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3,5-Dibenzoyl-1,4-dihydropyridines: Synthesis and MDR Reversal in Tumor Cells

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Abstract—Fifteen 4-phenyl-3,5-dibenzoyl-1,4-dihydropyridines (BzDHPs) (**1**–**15**) substituted at the 4-phenyl ring were synthesized and compared to their cytotoxic activity and multidrug resistance (MDR)-reversing activity in in vitro assay systems. Among them, 2-CF_3 (**5**) ($IC_{50} = 8.7\,\mu M$), 2-Cl (**11**) ($IC_{50} = 7.0\,\mu M$) and 3-Cl (**12**) ($IC_{50} = 7.0\,\mu M$) derivatives showed the highest cytotoxic activity against human oral squamous carcinoma (HSC-2) cells. The activity of P-glycoprotein (Pgp) responce for MDR in tumor cells was reduced by some of derivatives (**3, 4, 8, 12**), verapamil (VP) and nifedipine (NP). These data suggest that 3,5-dibenzoyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (**12**) can be recommended as a new drug candidate for MDR cancer treatment.

Introduction

Multidrug resistance (MDR) of cancer cells has often been correlated with the overexpression of P-glycoprotein (Pgp). An ABC transporter ATP-binding cassette acts as a cellular pump membrane transporter by extruding the anticancer agents and preventing their antitumor effect.[1] Therefore, it is important to develop molecules that can inhibit Pgp activity.[2,3] A variety of compounds have been shown to inhibit Pgp-mediated drug efflux.[4] Characterizing common structural features of Pgp-blocking agents is challenging. In general, MDR active compounds are highly lipophilic and have aromatic ring systems in the molecule[5] and a cationic or dicationic side chain.[4] Most compounds also possess a tertiary nitrogen atom with positive charge at a physiological pH.[6] Among the possible resistance modifiers, the dihydropyridines (DHPs), calcium antagonists, have been studied extensively as the analogue of verapamil (VP).[7] In a combination treatment with antitumor agents, such as *vinca* alkaloids or anthracyclines, and VP, cardiovascular side effects were found.[8] It is very important finding that DHPs without calcium antagonistic activity possess MDR reversal activity.[7] Structure–activity relationship of DHP calcium channel antagonists suggests that two acyl substituents at the 3- and 5-positions in DHP ring might affect the activity of DHP calcium channel antagonists.[9] Actually, the antagonist activity is optimized by ester substituents at the 3- and 5-positions and is reduced by their replacement with acetyl group.[9] By a rhodamine 123 fluorescent assay, we have demonstrated that 3,5-diacetyl-1,4-dihydropyridines are the efficient Pgp inhibitors.[10] Studies of structure–activity relationships have demonstrated that the most hydrophobic compound shows the highest MDR reversing effect, but, the lipophilicity is not the only determinant of MDR-modulating activity. Furthermore, alterations in the substituents at 4-phenyl ring or substituent's position reduced the activity. In this paper, we investigated the MDR-reversal activities of 3,5-dibenzoyl-2,6-dimethyl-1,4-dihydro-4-phenylpyridine derivatives (BzDHPs) (**1**–**15**) against mouse lymphoma cells transfected with human *MDR 1* gene, and their cytotoxic activity against human oral tumor cell lines.

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Results and Discussion

Chemistry

BzDHP derivatives (**1**–**15**) were prepared by the variations of the Hantzch reaction (Scheme 1).[11] Thus, refluxing the mixture of aqueous ammonia (excess), aldehyde and 2 equiv of benzoylacetone in MeOH gave BzDHPs (**1**–**15**) in moderate yields.

Cytotoxicity

Fifteen BzDHP derivatives (**1**–**15**) showed significantly varied cytotoxic activity against two human oral tumor cell lines (HSC-2 and HSG), depending on the substituents at the 4-phenyl ring and the substituent's position. As shown in Table 1, compounds **5** ($IC_{50}=8.7\,\mu M$), **11** ($IC_{50}=7.0\,\mu M$) and **12** ($IC_{50}=7.0\,\mu M$) showed the highest cytotoxic activity against HSC-2. The cytotoxicity was nearly the same to that of doxorubicine ($IC_{50}=4.1\,\mu M$). On the other hand, cytotoxic activity of **5** ($IC_{50}=8.7\,\mu M$) against HSG was higher than those of **11** (28 μM) or **12** (150 μM). Normal fibroblasts (HGF) were relatively resistant to **5**, **11** and **12**, as judged by the higher selectivity index (SI = HGF/HSC-2) ratio (60–>143) suggesting a tumor-selective cytotoxicity of these compounds.

MDR reversal on tumor cells

Rhodamine 123 assay has been widely accepted as a direct and reproducible assay for measuring Pgp-dependent drug efflux.[12,13] Therefore, a series of BzDHPs (**1**–**15**) have been evaluated for the ability to inhibit the Pgp-mediated drug-efflux by the rhodamine 123 fluorescent assay in the human *MDR1* gene transfected T cell mouse lymphoma cell. Among fifteen BzDHPs (**1**–**15**), 2-nitro- (**3**), 3-phenoxy- (**4**), 4-methylthio- (**8**) and 3-chloro- (**12**) derivatives were more potent than that of **VP**. A recent study conducted with 3,5-diacetyl-DHPs has also shown that the presence of 3-phenoxy substituent on the 4-phenyl ring considerably enhanced the MDR-reversing activity.[10] Among three 2- (**11**), 3- (**12**), and 4-chloro (**13**) derivatives, compound **12** was found to be more active than **11** or **13**, meaning that chloro-substitution at 3 position on phenyl ring brings a higher cytotoxic activity and MDR activity than that at the 2- or 4-position. However, 3-bromo derivative (**14**) was inactive. Two 2-methoxy (**9**) and 4-methoxy (**10**) derivatives have comparable activity with the parent DHP (**1**). The introduction of 2-nitro (**3**), or 4-methylthio (**8**) groups at 4-phenyl ring led to much more active derivatives when compared to **1**.

Lipophilicity

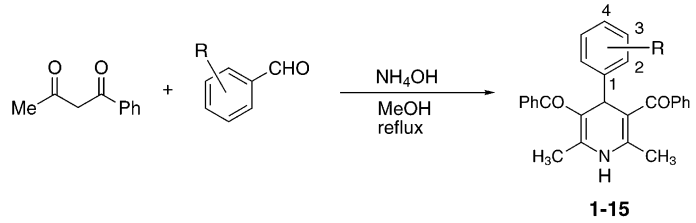
Lipophilicity is one of the important parameters affecting MDR-modulating efficiency in the structure–activity relationship studies of MDR modulating drugs.[4,6] The log P values of **1**–**15** were calculated by CLOGP[14] and were found to be higher (log P = 4.26–7.47) when compared to VP or nifedipine (NP) (Table 1). However, lipophilicity alone was not an essential parameter of direct MDR-modulating activity of the present series of compounds.

In conclusion, we synthesized and evaluated a new series of MDR-modulators derived from BzDHPs. The potency of MDR reversal was dependent on the nature of substituents and their positions at the 4-phenyl ring of BzDHPs. Compound **12** with the 3-chloro group on 4-phenyl ring showed the high MDR-modulating activity and the tumor-specific cytotoxicity, indicating a new drug candidate for MDR cancer chemotherapy.

Experimental

Melting points of BzDHPs (**1**–**15**) were determined in open glass capillaries in a paraffin bath and are uncorrected. ^1H NMR spectra were performed on a JEOL JNM-GSX 500 (500 MHz) spectrophotometer using tetramethylsilane as an internal standard. Mass spectra (MS) were recorded on a JEOL-JMS-DX300 spectrophotometer with direct inlet system at 70 eV. Combustion analyses were carried out on Coleman elemental analyser at Vadodara, India. Thin layer chromatography (TLC) was performed on a Merck Kieselgel 60 F254 (Merck 5549, USA).

The following chemicals were obtained from each indicated company: VP (Aldrich Chem. Comp. Inc., Milwarkee, WI, USA); NP (Wako Pure Chem. Ind.,



1-15

Compound	R	Compound	R	Compound	R
1	H	**6**	3-CF_3	**11**	2-Cl
2	3-NO_2	**7**	4-CF_3	**12**	3-Cl
3	2-NO_2	**8**	4-MeS	**13**	4-Cl
4	3-PhO	**9**	2-MeO	**14**	3-Br
5	2-CF_3	**10**	4-MeO	**15**	3,4,5-$(MeO)_3$

Scheme 1.

Table 1. Cytotoxic activity, MDR modulating activity and log P of BzDHPs (**1**–**15**)

Compound	Cytotoxic activity (IC_{50} μM)			SI (HGF/HSC-2)	MDR modulating activity[a]			Fluoresence activity ratio	Calcd log P
	HSC-2	HSG	HGF		FSC[b]	SSC[b]	FL-1[b]		
Par (control)[c]	—	—	—	—	513	269	7006	42.0	—
MDR + R123[d]	—	—	—	—	658	233	167	1.0	—
(±)-Verapamil	399	403	424	1.1	550	266	1960	11.8	3.71
Nifedipine	543	636	890	1.6	—	—	—	—	2.35
1	265	397	420	1.6	560	312	571	3.4	5.37
2	103	390	>1000	>9.7	547	267	407	2.4	5.11
3	285	315	929	3.3	514	263	2792	16.8	5.11
4	639	722	>1000	>1.6	555	269	2416	14.5	7.47
5	8.7	8.7	978	112	541	298	617	3.7	6.25
6	117	193	397	3.4	562	285	758	4.6	6.25
7	236	>1000	>1000	>4.2	567	298	822	4.9	6.25
8	>1000	>1000	>1000	><1.0	525	267	1660	10.0	5.93
9	>1000	>1000	>1000	><1.0	547	403	607	3.7	5.29
10	>1000	>1000	>1000	><1.0	562	283	538	3.3	5.29
11	7.0	28	421	60.1	534	267	565	3.4	6.08
12	7.0	150	>1000	>143	567	275	1882	11.3	6.08
13	37	365	716	19.4	540	270	328	2.0	6.08
14	434	>1000	>1000	>2.3	543	283	871	5.2	6.23
15	>1000	>1000	>1000	><1.0	550	259	434	2.6	4.26
Doxorubicin·HCl	4.1	5.3	>100	>24.4	—	—	—	—	—

[a]The final concentration of each compounds was 8 μg/mL in culture medium containing cell suspension and 0.4% DMSO.
[b]FSC, forward scatter count; SSC, side scatter count; FL-1, fluorescence intensity.
[c]Par, a parental cell without transfection of MDR gene.
[d]MDR, a parental cell transfected with MDR gene.

Ltd., Osaka, Japan); Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci., Lenexa, KS, USA).

General procedure for the preparation of BzDHPs (1–15)

A solution of benzoylacetone (1.0 g, 10 mmol) and liquid ammonia (sp. gr. 0.90) (0.32 mL, 20 mmol) in MeOH (10 mL) was treated with respective aldehyde (5 mmol), and the mixture was refluxed for 20–24 h. The separated solid was collected by suction and then, after charcoal treatment, recrystallized from MeOH.

3,5-Dibenzoyl-1,4-dihydro-2,6-dimethyl-4-phenylpyridine (1). Mp 220–222 °C (MeOH) (lit.[11] mp 229–231 °C), yield 28%. ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 6H), 5.14 (s, 1H), 5.67 (s, 1H), 6.94 (d, 2H, J = 7.4 Hz), 7.05 (t, 1H, J = 7.4 Hz), 7.12 (t, 2H, J = 7.4 Hz), 7.33 (t, 4H, J = 7.6 Hz), 7.42 (t, 2H, J = 7.6 Hz), 7.53 (d, 4H, J = 7.6 Hz). MS m/e 393 (M^+, 56%), 316 (100%).

3,5-Dibenzoyl-1,4-dihydro-2,6-dimethyl-4-(3′-nitrophenyl)-pyridine (2). Mp 210–212 °C (MeOH) (lit.[15] mp 205 °C), yield 32%. ^{1}H NMR ($CDCl_3$) δ 1.91 (s, 6H), 5.28 (s, 1H), 5.90 (s, 1H), 7.27 (t, 1H, J = 7.6 Hz), 7.32 (d, 1H, J = 7.3 Hz), 7.37 (t, 4H, J = 7.6 Hz), 7.46 (t, 2H, J = 7.3 Hz), 7.54 (d, 4H, J = 7.6 Hz), 7.93 (d, 1H, J = 7.3 Hz), 7.94 (s, 1H). MS m/e 438 (M^+, 25%), 316 (100%).

3,5-Dibenzoyl-1,4-dihydro-2,6-dimethyl-4-(2′-nitrophenyl)-pyridine (3). Mp 192–194 °C (dioxane), yield 30%. ^{1}H NMR ($CDCl_3$) δ 1.87 (s, 6H), 5.66 (s, 1H), 5.77 (s, 1H), 7.17–7.21 (m, 1H), 7.34 (t, 4H, J = 7.3 Hz), 7.42–7.46 (m, 3H), 7.52–7.55 (m, 2H), 7.58 (d, 4H, J = 7.6 Hz). MS m/e 438 (M^+, 6%), 421 (100%). Anal. calcd for $C_{27}H_{22}N_2O_4$: C, 73.97; H, 5.02; N, 6.39. Found: C, 73.99; H, 5.05; N, 6.40.

3,5-Dibenzoyl-1,4-dihydro-2,6-dimethyl-4-(3′-phenoxy-phenyl)pyridine (4). Mp 190 °C (MeOH), yield 32%. ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 6H), 5.13 (s, 1H), 5.63 (s, 1H), 6.65 (t, 1H, J = 2.1 Hz), 6.69 (dd, 2H, J = 2.1, 7.6 Hz), 6.82–6.85 (m, 2H), 7.03 (t, 1H, J = 7.4 Hz), 7.07 (t, 1H, J = 7.8 Hz), 7.24 (t, 2H, J = 7.8 Hz), 7.33 (t, 4H, J = 7.4 Hz), 7.42 (dt, 2H, J = 1.5, 7.4 Hz), 7.52 (dt, 4H, J = 1.5, 7.4 Hz). MS m/e: 485 (M^+, 62%), 316 (100%). Anal. calcd for $C_{33}H_{27}NO_3$: C, 81.63; H, 5.60; N, 2.88. Found: C, 81.67; H, 5.62; N, 2.85.

3,5-Dibenzoyl-4-(2′-trifluoromethylphenyl)-1,4-dihydro-2,6-dimethylpyridine (5). Mp 190–192 °C (MeOH), yield 18%. ^{1}H NMR ($CDCl_3$) δ 1.89 (s, 6H), 5.39 (s, 1H), 5.54 (s, 1H), 7.19 (t, 1H, J = 7.6 Hz), 7.34 (t, 4H, J = 7.6 Hz), 7.45 (t, 2H, J = 7.3 Hz), 7.52 (t, 1H, J = 7.6 Hz), 7.57 (d, 1H, J = 7.6 Hz), 7.65 (dd, 4H, J = 1.2, 7.6 Hz), 7.79 (d, 1H, J = 7.6 Hz). MS m/e 461 (M^+, 14%), 316 (100%). Anal. calcd for $C_{28}H_{22}F_3NO_2$: C, 72.88; H, 4.80; N, 3.04. Found: C, 72.89; H, 4.80; N, 3.06.

3,5-Dibenzoyl-4-(3′-trifluoromethylphenyl)-1,4-dihydro-2,6-dimethylpyridine (6). Mp 188–190 °C (MeOH), yield 26%. ^{1}H NMR ($CDCl_3$) δ 1.93 (s, 6H), 5.23 (s, 1H), 5.71 (s, 1H), 7.15 (br d, 1H, J = 8.2 Hz), 7.19–7.23 (m, 2H), 7.26–7.33 (m, 1H), 7.35 (t, 4H, J = 7.6 Hz), 7.44 (tt, 2H, J = 1.5, 7.6 Hz), 7.52 (dd, 4H, J = 1.2, 7.6 Hz). MS m/e 461 (M^+, 65%), 316 (100%). Anal. calcd for $C_{28}H_{22}F_3NO_2$: C, 72.88; H, 4.80; N, 3.04. Found: C, 72.87; H, 4.82; N, 3.05.

3,5-Dibenzoyl-4-(4′-trifluoromethylphenyl)-1,4-dihydro-2,6-dimethylpyridine (7). Mp 226–227 °C (MeOH), yield 20%. ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 6H), 5.21 (s, 1H), 5.67 (s, 1H), 7.09 (d, 2H, J = 8.2 Hz), 7.30 (t, 1H, J = 7.9 Hz), 7.36 (t, 4H, J = 7.6 Hz), 7.39 (d, 1H, J = 7.9 Hz), 7.45 (tt, 2H, J = 1.5, 7.6 Hz), 7.54 (dd, 4H, J = 1.5, 7.6 Hz). MS m/e 461 (M^+, 68%), 316 (100%). Anal. calcd for $C_{28}H_{22}F_3NO_2$: C, 72.88; H, 4.80; N, 3.04. Found: C, 72.90; H, 4.78; N, 3.02.

3,5-Dibenzoyl-1,4-dihydro-2,6-dimethyl-4-[4′-(methylthio)-phenyl]pyridine (8). Mp 144–146 °C (MeOH), yield 29%. ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 6H), 2.48 (s, 3H), 5.09 (s, 1H), 5.66 (s, 1H), 6.75 (d, 1H, J = 8.2 Hz), 6.85 (d, 1H, J = 8.2 Hz), 6.87 (d, 1H, J = 8.2 Hz), 7.02 (d, 1H, J = 8.2 Hz), 7.28 (t, 2H, J = 7.6 Hz), 7.34 (t, 2H, J = 7.6 Hz), 7.41–7.45 (m, 2H), 7.52–7.56 (m, 4H). MS m/e 439 (M^+, 88%), 316 (100%). Anal. calcd for $C_{28}H_{25}NO_2S$: C, 76.51; H, 5.73; N, 3.19. Found: C, 76.54; H, 5.72; N, 3.16.

3,5-Dibenzoyl-1,4-dihydro-4-(2′-methoxyphenyl)-2,6-dimethylpyridine (9). Mp 238–240 °C (MeOH), yield 22%. ^{1}H NMR ($CDCl_3$/DMSO-d_6, 4:1) δ 1.38 + 1.39 (s, 6H), 2.67 + 2.68 (s, 3H), 4.58 (s, 1H), 5.96–6.10 (m, 1H), 6.15–6.25 (m, 1H), 6.33–6.40 (m, 1H), 6.45–6.50 (m, 1H), 6.73–6.82 (m, 4H), 6.82–6.92 (m, 2H), 6.96–7.05 (m, 4H), 7.98 (s, 1H). MS m/e 423 (M^+, 7%), 392 (100%). Anal. calcd for $C_{28}H_{25}NO_3$: C, 79.41; H, 5.95; N, 3.31. Found: C, 79.42; H, 5.96; N, 3.33.

3,5-Dibenzoyl-1,4-dihydro-4-(4′-methoxyphenyl)-2,6-dimethylpyridine (10). Mp 191–192 °C (MeOH), yield 20%. ^{1}H NMR ($CDCl_3$) δ 1.93 (s, 6H), 3.68 (s, 3H), 5.07 (s, 1H), 5.63 (s, 1H), 6.42 + 6.65 (d, 2H, J = 8.8 Hz), 6.85 + 6.87 (d, 2H, J = 8.8 Hz), 7.27 (t, 1H, J = 7.6 Hz), 7.33 (t, 3H, J = 7.6 Hz), 7.39–7.44 (m, 2H), 7.52–7.55 (m, 4H). MS m/e 423 (M^+, 90%), 316 (100%). Anal. calcd for $C_{28}H_{25}NO_3$: C, 79.41; H, 5.95; N, 3.31. Found: C, 79.43; H, 5.94; N, 3.29.

3,5-Dibenzoyl-4-(2′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (11). Mp 216–218 °C (MeOH), yield 31%. ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 6H), 5.62 (s, 2H), 6.97 (dt, 1H, J = 1.5, 7.6 Hz), 7.06–7.10 (m, 2H), 7.18 (dd, 1H, J = 1.5, 7.8 Hz), 7.28–7.35 (m, 4H), 7.40–7.47 (m, 2H), 7.55–7.62 (m, 4H). MS m/e 427 (3.2%) + 429 (1.2%) (3:1, M^+), 316 (100%). Anal. calcd for $C_{27}H_{22}ClNO_2$: C, 75.78; H, 5.18; N, 3.27. Found: C, 75.80; H, 5.19; N, 3.25.

3,5-Dibenzoyl-4-(3′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (12). Mp 184–186 °C (MeOH), yield 35%. ^{1}H NMR ($CDCl_3$) δ 1.92 (s, 6H), 5.13 (s, 1H), 5.78 (s, 1H), 6.79–6.82 (m, 1H), 6.95–6.97 (m, 1H), 7.02–7.05 (m, 2H), 7.35 (t, 4H, J = 7.6 Hz), 7.44 (tt, 2H, J = 1.5, 7.6 Hz), 7.51–7.54 (m, 4H). MS m/e 427 (56%) + 429 (17%) (3:1, M^+), 316 (100%). Anal. calcd for $C_{27}H_{22}ClNO_2$: C, 75.78; H, 5.18; N, 3.27. Found: C, 75.79; H, 5.20; N, 3.29.

3,5-Dibenzoyl-4-(4′-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine (13). Mp 228–230 °C (MeOH), yield 28%. ^{1}H NMR ($CDCl_3$/DMSO-d_6, 4:1) δ 1.49 (s, 6H), 4.66 (s, 1H), 6.57 (d, 2H, J = 8.5 Hz), 6.70 (d, 2H, J = 8.5 Hz), 6.97 (t, 4H, J = 7.8 Hz), 7.06 (tt, 2H, J = 1.5, 7.6 Hz), 7.09–7.12 (m, 4H), 8.07 (s, 1H). MS m/e 427 (75%) + 429 (23%) (3:1, M^+), 316 (100%). Anal. calcd for $C_{27}H_{22}ClNO_2$: C, 75.78; H, 5.18; N, 3.27. Found: C, 75.80; H, 5.15; N, 3.26.

3,5-Dibenzoyl-4-(3′-bromophenyl)-1,4-dihydro-2,6-dimethylpyridine (14). Mp 182–184 °C (Dioxane), yield 38%. ^{1}H NMR ($CDCl_3$) δ 1.94 (s, 6H), 5.12 (s, 1H), 5.69 (s, 1H), 6.85 (dt, 1H, J = 1.2, 7.6 Hz), 6.97 (t, 1H, J = 7.6 Hz), 7.10 (t, 1H, J = 1.8 Hz), 7.18–7.19 (m, 1H), 7.30 (t, 1H, J = 7.6 Hz), 7.35 (t, 3H, J = 7.6 Hz), 7.42–7.47 (m, 2H), 7.52–7.56 (m, 4H). MS m/e 471 (45%) + 473 (30%) (1:1, M^+), 316 (100%). Anal. calcd for $C_{27}H_{22}BrNO_2$: C, 68.65; H, 4.69; N, 2.97. Found: C, 68.66; H, 4.66; N, 2.94.

3,5-Dibenzoyl-1,4-dihydro-4-(3′,4′,5′-trimethoxyphenyl)-2,6-dimethylpyridine (15). Mp 186 °C (MeOH), yield 17%. ^{1}H NMR ($CDCl_3$) δ 1.96 (s, 6H), 3.63 (s, 6H), 3.71 (s, 3H), 5.11 (s, 1H), 5.67 (s, 1H), 6.12 (s, 2H), 7.35 (t, 4H, J = 7.6 Hz), 7.44 (tt, 2H, J = 1.5, 7.3 Hz), 7.56–7.58 (m, 4H). MS m/e 483 (M^+, 74%), 316 (100%). Anal. calcd for $C_{30}H_{29}NO_5$: C, 74.52; H, 6.04; N, 2.90. Found: C, 74.55; H, 6.01; N, 2.89.

Calculation of distribution coefficient. The log P values of **1**–**15** were calculated by CLOGP.[14]

Cell culture. Human oral squamous cell carcinoma (HSC-2) cells and human salivary gland tumor (HSG) cells were maintained as a monolayer culture at 37 °C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization. Human gingival fibroblasts (HGF) were isolated from healthy gingival biopsies of a 10-year-old female, as described previously.[16] Cells between the fifth and seventh passages were used.

Cytotoxic activity

Cells were incubated for 24 h with the indicated concentrations of test samples in culture medium, and the viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[16] The A_{540} values of control HSC-2, HSG and HGF cells were 0.805, 0.623 and 0.252, respectively.

Cell and fluorescence uptake. *MDR1*/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype.[17] The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium, and 0.5 mL aliquots of the cell suspension were distributed into each Eppendorf centrifuge tube. Then, 2 μL of 2 mg/mL test compounds were added and incubated for 10 min at room temperature. Then, 50 μL rhodamine 123 (R123) as indicator was added to the samples (5.2 μM final

concentration) and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) (pH 7.4) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument (cell sorter). (±)-Verapamil (VP) was used as the positive control in R123 accumulation experiments.[17] The R123 accumulation was calculated from fluorescence of one height value using the second equation $y = 10^{X/256}$. In the case of logarithmic transformation, the 1024 digital channels were switched to one decade at each 256 ($=2^8$) channels. Then, the percentage of mean fluorescence intensity was calculated in parental and MDR cell lines, compared to untreated cells. The fluorescence activity ratio was calculated by the following equation:[17,18]

MDR reversal activity = (MDR treated/MDR control)/(parental treated/parental control)

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