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Design, synthesis and evaluation of naphthalene-2-carboxamides as reversal agents in MDR cancer ☆

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Abstract—A novel class of molecules with structure *N*-[3-(4-substituted-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamides were designed by generating a pharmacophore for potent MDR reversal activity, using Elacridar (GF 120918) as a query molecule and using MOE software. They were synthesized by condensing 6-methoxynaphthalene-2-carboxylic acid with *N*-[3-(4-substituted-1-piperazinyl) propyl] amines in the presence of DCC in DMF. They were evaluated in P388 murine lymphocytic leukemia cell line (P388) in vitro using SRB assay for cytotoxicity and in adriamycin-resistant P388 murine lymphocytic leukemia cell line (P388/ADR) using MTT assay for resistant reversal activity. Test compounds were non-toxic at the doses studied (upto 80 μg/ml). They effectively reversed adriamycin resistance at the doses studied (40 and 80 μg/ml). The percentage enhancement in adriamycin activity was in the range 33.58 −90.67 (at 40 μg/ml) and 8.80–46.04 (at 80 μg/ml) and the corresponding reversal potency values were in the range 1.33–1.90 and 1.08–1.46, respectively. Test compounds **2**, **3**, and **5** exhibited better activity as compared to the standard resistant reversal agent (Verapamil), at same concentration.

1. Introduction

Multidrug resistance is a serious problem in the effective management of cancer. P-glycoprotein (P-gp), a membrane protein, may be involved in the efflux of cytotoxic agents from cells leading to development of resistance.[1] Drugs like Verapamil, Propafenone, and many others were evaluated as reversal agents and showed promise. However, they are non-specific in their action and produced toxicity when evaluated in vivo.[2–4] This led to the development of elacridar (GF120918), which is more specific in its action and is free from toxic potential.[5] Additionally structural features and physico-chemical properties required for exhibiting potent P-gp inhibitory activity have been identified.[6] In the present work, it is proposed to first generate a pharmacophore for potent P-gp inhibitory activity and then design a novel series of compounds, synthesize them, and evaluate them in adriamycin-resistant P388 murine lymphocytic leukemia cell line (P388/ADR) for resistance reversal activity.

2. Results and discussion

A pharmacophore for MDR reversal activity was generated using GF120918 as a query molecule and based on this a novel class of compounds were designed with general structure N-substituted-6-methoxynaphthalene-2-carboxamides (Fig. 1).

They were synthesized by an efficient synthetic process (yield 66.8–75.7%). They were evaluated for cytotoxicity in vitro in P388 murine lymphocytic leukemia cell line using SRB assay and test compounds were found to be non-toxic upto a concentration of 80 μg/ml as percentage growth inhibition was very low (0–10.2%).

Cytotoxicity evaluation data are given in Table 1.

The % of cells killed, that is cytotoxicity index (CI), was calculated as follows:

Keywords: 6-Methoxy naphthalene-2-carboxamides; MDR reversal agents; P-glycoprotein; Chemosensitizers.

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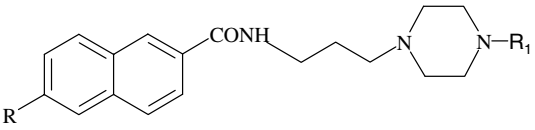

Compds	R	R_1
1	-OCH_3	-CH_3
2	-OCH_3	-n-C_4H_9
3	-OCH_3	-t-C_4H_9
4	-OCH_3	-C_6H_5
5	-OCH_3	-H
6	-H	-CH_3

Figure 1. General structure for test compounds **1**–**6**.

$$CI = \frac{\text{absorbance of drug treated cells}}{\text{absorbance of control cells}} \times 100$$

Test compounds **1**–**6** exhibited chemosensitizing activity at the doses studied, and they effectively reversed adriamycin resistance when evaluated in vitro in adriamycin-resistant P388 murine lymphocytic leukemia cell line (P388/ADR) at 40 and 80 μg/ml (Table 2).

The percentage enhancement in adriamycin activity was calculated by considering the difference in the percentage inhibition due to test compounds+adriamycin and that due to adriamycin alone, expressing it as percentage, while the reversal potency is the ratio of the percentage inhibition due to test compounds+adriamycin and that due to adriamycin.

Table 1. In vitro cytotoxicity on P388 murine lymphocytic leukemia cell line for test compounds **1**–**6**

Compound	Concentration (μg/ml)	% cell killed
1	10	0.0
	20	0.0
	40	2.3
	80	10.2
2	10	0.0
	20	0.0
	40	0.0
	80	0.0
3	10	0.0
	20	0.0
	40	0.0
	80	0.0
4	10	9.4
	20	9.8
	40	10.2
	80	8.1
5	10	0.0
	20	2.6
	40	4.0
	80	4.6
6	10	0.0
	20	0.0
	40	0.0
	80	0.0

The percentage enhancement in adriamycin activity was in the range 33.58–90.67 and reversal potency was in the range of 1.33–1.90 at 40 μg/ml dose of test compounds, the corresponding values were 8.80–46.04% and 1.08–1.46 at 80 μg/ml dose of test compounds.

Test compounds **2**, **3**, and **5** exhibited better activity as compared to the standard resistant reversal agent (Verapamil), at same concentration.

Test compounds **1**–**6** were more effective as chemosensitizers at the lower dose (40 μg/ml), compared to the activity at 80 μg/ml, a quality that is desired in a good chemosensitizing agent.

3. Conclusion

The present work led to the development of novel and potent resistant reversal agents for MDR cancer and three of the novel agents have shown better activity as compared to the standard resistant reversal agent (Verapamil), at the same concentration.

4. Experimental

4.1. Pharmacophore generation

Chemical Computing Group Inc.'s Molecular Operating Environment (MOE) 2001.02 and 2002.03 versions were used for pharmacophore generation. The computations were done on a Windows platform with a processor of 1.6 GHz and memory 512 RAM. A database of 2000 compounds was prepared by using structures reported in the literature and elacridar was used as a query molecule for pharmacophore generation.[5] A similarity searching protocol was applied by using elacridar as the query molecule. This was done on the basis of the fingerprints matching of the query molecule with each of the compounds in the database. On the basis of Tanimoto coefficient, best matched compounds were short-listed. Each of these molecules was aligned with elacridar by using MOE-FlexAlign, by taking the descriptors in various permutations and combinations. From the scores obtained, the important factors identified for P-gp inhibition were log *P*, H-bond donor, H-bond acceptor, aromaticity, and molar volume. By using the pharmacophoric tools, a pharmacophore was built by using Pharmacophore Query Editor based on the information on these short-listed molecules. The distances between the common centers (pharmacophoric points) were measured and are given in Figure 2.

On the basis of pharmacophoric distances, a novel naphthalene carboxamide molecule was designed. It consists of a naphthalene ring connected by an alkyl

Table 2. In vitro MDR reversal activity in P388 murine lymphocytic leukemia adriamycin-resistant cell line for test compounds **1**–**6**

Test system	Concn μg/ml (μmol)	% cell killed	% enhancement in ADR activity	Reversal potency	Concn μg/ml (μmol)	% cell killed	% enhancement in ADR activity	Reversal potency
Compd-**1**	80 (0.234)	1.80			40 (0.117)	2.30		
ADR	60	24.43			50	17.48		
Compd-**1** + ADR	80 + 60	35.68	46.04	1.46	40 + 50	27.13	55.20	1.55
Compd-**2**	80 (0.208)	1.30			40 (0.104)	2.23		
ADR	60	24.43			50	17.48		
Compd-**2** + ADR	80 + 60	34.28	44.31	1.40	40 + 50	32.85	87.92	1.87
Compd-**3**	80 (0.208)	3.15			40 (0.104)	3.33		
ADR	60	24.43			50	17.48		
Compd-**3** + ADR	80 + 60	34.10	39.58	1.39	40 + 50	33.33	90.67	1.90
Compd-**4**	80 (0.198)	2.10			40 (0.099)	2.05		
ADR	60	24.43			50	17.48		
Compd-**4** + ADR	80 + 60	32.40	32.62	1.32	40 + 50	29.78	70.36	1.70
Compd-**5**	80 (0.244)	1.80			40 (0.122)	1.63		
ADR	60	24.43			50	17.48		
Compd-**5** + ADR	80 + 60	32.15	31.60	1.31	40 + 50	30.83	76.37	1.76
Compd-**6**	80 (0.256)	0.00			40 (0.128)	0.30		
ADR	60	24.43			50	17.48		
Compd-**6** + ADR	80 + 60	26.58	8.80	1.08	40 + 50	23.35	33.58	1.33
VRP	80 (0.162)	10.8			40 (0.081)	5.1		
ADR	60	27.6			50	27.6		
VRP + ADR	80 + 60	52.5	90.21	1.90	40 + 50	47.3	71.37	1.71

ADR, Adriamycin; VRP, Verapamil.

linker to N^1 of a piperazine ring. The alkyl linker was optimized to a propyl group to satisfy the pharmacophoric distances. Substitutions were then made (maintaining the pharmacophoric distances) on the N^4 of piperazine to achieve the desired lipophilicity for P-gp inhibition.

4.2. Chemistry

All melting points were recorded on Thermonik melting point apparatus and are uncorrected; IR spectra were recorded in Jasco FT/IR 5300 spectrophotometer; ^{1}H NMR spectra were recorded in Varian spectrophotometer at 300 MHz in $CDCl_3$ (TMS as internal reference). Column chromatography was performed using Silica gel, 60–120 mesh, Qualigens fine chemicals, India. Elemental analyses values for final compounds were within ±0.4% of theoretical value.

N-[3-(4-Substituted-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamides were prepared by condensing 6-methoxy-2-naphthoic acid with *N*-[3-(4-substituted-1-piperazinyl) propyl] amines in the presence of DCC in *N*,*N*-dimethylformamide (DMF) as solvent. 6-Methoxy-2-naphthoic acid was prepared by oxidizing 6-methoxy-2-naphthaldehyde with ammonical silver nitrate. 6-Methoxy-2-naphthaldehyde was prepared from 2-naphthol by bromination followed by methylation using dimethyl sulfate in alkaline condition. The dry product was then treated with Mg in dry THF to form a Grignard reagent, which on reaction with DMF gave the aldehyde.[7]

The *N*-[3-(4-substituted-1-piperazinyl) propyl] amines were prepared by reacting *N*-(3-bromopropyl) phthalimide with mono-substituted piperazines (*N*-methyl piperazine, *N*-butyl piperazine, *N*-*tert*-butyl piperazine, and *N*-phenyl piperazine) and unsubstituted piperazine in DMF in the presence of anhydrous K_2CO_3, followed by reaction with hydrazine hydrate and acidification with HCl. After removal of phthalyl hydrazide, the filtrate was made alkaline and the free amine was extracted in chloroform/ethyl acetate mixture (1:2). Removal of solvent gave the desired amines.[8]

2-Naphthoic acid required for the synthesis of compound **6** was prepared from naphthalene by acetylation using acetyl chloride and anhydrous $AlCl_3$ in nitrobenzene,[9] followed by oxidation using sodium hypobromite (NaOBr).[10] Yield: 60%; mp: 182–184 °C (184–185 °C).[9,10] IR (KBr, cm^{-1}): 3053, 2839 (C–H stretch), 2646, 2569 (O–H stretch), 1686 (C═O stretch).

2-Naphthoic acid was condensed with *N*-[3-(4-methyl-1-piperazinyl) propyl] amine in the presence of DCC in DMF to obtain *N*-[3-(4-methyl-1-piperazinyl) propyl] naphthalene-2-carboxamide (compound **6**) (Section 4.2.8).

4.2.1. Synthesis of 6-methoxy-2-naphthoic acid. To 20 g (115 mM) of silver nitrate in 400 ml of distilled water, 7.5 ml of 10% sodium hydroxide was added, followed by addition of 2% ammonia solution to dissolve the silver oxide. To the solution 20 g (105 mM) of 6-methoxy-2-naphthaldehyde was added and stirred for 5 h with temperature maintained at 50–60 °C. The solution was concentrated, cooled to room temperature and the product formed was digested with 3× 75 ml of 10% sodium hydroxide solution. Extract on acidification gave the free acid, which was purified by recrystallization from alcohol.

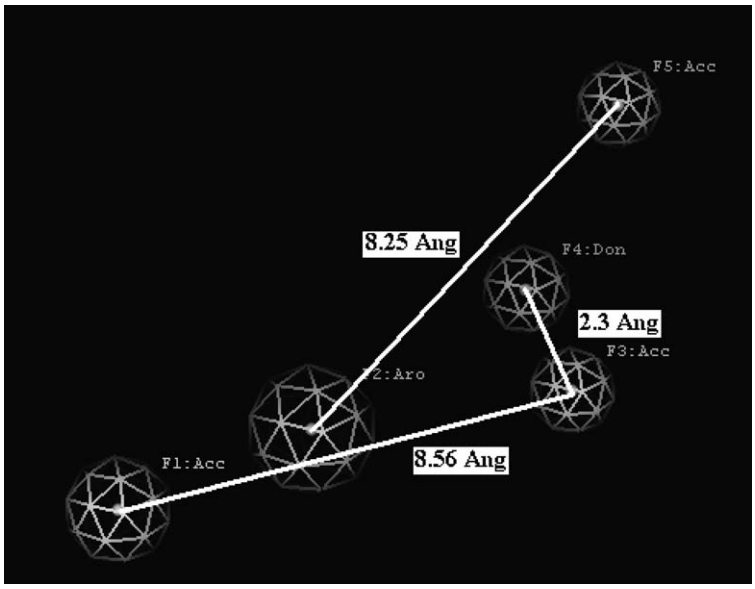

Figure 2. Pharmacophore (Acc, acceptor; Don, donor; Aro, aromatic).

Yield: 60%; mp: 203–204 °C (201–202°C).[11] IR (KBr, cm^{-1}): 3013, 2966, 2924 (C–H stretch), 2845, 2565 (O–H stretch), 1686 (C═O stretch), 1209 (C–O stretch, ether) ^1H NMR ($CDCl_3$) δ ppm: 3.96 (3 H, s, $–OCH_3$), 7.17–7.26 (3× 1H, m, Ar-H), 7.78 (1H, d, Ar-H), 7.89 (1H, d, Ar-H), 8.10 (1H, dd, Ar-H), 8.63 (1H, s, –COOH).

4.2.2. General method for preparation of *N*-[3-(4-substituted-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamides. 6-Methoxy-2-naphthoic acid (10 mM) was condensed with five *N*-[3-(4-substituted-1-piperazinyl) propyl] amines (12 mM) in turn in the presence of DCC (12 mM) in DMF to get compounds **1**–**5**. Dicyclohexylurea formed was removed by filtration and DMF was removed under vacuum. The residue obtained was washed with water to remove excess of amine and traces of DMF. The residue was purified by column chromatography using chloroform/ethyl acetate, 80:20 as eluent and then recrystallized from alcohol.

4.2.3. *N*-[3-(4-Methyl-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamide (1). Yield: 70.2%; mp: 137–138 °C. IR (KBr, cm^{-1}): 3327 (N–H stretch), 1684 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.2 (3H, s, $–CH_3$), 2.8 (4H, m, 2× $–(CH_2)–$, propyl), 3.8 (3H, s, $–OCH_3$), 6.2 (1H, s, –NH), 7.1–7.2 (2H, m, Ar-H), 7.6 (1H, s, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.2.4. *N*-[3-(4-Butyl-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamide (2). Yield: 68%; mp 130–132 °C. IR (KBr, cm^{-1}): 3283 (N–H stretch), 1707 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 0.8 (3H, t, CH_3, *n*-butyl), 1.2 (6H, m, 3× $–(CH_2)–$, *n*-butyl), 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.8 (4H, m, 2× $–(CH_2)–$, propyl), 3.8 (3H, s, $–OCH_3$), 6.2 (1H, s, –NH), 7.1–7.2 (2H, m, Ar-H), 7.6 (1H, s, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.2.5. *N*-[3-(*tert*-Butyl-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamide (3). Yield: 67.4%; mp 135–137 °C. IR (KBr, cm^{-1}): 3296 (N–H stretch), 1707 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 1.2 (9H, s, $–C(CH_3)_3$), 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.8 (4H, m, 2× $–CH_2–$, propyl), 3.8 (3H, s, $–OCH_3$), 6.2 (1H, s, –NH), 7.1–7.2 (2H, m, Ar-H), 7.6 (1H, s, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.2.6. *N*-[3-(4-Phenyl-1-piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamide (4). Yield: 66.8%; mp 138–140 °C. IR (KBr, cm^{-1}): 3287 (N–H stretch), 1707 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.8 (4H, m, 2× $–CH_2–$, propyl), 3.8 (3H, s, $–OCH_3$), 6.2 (1H, s, –NH), 6.5 (2H, d, Ar-Ph), 6.60 (1H, t, Ar-Ph), 6.80 (2H, t, Ar-Ph), 7.1–7.2 (2H, m, Ar-H),7.6 (1H, s, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.2.7. *N*-[3-(1-Piperazinyl) propyl]-6-methoxy naphthalene-2-carboxamide (5). Yield: 75.7%; mp 143–145 °C. IR (KBr, cm^{-1}): 3288 (N–H stretch), 1707 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.1 (1H, s, –N–H–, piperazine), 2.8 (4H, m, 2× $–CH_2–$, propyl), 3.9 (3H, s, $–OCH_3$), 6.2 (1H, s, –NH, amide), 7.1–7.2 (2H, m, Ar–H), 7.6 (1H, s, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.2.8. *N*-[3-(4-Methyl-1-piperazinyl) propyl]-naphthalene-2-carboxamide (6). Yield: 71.2%; mp 184–185 °C. IR (KBr, cm^{-1}): 3327 (N–H stretch), 1684 (C═O stretch); ^1H NMR ($CDCl_3$) δ ppm: 1.5 (2H, m, $–(CH_2)–$, propyl), 1.8 (8H, m, piperazine), 2.2 (3H, s, $–CH_3$), 2.8 (4H, m, 2× $–CH_2–$, propyl), 6.2 (1H, s, –NH), 7.10–7.29 (2H,

m, Ar-H), 7.67 (2H, d, Ar-H), 7.8 (2H, dd, Ar-H), 8.0 (1H, s, Ar-H).

4.3. Pharmacological evaluation

Test compounds were evaluated for cytotoxicity in vitro in P388 murine lymphocytic leukemia cell line (P388) using SRB assay[12] and resistance reversal activity in vitro in adriamycin-resistant P388 murine lymphocytic leukemia cell line (P388/ADR) using MTT assay.[13]

Parental P388 cell line was grown and maintained in the BDF_1 mice by intraperitoneal weekly transplantation of the cells. Resistant cell line (P388/ADR) was developed by giving intraperitoneal injections of 0.65 mg/kg adriamycin with days 1–9 schedule, for 20 passages in BDF_1 mice. For evaluation, tumor cells were obtained from mice bearing seven-day-old tumor. The ascitic fluid from the intraperitoneal cavity of donor mice was aspirated asceptically into 0.9% sodium chloride. The final cell density was adjusted to 10^7 cells/ml.

4.3.1. Preparation of cell suspension. Culture cells were suspended in sterile saline and centrifuged at 1000 rpm for 10 min. The tumor cells were subjected to cold hypotonic shock (4 °C) of ammonium chloride buffer (pH 7.2) to remove erythrocyte. The cells were centrifuged and washed twice with minimal essential medium (MEM) and finally suspended in MEM to achieve a concentration 5×10^5 cells/ml.

Test compounds **1**–**6** were evaluated for cytotoxicity at 10, 20, 40 and 80 μg/ml. MDR reversal activity was evaluated at 40 and 80 μg/ml, and the corresponding adriamycin concentrations were 50 and 60 μg/ml, respectively. Verapamil (VRP) was used as a standard chemomodulator and was evaluated at 40 and 80 μg/ml. All experiments were carried out in quadruplicate.

4.3.2. MTT assay. Cell suspension 90 μl/well was added to 96 wells and incubated at 37 °C for 24 h in 5% CO_2 incubator. Ten microlitres of either solutions of test compounds or solution of adriamycin or combination of a solution of adriamycin and solution of test compounds was added to the wells and the plates were incubated at 37 °C for 48 h in 5% CO_2 incubator. MTT reagent 10 μl was then added to the wells and further incubated for 6 h. The supernatant was then carefully removed and the formazan crystals were dissolved in 100 μl of acidified isopropanol (0.04 N HCl in isopropanol) and the absorbance of the solution was then read on spectrophotometer at a wavelength of 540 nm.

4.3.3. SRB assay. Procedure was same upto addition of test solutions to the microtiter plate. The cells were then fixed with 50 μl of cold (4 °C) 50% trichloroacetic acid (TCA) and were further incubated at 37 °C for 1 h, washed five times with distilled water, and plates were air-dried and stored until use. The TCA fixed cells were stained for 30 min with SRB reagent, at the end excess stain was removed by quickly rinsing the cells four times with 1% acetic acid. Residual wash solution was removed by sharply flicking the plates over the sink. The wells were air-dried until no moisture is visible. The bound dye was solubilized in 100 μl of 10 mM unbuffered Tris base and absorbance of the solution was recorded on spectrophotometer at a wavelength of 565 nm.

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