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ORIGINAL RESEARCH

Synthesis, anticancer and MRP1 inhibitory activities of 4-alkyl/aryl-3,5-bis(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines

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Abstract Fourteen new 4-alkyl/aryl-3,5-bis-(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines (**4a**–**4n**) have been prepared by conventional and microwave irradiation (MWI) methods from a three component reaction mixture viz., alkyl acetoacetate (**1**), appropriate aldehyde (**2**) and ammonium acetate (**3**). The compounds prepared have been purified and characterized by their spectral (FTIR, ^{1}H NMR and MS) data. The two synthetic methods employed have been compared in terms of relative yields and reaction times. On comparison, the MWI method has been found to be easy, simple, eco-friendly, rapid and high yielding. The synthesized compounds have been evaluated for their cytotoxic activity against HT-29 (colon cancer) and MDA-MB (breast cancer) cell lines and MRP1 inhibitory activity using the insect cell membrane MRP 1 ATPase assay. Though some of the compounds could exhibit some degree of cytotoxicity it was found to be low in comparison to standard. Among the compounds tested **4g** was relatively better in its MRP1 inhibitory action (IC_{50} = 16 μM) but not comparable to that of benzbromarone (IC_{50} = 4 μM).

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Introduction

Multidrug resistance associated protein 1 (MRP1) is a membrane-bound, 1531 amino acid containing (190-kDa) energy-dependent efflux transporter belonging to the super family of ATP-binding cassette (ABC) transporters involved in cancer cell multidrug resistance (MDR phenotype). It is a membrane-bound, energy-dependent efflux pump which is structurally and functionally related to the MDR protein, P-glycoprotein (P-gp/ABCB1) (Teodori *et al*., 2006). MRP1 is over expressed in the membranes of cancer cells and effectively extrudes various cytotoxic agents out of tumor cells, leading to a decrease in cellular drug concentrations. It is widely expressed in all tissues except in the human liver, it plays a significant role in tissue defense from toxic agents (Okamura *et al*., 2009; Leslie *et al*., 2005) in combination with other ABC transporters. Like P-gp, MRP1 confers resistance on cancer cells by using the energy of ATP binding and hydrolysis to efflux anticancer drugs.

MDR is a tremendous problem in the treatment of many types of cancer; the patients who overexpress multidrug resistance proteins such as P-gp and MRP1 in their tumors are usually not responsive to the treatment associated with anticancer agents (Bellamy and Dalton, 1994). Thus, many of these patients progress to an advanced stage of disease and have poorer prognoses. It has been hypothesized that inhibition of MDR transporters can restore sensitivity to some oncolytics, allowing patients with drug-resistant

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tumors to become responsive to their drug therapy (Avendano and Menendez, 2002). This hypothesis is presently being tested in the clinic for P-gp (Coley, 2010). However, the search for MRP1 inhibitors (or modulators/chemosensitizers) to overcome MRP1/ABCC1-mediated MDR began just a few years ago and few potent and selective modulators have been investigated (Boumendjel *et al*., 2005; Yang *et al*., 2010).

1,4-Dihydropyridines (DHPs), a class of calcium channel blockers are used as therapeutic agents to treat angina and hypertension (Bernard *et al*., 1974). Several of them are also reported to exhibit a variety of biological and pharmacological activities, viz., bronchodilatory (Suresh *et al*., 2007), vasodilatory (Iwanami *et al*., 1979), hepatoprotective (Isaac *et al*., 1998), neuroprotectant (Klusa, 1995), platelet aggregation inhibitory (Carlos *et al*., 1988) and cerebral anti-ischemic (Sircar *et al*., 1991). They are also reported to possess potential anticancer properties (Boer and Gekeler, 1995). Shortly after recognizing the chemosensitizing properties of a calcium antagonist verapamil, lead to a thinking allowed by finding that the other calcium antagonists, the DHP derivatives were also potent chemosensitizing compounds in vitro (Avendano and Menendez, 2002). Hence, this field has attracted the attention of several medicinal chemists, pharmacologists and oncologists to investigate further in view of the growing resistance to chemotherapy by cancer cells.

A series of 4-phenyl-3,5-dibenzoyl-1,4-dihydropyridines (Fig. 1, Structure 1) differently substituted at the 4-phenyl ring have been reported to show a varied cytotoxicity against two human oral tumor cell lines (HSC-2 and HSG) (Kawase *et al*., 2002). Among such compounds, those substituted with 2-CF_3 (IC_{50} = 8.7 μM) and 2-Cl (IC_{50} = 7.0 μM) were reported to show the highest cytotoxic activity against HSC-2. On the other hand, cytotoxic activity of 2-CF_3 analog (IC_{50} = 8.7 μM) against HSG was reported higher than 2-Cl analog (IC_{50} = 28 μM). These data suggested that-3,5-dibenzoyl-1,4-dihydropyridines can be considered as leads for the development of new drug for cancer treatment.

Shigeyuki *et al*., (2001) reported the structure–activity relationships of some newly synthesized 1,4-dihydropyridine derivatives (Fig. 1, Structure 2). The compound having a 1-pentyl group at the 4-position and 3-pyridylpropyl ester groups at 3- and 5-positions was found to be effective in overcoming P-glycoprotein-mediated multidrug resistance (MDR) in cultured human cancer cells, in vitro.

Another member of dihydropyridine derivative B-859-35 (Fig. 1, Structure 3) was reported to show a selective carcinostatic effect on some tumors (Johann *et al*., 1991).

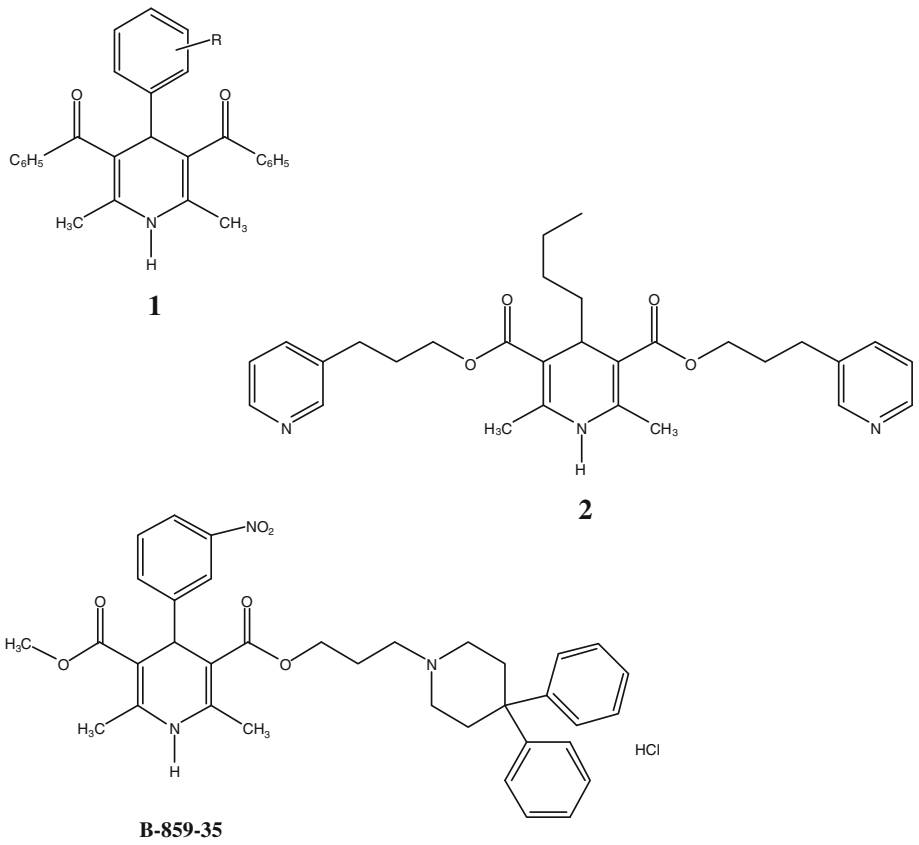


Fig. 1 Some of the 1,4-dihydropyridine molecules as anticancer agents

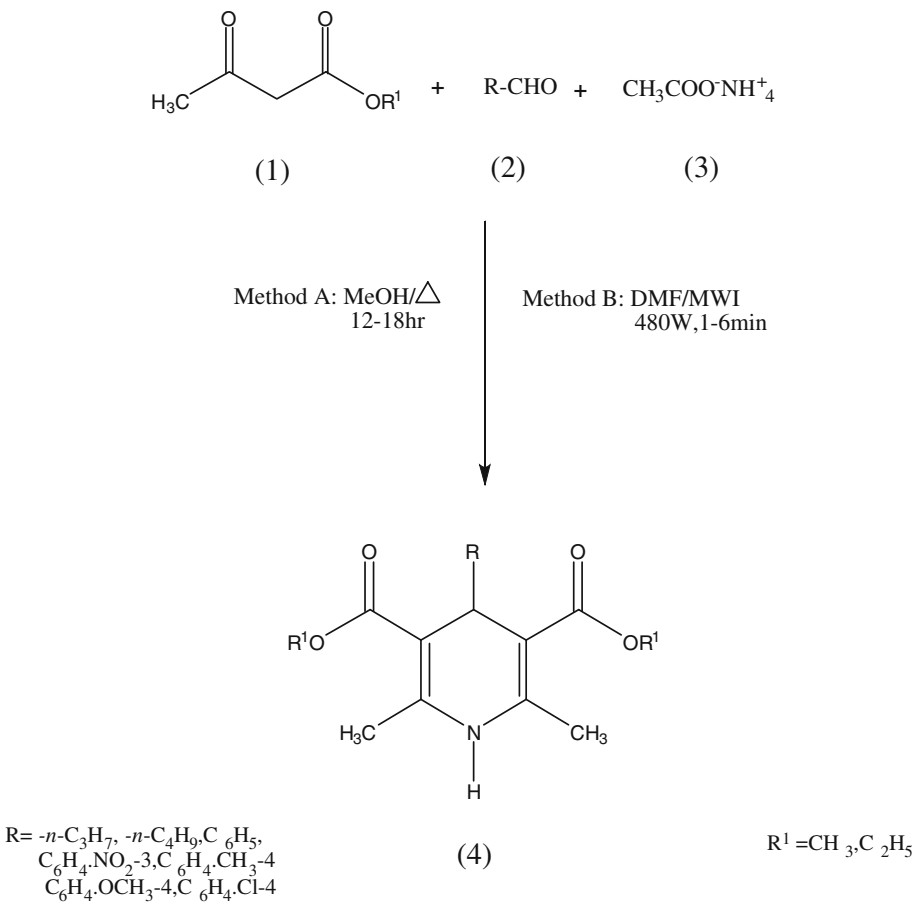
In order to evaluate whether the anticancer activity of B-859-35 can be modulated, the new molecule was combined with several established antitumor drugs. A combination of B-859-35 with VP-16 (etoposide) in MDR expressing Walker rat carcinoma cells has been reported to exhibits synergetic effect. A combination of B-859-35 with doxorubicin was shown to result in stronger synergism than verapamil/doxorubicin, especially at low concentrations of B-859-35 (Johann *et al*., 1991). These findings indicated that an antitumor agent B-859-35 reversed multidrug resistance and thus this molecule could emerge as an interesting drug for cancer treatment. Figure 1 has included some more dihydropyridines as anticancer and MRP1 inhibitory agents.

Therefore, in view of growing incidents of different cancer cells resistance to various potent anticancer agents and DHPs exhibiting an ability to chemosensitize such cells it has been thought worthwhile to synthesize some new dihydropyridine derivatives bearing different alkyl and aryl groups at 4-position and methyl/ethyl ester groups at the 3- and 5-positions of the DHP system and evaluate them for their possible anticancer as well as MRP1 inhibitory activities. The proposed compounds are synthesized by two different synthetic methods and characterized by their spectral and analytical properties and evaluated them for their possible anticancer activity against human cancer cell lines (HT-29 and MDA-MB) and MRP1 inhibitory activity by standard experimental methods and the results are presented in this communication.

Materials and methods

Chemistry

Fourteen new 4-alkyl/aryl-3,5-bis-(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines (**4a**–**4n**, Scheme 1) have been synthesized by a modified Hantzsch one-pot synthesis involving the three-component condensation reaction of alkyl acetoacetate (**1**; 2 M) with appropriate aldehyde (**2**; 1 M) and ammonium acetate (**3**; 3 M) by conventional as well as microwave-induced methods (Anniyappan and Perumal, 2002). Completion of the reaction was monitored by TLC. The title compounds **4a**–**4n** were obtained in good to excellent yields (62–90%). But however MWI method, in comparison to conventional heating was simple, easy, eco-friendly and the reactions were rapid and high yielding.



Scheme 1 Synthesis of 4-alkyl/aryl-3,5-bis (carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines

Experimental procedures are given as general methods. All M.P. were determined in open capillaries using Toshnwal melting point apparatus. Infra red spectra of the compounds were recorded in KBr pellet using Shimdzu FTIR-8700 spectrophotometer, ^{1}HNMR spectra were recorded on Avance-300 MHz spectrophotometer using TMS as an internal standard and Mass spectra by direct inlet method on VG Micro Mass 7070 H spectrophotometer operating at 70 eV. Elemental analyses of the compounds were carried out Elementar instrument.

General procedure for the synthesis of 4-alkyl/aryl-3,5-bis-(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines (**4a**–**4n**)

Conventional method

Ethyl/methyl acetoacetate (**1**; 0.05 M) and an appropriate aldehyde (**2**; 0.025 M) were taken into a RB flask and dissolved in ethanol (25 cm^3) by shaking. Ammonium acetate (**3**; 0.025 M) was added, stirred and the reaction mixture was heated under reflux for 12–18 h, on a hot water-bath. After completion of the reaction (monitored by TLC), ethanol was removed to a possible extent by distillation and the residue was cooled, triturated with crushed ice ($\sim$100 g). The product was filtered, washed with small portions of cold water (3 × 10 cm^3) and dried. The isolated product was purified by recrystallization from 90% aqueous ethanol.

Microwave irradiation method

Ethyl/methyl acetoacetate (**1**; 0.05 M) and an appropriate aliphatic aldehyde or aromatic aldehyde (**2**; 0.025 M) were taken into a beaker and dissolved in minimum quantity of dimethylformamide (10 cm^3). To this solution, ammonium acetate (**3**; 0.025 M) was added while stirring. A funnel was inversely hanged over the beaker and the reaction mixture was subjected to microwave irradiation at 480 W for 2–6 min, with a pulse rate of 60 s each in a microwave oven. Solvent was removed by distillation under reduced pressure after completion of the reaction (TLC). The residue was cooled and triturated with crushed ice ($\sim$100 g). The resultant product was filtered, washed with small portions of cold water (3 × 10 cm^3), dried and purified by recrystallization from 90% aqueous ethanol.

Adopting the above two procedures the following fourteen different 4-alkyl/aryl-3,5-bis-(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines (**4a**–**4n**) were prepared and characterized:

*(a) 4-n-Propyl-3,5-bis (carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4a**; $R = \text{-}n\text{-}C_3H_7$, $R^1 = C_2H_5$)* Yield: conventional method 47%, microwave method 90%; m.p. 122–124°C; IR (KBr) cm^{-1}: 3351 (–NH), 2956 (C–H, aliphatic), 1644 (C=O, CO_2 Et); ^{1}HNMR ($CDCl_3$): δ (ppm) 0.85 (t, 3H, $-CH_2CH_2C\underline{H}_3$ at C_4), 1.30 (m, 8H, $-CO_2CH_2C\underline{H}_3$ at C_3 & C_5 and $CH_2C\underline{H}_2CH_3$ at C_4), 2.32 (s, 6H, $-C\underline{H}_3$ at C_2 & C_6 of DHP), 3.94–4.22 (m, 6H, $-C\underline{H}_2CH_2CH_3$ at C_4 & $-CO_2C\underline{H}_2CH_3$ at C_3, C_5), 5.62 (s, 1H, –N$\underline{H}$), 7.26 (s, 1H, –C$\underline{H}$ at C_4); MS (*m/z*): 296 (M+); Elemental analyses: Calcd. For $C_{16}H_{25}N_1O_4$: C, 65.06; H, 8.53; N, 4.74. Found C, 65.01; H, 8.42; N, 4.64.

*(b) 4-n-Butyl-3,5-bis (carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4b**; $R = \text{-}n\text{-}C_4H_9$, $R^1 = C_2H_5$)* Yield: conventional method 39%, microwave method 72%; m.p. 92–94°C; IR (KBr) cm^{-1}: 3344 (–NH), 2932 (C–H, aliphatic), 1651 (C=O, CO_2 Et); ^{1}HNMR ($CDCl_3$): δ (ppm) 0.82 (t, 3H, $-CH_2CH_2CH_2C\underline{H}_3$ at C_4), 1.30 (m, 10H, $-CO_2CH_2C\underline{H}_3$ at C_3, C_5 & $-CH_2C\underline{H}_2C\underline{H}_2CH_3$ at C_4), 2.38 (s, 6H of $-CH_3$ at C_2 & C_6), 4.22 (m, 6H, $-C\underline{H}_2CH_2CH_2CH_3$ at C_4 & $-CO_2C\underline{H}_2CH_3$ at C_3, C_5), 5.52 (s, 1H, –N$\underline{H}$), 7.28 (s, 1H, –CH at C_4); MS (*m/z*): 310 (M+); Elemental analyses: Calcd. for $C_{17}H_{27}N_1O_4$: C, 65.99; H, 8.80; N, 4.53. Found C, 65.84; H, 8.72; N, 4.41.

*(c) 4-Phenyl-3,5-bis (carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4c**; $R = C_6H_5$, $R^1 = C_2H_5$)* Yield: conventional method 52%, microwave method 93%; m.p. 150–152°C; IR (KBr) cm^{-1}: 3340 (–NH), 3068 (C–H, aromatic), 2985 (C–H, aliphatic), 1659 (C=O, CO_2Et), 1591 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 1.24 (t, 6H, $-CO_2CH_2C\underline{H}_3$ at C_3 & C_5), 2.26 (s, 6H, $-C\underline{H}_3$ at C_2 & C_6), 4.10 (q, 4H, $-CO_2C\underline{H}_2CH_3$ at C_3 & C_5), 4.96 (s, 1H, –N$\underline{H}$), 5.62 (s, 1H, –C$\underline{H}$ at C_4), and 7.10 to 7.32 (m, 5H, Ar–H at C_4); MS (*m/z*) 328 (M+); Elemental analyses: Calcd. for $C_{19}H_{23}N_1O_4$: C, 69.28; H, 7.04; N, 4.25. Found C, 69.16; H, 6.98; N, 4.18.

*(d) 4-(3-Nitrophenyl)-3,5-bis(carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4d**; $R = C_6H_4{\cdot}NO_2\text{-}3$, $R^1 = C_2H_5$)* Yield: conventional method 54%, microwave method 92%; m.p. 160–162°C; IR (KBr) cm^{-1}: 3345 (–NH), 3080 (C–H, aromatic), 2991 (C–H, aliphatic), 1645 (C=O, CO_2Et), 1525 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 1.29 (t, 6H, $-CO_2CH_2C\underline{H}_3$ at C_3 & C_5), 2.28 (s, 6H, $-C\underline{H}_3$ at C_2 & C_6), 4.20 (q, 4H, $-CO_2C\underline{H}_2CH_3$ at C_3 & C_5), 4.98 (s, 1H, –N$\underline{H}$), 5.87 (s, 1H, –C$\underline{H}$ at C_4), and 7.62 to 8.12 (m, 5H, Ar–$\underline{H}$ at C_4); MS (*m/z*) 374 (M+); Elemental analyses: Calcd. for $C_{19}H_{22}N_2O_6$: C, 60.95; H, 5.92; N, 7.48. Found C, 60.76; H, 5.93; N, 7.39.

*(e) 4-(4-Methoxyphenyl)-3,5-bis(carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4e**; $R = C_6H_4{\cdot}OCH_3\text{-}4$, $R^1 = C_2H_5$)* Yield: conventional method 41%, microwave method 86%; m.p. 148–150°C; IR (KBr) cm^{-1}: 3342

(–NH), 3064 (C–H, aromatic), 2984 (C–H, aliphatic), 1650 (C=O, CO_2Et), 1491 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 1.25 (t, 6H, –$CO_2CH_2C\underline{H}_3$ at C_3 & C_5), 3.83 (s, 3H, –$C\underline{H}_3$ at C_4), 2.26 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 4.30 (q, 4H, –$CO_2C\underline{H}_2CH_3$ at C_3 & C_5), 5.46 (s, 1H, –N$\underline{H}$), 5.82 (s, 1H, –C$\underline{H}$ at C_4), and 6.87 to 7.12 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*) 359 (M+); Elemental analyses: Calcd. for $C_{20}H_{25}N_1O_5$: C, 66.83; H, 7.01; N, 3.90. Found C, 66.68; H, 6.92; N, 3.84.

*(f) 4-(4-Methylphenyl)-3,5-bis(carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4f**; $R = C_6H_4{\cdot}CH_3$-4, $R^1 = C_2H_5$)* Yield: conventional method 53%, microwave method 89%; m.p. 140–142°C; IR (KBr) cm^{-1}: 3360 (–NH), 3076 (C–H, aromatic), 2987 (C–H, aliphatic), 1697 (C=O, CO_2Et), 1487 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 1.54 (t, 6H, –$CO_2CH_2C\underline{H}_3$ at C_3 & C_5), 2.34 (s, 3H, –$C\underline{H}_3$ at C_4), 2.90 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 4.50 (q, 4H, –$CO_2C\underline{H}_2CH_3$ at C_3 & C_5), 5.06 (s, 1H, –N$\underline{H}$), 5.92 (s, 1H, –C$\underline{H}$ at C_4), and 7.20 to 7.42 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*) 343 (M+); Elemental analyses: Calcd. for $C_{20}H_{25}N_1O_4$: C, 69.95; H, 7.34; N, 4.08. Found C, 69.86; H, 7.23; N, 4.02.

*(g) 4-(4-Chlorophenyl)-3,5-bis (carboethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4g**; $R = C_6H_4{\cdot}Cl$-4, $R^1 = C_2H_5$)* Yield: conventional method 59%, microwave method 85%; m.p. 144–146°C; IR (KBr) cm^{-1}: 3343 (–NH), 3046 (C–H, aromatic), 2982 (C–H, aliphatic), 1651 (C=O, CO_2Et), 1591 (C=C, aromatic) and 1092 (C–Cl); ^{1}HNMR ($CDCl_3$): δ (ppm) 1.30 (t, 6H, –$CO_2CH_2C\underline{H}_3$ at C_3 & C_5), 2.34 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 4.10 (q, 4H, –$CO_2C\underline{H}_2CH_3$ at C_3 & C_5), 4.94 (s, 1H, –N$\underline{H}$), 5.70 (s, 1H, –C$\underline{H}$ at C_4), and 7.10 to 7.36 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*): 363 (M+); Elemental analyses: Calcd. for $C_{19}H_{22}N_1O_4Cl$: C, 62.72; H, 6.09; N, 3.85. Found C, 62.68; H, 6.02; N, 3.74.

*(h) 4-n-Propyl-3,5-bis (carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4h**; $R = \text{-}n\text{-}C_3H_7$, $R^1 = CH_3$)* Yield: conventional method 53%, microwave method 72%; m.p. 118–120°C; IR (KBr) cm^{-1}: 3363 (–NH), 2953 (C–H, aliphatic), 1652 (C=O, CO_2Et), 1490 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 0.90 (t, 3H of –$CH_2CH_2C\underline{H}_3$ at C_4), 3.74 (s, 6H of –$CO_2C\underline{H}_3$ at C_3 & C_5), 2.28 (s, 6H of –$C\underline{H}_3$ at C_2 & C_6), 1.08–1.31 (q, 4H of –$C\underline{H}_2CH_2CH_3$ at C_4), 5.62 (s, 1H of –N$\underline{H}$), 7.26 (s, 1H of –C$\underline{H}$ at C_4); MS (*m/z*): 267 (M+); Elemental analysis: Calcd. for $C_{14}H_{21}N_1O_4$: C, 62.90; H, 7.92; N, 5.24. Found C, 62.78; H, 7.90; N, 5.18.

*(i) 4-n-Butyl-3,5-bis (carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4i**; $R = \text{-}n\text{-}C_4H_9$, $R^1 = CH_3$)* Yield: conventional method 43%, microwave method 76%; m.p. 92–94°C; IR (KBr) cm^{-1}: 3334 (–NH), 2927 (C–H, aliphatic), 1652 (C=O, CO_2Et), 1490 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 0.90 (t, 3H, –$CH_2CH_2C\underline{H}_3$ at C_4), 3.74 (s, 6H, –$CO_2C\underline{H}_3$ at C_3 & C_5), 2.28 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 1.10 (t, 3H, –$C\underline{H}_2CH_2CH_2CH_3$ at C_4), 1.28–1.34 (q, 4H, –$CH_2C\underline{H}_2C\underline{H}_2CH_3$ at C_4), 5.58 (s, 1H, –N$\underline{H}$), 6.98 (s, 1H, –C$\underline{H}$ at C_4); MS (*m/z*): 281 (M+); Elemental analyses: Calcd. for $C_{15}H_{23}N_1O_4$: C, 64.03; H, 8.24; N, 4.98. Found C, 63.98; H, 8.17; N, 4.84.

*(j) 4-Phenyl-3,5-bis (carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4j**; $R = C_6H_5$, $R^1 = CH_3$)* Yield: conventional method 61%, microwave method 78%; m.p. 170–172°C; IR (KBr) cm^{-1}: 3346 (–NH), 3062 (C–H, aromatic), 2951 (C–H, aliphatic), 1698 (C=O, CO_2Et), 1490 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 2.28 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 3.55 (s, 6H, –$CO_2C\underline{H}_3$ at C_3 & C_5), 4.98 (s, 1H, –N$\underline{H}$), 5.42 (s, 1H, –C$\underline{H}$ at C_4), and 7.17 to 7.37 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*): 301 (M+); Elemental analyses: Calcd. for $C_{17}H_{19}N_1O_4$: C, 67.76; H, 6.36; N, 4.65. Found C, 67.68; H, 6.25; N, 4.52.

*(k) 4-(3-Nitrophenyl)-3,5-bis(carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4k**; $R = C_6H_4{\cdot}NO_2$-3, $R^1 = CH_3$)* Yield: conventional method 63%, microwave method 89%; m.p. 140–142°C; IR (KBr) cm^{-1}: 3357 (–NH), 3062 (C–H, aromatic), 2954 (C–H, aliphatic), 1652 (C=O, CO_2Et), 1485 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 2.30 (s, 6H, –$C\underline{H}_3$ at C_2 & C_6), 3.15 (s, 6H, –$CO_2C\underline{H}_3$ at C_3 & C_5), 4.96 (s, 1H, –N$\underline{H}$), 5.30 (s, 1H, –C$\underline{H}$ at C_4), and 7.10 to 7.36 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*): 347 (M+); Elemental analyses: Calcd. for $C_{17}H_{18}N_2O_6$: C, 58.96; H, 5.24; N, 8.09. Found C, 58.82; H, 5.18; N, 7.98.

*(l) 4-(4-Methoxyphenyl)-3,5-bis(carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4l**; $R = C_6H_4{\cdot}OCH_3$-4, $R^1 = CH_3$)* Yield: conventional method 58%, microwave method 84%; m.p. 166–168°C; IR (KBr) cm^{-1}: 3366 (–NH), 2951 (C–H, aliphatic), 1682 (C=O, CO_2Et), 1487 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 2.32 (s, 6H, –CH_3 at C_2 & C_6), 3.24 (s, 6H, –$CO_2C\underline{H}_3$ at C_3 & C_5), 3.83 (s, 3H, –O$C\underline{H}_3$ at C_4), 5.10 (s, 1H, –N$\underline{H}$), 5.84 (s, 1H, –C$\underline{H}$ at C_4), and 6.87 to 7.12 (m, 4H, Ar–$\underline{H}$ at C_4); MS (*m/z*): 331 (M+); Elemental analyses: Calcd. for $C_{18}H_{21}N_1O_5$: C, 65.24; H, 6.39; N, 4.23. Found C, 65.18; H, 6.25; N, 4.18.

*(m) 4-(4-Methylphenyl)-3,5-bis (carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4m**; $R = C_6H_4{\cdot}CH_3$-4, $R^1 = CH_3$)* Yield: conventional method 46%, microwave method 62%; m.p. 140–142°C; IR (KBr) cm^{-1}: 3349 (–NH), 2949 (C–H, aliphatic), 1697 (C=O, CO_2Et), 1490 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 2.26 (s, 6H, –$C\underline{H}_3$ at C_2

& C_6), 3.77 (s, 6H, $-CO_2C\underline{H}_3$ at C_3 & C_5), 2.34 (s, 3H, $-C\underline{H}_3$ at C_4), 4.98 (s, 1H, –N<u>H</u>), 5.67 (s, 1H, –C<u>H</u> at C_4), and 7.02 to 7.12 (m, 4H, Ar–<u>H</u> at C_4); MS (*m/z*): 315 (M+); Elemental analyses: Calcd. for $C_{18}H_{21}N_1O_4$: C, 68.55; H, 6.71; N, 4.44. Found C, 68.42; H, 6.68; N, 4.14.

*(n) 4-(4-Chlorophenyl)-3,5-bis(carbomethoxy)-1,4-dihydro-2,6-dimethylpyridine (**4n**; $R = C_6H_4$·Cl-4, $R^1 = CH_3$)* Yield: conventional method 57%, microwave method 85%; m.p. 190–192°C; IR (KBr) cm^{-1}: 3340 (–NH), 3068 (C–H, aromatic), 2984 (C–H, aliphatic), 1696 (C=O, CO_2Et), 1498 (C=C, aromatic); ^{1}HNMR ($CDCl_3$): δ (ppm) 2.24 (s, 6H of $-C\underline{H}_3$ at C_2 & C_6), 3.77 (s, 6H, $-CO_2C\underline{H}_3$ at C_3 & C_5), 4.84 (s, 1H, –N<u>H</u>), 5.32 (s, 1H, –C<u>H</u> at C_4), and 7.12 to 7.40 (m, 4H, Ar–<u>H</u> at C_4); MS (*m/z*): 335 (M+); Elemental analyses: Calcd. for $C_{17}H_{18}N_1O_4Cl$: C, 60.81; H, 5.40; N, 4.17. Found C, 60.75; H, 5.48; N, 4.08.

Biological activities

Evaluation of cell cytotoxicity

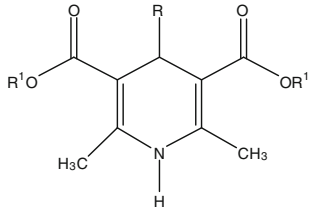
Cell culture

Colon cancer (HT-29) cells and breast cancer (MDA-MB) cells were maintained as a monolayer culture in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2 atmosphere and sub-cultured after trypsinization (0.5% trypsin/2.6 mM EDTA).

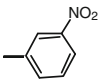
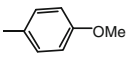
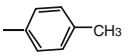
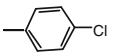
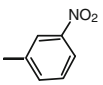
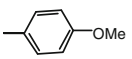
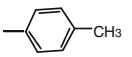
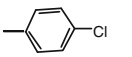
Cytotoxicity assay

Cell suspension (1×10^4 cells, counted by Tryphan blue exclusion dye method) with series of concentrations tested (10, 50, 100, and 200 μg/cm^3 in DMSO such that the final concentration of DMSO in media is less than 1%) were placed in 96-well plates and incubated in carbon dioxide incubator with 5% CO_2 at 37°C for 48 h in DMEM with 10% FBS medium. Control wells contain 1% DMSO and cell suspension. Then, the above media was replaced with 90 μl of fresh serum-free media and 10 μl of MTT reagent (5 mg/cm^3) and plates were incubated at 37°C for 4 h. There after the above media was replaced with 200 μl of DMSO and incubated further at 37°C for 10 min. The absorbance at 570 nm was measured on a spectrophotometer. The readings were averaged and viability of the test samples was compared with DMSO control. IC_{50} values were determined from plot: %inhibition (from control) versus concentration and the results are presented in Table 1.

Table 1 Anticancer activity of 4-alkyl/aryl-3,5-bis(carboethoxy/carbomethoxy)1,4-dihydro-2,6-dimethylpyridines



4a-n

S. no.	Compound code	–R	–R^1	IC_{50} (μM)	
				MDA-MB	HT-29
1	**4a**	-*n*-C_3H_7	$-C_2H_5$	500	NA
2	**4b**	-*n*-C_4H_9	$-C_2H_5$	350.1	NA
3	**4c**		$-C_2H_5$	NA	NA
4	**4d**		$-C_2H_5$	244.1	500
5	**4e**		$-C_2H_5$	330.6	NA
6	**4f**		$-C_2H_5$	500	310.2
7	**4g**		$-C_2H_5$	268.0	261.7
8	**4h**	-*n*-C_3H_7	$-CH_3$	NA	NA
9	**4i**	-*n*-C_4H_9	$-CH_3$	NA	500
10	**4j**		$-CH_3$	NA	NA
11	**4k**		$-CH_3$	500	368.2
12	**4l**		$-CH_3$	NA	451.9
13	**4m**		$-CH_3$	500	NA
14	**4n**		$-CH_3$	460.2	NA
15	Cisplatin	–	–	14.70	6.56

NA no activity

MRP1 ATPase assay

The MRP1 ATPase studies were carried out using purified vesicles from Sf9 (*Spodoptera frugiperda*) (Solvo Biotechnology, Budapest, Hungary). The colorimetric assay for measuring transporter-associated ATPase activity was performed as per the instructions of the manufacturer, a modification of the method of Sarkadi *et al*. (1992).

In this assay two variants were employed. The activation assay measured the increase in vanadate-sensitive ATPase activity in the presence of a range of test compound concentrations through spectrophotometric quantification of the amount of inorganic phosphate generated as a product of ATPase-mediated conversion of ATP to ADP. This assay gave a measure of the ability of an agent to stimulate transporter activity (from basal levels), a characteristic common to substrates of such ATPase transporters.

In the inhibition assay, ATPase activity is maximally stimulated using a saturating concentration of an activator of the transporter being studied. MRP-1 ATPase activity stimulated by 10 mM N-ethyl-maleimide-glutathione (NEM-GS) mix, and the decrease in this maximum vanadate-sensitive ATPase activity was measured in the presence of a range of test compound concentrations. Agents which inhibit MRP-1 function will typically reduce the ATPase activity of the pump, leading to a decrease in the activator-stimulated production of inorganic phosphate. All MRP-1 ATPase studies were carried out in the presence of 2 mM glutathione. Benzbromarone(3,5-dibromo-4-hydroxyphenyl)-(2-ethyl-3-benzofuranyl) methanone, a known specific MRP1 inhibitor was used as a positive control (Bobrowoska *et al*., 2003). Positive control was performed in the presence of an ATPase activator, NEM-GS (10 mM). In the activation assay, the maximal stimulatory effect of the test compounds is expressed on a scale where 0% is the basal ATPase activity and 100% is the activity in the presence of the reference activator, NEM-GS. In the ATPase inhibition assays, membrane was incubated with NEM-GS in the presence or absence of increasing concentrations of test compounds (1–1000 μM). Here, IC_{50} is defined as the concentration of the test compounds that inhibits by 50% the maximally stimulated ATPase.

Results and discussion

The new DHPs (**4a**–**4n**) were evaluated for cytotoxic activity against both the cell lines: colon cancer (HT-29) and breast cancer (MDA-MB) using the MTT assay method. The inhibitory potencies (IC_{50}) of the new DHPs are listed in Table 1. The data indicates clearly that three of the compounds: **4c** ($R = C_6H_5$ & $R_1 = C_2H_5$), **4h** ($R = n\text{-}C_3H_7$ & $R_1 = CH_3$) and **4j** ($R = C_6H_5$ & $R_1 = CH_3$) were inactive against both the cell lines tested, based on the cell viability, whereas the compounds **4d** ($R = C_6H_4{\cdot}NO_2$-3 & $R_1 = C_2H_5$), **4g** ($R = C_6H_4{\cdot}$Cl-4 & $R_1 = C_2H_5$) and **4k** ($R = C_6H_4{\cdot}NO_2$-3 & $R_1 = CH_3$) were uniquely active against both the cell lines. The test compounds **4a** ($R = n\text{-}C_3H_7$ & $R_1 = C_2H_5$), **4b** ($R = n\text{-}C_4H_9$ & $R_1 = C_2H_5$), **4e** ($R = C_6H_4{\cdot}OCH_3$-4 & $R_1 = C_2H_5$), **4m** ($R = C_6H_4{\cdot}CH_3$-4 & $R_1 = CH_3$) and **4n** ($R = C_6H_4{\cdot}$Cl-4 & $R_1 = CH_3$) were selectively active against only the breast cancer cell lines MDA-MB, while two of the test compounds **4i** ($R = n\text{-}C_4H_9$ & $R_1 = CH_3$), and **4l** ($R = C_6H_4{\cdot}OCH_3$-4 & $R_1 = CH_3$) were selective against the colon cancer cell lines HT-29. But, however, none of the present series of dihydropyridines were comparable in their potency with Cisplatin, the standard.

But, among these compounds, compound **4d** with *m*-nitrophenyl and ethyl ester groups was found to be relatively more potent ($IC_{50} = 244.1$ μM) particularly against the breast cancer cell lines (MDA-MB) and close to it was the compound **4g** with 4-chlorophenyl and ethyl ester groups ($IC_{50} = 268.2$ μM) as the substituents. Similarly, the test compound **4g** was also relatively more potent against the colon cancer cell lines (HT-29) with an IC_{50} value of 261.7 μM, whereas reference drug Cisplatin was many times more potent against both the types of cancer cell lines with the IC_{50} values 14.70 and 6.56 μM, respectively.

Only eight new DHPs which showed anticancer activity were investigated for their MRP1 inhibitory activity. In the screening program, the capacity of the compounds either to activate or to inhibit the ATPase activity on isolated Sf9 cell membranes expressing human MRP1 transporter were studied. In the activation study, it was observed that only one test compound, **4g** induced inhibition on MRP1 basal ATPase activity at concentrations above 5 μM. In theory, it is assumed that good substrates activate transporter's basal ATPase activity, whereas slowly transported substrates rather inhibit it. In agreement with this information, the compound **4g** has been shown to be slowly transported substrates of MRP1. In addition, in the present study, **4g** was able to inhibit the NEM-GS-activated MRP1 ATPase with respective IC_{50} value of 16 μM (mean of three experiments) as comparable to a standard MRP1 inhibitor, benzbromarone ($IC_{50} = 4$ μM). However, the rest of the seven molecules failed to show inhibition at the concentrations tested.

The inhibition data indicates the % of MRP1 inhibited by the test compound at a given concentration. The percentage of MRP1 inhibition observed with compound **4g** at 10 μM concentration was found to be 47 (Fig. 2); and its maximum% inhibition of MRP1 was observed to be 65 at 50 μM concentration. These results clearly indicate that the test compound **4g** specifically interact with MRP1 to inhibit, in comparison to the reference drug benzbromarone.

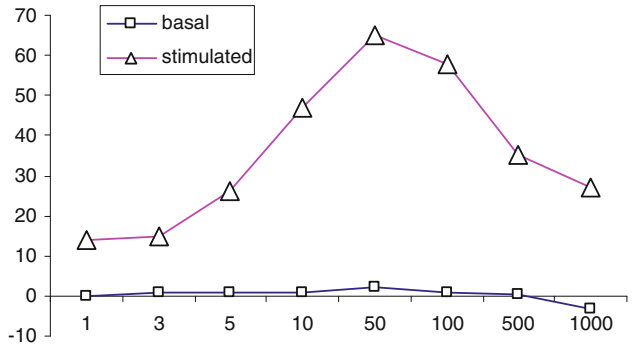


Fig. 2 Modulatory effect of test compound **4g** on ATPase activity in isolated Sf9 cell membranes expressing human MRP1 was tested in the 1–1000 μM concentration range for their capability to alter the ATPase activity in isolated membrane vesicles. The action of **4g** on MRP1 was checked in an activation assay (*square*) and in an inhibition assay (*triangle*). The activation assay was performed on basal ATPase activity, which was 5.2 nmol Pi/mg protein/min for MRP1. In the inhibition assays, NEM-GS at 10 mM for MRP1 was used as the reference activator. The maximal stimulatory effect of this activator corresponded to ATPase activity of 9.6 nmol Pi/mg protein/min. The ATPase activities were expressed on a scale where 0% corresponded to the basal activity and 100% was defined as the activity observed in the presence of NEM-GS. In the inhibition assays, IC_{50} was defined as the concentration of test compounds that inhibits by 50% the maximally stimulated ATPase. Data presented were means ± SD of three experimental points of one representative experiment

Conclusions

4-Alkyl/Aryl-3,5-bis-(carboethoxy/carbomethoxy)-1,4-dihydro-2,6-dimethylpyridines could be successfully synthesized in better purity and good yields (62–90%) by modified Hantzsch method. The MWI method has resulted in increase yields with a shorter reaction times.

Novel 1,4-DHPs synthesized in our laboratory were evaluated for their anticancer and MRP1 inhibitory activities. Among the series of molecules evaluated, 8 molecules were found to be relatively active with anticancer property of course with no comparable potency with that of standard, Cisplatin in their anticancer property. These molecules were specifically selected for MRP1 inhibitory effect by MRP1 ATPase kit experimentally. In conclusion, the present findings identify the test compound **4g** as the only inhibitor of MRP1 (ABCC1)-mediated substrate transport. This data could be useful to develop a pharmacophore model to design some new MRP1 antagonists.

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