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

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Abstract. *Eleven 4-phenyl-3,5-diacetyl-1,4-dihydropyridines (AcDHPs) [**G1-11**] substituted at the phenyl ring were synthesized and compared for their cytotoxic activity and multidrug resistance (MDR)-reversing activity in in vitro assay systems. Among them, compound [**G7**] showed the highest cytotoxic activity against human promyelocytic leukemia HL-60 and human squamous cell carcinoma HSC-2 cells. However, no compounds tested produced radicals at pH 7.4-12.5. The activity of P-glycoprotein (Pgp) responsible for MDR in tumor cells was reduced by compounds [**G2, 3, 6, 5, 8, 1, 11**], verapamil [**VP**] and nifedipine [**NP**]. However, compounds [**G4, 7, 10**] were hardly active while G9 did not show a MDR reversing effect at 2.0-20.0 μg/mL. These data show a relationship between chemical structures and MDR-reversing effect on tumor cells.*

The development of multidrug resistance (MDR)-tumor cell populations is a major problem in the chemotherapy of human cancer (1). When tumor cells become resistant to anticancer agents such as *vinca* alkaloids or anthracyclines, they often show resistance to other antitumor agents with different structures and mechanisms of action. One of the MDR types was proved to involve a membrane-bound protein, P-glycoprotein (Pgp) in MDR cancer cells, protozoa and bacteria. This protein acts as an efflux pump for anticancer drugs (2). Recently, various compounds have been shown to inhibit Pgp-mediated drug efflux (3). These compounds include ion channel blockers such as verapamil (**VP**) (4), dihydropyridines (DHPs) (5), propafenone (6), antipsychotic drugs like phenothiazines (7), cyclosporins (8), thiazinopiperidine S9788 (9), acridone carboxamide GF120918 (10), quinoline MS209 (11), and stipiamide (12). Among them, DHPs, calcium antagonists, have been studied extensively for the analogy to **VP** (5). In a combination treatment with antitumor agents, such as *vinca* alkaloids or anthracyclines, calcium antagonist **VP** caused cardiovascular side effects (4). It is very important finding that DHPs, which do not have any calcium antagonistic activity, possess MDR reversal activity (5). From the structure-activity relationship on DHP calcium channel antagonists, the role of C-3 and C-5 substituents in the DHP ring has received attention (13). Generally, the antagonist activity is optimized by ester substituents at 3- and 5-positions and is reduced by their replacement with an acetyl group (13). In this paper, we investigate the cytotoxic and the MDR-reversal activities of 3,5-diacetyl-2,6-dimethyl-1,4-dihydropyridine (AcDHP) derivatives against mouse lymphoma cells transfected with MDR 1 gene.

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Materials and Methods

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

Chemicals. AcDHPs were newly synthesized (14) and the structures are shown in Figure 1. The following chemicals were obtained from each indicated company: **VP** (Aldrich); **NP** (Wako Pure Chem. Ltd., Osaka); RPMI1640 medium (GIBCo, Grand Island, NY, USA); ascorbic acid (Tokyo Kasei Kogyo Co. Ltd., Japan); fetal bovine serum (FBS) (JRH Biosci., Lenexa, KS, USA).

General procedure for the preparation of AcDHPs [G1-11]. A solution of acetylacetone (1.0 g, 10 mmol) and liquor 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 hours. The separated solid was collected by suction. The reaction mass was kept for 24-36 hours and then, after charcoal treatment, recrystallized from MeOH.

The following compounds were obtained:

a) 3,5-Diacetyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine [**G1**]. mp: 204-206°C (MeOH) (Lit. 15: mp 211-212°C). yield: 42%.

b) 3,5-Diacetyl-1,4-dihydro-2,6-dimethyl-4-(3-phenoxyphenyl)pyridine [**G2**]. mp: 175-176°C (MeOH). yield: 37%. ^{1}H NMR ($CDCl_3$) δ: 2.25 (s, 6H), 2.28 (s, 6H), 5.06 (s, 1H), 5.82-5.92 (br, 1H), 6.73 (dd, 1H, *J*=2.4, 8.2 Hz), 6.86+6.87 (d, 1H, *J*=2.1 Hz), 6.94 (d, 2H, *J*=7.9 Hz), 6.97 (d, 1H, *J*=7.6 Hz), 7.06 (t, 1H, *J*=7.3 Hz), 7.15 (t, 1H, *J*=7.9 Hz), 7.28 (t, 2H, *J*=7.9 Hz). MS m/e: 361 (M^+, 26%), 192 (100%). Anal Calcd for $C_{23}H_{23}NO_3$: C, 76.45; H, 6.37; N, 3.87. Found: C, 76.52; H, 6.42; N, 3.81.

c) 3,5-Diacetyl-4-(3-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine [**G3**]. mp: 209-210°C (MeOH). yield: 47%. ^{1}H NMR ($CDCl_3$) δ: 1.54 (s, 6H), 1.63 (s, 6H), 4.40 (s, 1H), 6.38-6.50 (m, 4H), 8.08 (s, 1H). MS m/e: 305+303 (1:3) (M^+, 7%:22%), 192 (100%). Anal Calcd for $C_{17}H_{18}ClNO_2$: C, 67.21; H, 5.93; N, 4.61. Found: C, 67.26; H, 5.90; N, 4.70.

d) 3,5-Diacetyl-1,4-dihydro-4-(4-hydroxy-3-methoxyphenyl)-2,6-dimethylpyridine [**G4**]. mp: 195-197°C (MeOH). yield: 53%. ^{1}H NMR ($CDCl_3$) δ: 2.25 (s, 6H), 2.31 (s, 6H), 3.82 (s, 3H), 5.03 (s, 1H), 5.40-5.52 (br, 1H), 5.70 (s, 1H), 6.74 (dd, 1H, *J*=2.1, 8.2 Hz), 6.74 (d, 1H, J=8.2 Hz), 6.81 (d, 1H, *J*=2.1 Hz). MS m/e: 315 (M^+, 32%), 192 (100%). Anal Calcd for $C_{18}H_{21}NO_4$: C, 68.57; H, 6.66; N, 4.44. Found: C, 68.63; H, 6.60; N, 4.51.

e) 3,5-Diacetyl-1,4-dihydro-2,6-dimethyl-4-(4-dimethylaminophenyl)pyridine [**G5**]. mp: 219-220°C (MeOH). yield: 33%. ^{1}H NMR ($CDCl_3$) δ: 2.25 (s, 6H), 2.29 (s, 6H), 2.87 (s, 6H), 4.94 (s, 1H), 5.82 (s, 1H), 6.60 (d, 2H, *J*=8.5 Hz), 7.07 (d, 2H, *J*=8.5 Hz). MS m/e: 314 (M^+, 2%), 311 (100%). Anal Calcd for $C_{19}H_{24}N_2O_2$: C, 73.07; H, 7.69; N, 8.97. Found: C, 73.14; H, 7.76; N, 8.93.

f) 3,5-Diacetyl-1,4-dihydro-4-(4-methoxyphenyl)-2,6-dimethylpyridine [**G6**]. mp: 171-172°C (MeOH). yield: 58%. ^{1}H NMR ($CDCl_3$) δ: 2.24 (s, 6H), 2.29 (s, 6H), 3.73 (s, 3H), 5.01 (s, 1H), 5.80-5.86 (br, 1H), 6.74 (d, 2H, *J*=8.8 Hz), 7.13 (d, 2H, *J*=8.8 Hz). MS m/e: 299 (M^+, 41%), 192 (100%). Anal Calcd for $C_{18}H_{21}NO_3$: C, 72.24; H, 7.02; N, 4.68. Found: C, 72.29; H, 7.10; N, 4.73.

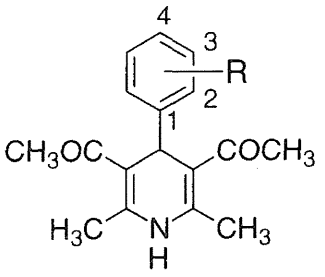
g) 3,5-Diacetyl-4-(4-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine [**G7**]. mp: 180-182°C (MeOH). yield: 57%. ^{1}H NMR ($CDCl_3$) δ: 2.26 (s, 6H), 2.33 (s, 6H), 5.12 (s, 1H), 5.87-5.96 (br, 1H), 7.16-7.21 (m, 4H). MS m/e: 305+303 (1:3) (M^+, 9%:26%), 192 (100%). Anal Calcd for $C_{17}H_{18}ClNO_2$: C, 67.21; H, 5.93; N, 4.61. Found: C, 67.20; H, 5.87; N, 4.56.

h) 3,5-Diacetyl-1,4-dihydro-2,6-dimethyl-4-[4-(methylthio)phenyl]pyridine [**G8**]. mp: 181-182°C (MeOH). yield: 46%. ^{1}H NMR ($CDCl_3$) δ: 2.24 (s, 6H), 2.30 (s, 6H), 2.41 (s, 3H), 5.05 (s, 1H), 5.89 (s, 1H), 7.09 (d, 2H, *J*=8.5 Hz), 7.13 (d, 2H, *J*=8.5 Hz). MS m/e: 315 (M^+, 44%), 192 (100%). HR MS Calcd for $C_{18}H_{21}NO_2S$: 315.1293. Found: 315.1294.

i) 3,5-Diacetyl-4-(3-bromophenyl)-1,4-dihydro-2,6-dimethylpyridine [**G9**]. mp: 224-226°C (MeOH). yield: 51%. ^{1}H NMR ($CDCl_3$ + DMSO-d_6) δ: 1.73 (s, 6H), 1.82 (s, 6H), 4.59 (s, 1H), 6.59 (t, 1H, *J*=7.8 Hz), 6.66 (d, 1H, *J*=7.9 Hz), 6.72 (d, 1H, *J*=7.6 Hz), 6.81 (s, 1H0), 8.14 (s, 1H). MS m/e: 349+347 (1:1) (M^+, 13%:15%), 192 (100%). HR MS Calcd for $C_{17}H_{18}{}^{81}BrNO_2$): 347.0521 (349.0501). Found: 347.0506 (349.0482).

j) 3,5-Diacetyl-4-(2-chlorophenyl)-1,4-dihydro-2,6-dimethylpyridine [**G10**]. mp: 200-202°C (MeOH) (Lit. 15: mp 178°C). yield: 53%. ^{1}H NMR ($CDCl_3$) δ: 2.24 (s, 6H), 2.29 (s, 6H), 5.40 (s, 1H), 5.79 (s, 1H), 7.06 (dt, 1H, *J*=1.5, 7.6 Hz), 7.12 (dt, 1H, *J*=1.2, 7.6 Hz), 7.23-7.25 (m, 2H). MS m/e: 305+303 (1:3) (M^+, 2%:5%), 192 (100%). Anal Calcd for $C_{17}H_{18}ClNO_2$: C, 67.21; H, 5.93; N, 4.61. Found: C, 67.17; H, 5.87; N, 4.58.

k) 3,5-Diacetyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine [**G11**]. mp: 233-235°C (MeOH) (Lit. 15: mp 218°C). yield: 28%. ^{1}H NMR ($CDCl_3$+DMSO-d_6) δ: 1.40 (s, 6H), 1.44 (s, 6H), 4.82 (s, 1H), 6.47 (t, 1H, *J*=7.6 Hz), 6.60 (d, 1H, *J*=7.6 Hz), 6.68 (t, 1H, *J*=7.6 Hz), 6.80 (d, 1H, *J*=7.6 Hz), 8.02 (s, 1H). MS m/e: 314 (M^+, 9%), 252 (100%). HR MS Calcd for $C_{17}H_{18}N_2O_4$: 314.1266. Found: 314.1278.



G1-G11

Compound	R
G1	3-NO_2
G2	3-PhO
G3	3-Cl
G4	4-OH, 3-MeO
G5	4-Me_2N
G6	4-MeO
G7	4-CI
G8	4-MeS
G9	3-Br
G10	2-CI
G11	2-NO_2

Figure 1. *Structures of AcDHPs [G1-11].*

Cell culture. Human promyelocytic leukemia HL-60 cells were maintained at 37°C in RPMI1640 medium supplemented with 10% FBS in a humidified 5% CO_2 atmosphere (14). Human squamous cell carcinoma HSC-2 cells were maintained as a monolayer culture in DMEM supplemented with 10% FBS, and subcultured by trypsinization (16).

Cytotoxic activity. HL-60 cells (1 x 10^6/mL) were incubated for 24 hours with various concentrations of test samples in the culture medium, and the viable cell number was determined by trypan blue dye exclusion (16). The 50% cytotoxic concentration (CC_{50}) of each compound was determined by dose-response curve. The cytotoxic activity of each compound was also assayed with HSC-2 cells. In brief, near-confluent HSC-2 cells were treated for 24 hours with various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm) was determined by the crystal violet method (17).

Assay for radical intensity. The radical intensity of test samples was determined at 25°C exactly 1 min after mixing samples in 0.1M Tris-HCl (pH 7.4), 0.1M Tris-HCl (pH 8.0)(only for experiment with ascorbate), 0.1M $NaHCO_3$-Na_2CO_3 (pH 9.5) or 0.1M KOH (pH 12.5) with electron spin resonance (ESR) spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 335.6 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 2-10 x 100; time constant, 0.1 sec; scanning time, 4 min. The radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (18).

Cell and fluorescence uptake. MRD1/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of 2 x 10^6/mL and resuspended in serum-free McCoy's 5A medium, and 0.5 mL aliquot of the cell suspension were distributed into each Eppendorf centrifuge tube. Then, 2 μL of 2 mg/mL tested compounds were added and incubated for 10 min at room temperature. Then, 50 μL rhodamine 123 (R123) as an 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) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument (cell sorter). **VP** was used as the positive control in the R123 accumulation experiments (17, 18). R123 accumulation was calculated from the fluorescence of one height value using the 2nd 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 (18, 19):

Ratio=(MDR treated/MDR control)/(parental treated/parental control)

Calculation of distribution coefficient. The log P values were calculated by CLOGP (20).

Results

Cytotoxicity. Eleven AcDHPs [**G1-11**], **VP** and **NP** were at first tested for their cytotoxicity against HL-60 and HSC-2 cell lines. The cytotoxic activity of **G7** against HL-60 (CC_{50}=20 μM) and HSC-2 (CC_{50}=41 μM) was the greatest among 11 AcDHPs, and was comparable with that of **VP** and **NP** (Table I). Compounds [**G8**] and [**G10**] showed moderate cytotoxicity *in vitro.* On the other hand, the cytotoxic activity of nine AcDHPs were much less. ESR spectroscopy showed that these compounds did not produce radicals at pH 7.4-12.5 and also, neither enhanced the radical intensity of sodium ascorbate (Table I). Therefore, it seems likely that the induction of cytotoxic activity by **G7** does not relate to their radicals or prooxidant actions.

MDR reversal on tumor cells. The Rhodamine 123 assay has been widely documented as a direct and reproducible assay for measuring Pgp-dependent efflux (21). The substituent effect at the benzene ring of AcDHPs was examined. Table I summarizes the activity of compounds with different aromatic substituents at the 4-position. Their activity depended on the C-4 aryl group substitution. In the present series of compounds, the most lipophilic compound **G2** with 3-phenoxy group on the phenyl ring gave the highest activity. Among three *ortho* [**G10**]-, *meta* [**G3**]- and *para* [**G7**]-chloro derivatives, *meta*-chloro compound [**G3**] had the highest activity. However, *meta*-bromo substituent [**G9**] was inactive. Two *ortho* [**G11**]- and *meta* [**G1**]-nitro compounds had almost similar activity. Strong electron-donating groups such as methoxy [**G6**], dimethylamino [**G5**] and methylthio [**G8**] in the *para* position generally gave high activity. However, no relation between MDR-modulating activity and structural feature could be observed from these studies.

Lipophilicity. Lipophilicity is an important parameter affecting MDR-modulating efficiency on the structure-activity relationship of MDR drugs (22). Their log P values were calculated by CLOGP (Table I) (20). Among 11 AcDHPs, 3-phenoxy derivative [**G2**] with the highest lipophilicity had the highest activity. On the other hand, the activities of more hydrophobic 2-bromo [**G9**], 2-chloro [**G10**] and 4-chloro [**G7**] compounds were lower than those of more hydrophilic 4-methoxy [**G6**], 4-dimethylamino [**G5**], 3-nitro [**G1**] and 2-nitro [**G11**] compounds. It was concluded that MDR-modulating activity of the present series of compounds could not be determined by lipophilicity alone, but lipophilicity may be regarded as a favorable parameter within a homologous series.

Discussion

Effects of calcium channel blockers with structure distinct from **VP** have been studied for anti-MDR activity. The DHP analogs, niludipine, nimodipine and nicardipine were found to be potent antagonists of MDR, *i.e.,* its combination with 3.5-10 μM nicardipine fully reversing vincristine resistance in P388/VCR and K562/VCR cells and partially reversing doxorubicin resistance in P388/ADR cells (23). It is suggested that Pgp seems to be a good acceptor, as judged by the inhibition of photolabeling of Pgp by [^{3}H]azidopine by DHP analogs (5d, 5e). Additionally, the inhibition was significantly correlated with their reversing activity (5d, 5e). To find an agent which lacks calcium-antagonistic activity, but still possesses MDR reversal activity, several DHPs were screened on their ability to reverse MDR. However, no specific structural features could be identified by chemosensitizing activity (5). As modified DHP calcium channel blockers, the role of the C-3 and C-5 substituents in DHP ring has received attention, because derivatives with 3,5-diacetyl groups have reduced calcium antagonistic activity. Compounds **G1, G10** and **G11** have been reported to possess an approximately equivalent potency of calcium antagonistic activities, as assessed by their ability to relax established constractions induced by 1 mmol/L Ca^{2+} (15). Comparison of the calcium antagonistic potency of these three compounds and **NP** shows that these compounds are about 100 times less potent than

Table I. *Relationship between radical intensity, biological activity, MDR-modulating activity and calculated log P of AcDHPs [G1-11].*

Compd No	Cytotoxic activity CC_{50} μM HL-60	HSC-2	Radical intensity[a] (pH 7.4)	(pH 9.5)	(pH 12.5)	+Ascorbate (3mM)[b] (pH 12.5)	Fluorescence activity ratio 2.0 μg/mL	20.0 μg/mL	Calcd log P
Ascorbate	-	-	-	-	-	0.367 ± 0.009	-	-	-
MDR + R123	-	-	-	-	-	-	-	-	-
(+)-Verapamil	21	76	<0.03	<0.03	<0.03	0.401 ± 0.020	3.8	7.4	3.71
Nifedipine	19	42	<0.03	<0.03	<0.03	0.398 ± 0.014	2.2	7.9	2.35
G1	156	430	<0.03	<0.03	<0.03	0.362 ± 0.007	2.4	12.6	1.19
G2	292	500	<0.03	<0.03	<0.03	0.423 ± 0.054	25.9	28.8	3.54
G3	203	473	<0.03	<0.03	<0,03	0.380 ± 0.009	2.4	34.4	2.16
G4	229	398	<0.03	<0.03	<0.03	0.458 ± 0.006	2.6	4.6	0.62
G5	215	236	<0.03	<0.03	<0.03	0.356 ± 0.010	2.5	15.8[c]	1.64
G6	99	233	<0.03	<0.03	<0.03	0.381 ± 0.010	2.9	40.6[c]	1.36
G7	20	41	<0.03	<0.03	<0.03	0.396 ± 0.008	4.1	5.5	2.16
G8	56	164	<0.03	<0.03	<0.03	0.389 ± 0.012	5.5	12.5	2.00
G9	205	358	<0.03	<0.03	<0.03	0.350 ± 0.011	1.1	1.3[d]	2.31
G10	50	156	<0.03	<0.03	<0.03	0.375 ± 0.013	2.5	3.1[d]	2.16
G11	103	180	0.267	<0.03	<0.03	0.444 ± 0.002	2.1	19.6	1.11

a) The radical intensity of each test compound (5 mM) at the highest peak was determined by ESR spectroscopy in 50% DMSO. b) The radical intensity of sodium ascorbate (3 mM) was determined in the presence of 5 mM each test compound. c) Increased intracellular granulation. d) Decreased cell size and granulation.

NP (15). Thus, 3,5-diacetyl substitutions caused a decrease in the calcium antagonistic activity.

The lipophilicity of MDR-modulating agents was calculated as parameter for one predictable activity. The MDR-modulating activity might be closely correlated with the lipophilicity of the molecule (21). The most active reversing compound **G2** was more hydrophobic than any other compounds. In contrast, compounds **G3, G7, G9,** and **G10**, which displayed similar lipophilicity, showed markedly different effects on Rhodamine 123 accumulation. This discrepant result suggests that lipophilicity is not the only determinant of MDR-modulating activity. Nevertheless, alteration of either the substituents at 4-phenyl group or the substituents' position reduced the activity, further supporting the *claim* that lipophilicity is not the only determinant on the structure-activity relationship of drugs (22). Further studies with other substituted-DHP analogs are necessary to confirm the relationship between lipophilicity and DHPs.

We synthesized and evaluated a new series of MDR-modulators derived from AcDHPs. Compound [**G2**] with a phenoxy group on 4-phenyl ring showed the highest MDR-modulating activity among AcDHPs [**G1-G11**] and was more potent than **VP.** Since these AcDHPs have a low calcium-antagonistic activity, their activity might be associated with DHP derivatives.

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