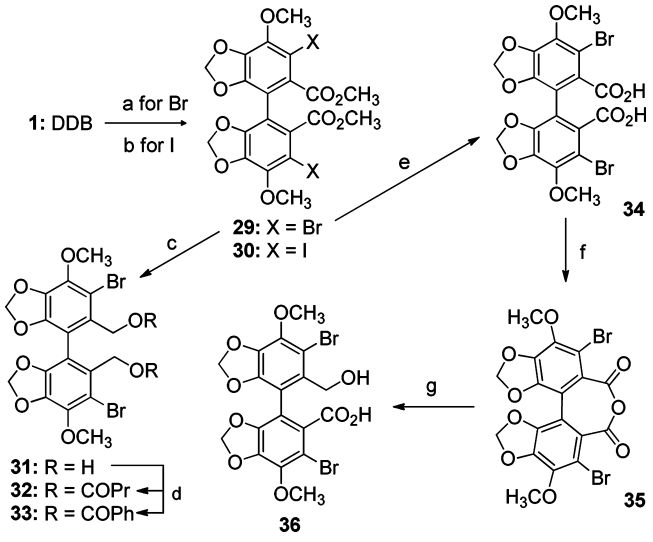
Scheme 2. Syntheses of Dihalogenated DDB Analogues[a]



[a]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 **32**, and $PhCO_2H$ for **33**, (e) KOH, EtOH, reflux, (f) Ac_2O, reflux, (g) $NaBH_4$, THF, MeOH.

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 = 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. 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 1 compounds (**4**−**11**), except for **3** and **12**, the group II and III compounds (**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**, **35**, **36**), which generally had weaker reversal ability, as well as compounds with inherent cytotoxicity (**28**) were eliminated from further testing.

Chemoreversal Ability of DDB Analogues with TAX, VCR, and Doxorubicin (DOX). A quantitative evaluation of the reversal ability of DDB analogues **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 IC_{50} value of anticancer drugs in the presence of test compounds 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 drugs plus DDB analogue. 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 analogues, 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** and **14**) were more effective than noncyclic 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 = isobutyl) **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 analogue **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 moderately 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 *p*-methyl (**20**) and *p*-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 the phenyl ring resulted in the following order of potency: 3,4-di-OMe (**22**) ≈ 3,4,5-tri-OMe (**23**) > H (**19**) ≈ 4-Me (**20**) ≈ 4-OMe (**21**). In conclusion, analogues **16** and **23** showed significant TAX and VCR cytotoxic reversal ability, and analogue **6** exhibited the most potent DOX cytotoxic reversal ability.

On the basis of 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 μM 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 IC_{50} values over 1000 nM. When 10 μM 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 that of VRP. KBvin became even

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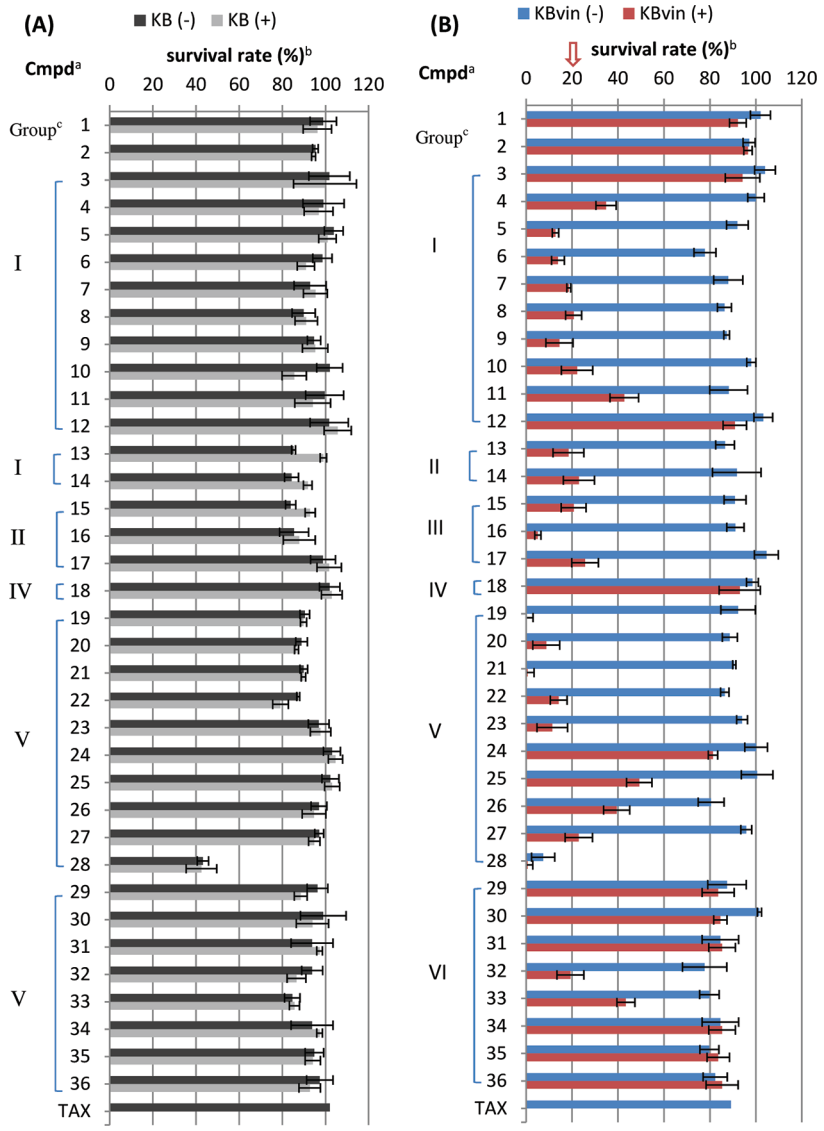

Figure 2. Screening of reversal abilities against KB (A) and KBvin (B). Note: [a]Concentration of compounds: 10 μM; [b]survival rate (%) was measured by SRB method using KB and KBvin cells in the presence (+) or absence (−) of paclitaxel (TAX). Compounds with survival rates below 20% were considered very potent and moved to further experiments. [c]Group (I) saturated acyclic alkyl; (II) cyclic alkyl ; (III) unsaturated; (IV) polar; (V) aromatic; (VI) halogenated.

more sensitive to VCR than KB, when KBvin cells were cotreated with 10 μM **23** and VCR. These results demonstrated that 10 μM 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 nontoxic 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 (EC_{50}) value of **6** (2.81 μM) was similar to that of VRP (2.71 μM), compound **23** (1.87 μM) exhibited a lower EC_{50} than VRP (*P* values are given in Supporting Information Table S3). These results demonstrate that **23** could be more effective than VRP in chemosensitizing the MDR cells to TAX.

The Effect of DDB Analogues on P-gp Function in KBvin Cells. To confirm our hypothesis that DDB analogues 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, Supporting Information Table S4). Dose-dependent intracellular accumulation of calcein was observed in the presence of these compounds. Although **6** was slightly less potent than VRP, **23** was up to 2-fold more potent than VRP, especially at concentrations around the EC_{50} value (1.87 μM) of **23**. Therefore, these results clearly indicated that DDB analogues, 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 pretreated with compounds followed by addition of DOX. Intracellular accumulation of DOX was measured as the fluorescence intensity and standardized as fold ratio. All DDB analogues induced DOX accumulation in KBvin cells at 1.2- to 2.4-fold. The cellular accumulation of DOX by DDB analogues was

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Table 1. Reversal Effects with Paclitaxel (TAX), Vincristine (VCR), and Doxorubicin (DOX) in KBvin

group	compd[a]	IC_{50} of TAX (nM)[b]	fold[c]	IC_{50} of VCR (nM)	fold	IC_{50} of DOX (nM)	fold
		976.8 (TAX alone)	–	2520.7 (VCR alone)	–	1942.3 (DOX alone)	–
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
	11	39.11	25	47.31	53	61.9	31
II	**13**	48.65	20	8.14	310	86.3	23
	14	39.89	24	10.55	239	65.8	30
III	**15**	9.55	102	10.98	230	547.8	4
	16	2.99	326	4.50	560	291.5	7
	17	47.45	21	46.53	54	573.9	3
V	**19**	7.65	167	20.21	101	157.7	9
	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

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

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 analogues 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 analogues interfere with the drug efflux function of P-gp.

Hydrophobicity Evaluation of Active DDB Analogues. P-gp contains a large drug-binding pocket with a volume of around 6000 Å.[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 analogues could influence their MDR reversal activity. Table 2 shows the clogP values of synthesized compounds. Although a few exceptions were present, the chemosensitizing effects of compounds were moderately correlated with their clogP values. Active compounds had clogP values of 4–8; those with clogP lower than 4 tended to lose chemosensitizing activity. The clogP 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, the reduced 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, partly because of their toxicity. We selected DDB, a clinically used hepatoprotective compound, as a lead, and 33 new DDB analogues were newly designed and synthesized. All synthesized analogues 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′-methyl ester group to DDB, and SAR studies are summarized in Figure 7. Insertion of a 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 analogues. Among all tested compounds, DDB derivatives **16** and **23** were 5–10 times more effective than VRP for TAX and VCR reversal ability. Analogue **6** also showed 5-fold greater chemosensitizing effect against DOX than VRP. Importantly, active DDB analogues displayed no cytotoxicity against tumor cells established from different tissues, suggesting that our novel DDB analogues 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 analogues interfere with the P-gp drug efflux pump. To conclude, newly synthesized DDB analogues were identified as P-gp inhibitors possessing a new scaffold for nontoxic chemosensitizer drug development.

EXPERIMENTAL SECTION

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

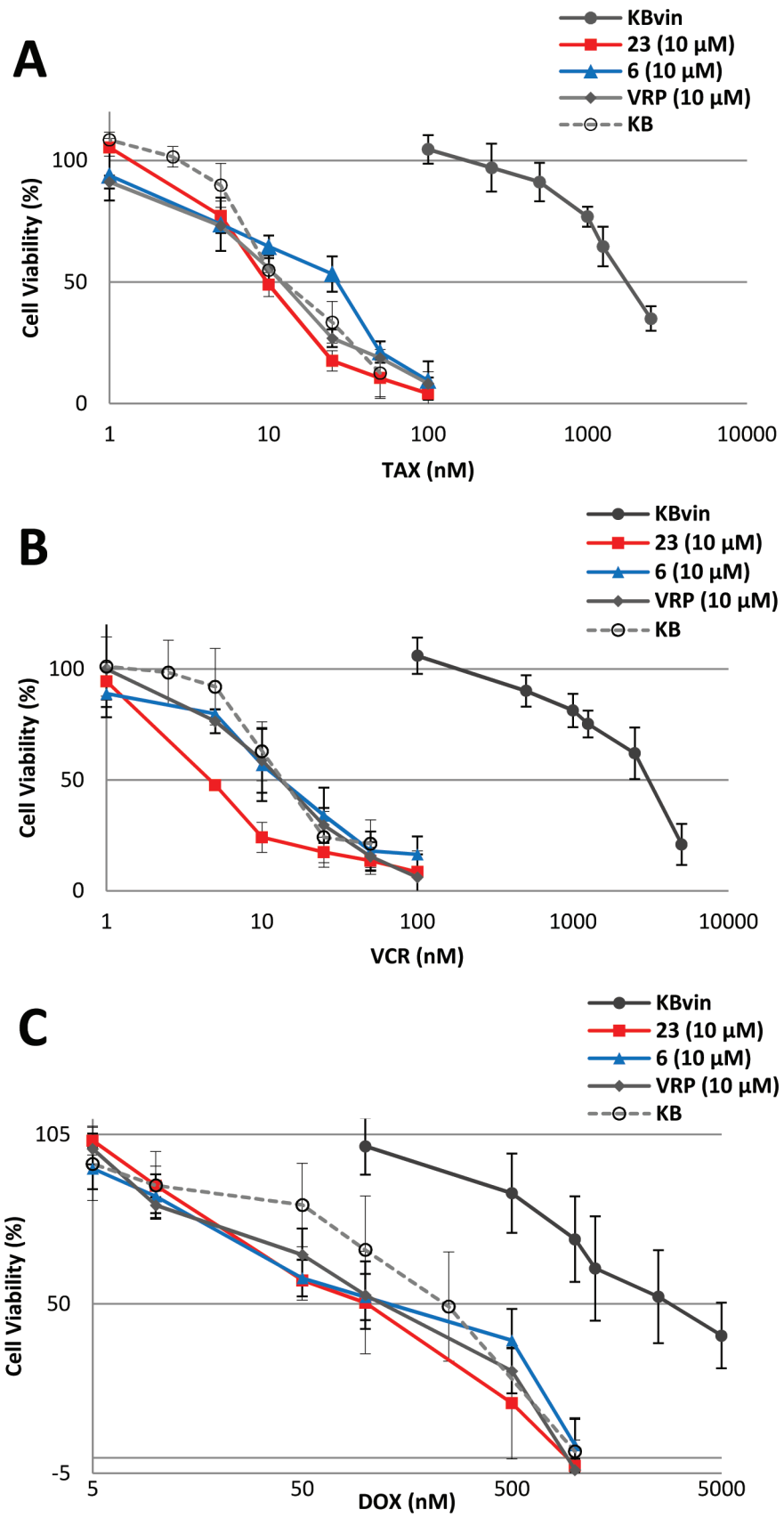

Figure 3. Reversal of chemosensitivity of KBvin by **6** or **23**. Chemoresistant KBvin cells were incubated with various concentrations of anticancer drugs TAX (A), VCR (B), or DOX (C) in the presence of test compounds, as indicated, for 72 h to evaluate the effect on chemosensitization. KB cells (○) 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 cotreated with 10 μM of **6** (▲), **23** (■), or VRP (♦).

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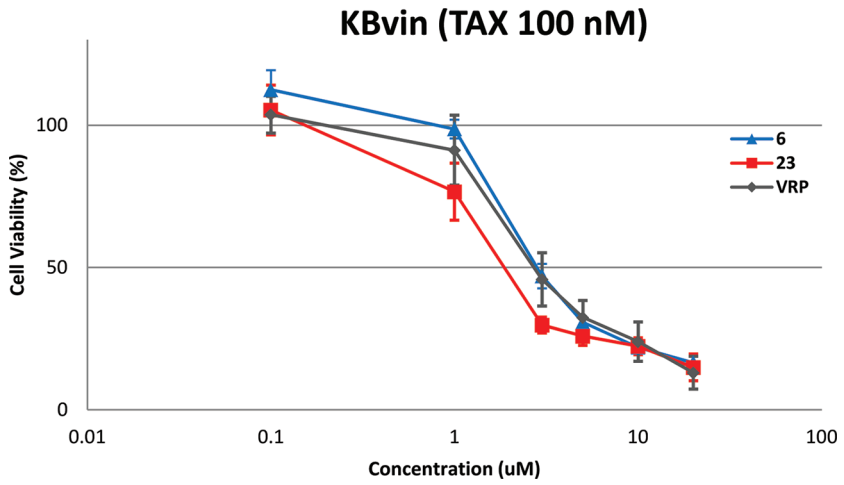

Figure 4. Dose−response effect of compounds **6** and **23** on sensitization of KBvin to TAX. Multidrug-resistant KBvin cells were treated with various concentrations of compounds **6** or **23** in the presence of 100 nM TAX, an absolutely nontoxic concentration for KBvin. Data are expressed as mean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

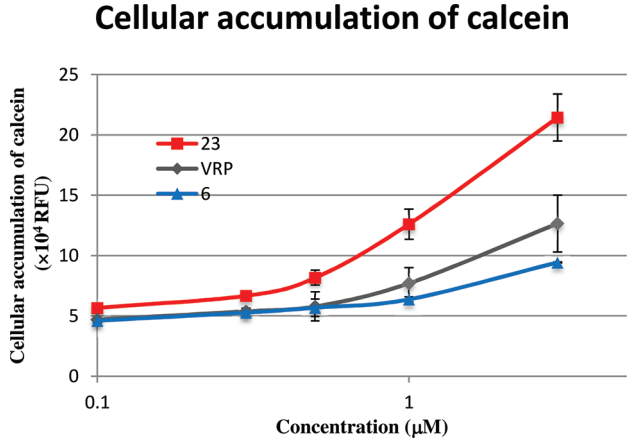

Figure 5. Effect of compounds on P-gp function in KBvin cells. KBvin cells were pretreated with compounds followed by addition of calcein-AM. The cellular accumulation of calcein is represented by the relative fluorescent unit (× 10^4 RFU). Cellular accumulation of calcein demonstrates inhibition of efflux activity of P-gp. Data with mean ± SD of three independent experiments are shown in Supporting Information Table S2.

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 CH_2Cl_2 were added an appropriate carboxylic acid (5 equiv mol), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (5 equiv mol), and 4-(dimethylamino)-pyridine (1 equiv mol) and stirred overnight. The reaction mixture was then applied directly to preparative TLC (hexane−EtOAc) without workup.

*(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)-bis(methylene) Diacetate (**3**).* 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 (**7**).* 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) (**8**).* 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) (**9**).* 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 (**10**).* 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) (**11**).* 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 (**12**).* 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.

*(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}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) (**16**).* 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

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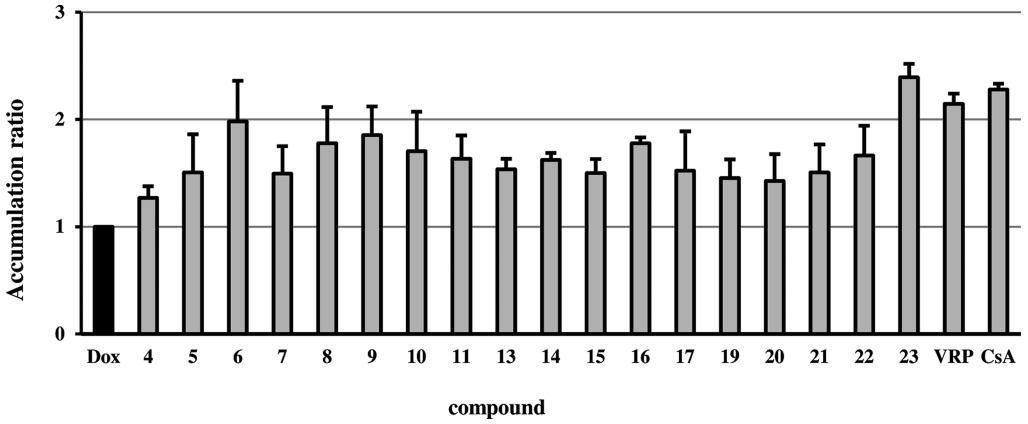

Figure 6. Recovered DOX accumulation in DDB analogue-treated drug resistant KBvin cells. KBvin cells were incubated in DOX medium (final concn 10 μM) for 3 h in the presence of 10 μM DDB analogues, and then cellular accumulation of DOX was measured as the intrinsic fluorescence intensity of DOX. The 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 are represented as mean ± SD, n = 3. P-gp inhibitor VRP (10 μM) or cyclosporine (CsA) (5 μM) was used as a positive control.

Table 2. cLogP Values of Synthesized DDB-Derived Compounds

compd	clogP[a]	compd	clogP
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.

= 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) (**17**).* 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) (**22**).* 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 4−6, 13, 14, 19−21, and 23−28. To a flask with anhydrous dichloromethane were added compound **2** and triethylamine (5−10 equiv mol) first, and then the appropriate acyl chloride (2.2 equiv mol) was added at 0 °C under nitrogen. The reaction was warmed 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 performed 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}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) (**5**).* 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) (**6**).* 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 (**13**).* 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 (**14**).* 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 (**19**).* 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) (**20**).* 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.

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*(4,4′-Dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-diyl)-bis(methylene) Bis(4-methoxybenzoate) (**21**).* 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) (**23**).* 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) (**24**).* 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) (**25**).* 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) (**26**).* 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] (**27**).* 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) (**28**).* 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) (**18**).* To a solution of **2** (26 mg, 0.072 mmol) in anhydrous THF (2.0 mL) were added succinic anhydride (9 mg, 0.090 mmol) and DMAP (5 mg, 0.040 mmol). The mixture was refluxed overnight. After cooling to rt, the whole was acidified with 1 N 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 (**30**).* 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 performed by preparative TLC (hexane−EtOAc: 7:3). Mono- and diiodo products were found under these conditions. Yield 7%; colorless amorphous; ^{1}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 $C_{20}H_{16}I_2NaO_{10}$ $(M + Na)^+$ 692.8731, found 692.8704.

*3,3′-Dibromo-4,4′-dimethoxy-5,6,5′,6′-bis(methylenedioxy)-biphenyl-2,2′-dimethanol (**31**).* To a stirred solution containing **1** (95.2 mg, 0.165 mmol) and 1 mL of anhydrous CH_2Cl_2 under nitrogen at around −20 °C (ice with brine) was added diisobutylaluminum hydride (DIBAL) (0.83 mL, 0.83 mmol) dropwise. After 1 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 was 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 (**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), and 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 (**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; ^{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 (**36**).* To a stirred solution containing anhydrous THF (5 mL) and **35** (69.4 mg, 0.13 mmol) under nitrogen was added sodium borohydride (15 mg, 0.39 mmol), followed by MeOH (0.1 mL), 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 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 of 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% CO_2 atmosphere.

Cytotoxicity Analysis (SRB assay). Cytotoxicity was determined by the sulforhodamine B (SRB) colorimetric assay. Cells (3−5 × 10^3 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 of 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 μM 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 IC_{50} values of anticancer drugs (vincristine, paclitaxel, and doxorubicin) in the absence or presence of 10 μM test compound. IC_{50} 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 value, as the potency parameter of test compounds, was calculated as follows: reversal fold = IC_{50} (anticancer drug alone)/IC_{50} (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 μM calcein-AM and 10 μM 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 the 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 compared with vehicle (DMSO) treatment.

■ ASSOCIATED CONTENT

Supporting Information

Cytotoxicity of DDB analogues (Table S1). IC_{50} ± 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.

■ AUTHOR INFORMATION

Corresponding Author

*(K.H.L.): phone: 919-962-0066; fax: 919-966-3893; e-mail: khlee@unc.edu. (K.N.G.): phone: 919-843-6325; e-mail: goto@email.unc.edu.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DDB, dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate; MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; TAX, paclitaxel; VCR, vincristine; DOX, doxorubicin; VRP, verapamil; EDCI, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine; DIBAL, diisobutylaluminium hydride

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