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QUARTERLY

The ability to overcome multidrug resistance of tumor cell lines by novel acridine cytostatics with condensed heterocyclic rings★✪

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Two recently synthesized groups of acridine cytostatics containing fused heterocyclic ring(s): pyrazoloacridines (PAC) and pyrazolopyrimidoacridines (PPAC) were tested in regard to their *in vitro* cytotoxic activity towards a panel of sensitive and resistant human tumor cell lines. The obtained results corroborate our earlier hypothesis on the essential role of heterocyclic ring fused to the acridine moiety in the ability of acridine cytostatics to overcome multidrug resistance of tumor cells. The presence, location and kind of substituents considerably influenced both the cytotoxic activity of the derivatives and their ability to overcome multidrug resistance. The same factors also affected the cytostatics ability to differentiate between tumor cell lines with various types of drug exporting pumps.

The development of multidrug resistance (MDR) of tumor cells due to the overexpression of genes coding for plasma membrane drug efflux pumps such as P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) has become a serious

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Abbreviations: DX, Doxorubicin; LRP, **l**ung **r**esistance related **p**rotein dependent resistance; MDR, multidrug resistance; MIT, Mitoxantrone; MRP, **m**ultidrug **r**esistance-associated **p**rotein dependent resistance; PAC, pyrazoloacridines; P-gp, P-glycoprotein; PPAC, pyrazolopyrimidoacridines; RI, resistance index; VINC, Vincristine.

problem in clinical oncology [1–3]. A worldwide effort has been made to design novel cytostatics able to overcome this undesirable effect. In this endeavor, the identification of structural factors of modified cytostatics, crucial for exhibiting activity towards the multidrug resistant cells, is of utmost importance.

In our previous studies, we postulated that for anthracenedione and related acridine cytostatics the introduction to these molecules of a fused five- or six-membered heterocyclic ring yields compounds able to overcome multidrug resistance, due to fast influx exceeding the velocity of ABC proteins mediated efflux [4, 5]. It was shown that the kind and location of substituents influenced the cytotoxic potential of these compounds[1] [6]. In order to optimize the advantageous properties of such compounds, more detailed structure-activity relationship studies are indispensable.

In this paper, we present biological characteristics of two recently synthesized groups of acridine cytostatics. These are pyrazoloacridines (PAC) and pyrazolopyrimidoacridines (PPAC) (Fig. 1) [7–9]. PAC compounds contain a fused five-membered heterocyclic pyrazole ring, however, the formation of an additional hydrogen bonded six-membered ring can be also expected. The PPAC compounds contain two fused covalently bonded heterocyclic rings; pyrazole and pyrimidine one.

In vitro cytotoxic activity of these compounds, together with the reference cytostatics, was examined against sensitive human

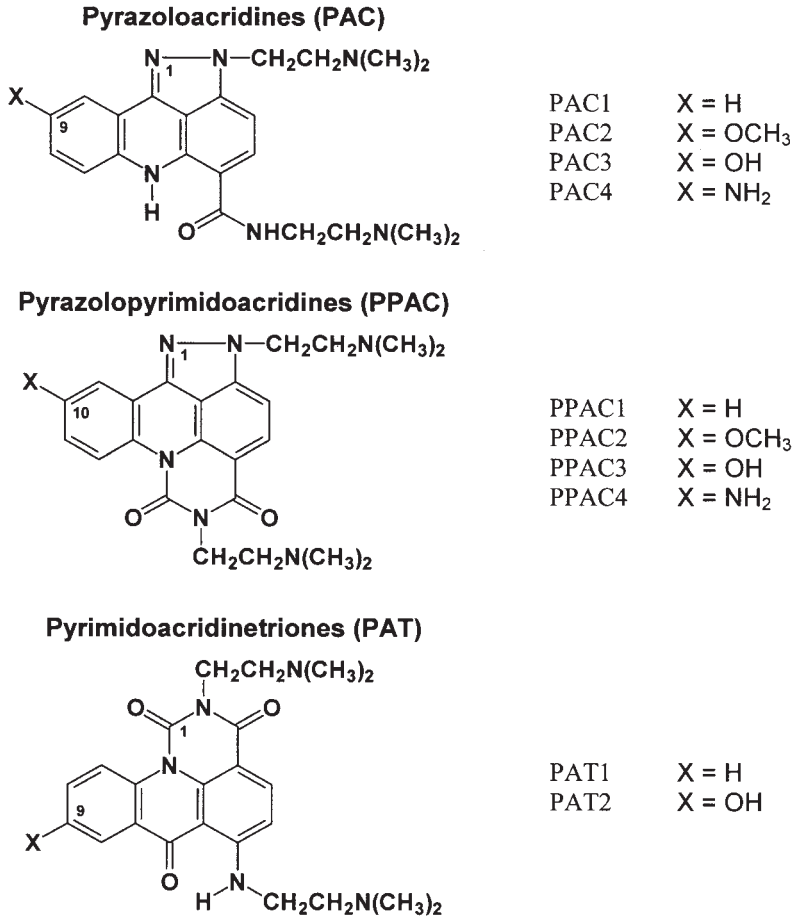

Figure 1. Structures of compounds.

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leukemia cell lines MOLT-4, K562, HL60, and their resistant sublines K562/DX, HL60/VINC with P-gp dependent resistance and HL60/DX with MRP-1 dependent resistance. Also, the cytotoxic activity was examined towards human small-lung carcinoma sensitive cell line GLC_4 and its resistant subline GLC_4/DX with MRP/LRP dependent resistance.

MATERIALS AND METHODS

All reagents for tissue culture were purchased from Gibco Bio-Cult (U.K.). The reference compounds were kindly donated by the Nazionale Istituto Tumori (Milan, Italy, Doxorubicin) and the Institute of Pharmaceutical Research (Warszawa, Poland, Mitoxantrone). Vincristine was purchased from Pierre Fabre.

Cell lines. Human acute lymphoblastic leukemia cell line MOLT-4 (University of Manchester School of Biology, U.K.) was grown in RPMI 1640 medium supplemented with 10% FBS (foetal bovine serum), penicillin G (100 000 units/l), and streptomycin (100 mg/l). Human myelogenous leukemia sensitive cell line K562 and Doxorubicin resistant subline K562/DX (Institut de Cancerologie et d'Immunogénétiques, Villejuif, France) were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 000 units/l), streptomycin (100 mg/l), and 2 mM L-glutamine. Reselection of the resistant cell line was performed once a month by exposure to 500 nM Doxorubicin. Human promyelocytic leukemia sensitive cell line HL60 and resistant sublines: Vincristine resistant HL60/VINC and Doxorubicin resistant HL60/DX (Kansas State University, Manhattan, KS, U.S.A.), were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 000 units/l), and streptomycin (100 mg/l). Reselection of the resistant cell lines was performed once a month by exposure to 200 nM Doxorubicin and 1 μM Vincristine for HL60/DX and HL60/VINC, respectively. Human small-lung carcinoma sensitive cell line GLC_4 and Doxorubicin resistant subline GLC_4/DX (University of Groningen, The Netherlands) were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 000 units/l), and streptomycin (100 mg/l). Reselection of the resistant cell line was performed once a month by exposure to 1 μM Doxorubicin.

Cell lines were grown in a controlled (air/5% CO_2) humidified atmosphere at 37°C and were transplanted two–three times a week. For the experiments only the cells in the logarithmic phase of growth were used. The resistant cell lines were maintained without the reselection drugs at least one week before the experiments.

In vitro *cytotoxic activity*. Cells of required density were seeded and different concentrations of the drugs were added. The experiments were carried out in a controlled (air/5% CO_2) humidified atmosphere at 37°C. The cytotoxic activity (IC_{50} value) of the compound was defined as its *in vitro* concentration causing 50% inhibition of cell growth after continuous exposure to the drug (72 h), as measured by cell counting with a Zb_I Coulter Counter (Coulter Electronics, Ltd., U.K.). The results are given as mean of at least three independent experiments ± standard error of the mean (S.E.M.). The resistance index (RI) was defined as the ratio of the IC_{50} value for a resistant cell line to the IC_{50} value for a sensitive one.

Examined compounds. All compounds studied were synthesized in our laboratories. Their structures are presented in Fig. 1.

RESULTS AND DISCUSSION

The cytotoxic activity of tested compounds on a panel of human sensitive and resistant cell lines is presented in Table 1.

The cytotoxic potentials of pyrazoloacridines (PAC) and pyrazolopyrimidoacridines (PPAC) towards sensitive cell lines indicate

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Table 1. *In vitro* cytotoxic activity of examined compounds towards sensitive and resistant human cell lines

Compound	Cell line *(type of resistance)* IC_{50} [nM]±S.E.M.// *RI*							
	MOLT-4	HL60	HL60/DX *(MRP)*	HL60/VINC *(P-gp)*	K562	K562/DX *(P-gp)*	GLC_4	GLC_4/DX *(MRP/LRP)*
PAC1	124 ± 24	217 ± 24	161 ± 12 *0.7*	455 ± 45 *2.1*	863 ± 70	4409 ± 312 *5.1*	1165 ± 164	704 ± 84 *0.6*
PAC2	240 ± 25	322 ± 57	297 ± 32 *0.9*	346 ± 20 *1.1*	1192 ± 127	3456 ± 252 *2.9*	1884 ± 110	1013 ± 7 *0.5*
PAC3	63 ± 0.5	125 ± 17	545 ± 38 *4.4*	1797 ± 62 *14.4*	1012 ± 136	16372 ± 4309 *16.2*	1095 ± 80	1127 ± 151 *1.0*
PAC4	815 ± 95	507 ± 75	723 ± 102 *1.4*	8048 ± 1891 *15.9*	1466 ± 66	35158 ± 5520 *24.0*	2859 ± 324	1948 ± 356 *0.7*
PPAC1	4.8 ± 0.5	21 ± 5	84 ± 11 *4.0*	28 ± 2 *1.3*	74 ± 13	268 ± 39 *3.6*	90 ± 18	165 ± 12 *1.8*
PPAC2	28 ± 7	58 ± 12	55 ± 2 *0.9*	33 ± 4 *0.6*	214 ± 37	359 ± 41 *1.7*	332 ± 52	201 ± 41 *0.6*
PPAC3	10 ± 3	11 ± 0.3	165 ± 12 *15.0*	258 ± 38 *23.5*	120 ± 6	3850 ± 858 *32.1*	353 ± 38	274 ± 39 *0.8*
PPAC4	20 ± 4	20 ± 6	48 ± 7 *2.4*	68 ± 8 *3.4*	229 ± 35	1155 ± 165 *5.0*	387 ± 93	155 ± 17 *0.4*
DX	30 ± 6	20 ± 3	3928 ± 263 *196.4*	647 ± 60 *32.4*	42 ± 3	7031 ± 542 *167.4*	59 ± 12	3009 ± 413 *51.0*
MIT	1.6 ± 0.3	2.2 ± 0.3	1179 ± 203 *535.9*	57 ± 4 *25.9*	21 ± 4	554 ± 50 *26.4*	45 ± 10	178 ± 38 *4.0*

that in general PPAC are much more active than PACs. The most active derivative PPAC1 showed the activity similar to that obtained for the reference cytostatics Mitoxantrone and Doxorubicin. The cytotoxic activity was influenced by the kind of substituents at position 9 or 10 for PAC and PPAC, respectively. Non-substituted compounds (PAC1 and PPAC1) and their hydroxy analogues (PAC3 and PPAC3) were in general more active than methoxy or amino derivatives.

Both PAC and PPAC exhibited excellent activity towards the cell lines with induced multidrug cross-resistance of various types (P-gp, MRP-1, MRP/LRP), as opposed to the reference compounds used. The resistance indexes (RI) for the examined compounds were very low. However, the nature of the substituents considerably influenced the ability of the derivatives to overcome multidrug resistance and to differentiate between tumor cell lines with various types of drug exporting pumps. Non-substituted compounds (PAC1 and PPAC1) showed the most interesting properties: very high activity and ability to overcome multidrug resistance in all cases. The compounds with hydrophobic substituents (OCH_3: PAC2 and PPAC2) were optimal for the low resistance indexes for all types of protein pumps (Fig. 2). It should be stressed that discrimination between the P-gp and MRP type resistant cells could be achieved by appropriate substitution. A hydrophilic substitution (OH, NH_2) allows the retaining of the cytotoxic activity against MRP and MRP/LRP cell lines. However, it leads to decreased cytotoxicity towards the cell lines with P-gp dependent resistance (Fig. 2). We demonstrated earlier similar effects for other acridine derived compounds, pyrimidoacridinetrione PAT1 and its 9-hydroxy analogue PAT2 (Fig. 1) [6]. The cytotoxic activity of PAT1 towards several sensitive and resistant cell lines with various types of resistance was high

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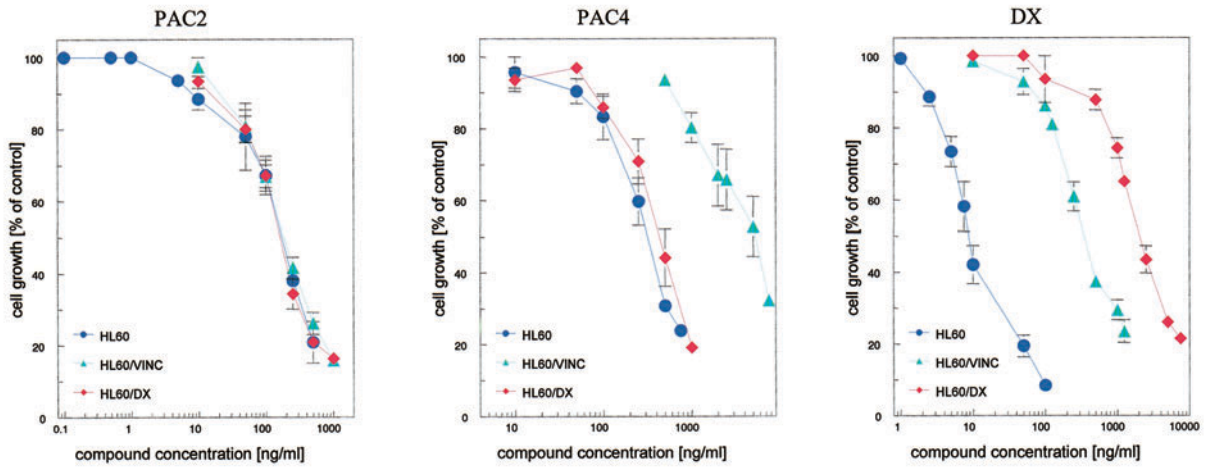

Figure 2. Dose/response curves for PAC2 (hydrophobic substituent), PAC4 (hydrophilic substituent) and Doxorubicin.

Human promyelocytic leukemia sensitive cell line HL60 and resistant sublines HL60/VINC (P-gp) and HL60/DX (MRP).

(comparable to those of Mitoxantrone and Doxorubicin) and the resistance indexes were low (1.4÷1.9). The hydroxy substituent influenced the ability of PAT2 to overcome multidrug resistance. The activity was retained only for the MRP/LRP cell line (RI = 2) and decreased (several fold) for all the lines with P-gp dependent resistance, although the activity against sensitive lines was similar to that for PAT1.

The obtained results corroborate our hypothesis on the essential role of heterocyclic ring(s) fused to the acridine moiety in the ability of acridine cytostatics to overcome multidrug resistance of tumor cells. Moreover, the presence and kind of the substituent considerably influenced the cytotoxic activity of the derivatives and their ability to overcome multidrug resistance. The substituents also seem to affect the differentiation between tumor cell lines with various types of drug exporting pumps.

We suggest that the ability of examined acridines to overcome the MRP type resistance results from the fact that they are poor substrates for transferases participating in the formation of anionic conjugates (the substrates of MRP pumps) or they cannot be co-transported with glutathione or glucuronate. The hydrophobic subtituent OCH_3 is optimal for low resistance indexes for P-gp and MRP dependent resistant cell lines.

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