Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 739–748
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pyrazolotriazolopyrimidine derivatives sensitize melanoma cells to the chemotherapic drugs: taxol and vindesine

Stefania Merighi[a,1], Prisco Mirandola[b,c,1], Katia Varani[a], Stefania Gessi[a], Silvano Capitani[b,d], Edward Leung[e], Pier Giovanni Baraldi[f], Mojgan Aghazadeh Tabrizi[f], Pier Andrea Borea[a,d,*]

[a]*Department of Clinical and Experimental Medicine, Pharmacology Unit, University of Ferrara, 44100 Ferrara, Italy*
[b]*Department of Morphology and Embryology, Institute of Human Anatomy, University of Ferrara, 44100 Ferrara, Italy*
[c]*Department of Human Anatomy, Pharmacology and Forensic Medicine, Institute of Normal Human Anatomy, Ospedale Maggiore, University of Parma 43100, Parma, Italy*
[d]*Centro Nazionale di Eccellenza per lo Sviluppo di Metodologie innovative per lo studio ed il trattamento delle patologie infiammatorie, University of Ferrara, 44100 Ferrara, Italy*
[e]*King Pharmaceuticals, 27513 Cary, NC, USA*
[f]*Department of Pharmaceutical Sciences, University of Ferrara, 44100 Ferrara, Italy*

Received 3 April 2003; accepted 15 May 2003

Abstract

In this study, we have evaluated the "*in vitro*" modulatory activity of a series of pyrazolotriazolopyrimidine derivatives (PTP-d) in sensitizing malignant melanoma cells to the chemotherapic drugs: taxol and vindesine. To that end, we have described the impact of chemotherapeutic agents on the cell cycle and on the induction of apoptosis when used alone or in combination with PTP-d. We have demonstrated that four PTP-d reduced chemotherapic drugs EC_{50} doses of the G_2/M accumulation with an average of 1.7-fold for taxol and 9.5-fold for vindesine when challenged on A375 human melanoma cell line. This sensitization activity was also confirmed by analyzing the apoptosis degree induced by the chemotherapic drugs. Interestingly, PTP-d had no effects on the response to cytotoxic agents by skin-derived human keratinocyte cells, NCTC 2544. Therefore, we have investigated the signaling pathway sustaining the sensitizing effect of PTP-d, providing functional evidence that active compounds are able to inhibit multidrug resistance-associated ATP-binding cassette drug transporter. These results suggested that PTP-d hold great promise for the treatment of multidrug resistance in cancers, leading to potential new therapies for melanoma.

Keywords: Adenosine; Keratinocytes; Melanoma; Pyrazolotriazolopyrimidine derivatives; Taxol; Vindesine

1. Introduction

The incidence of cutaneous melanoma has been rapidly increasing, with an estimate of 47,700 new cases diagnosed in 2000 in the United States [1]. Melanoma is cured in most cases by surgery, but once the metastatic phase develops, it is almost always fatal. Patients with metastatic melanoma survive an average of 4–6 months [2]. There have been

* Corresponding author. Tel.: +39-0532-291214; fax: +39-0532-291205.
E-mail address: bpa@dns.unife.it (P.A. Borea).
[1] These two authors contributed equally to this paper.

Abbreviations: DAPI, 4′,6′,-diamino-2-phenyl-indole; DMEM, Dulbecco's Modified Eagle's Medium; EC_{50}, dose exerting the 50% of the G_2/M arrest; EC_{MAX}, dose exerting the maximal apoptosis; EMEM, Minimum Essential Medium Eagle's with Earle's Salts; FACS, fluorescence-activated cell sorter; F_{MAX}, maximal fluorescence; F_{RES}, residual fluorescence; MDR, multidrug resistance; MRE 3008F20, 5-*N*-(4-methoxy-phenyl-carbamoyl)amino-8-propyl-2(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3042F20, 5-*N*-(4-sulphonyl-phenyl-carbamoyl)amino-8-methyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3046F20, 5-*N*-(4-methylphenyl-carbamoyl)amino-8-methyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3056F20, 5-*N*-(4-sulphonyl-phenyl-carbamoyl)amino-8-propyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3062F20, 5-*N*-(4-phenyl-carbamoyl)amino-8-butyl-2-(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3100F20, 5-*N*-(4-diethylamino-phenyl-carbamoyl)amino-8-methyl-(2(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5-*c*]pyrimidine); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Pgp, P-glycoprotein; PTP-d, pyrazolotriazolopyrimidine derivatives; Rh123, rhodamine 123; RNase, ribonuclease; SEC_{50}, the concentrations of PTP-d exerting the 50% of the sensitizing activity.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00400-3

relatively few new chemotherapeutic agents in the past several years that have demonstrated any activity in this disease. Taxol and vindesine have demonstrated antitumor activity in patients with melanoma [2,3]. Unfortunately, taxol and vindesine are subject to the problem of multidrug resistance (MDR), which results from the overexpression of the P-glycoprotein (Pgp). It is noteworthy that MDR is a common phenomenon in malignant melanoma [4]. As mechanisms of resistance are identified, new drugs may increase the sensitivity of melanoma to chemotherapeutic treatments.

Recently, there has been considerable interest in the involvement of adenosine in tumor priming and progression [5–8]. Adenosine can be released from a variety of cells throughout the body, as the result of increased metabolic rates, in concentrations that can have a profound impact on the vasculature, on immuno-escaping, and on the growth of tumor masses [9]. It is recognized that the concentrations of this nucleoside are increased in cancer tissues and in hypoxia [10,11].

Therefore, it is not surprising that adenosine has been shown to be a crucial factor in determining the cell progression pathway, either to apoptosis or a cytostatic state [12–19]. In particular, it has been demonstrated that adenosine may arrest keratinocyte proliferation [5].

Four different adenosine receptors have been identified and pharmacologically characterized: A_1, A_{2A}, A_{2B}, and A_3 [9]. In particular, activation of A_3 adenosine receptor blocks ultraviolet (UV)-irradiation-induced apoptosis [16], impairs cell proliferation, but also improves cell survival of human melanoma cells [7].

This has important consequences: it seems likely that the A_3 adenosine receptor subtype may be the target for the development of a new series of antitumoral drugs [20–23].

Thus, the goal of our work was to characterize the sensitizing activity to taxol and to vindesine by a series of selective A_3 receptor antagonists PTP-d. The results obtained with the human malignant melanoma cell line A375 have been compared with the human skin-derived keratinocyte cell line NCTC 2544. The aim was to gain insight into the direct potential role of A_3 adenosine receptor blockage in counteracting the occurrence of MDR, frequently observed in malignant skin-derived cells.

2. Materials and methods

2.1. Chemicals and reagents

A375 and NCTC 2544 cells were obtained from American Tissue Culture Collection (ATCC). Tissue culture media and growth supplements were obtained from Bio-Whittaker. Unless otherwise noted, all other chemicals were purchased from Sigma. MRE 3046F20, 5-*N*-(4-methylphenyl-carbamoyl)amino-8-methyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3100F20, 5-*N*-(4-diethylamino-phenyl-carbamoyl)amino-8-methyl-(2(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5-*c*]pyrimidine); MRE 3008F20, 5-*N*-(4-methoxy-phenyl-carbamoyl)-amino-8-propyl-2(2-furyl)-pyrazolo-[4,3*e*]1,2,4-triazolo[1,5*c*]pyrimidine, MRE 3042F20, 5-*N*-(4-sulphonyl-phenyl-carbamoyl)amino-8-methyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5*c*]pyrimidine; MRE 3056F20, 5-*N*-(4-sulphonyl-phenyl-carbamoyl)amino-8-propyl-2(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRE 3062F20, 5-*N*-(4-phenyl-carbamoyl)amino-8-butyl-2-(2-furyl)-pyrazolo-[4,3*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine were synthesized by Prof. P.G. Baraldi, University of Ferrara, Italy. Ribonuclease (RNAse) was purchased from Boehringer.

2.2. Cell culture

Cells were maintained in DMEM (A375) or EMEM (NCTC 2544) medium containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM).

2.3. Colony formation assay

Exponentially growing A375 cells were seeded at 300 cells per well in 6-well plates and treated with taxol and A_3 adenosine receptor antagonists. After 7 days of growth the cells were fixed and stained with 1/10 Giemsa/PBS. Colonies of greater than 30 cells were scored as survivors. For each treatment, six individual wells were scored.

2.4. Morphological analysis

To recover all seeded cells, the adherent culture fraction was trypsinized and mixed with the supernatant fraction. Then, the cell suspension was spun to a slide fixed, permeabilized, and stained with DAPI [7]. Slides were observed on Zeiss Axiophot fluorescent microscope.

2.5. Flow cytometry analysis

Cells were permeabilized in 70% (v/v) ethanol/PBS and stained with a PBS solution containing 20 μg/mL propidium iodide and 100 μg/mL RNAse and then analyzed by FACS (Becton-Dickinson). The content of DNA was evaluated by the Cell-LISYS program (Becton-Dickinson). Cell distribution among cell cycle phases and apoptotic cells were evaluated as previously described [7].

2.6. Rhodamine 123 (Rh123) efflux assay

Cells were loaded with 50 ng/mL Rh123 for 30 min at 37°. The cells were washed and resuspended in dye-staining agent-free medium for 3 hr at 37° to allow Rh123 efflux. The fluorescence of Rh123 was analyzed by flow

cytometer (residual fluorescence (F_{RES})). The fluorescence was compared with Rh123-loaded cells maintained at 4° to prevent drug export (maximal fluorescence (F_{MAX})). To evaluate the ability of PTP-d to interfere with drug efflux activity of MDR-associated ATP-binding cassette drug transporters, Rh123-loaded cells were resuspended in dye-staining agent-free medium in the presence of PTP-d (10 μM). Cells from each cell line that had not been exposed to Rh123 were used as negative controls.

2.7. ATPLite assay

The intracellular ATP concentration was determined with a luminescent ATP detection kit (ATPLite-M by Packard) according to the manufacturer's directions. Light units generated by ATP in each sample were normalized to control (solution with known ATP concentration) and expressed as the absolute ATP levels.

2.8. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The number of living cells was determined evaluating the mitochondrial dehydrogenase activity by using MTT, as previously described [7]. All individual drug concentrations were done in four separate wells, and each experiment repeated three times.

2.9. Statistical analysis

All values in the figures and text are expressed as mean ± SD of N observation (with $N \geq 3$). Data sets were examined by ANOVA and Dunnett's test (when required). A P value less than 0.05 was considered statistically significant. Representative images obtained by FACS and by fluorescent microscope observation are reported, with similar results having been obtained in at least three different experiments.

Dose–response curves of cell cycle alteration were analyzed with the nonlinear least-squares curve fitting program Prism (GraphPAD). The G_2/M phase accumulations were fitted with sigmoidal dose–response function obtaining the dose exerting the 50% of the G_2/M arrest (EC_{50} and SEC_{50}). The sub-G_1 (apoptosis) accumulation curves were fitted with Gaussian distribution obtaining the dose exerting the maximal apoptosis (EC_{MAX}) by mean value.

3. Results

At first, we evaluated whether the A_3 adenosine receptor antagonist MRE 3008F20 [24] could enhance the toxic effect of a conventional chemotherapic drug. We performed colony formation assay experiments on A375 melanoma cells treated with increased concentrations of taxol (0.75, 2.5, and 7.5 ng/mL).

Taxol at 0.75 ng/mL reduced the number of colonies (64 ± 3% respect to untreated cells), whereas at 2.5 ng/mL it abrogated the colony formation ability of A375 cells. MRE 3008F20 showed low toxicity at 0.1 and 1 μM, reducing the number of colonies by 13 ± 5% and 20 ± 4%, respectively. When 0.1 μM MRE 3008F20 was assayed with taxol at 0.75 ng/mL, we observed a strong reduction of the number of the colonies (29 ± 5% respect to untreated cells); furthermore, MRE 3008F20 at 1 μM completely abolished the colony formation ability of A375 cells simultaneously treated with 0.75 ng/mL taxol (Fig. 1).

Prompted by these observations, we performed an acute treatment of A375 cells with MRE 3008F20 in combination with antimitotic drugs. After 24 hr, we observed that the antimitotic drug taxol (25 ng/mL) induced a profound modification of the nuclei morphology of A375 cells. The nuclei of treated cells were irregular, fragmented, and the chromatin was often condensed (Fig. 2B). When taxol and MRE 3008F20 (10 μM) were used in combination, we observed an increased number of cells in mitosis (Fig. 2C).

To better quantify the amount of cells blocked in G_2/M phases, we performed flow cytometer analysis.

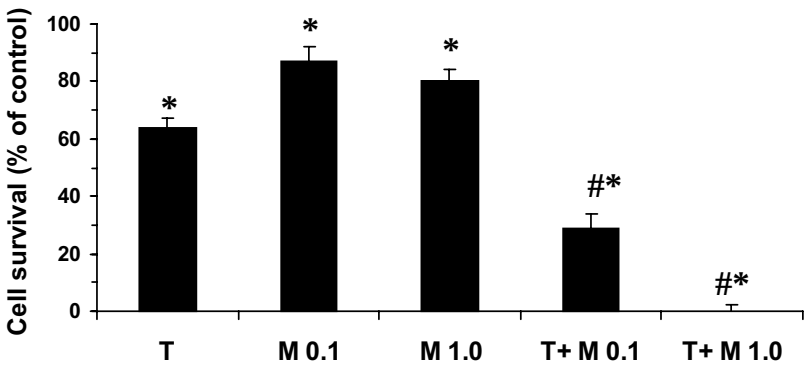

Fig. 1. Colony formation assay of A375 cells. The values represent the mean ± SEM of four independent experiments. T: taxol, 0.75 ng/mL; M0.1: MRE 3008F20, 0.1 μM; M1.0: MRE 3008F20, 1.0 μM; T + M0.1: taxol plus MRE 3008F20, 0.1 μM; T + M1.0: taxol plus MRE 3008F20, 1.0 μM treated cells. $^{*}P < 0.01$ vs. control DMSO-treated cells; $^{\#}P < 0.01$ vs. taxol-treated cells. Analysis was by ANOVA followed by Dunnett's test.

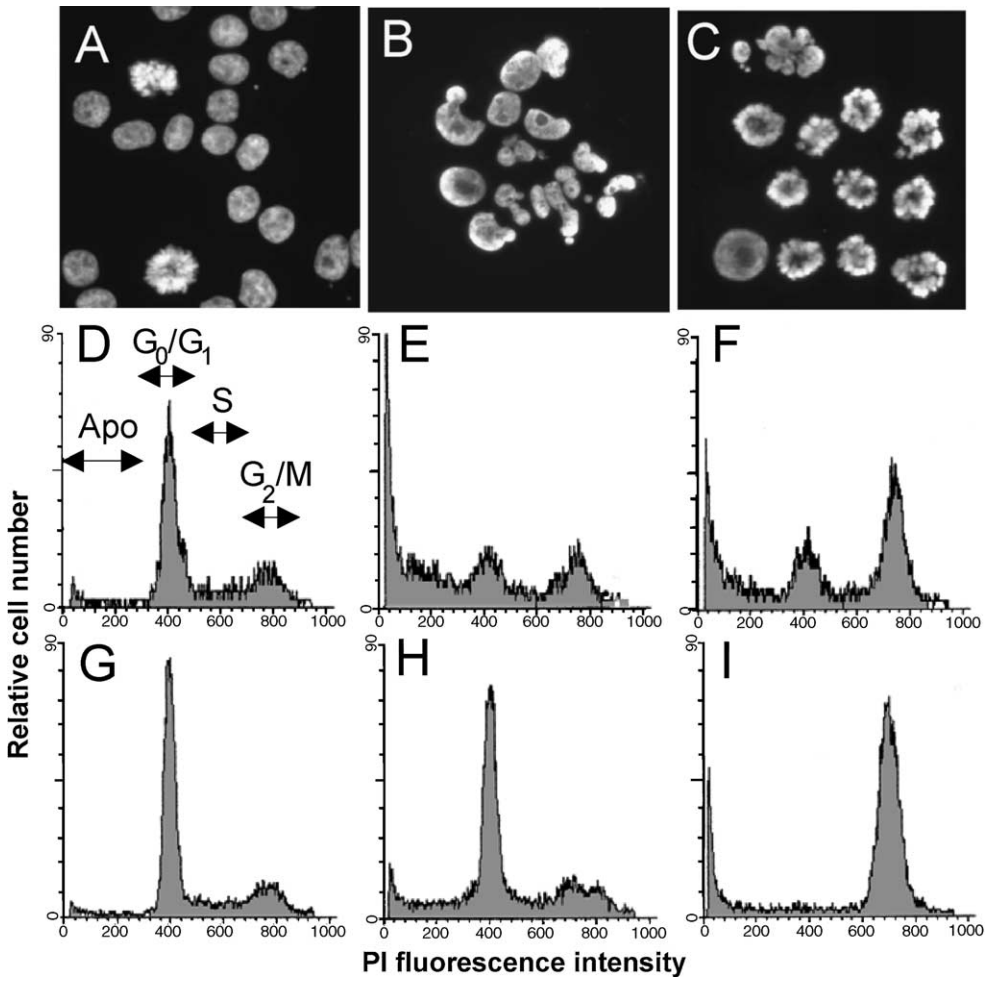

Fig. 2. Cytospun of A375 cells treated with DMSO (A), taxol, 25 ng/mL (B); taxol, 25 ng/mL plus MRE 3008F20, 10 μM (C). Flow cytometry analysis of A375 cells treated with drug vehicle as control (D and G) or with vindesine, 1 nM (H) alone; taxol, 25 ng/mL (E) alone. Cells were treated with vindesine plus MRE, 3008F20 10 μM (I) or with taxol plus MRE 3008F20, 10 μM (F).

Taxol (25 ng/mL) reduced the percentage of cells in G_0/G_1 phases and increased the number of cells in G_2/M phases (Fig. 2E), whereas 1 nM vindesine did not alter significantly cell proliferation if compared with drug vehicle-treated cells (Fig. 2H and G, respectively). When MRE 3008F20 was used in combination with taxol (Fig. 2F) or with vindesine (Fig. 2I), A375 cells accumulated in G_2/M phases.

We analyzed the sensitivity of the A375 cell line to taxol and vindesine. At 24 hr post-exposure, taxol and vindesine induced a dose-dependent cell accumulation into G_2/M cell cycle phases (Fig. 3A and C, empty symbols) and a parallel decrease of the G_0/G_1 population (data not shown). We found that the concentration exerting the 50% of the G_2/M accumulation (EC_{50}) was 16.60 ± 2.00 ng/mL and 1.90 ± 0.20 nM for taxol and vindesine, respectively. Furthermore, our analyses showed that EC_{50} values of taxol and vindesine for the decrease of G_0/G_1 population were not significantly changed with respect to EC_{50} values of G_2/M arrest. The S-phase population, representative of replicating DNA, was uninformative because it was not appreciably changed. In A375 cells, the percentage of apoptotic cells increased progressively with taxol concentration, reaching the maximum value (ranging from 35 to 53% of total culture, on different experiments) at 6 ng/mL (Fig. 3B, empty symbols). An increase of the taxol concentration resulted in a decrease of A375 cells at sub-G1. The taxol concentration exerting the maximal apoptosis (EC_{MAX}) was 6.00 ± 0.63 ng/mL. A similar analysis was performed with vindesine obtaining EC_{MAX} value of 3.54 ± 0.42 nM.

After this preliminary analysis A375 cells were treated with vindesine and taxol in the presence of 10 μM MRE 3008F20 (Fig. 3A and C). MRE 3008F20 reduced taxol and vindesine EC_{50} values by 1.9- and 4.0-fold, respectively. Similar results were obtained analyzing EC_{50} values calculated for the G_0/G_1 population (data not shown).

Furthermore, MRE 3008F20 (10 μM) reduced EC_{MAX} value by 2.0- and 2.1-fold, for taxol and vindesine, respectively (Fig. 3B and D).

We quantified the ability of other PTP-d (MRE 3062F20, MRE 3046F20, MRE 3100F20, MRE 3042F20, and MRE

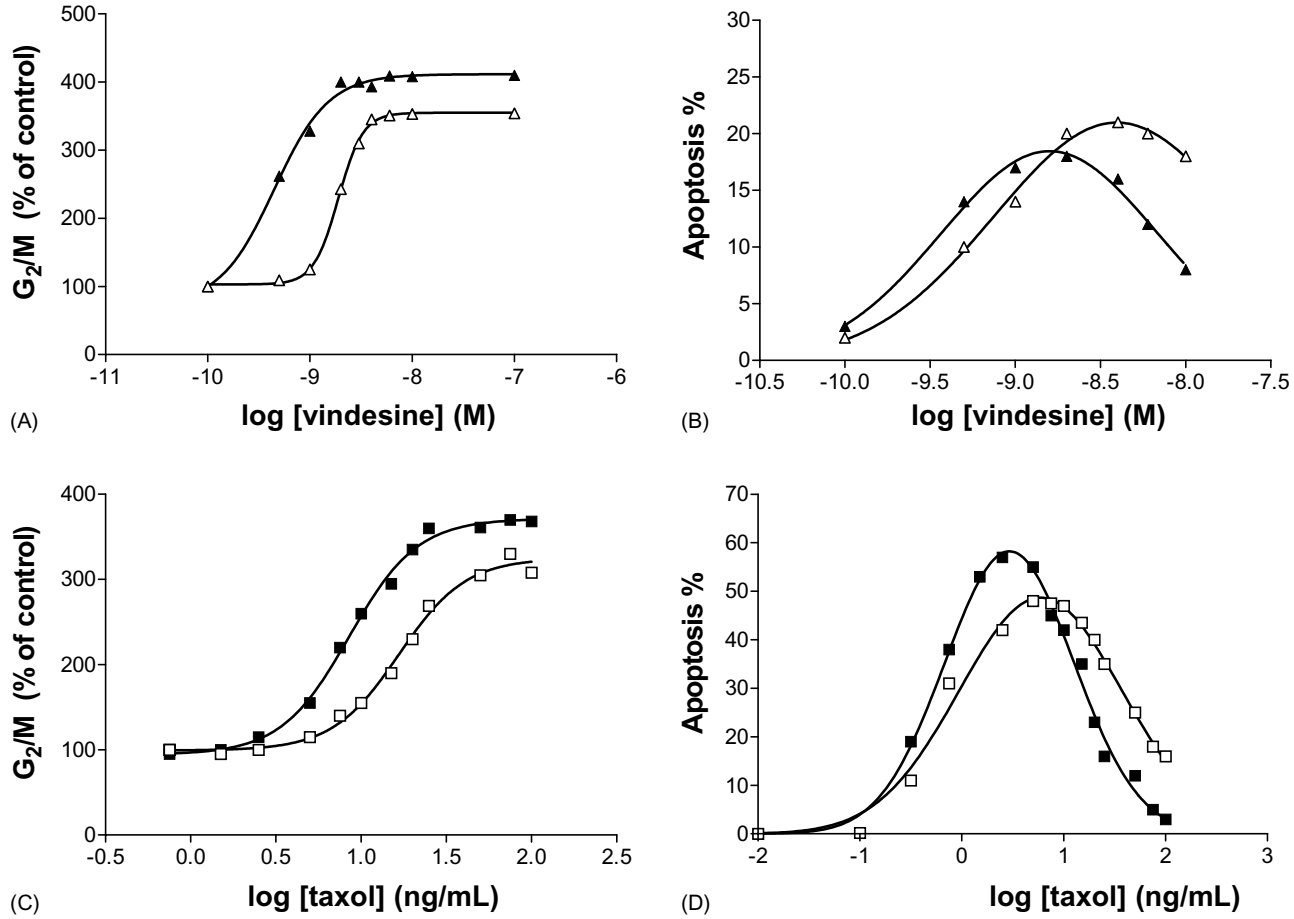

Fig. 3. Typical dose–response curve of A375 cells exposed to vindesine (A and B) and taxol (C and D) alone (open symbols) or in the presence of 10 μM MRE 3008F20 (filled symbols). Panels A and C show the number of cells arrested in G_2/M phases calculated as percentage of untreated cells (control), panels B and D the accumulation (% of total living cells) of the sub-G_1 (apoptosis) population.

3056F20) [25] to sensitize A375 cells to vindesine and taxol (Table 1A and B). We verified whether the sensitizing activity observed by malignant melanoma cells was reproducible in the principal epidermis cytotype: the human keratinocyte.

For this, we chose human normal keratinocytic cell line NCTC 2544, having a pattern of surface A_3 adenosine receptor expression similar to the A375 cell line. In particular, we have previously demonstrated that the density of A_3 receptors is 291 ± 50 fmol/mg and

Table 1A
Induction of G_2/M accumulation of A375 and NCTC 2544 cells by taxol and vindesine with or without PTP-d and verapamil treatment

	A375		NCTC 2544	
	Taxol (EC_{50})[a]	Vindesine (EC_{50})[b]	Taxol (EC_{50})[a]	Vindesine (EC_{50})[b]
DMSO	16.60 ± 2.00	1.90 ± 0.20	2.69 ± 0.28	0.89 ± 0.08
MRE 3008F20	8.60 ± 0.81[*]	0.48 ± 0.05[#]	2.41 ± 0.30	0.70 ± 0.06
MRE 3062F20	8.26 ± 0.75[*]	0.10 ± 0.02[#]	2.50 ± 0.31	0.72 ± 0.06
MRE 3046F20	11.71 ± 1.15[*]	0.39 ± 0.03[#]	2.63 ± 0.28	0.60 ± 0.07
MRE 3100F20	10.27 ± 0.94[*]	0.19 ± 0.02[#]	2.72 ± 0.26	0.80 ± 0.07
MRE 3042F20	17.01 ± 1.54	1.80 ± 0.20	2.69 ± 0.24	0.82 ± 0.09
MRE 3056F20	16.30 ± 2.47	1.95 ± 0.21	2.41 ± 0.29	0.76 ± 0.07
Verapamil	7.50 ± 0.94[*]	0.49 ± 0.04[#]	2.50 ± 0.30	0.73 ± 0.08

Values are expressed as mean ± SE of four independent experiments. PTP-d and verapamil were used at 10 μM. EC_{50} values were obtained by analyzing G_2/M accumulation dose–response curve.

[a] Values expressed as ng/mL of taxol.

[b] Values expressed as nM of vindesine.

[*] $P < 0.01$ vs. taxol DMSO; analysis by ANOVA followed by Dunnett's test.

[#] $P < 0.01$ vs. vindesine DMSO; analysis by ANOVA followed by Dunnett's test.

Table 1B
Induction of apoptosis of A375 and NCTC 2544 cells by taxol and vindesine with or without PTP-d and verapamil treatment

	A375		NCTC 2544	
	Taxol (EC_{MAX})[a]	Vindesine (EC_{MAX})[b]	Taxol (EC_{MAX})[a]	Vindesine (EC_{MAX})[b]
DMSO	6.00 ± 0.63	3.54 ± 0.42	2.69 ± 0.23	2.50 ± 0.27
MRE 3008F20	3.04 ± 0.32[*]	1.66 ± 0.20[#]	2.55 ± 0.24	2.34 ± 0.25
MRE 3062F20	3.28 ± 0.34[*]	2.04 ± 0.21[#]	2.57 ± 0.27	2.48 ± 0.15
MRE 3046F20	4.32 ± 0.45[*]	1.63 ± 0.18[#]	2.65 ± 0.21	2.53 ± 0.14
MRE 3100F20	4.10 ± 0.47[*]	1.99 ± 0.17[#]	2.43 ± 0.29	2.39 ± 0.21
MRE 3042F20	6.45 ± 0.78	3.33 ± 0.34	2.56 ± 0.23	2.00 ± 0.23
MRE 3056F20	6.30 ± 0.81	3.60 ± 0.37	2.48 ± 0.24	2.37 ± 0.24
Verapamil	3.51 ± 0.29[*]	2.29 ± 0.34[#]	2.70 ± 0.21	2.44 ± 0.20

Values are expressed as mean ± SE of four independent experiments. PTP-d and verapamil were used at 10 μM. EC_{MAX} values were obtained by analyzing sub-G_1 accumulation dose–response curve.
[a] Values expressed as ng/mL of taxol.
[b] Values expressed as nM of vindesine.
[*] $P < 0.01$ vs. taxol DMSO; analysis by ANOVA followed by Dunnett's test.
[#] $P < 0.01$ vs. vindesine DMSO; analysis by ANOVA followed by Dunnett's test.

337 ± 26 fmol/mg of protein on A375 and NCTC 2544[2] cell lines, respectively [26].

We identified the EC_{50} values of vindesine- and taxol-mediated cell cycle distribution alteration (G_2/M accumulation) under treatment with the PTP-d series or with drug vehicle (Table 1A). The values of EC_{MAX} were also reported for each cell line in the Table 1B.

On one hand, in A375 cells, MRE 3042F20 and MRE 3056F20 did not enhance taxol and vindesine antiproliferative and apoptotic activity, while MRE 3008F20, MRE 3062F20, MRE 3046F20, and MRE 3100F20 reduced EC_{50} and EC_{MAX} values with similar efficacy.

On the other hand, NCTC 2544, despite higher sensitivity to antimitotic drugs than melanoma cells did not show any increased sensitivity by PTP-d treatment. To better investigate the ability of PTP-d to sensitize malignant melanoma cells to taxol and vindesine, A375 cells were treated with vindesine (1 nM) with increasing concentrations of PTP-d. The rank order of potency was: MRE 3062F20 > MRE 3008F20 > MRE 3046F20 > MRE 31-SEC_{50}) were reported in Table 2. Surprisingly, SEC_{50} values did not agree with inhibitory equilibrium binding constant (K_i) of PTP-d observed in binding experiments for the adenosine A_3 receptor [25,26]. These experimental evidences suggested the involvement of a cell-specific factor that was present in A375 while absent in NCTC 2544 cells. Due to the occurrence of MDR in melanoma, in order to evaluate the involvement of MDR-associated ATP-binding cassette drug transporter, A375 and NCTC 2544 cells were treated with verapamil, a well-known MDR phenotype reverter and a Pgp inhibitor [27]. As shown in Table 1A and B verapamil reduced the EC_{50} and EC_{MAX} doses of chemotherapic drugs in A375 cell line but not in NCTC 2544. Therefore, we have evaluated the involvement of Pgp-like activity by Rh123 retention assay: a functional assay for MDR-associated ATP-binding cassette drug transporter. A375 cells, loaded with Rh123 and then cultured in Rh123-free medium, show to be composed by two populations characterized by a F_{RES} having a mean fluorescence intensity lower than F_{MAX} (Fig. 4A, black filled area). The population with the lowest fluorescence, accounting for 30 ± 5% of total cells, represents the cells expressing functional MDR-associated ATP-binding cassette drug transporter and having low intracellular level of Rh123 (Fig. 4A, gate i). On the contrary, NCTC 2544 cells show a F_{RES} chromatogram comparable to F_{MAX} (Fig. 4B, black filled area and gray filled area, respectively), consistent to the absence or undetectable level of drug pumping activity in these cells.

To ensure that the Rh123 efflux observed in A375 cells was mediated by the Pgp-like pumping activity and not by other transporters, we performed a new set of Rh123 retention experiments in the presence of probenecid (1 mM), inhibitor of MRP [28] and verapamil as specific Pgp inhibitor. We found that probenecid did not inhibit Rh123 efflux (Fig. 4D) while verapamil abrogated Rh123

Table 2
Adenosine receptor antagonist parameters of sensitizing activity to vindesine and binding affinity to A_3 adenosine receptor in A375 cells

	SEC_{50} (μM)	K_i (nM)
MRE 3008F20	0.55 ± 0.06	3.1 ± 0.3[a]
MRE 3062F20	0.43 ± 0.04	2.3 ± 0.2[a]
MRE 3046F20	0.57 ± 0.06	3.0 ± 0.3[a]
MRE 3100F20	0.97 ± 0.10	30.0 ± 2.5[b]
MRE 3042F20	>10	25.0 ± 3.0[b]
MRE 3056F20	>10	30.0 ± 3.1[b]

Values are expressed as mean ± SE of four independent experiments. SEC_{50}: adenosine receptor antagonist dose that induces the 50% of the sensitizing activity to vindesine, calculated on G_2/M accumulation dose–response curve. K_i: equilibrium constant of binding affinity at human A_3 adenosine receptor.
[a] Merighi *et al.* [26].
[b] Baraldi and Borea [25].

[2] Personal communication.

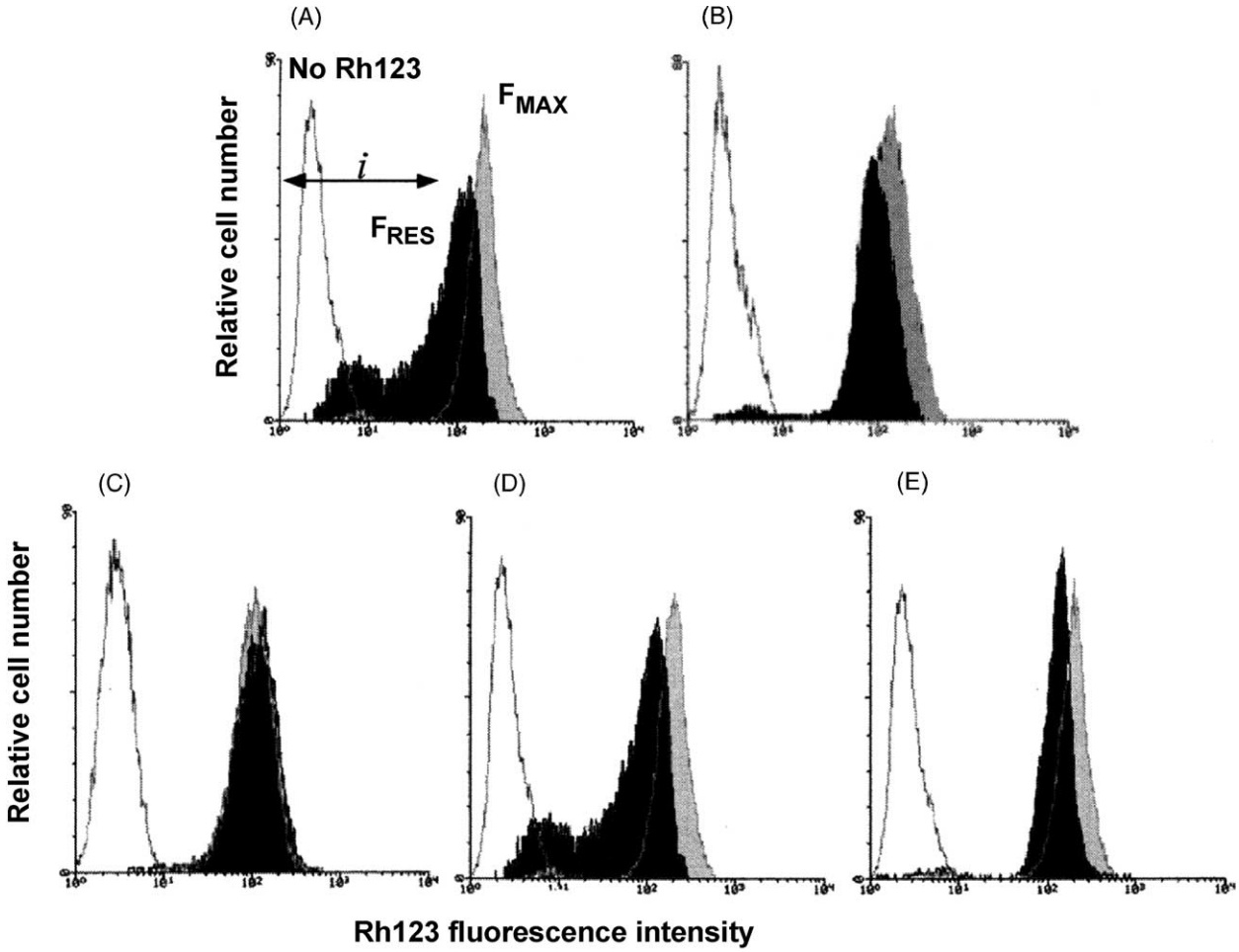

Fig. 4. Representative flow chromatograms of Rh123 accumulation by A375 cells (A and C–E) and NCTC 2544 cells (B) in the presence of verapamil, 10 μM (C); MRE 3008F20, 10 μM (E); probenecid, 1 mM (D); or drug vehicle (A and B). F_{MAX}: gray filled area; F_{RES}: black filled area. Rh123 unstained cell chromatogram is reported as unfilled area. Cells with active Rh123 efflux were scored by gate "i".

efflux (Fig. 4C), suggesting that Rh123 exits from A375 cells *via* Pgp-like mediated transport.

We report that PTP-d were able to inhibit Pgp-like mediated Rh123 efflux. In the presence of MRE 3008F20, Rh123 yielded a F_{RES} chromatogram comparable to F_{MAX} (Fig. 4E, black filled area and gray filled area, respectively), consistent with a complete blockage of Pgp-like mediated Rh123 transport in A375 cell line.

Table 3 summarizes the percentage of cells expressing Pgp-like activity (% of Rh123 negative cells) in the presence of adenosine receptor antagonists. We observed that MRE 3100F20, MRE 3008F20, MRE 3062F20, and MRE 3046F20 are strong inhibitors of Pgp activity, while MRE 3042F20 and MRE 3056F20 did not interfere with drug efflux. These data strongly suggest the existence of a structure–activity relationship for the inhibition of Pgp-like drug expulsion activity mediated by adenosine receptor antagonists.

To further investigate the role of PTP-d in modulating the Pgp-like transport of chemotherapic drugs, we measured the intracellular ATP content of A375 cells (12 ± 1 nmol/10^6 cells). We found that PTP-d did not modify the intracellular concentration of ATP (10 ± 2 nmol/10^6 cells, 10 μM for 24 hr).

To exclude that Rh123 efflux could be consequent to strong cell vitality reduction, we evaluated the toxicity of PTP-d by MTT assay. No significant reduction of cell viability was found under PTP-d (up to 10 μM for 24 hr) treatment.

Table 3
Rhodamine 123 efflux (Rh123) assay in A375 cells in the presence and absence of PTP-d and verapamil

Treatment	Concentration (μM)	% of Rh123 negative cells
DMSO		30 ± 5
MRE 3100F20	10	0.4 ± 0.5*
MRE 3008F20	10	0.5 ± 0.5*
MRE 3062F20	10	1.0 ± 0.5*
MRE 3046F20	10	7 ± 2*
MRE 3042F20	10	31 ± 3
MRE 3056F20	10	28 ± 4
Verapamil	10	0.2 ± 1.0*

Values are expressed as mean ± SE of four independent experiments.
* $P < 0.01$ vs. DMSO; analysis by ANOVA followed by Dunnett's test.

4. Discussion

This is the first study to show that various ligands of the A_3 adenosine receptor (pyrazolo[4,3*e*]1,2,4-triazolo[1,5-*c*]pyrimidine derivatives) exert sensitizing activity to taxol and vindesine in human melanoma A375 cell line. Conversely, this effect has not been observed in normal keratinocytic NCTC 2544 cell line. Furthermore, sensitizing enhancement by active PTP-d was only observed at a concentration about 1000-fold higher than that required for their specific binding to the high-affinity A_3 receptor [25,26], thus questioning the relationship between the sensitizing effect of active PTP-d and their receptor binding. Moreover, no sensitizing activity to taxol and vindesine by MRE 3042F20 and MRE 3056F20 (PTP-d with high affinity to A_3 adenosine receptor) was found. This would emphasize that A_3 adenosine receptor expression is not a prerequisite for PTP-d sensitizing activity.

We have characterized, for the first time, the sensitivity of melanoma and keratinocytic cell lines to taxol and to vindesine analyzing the apoptotic degree (EC_{MAX}) and the alteration of cell distribution among the different cell cycle phases (EC_{50}). Taxol and vindesine are microtubule interfering agents that cause apoptosis and alter cell proliferation arresting cell progression into G_2/M cell cycle phases.

We have demonstrated that four PTP-d reduced chemotherapic drugs EC_{50} doses of the G_2/M accumulation with an average of 1.7-fold for taxol and 9.5-fold for vindesine when challenged on A375 cell line. This sensitization activity was also confirmed by analyzing the degree of apoptosis induced by the chemotherapic drugs. In contrast, sensitizing activity of PTP-d was completely absent in NCTC 2544 cells.

Furthermore, we have demonstrated that the presence of MDR-associated ATP-binding cassette drug transporter activity is necessary to assure the PTP-d-induced sensitization to taxol and to vindesine. Previous studies reported that melanoma cell line expresses functional Pgp [4,29,30]. Similarly, in our work, A375 cells produced a drug efflux activity, whereas in NCTC 2544 cells we were unable to demonstrate any Rh123 efflux. The use of the MRP inhibitor probenecid (1 mM) in Rh123 experiments confirmed that Rh123 efflux was mediated by Pgp-like pump. Treatment with a reverter agent (verapamil) showed the importance of an ATP-dependent drug transporter in inducing resistance to taxol and vindesine in A375 cell line. Furthermore, these findings underline the ''reverter-like'' behavior of the active PTP-d. Finally, different expression of MDR-associated ATP-binding cassette drug transporter activity in A375 and NCTC 2544 cell lines may explain the inability of verapamil and PTP-d to sensitize to chemotherapic drugs NCTC 2544 cells lacking Pgp-like expression.

To further investigate the role of PTP-d in modulating transport of chemotherapic drugs, we measured the intracellular ATP content and cell viability under PTP-d treatment. We found that PTP-d did not modify the intracellular concentration of ATP. Consequently, we can exclude that PTP-d block Pgp-like activity by reducing the energetic intracellular stores necessary for active drug efflux. In view of the documented evidences suggesting that allosteric regulatory sites are distinct from transport sites on Pgp [31], we hypothesize that active PTP-d may be able to interact with a binding site on MDR-associated ATP-binding cassette drug transporter as modulators or substrates. Additionally, active PTP-d may be able to control ATP hydrolysis produced during the drug transport.

Further studies are required to underscore the nature of the interaction of PTP-d with MDR-associated ATP-binding cassette drug transporter and to characterize their potential sites of binding.

In contrast to the four active PTP-d, MRE 3042F20 and MRE 3056F20 were unable to inhibit Pgp-like activity in A375 cells. These findings suggest the existence of a structure–activity relationship of PTP-d interaction with MDR-associated ATP-binding cassette drug transporter. It is not an easy task to rationalize the data collected in the present work into sound and consistent structure–activity relationships. Nevertheless, some useful preliminary conclusions can be extracted from the limited series of compounds studied. First, we observed that the presence of a phenyl-carbamoyl-amino derivative on N5 of the 2-furyl-pyrazolo-triazolo-pyrimidine basal structure is a common feature of this series of PTP-d: no conclusion can be drawn about this substituent *per se*, since it is an invariant feature in all compounds tested. Second, we underline that MRE 3042F20 and MRE 3056F20 have a SO_3H substituent that confers to them a higher hydrophilic character with respect to active PTP-d (Fig. 5). Accordingly, it has been reported that lipophilicity is one of the common features of MDR reverting agents [32,33]. Nevertheless, there is no evidence to decide whether the detrimental effect of MRE 3042F20 and MRE 3056F20 is due to hydrophilicity as such or results from an unfavorable pharmacophoric contribution of an anionic group such as SO_3^-.

These observations can be useful in the design of more potent PTP-d molecules able to block MDR-associated ATP-binding cassette drug transporters.

Although in this study, we have performed experiments of PTP-d and antitumor drug co-treatment in human tumor and normal cell lines ''*in vitro*'', and there may be important differences with ''*in vivo*'' system, our data strengthen the notion that PTP-d may represent a potential new class of pharmacologic drugs, improving conventional anticancer therapies in patients affected by skin-derived cell malignancies. Moreover, we have provided a molecular mechanism to explain how PTP-d can sensitize melanoma cells to conventional chemotherapic drugs, such as taxane, and ''Vinca'' alkaloid derivatives by impairing Pgp-like activity. If our present findings are confirmed with ''*in vivo*'' studies, they might suggest that a combination of PTP-d with chemotherapic drugs would be beneficial to

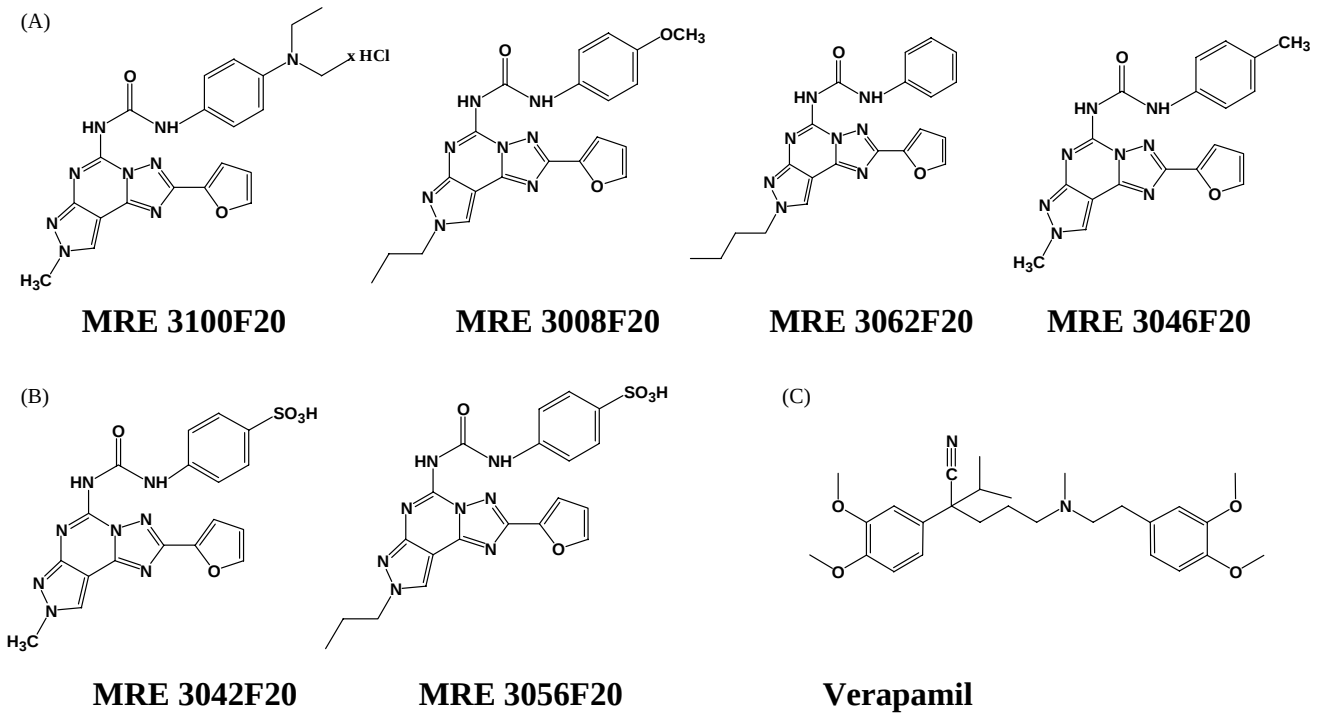

Fig. 5. Chemical structures of the compounds tested in this study. (A) Pyrazolotriazolopyrimidine derivatives found to enhance chemotherapic drugs. (B) Pyrazolotriazolopyrimidine derivatives that did not enhance chemotherapic drugs. (C) Verapamil.

counteract the occurrence of MDR, frequently observed in melanoma.

Acknowledgments

The authors are grateful to Dr. Kristi Bemis (Institute of Human Virology, University of Maryland, Baltimore, MD) for reviewing the manuscript.

References

[1] Bajetta E, Del Vecchio M, Bernard-Marty C. Metastatic melanoma: chemotherapy. Semin Oncol 2002;29:427–45.
[2] Feun LG, Savaraj N, Hurley J, Marini A, Lai S. A clinical trial of intravenous vinorelbine tartrate plus tamoxifen in the treatment of patients with advanced malignant melanoma. Cancer 2000;88:584–8.
[3] Nathan FE, Berd D, Sato T, Mastrangelo MJ. Paclitaxel and tamoxifen: an active regimen for patients with metastatic melanoma. Cancer 2000;88:79–87.
[4] Molinari A, Calcabrini A, Meschini S, Stringaro A, Del Bufalo D, Cianfriglia M, Arancia G. Detection of P-glycoprotein in the Golgi apparatus of drug-untreated human melanoma cells. Int J Cancer 1998;75:885–93.
[5] Brown JR, Cornell K, Cook PW. Adenosine- and adenine-nucleotide-mediated inhibition of normal and transformed keratinocyte proliferation is dependent upon dipyridamole-sensitive adenosine transport. J Invest Dermatol 2000;115:849–59.
[6] Ohana G, Bar-Yehuda S, Barer F, Fishman P. Differential effect of adenosine on tumor and normal cell growth: focus on the A_3 adenosine receptor. J Cell Physiol 2001;186:19–23.
[7] Merighi S, Mirandola P, Milani D, Varani K, Gessi S, Klotz KN, Leung E, Baraldi PG, Borea PA. Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. J Invest Dermatol 2002;119:923–33.
[8] Fishman P, Bar-Yehuda S. Pharmacology and therapeutic applications of A_3 receptor subtype. Curr Top Med Chem 2003;3:463–9.
[9] Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.
[10] Blay J, White TD, Hoskin DW. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. Cancer Res 1997;57:2602–5.
[11] Barry CP, Lind SE. Adenosine-mediated killing of cultured epithelial cancer cells. Cancer Res 2000;60:1887–94.
[12] Kohno Y, Sei Y, Koshiba M, Kim HO, Jacobson KA. Induction of apoptosis in HL-60 human promyelocytic leukemia cells by adenosine A_3 receptor agonists. Biochem Biophys Res Commun 1996;219:904–10.
[13] Yao Y, Sei Y, Abbracchio MP, Jiang JL, Kim YC, Jacobson K. Adenosine A_3 receptor agonists protect HL60 and U-937 cells from apoptosis induced by A_3 antagonists. Biochem Biophys Res Commun 1997;232:317–22.
[14] Brambilla R, Cattabeni F, Ceruti S, Barbieri D, Franceschi C, Kim YC, Jacobson KA, Klotz KN, Lohse MJ, Abbracchio MP. Activation of the A_3 adenosine receptor affects cell cycle progression and cell growth. Naunyn Schmiedebergs Arch Pharmacol 2000;361:225–34.
[15] Shneyvays V, Jacobson KA, Li AH, Nawrath H, Zinman T, Isaac A, Shainberg A. Induction of apoptosis in rat cardiocytes by A_3 adenosine receptor activation and its suppression by isoproterenol. Exp Cell Res 2000;257:111–26.
[16] Gao Z, Li BS, Day YJ, Linden J. A_3 adenosine receptor activation triggers phosphorylation of protein kinase B and protects rat basophilic leukemia 2H3 mast cells from apoptosis. Mol Pharmacol 2001;59:76–82.
[17] Schrier SM, van Tilburg EW, van der Meulen H, Ijzerman AP, Mulder GJ, Nagelkerke JF. Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells: studies on involvement of adenosine

receptors and adenosine uptake. Biochem Pharmacol 2001;61:417–25.

[18] Fishman P, Bar-Yehuda S, Vagman L. Adenosine and other low molecular weight factors released by muscle cells inhibit tumor cell growth. Cancer Res 1998;58:3181–7.

[19] Fishman P, Madi L, Bar-Yehuda S, Barer F, Del Valle L, Khalili K. Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. Oncogene 2002;21:4060–4.

[20] Fishman P, Bar-Yehuda S, Barer F, Madi L, Multani AS, Pathak S. The A_3 adenosine receptor as a new target for cancer therapy and chemoprotection. Exp Cell Res 2001;269:230–6.

[21] Gessi S, Varani K, Merighi S, Morelli A, Ferrari D, Leung E, Baraldi PG, Spalluto S, Borea PA. Pharmacological and biochemical characterization of A_3 adenosine receptors in Jurkat T cells. Br J Pharmacol 2001;134:116–26.

[22] Merighi S, Baraldi PG, Gessi S, Iannotta V, Klotz KN, Leung E, Mirandola P, Tabrizi MA, Varani K, Borea PA. Adenosine receptors and human melanoma. Drug Dev Res 2003;58:377–85.

[23] Merighi S, Mirandola P, Varani K, Gessi S, Leung E, Baraldi PG, Tabrizi MA, Borea PA. A glance at adenosine receptors: novel target for antitumor therapy. Pharmacol Ther, in press.

[24] Varani K, Merighi S, Gessi S, Klotz KN, Leung E, Baraldi PG, Cacciari B, Romagnoli R, Spalluto G, Borea PA. [^{3}H]MRE 3008F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A_3 adenosine receptors. Mol Pharmacol 2000;57:968–75.

[25] Baraldi PG, Borea PA. New potent and selective human adenosine A_3 receptor antagonists. Trends Pharmacol Sci 2000;21:456–9.

[26] Merighi S, Varani K, Gessi S, Cattabriga E, Iannotta V, Uluoglu C, Leung E, Borea PA. Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. Br J Pharmacol 2001;134:1215–26.

[27] Wiese M, Pajeva IK. Structure–activity relationships of multidrug resistance reversers. Curr Med Chem 2001;8:685–713.

[28] Bakos E, Evers R, Sinko E, Varadi A, Borst P, Sarkadi B. Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. Mol Pharmacol 2000;57:760–8.

[29] Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 1989;81:116–24.

[30] Berger W, Elbling L, Minai-Pour M, Vetterlein M, Pirker R, Kokoschka EM, Micksche M. Intrinsic MDR-1 gene and P-glycoprotein expression in human melanoma cell lines. Int J Cancer 1984;59:717–23.

[31] Martin C, Berridge G, Higgins CF, Mistry P, Charlton P, Callaghan R. Communication between multiple drug binding sites on P-glycoprotein. Mol Pharmacol 2000;58:624–32.

[32] Zamora JM, Pearce HL, Beck WT. Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Mol Pharmacol 1988;33:454–62.

[33] Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 1990;42:155–99.