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5-ARYLIDENE(THIO)HYDANTOIN DERIVATIVES AS MODULATORS OF CANCER EFFLUX PUMP*

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Multidrug resistance has become a factor seriously limiting treatment of various diseases, including bacterial (1–3) and fungal (4) infections and cancer (5–7). The major mechanism of multidrug resistance (MDR) in cancer is the over-expression of ATP-dependent drug-efflux pumps (drug transporters), which reduces the accumulation of agents. One of the most important mechanisms of MDR in cancer is the over-expression of ABC multidrug transporters, e.g., P-glycoprotein (P-gp, ABCB1). Various chemical groups possessing efflux pump inhibitor (EPI) properties in P-gp have been described (7–9), including three generations of P-gp modulators, but none of them has passed the phase of clinical trials because of the undesirable side effects (5). Consequently, it is a big challenge to search for new successful P-gp inhibitors which are active during chemotherapy and produce minimal side effects.

In this context, our interest has been focused on potential EPI activity of arylidene(thio)hydantoin derivatives. Particularly, as recent lines of evidence distinctly identified and described a presence of benzyl-hydantoin binding site in protein transporters (10, 11). Recently, there is an increasing interest in hydantoin derivatives as potential anticancer agents (12–14). It has been shown that hydantoin derivatives inhibit autophosphorylation and proliferation of some human cancer cells that over-express EGFR (12, 13). Furthermore, phenylmethylene-hydantoins with anti-metastatic activity were described as well (14).

Derivatives of (thio)hydantoin are one of our main interests for more than twenty years. Our previous works described a number of aromatic hydantoin derivatives which demonstrated various pharmacological properties as antimicrobial (15–17), hypotensive, antiarrhythmic- or/and GPCR-agents

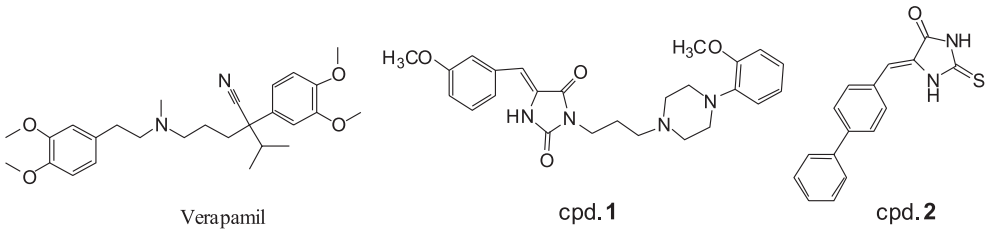

Figure 1. Compounds with confirmed P-gp inhibiting properties

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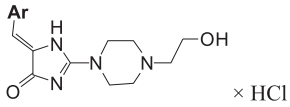

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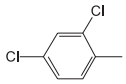
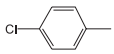
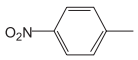
3		**7**	
4		**8**	
5		**9**	
6			

Figure 2. Arylidene-imidazolone derivatives **3–9**

(18–20). Our recent studies (21) indicated that some arylidene(thio)hydantoin derivatives (Fig.1, **1** and **2**) possessed P-gp modulating properties in cancer cells in the range of verapamil or higher (Fig. 1). It suggested that further studies on arylidenehydantoin derivatives as modulators of MDR efflux pumps of cancer cells should be performed. Thus, the present work is focused on derivatives of thiohydantoin: 5-arylideneimidazolones with piperazine substituent. A series of new hydroxyethylpiperazine arylideneimidazolone derivatives **3–9** (Fig. 2) were synthesized and tested on their efflux modulating effects in T-lymphoma cancer cells as well as structure-activity relationship was analyzed.

EXPERIMENTAL

Chemistry

^{1}H-NMR spectra were recorded on a Varian Mercury VX 300 MHz PFG instrument (Varian Inc., Palo Alto, CA, USA) in DMSO-d_6 at ambient temperature using the solvent signal as an internal standard. IR spectra were recorded on a Jasco FT/IR-410 apparatus using KBr pellets and are reported in cm^{-1}. Thin-layer chromatography was performed on precoated Merck silica gel 60 F_{254} aluminium sheets, the used solvent systems were: (I) chloroform/isopropanol/NH_3 9:11:3; (II) methylene chloride/methanol (1:1, v/v). Melting points were determined using Mel-Temp II apparatus and are uncorrected. Elemental analyses were within ± 0.4% of the theoretical values unless stated otherwise. Syntheses under microwave irradiation were performed in household microwave oven Samsung M1618. Methods of synthesis of compounds **11-24** were performed according to the methods described earlier (15–17).

General method for synthesis of compounds 3–9

1-(2-Hydroxyethyl)piperazine (10 mmol, 1.30 g) and a suitable 2-(methylthio)-5-arylidene-1H-imidazol-4(5H)-on **18–24** (5 mmol) were put into roumd bottom flask and melted at 110–130˚C on oil bath under reflux linked to an absorber with concentrated HNO_3. The melting was continued for 40–90 min. Then, ethanol (10–15 mL) was added and the mixture was refluxed for 5–6 h. The mixture was left at room temperature overnight. The precipitate of products **3b–9b** was separated by filtration, washed with ethanol, purified by crystallization with ethanol or by column chromatography. Compounds in basic form **3b–9b** were suspended in dry ethanol (10 mL) and saturated with gaseous HCl to give corresponding hydrochlorides **3–9**.

Synthesis of (Z)-5-benzylidene-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (3)

(Z)-5-benzylidene-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**3b**)

1-(2-Hydroxyethyl)piperazine with (Z)-5-benzylidene-2-(methylthio)-1H-imidazol-4(5H)-one **18** (5 mmol, 1.09 g) were melted at 120^{o}C for 60 min. The mixture with ethanol (10 mL) was stirred and refluxed for 6 h. Pure white-grey crystals of product **3b** were obtained after filtration (3 mmol, 0.95 g,

63%); m.p. 204–207°C; R_f (I) 0.41. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.44 (t, *J* = 6.16 Hz, 2H, Pp-CH_2), 2.48–2.51 (m, 4H, Pp-3,5-H), 3.48–3.54 (m, 4H, Pp-2,6-H), 3.58 (br s, 2H, C$\underline{H_2}$-OH), 4.42 (t, *J* = 5.20 Hz, 1H, OH), 6.29 (s, 1H, CH=C), 7.18–7.23 (t def., 1H, Ar-4-H), 7.31–7.36 (t def., 2H, Ar-3,5-H), 8.00 (br s, 2H, Ar-2,6-H), 11.25 (br. s, 1H, NH).

(Z)-5-benzylidene-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**3**)

Compound **3b** (0.86 mmol, 0.257 g) in dry ethanol (6 mL) was converted into hydrochloride to give white powder of **3** (0.64 mmol, 0.24 g, 75%); m.p. 255–260°C; Rf (I) 0.41. Analysis: calcd. for $C_{16}H_{20}N_4O_2 \times 1.75\ HCl \times 0.5\ H_2O$: C, 51.50; H, 6.14; N, 15.01%; found: C, 51.60; H, 5.83 ; N, 15.17%.

Synthesis of (Z)-5-(4-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (4)

(Z)-5-(4-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**4b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(4-chlorobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one **19** (5 mmol, 1.26 g) were melted at 120°C for 90 min. The mixture with ethanol (10 mL) was stirred and refluxed for 5.5 h. Pure yellow crystals of product **4b** was obtained (2 mmol, 0.61g, 36%); m.p. 197–199°C; Rf (I) 0.48. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.41 (t, *J* = 6.61 Hz, 2H, Pp-CH_2), 2.48 (dd, J_1 = 3.85 Hz, J_2 = 1.80 Hz, 4H, Pp-3,5-H), 3.49 (dd, J_1 = 11.28 Hz, J_2 = 5.90 Hz, 2H, C$\underline{H_2}$-OH), 3.59 (br. s, 4H, Pp-2,6-H), 4.42–4.45 (t def., 1H, OH), 6.27 (s, 1H, CH=C), 7.37 (d, *J* = 8.46 Hz, 2H, Ar-3,5-H), 8.02(d, *J* = 7.44 Hz, 2H, Ar-2,4-H), 11.28 (br. s, 1H, N1-H).

(Z)-5-(4-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**4**)

Compound **4b** (0.93 mmol, 0.31 g) in dry ethanol (6 mL) was converted into hydrochloride to give white powder of **4** (0.90 mmol, 0.36 g , 98%); m.p. 263–265°C; Rf (I) 0.48. Analysis: calcd. for $C_{16}H_{19}N_4O_2Cl \times HCl \times 1.5\ H_2O$: C, 48.25; H, 5.82; N, 14.07%; found: C, 48.59; H, 5.82; N, 14.03%. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.19 (br s, 4H, Pp-2,6-H), 3.56–3.64 (t def., 5H, Pp-3,5-H, Pp-CH_2a), 3.77–3.81 (t def., 3H, Pp-CH_2b, C$\underline{H_2}$-OH), 4.31 (br. s, 1H, OH), 6.43 (s, 1H, CH=C), 7.39–7.50 (dt def., 2H, Ar-3,5-H), 8.03 (d, *J* = 8.72 Hz, 2H, Ar-2,4-H), 10.82 (br. s, 1H, N^1-H). IR (KBr, cm^{-1}): 3343 (N^1-H), 2997 (OH), 2939 (CH), 2850 (C-CH_2-C), 2633 (NH^+), 1761 (C=O), 1686 (Ar-CH=H), 1602 (Ar, C=C).

Synthesis of (Z)-5-(3-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (5)

(Z)-5-(3-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**5b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(3-chlorobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one (5 mmol, 1.26 g) were melted at 130°C for 60 min. The mixture with ethanol (10 mL) was stirred and refluxed for 5 h. Pure yellow crystals of product **5b** was obtained (0.8 mmol, 0.27g, 16%); m.p. 183–184°C; Rf (I) 0.39. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.41–2.52 (m, 6H, Pp-3,5-H, Pp-CH_2), 3.49–3.69 (m, 6H, Pp-2,6-H, C$\underline{H_2}$-OH), 4.42 (t, *J* = 5.39 Hz, 1H, OH), 6.26 (s, 1H, CH=C), 7.22–7.25 (d def., 1H, Ar-4-H), 7.32–7.38 (t def., 1H, Ar-5-H), 7.91–7.93 (d def., 1H, Ar-6-H), 8.17 (br s, 1H, Ar-2-H), 11.34 (br s, 1H, N^1-H).

(Z)-5-(3-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**5**)

Compound **5b** (0.6 mmol, 0.20 g) in dry ethanol (6 mL) was converted into hydrochloride to give yellow powder of **5** (0.35 mmol, 0.13 g , 58%); m.p. 253–255°C; Rf (I) 0.39. Analysis: calcd. for $C_{16}H_{20}N_4O_2 \times HCl \times 0.25\ H_2O$: C, 51.14; H, 5.50; N, 14.91%; found: C, 51.03; H, 5.50; N, 14.64. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.19 (s, 2H, Pp-CH_2), 3.39–3.43(t def., 4H, Pp-3,5-H), 3.60 (s def., 4H, Pp-2,6-H), 3.76–3.80 (t def., 2H, C$\underline{H_2}$-OH), 4.28 (br s, 1H, OH), 6.38 (s, 1H, CH=C), 7.26 (d def., *J* = 8.98 Hz, 1H, Ar-4-H), 7.34-7.39 (t def., 1H, Ar-5-H), 7.95 (d, *J* = 7.69 Hz, 1H, Ar-6-H), 8.14 (s, 1H, Ar-2-H), 10.60 (br s, 1H, N^1-H), 11.58 (br s, 1H, NH^+). IR (KBr, cm^{-1}): 3303 (N^1-H), 3114 (OH), 3045 (CH), 2851 (C-CH_2-C), 2464 (NH^+), 1706 (C=O), 1653 (Ar-CH=H), 1580 (Ar, C=C).

Synthesis of (Z)-5-(2-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (6)

(Z)-5-(2-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**6b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(2-chlorobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one (5 mmol, 1.26 g) were melted at 130°C for 60 min. The mixture with ethanol (10 mL) was stirred and refluxed for 5 h. Pure yellow crystals of **6b** were obtained (4 mmol, 1.41g, 80%); m.p. 237–238°C; Rf (I) 0.43. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.40 (t, *J* = 6.16 Hz, 2H, Pp-CH_2), 2.48–2.52(m, 4H, Pp-3,5-H), 3.52 (br s, 2H, C$\underline{H_2}$-OH), 3.69 (br s, 4H, Pp-2,6-H), 4.45 (br s, 1H, OH),

6.58 (s, 1H, CH=C), 7.18–7.24 (m, 1H, Ar-4-H), 7.32–7.37 (m, 1H, Ar-5-H), 7.42–7.45 (m, 1H, Ar-3-H), 8.79 (br s, 1H, Ar-6-H), 11.35 (br s, 1H, NH).

(Z)-5-(2-chlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**6**)

Compound **6b** (1.22 mmol, 0.41 g) in dry ethanol (7 mL) was converted into hydrochloride to give bright powder of **6** (0.93 mmol, 0.38 g, 76%); m.p. 230–233°C; Rf (I) 0.43. Analysis: calcd. for $C_{16}H_{19}ClN_4O_2 \times 2HCl$: C, 47.13; H, 5.19; N, 13.74%; found: C, 47.15; H, 4.94; N, 13.76%. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.19 (br s, 4H, Pp-3,5-H), 3.61–3.64 (m, 5H, Pp-2,6-H, Pp-CH_2a), 3.71–3.81 (t def., 3H, Pp-CH_2b, C$\underline{H_2}$-OH), 4.35 (br s, 1H, OH), 6.70 (s, 1H, CH=C), 7.24–7.30 (m, 1H, Ar-4-H), 7.34–7.39 (m, 1H, Ar-5-H), 7.46–7.49 (m, 1H, Ar-3-H), 8.66 (d, J = 7.70 Hz, 1H, Ar-6-H), 10.90 (br s, 1H, N^1-H). IR (KBr, cm^{-1}): 3416 (N^1-H), 3307 (OH), 3019 (CH), 2931 (C-CH_2-C), 2685 (NH$^+$), 1759 (C=O), 1684 (Ar-CH=H), 1605 (Ar, C=C).

Synthesis of (Z)-5-(2,4-dichlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**7**)

(Z)-5-(2,4-dichlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**7b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(2,4-dichlorobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one (5 mmol, 1.44 g) were melted at 130°C for 40 min. The mixture with ethanol (10 mL) was stirred and refluxed for 5 h. Pure yellow crystals of product **7b** was obtained (4 mmol, 1.48 g, 80%); m.p. 210–213°C; Rf (I) 0.31. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.41 (t, J = 6.16 Hz, 2H, Pp-CH_2), 2.47–2.52 (m, 4H, Pp-3,5-H), 3.51 (br s, 4H, Pp-2,6-H), 3.61 (br s, 2H, C$\underline{H_2}$-OH), 4.45 (br s, 1H, OH), 6.49 (s, 1H, CH=C), 7.41–7.44 (dd def., 1H, Ar-5-H), 7.58 (s, 1H, Ar-3-H), 8.80 (d, J = 7.95 Hz, 1H, Ar-6-H), 11.41 (br s, 1H, N1-H).

(Z)-5-(2,4-dichlorobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**7**)

Compound **7b** (0.69 mmol, 0.26 g) in dry ethanol (6 mL) was converted into hydrochloride to give yellow powder of **7** (0.54 mmol, 0.24 g, 78%); m.p. 235–240°C; Rf (I) 0.31. Analysis: calcd. for $C_{16}H_{18}Cl_2N_4O_2 \times 1.75\ HCl \times 0.5\ H_2O$: C, 43.47; H, 4.73; N, 12.67%; found: C, 43.49; H, 4.73; N, 12.43%. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.19 (br s, 4H, Pp-3,5-H), 3.60–3.64 (d def., 5H, Pp-CH_2a, Pp-2,4-H), 3.78 (t, J = 5.10 Hz, 3H, Pp-CH_2b, C$\underline{H_2}$-OH), 4.38 (br s, 1H, OH), 6.61 (s, 1H, CH=C), 7.41–7.44 (dd def., 1H, Ar-5-H), 7.63 (s, 1H, Ar-3-H), 8.73 (d, J = 8.70 Hz, 1H, Ar-6-H), 10.89 (br s, 1H, N^1-H). IR (KBr, cm^{-1}): 3321 (N^1-H), 3002 (OH), 2936 (CH), 2800 (C-CH_2-C), 2633 (NH$^+$), 1764 (C=O), 1683 (Ar-CH=H), 1579 (Ar, C=C).

Synthesis of (Z)-5-(4-nitrobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**8**)

(Z)-5-(4-nitrobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one (**8b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(4-nitrobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one (5 mmol, 1.32 g) were melted at 110°C for 60 min. The mixture with ethanol (15 mL) was stirred and refluxed for 5.5 h. Pure orange crystals of **8b** were obtained (4 mmol, 1.43 g, 80%); m.p. 224–226°C; Rf (I) 0.30. ^{1}H-NMR (DMSO-d_6, δ, ppm): 2.42 (t, J = 6.16 Hz, 2H, Pp-CH_2), 2.47–2.53 (m, 4H, Pp-3,5-H), 3.50 (t, J = 6.16 Hz, 2H, C$\underline{H_2}$-OH), 3.65 (br s, 4H, Pp-2,6-H), 4.43 (br s, 1H, OH) 6.33 (s, 1H, CH=C), 8.14–8.17 (d def., 2H, Ar-2,6-H), 8.23–8.26 (d def., 2H, Ar-3,5-H), 11.47 (br s, 1H, N1-H).

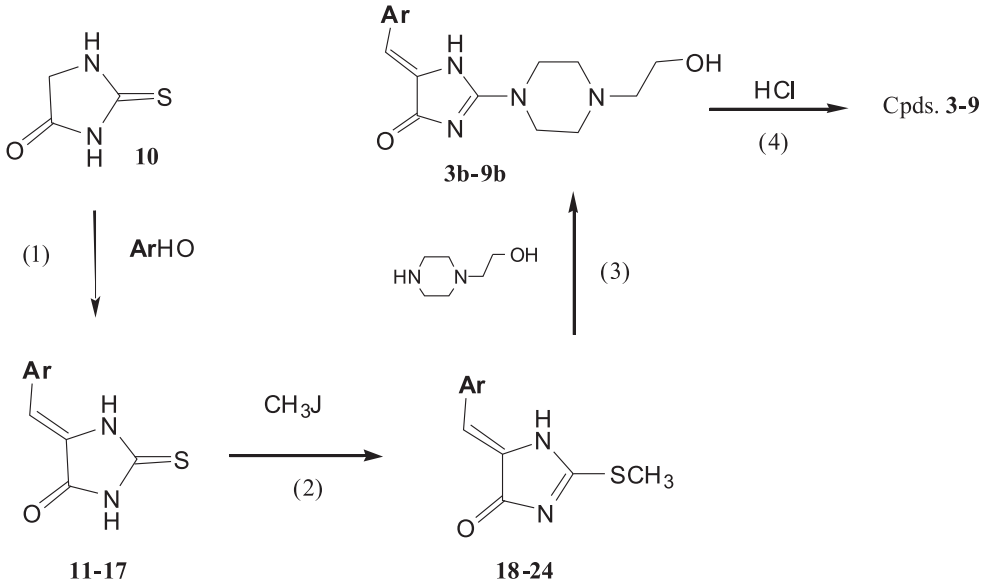
(Z)-5-(4-nitrobenzylidene)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-1H-imidazol-4(5H)-one hydrochloride (**8**)

Compound **8b** (0.75 mmol, 0.26 g) in dry ethanol (6 mL) was converted into hydrochloride to give orange powder of **8** (0.55 mmol, 0.23 g , 73%); m.p. 272–275°C; Rf (I) 0.30. Analysis: calcd. for $C_{16}H_{19}N_5O_4 \times 2HCl$: C, 45.94; H, 5.06; N, 16.74%; found: C, 46.02; H, 4.87; N, 16.59%. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.21 (br s, 4H, Pp-3,5-H), 3.62–3.66 (m, 5H, Pp-CH_2a, Pp-2,6-H), 3.79–3.82 (t def., 3H, Pp-CH_2b, C$\underline{H_2}$-OH), 4.50 (br s, 1H, OH) 6.47 (s, 1H, CH=C), 8.15–8.29 (m, 4H, Ar), 10.97 (br s, 1H, N1-H). IR (KBr, cm^{-1}): 3266 (N^1-H), 3107 (OH), 2998 (CH), 2933 (C-CH_2-C), 2683 (NH$^+$), 1761 (C=O), 1686 (Ar-CH=H), 1662 (Ar, C=C).

Synthesis of (Z)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-5-(naphthalen-2-ylmethylene)-1H-imidazol-4(5H)-one hydrochloride (**9**)

(Z)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-5-(naphthalen-2-ylmethylene)-1H-imidazol-4(5H)-one (**9b**)

1-(2-Hydroxyethyl)piperazine and (Z)-5-(4-nitrobenzylidene)-2-(methylthio)-1H-imidazol-4(5H)-one (5 mmol, 1.342 g) were melted at 120°C for 60 min. The mixture with ethanol (10 mL) was stirred and refluxed for 5.5 h. Column chromatogra-



Scheme 1. Synthetic way: (1) Knoevenagel condensation; (2) S-methylation; (3) substitution with hydroxyethylpiperazine; (4) convertion into hydrochlorides **3–9** with gaseous HCl

phy using solvent (II) gave pure yellow crystals of product **9b** (2 mmol, 0.82 g, 47%); m.p. 306–308°C; Rf (I) 0.29. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.18–3.34 (m, 4H, Pp-3,5-H), 3.60 (d, *J* = 9.75 Hz, 5H, Pp-2,6-H, Pp-CH_2a), 3.80 (br s, 3H, Pp-CH_2b, CH_2-OH), 4.35 (br s, 1H, OH), 7.15 (s, 1H, CH=C), 7.51–7.59 (m, 3H, naphth-4,5,9-H), 7.83–7.97 (dt def., 2H, naphth-3,6-H), 8.22 (br s, 1H, naphth-10-H), 8.90 (br s, 1H, naphth-8-H), 11.07 (br s, 1H, N1-H).

(Z)-2-(4-(2-hydroxyethyl)piperazin-1-yl)-5-(naphthalen-2-ylmethylene)-1H-imidazol-4(5H)-one hydrochloride (**9**)

Compound **9b** (1.43 mmol, 0.500 g) in dry ethanol (8 mL) was converted into hydrochloride to give orange powder of **9** (1.13 mmol, 0.48 g , 80%); m.p. 285–287°C; Rf (I) 0.29. Analysis: calcd. for $C_{20}H_{22}N_4O_2 \times 1.5$ HCl × H_2O: C, 56.77; H, 6.07; N, 13.24%; found: C, 57.01; H, 6.33; N, 13.01%. ^{1}H-NMR (DMSO-d_6, δ, ppm): 3.19 (br s, 4H, Pp-3,5-H), 3.63–3.71 (t def., 5H, Pp-2,6-H, Pp-CH_2a), 3.79–3.82 (t def., 3H, Pp-CH_{2b}, CH_2-OH), 4.39 (br s, 1H, OH), 7.24 (s, 1H, CH=C), 7.53–7.62 (m, 2H, naphth-3,6-H), 7.89–7.98 (q def., 1H, naphth-10-H), 8.51 (br s, 1H, naphth-8-H), 11.29 (br s, 1H, N^1-H). IR (KBr, cm^{-1}): 3415 (N^1-H), 3312 (OH), 2997 (CH), 2661 (C-CH_2-C), 2450 (NH^+), 1758 (C=O), 1681 (Ar-CH=H), 1590 (Ar, C=C).

PHARMACOLOGY

Cell lines

L5178Y mouse T-cell lymphoma cells (ECACC cat. no. 87111908, U.S. FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus, as described elsewhere (22, 23).

The *ABCB1*-expressing cell lines were selected by culturing the infected cells with 60 ng/mL of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maintain the MDR phenotype. L5178 mouse T-cell lymphoma cells (parental) and the human *ABCB1*-gene transfected sub-line were cultured in McCoy's 5A medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (penicillin, streptomycin) at 37°C and in a 5% CO_2 atmosphere.

Flow cytometry assay for evaluation of a compound on the retention of rhodamine 123 by MDR in tumor cells.

This assay has been fully described previously (24). Briefly, the cells were adjusted to a density of 2×10^6/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. Volumes of 2–20 μL of

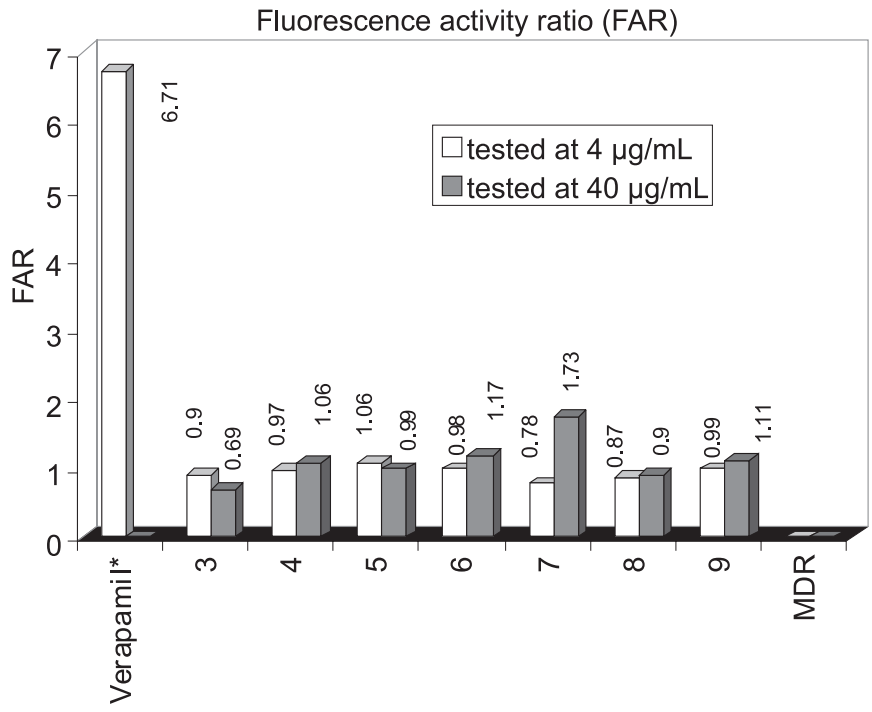


Figure 3. Fluorescence activity ratios (FARs) for compounds **3**–**9** in comparison to verapamil (tested at dose of 10 ěg/mL) using rhodamine 123 accumulation assay

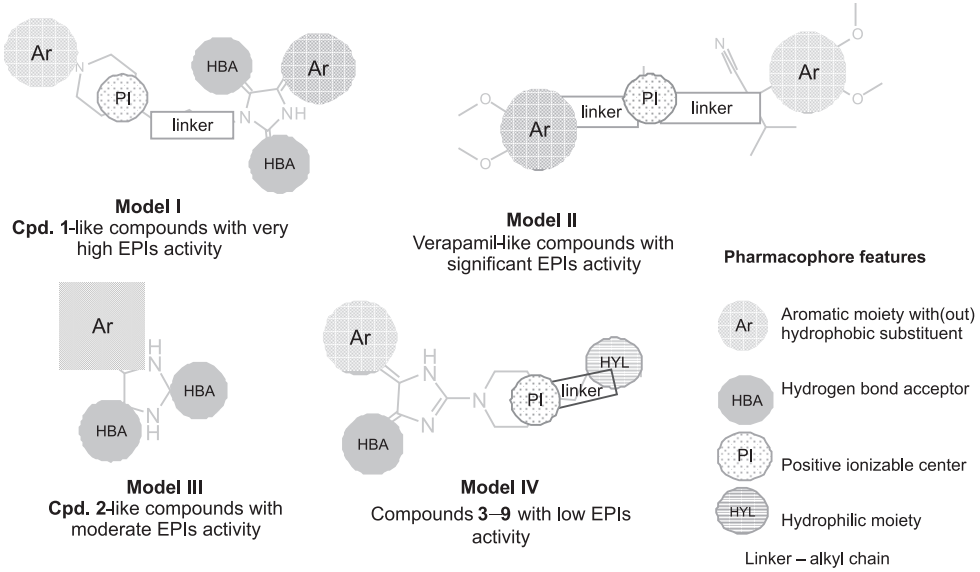


Figure 4. Pharmacophore hypotheses for efflux modulators in mouse T-lymphoma cells overexpressing the ABCB1 system

test compounds were added at various concentrations, and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 mM final concentration) of rhodamine 123 was added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and re-suspended in 0.5 mL of phosphate-buffered saline (PBS) for analysis. The fluorescence uptake of the cell population

was measured with FACStar Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared to untreated cells. A fluorescence activity ratio (FAR) was calculated on the basis of the measured fluorescence values *via* the following equation:

$$FAR = \frac{\text{MDR treated/MDR control}}{\text{parental treated/parental control}}$$

The results presented are obtained from a representative flow cytometry experiment in which 10,000 individual cells of the population were evaluated for amount of rhodamine 123 retained. They were originally presented by the Beckton Dickinson FACStar flow cytometer as histograms and the data were converted to FAR units that define fluorescence intensity, standard deviation, peak channel in the total- and in the gated- populations .

RESULTS

Compounds **3–9** were obtained within 3-step synthesis according to the Scheme 1. In the first step, 5-arylidene-2-thiohydantoins **11–17** were synthesized using two variants of Knoevenagel's condensation for 2-thiohydantoin with suitable aromatic aldehydes that was described in previous works (15–17). Compounds **11–17** were methylated in the second step to give intermediates **18–24** (15–17). Final products in basic form (**3b–9b**) were obtained by the replacement of S-methyl group with hydroxyethylpiperazine. The process was performed by melting of intermediates **18–24** with hydroxyethylpiperazine on oil-bath by the use of 2-fold excess of the amine. Compounds **3b–9b** were suspended in ethanol and converted into hydrochlorides **3–9** by the use of gaseous HCl (21, 22).

Compounds **3–9** were tested for their efflux modulating effects in mouse T-lymphoma cells with over-expressed ABCB1 system using fluorescence activated cell sorting. The modulation of intracellular drug accumulation was evaluated by flow cytometry using rhodamine 123 accumulation assay according to the method described previously (5, 6). Results are presented in Figure 3.

Structure-activity relationship was analyzed basing on chemical features of both, potential (**3–9**) and confirmed (**1, 2** and verapamil) modulators of PgP. The following features, important for ligand-protein interactions, were considered: aromatic moiety with(out) hydrophobic substituent (Ar), hydrogen bond acceptor (HBA), positive ionizable center (PI), hydrophilic moiety (HYL) as well as alkyl linkers which influence conformational flexibility of ligands (Figure 4).

DISCUSSION AND CONCLUSION

Compounds **3–9** displayed weak cancer efflux pump inhibitors properties, weaker than those of verapamil and compound **2** and much weaker than that of compound **1** (24). Among the new compounds, compound **7** was the most promising one, showing dose-dependent fluorescence activity ratio (FAR), with a maximum FAR value of 1.73 at 40 μg/mL. Concerning compounds **3–6** and **8**, **9** the increase of concentration caused very low increase-(**4, 6, 8** and **9**) or some decrease (**3** and **5**) of FAR. Compounds with the highest efflux pump inhibitors potency include two aromatic fragments Ar (Model I and II, Fig. 4). Pharmacophore features PI, HBA and alkyl linker(s) seem to be profitable for EPI activity but their influence is not very strong. Hydrophilic hydroxyl group (HYL), occurring in compounds **3–9,** is probably a main factor, which decreased their activity comparing to that of compounds **1**, **2** and verapamil**.** A conversion of hydantoin moiety into imidazolone as well as an exchange of amine-alkyl substituent place from position 3 into position 2 is supposed to be an additional factor limiting the activity.

The highest activity among 5-arylideneimidazolones **3-9** was observed for 2,4-dichlorobenzylidene derivative **7** which includes the most lipophilic aromatic fragment (two chlorides at phenyl ring). These results confirm an important role of hydrophobic aromatic fragments for cancer efflux pumps modulator properties.

Results of our study indicated that hydrophobic aromatic moieties (Ar) were the most favorable features for cancer EPIs activity, whereas hydrophilic hydroxyl groups (HYL) caused a significant decrease of the activity.

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