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Design and synthesis of new symmetrical derivatives of dihydropyridine containing a pyridyl group on the 3, 5-positions and evaluation of their cytotoxic and multidrug resistance reversal activity

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Abstract

Today, chemotherapy is an important part in the treatment of several kinds of cancer; however, the development of drug resistance remains one of the major obstacles in successful chemotherapy. Several types of agents have been recognized as multidrug resistance (MDR) inhibitors, among which the 1,4-dihydropyridines (DHPs) have been investigated the most. P-glycoprotein inhibition has been reported as the main MDR reversal mechanism of DHPs, whilst other mechanisms such as inhibition of topoisomerase II have received less attention. Therefore, in this study new derivatives of DHP have been synthesized. Their cytotoxic activity and their effects in reversing atypical MDR have been evaluated. The results confirmed the appropriate effect of these compounds on atypical MDR. Although it was observed that these compounds had a moderate cytotoxic effect, the cytotoxicity of one compound on the K562 cell line (IC50 = 6.61 μM) was comparable with that of doxorubicin (IC50 = 4.17 μM). Finally, the Ca^{2+}-channel antagonistic activity, an undesired effect for these compounds, was evaluated.

Introduction

Today, chemotherapy is an important part in the treatment of several kinds of cancer. However, multidrug resistance (MDR) development limits the effectiveness of chemotherapy and remains a clinical obstacle in cancer treatment (Liscovitch & Lavie 2002; Thomas & Coley 2003). Several different mechanisms have been suggested for the development of MDR. It has been shown that over-expression of ATP-binding cassette (ABC) transporters is mainly responsible for MDR development, which mediates the ATP-dependent efflux of a variety of structurally and functionally diverse compounds across different biological membranes (Ueda et al 1986; Ozben 2006). This type was classified as classical or typical MDR (Morrow & Cowan 1997; Ambudkar et al 1999). Some of the most active drugs currently available for the treatment of cancer act on topoisomerase II as their primary target and are mentioned as topoisomerase II poisons (Burden & Osheroff 1998; Walker & Nitiss 2002). A multidrug resistance pattern has been described for the resistance of cells to several topoisomerase poisons, which has been named atypical or non-classical MDR (Liscovitch & Lavie 2002).

One of the most investigated strategies to overcome resistance to anticancer drugs is administration of agents that could inhibit the MDR mechanisms. Therefore, studies have been focused on the development of agents capable of inhibiting MDR mechanisms. Many compounds with diverse structures have been introduced as MDR modulators (Teodori et al 2002). Among the possible resistance modifiers, 1,4-dihydropyridines (DHPs), Ca^{2+}-channel antagonists, have been investigated extensively (Kawase & Motohashi 2003; Fusi et al 2006). However, the clinical use of DHPs as MDR reversal agents has been limited due to their potential cardiovascular side effects (Philip et al 1992). Attempts have been made to develop DHP molecules with high MDR reversal activity but low Ca^{2+}-channel antagonistic activity. It has been revealed that the main mechanism of DHPs as MDR-modulators is inhibition of P-glycoprotein (P-gp), which belongs to the ABC transporter family (Nogae et al 1989; Tanabe et al 1998; Voigt et al 2007). Also, there are some reports

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about effects of DHPs on MDR associated to other ABC transporters, such as multidrug resistance associated protein (MRP) and breast cancer resistance protein (BCRP) (Zhou et al 2005a, b). As previously mentioned, some classes of atypical MDR are associated with altered topoisomerase II levels and activity. Recently, we reported the effectiveness of some DHP derivatives in reversing atypical MDR (Mehdipour et al 2007). Furthermore, it was reported that dexniguldipine exerted its cytotoxicity by inhibition of DNA topoisomerase I through a non-competitive mechanism (Straub et al 1997).

Those findings raised the hypothesis that some other DHP derivatives with similar structure to our previously synthesized derivatives may also be effective against topoisomerase II-mediated MDR (atypical MDR). Hence, in this study, we have evaluated the potentiation effect of some newly synthesized DHP derivatives on the IC50 value (the concentration resulting in 50% inhibition) of mitoxantron in an HL60/MX1 cell line, whose MDR mechanism was postulated to be an alteration in topoisomerase II levels or activity (Harker et al 1989). Furthermore, the intrinsic cytotoxic effect of these derivates was investigated, as was their calcium channel antagonistic activity, an undesirable effect of the synthesized compounds.

Materials and Methods

Physical measurements

The melting points were measured on a hot stage apparatus (Electrothermal, Essex, UK) and were uncorrected. The ^{1}H NMR spectra were performed on a Burker-AvanceDPX-500 MHz in d_1-chloroform. Tetramethysilane (TMS) was used as the internal standard. The mass spectra were obtained with a Hewlett-Packard spectrometer (Hewlett-Packard (HP) 6890, Böblingen, Germany) with a direct inlet system at 70 eV. The IR spectra were measured with a Perkin-Elmer spectrometer (KBr disk) (Perkin-Elmer, Waltham, MA, USA). All spectra confirmed the structure of the synthesized compounds.

Synthesis of arylacetoacetate (3a–c)

A mixture of 16.67 mmol of corresponding alcohol (Sigma-Aldrich Chemie GmbH, Deisenhofon, Germany) and 16.67 mmol 2,2,6-trimethyl-4H-1,3-dioxin-4-one (Sigma-Aldrich Chemie GmbH, Deisenhofon, Germany) were refluxed in 10 mL xylene stirred vigorously at a temperature of 150°C for 75 min. The formation of final product was confirmed using thin layer chromatography (TLC) (>90%). Afterwards, the reaction mixture was cooled and the xylene removed. The product was purified by TLC on silica gel with chloroform–methanol (90/10) mobile phase to give pure compounds **3a–c**.

*Synthesis of pyridine-3-yl-propyl-3-oxobutanate (**3a**)*
Yield 95% Anal. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.12; H, 6.81; N, 6.35. ^{1}H NMR ($CDCl_3$): δ 1.98 (m, 2H, $COCH_2CH_2CH_2$), 2.12 (s, 3H, CH_3), 2.77 (t, 2H, $COCH_2CH_2CH_2$), 3.43 (s, 3H, $COCH_2CO$), 4.08 (t, 2H, $COCH_2CH_2CH_2$), 7.23–7.70 (m, 3H, $H_{4,5,6}$-pyridyl), 8.53 (d, 1H, H_2-pyridyl). MS: m/z (%) 221(4), 206(4), 136(100), 106(90), 85(21), 78(33), 43(70). IR (KCl): ν 1745(CO, ester), 1717 (CO, ketone).

*Synthesis of pyridine-4-yl-propyl-3-oxobutanate (**3b**)*
Yield 92% Anal. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.15; H, 6.80; N, 6.34. ^{1}H NMR ($CDCl_3$): δ 1.93 (m, 2H, $COCH_2CH_2CH_2$), 2.05 (s, 3H, CH_3), 2.45 (t, 2H, $COCH_2CH_2CH_2$), 3.41 (s, 3H, $COCH_2CO$), 4.21 (t, 2H, $COCH_2CH_2CH_2$), 7.29–7.69 (m, 2H, $H_{5,6}$-pyridyl), 8.59 (m, 2H, $H_{2,4}$-pyridyl). MS: m/z (%) 221(7), 206(9), 178(18), 136(100), 122(92), 92(85), 85(24), 65(42), 43(68). IR (KCl): ν 1741 (CO, ester), 1717 (CO, ketone).

*Synthesis of pyridine-2-yl-propyl-3-oxobutanate (**3c**)*
Yield 90% Anal. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.11; H, 6.82; N, 6.36. ^{1}H NMR ($CDCl_3$): δ 1.96 (m, 2H, $COCH_2CH_2CH_2$), 2.10 (s, 3H, CH_3), 2.65 (t, 2H, $COCH_2CH_2CH_2$), 3.58 (s, 3H, $COCH_2CO$), 4.12 (t, 2H, $COCH_2CH_2CH_2$), 7.32 (m, 2H, $H_{2,6}$-pyridyl), 8.64 (m, 2H, $H_{3,5}$-pyridyl). MS: m/z (%) 221(10), 206(12), 136(99), 122(100), 106(99), 92(80), 65(45), 43(72). IR (KCl): ν 1744 (CO, ester), 1717 (CO, ketone).

General procedure for the synthesis of symmetrical derivatives of DHP

A mixture of 1-methyl-5-nitroimidazole-2-carboxaldehyde (1 mmol) or nitrobenzaldehyde (1 mmol), arylacetoacetate (2 mmol), and ammonium hydroxide (excess) were refluxed in methanol (40 mL) overnight. DHP derivatives are photosensitive (Shamsipur et al 2003) and so the reaction was carried out under the light of a sodium lamp to prevent the degradation of compounds during the process. The formation of compound was evaluated using TLC. The solution was cooled and concentrated under reduced pressure and purified by TLC on silica gel with chloroform–methanol (85/15) mobile phase. The products were recrystallized from ether or a mixture of chloroform/n-hexan (80/20) to give pure compounds **4a–h** and **6a–b**.

*3,5-Di-(pyridin-3-yl-propyl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4a**)*
Yield 36% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.91; H, 5.80; N, 10.06. ^{1}H NMR ($CDCl_3$): δ 1.93 (m, 4H, $COCH_2CH_2CH_2$), 2.38 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.61 (t, 4H, $COCH_2CH_2CH_2$), 4.09 (t, 4H, $COCH_2CH_2CH_2$), 5.14 (s, 1H, C_4-H), 6.77 (s, 1H, NH), 7.27 (d, 1H, H_5-pyridyl), 7.38 (m, 1H, H_5-phenyl), 7.48 (d, 1H, H_6-phenyl), 7.64 (d, 2H, H_6-pyridyl), 7.95 (d, 1H, H_4-phenyl), 8.17 (s, 1H, H_2-phenyl), 8.41 (m, 4H, $H_{2,4}$-pyridyl). MS: m/z (%) 539(5), 508(2), 434(6), 297(18), 281(5), 226(8), 196(13), 136(24), 120(55), 106(46), 92(14), 77(31), 51(8). IR (KBr): ν 3266 (NH), 1688 (CO), 1499, 1348 cm^{-1} (NO_2).

*3,5-Di-(pyridin-3-yl-propyl)-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4b**)*
Yield 30% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.90; H, 5.77; N, 10.05. ^{1}H NMR ($CDCl_3$): δ 1.92 (m, 4H, $COCH_2CH_2CH_2$), 2.37 (s, 6H, C_2-CH_3 and

C_6-CH_3), 2.61 (t, 4H, $COCH_2CH_2CH_2$), 4.09 (t, 4H, $COCH_2CH_2CH_2$), 5.15 (s, 1H, C_4-H), 6.64 (s, 1H, NH), 7.27 (m, 4H, $H_{2,6}$-phenyl and H_5-pyridyl), 7.53 (d, 2H, H_6-pyridyl), 8.13 (d, 2H, $H_{3,5}$-phenyl), 8.41 (m, 4H, $H_{2,4}$-pyridyl). MS: m/z (%) 538(0.8), 508(1), 434(1), 297(4), 224(5), 136(11), 118(63), 92(58), 71(42), 57(88), 43(100). IR (KBr): ν 3270 (NH), 1692 (CO), 1518, 1346 cm^{-1} (NO_2).

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

Yield 40% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.90; H, 5.78; N, 10.09. ^{1}H NMR ($CDCl_3$): δ 1.95 (m, 4H, $COCH_2CH_2CH_2$), 2.34 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.53 (t, 4H, $COCH_2CH_2CH_2$), 4.1 (t, 4H, $COCH_2CH_2CH_2$), 5.85 (s, 1H, C_4-H), 5.9 (s, 1H, NH), 7.2 (m, 4H, $H_{4,6}$-phenyl and H_5-pyridyl), 7.44 (m, 1H, H_5-phenyl), 7.55 (d, 2H, H_6-pyridyl), 7.7 (d, 1H, H_3-phenyl), 8.35 (m, 4H, $H_{2,4}$-pyridyl). MS: m/z (%) 537(8), 433(8), 388(67), 297(17), 224(88), 211(14), 196(27), 136(21), 118(100), 106(68), 92(71), 77(18), 51(21). IR (KBr): ν 3195 (NH), 1692 (CO), 1499, 1352 cm^{-1} (NO_2).

*3,5-Di-(pyridin-4-yl-propyl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4d**)*

Yield 35% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.91; H, 5.81; N, 10.06. ^{1}H NMR ($CDCl_3$): δ 1.93 (m, 4H, $COCH_2CH_2CH_2$), 2.36 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.58 (t, 4H, $COCH_2CH_2CH_2$), 4.24 (t, 4H, $COCH_2CH_2CH_2$), 5.08 (s, 1H, C_4-H), 7.08 (d, 1H, NH), 7.38 (d, 4H, $H_{2,6}$-pyridyl), 7.4 (m, 1H, H_5-phenyl), 7.65 (s, 1H, H_2-phenyl), 7.92 (d, 1H, H_6-phenyl), 8.1 (d, 1H, H_4-phenyl), 8.40 (d, 4H, $H_{3,5}$-pyridyl). MS: m/z (%) 539(17), 434(51), 403(53), 391(10), 376(34), 329(100), 297(87), 271 (61), 196(5), 136(9), 120(45), 106(27), 92(24), 77(8), 51(4). IR (KBr): ν 3276 (NH), 1696 (CO), 1508, 1352 cm^{-1} (NO_2).

*3,5-Di-(pyridin-4-yl-propyl)-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4e**)*

Yield 29% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.90; H, 5.80; N, 10.09. ^{1}H NMR ($CDCl_3$): δ 2.01 (m, 4H, $COCH_2CH_2CH_2$), 2.36 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.59 (t, 4H, $COCH_2CH_2CH_2$), 4.08 (t, 4H, $COCH_2CH_2CH_2$), 5.12 (s, 1H, C_4-H), 6.23 (s, 1H, NH), 7.26 (d, 4H, $H_{2,6}$-pyridyl), 7.50 (d, 2H, $H_{2,6}$-phenyl), 8.1 (d, 2H, $H_{3,5}$-phenyl), 8.4 (d, 4H, $H_{3,5}$-pyridyl). MS: m/z (%) 539(8), 508(8), 434(20), 403(15), 329(16), 297(37), 271(1), 226(3), 210(20), 136(24), 118(100), 106(35), 93(100), 77(21), 51(23). IR (KBr): ν 3267 (NH), 1693 (CO), 1500, 1350 cm^{-1} (NO_2).

*3,5-Di-(pyridin-4-yl-propyl)-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4f**)*

Yield 47% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.91; H, 5.81; N, 10.10. ^{1}H NMR ($CDCl_3$): δ 2.05 (m, 4H, $COCH_2CH_2CH_2$), 2.36 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.78 (t, 4H, $COCH_2CH_2CH_2$), 4.10 (t, 4H, $COCH_2CH_2CH_2$), 5.13 (s, 1H, C_4-H), 6.09 (s, 1H, NH), 6.99 (d, 1H, H_6-phenyl), 7.07 (d, 1H, H_4-phenyl), 7.26 (d, 4H, $H_{2,6}$-pyridyl), 7.53 (m, 1H, H_5-phenyl), 8.4 (d, 4H, $H_{3,5}$-pyridyl). MS: m/z (%) 537(8), 508(1), 432(11), 388(35), 376(41), 297(8), 240(26), 226(49), 210(25), 196(30), 137(15), 119(18), 106(61), 93(13), 77(27), 51(18). IR (KBr): ν 3267 (NH), 1692 (CO), 1502, 1349 cm^{-1} (NO_2).

*3,5-Di-(pyridin-2-yl-propyl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4g**)*

Yield 45% Anal. Calcd for $C_{31}H_{32}N_4O_6$: C, 66.89; H, 5.79; N, 10.07. Found: C, 66.92; H, 5.81; N, 10.09. ^{1}H NMR ($CDCl_3$): δ 2.05 (m, 4H, $COCH_2CH_2CH_2$), 2.36 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.78 (t, 4H, $COCH_2CH_2CH_2$), 4.10 (t, 4H, $COCH_2CH_2CH_2$), 5.13 (s, 1H, C_4-H), 6.05 (s, 1H, NH), 7.15 (m, 2H, H_4-pyridyl), 7.26 (d, 2H, H_6-pyridyl), 7.45 (m, 1H, H_5-phenyl), 7.47 (d, 1H, H_6-phenyl), 7.58 (m, 2H, H_5-pyridyl), 7.85 (s, 1H, H_2-phenyl), 8.15 (d, 1H, H_4-phenyl), 8.52 (dd, 2H, H_3-pyridyl). MS: m/z (%) 538(1), 508(2), 434(5), 403(3), 376(4), 357(8), 329(11), 313(15), 272(22), 254(21), 242(69), 224(61), 198(65), 120(100), 106(19), 93(28), 78(11), 51(8). IR (KBr): ν 3514 (NH), 1699 (CO), 1497, 1347 cm^{-1} (NO_2).

*3,5-Di-(pyridin-2-yl-propyl)-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**4h**)*

Yield 20% Anal. Calcd for $C_{29}H_{32}N_6O_6$: C, 62.02; H, 5.92; N, 14.96. Found: C, 62.05; H, 5.90; N, 14.99. ^{1}H NMR ($CDCl_3$): δ 1.95 (m, 4H, $COCH_2CH_2CH_2$), 2.34 (s, 6H, C_2-CH_3 and C_6-CH_3), 2.53 (t, 4H, $COCH_2CH_2CH_2$), 4.1 (t, 4H, $COCH_2CH_2CH_2$), 5.85 (s, 1H, C_4-H), 5.9 (s, 1H, NH), 7.15 (m, 1H, H_4-pyridyl), 7.28 (d, 1H, H_6-pyridyl), 7.47 (d, 4H, $H_{2,6}$-phenyl), 7.84 (m, 2H, H_5-pyridyl), 8.15 (d, 2H, $H_{3,5}$-phenyl), 8.51 (d, 2H, H_3-pyridyl). MS: m/z (%) 538(0.8), 508(0.8), 434(1), 537(10), 272(32), 254(31), 242(58), 224(77), 196(23), 120(10), 106(19), 93(28), 78(11), 51(8). IR (KBr): ν 3513 (NH), 1699 (CO), 1497, 1347 cm^{-1} (NO_2).

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

Yield 24% Anal. Calcd for $C_{29}H_{32}N_6O_6$: C, 62.02; H, 5.92; N, 14.96. Found: C, 62.04; H, 5.93; N, 14.99. ^{1}H NMR ($CDCl_3$): δ 1.9 (m, 4H, $COCH_2CH_2CH_2$) 2.2 (s, 6H, C_2-CH_3 and C_6-CH_3) 2.7 (t, 4H, $COCH_2CH_2CH_2$) 4.2 (t, 4H, $COCH_2CH_2CH_2$) 4.3 (s, 3H, N-CH_3) 5.1 (s, 1H, C_4-H), 7.1 (m, 2H, H_5-pyridyl), 7.2 (s, 1H, NH), 7.4 (d, 2H, H_6-pyridyl), 7.9 (s, 1H, $NH_{imidazolyl}$), 8.5 (m, 4H, $H_{2,4}$-pyridyl). MS: m/z (%) 541(3), 512(11), 433(32), 405(8), 349(25), 315(27), 297(46), 270(27), 228(3), 136(32), 119(100), 106(58), 92(60), 77(11), 51(5). IR (KBr): ν 3270 (NH), 1698 (CO), 1498, 1379 cm^{-1} (NO_2).

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

Yield 12% Anal. Calcd for $C_{29}H_{32}N_6O_6$: C, 62.02; H, 5.92; N, 14.96. Found: C, 62.05; H, 5.90; N, 14.98. ^{1}H NMR ($CDCl_3$): δ 2 (m, 4H, $COCH_2CH_2CH_2$) 2.2 (s, 6H, C_2-CH_3 and C_6-CH_3) 2.7 (t, 4H, $COCH_2CH_2CH_2$) 4.2 (dd, 4H, $COCH_2CH_2CH_2$) 4.3 (s, 3H, N-CH_3) 5.2 (s, 1H, C_4-H) 7 (d, 4H, $H_{2,6}$-pyridyl), 7.2 (s,1H,NH), 7.9 (s, 1H, $NH_{imidazolyl}$), 8.5 (d, 4H, $H_{3,5}$-pyridyl). MS: m/z (%) 541(1), 512(2), 432(50), 405(4), 349(13), 329(19), 315(8), 297(31), 271(23), 136(14), 119(100), 106(63), 93(88), 77(21), 51(15). IR (KBr): ν 3220 (NH), 1703 (CO), 1515, 1357 cm^{-1} (NO_2).

Pharmacological testing

Male albino guinea-pigs were obtained from the Animal House Department of Shiraz University of Medical Sciences. The animals were housed in a room maintained at a temperature of 23 ± 2°C, 55 ± 10% humidity, on a 12-h dark/light cycle. They had free access to standard rodent chow and tap water at all times. The animals were fasted from one day before the test (Amini et al 2002). Guinea-pigs (300–450 g) were killed by a blow on the head and the intestines were removed above the ileocaecal junction. Smooth muscle segments of approximately 1-cm length were mounted in an adequate amount of oxygenated physiological saline solution at 4°C for approximately 4–18 h. Afterwards, the muscle segments were suspended in a jacketed organ bath containing oxygenated (95% O_2 and 5% CO_2) physiological saline solution of the following composition (in mM: NaCl 137, $CaCl_2$ 1.8, KCl 2.7, $MgSO_4$ 1.1, $NaHPO_4$ 0.4, $NaHCO_3$ 12, and glucose 5) at 37°C. The physiological saline solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 and its temperature was kept at 37°C. A resting tension of 0.5 g was applied to the ileal segments. The organ bath fluid was changed every 15 min, allowing the muscle segments to equilibrate for 1 h. Contractions of ileal segments were recorded with a force-displacement transducer (Hugo Sachs, March-Hugstetten, Germany) on a physiograph (Hugo Sachs).

To investigate the effects of the synthesized DHPs on KCl-induced contractions of the ileum, several contractions were elicited by KCl (40 mM). The mean value of these was considered as 100%. When no significant differences between KCl-induced contractions were seen, tissue was pre-incubated with one particular concentration of one of the compounds for 15 min. The KCl-induced contraction was assessed. The IC50 value of each compound was calculated from the contraction–response curve (Dagnino et al 1987; Miri et al 2006).

To obtain the highest concentration, all compounds and nifedipine (the reference drug) were dissolved in dimethyl sulfoxide (DMSO); however, other concentrations were made by dissolving drugs in water.

Statistical analysis

Results were reported as mean ± s.e.m. To analyse data, one-way analysis of variance following the Tukey–Dunnet test was performed. A P value under 0.05 was considerd significant.

Cell lines

Five cell lines were used in this study: Jurkat (human, peripheral blood, leukaemia, and T cell), K562 (chronic myelogenous leukaemia), Fen (bladder carcinoma cell line), Raji (lymphoblast-like cells), and HL60/MX1 (acute promyelocytic leukaemia). All cell lines were purchased from the Iran Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI-1640 (Biosera, East Sussex, UK) supplemented with 10% or 20% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Biosera, East Sussex, UK) in a humidified 5% CO_2 incubator at 37°C.

Cytotoxicity evaluation

An amount of each of the compounds was dissolved in DMSO. Four concentrations (10, 100, 500 and 1000 μM) were prepared through serial dilution in RPMI-1640. Similar amounts of DMSO, as a negative control, and doxorubicin, as a positive control, were made in the same way. In the experiments 96-well plates were used. Each well was filled with 1–5 × 10^4 cells (depending on the cell line) in 100 μL culture media. Afterwards, 11.1 μL stock solutions of the compounds, or the negative or positive controls, were added to the wells to get the final concentrations of 1, 10, 50 or 100 μM. The experiments were performed in triplicate. Three wells containing only the same number of cells were left in each plate. Plates were incubated in a 5% humidified incubator at 37°C for 48 h. MTT solution (3-(4,5-dimethlthiazide-2yl)-2,5-diphenyl tetrazolium bromide; Sigma, USA), 10 μL, in phosphate-buffered saline at a concentration of 5 mg mL^{-1} was then added to the wells and the MTT assay was performed as described by Jabbar et al (1989). Before calculating the IC15, IC30, and IC50, percents of inhibition of DMSO were subtracted from those of compounds.

MDR reversal activity

MDR reversal activity of DHP compounds was reported as IC50 values and ratio factor (RF) in the HL60/MX1 cell line. Cells were seeded into 96-well plates at 3 × 10^4 cells/well. Various concentrations of mitoxantron and DHP derivatives were gradually added and plates were incubated in 5% humidified incubator at 37°C for 48 h (Ji et al 2005). Finally, the MTT assay was performed as mentioned before. IC50 values of mitoxantron (concentration resulting in 50% inhibition of cell growth) were calculated in the presence of four concentrations of DHP derivatives (10 nM, 100 nM, 1 μM and 10 μM). The RF was calculated by the following equation (Gudmundsson & Stouch 2002):

$$\text{MDR ratio (RF)} = \text{IC50 (cytotoxic drug alone)}/\text{IC50 (cytotoxic drug + inhibiting agent)}$$

QSAR studies

The chemical structure of the molecules was drawn by Hyperchem software (Hypercube Inc. version 7). Semi-empirical AM_1 calculations were used for geometric optimization of the molecules by this software. Some QSAR descriptors such as geometrical descriptors, constitutional descriptors, topological

descriptors, and 3D-MoRSE descriptors were calculated by DRAGON software (see http://www.disat.unimib.it/chm). The MLR equations were obtained by the stepwise variable selection method of SPSS software (SPSS Inc., version 13.5).

Results and Discussion

Chemistry

DHP derivatives **4a–4h** and **6a–6b** were synthesized using the modified Hantzsch classical condensation procedure as outlined in Figure 1. The acetoacetate esters **3a–c** (> 90% yields) were obtained through the reaction of corresponding alcohols **1a–c** with 2,2,6-trimethyl-4-H-1,3-dioxin-4-one (Clemens & Hyatt 1985). Refluxing the mixture of aqueous ammonia, aldehyde and 2 equiv. acetoacetate ester in methanol gave compounds **4a–4h** and **6a–6b** in moderate yields. These derivatives are listed in Table 1.

Pharmacology

The in-vitro calcium channel antagonistic activity of compounds was determined as the molar concentration of test compounds needed to produce 50% inhibition of guinea-pig ileal longitudinal smooth muscle (GPILSM) contractility (Hosseini et al 2007). Results are summarized in Table 1.

Compounds **6a** and **6b** exhibited the weakest calcium-channel antagonistic activity (10^{-6} M range), showing a considerable reduction in the activity relative to the reference drug nifedipine (IC50 = 3.55×10^{-8}). Therefore, substitution of 1-methyl-5-nitro-2-imidazole on the 4-position decreased the calcium-channel antagonistic effect.

Cytotoxicity

The cytotoxicity data are presented in Table 2 as IC15 values. All of the compounds had IC15 values against all cell lines. Also, all compounds had IC30 values at least on one cell line, whereas just six compounds had IC50 values in concentrations lower than 100 μM. Compounds **4b** and **4c** could be described as the best cytotoxic agents because of having an IC50 against all cell lines. According to the results, the cytotoxic effect of compound **4b** (IC50 = 6.61) on the K562 cell line was comparable with that of doxorubicin (IC50 = 4.17). The most cytotoxic effects were seen on the K562 cell line while the least activity was observed on the Raji cell line (Table 3).

MDR reversal assay

Generally MDR active compounds are highly lipophilic and have aromatic ring systems in their molecules (Molnár et al 1998). Studies have reported that many 1,4-DHP derivatives, which have an aromatic ring at the 4-position, could potentiate the cytotoxic action of anticancer agents (Kiue et al 1990; Kawase et al 2002). Therefore, we examined the ability of the synthesized DHPs with an aromatic ring at the 4-position to overcome MDR.

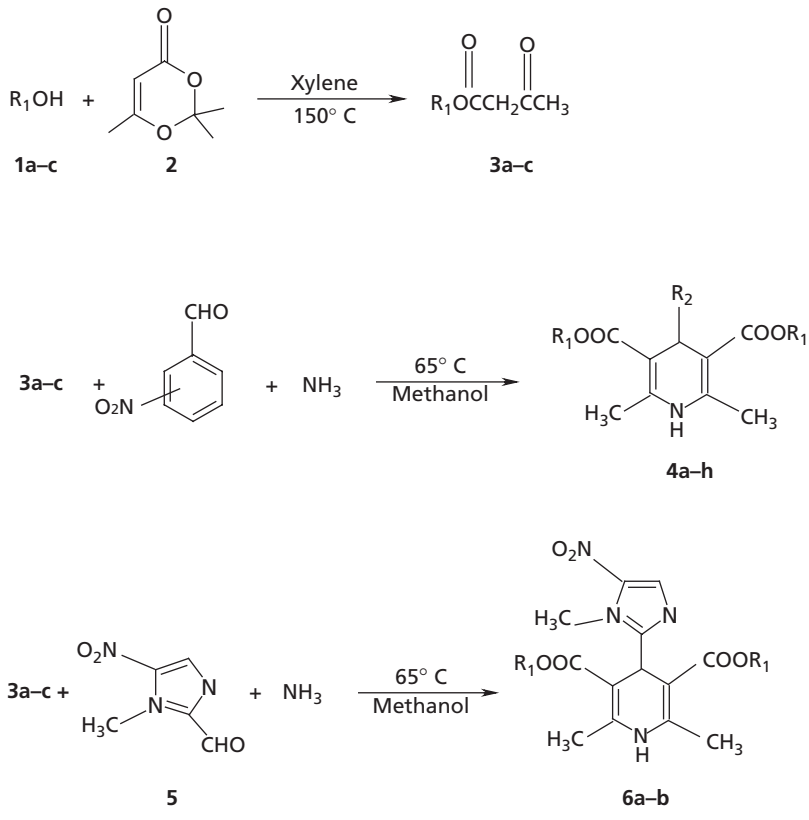
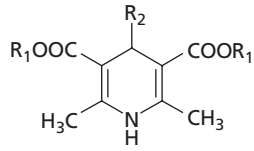
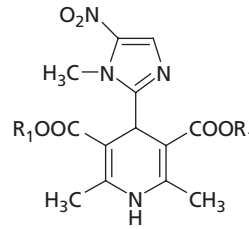


Figure 1 Synthetic pathway of DHP derivatives.

Table 1 Physical and calcium-channel antagonism data for compounds **4a–h** and **6a–b**



4a–h



6a–b

Compound	R_1	R_2	Melting points (°C)	Yield (%)	IC50 ± s.e.m. (M) (n = 3)
4a	Pyridine-3-yl-propyl	3-nitrophenyl	134–136	36	$1.49 \pm (0.91) \times 10^{-7}$
4b	Pyridine-3-yl-propyl	4-nitrophenyl	102–104	30	$1.40 \pm (0.5) \times 10^{-7}$
4c	Pyridine-3-yl-propyl	2-nitrophenyl	105–108	40	$1.87 \pm (0.69) \times 10^{-7}$
4d	Pyridine-4-yl-propyl	3-nitrophenyl	Decomposed at 180	35	$3.08 \pm (2.77) \times 10^{-7}$
4e	Pyridine-4-yl-propyl	4-nitrophenyl	Decomposed at 160	29	$3.42 \pm (0.25) \times 10^{-7}$
4f	Pyridine-4-yl-propyl	2-nitrophenyl	Decomposed at 152	47	$1.2 \pm (0.14) \times 10^{-7}$
4g	Pyridine-2-yl-propyl	3-nitrophenyl	67–70	45	$0.75 \pm (0.35) \times 10^{-7}$
4h	Pyridine-2-yl-propyl	4-nitrophenyl	71–74	20	$3.7 \pm (2.22) \times 10^{-7}$
6a	Pyridine-3-yl-propyl	–	Decomposed at 134	24	$2.59 \pm (0.5) \times 10^{-6}$
6b	Pyridine-4-yl-propyl	–	130–132	12	$2.38 \pm (1.16) \times 10^{-6}$
	Nifedipine				$3.55 \pm (1.62) \times 10^{-8}$

Table 2 Cell growth inhibitory activity of compounds **4a–h** and **6a–b** in-vitro (IC15)

Compound	IC15 (μM)				
	Jurkat	Raji	K562	Fen	HL60/MX1
4a	48.98 ± 8.7	63.09 ± 5.6	50.12 ± 6.5	7.24 ± 0.90	12.59 ± 1.5
4b	8.91 ± 0.78	7.08 ± 0.70	1.23 ± 0.43	14.12 ± 0.12	7.76 ± 0.35
4c	4.36 ± 0.98	3.16 ± 1.1	3.39 ± 0.66	11.22 ± 1.6	2.24 ± 0.28
4d	97.72 ± 23.2	54.95 ± 4.2	46.77 ± 10.4	42.66 ± 6.1	19.05 ± 1.5
4e	64.56 ± 12.3	20.42 ± 4.1	39.81 ± 7.6	21.38 ± 0.68	4.26 ± 0.14
4f	19.95 ± 8.5	25.70 ± 3.4	1>	61.66 ± 6.8	52.48 ± 3.7
4g	53.70 ± 10.7	46.77 ± 1.8	1>	36.31 ± 1.1	15.85 ± 0.24
4h	17.38 ± 2.3	64.56 ± 1.6	5.5 ± 2.1	18.2 ± 1.2	12.89 ± 0.21
6a	38.02 ± 2.4	2.63 ± 0.18	7.08 ± 1.2	7.58 ± 0.31	31.62 ± 0.44
6b	45.71 ± 1.2	48.98 ± 8.1	3.02 ± 0.98	7.58 ± 0.79	5.25 ± 0.08
Doxorubicin	0.002 ± 0.00	0.32 ± 0.01	0.004 ± 0.00	0.008 ± 0.00	0.017 ± 0.00

Table 3 Cell growth inhibitory activity of compounds **4a–h** and **6a–b** in-vitro (IC50)

Compound	IC50 (μM)				
	Jurkat	Raji	K562	Fen	HL60/MX1
4b	40.74 ± 1.1	66.07 ± 8.3	6.61 ± 1.5	54.95 ± 2.7	20.42 ± 0.6
4c	69.18 ± 5.7	33.88 ± 3.1	12.88 ± 3.2	47.86 ± 7.2	9.55 ± 1.1
4e	–	–	–	97.72 ± 11.4	–
4f	44.67 ± 8.7	–	66.07 ± 17.8	91.2 ± 3.6	37.15 ± 0.92
6a	–	–	–	81.28 ± 8.8	83.18 ± 1.2
6b	–	–	–	–	97.72 ± 8.1
Doxorubicin	1.82 ± 0.45	9.59 ± 0.38	4.17 ± 0.75	5.13 ± 0.18	0.40 ± 0.01

To perform this investigation, a cultured MDR cell line, HL60/MX1, a mitoxantron-resistant derivative of the HL60 cell line, which was derived from a patient with acute promyelocytic leukaemia, was used. The MDR reversal ability was shown as the IC50 value, the concentration resulting in 50% inhibition of cell growth in the presence of four concentrations of DHP derivatives, and the RF. RF was determined by dividing the IC50 of a cytotoxic drug alone (mitoxantron) against HL60/MX1 cells by the IC50 of a cytotoxic drug with inhibiting agent (DHP derivatives). As summarized in Table 4, compound **6a** had the strongest MDR reversing activity, based on the RF value (RF_{max} = 22.5), whereas compound **4e** showed the lowest MDR reversing activity (RF_{max} = 2.14). Previous studies have determined that P-gp inhibiting activity is the main mechanism of DHPs as MDR reversal agents, but, in this study, we examined the appropriate effect of some DHP derivatives on the atypical MDR. Our results confirmed the effectiveness of these compounds on this type of MDR. Consequently, we suggested compound **6a** as a good candidate for overcoming MDR since it exerted the strongest MDR reversal activity and the weakest calcium-channel antagonistic effect. We also recommended compound **4g** (RF_{max} = 17.78), which had a high MDR reversal effect but relatively low calcium-channel antagonistic activity.

Due to the fact that the cytotoxicity assay was based on general assessments, more than one mechanism may have been involved in the MDR activity of these compounds; therefore, more investigations are needed to determine the exact MDR mechanism of these agents.

QSAR studies

To obtain the relationship between structural parameters of these derivatives and their calcium-channel blocking and cytotoxic activity, a quantitative structure–activity relationship (QSAR) with different types of molecular descriptors including constitutional descriptors, topological descriptors, geometrical descriptors, 2D autocorrelation descriptors, and 3D-MoRSE descriptors were performed. The activity data were expressed in logarithmic scale (i.e. pIC50 for calcium-channel antagonistic activity, and pIC15 for cytotoxicity effect). Physicochemical properties such as molecular surface area (SA), molar volume (MV), molar refractivity (MR), lipophilicity (LogP), and hydration energy (HE) were calculated for each molecule. HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) energies and dipole moment were used to account for the electronic feature of the molecules.

Firstly, the correlation of calculated descriptors with each other and with the dependent variable was examined. Among the co-linear descriptors ($r > 0.9$), the one with the highest correlation with the dependent variable was retained and the others were removed. The remaining descriptors were used to find the best multilinear regression equation using stepwise selection-based multiple linear regression (MLR) subroutine of SPSS software. To validate the prediction ability of the models, the leave-one-out cross-validation method was employed. Square of cross-validate correlation coefficient (r^2cv) was used to measure model predictivity.

The resulting QSAR model for cytotoxicity on the K562 cell line was as follows:

$$\text{pIC15} = 2.547 - 3.964(\text{mor28m}) + 1.486(\text{mor18u})$$
$$n = 8 \quad r^2 = 0.919 \quad r^2_{cv} = 0.834 \quad \text{s.e.} = 0.21 \qquad (1)$$

In this equation, n is the number of components, and r^2, r^2_{cv}, and s.e. are correlation coefficients, square of cross-validate correlation coefficient, and standard error of regression, respectively. That this two-parametric equation contained 3D-MoRSE parameters (mor28m and mor18u) showed the importance of those parameters in the cytotoxicity of the compounds.

The resulting QSAR model for cytotoxicity on the Jurkat cell line was as follows:

$$\text{pIC15} = 2.866 + 8.02(\text{PJI3}) - 23.244(\text{ATS4m})$$
$$n = 10 \quad r^2 = 0.906 \quad r^2_{cv} = 0.814 \quad \text{s.e.} = 0.15 \qquad (2)$$

In this two-parametric equation, PJI3 (3D Petijean shape index) and ATS4m (Borto-Moreau autocorrelation) implied

Table 4 MDR reversing activity of compounds **4a–h** and **6a–b** on HL60/MX1

Compound	IC50 of mitoxantrone and ratio factor (RF)							
	10 nM	RF	100 nM	RF	1 μM	RF	10 μM	RF
4a	269.1 ± 12.2[a]	1.35	54.9 ± 5.3	6.61	87.1 ± 12.5	4.17	89.1 ± 8.8	4.07
4b	605.1 ± 45.3	0.35	162.1 ± 6.7	2.24	182.4 ± 20.2	1.99	147.6 ± 12.3	2.46
4c	138.0 ± 21.7	2.63	100.0 ± 19.8	3.63	95.5 ± 8.8	3.8	66.1 ± 10.2	5.5
4d	199.5 ± 22.6	1.82	208.6 ± 21.8	1.74	87.1 ± 4.5	4.17	87.1 ± 10.2	4.17
4e	526.2 ± 18.4	0.69	245.3 ± 12.3	1.48	240.4 ± 12.3	1.51	169.6 ± 8.9	2.14
4f	315.7 ± 32.0	1.15	67.6 ± 6.6	5.37	58.9 ± 6.7	6.16	28.2 ± 4.5	12.88
4g	155.2 ± 12.9	2.34	102.3 ± 6.7	3.55	20.4 ± 4.3	17.78	56.2 ± 0.5	6.46
4h	370.5 ± 10.9	0.98	315.7 ± 11.2	1.15	191.1 ± 4.9	1.90	52.5 ± 2.2	6.92
6a	103.7 ± 8.7	3.5	20.7 ± 2.4	17.52	16.1 ± 1.2	22.53	82.5 ± 12.1	4.4
6b	310.3 ± 32.5	1.17	144.6 ± 8.9	2.51	138.0 ± 12.3	2.63	224.1 ± 34.3	1.62
–	363.0 ± 37.1[b]							

[a]IC50 of mitoxantron in the presence of DHP derivatives; [b]IC50 of mitoxantron alone (control IC50).

the importance of geometrical and topological parameters in the cytotoxic activity of the compounds on the Jurkat cell line.

The next equation was obtained for cytotoxicity of the HL60/MX1 cell line:

$$\text{pIC15} = -3.5 - 4.524(\text{mor32v}) - 0.423(\text{mor10u})$$
$$n = 10 \quad r^2 = 0.759 \quad r^2_{cv} = 0.609 \quad \text{s.e.} = 0.23 \qquad (3)$$

The next equation was obtained for cytotoxicity of the Fen cell line:

$$\text{pIC15} = 2.158 + 1.193(\text{mor18u}) + 0.769(\text{mor21v})$$
$$n = 10 \quad r^2 = 0.918 \quad r^2_{cv} = 0.892 \quad \text{s.e.} = 0.11 \qquad (4)$$

This two-parametric QSAR equation for the Fen cell line had correlation coefficients for calibration and cross-validation equal to 0.918 and 0.892, respectively, which meant that this equation could reproduce 89% of variances in the cytotoxicity of the Fen cell line.

The obtained QSAR models for cytotoxicity on the Raji cell line and calcium-channel blocking effect had not implied adequate predictivity, since their r^2 and r^2_{cv} were too different.

The 3D molecule representation of the structure based on electron diffraction (MoRSE) descriptors provided 3D information from the 3D coordinates by using the same transform as in electron diffraction. 3D descriptors, especially 3D-MoRSE, afforded the possibility for choosing an appropriate atomic property (Todeschini & Consonni 2000). As represented above, 3D-MoRSE parameters were present in almost all QSAR models and showed the importance of these parameters in the activity of the synthesized compounds. Three of the obtained QSAR equations with 3D-MoRSE descriptors were considered as the best model for cytotoxicity on different cell lines including HL60/MX1. Interestingly, this phenomenon had occurred for our previous derivatives in which MoRSE parameters were included in equations of different cytotoxicity values (Mehdipour et al 2007).

Conclusion

P-gp inhibition has been considered as the most well-known mechanism of DHPs in reversing MDR to anticancer drugs; however, this study has provided, for a second time, the effect of DHP derivatives on atypical MDR. Based on our results, compound **6a** was the most promising, as it had the strongest MDR reversal activity but the lowest calcium-channel antagonistic effect. According to these results, it seemed that the presence of a nitro-imidazole group on the 4-position as well as 2-pyridyl group on 3, 5-positions increased the MDR reversal effect; therefore, we propose compounds **6a** and **4g** for further in-vitro and in-vivo investigation. We suggest also the synthesis of a compound with a 2-pyridyl group on the 3, 5-positions and a nitroimidazole group on the 4-position.

Since the proposed MDR mechanism in the HL60/MX1 cell line was resistant to topoisomerase poisons, it could be postulated that these derivatives acted as MDR reversal agents through inhibiting this mechanism. However, the MTT assay is a general assessment; performing more accurate assays especially at the molecular level could be helpful in determining the exact effect of DHPs on atypical MDR.

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