Unsymmetric dihydropyridines bearing 2-pyridyl methyl carboxylate as modulators of P-glycoprotein; synthesis and biological evaluation in resistant and non-resistant cancer cells

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Abstract

Multidrug resistance (MDR) in cancer cells is often associated with overexpression of P-glycoprotein (P-gp or ABCB1 or MDR1), therefore, modulators of this transporter might be helpful in overcoming MDR. In this study, 16 novel unsymmetrical dihydropyridine (DHP) derivatives bearing 2-pyridyl methyl carboxylate at C_3 and nitroimidazole or nitrophenyl ring at C_4 positions of the DHP ring were synthesized. Their cytotoxicity was tested against 4 human cancer cells by MTT assay. MDR reversal capacity was examined in P-gp overexpressing cells (MES-SA/DX5) by measuring alteration of doxorubicin's IC_{50} and performing flow cytometric determination of intracellular rhodamine 123 accumulation. Calcium channel blocking (CCB) activity, as a side effect of DHPs, was tested on guinea pig ileum. Molecular docking was performed to explain the binding mode of compounds. Two derivatives, **4a** and **4c**, containing 4-nitrophenyl at C_4 while possessing methyl (**4a**) and *iso*-propyl carboxylates (**4c**) at C_5 position of DHP core demonstrated superior cytotoxic and MDR reversal activities and lower CCB effect. Docking analysis confirmed the importance of 4-nitrophenyl ring for P-gp inhibitory activity. Some of the synthesized DHP derivatives with considerable MDR reversal capacity, could be promising compounds for further discovery of useful agents for management of drug resistant cancer.

Keywords: Cancer, ABC transporters, Antineoplastic Agents, Molecular docking, Structure-activity relationship

1. Introduction

Cancer is the main cause of death in the world and the annual number of new cancer cases is estimated to rise from 14 million in 2012 to 22 million within the next 2 decades [1]. Multidrug resistance (MDR) in cancer, defined as the resistance of cancer cells to several different chemotherapeutic agents, is an important cause of treatment failure [2,3]. MDR appears to have diverse and complex mechanisms. One of the most accepted classifications is the division to classical or ATP-binding cassette (ABC) transporters-mediated MDR and atypical MDR that involves transporter–independent mechanisms [4].

ABC efflux transporter proteins pump the anticancer agent out of the cancer cell and do not allow its intracellular accumulation [2,5-8]. One of these efflux pumps, P-glycoprotein (P-gp, ABCB1 or MDR1), belongs to the family of ATP-binding cassette (ABC) transporters and constitutes a major cause of MDR [9,10]. This membrane protein is responsible for the efflux of several compounds across the plasma membrane, including anticancer agents [11]. There has been much effort focused towards the development of efficient MDR reversal agents in order to restore the sensitivity of cancer cells to chemotherapeutic agents [6].

1,4-Dihydropyridine (1,4-DHP) derivatives have several pharmacological activities and a number of them are being used for management of cardiovascular diseases due to their calcium channel blocking (CCB) capacity [12]. Aside from their CCB effect, these compounds have been also studied for their MDR reversal activity [13-16] and their interactions with P-gp have been assessed in previous reports [17-20]. These compounds have also shown cytotoxic effects against different cancer cells [18,21,22] and therefore, represent high potential for discovery of anticancer and MDR reversal agents. Since the CCB capacity of DHPs may

cause undesired cardiovascular effects, investigators have attempted to synthesize DHPs with better cytotoxic and MDR reversal profile, while possessing low CCB effect [23,24].

According to a previous study, DHPs with pyridine ring at C_3 and C_5 positions had better MDR reversal activities [15]. The previous studies of our group have also shown that symmetric and fused DHPs containing pyridyl alkyl carboxylate moieties are potent P-glycoprotein modulators [25-27].

In continuation of our ongoing efforts on the structure-activity relationship (SAR) of DHPs as cytotoxic and MDR reversal agents, in the present study, novel unsymmetric DHP derivatives with 2-pyridyl methyl carboxylate at C_3 while containing nitrophenyl or nitroimidazole moieties at C_4 and different alkyl carboxylates at C_5 were synthesized and their CCB, cytotoxic and MDR reversal activities were examined. Finally, in order to get perception about the binding mode of the derivatives in the active site of P-gp, molecular docking analysis was carried out.

2. Experimental section

2.1. General

Nifedipine, rhodamine 123, MTT (thiazolyl blue tetrazolium bromide) and 2,2,6-trimethyl-4H-1,3-dioxin-4-one were purchased from Sigma-Aldrich. Alkyl acetoacetates (methyl, ethyl, *iso*-propyl and *tert*-butyl acetoacetate), dimethyl sulfoxide and 2-pyridyl methyl alcohol were obtained from Merck, Darmstadt, Germany. Penicillin-G/streptomycin and RPMI 1640 were from Biosera, Ringmer, UK. FBS and doxorubicin were from Invitrogen, San Diego, CA and Ebewe Pharma, Unterach, Austria, respectively. Melting points were determined by using an electrothermal 9100 digital melting point apparatus and were uncorrected (Electrothermal, Essex, UK). The reaction progress and purity of the synthesized compounds were checked on

Merck aluminum plates precoated with silica gel 60 F-254Silica gel column chromatography was performed with Silica gel 60G. ^{1}H NMR spectra were recorded on a Bruker 500 spectrometer with TMS (tetramethylsilane) as an internal standard. IR spectra were measured in KBr with a Perkin-Elmer FT-IR in the range of 600–4000 cm^{-1}. Mass spectra were recorded on an Agilent GC-MS.

2. 2. Synthesis

2.2.1. Synthesis of 2-pyridyl methyl-3-oxobutanoate (III)

2-pyridyl methyl alcohol **I** (16. 67mmol, 1.82 g) was added to the stirred solution of 2,2,6-trimethyl-4H-1,3-dioxin-4-one **II** (16. 67mmol, 2.37 g) in xylene. The resulting mixture was refluxed for 1 hr. After TLC (chloroform: methanol, 9:1) showed complete conversion the solvent was removed under the reduced pressure. The final product **III** was purified using column chromatography (petroleum ether:ethylacetate, 4:1).

IR (KCl) 3020 (C-H aromatic), 1747 (CO-ester), 1720 (CO-ketone) cm^{-1}

2.2.2. General procedure for the synthesis of unsymmetrical derivatives of DHP

Compounds were synthesized using modified Hantzsch reaction [28]. Two mmol (0.23-0.32 g) of appropriate alkyl acetoacetate **IVa-d** and 3 mmol (0.23 g) of ammonium acetate were dissolved in appropriate alkyl alcohol. The reaction mixture was refluxed for 24 hrs. After completion of the reaction as indicated by TLC, aryl aldehyde **VIIa-d** (2 mmol, 0.30 g) and 2-pyridyl methyl-3-oxobutanoate **III** (2 mmol, 0.38 g) were added to the solution. After refluxing for further 24 hrs, the solvent was evaporated under reduced pressure. The products

were purified by column chromatography and preparative TLC on silica gel, affording pure materials **1a-d**, **2a-d**, **3a-d** and **4a-d**. The final products were recrystallized from diethyl ether and petroleum ether [29]. Characteristic data of synthesized compounds are as follows:

*3-pyridin-2-yl methyl 5-methyl 2,6-dimethyl-4-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,4-dihydropyridine-3,5-dicarboxylate (**1a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.61 (1H, brs, N***H***), 8.53 (1H, d, C_6-***H***-pyridyl, *J*=3.9 Hz), 7.92 (1H, s, C_4-***H***-imidazol), 7.62 (1H, t, C_4-***H***-pyridyl, *J*=7.3 Hz), 7.21 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.08 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.35 and 5.09 (2H, dd, O-C***H***$_2$-pyridyl, *J*=12.8 Hz), 5.18 (1H, s, C_4-***H***-DHP), 3.94 (3H, s, N_1-C***H$_3$***-imidazol), 3.66 (3H, s, O-C***H$_3$***), 2.29 and 2.23 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***). MS (EI): m/z 428 ($[MH]^+$, 8), 410 (39), 335 (53), 291 (84), 259 (100), 192 (56), 93 (93). IR (KBr) 3264 (NH), 3056 (CH-aromatic), 2934 (CH-aliphatic), 1695 (CO), 1536, 1378 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-ethyl 2,6-dimethyl-4-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,4-dihydropyridine-3,5-dicarboxylate (**1b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.94 (1H, brs, N***H***), 8.53 (1H, d, C_6-***H***-pyridyl, *J*=4.0 Hz), 7.92 (1H, s, C_4-***H***-imidazol), 7.61 (1H, t, C_4-***H***-pyridyl, *J*=7.3 Hz), 7.21 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.09 (1H, d, C_3-***H***-pyridyl, *J*=7.7 Hz), 5.35 and 5.10 (2H, dd, O-C***H***$_2$-pyridyl, *J*=12.9 Hz), 5.19 (1H, s, C_4-***H***-DHP), 4.12 (2H, q, O-C***H$_2$***CH_3, *J*=7.0 Hz), 3.97 (3H, s, N_1-C***H$_3$***-imidazol), 2.22 and 2.27 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***), 1.22 (3H, t, O-CH_2C***H$_3$***, *J*=7.0 Hz). MS (EI): m/z 442 ($[MH]^+$, 29), 424 (56), 349 (84), 305 (80), 259 (100), 93 (96). IR (KBr) 3270 (NH), 3058 (CH-aromatic), 2933 (CH-aliphatic), 1700 (CO), 1506, 1373 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-isopropyl 2,6-dimethyl-4-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,4-dihydropyridine-3,5-dicarboxylate (**1c**)*

^{1}H NMR (500MHz, $CDCl_3$) δ_H 8.60 (1H, brs, N***H***), 8.53 (1H, d, C_6-***H***-pyridyl, *J*=3.8Hz), 7.92 (1H, s, C_4-***H***-imidazol), 7.62 (1H, t, C_4-***H***-pyridyl, *J*=7.4Hz), 7.21 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.09 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.35 and 5.11 (2H, dd, O-C***H***$_2$-pyridyl, *J*=12.9 Hz), 5.17 (1H, s, C_4-***H***-DHP), 5.01 (1H, sep, O-C***H***$(CH_3)_2$, *J*=6.1 Hz), 3.99 (3H, s, N_1-C***H*****$_3$**-imidazol), 2.23 and 2.28 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.17and 1.20(6H, 2d, OCH(C***H*****$_3$**$)_2$, *J*= 6.1 Hz). MS (EI): m/z 456($[MH]^+$, 90), 438(20), 363(46), 329(64), 259(85), 233(68), 178(38), 93(100). IR (KBr) 3280(NH), 3135, 3051(CH-aromatic), 2982, 2930(CH-aliphatic), 1707, 1658(CO), 1540, 1339(NO_2) cm^{-1}

*3-pyridin-2-yl methyl 5-tert-butyl 2,6-dimethyl-4-(1-methyl-5-nitro-1H-imidazol-2-yl)-1,4-dihydropyridine-3,5-dicarboxylate (**1d**)*

^{1}H NMR (500MHz,$CDCl_3$) δ_H 8.88 (1H, brs, N***H***), 8.55 (1H, d, C_6-***H***-pyridyl, *J*=3.8 Hz), 7.93 (1H, s, C_4-***H***-imidazol), 7.65 (1H, t, C_4-***H***-pyridyl, *J*=7.4 Hz), 7.25 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.11 (1H, d, C_3-***H***-pyridyl, *J*=7.6Hz), 5.35 and 5.11 (2H, dd, O-C***H***$_2$-pyridyl, *J*=13.0 Hz), 5.14 (1H, s, C_4-***H***-DHP), (1H, d, O-C***H***$_2$-pyridyl, *J*=13.0 Hz),3.97 (3H, s, N_1-C***H*****$_3$**-imidazol), 2.28 and 2.18(6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.41 (9H, s, O-C(C***H*****$_3$**$)_3$). MS (EI): m/z 470($[MH]^+$, 16), 377 (52), 352(34), 321(54), 259(62), 233(100), 93(78). IR (KBr) 3298(NH), 3070(CH-aromatic), 2972, 2934(CH-aliphatic), 1679(CO), 1514, 1339 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-methyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**2a**)*

^{1}H NMR (500 MHz, DMSO-d_6) δ_H 9.07 (1H, s, N***H***), 8.44 (1H, d, C_6-***H***-pyridyl, *J*=4.4 Hz), 7.67 (1H, d, C_3-***H***-phenyl, *J*=7.4 Hz), 7.61 (2H, m,C_4-***H***-pyridyl and C_5-***H***-phenyl), 7.50 (1H, d, C_6-***H***-phenyl, *J*=7.7 Hz), 7.37 (1H, t, C_4-***H***-phenyl, *J*=7.6 Hz), 7.23 (1H, m, C_5-***H***-pyridyl), 6.82 (1H, d, C_3-***H***-pyridyl, *J*=7.8 Hz), 5.60 (1H, s, C_4-***H***-DHP), 5.04 (2H, s, O-C***H$_2$***-pyridyl), 3.42 (3H, s, O-C***H$_3$***), 2.22 and 2.28 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***). MS (EI): m/z 424 ([MH]$^+$, 12), 406 (20), 331 (100), 284 (18),192 (16), 93 (25). IR (KBr) 3265 (NH), 3076 (CH-aromatic), 2947 (CH-aliphatic), 1713, 1698 (CO), 1530, 1351 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-ethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**2b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 9.04 (1H, s, N***H***), 8.44 (1H, m, C_6-***H***-pyridyl), 7.69 (1H, m, C_3-***H***-phenyl), 7.60-7.64 (2H, m, C_4-***H***-pyridyl and C_5-***H***-phenyl), 7.50 (1H, m, C_6-***H***-phenyl), 7.38 (1H, m, C_4-***H***-phenyl), 7.24 (1H, m, C_5-***H***-pyridyl), 6.85 (1H, d, C_3-***H***-pyridyl, *J*=7.8 Hz), 5.66 (1H, s, C_4-***H***-DHP), 5.04 and 5.02 (2H, dd, O-C***H$_2$***-pyridyl, *J*=13.8 Hz), 3.81-4.00 (2H, m, O-C***H$_2$***CH_3), 2.23 and 2.26 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***), 1.04 (3H, t, O-CH_2C***H$_3$***, *J*=7.0 Hz). MS (EI): m/z 438 ([MH]$^+$, 39), 420 (15), 345 (100), 329 (20), 92 (40). IR (KBr) 3258 (NH), 3073 (CH-aromatic), 2984 (CH-aliphatic), 1700 (CO), 1531, 1350 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-isopropyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**2c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.50 (1H, d, C_6-***H***-pyridyl, *J*=3.6 Hz), 7.70 (1H, d, C_3-***H***-phenyl, *J*=8.0 Hz), 7.55 (2H, m, C_6-***H***-phenyl and C_4-***H***-pyridyl), 7.46 (1H, t, C_5-***H***-phenyl, *J*=7.3 Hz), 7.25 (1H, t, C_4-***H***-phenyl, *J*=6.0 Hz), 7.14 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.00 (1H, d, C_3-***H***-pyridyl, *J*=7.7 Hz), 5.94 (1H, s, C_4-***H***-DHP), 5.76 (1H, s, N***H***), 5.24 and 5.13 (2H, dd, O-C***H₂***-pyridyl, *J*=13.6 Hz), 4.95 (1H, m, O-C***H***$(CH_3)_2$), 2.29 and 2.33 (6H, 2s, C_2-C***H₃*** and C_6-C***H₃***), 1.21 and 0.97 (6H, 2d, O-CH(C***H₃***)$_2$, *J*=6.6 Hz). MS (EI): m/z 452 ($[MH]^+$, 6), 434 (25), 359 (100), 317 (47), 270 (29), 92 (36). IR (KBr) 3267 (NH), 3067 (CH-aromatic), 2980 (CH-aliphatic), 1694 (CO), 1522, 1349 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-tert-butyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**2d**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.53 (1H, d, C_6-***H***-pyridyl, *J*=3.9 Hz), 7.65-7.68 (2H, m, C_3-***H***-phenyl and C_4-***H***-pyridyl), 7.56 (1H, d, C_6-***H***-phenyl, *J*=7.7 Hz), 7.48 (1H, t, C_5-***H***-phenyl, *J*=7.4 Hz), 7.27 (1H, m, C_4-***H***-phenyl), 7.22 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.09 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.87 (1H, s, C_4-H-DHP), 5.78 (1H, brs, N***H***), 5.31 and 5.16 (2H, dd, O-C***H₂***-pyridyl, *J*=13.8), 2.31 and 2.28 (6H, 2s, C_2-C***H₃*** and C_6-C***H₃***), 1.36 (9H, s, O-C(C***H₃***)$_3$). MS (EI): m/z 466 ($[MH]^+$, 58), 392 (36), 373 (48), 317 (100), 287 (25), 93 (39), 57 (53). IR (KBr) 3227 (NH), 3080, 3024 (CH-aromatic), 2974 (CH-aliphatic), 1703, 1694 (CO), 1526, 1353 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**3a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.56 (1H, m, C_6-***H***-pyridyl), 8.08 (1H, m, C_2-***H***-phenyl), 8.00 (1H, m, C_4-***H***-phenyl), 7.58-7.63 (2H, m, C_6-***H***-phenyl and C_4-***H***-pyridyl), 7.34 (1H, t, C_5-***H***-phenyl, *J*=7.9 Hz), 7.20 (1H, m, C_5-***H***-pyridyl), 7.05 (1H, d, C_3-***H***-pyridyl, *J*=7.8 Hz), 5.86 (1H, s, N***H***), 5.30 and 5.14 (2H, dd, O-C***H***$_2$-pyridyl, *J*=13.4 Hz), 5.18 (1H, s, C_4-***H***-DHP), 3.65 (3H, s, O-C***H*****$_3$**), 2.42 and 2.38 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**). MS (EI): m/z 424 ([MH]$^+$, 10), 331 (100), 301 (31), 192 (44), 93 (55). IR (KBr) 3263 (NH), 3072 (CH-aromatic), 2949 (CH-aliphatic), 1695 (CO), 1529, 1348 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-ethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**3b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.56 (1H, d, C_6-***H***-pyridyl, *J*=4.7 Hz), 8.09 (1H, s, C_2-***H***-phenyl), 8.00 (1H, d, C_4-***H***-phenyl, *J*=8.1 Hz), 7.58-7.63 (2H, m, C_6-***H***-phenyl and C_4-***H***-pyridyl), 7.34 (1H, t, C_5-***H***-phenyl, *J*=8.1 Hz), 7.20 (1H, t, C_5-***H***-pyridyl, *J*=6.1 Hz), 7.04 (1H, d, C_3-***H***-pyridyl, *J*=7.7 Hz), 5.78 (1H, s, N***H***), 5.29 and 5.14 (2H, dd, O-C***H***$_2$-pyridyl, *J*=13.4 Hz), 5.18 (1H, s, C_4-***H***-DHP), 4.10 (2H, m, O-C***H*****$_2$**CH_3), 2.42 and 2.38 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.23 (3H, t, O-CH_2C***H*****$_3$**, *J*=7.0). MS (EI): m/z 438 ([MH]$^+$, 44), 345 (100), 329 (27), 315 (40), 206 (39), 93 (87). IR (KBr) 3261 (NH), 3071 (CH-aromatic), 2979 (CH-aliphatic), 1699 (CO), 1525, 1348 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-isopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**3c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.54 (1H, d, C_6-***H***-pyridyl, *J*=4.0 Hz), 8.08 (1H, s, C_2-***H***-phenyl), 7.98 (1H, d, C_4-***H***-phenyl, *J*=7.8 Hz), 7.57-7.62 (2H, m, C_6-***H***-phenyl and C_4-***H***-pyridyl), 7.33 (1H, t, C_5-***H***-phenyl, *J*=7.8 Hz), 7.19 (1H, t, C_5-***H***-pyridyl, *J*=5.7 Hz), 7.03 (1H, d, C_3-***H***-pyridyl, *J*=7.6), 5.76 (1H, s, N***H***), 5.27 (1H, d, O-C***H***$_2$-pyridyl, *J*=13.4 Hz), 5.13 (2H, m, O-C***H***$_2$-pyridyl and C_4-***H***-DHP), 4.95 (1H, sep, O-C***H***$(CH_3)_2$, *J*=6.2 Hz), 2.40 and 2.36 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***), 1.25 and 1.09 (6H, 2d, O-CH(C***H$_3$***)$_2$, *J*=6.0 Hz). MS (EI): m/z 452 ($[MH]^+$, 19), 359 (100), 317 (92), 287 (43), 178 (45), 93 (75). IR (KBr) 3269 (NH), 3068 (CH-aromatic), 2980 (CH-aliphatic), 1695 (CO), 1522, 1348 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-tert-butyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**3d**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.56 (1H, d, C_6-***H***-pyridyl, *J*=3.6 Hz), 8.09 (1H, s, C_2-***H***-phenyl), 8.00 (1H, d, C_4-***H***-phenyl, *J*=7.8 Hz), 7.62-7.64 (m, 2H, C_6-***H***-phenyl and C_4-***H***-pyridyl), 7.35 (1H, t, C_5-***H***-phenyl, *J*=7.8 Hz), 7.23 (1H, t, C_5-***H***-pyridyl, *J*=5.7 Hz), 7.09 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.83 (1H, s, N***H***), 5.29 and 5.18 (2H, dd, O-C***H$_2$***-pyridyl, *J*=13.6 Hz), 5.13 (1H, s, C_4-***H***-DHP), 2.41 and 2.34 (6H, 2s, C_2-C***H$_3$*** and C_6-C***H$_3$***), 1.40 (9H, s, O-C(C***H$_3$***)$_3$). MS (EI): m/z 466 ($[MH]^+$, 5), 373 (20), 317 (100), 287 (48), 178 (32), 93 (42). IR (KBr) 3266 (NH), 3065, 3012 (CH-aromatic), 2980 (CH-aliphatic), 1693 (CO), 1521, 1348 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-methyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4a**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.56 (1H, d, C_6-***H***-pyridyl, *J*=3.6 Hz), 8.02 (2H, d, C_3-***H***-phenyl and C_5-***H***-phenyl, *J*=8.2 Hz), 7.58 (1H, t, C_4-***H***-pyridyl, *J*=7.2 Hz), 7.40 (2H, d, C_2-***H***-phenyl and C_6-***H***-phenyl, *J*=8.2 Hz), 7.20 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.02 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.72 (1H, s, N***H***), 5.30 and 5.13 (2H, dd, O-C***H***$_2$-pyridyl, *J*=13.3 Hz), 5.17 (1H, s, C_4-***H***-DHP), 3.63 (3H, s, O-C***H*****$_3$**), 2.40 and 2.36 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**). MS (EI): m/z 424 ($[MH]^+$, 46), 331 (100), 301 (38), 192 (65), 93 (93). IR (KBr) 3259 (NH), 3066 (CH-aromatic), 2948 (CH-aliphatic), 1699 (CO), 1513, 1342 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-ethyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4b**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.55 (1H, d, C_6-***H***-pyridyl, *J*=4.4 Hz), 8.02 (2H, d, C_3-***H***-phenyl and C_5-***H***-phenyl, *J*=8.8 Hz), 7.58 (1H, m, C_4-***H***-pyridyl), 7.41 (2H, d, C_2-***H***-phenyl and C_6-***H***-phenyl, *J*=8.8 Hz), 7.20 (1H, m, C_5-***H***-pyridyl), 7.02 (1H, d, C_3-***H***-pyridyl, *J*=7.8 Hz), 5.80 (1H, s, N***H***), 5.29 and 5.13 (2H, dd, O-C***H***$_2$-pyridyl, *J*=13.4 Hz), 5.17 (1H, s, C_4-***H***-DHP), 4.08 (2H, m, O-C***H*****$_2$**CH_3), 2.39 and 2.35 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.20 (3H, m, O-CH_2C***H*****$_3$**). MS (EI): m/z 438 ($[MH]^+$, 7), 345 (100), 315 (34), 206 (30), 93 (59). IR (KBr) 3258 (NH), 3066 (CH-aromatic), 2973, 2937 (CH-aliphatic), 1700 (CO), 1510, 1342 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-isopropyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4c**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.55 (1H, d, C_6-***H***-pyridyl, *J*=3.6 Hz), 8.02 (2H, d, C_3-***H***-phenyl and C_5-***H***-phenyl, *J*=8.4 Hz), 7.57 (1H, t, C_4-***H***-pyridyl, *J*=7.4 Hz), 7.41 (2H, d, C_2-***H***-phenyl and C_6-***H***-phenyl, *J*=8.4 Hz), 7.19 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.03 (1H, d, C_3-***H***-pyridyl, *J*=7.7 Hz), 5.80 (1H, s, N***H***), 5.28 (1H, d, O-C***H***$_2$-pyridyl, *J*=13.4 Hz), 5.14 (2H, m, O-C***H***$_2$-pyridyl and C_4-***H***-DHP), 4.94 (1H, sep, O-C***H***CH_3, *J*=6.1 Hz), 2.38 and 2.35 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.23 and 1.09 (6H, 2d, O-CH(C***H*****$_3$**)$_2$, *J*=6.1 Hz). MS (EI): m/z 452 ($[MH]^+$, 58), 343 (30), 317 (29), 178 (40), 93 (100). IR (KBr) 3264 (NH), 3072 (CH-aromatic), 2971, 2930 (CH-aliphatic), 1693 (CO), 1508, 1342 (NO_2) cm^{-1}.

*3-pyridin-2-yl methyl 5-tert-butyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4d**)*

^{1}H NMR (500 MHz, $CDCl_3$) δ_H 8.57 (1H, d, C_6-***H***-pyridyl, *J*=3.6 Hz), 8.04 (2H, d, C_3-***H***-phenyl and C_5-***H***-phenyl, *J*=8.3 Hz), 7.63 (1H, t, C_4-***H***-pyridyl, *J*=7.2 Hz), 7.42 (2H, d, C_2-***H***-phenyl and C_6-***H***-phenyl, *J*=8.3 Hz), 7.25 (1H, t, C_5-***H***-pyridyl, *J*=5.9 Hz), 7.08 (1H, d, C_3-***H***-pyridyl, *J*=7.6 Hz), 5.78 (1H, s, N***H***), 5.31 and 5.19 (2H, dd, O-C***H*****$_2$**-pyridyl, *J*=13.5 Hz), 5.13 (1H, s, C_4-***H***-DHP), 2.39 and 2.34 (6H, 2s, C_2-C***H*****$_3$** and C_6-C***H*****$_3$**), 1.39 (9H, s, O-C(C***H*****$_3$**)$_3$). MS (EI): m/z 466 ($[MH]^+$, 30), 317 (100), 287 (45), 178 (32), 93 (77). IR (KBr) 3239 (NH), 3095 (CH-aromatic), 2976, 2933 (CH-aliphatic), 1701, 1659 (CO), 1512, 1342 (NO_2) cm^{-1}.

2.3. Cell lines

Cancer cell lines including K562 (human chronic myelogenous leukemia), LS180 (human colon adenocarcinoma), MCF-7 (human breast adenocarcinoma), and also cell lines used for MDR reversal tests, MES-SA and MES-SA/DX5 (human uterine sarcoma), were maintained in RPMI 1640 supplemented with 10% FBS, and 100 units/ml penicillin-G and 100 μg/ml streptomycin. The percentage of FBS for HL-60 (human acute promyelocytic leukemia) was 20%. For MES-SA/DX5 cells, 100 nM doxorubicin was added to the maintenance flasks and it was removed 24 hrs before starting the tests. LS180, MCF-7, MES-SA and MES-SA/DX5 cells were grown in monolayer cultures, while K562 and HL-60 cells were grown in suspension, at 37°C in humidified air containing 5% CO_2.

2.4. Cytotoxicity assay

Cytotoxicity of synthesized compounds was measured by MTT reduction assay [30,31]. Suspensions of HL-60, K562, LS180 and MCF-7 with appropriate densities were prepared and seeded in 96-well plates. The cytotoxicity assay was performed as described in our previous study [32]. After adding DMSO to solubilize the produced formazan, the absorbance of each well was read at 570 nm with background correction at 650 nm by a microplate reader (model 680, Bio-Rad, Japan).

2.5. MDR reversal measured by alteration of sensitivity to doxorubicin in resistant cells

MES-SA and MES-SA/DX5 cells were seeded in 96-well plates and maintained overnight at 37°C. Then the compounds at different concentrations were added in the absence or presence

of doxorubicin. There was only doxorubicin in reference wells. After 3 days of incubation at 37°C, MTT assay was conducted as described in our previous study [32,33].

2.6. Rhodamine 123 efflux assay for measurement of P-glycoprotein inhibition

R123 is a selective substrate for P-gp [34]. The assay that is used here was a modified version of the assay that was described in a previous study [32,35].

The MES-SA/DX5 cells were trypsinized, suspended in fresh medium and counted. Nine-hundred µL of the suspension containing 2.5 x 10^5 cells was placed in micro tubes and 100 µL of 5 µM R123 was added. Cells were kept at 37°C for 30 min and then were centrifuged and washed twice with ice-cold PBS. Five-hundred µL of three different concentrations of the synthesized compounds (ranging from 2.5 to 25 µM, final concentration) were added to the cells alongside with two concentrations of verapamil (2.5 and 10 µM), which was used as a reference P-gp inhibitor compound. The cells were incubated at 37°C for another 30 min. Finally, suspensions (5.0 x 10^5cells/ml) were analyzed by a flow cytometer (BD FACS Calibur, Becton Dickenson, USA). The number of cells counted for each sample was 10,000.

2.7. CCB activity evaluation

The CCB evaluation was carried out as previously described [32,36]. Male guinea pigs (300-450g) were purchased from Animal House Department, Shiraz University of Medical Sciences, fasted for 24 hrs and sacrificed by a blow on the neck. One-cm segments of ileum were installed in oxygenated tyrode solution [32] in jacketed organ bath (Pan Lab (Letica), Spain) at 37°C. KCl (40 mM) was added to the solution in presence or absence of the

synthesized compounds, nifedipine and DMSO as blank, final concentrations of which were 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M (x3, at 10 min intervals). The inhibitory effect of each compound was measured by percentile of the heights of the corresponding peaks in contrast to the peaks made by KCl in the absence of the compound. IC_{50} of each tested compound were calculated based on these percentages.

2.8. P-gp docking analysis

In this study, we applied AutoDock 4.2 package software to inquire the affinity and binding modes of the synthesized derivatives to the binding pocket of P-gp. Since the high resolution crystal structure of P-gp is not available, we applied the 3D structure of human P-gp which has been recently generated by homology modeling and MD simulation studies on X-ray structure of Apo murine P-gp (PDB entry: 3G5U) by our research group [37,38]. All the docked compounds were sketched and minimized (by molecular mechanics, MM+, and semi-empirical, AM1, methods) using HYPERCHEM 7.0 software. AutoDock Tools 4.2 was used to prepare ligands and protein for docking process. The PDBQT files were generated by adding charges and defining the degree of torsions. The grid dimensions were set to 60 × 60 × 60 Å with points separated by 0.375 Å and the grid was centered at 93.12, 68.17 and 124.91 Å (X, Y and Z) that involved the active site of DHPs. Docking was performed using Lamarckian Genetic Algorithm as the docking algorithm with 100 runs. Other parameters were left as default.

3. Results and Discussion

3.1. Synthesis

Unsymmetrical DHP derivatives (**1a-d**, **2a-d**, **3a-d** and **4a-d**) were synthesized based on the described method in "Experimental" section. The step-by-step method is depicted in Fig. 1.

Reaction of 2-pyridyl methyl alcohol **I** with 2,2,6-trimethyl-4-H-1,3-dioxin-4-one **II** produced 2-pyridyl methyl-3-oxobutanoate **III** in a good yield (95%). The final unsymmetric DHPs were synthesized according to a modified Hanstzch reaction. Hanstzch reaction has been one of the major methods of DHPs synthesis since its introduction in 1882 [39]. Chemical reaction of different alkyl acetoacetates **IVa-d** and ammonium acetate **V** in refluxing alkyl alcohol resulted in the formation of different aminocrotonates **VIa-d**. The aforementioned aminocrotonates then reacted with different aryl aldehydes and 2-pyridyl methyl-3-oxobutanoate **III** in order to produce the final unsymmetric DHPs. Synthesized compounds were then purified by preparative TLC and the chemical structures of the desired compounds were confirmed by ^{1}H NMR and EI-MS. The structures and physical properties of the synthesized compounds are summarized in Fig. 1 and Table 1, respectively.

3.2. Cytotoxicity

In order to investigate the anticancer potential of the synthesized compounds, the cytotoxic activity evaluations were performed against 4 human cancer cell lines including HL-60, K562, LS180 and MCF-7 by using MTT assay. The results are presented as IC_{50} values in Table 2.

In general, compounds bearing nitrophenyl moiety at C_4 (**2a-d**, **3a-d** and **4a-d**) showed cytotoxic activities, while the agents with nitroimidazole group at this position (**1a-d**) had no considerable cytotoxicity against the cell lines.

As for the substituent at C_5 position, the compounds that possess *iso*-propyl and *tert*-butyl (**2c-d**, **3c-d** and **4c-d**) had lower IC_{50} values, i.e., higher cytotoxic effects. It should be noted that since all compounds with nitrophenyl groups had similar IC_{50} values, it can be concluded that the aromatic group at C_4 position is a more influential factor compared to the alkyl group at C_5 position. An exception is **2d** bearing 2-nitrophenyl and *tert*-butyl at C_4 and C_5 positions, respectively, which demonstrated low cytotoxic activity. This is probably due to the change in the orientation caused by NO_2 group and the bulk of *tert*-butyl substitute that probably prevents the molecule from approaching its target site.

The cytotoxic potency of the compounds synthesized in the present study, were far better than the potencies of the symmetrical molecules containing pyridyl, reported in our previous studies, where only a few compounds showed IC_{50} values of lower than 100 μM [40,41]. Therefore, it can be stated that cytotoxicity will be improved by adding carbon to the carboxylate moieties at C_3 and C_5 positions.

3.3. MDR reversal measured by alteration of sensitivity to doxorubicin in resistant cells

MDR reversal activity was tested in P-gp overexpressing MES-SA/DX5 cells and the results were compared to those obtained in the parental cell line, MES-SA. The findings related to all synthesized compounds are presented in Tables 3 and 4, while one representative agent from each of the 4 groups of derivatives are shown in Figs. 2 and 3. P-gp expression in MES-SA and MES-SA/DX5 cells was examined by western blot and it was observed that while MES-SA-DX5 cells express a considerable amount of P-gp, the expression of this protein is below the detection limit in the parental MES-SA cells (data not shown).

MDR reversal activity measurement in P-gp overexpressing MES-SA/DX5 cells revealed that compounds with 4-nitrophenyl (**4a-d**) and 3-nitrophenyl (**3a-d**) at C_4 position,

generally possessed higher MDR reversal activities than their counterparts having nitroimidazole **(1a-d)** and 2-nitrophenyl (**2a-d**) rings (Table 3). Some of these compounds (**3c**, **3d**, **4b**, **4c** and **4d**) caused a significant reduction in the IC_{50} of doxorubicin at concentrations as low as 1 μM. Therefore, considering Log P values in Table 1, it can be deduced that less lipophilic nitroimidazole substituents were worse P-gp modulators than 3- and 4-nitrophenyl substituents. Moreover, a significant reduction of doxorubicin IC_{50} was observed in the presence of compounds **3a**, **3b** and **4a** at 2.5 μM concentrations and above. In our previous studies, compounds bearing nitroimidazole showed MDR reversal effect in HL60/MX1 (acute promyelocytic leukemia) cell line as a cell model of atypical MDR, which is a mitoxantrone resistant derivative of the human leukemia HL60 cell line with altered topoisomerase II catalytic activity and reduced levels of topoisomerase II alpha and beta proteins [40,41]. So, it is possible that these compounds are only effective on topoisomerase II and not P-gp.

Comparing the alkyl groups at C_5 position shows that the more lipophilic *tert*-butyl and *iso*-propyl substitutes confer higher MDR reversal activities than methyl and ethyl substitutions (Log P values are reported in Table 1). As expected, none of the tested compounds significantly increased the sensitivity of non-resistant MES-SA cells to doxorubicin (Table 4).

3.4. Rhodamine 123 efflux assay for measurement of P-glycoprotein inhibition

The efflux of Rh123 in MES-SA/DX5 cells was measured by a flow cytometric method. One representative diagram is depicted in Fig. 4 and the geometric means of all experiments are shown in Fig. 5.

Rh123 is selectively pumped out of the cell by P-gp, therefore, inhibitors of P-gp can prevent this process and increase intracellular accumulation of this fluorescent probe [34]. Rh123 efflux assay was used to confirm the results obtained from MTT method and to specifically show that the compounds work through P-gp pump inhibition.

Flow cytometric determination of Rh123 efflux in MES-SA/DX5 cells showed that in general, the compounds with 4-nitrophenyl (**4a-d**) or 3-nitrophenyl at C_4 position (**3a-d**) were better modulators of P-gp, because they promoted intracellular Rh123 retention more strongly and at lower concentrations compared to compounds with nitroimidazole (**1a-d**) or 2-nitrophenyl (**2a-d**). **3c**, **4a**, **4b** and **4c** were the most promising P-gp inhibitors which caused significant Rh123 accumulation compared to control in MES-SA/DX5 cells at 5 μM concentration.

Generally, the Rh123 efflux results were in agreement with the findings of MDR reversal assay obtained by MTT reduction method. However, as these two techniques measure cellular events at different incubation times, some dissimilarities were observed in the order of compound activities; While, **3d** and **4d** demonstrated significant reduction in the IC_{50} of doxorubicin at concentrations as low as 1 μM (Table 3), these compounds exhibited lower P-gp inhibitory activities in flow cytometric Rh123 determination assay compared to the other derivatives in their subgroups, 3-nitrophenyl and 4-nitrophenyl bearing derivatives at C4, respectively (Fig. 5).

Regarding the substitution at C_5 position, it is noticeable that in the most potent compound sets, the *iso*-propyl containing compounds (**3c** and **4c**) showed greater P-gp inhibitory activities compared to other C_5 substituents, but in the case of the least potent series, the compounds containing *tert*-butyl at C_5 position (**1d** and **2d**) had better effects.

In our previous studies, 3-nitrophenyl and 4-nitrophenyl at C_4 position of the DHP ring resulted in slightly higher activity compared to the compounds with nitroimidazole as the aromatic ring at C_4 position. Moreover, as noted in our former studies, the presence of larger alkyl groups such as *iso*-propyl and *tert*-butyl at C_5 position leads to more active compounds [32]. These consequences are consistent with the results of the current study.

3.5. CCB activity evaluation

The CCB activity of synthesized compounds, as a potential side effect of compounds with DHP structure, was evaluated on guinea pig ileal longitudinal smooth muscle (GPLISM) and the IC_{50} related to each compound was calculated. The results are shown in Table 5.

Among the synthesized compounds, those bearing nitroimidazolyl or 4-nitrophenyl group at C_4 (**4a-d** and **1a-d**) demonstrated the lowest CCB activity. Compound **1a** with an IC_{50} value of 8.1 x 10^{-6} M was 76 times less active than nifedipine as the reference CCB agent. On the other hand, compounds possessing 2-nitrophenyl or 3-nitrophenyl at C_4 (**2a-d** and **3a-d**) had the lowest IC_{50} values. Some of these compounds (**3a-c** and **2a**) were even stronger than nifedipine. These structure-activity relationship findings are in agreement with the outcomes of our previous study [41].

In addition, introducing *iso*-propyl or *tert*-butyl groups at C_5 position reduced the CCB activity, while the opposite happened by methyl or ethyl substitutions. For example, **4c-d** blocked the calcium channels less than **4a-b** and similarly **2c-d** had higher IC_{50} values than **2a-b**. Although C_5 moiety had some effects on the IC_{50} values, the most influential factor to determine the CCB activity appeared to be the aromatic group at C_4 position. This is in line with the previous studies and modeling of the 1,4-dihydropyridines as calcium channel blockers [42,43].

3.6. Molecular docking study

It has been reported that DHPs bind near the Nucleotide Binding Domain (NBD) of P-gp. Borchers and colleagues determined the active site of DHPs by photo affinity labeling and mass spectrometry [44]. They suggested that DHPs bind to the P-gp 468 –527 residues. Szabon-Watola and coworkers predicted the binding location of the DHPs resides near the conserved Val907 of coupling helix 2, and an essential Mg^{2+} binding residue Gln475 of the Walker domain [45]. They suggested that a ligand binding in this location would be expected to limit conformational reorganization necessary for xenobiotic efflux. The molecular docking analysis of **1c**, **2c**, **4a** and **4c** suggested that the most active derivative, **4c**, possessed the lowest estimated free energy of binding (-10.59 kcal/mol) and estimated inhibition constant (17.30 nM) (Table 6). Oxygen atoms of NO_2 group on phenyl ring made two strong hydrogen bond interactions with Arg543 and Ser474 and a key hydrogen bond could be seen between NH of DHP ring and carbonyl side chain of Gln441. Carbonyl functions of 2-pyridyl methyl carboxylate and *iso*-propyl carboxylate moieties involved in hydrogen bond interactions with hydroxyl groups of Ser474 and Ser909, respectively. Additionally, isopropyl appeared to be well accommodated into the hydrophobic pocket surrounded by Il470, Val471 and Tyr490 (Fig. 6. A). Compound **4a**, having methyl carboxylate moiety, demonstrated the second best estimated free energy of binding (-9.74 kcal/mol) and estimated inhibition constant (71.97 nM) (Table 6). It seems that the binding pose of **4c** (containing *iso*-propyl carboxylate at C_5) is slightly different from that of compound **4a** (containing methyl carboxylate at C_5) (Fig. 6. B). Consequently, **4c** established hydrogen bond interaction between nitrogen atom of pyridine ring and Asn903 but in the case of **4a**, this interaction was not observed. Moreover, unlike the bulky *iso*-propyl group in **4c**, the small methyl function in **4a** did not well orientate

toward the mentioned hydrophobic residues. Compounds **1c** and **2c**, bearing nitroimidazole and 2-nitrophenyl at C_4, respectively, showed the lowest estimated free energies of binding (-9.67 and kcal/mol, respectively) (Table 6). As illustrated in Fig. 6. C and Fig. 6. D, the binding orientation of **2c** and **1c** is totally different from that of **4a** and **4c**. Therefore, for compound **2c**, 2-pyridyl methyl carboxylate moiety did not have any interaction with the receptor, while NH of DHP ring involved in a hydrogen bond interaction with Ser909 and NO_2 formed two hydrogen bonds with Arg547 and Val472. Another hydrogen bond interaction was established between Arg543 and carbonyl group of the methyl carboxylate moiety Fig. 6. C. Binding interactions of compound **1c** is illustrated in Fig. 6 .D.

Generally, molecular docking analysis of the compounds in the active site of P-gp indicated that substitutions on C_4 and C_5 positions of DHP ring played an important role in binding orientations of the tested compounds. The presence of 4-nitrophenyl (as in **4c**) at C_4 is more favored than 2-nitrophenyl (as in **2c**) and nitroimidazole (as in **1c**) and could probably lead to more favorable interactions and better orientations. According to docking results compound **4c**, bearing *iso*-propyl carboxylate substitution at C_3, might have better binding affinity to P-gp active site than its counterpart, **4a**, with methyl carboxylate moiety due to the bulk of *iso*-propyl group, which provided better orientation of **4c** in the pocket comprising some lipophilic residues.

4. Conclusions

In conclusion, 16 novel DHP derivatives were synthesized and examined for cytotoxicity, MDR reversal capacities and also CCB activity as a potential side effect. The compounds with noticeable inherent cytotoxicity, remarkable MDR reversal and low CCB effects were identified. Consequently, **4a** and **4c** (both possessing 4-nitrophenyl at C_4 and methyl (**4a**) and

iso-propyl carboxylates (**4c**) at C_5) were recognized as the most promising compounds with low CCB activity which had moderate inherent cytotoxicity against cancer cells and significantly increased the sensitivity of P-gp overexpressing MES-SA-DX5 cells to doxorubicin at the low concentrations of 1 and 2.5 μM (**4c** and **4a**, respectively). Furthermore, the two compounds remarkably increased the intracellular accumulation of Rh123 in MES-SA-DX5 cells comparable to that of verapamil.

Compound **3c** (bearing 3-nitrophenyl at C_4 and *iso*-propyl carboxylate at C_5) was the most effective MDR reversal derivative; however, its CCB activity did not differ much from that of nifedipine. Compounds with nitroimidazole as the aromatic moiety at C_4, showed neither cytotoxicity nor MDR reversal effect.

The binding modes of potent compounds, **4a** and **4c** inside the P-gp active site indicated that these compounds could be well accommodated in the active site and key hydrogen bond interactions were observed between ligands and Gln441, Ser474, Arg543, Ser909 and Asn903 of the active site.

Some of the synthesized DHP derivatives presented in this study, have cytotoxic and MDR reversal effects, while possessing low CCB activity, and could represent promising compounds for further development of novel agents useful for treatment of resistant types of cancer.

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Table and figure caption list:

Table 1. Physical properties of synthesized unsymmetrical dihydropyridine compounds

Table 2. Cytotoxic activity of synthesized dihydropyridine compounds on various human cancer cell

Table 3. Effects of synthesized dihydropyridine compounds on the sensitivity of multidrug-resistant MES-SA/DX5 cells to doxorubicin assessed by MTT assay.

Table 4. Effects of synthesized dihydropyridine compounds on the sensitivity of non-resistant MES-SA cells to doxorubicin assessed by MTT assay.

Table 5. Calcium channel blocking activities of synthesized unsymmetrical dihydropyridine compounds.

Table 6. Docking results of selected compounds with P-gp.

Fig. 1. Synthesis procedure of 1,4-dihydropyridine derivatives.

Fig. 2. Effects of representative synthesized 1,4-dihydropyridine compounds on the sensitivity of multidrug-resistant MES-SA/DX5 cells to doxorubicin examined by MTT assay. MES-SA/DX5 cells were seeded in 96-well plates and compounds 1d (a), 2d (b), 3c (c) and 4c (d) were added at 0.5–5 μM concentrations after 24 hrs. Doxorubicin was also added at 100-1000 nM concentrations right after the test compound and cells were further incubated for 72 hrs. MTT test was performed and the data were presented as % viability compared to control (non-treated) wells. Compounds 3c (c) and 4c (d) caused significant changes at the concentration of 1 μM, while compounds 1d (a) and 2d (b) did not differ from control (doxorubicin alone) at 1 μM (data not shown). Data are mean ± S.E.M. of 3-5 independent experiments and differences were analysed by using the one-way ANOVA followed by LSD test.

Fig. 3. Effects of representative synthesized compounds against non-resistant parental MES-SA cells to doxorubicin measured by MTT assay. MES-SA cells were seeded in 96-well plates after 24 hrs of incubation the compounds were added at 0.5–5 μM concentrations immediately followed by doxorubicin addition at 100-1000 nM. After further incubation for 72 hrs, MTT test was performed and the data were presented as %viability compared to control (non-treated) wells. Data are mean ± S.E.M. of 3-5 independent experiments. Differences were analysed by using the one-way ANOVA followed by LSD test.

Fig. 4. Effect of compound 4c on the fluorescence intensity of Rh123 in MES-SA/DX5 cells. MES-SA/DX5 cells were trypsinized and resuspended in fresh medium. 2.5 x 10^5 cells in a micro tube were treated with Rh123 (5 μM, final concentration) and incubated at 37 °C for 30 min. The cells were centrifuged and washed twice with ice-cold PBS and then treated with 3 different concentrations of the synthesized compounds (5, 10 and 25 μM). The cells were incubated at 37°C for another 30 min and finally, 10,000 of them were injected to a flow cytometer.

Fig. 5. Inhibition of Rh123 efflux shown as mean fluorescence intensity in MES-SA/DX5 cells in the presence or absence of synthesized compounds and verapamil. MES-SA/DX5 cells were trypsinized and resuspended in fresh medium. 2.5 × 10^5 cells in a micro tube were treated with Rh123 (5 μM, final concentration) and incubated at 37 °C for 30 min. The cells were centrifuged and washed twice with ice-cold PBS and then treated with 3 different concentrations of the synthesized compounds (ranging from 2.5 to 25 μM). The cells were incubated at 37°C for another 30 min and finally, 10,000 of them were injected to a flow cytometer. Ctrl: Control sample, VP: verapamil. Data are mean ± S.E.M. of 3-5 independent experiments, and differences were analyzed by using the one-way ANOVA followed by LSD

test.* The difference between Gmean in the absence and the presence of the test compound is significant (P value< 0.05).

Fig. 6. Docking of compounds 4c (A), 4a (B), 2c (C) and 1c (D) in the active site of P-gp. Ligands are displayed as yellow sticks, while the core amino acid residues are displayed as grey sticks and hydrogen bonds are illustrated by green lines.

Table 1. Physical properties of synthesized unsymmetrical dihydropyridine compounds.

Compound	MW[a] (g/mol)	mp[b] (°C)	Yield	Log P[c]	R_f
1a	427	203-206	24%	1.70	0.40
1b	441	200-206	18%	2.12	0.38
1c	455	190-192	12%	2.59	0.40
1d	469	204-207	17%	3.00	0.40
2a	423	200-202	19%	2.56	0.54
2b	437	194-196	20%	2.98	0.52
2c	451	176-181	31%	3.44	0.44
2d	465	137-139	28%	3.86	0.56
3a	423	106-108	14%	2.56	0.58
3b	437	137-139	38%	2.98	0.56
3c	451	167-172	21%	3.44	0.52
3d	465	198-199	39%	3.86	0.56
4a	423	136-138	21%	2.56	0.60
4b	437	117-118	41%	2.98	0.64
4c	451	123-124	15%	3.44	0.52
4d	465	155-160	25%	3.86	0.60
Nifedipine					

[a] Molecular weight. [b] Melting point. [c] Logarithm of partition coefficient between n-octanol and water (LogP) calculated by DruLiTo 1 software.

Table 2. Cytotoxic activity of synthesized dihydropyridine compounds on various human cancer cell lines.

Compound	**IC_{50} (μM)**			
	HL-60	**K562**	**LS180**	**MCF-7**
1a	>200	>200	>200	102.8 ± 24.2*
1b	151.7 ± 75.4	>200	>200	100.0 ± 30.9
1c	107.0 ± 17.5	>200	128.6 ± 33.7	106.1 ± 28.9
1d	133.4 ± 6.5	>200	115.7 ± 51.8	39.7 ± 4.4
2a	22.5 ± 4.5	34.7 ± 2.6	43.0 ± 5.9	44.1 ± 5.2
2b	44.2 ± 6.8	23.4 ± 1.6	27.4 ± 4.5	36.1 ± 3.5
2c	30.4 ± 2.4	11.1 ± 2.2	37.0 ± 2.1	24.1 ± 3.8
2d	36.4 ± 5.7	52.6 ± 13.9	54.2 ± 3.0	84.7 ± 16.4
3a	43.3 ± 1.3	78.5 ± 4.4	56.9 ± 5.4	46.2 ± 10.0
3b	44.6 ± 1.6	32.4 ± 0.9	46.5 ± 5.9	38.1 ± 2.3
3c	38.0 ± 2.0	21.8 ± 2.2	89.6 ± 10.4	26.8 ± 3.5
3d	11.7 ± 0.4	21.8 ± 1.8	32.5 ± 4.3	32.4 ± 3.9
4a	40.9 ± 4.1	49.6 ± 4.2	65.0 ± 10.7	55.8 ± 13.4
4b	35.0 ± 5.6	35.5 ± 7.6	50.6 ± 11.7	35.6 ± 4.0
4c	24.0 ± 2.5	22.1 ± 6.5	32.5 ± 6.4	24.9 ± 5.8
4d	25.0 ± 2.3	16.0 ± 2.0	30.6 ± 8.5	20.4 ± 5.5
Doxorubicin	$(6.9 \pm 0.9)\times10^{-3}$	$(68.3 \pm 13.4)\times10^{-3}$	$(51.1 \pm 12.3)\times10^{-3}$	$(32.8 \pm 8.3)\times10^{-3}$

* The values higher than 100 μM are obtained by extrapolation of the dose-response curve. Values are presented as mean ± S.E.M. of at least 4 experiments.

Table 3. Effects of synthesized dihydropyridine compounds on the sensitivity of multidrug-resistant MES-SA/DX5 cells to doxorubicin assessed by MTT assay.

Compound	IC_{50} of doxorubicin in the presence of different concentrations of test compound						
	0 µM	**0.5 µM**	**1 µM**	**2.5 µM**	**5 µM**	**10 µM**	**25 µM**
1a	702.1 (97.4)	-	-	-	664.4 (135.9)	435.5 (92.6)	551.1 (60.5)
1b	725.5(82.3)	-	-	-	548.8 (81.7)	500.4 (51.2)	180.9* (17.1)
1c	770.4 (109.2)	-	-	-	918.5 (59.0)	561.1 (127.5)	111.6* (12.0)
1d	695.3 (192.9)	-	-	437.8 (58.1)	198.8* (45.2)	-	-
2a	629.5 (125.7)	-	-	-	1017.2 (83.5)	674.2 (141.5)	-
2b	837.0 (90.4)	-	-	1004.6 (131.1)	682.3 (80.9)	425.8*(45.7)	-
2c	862.4 (110.8)	-	-	681.7 (74.8)	530.4 (146.9)	269.9* (220.4)	-
2d	1005.7(169.3)	642.3 (111.3)	426.7* (43.7)	306.3*(53.4)	72.6* (22.9)	-	-
3a	828.5 (73.4)	-	852.8 (204.0)	442.0*(107.1)	88.4*(25.7)	-	-
3b	798.3 (77.4)	-	585.6 (77.3)	378.6* (65.9)	112.3* (50.8)	-	-
3c	964.5 (97.6)	737.7 (125.7)	615.5* (70.1)	334.5* (31.3)		-	-
3d	838.6 (60.0)	-	515.0*(195.4)	429.3* (37.9)	303.5* (51.2)	-	-
4a	563.2 (115.7)	-	434.0 (40.8)	303.7* (47.3)	-	-	-
4b	777.2 (148.4)	-	386.8* (16.8)	148.3* (24.9)	-	-	-
4c	748.2 (81.1)	683.9 (65.7)	454.4* (127.9)	219.5* (39.7)	-	-	-
4d	815.1 (45.5)	667.4 (106.9)	537.1* (65.4)	239.5* (14.5)	69.0* (26.0)	-	-

* The difference between the IC_{50} value of doxorubicin (expressed in nM) in the absence of the test compound (0 µM) and its presence at different concentrations (0.5-25 µM) was significant ($p < 0.05$). Values are presented as mean (S.E.M.) of at least 4 experiments and differences were analysed by using the one-way ANOVA followed by LSD test.

Table 4. Effects of synthesized dihydropyridine compounds on the sensitivity of non-resistant MES-SA cells to doxorubicin assessed by MTT assay.

Compound	IC_{50} of doxorubicin at the presence of different concentrations of test compound						
	0 µM	**0.5 µM**	**1 µM**	**2.5 µM**	**5 µM**	**10 µM**	**25 µM**
1a	28.9 (4.8)	-	-	-	32.8 (14.0)	32.1 (6.6)	21.2 (3.9)
1b	42.0 (9.1)	-	-	-	21.8 (8.8)	29.0 (8.3)	21.9 (6.4)
1c	36.7 (10.2)	-	-	-	30.6 (10.6)	26.2 (9.1)	17.3 (7.7)
1d	40.9 (8.1)	-	-	43.2 (11.8)	35.1 (8.0)	-	-
2a	29.9 (3.5)	-	-	-	30.1 (4.8)	20.4 (2.7)	-
2b	28.3 (2.5)	-	-	36.9 (6.1)	33.6 (7.2)	24.4 (5.0)	-
2c	25.2 (3.4)	-	-	33.0 (14.8)	26.2 (7.6)	11.8 (3.3)	-
2d	33.0 (5.4)	26.8 (8.8)	30.6 (9.8)	29.0 (7.7)	24.1 (3.6)	-	-
3a	30.0 (4.4)	-	22.0 (1.0)	41.2 (4.6)	34.8 (7.4)	-	-
3b	30.0 (3.1)	-	35.0 (5.6)	43.7 (8.7)	27.1 (2.4)	-	-
3c	32.5 (2.7)	24.2 (1.8)	40.5 (5.1)	26.0 (4.2)	-	-	-
3d	26.5 (3.2)	-	17.2 (2.4)	30.5 (3.9)	22.2 (3.2)	-	-
4a	35.2 (3.3)	-	42.5 (3.6)	38.7 (4.7)	-	-	-
4b	39.8 (4.3)	-	33.5 (2.5)	30.4 (4.6)	-	-	-
4c	33.9 (6.0)	27.0 (1.8)	30.0 (4.5)	18.2 (2.1)	-	-	-
4d	33.3 (5.0)	31.2 (7.9)	31.1 (6.7)	23.4 (2.2)	23.0 (4.9)	-	-

* The differences between the IC_{50} value of doxorubicin (expressed in nM) in the absence of the test compound (0 µM) and its presence at different concentrations (0.5-25 µM) were not significant ($p < 0.05$). Values are presented as mean (S.E.M.) of at least 4 experiments and differences were analysed by using the one-way ANOVA followed by LSD test.

Table 5. Calcium channel blocking activities of synthesized unsymmetrical dihydropyridine compounds.

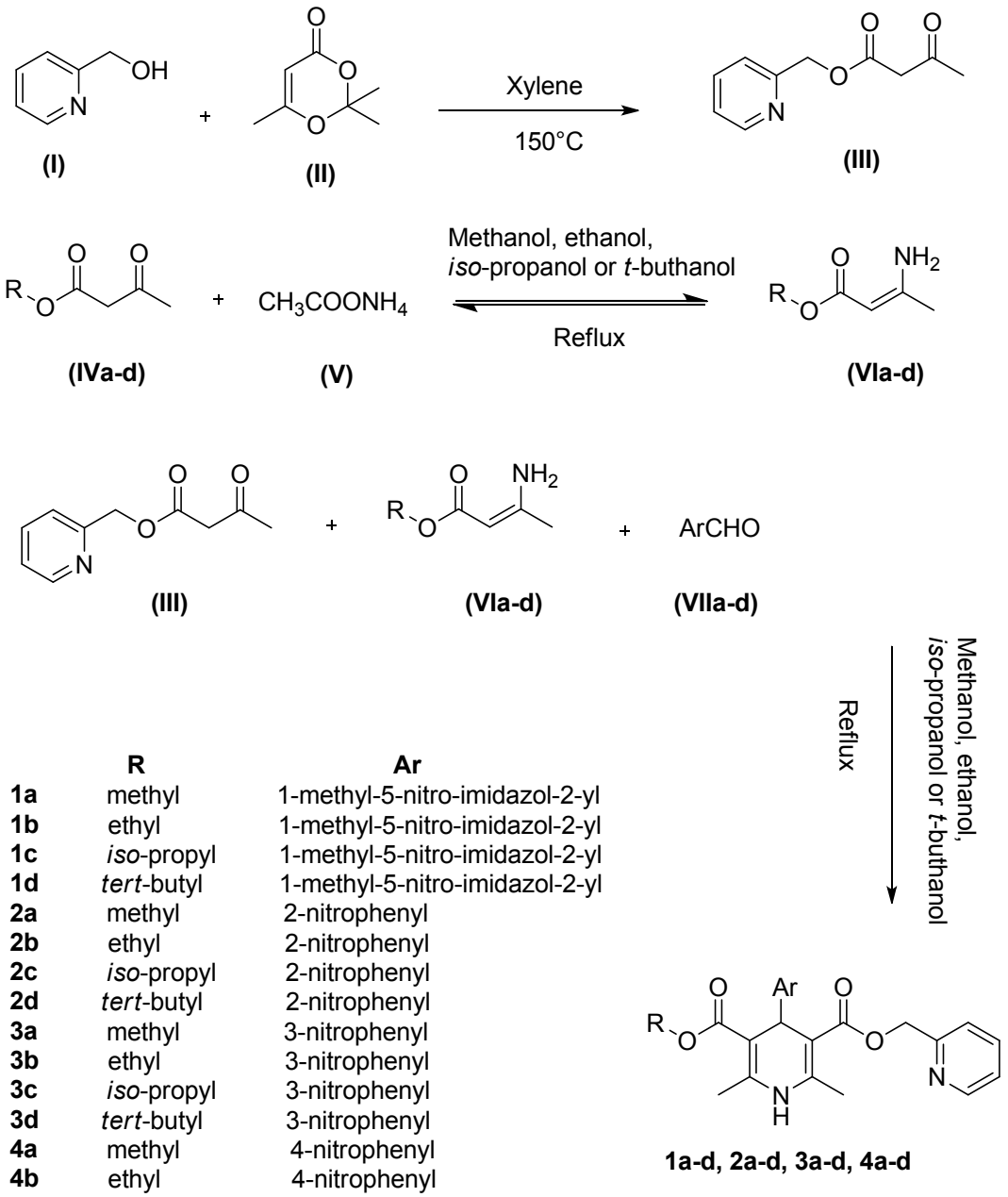
Compounds	IC_{50} (M)	P value*
1a	$(8.1 \pm 3.4)\times10^{-6}$	0.000
1b	$(4.6 \pm 0.5)\times10^{-7}$	0.163
1c	$(4.8 \pm 1.9)\times10^{-7}$	0.334
1d	$(1.3 \pm 0.6)\times10^{-6}$	0.008
2a	$(1.1 \pm 0.3)\times10^{-8}$	1.000
2b	$(1.1 \pm 0.4)\times10^{-7}$	1.000
2c	$(2.0 \pm 0.9)\times10^{-7}$	0.997
2d	$(6.8 \pm 1.8)\times10^{-7}$	0.036
3a	$(6.5 \pm 1.3)\times10^{-8}$	1.000
3b	$(9.5 \pm 2.2)\times10^{-8}$	1.000
3c	$(5.0 \pm 0.2)\times10^{-8}$	0.713
3d	$(1.5 \pm 0.4)\times10^{-7}$	0.997
4a	$(1.8 \pm 0.7)\times10^{-6}$	0.001
4b	$(5.2 \pm 1.0)\times10^{-7}$	0.086
4c	$(2.9 \pm 2.4)\times10^{-6}$	0.014
4d	$(2.8 \pm 0.9)\times10^{-6}$	0.000
Nifedipine	$(1.1 \pm 0.4)\times10^{-7}$	-

* Statistical analysis of the difference between IC_{50} values of test compounds and nifedipine.

Values are presented as mean ± S.E.M. of 3-4 experiments.

Table 6. Docking results of selected compounds with P-gp.

Compound	ΔG (kcal/mol)	Ki (nM)	Hydrogen bond interaction		
			Atom of ligand	Amino acid	Distance (Å)
1c	-9.55	99.25	Oxygen (NO_2)	Arg543	1.80
			Oxygen (NO_2)	Arg543	2.51
			C=O (*iso*-propyl carboxylate)	Ser909	2.55
			C=O (pyridyl carboxylate)	Arg547	2.19
			N<u>H</u>	Gln441	1.81
2c	-9.94	51.70	Oxygen (NO_2)	Arg547	2.02
			Oxygen (NO_2)	Val472	2.11
			C=O (pyridyl carboxylate)	Arg543	2.14
			N<u>H</u>	Ser909	2.05
4a	-10.04	43.95	Oxygen (NO_2)	Arg543	2.55
			Oxygen (NO_2)	Ser474	2.23
			C=O (methyl carboxylate)	Ser909	2.80
			C=O (pyridyl carboxylate)	Ser474	2.04
			N<u>H</u>	Gln441	1.97
4c	-10.30	28.31	Oxygen (NO_2)	Arg543	2.20
			Oxygen (NO_2)	Ser474	2.40
			C=O (*iso*-propyl carboxylate)	Ser909	2.78
			C=O (pyridyl carboxylate)	Ser474	2.04
			N<u>H</u>	Gln441	1.98
			Nitrogen (pyridine ring)	Asn903	2.00



	R	Ar
1a	methyl	1-methyl-5-nitro-imidazol-2-yl
1b	ethyl	1-methyl-5-nitro-imidazol-2-yl
1c	*iso*-propyl	1-methyl-5-nitro-imidazol-2-yl
1d	*tert*-butyl	1-methyl-5-nitro-imidazol-2-yl
2a	methyl	2-nitrophenyl
2b	ethyl	2-nitrophenyl
2c	*iso*-propyl	2-nitrophenyl
2d	*tert*-butyl	2-nitrophenyl
3a	methyl	3-nitrophenyl
3b	ethyl	3-nitrophenyl
3c	*iso*-propyl	3-nitrophenyl
3d	*tert*-butyl	3-nitrophenyl
4a	methyl	4-nitrophenyl
4b	ethyl	4-nitrophenyl
4c	*iso*-propyl	4-nitrophenyl
4d	*tert*-butyl	4-nitrophenyl

Fig. 1

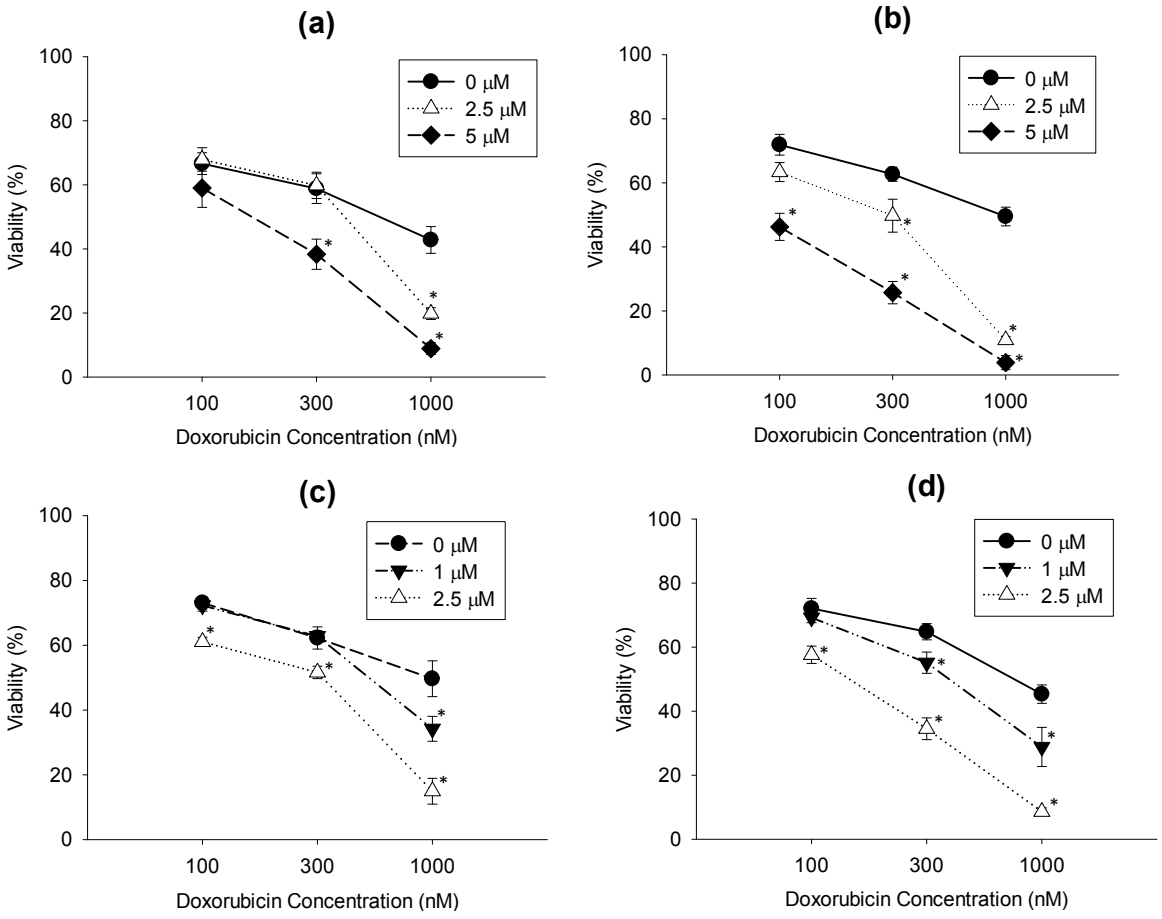
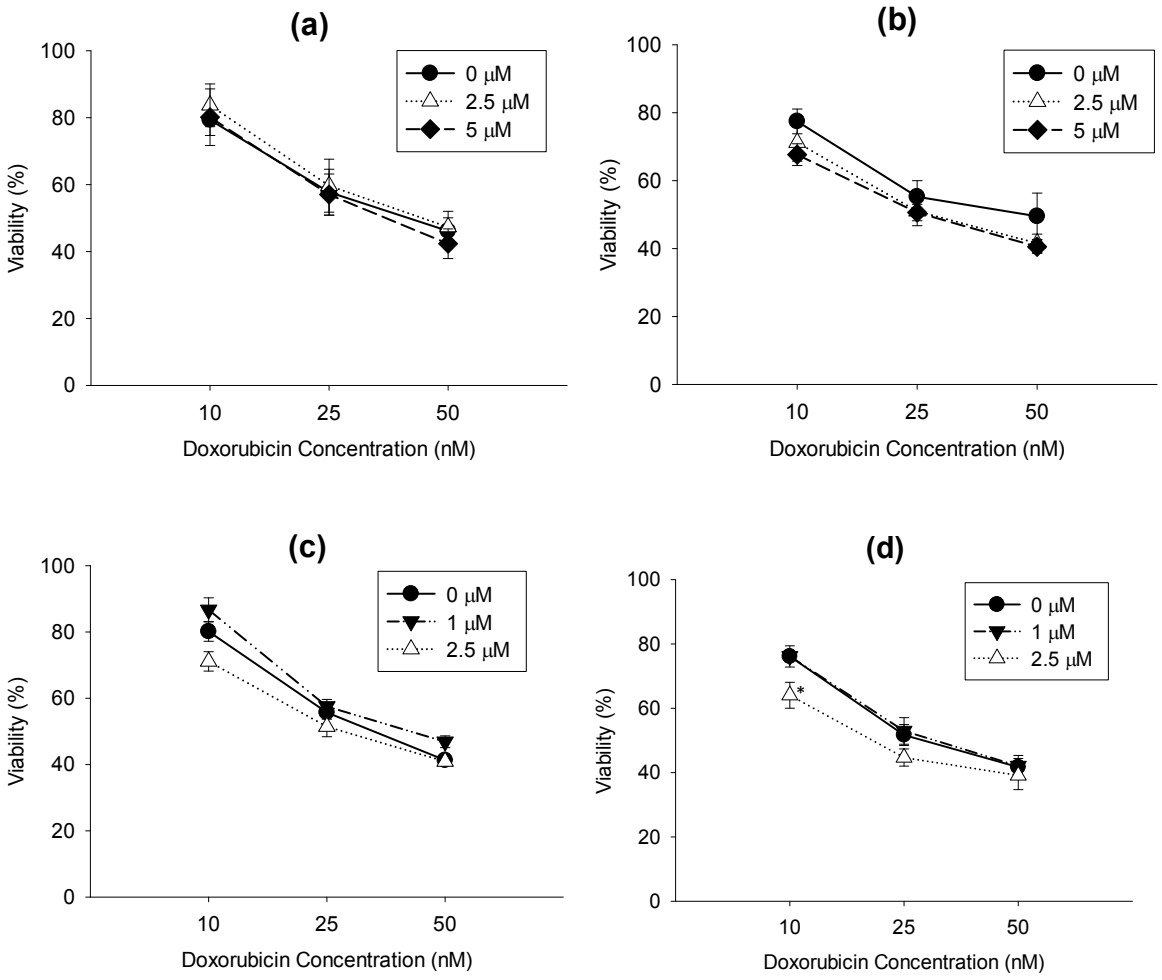


Fig. 2



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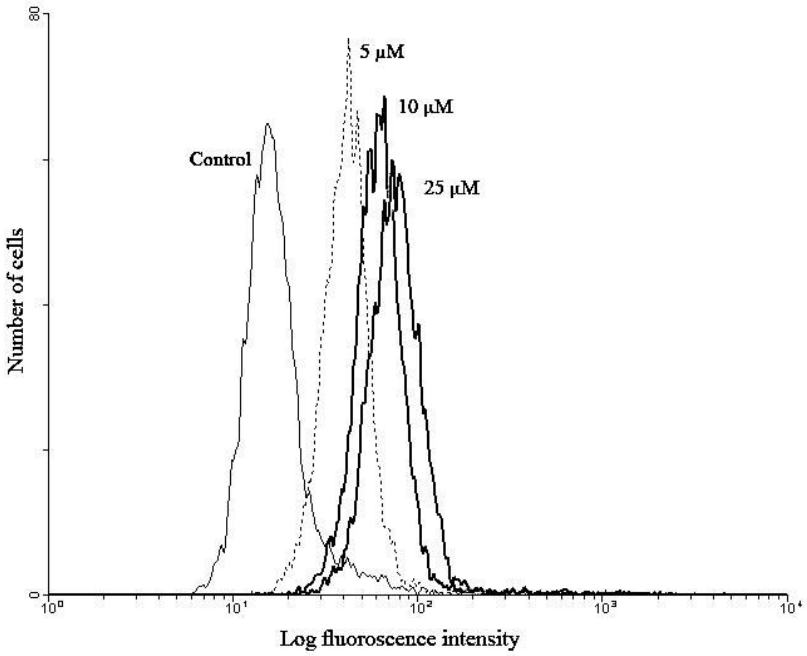

Fig. 4

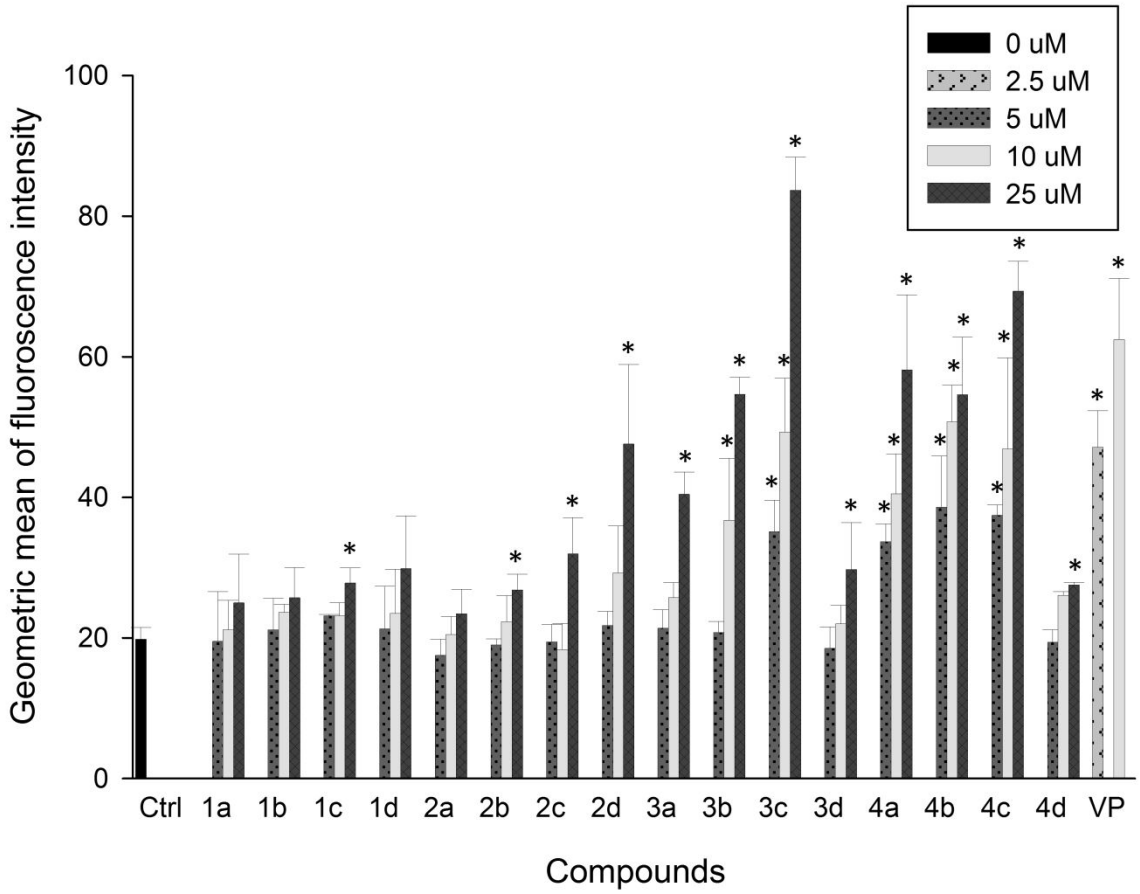

Fig. 5

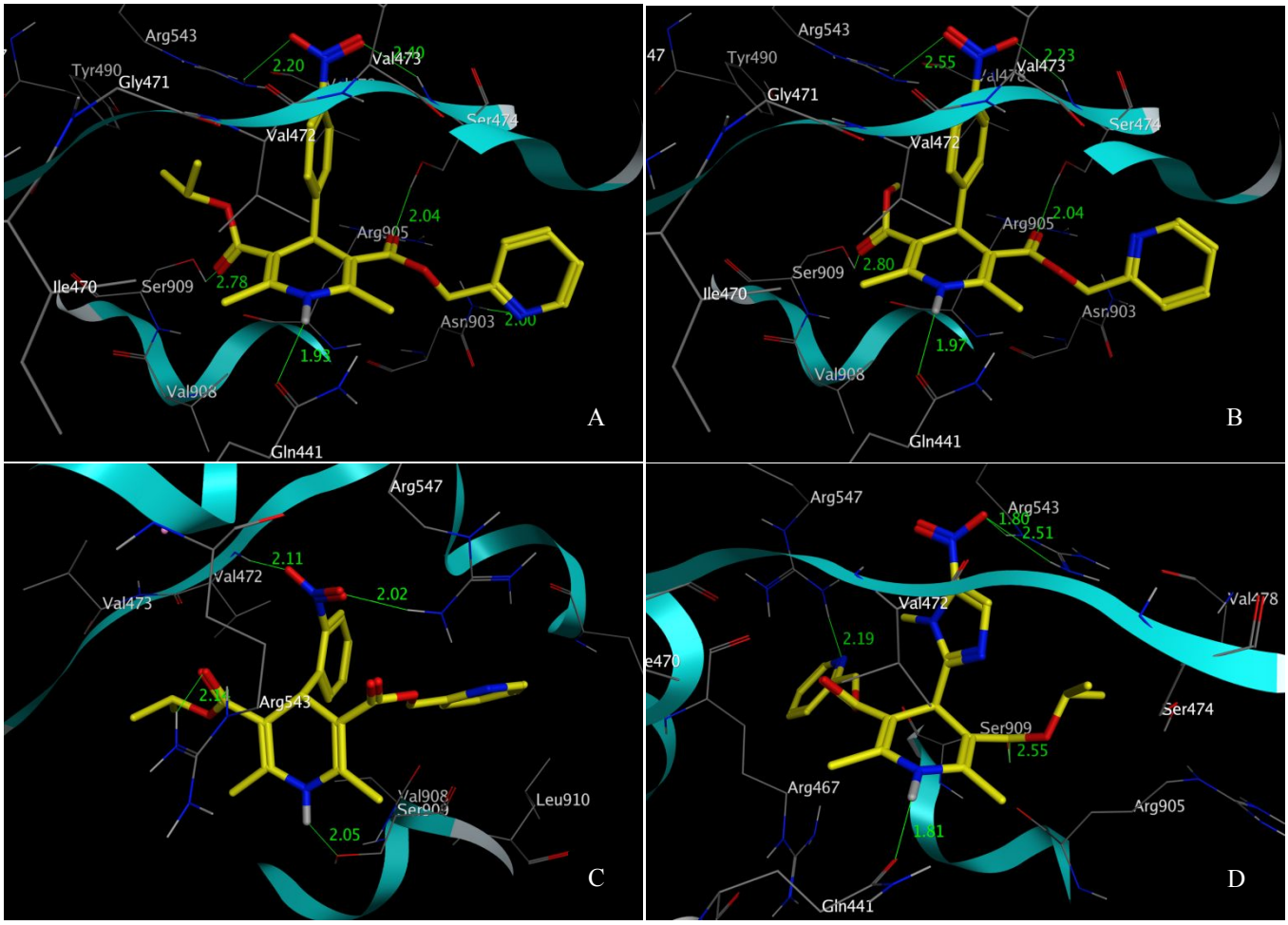

Fig. 6