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Enhanced 9-(2-Phosphonylmethoxyethyl)adenine Secretion by a Specific, Indomethacin-Sensitive Efflux Pump in a Mutant 9-(2-Phosphonylmethoxyethyl)adenine-Resistant Human Erythroleukemia K562 Cell Line

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ABSTRACT

We have investigated the molecular basis of the 100-fold resistance of mutant human erythroleukemia K562/PMEA-1 cells to the antiproliferative potential of 9-(2-phosphonylmethoxyethyl)adenine (PMEA). Upon exposure to high PMEA concentrations, comparable intracellular PMEA levels were initially observed in mutant K562/PMEA-1 and wild-type K562/0 cells, indicating that PMEA influx was unaltered. However, after 4 hr of exposure to 0.2 μM [^{3}H]bis(pivaloyloxymethyl)-PMEA [bis(POM)-PMEA], the total intracellular level of unphosphorylated and mono- and diphosphorylated PMEA was 2.8-fold lower in K562/PMEA-1 than in K562/0 cells. Increased PMEA secretion from K562/PMEA-1 cells (compared with K562/0 cells) became more pronounced upon prolonged exposure to bis(POM)-PMEA; after 24 hr, K562/PMEA-1 cells showed 65-fold lower total intracellular PMEA levels than K562/0 cells and at 48 hr, >400-fold less total PMEA was detected in K562/PMEA-1 cells. In addition, PMEA phosphorylation was 25- to 50-fold less efficient in K562/PMEA-1 than in K562/0 cells, pointing to an additional defect at the level of the metabolism of PMEA. The PMEA efflux mechanism was shown to be temperature- and azide-dependent, was markedly inhibited by indomethacin, and did not recognize adenine nucleotides or the phosphorylated metabolites of 3′-azido-3′-deoxythymidine. Also, over a 28-hr period, PMEA efflux was not affected by an inhibitor of RNA synthesis (actinomycin D) or protein synthesis (cycloheximide). Our studies revealed that resistance of K562/PMEA-1 cells to PMEA is the combined result of a severely impaired PMEA phosphorylation on the one hand, and an enhanced PMEA secretion by a highly specific, indomethacin-sensitive efflux pump, different from the classical P-glycoprotein- and multidrug resistance protein-mediated resistance mechanisms, on the other hand.

The acyclic nucleoside phosphonates are a unique class of drugs that were designed and developed as broad-spectrum agents for antiviral chemotherapy (De Clercq *et al*., 1986). The prototype compound of the acyclic nucleoside phosphonates, PMEA (Fig. 1), is endowed with strong antiviral activity against herpes viruses, human hepatitis B virus and retroviruses, including HIV (Naesens *et al*., 1997). Moreover, bis(POM)-PMEA, an oral prodrug of PMEA with improved bioavailability and pharmacokinetic properties, is currently being explored for its efficacy against hepatitis B virus and HIV infections in phase II and phase III clinical trials, respectively (Barditch-Crovo *et al*., 1997).

Recently, we discovered that PMEA and some closely related analogues are also strong inducers of tumor cell differentiation in several *in vitro* tumor cell lines (Balzarini *et al*., 1995; Hatse *et al*., 1995; 1998a). In addition, PMEA was found to exert a marked antitumor effect in choriocarcinoma-bearing rats (Hatse *et al*., 1998b). In this *in vivo* choriocarcinoma model, PMEA is able to completely suppress the development of new tumors and to cause regression of existing tumors (Hatse *et al*., 1998b). In light of these findings, PMEA may be considered as a possible drug candidate for the treatment of differentiation-susceptible neoplastic diseases.

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ABBREVIATIONS: PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEAp, monophosphorylated form of 9-(2-phosphonylmethoxyethyl)adenine; PMEApp, diphosphorylated form of 9-(2-phosphonylmethoxyethyl)adenine; AZT, 3′-azido-3′-deoxythymidine; bis(POM)-PMEA, bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; MDR, multidrug resistance; mono(POM)-PMEA, mono(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; PMEG, 9-(2-phosphonylmethoxyethyl)guanine; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.1% Tween-20; MRP, multidrug resistance protein; P-gp, P-glycoprotein.

The rapid emergence of drug-resistant virus strains represents a serious obstacle in antiviral chemotherapy. Compared with other antiretroviral nucleoside analogues, the development of HIV resistance to PMEA seems to occur rather slowly (Barditch-Crovo *et al.*, 1997). Likewise, tumor cell resistance to cytostatic agents imposes severe limitations to the clinical efficacy of many powerful anticancer agents. In view of a possible application of PMEA in anticancer chemotherapy, it is relevant to gain insight in the molecular mechanism(s) that may confer PMEA resistance to tumor cells upon prolonged exposure of the cells to PMEA. PMEA-resistant cell populations have already been selected from the murine leukemia L1210 cell line (Balzarini *et al.*, 1998) and from human lymphoid CEM (Robbins *et al.*, 1995a) and erythroleukemia K562 cells (Hatse *et al.*, 1996).

Cellular uptake of PMEA has been shown to occur via an endocytosis-like process (Palú *et al.*, 1991). Intracellularly, PMEA is metabolized to its mono- and diphosphorylated forms (designated PMEAp and PMEApp, respectively), the latter being the active metabolite that is responsible for the biological activities of the compound (Balzarini *et al.*, 1991; Pisarev *et al.*, 1997). Both adenylate kinase (Robbins *et al.*, 1995b) and PRPP synthetase (Balzarini *et al.*, 1991) have been found to convert PMEA to its phosphorylated form(s). PMEApp has a relatively long intracellular half-life (16–18 hr in MT-4 cells) and is eventually degraded by the ATP-catabolizing enzymes ATPase, phosphodiesterase, and nucleoside diphosphate kinase (Balzarini *et al.*, 1991). Unlike PMEA, the lipophilic ester prodrug bis(POM)-PMEA passively diffuses into the cell. Thus, in addition to its clinical advantages over the parent compound PMEA, bis(POM)-PMEA is a useful tool with which to study intracellular PMEA metabolism uncoupled from cellular PMEA uptake. PMEA is released from bis(POM)-PMEA by a two-step hydrolysis. The intermediate mono(POM)-PMEA may also be formed by chemical hydrolysis in the absence of cells or serum. Further breakdown of mono(POM)-PMEA to PMEA requires the involvement of esterases (Srinivas *et al.*, 1993).

In the present study, we characterized the PMEA-resistant variant of the human erythroleukemia K562 cell line, designated K562/PMEA-1, that was recently established at our laboratory. In a previous article, we reported that the mutant K562 cell line displays, in addition to a 100-fold resistance to the cytostatic and differentiation-inducing properties of PMEA, a 40-fold cross-resistance to bis(POM)-PMEA and a 4- to 10-fold cross-resistance to the closely related phosphonate derivatives PMEDAP and the *S*-enantiomers of 9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and 9-(3-fluoro-2-phosphonylmethoxypropyl)adenine (Hatse *et al.*, 1996). Interestingly, K562/0 and K562/PMEA-1 cells are equally sensitive to the antiproliferative activity of the guanine counterpart PMEG, which, unlike PMEA, is phosphorylated intracellularly by guanylate kinase (Ho *et al.*, 1991; Casara *et al.*, 1995; Navé *et al.*, 1996). K562/PMEA-1 cells also exhibited a 5- to 25-fold resistance to the differentiation-inducing, but not cytostatic, properties of a wide variety of structurally unrelated antimetabolites targeted at diverse cellular enzymes. We have now investigated in detail PMEA transport and metabolism in PMEA-resistant and wild-type K562 cells by the use of radiolabeled PMEA and bis(POM)-PMEA.

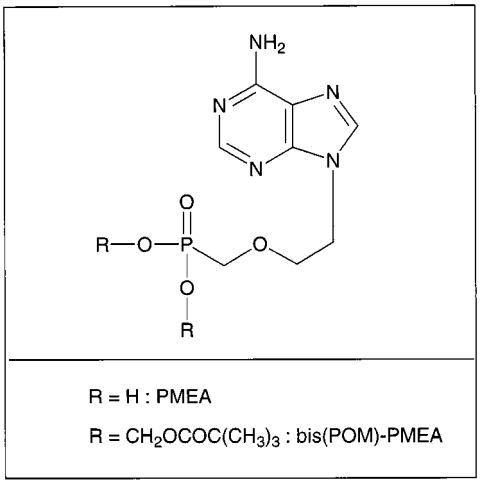

Fig. 1. Structural formulae of the acyclic nucleoside phosphonate PMEA and its oral prodrug bis(POM)-PMEA.

Materials and Methods

Compounds. The synthesis and antiretroviral activity of the acyclic nucleoside phosphonates used in this study have been described previously (De Clercq *et al.*, 1986; Holy and Rosenberg, 1987). PMEA and bis(POM)-PMEA were obtained from Dr. N. Bischofberger (Gilead Sciences, Foster City, CA). AZT (Zidovudine), indomethacin, cycloheximide and actinomycin D were purchased from Sigma Chemical (St. Louis, MO).

Cells. Human erythroleukemia K562 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were cultured in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine (Gibco) and 0.075% $NaHCO_3$ (Gibco). Subcultivations were performed every 3–4 days. The PMEA-resistant variant K562/PMEA-1 was selected from the wild-type K562/0 cell line as described previously (Hatse *et al.*, 1996) and was maintained in the presence of 1 mM PMEA. Before the experiments, the K562/PMEA-1 cells were kept in drug-free medium for at least 3 days. All cell cultures were maintained at 37° in a humidified, CO_2-controlled atmosphere.

Cytostatic assay (IC_{50} measurement). Exponentially growing K562/0 and K562/PMEA-1 cells were seeded in 96-well microtiter plates (Falcon, Becton Dickinson, Grenoble, France) at a final density of 2.5×10^5 cells/ml in RPMI-1640-based growth medium. Test compounds were added at 1:5 serial dilutions in the appropriate concentration range. In each well, the final volume was 200 μl. The cells were then allowed to proliferate for 72 hr (approximately three cell generations). At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics, Harpenden Herts, UK). The IC_{50} (50% inhibitory concentration for cell proliferation) was defined as the compound concentration required to inhibit cell proliferation by 50% compared with the untreated control.

Uptake and intracellular metabolism of radiolabeled PMEA and bis(POM)-PMEA by wild-type and PMEA-resistant K562 cells. Exponentially growing K562/0 and K562/PMEA-1 cells were seeded in 5-ml culture flasks in RPMI-1640-based growth medium at a final density of 3×10^5 cells/ml. The cells were incubated with [2,8-^{3}H]PMEA (radiospecificity, 40 Ci/mmol) or [8-^{3}H]bis-(POM)-PMEA (radiospecificity, 3 Ci/mmol) (Moravek Biochemicals, Brea, CA) at the appropriate extracellular concentrations, as indicated in Results. After the appropriate time intervals, cells were centrifuged at 4°, thoroughly washed with ice-cold RPMI-1640 medium (without serum), and precipitated with ice-cold 75% methanol. Aliquots of the cell-free culture medium were extracted with two volumes of ice-cold 100% methanol. After centrifugation, HPLC analysis of the supernatants was accomplished on a Partisphere SAX ion exchange column (column dimensions, 4.6 × 125 mm; Whatman,

Maidstone, UK). The buffer gradient system was as follows: 10 min at 2 mM $(NH_4)H_2PO_4$, pH 5.0 (flow, 2 ml/min); 5 min linear gradient to 7.5 mM $(NH_4)H_2PO_4$ + 5 mM KCl, pH 5.0; 15 min linear gradient to 0.15 M $(NH_4)H_2PO_4$ + 0.3 M KCl, pH 5.0; 25 min at 0.15 M $(NH_4)H_2PO_4$ + 0.3 M KCl, pH 5.0; 5 min linear gradient to 0.25 M $(NH_4)H_2PO_4$ + 0.5 M KCl pH 5.0; 5 min linear gradient to 2 mM $(NH_4)H_2PO_4$ pH 5.0; 20 min equilibration at the same buffer conditions. The retention times for PMEA and its mono- and diphosphorylated metabolites PMEAp and PMEApp were 10–11 min, 31 min, and 53–54 min, respectively. Quantification of radiolabeled metabolites was accomplished by liquid scintillation counting of the eluted fractions using OptiPhase 'HiSafe' 3 counting fluid (Wallac, Turku, Finland).

Efflux of PMEA from wild-type and PMEA-resistant K562 cells preincubated with radiolabeled bis(POM)-PMEA and effect of low temperature, azide, indomethacin, cycloheximide and actinomycin D. Exponentially growing K562/0 and K562/PMEA-1 cells were seeded in 5-ml culture flasks at a final density of 4–5 $\times$ 10^5 cells/ml in RPMI-1640-based growth medium. [8-^{3}H]bis(POM)-PMEA was added at 3 μCi/ml, which corresponds to a final extracellular concentration of 1 μM. After 4 hr, the cells were harvested by centrifugation at 4° and extracellular radioactivity was carefully removed by 2 subsequent washing steps at 4°. Thereafter, the cells were resuspended in RPMI-1640 medium (without serum) at a final density of 3 $\times$ 10^6 cells/ml and incubated at 37° or at 4°. At various time points (0, 0.5, 1, 2.5, and 24 hr), a 200-μl aliquot was removed and immediately cooled on ice. Cells were separated from the medium by centrifugation at 4° and total radioactivity in the cell-free medium was determined by liquid scintillation counting using UltimaGold counting fluid (Packard, Meriden, CT). Also, cell-free culture medium samples for HPLC analysis were precipitated by addition of 2 volumes of ice-cold 100% methanol. After centrifugation, the supernatants were injected on a Partisphere SAX ion exchange column and eluted, as described above. To the cell pellets, 100 μl of 1% Triton-X100 in water was added to disrupt cell membranes and total radioactivity in the crude cell extracts was measured.

The effect of azide treatment on PMEA efflux was assessed as follows. After preincubation of K562/0 and K562/PMEA-1 cells with radiolabeled bis(POM)-PMEA at 3 μCi/ml (i.e., a final concentration of 1 μM) for 90 min, the cells were thoroughly washed at 4° with glucose-free medium (Gibco) without serum. Then, the cells were resuspended at 2 $\times$ 10^6 cells/ml in normal RPMI-1640-based growth medium or in glucose-free medium containing 5% dialyzed serum, 10 mM sodium azide and 10 mM 2-deoxy-D-glucose (Sigma). After incubation for 15 min at 37° in the presence of azide, intracellular ATP pools were depleted to 3–10% of control values, as demonstrated by HPLC analysis, and the monitoring of PMEA efflux was started (zero time point). At 0, 1, 3 and 6 hr, 200 μl samples were removed and processed as described above, and the percentage of total radioactivity in the extracellular medium was determined.

The effects of indomethacin, cycloheximide and actinomycin D on PMEA secretion were investigated by preincubating the cells with radiolabeled bis(POM)-PMEA at 3 μCi/ml (i.e., a final concentration of 1 μM) in the presence or absence of 50 μM indomethacin, 100 μg/ml cycloheximide, or 200 ng/ml actinomycin D. After wash-out of the radiolabeled bis(POM)-PMEA, the cell cultures were further incubated in bis(POM)-PMEA-free medium (without serum) containing the inhibitors at the same concentrations as during the preincubation.

Efflux of AZT metabolites from wild-type and PMEA-resistant K562 cells. Exponentially growing K562/0 and K562/PMEA-1 cells were seeded as described above and incubated with 0.2 μCi/ml (final concentration, 1 μM) [*methyl*-^{3}H]AZT (Moravek Biochemicals) in the presence or absence of 50 μM indomethacin. After 4 hr, the extracellular AZT was carefully washed out and the secretion of radioactivity from the cells into the medium in the presence or absence of 50 μM indomethacin was followed in function of time, as described above.

Efflux of adenine nucleotides from wild-type and PMEA-resistant K562 cells preincubated with bis(POM)-PMEA. Exponentially growing K562/0 and K562/PMEA-1 cells were seeded as described above and preincubated in the presence or absence of 1 μM non-radioactive bis(POM)-PMEA for 24 hr. Then, [2,8-^{3}H]adenine (radiospecificity, 29 Ci/mmol) (Moravek Biochemicals) was added to the cell cultures at 1 μCi/ml (0.035 μM). After further incubation for 6 hr, the cells were centrifuged at 4°, samples of the cell-free culture medium were precipitated with methanol and analyzed on a Whatman Partisphere SAX column, and radioactivity in the eluted fractions was measured as described above.

Immunoblotting. Exponentially growing K562/0 and K562/PMEA-1 cells were harvested and crude protein extracts were prepared in cold radioimmunoprecipitation assay buffer [PBS (1$\times$ = 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate] with freshly added enzyme inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 30 μl/ml aprotinin, and 1 mM sodium orthovanadate) (Sigma Chemical). Protein concentrations of the extracts were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Fifty or 100 μg of protein were loaded on 4–15% Tris·HCl Ready Gels (Bio-Rad) and electrophoresis was accomplished at 60–100 V using Tris/Glycine/sodium dodecyl sulfate electrophoresis buffer (Bio-Rad). The separated proteins were then transferred to a Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) by electroblotting. Aspecific binding sites were blocked by immersing the membrane in 5% dry milk in PBST for 1 hr at room temperature. After rinsing in PBST, the membrane was incubated overnight at 4° with 2 μg/ml monoclonal mouse antihuman MRP antibody (clone QCRL-1; Chemicon International, Temecula CA) diluted in PBST containing 2% dried milk. Then, the membrane was washed using three changes of PBST and incubated for 30 min at room temperature with peroxidase-linked sheep antimouse Ig antibody (Amersham) diluted 1/2000 in PBST containing 2% dry milk. After thorough washing (5 times 10 min) in PBST, enhanced chemiluminescence detection (Amersham) was performed according to the manufacturer's instructions.

Flow cytometry. All manipulations were performed at 4°. Exponentially growing K562/0 and K562/PMEA-1 cells were harvested, washed once with PBS, and fixed in 1% paraformaldehyde in PBS for 30 min. Thereafter, the cells were washed once with PBS and once with blocking solution (PBS containing 1% bovine serum albumin and 5% normal goat serum). Then, 10^6 cells were permeabilized for 30 min in 200 μl of blocking solution containing 0.1% Tween-20, followed by direct addition of 10 μl (approximately 1 μg) of primary monoclonal antibody (either C494 mouse antihuman P-gp or QCRL-1 mouse antihuman MRP; Chemicon International). Irrelevant mouse IgG1 (Dako, Glostrup, Denmark) was used as a negative control. After incubation for 1 hr, the cells were washed twice in blocking solution containing 0.1% Tween-20 and subsequently incubated with secondary antibody (fluorescein isothiocyanate-conjugated goat antimouse Ig; Caltag Laboratories, San Francisco, CA) diluted 1/500 in blocking solution containing 0.1% Tween-20. Thereafter, the cells were washed twice with blocking solution containing 0.1% Tween-20, resuspended in 1% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson, Le Pont de Claix, France).

Results

Effect of the extracellular PMEA concentration on uptake and metabolism of radiolabeled PMEA and bis(POM)-PMEA by K562/0 cells. K562/0 cell cultures were seeded in growth medium containing PMEA at different con-

centrations (i.e., 0, 20, 100, 500, and 2500 μM). To each culture flask, 25 μCi (0.125 μM) of [2,8-^{3}H]PMEA was added. Table 1 shows the proportional increase in the total amount of intracellular PMEA and PMEA metabolites at 24 hr with increasing extracellular PMEA concentrations. The sum of PMEA, PMEAp, and PMEApp was 0.3, 25, 100, 495, and 1862 pmol/10^6 cells for K562/0 cells incubated with 0.125, 20, 100, 500, and 2500 μM PMEA, respectively. Also, the data in Table 1 indicate that a threshold concentration of PMEA is required to afford optimal intracellular PMEA phosphorylation. At 0.125 μM PMEA, only 17% of the intracellular PMEA is converted to the mono- and diphosphorylated forms, compared with 69–77% at drug concentrations ranging between 20 and 2500 μM. The proportional increase in the intracellular amounts of PMEAp and PMEApp with increasing extracellular PMEA concentrations indicate that the enzyme(s) responsible for PMEA phosphorylation in K562 cells are not saturated up to 2.5 mM PMEA (Table 1).

In another experiment, we demonstrated that the presence of free PMEA in the cell culture medium does not markedly influence the uptake of the prodrug bis(POM)-PMEA and subsequent metabolism of PMEA derived from it. In the presence of gradually increasing extracellular concentrations of unlabeled PMEA (i.e., 0, 20, 100, 500, or 2500 μM), the intracellular amounts of radiolabeled PMEA, PMEAp, and PMEApp derived from 3 μCi (0.2 μM) of [8-^{3}H]bis(POM)-PMEA varied between 2.5 and 5.0 pmol/10^6 cells, 1.6 and 4.0 pmol/10^6 cells, and 7.0 and 13.8 pmol/10^6 cells, respectively (data not shown). Also, the uptake and metabolism of bis(POM)-PMEA proved 100-fold more efficient than that of PMEA. Indeed, the total amount of intracellular PMEA in K562/0 cells incubated with 0.2 μM [^{3}H]bis(POM)-PMEA (without the addition of unlabeled PMEA) was 14 pmol/10^6 cells, and the contribution of the phosphorylated metabolites (PMEAp + PMEApp) was 63% (data not shown). A 100-fold higher concentration of [^{3}H]PMEA (i.e., 20 μM) was required to afford a comparable total intracellular amount of PMEA (19 pmol/10^6 cells) and a comparable proportion of phosphorylated metabolites (69%) (Table 1).

Influx of PMEA in wild-type versus PMEA-resistant K562 cells. K562/0 and K562/PMEA-1 cells were incubated in the presence of [2,8-^{3}H]PMEA at a low extracellular concentration of 0.125 μM. After 4 hr, the amounts of [^{3}H]PMEA, [^{3}H]PMEAp and [^{3}H]PMEApp detected in K562/0 cells were 3.8, 0.03, and 0.07 pmol/10^6 cells, respectively. In K562/PMEA-1 cells, 250-fold less [^{3}H]PMEA (i.e., 0.015 pmol/10^6 cells) was found, and the amounts of [^{3}H]PMEAp and [^{3}H]PMEApp were below the detection limit (data not shown). If, however, K562/0 and K562/PMEA-1 cells were incubated for 4 hr with a very high concentrations of nonradiolabeled PMEA (5 mM), comparable amounts of PMEA (i.e., 497 and 604 pmol/10^6 cells) were detected in K562/0 and K562/PMEA-1 cells, respectively. Although no phosphorylated PMEA metabolites could be detected in K562/PMEA-1 cells under these experimental conditions, a PMEApp peak (representing 262 pmol/10^6 cells) was clearly visible in the chromatogram of K562/0 cells (data not shown). Thus, although the amounts of unphosphorylated PMEA were comparable in both cell lines after 4 hr of incubation, the total amount of PMEA plus phosphorylated PMEA metabolites was higher in wild-type than in mutant K562 cells.

Intracellular amounts of PMEA, PMEAp, and PMEApp in K562/0 and K562/PMEA-1 cells as a function of time and extracellular concentration of bis(POM)-PMEA. Wild-type K562 cells were exposed to [8-^{3}H]bis(POM)-PMEA at 0.5 μM, which corresponds to the IC_{50} value of bis(POM)-PMEA for K562/0 cell proliferation, or at a 2.5-fold lower concentration (0.2 μM). In comparison, PMEA-resistant K562 cells were incubated with [8-^{3}H]bis(POM)-PMEA at its IC_{50} value in K562/PMEA-1 cells (i.e., 25 μM), at a 2.5-fold lower concentration (i.e., 10 μM) or at 0.2 μM. At different time points (i.e., 4, 24, and 48 hr) the intracellular amounts of PMEA and its phosphorylated metabolites were determined in the cell extracts. After 4 hr of incubation with 0.2 μM bis(POM)-PMEA, 18- to 25-fold less PMEAp and PMEApp were found in K562/PMEA-1 cells, compared with K562/0 cells (i.e., 0.5 and 1 pmol/10^6 K562/PMEA-1 cells for PMEAp and PMEApp, respectively, compared with 9 and 25 pmol/10^6 K562/0 cells), although comparable amounts of PMEA (i.e., 18 to 20 pmol/10^6 cells) were detected in both cell lines (Table 2). Also, K562/0 cells exposed to bis(POM)-PMEA at its IC_{50} value (i.e., 0.5 μM) contained 81 pmol PMEApp/10^6 cells at 4 hr. For K562/PMEA-1 cells, a 50-fold higher extracellular concentration of bis(POM)-PMEA was required to afford comparable intracellular amounts of PMEApp (72 pmol PMEApp/10^6 cells) (Table 2). A similar conclusion could be reached for the PMEApp levels measured in mutant and wild-type K562 cells at 24 hr (Table 2). These data are consistent with the fact that PMEApp is the active PMEA metabolite, because 25 μM indeed corresponds to the IC_{50} value of bis(POM)-PMEA in K562/PMEA-1 cells, compared with 0.5 μM in K562/0 cells.

In contrast to the observations made after 4 hr, when comparable amounts of PMEA were found in K562/0 and K562/PMEA-1 cells incubated with 0.2 μM bis(POM)-PMEA, 30-fold less PMEA was observed at 24 hr in K562/PMEA-1 cells exposed to 0.2 μM bis(POM)-PMEA, compared with K562/0 cells (0.16 pmol/10^6 cells for K562/PMEA-1 cells versus 5 pmol/10^6 cells for K562/0 cells) (Table 2). At 24 hr, compared with the 4-hr time point, the intracellular level of

TABLE 1
Intracellular amounts of PMEA, PMEAp, and PMEApp in K562/0 cells incubated for 24 hr with [2,8-^{3}H]PMEA at different extracellular concentrations

Extracellular [^{3}H]PMEA concentration	[^{3}H]PMEA	[^{3}H]PMEAp	[^{3}H]PMEApp	[^{3}H]PMEAp + [^{3}H]PMEApp (%) / Total intracellular [^{3}H]PMEA
μM		*pmol / 10^6 cells*		
0.125	0.282	0.017	0.041	17
20	5.8	4.1	8.9	69
100	28	29	43	72
500	119	187	189	76
2500	435	747	680	77

TABLE 2
Intracellular levels of PMEA, PMEAp, and PMEApp as a function of time in wild-type versus PMEA-resistant K562 cells incubated with [8-^{3}H]bis(POM)-PMEA at different extracellular concentrations
The data represent the means ± standard deviation for two independent experiments.

Cell line	[^{3}H]Bis(POM)-PMEA	4 hr			24 hr			48 hr		
		[^{3}H]PMEA	[^{3}H]PMEAp	[^{3}H]PMEApp	[^{3}H]PMEA	[^{3}H]PMEAp	[^{3}H]PMEApp	[^{3}H]PMEA	[^{3}H]PMEAp	[^{3}H]PMEApp
	μM	*pmol/10^6 cells*								
K562/0	0.2	20 ± 9	9.0 ± 5.7	25 ± 13	5.0 ± 0.1	1.8 ± 1.2	6.2 ± 2.5	3.2 ± 2.3	0.2 ± 0.1	0.7 ± 0.1
	0.5	68 ± 42	26 ± 16	81 ± 37	14 ± 1	8.0 ± 5.9	30 ± 15	5.0 ± 0.4	1.0 ± 0.6	3.7 ± 1.6
K562/PMEA-1	0.2	18 ± 15	0.5 ± 0.4	1.0 ± 0.5	0.16 ± 0.16	0.005 ± 0.007	0.043 ± 0.061	0.006 ± 0.008	0.004 ± 0.006	0.000
	10	1832 ± 598	16 ± 10	47 ± 10	26 ± 5	2.8 ± 0.3	14 ± 7	0.6 ± 0.8	0.0	0.0
	25	3150 ± 921	16 ± 7	72 ± 16	37	5	28	1.5 ± 2.1	0.0	0.0

PMEA in K562/0 cells had decreased 4- to 5-fold for both 0.2 and 0.5 μM bis(POM)-PMEA. However, in K562/PMEA-1 cells, the decrease in intracellular PMEA from 4 hr to 24 hr was at least 70- to 112-fold for each of the 3 concentrations of bis(POM)-PMEA (Table 2). After 48 hr of continuous exposure to 0.2 μM bis(POM)-PMEA, K562/0 cells still contained 3.2 pmol PMEA/10^6 cells, which represents 16% of the amount noted at 4 hr and which is >500-fold higher than in K562/PMEA-1 cells under the same conditions. Even at 25 μM bis(POM)-PMEA, the amount of PMEA detected after 48 hr in K562/PMEA-1 cells was only 0.05% of the PMEA level measured after 4 hr of incubation, whereas PMEAp and PMEApp could no longer be detected at 48 hr (Table 2).

Efflux of PMEA from K562/0 and K562/PMEA-1 cells in the presence or absence of indomethacin. Wild-type and PMEA-resistant K562 cells were loaded during 4 hr with 1 μM [8-^{3}H]bis(POM)-PMEA. After careful wash-out of extracellular radioactivity at 4°, the cells were further incubated in drug-free medium. At 0, 0.5, 1, 2.5, and 24 hr, aliquots were removed from the cell cultures to monitor the decrease in total intracellular radioactivity and the appearance of radioactivity in the extracellular medium as a function of time. The total amounts of radiolabeled substrate (either under the form of bis(POM)-PMEA, mono(POM)-PMEA, PMEA, PMEAp, or PMEApp) loaded in the cells during the 4-hr preincubation step were 190 ± 25 pmol/10^6 cells for K562/0 cells and 86 ± 5 pmol/10^6 cells for K562/PMEA-1 cells (zero time point). As shown in Fig. 2, efflux of radioactivity into the extracellular medium occurred with both K562/0 and K562/PMEA-1 cells. However, the rate at which radioactivity from the cells appeared in the culture medium was markedly higher for K562/PMEA-1 than for K562/0 cells. After 0.5, 1, 2.5, and 24 hr, 21, 26, 54, and 93%, respectively, of the total radioactivity measured in K562/PMEA-1 cell cultures was found extracellularly (Fig. 2B), compared with 13, 15, 22, and 65%, respectively, for K562/0 cells (Fig. 2A). HPLC analysis of the extracellular medium of K562/PMEA-1 cells at the 24-hr time point revealed that 95.7% of the extracellular radioactivity was associated with PMEA. The contributions of bis(POM)-PMEA and PMEAp were only 4% and 0.3%, respectively, whereas mono(POM)-PMEA and PMEApp were not detectable in the culture medium (data not shown).

To determine the effect of indomethacin on PMEA secretion from K562/0 and K562/PMEA-1 cells, an identical experiment was performed in the presence of 50 μM indomethacin. Immediately after wash-out of extracellular [8-^{3}H]bis(POM)-PMEA (zero time point), K562/0 and K562/PMEA-1 cells contained 243 ± 21 and 279 ± 36 pmol radiolabeled substrate per 10^6 cells, respectively. Fig. 2 clearly demonstrates that indomethacin markedly retarded PMEA secretion from both wild-type and PMEA-resistant K562 cells. In the presence of indomethacin, only 16% of the total radioactivity had appeared in the culture medium of K562/PMEA-1 cells at 2.5 hr, compared with 54% in the absence of indomethacin. Even at 24 hr, the proportion of radioactivity measured in the extracellular medium of K562/PMEA-1 cells exposed to indomethacin (i.e., 51%) was still markedly lower than in the absence of indomethacin (i.e., 93%) (Fig. 2B).

Reversal of the PMEA- and bis(POM)-PMEA resistance of K562/PMEA-1 cells by indomethacin. The IC$_{50}$ values of PMEA for K562/0 and K562/PMEA-1 cell proliferation were determined in the presence and absence of 50 μM

indomethacin. Whereas indomethacin did not significantly influence the cytostatic activity of PMEA in K562/0 cells (IC_{50}, 32 μM in the absence of indomethacin, compared with 21 μM in the presence of indomethacin), K562/PMEA-1 cells became 13-fold more sensitive to PMEA in the presence of indomethacin (IC_{50} was 4162 μM in the absence of indomethacin, compared with 331 μM in the presence of indomethacin) (Table 3). Thus, the presence of indomethacin reduced the PMEA resistance of K562/PMEA-1 cells from 130-fold to 16-fold (Table 3), and thus, resensitized the K562/PMEA-1 cells to PMEA by inhibiting PMEA efflux.

Effect of indomethacin on intracellular and extracellular amounts of PMEA and its metabolites in wild-type and PMEA-resistant K562 cells exposed to [8-^{3}H]bis(POM)-PMEA. K562/0 and K562/PMEA-1 cells were incubated with 1 μM [8-^{3}H]bis(POM)-PMEA in the presence or absence of 50 μM indomethacin. After 4 and 24 hr, the intracellular and extracellular amounts of PMEA, PMEAp, and PMEApp were determined by HPLC analysis. Fig. 3 shows that comparable amounts of PMEA were detected at 4 hr in wild-type and mutant K562 cells incubated without indomethacin (i.e., 143 ± 44 and 153 ± 80 pmol/10^6 cells, respectively). However, about 20-fold less PMEAp and PMEApp was found in K562/PMEA-1 cells, as compared with wild-type (2.0 ± 0.3 versus 40 ± 12 pmol/10^6 cells for PMEAp and 5.8 ± 1.3 versus 125 ± 25 pmol/10^6 cells for PMEApp). Thus, already after 4 hr, the sum of intracellular nonphosphorylated and mono- and diphosphorylated PMEA was approximately 2-fold lower in K562/PMEA-1 than in K562/0 cells. Indomethacin did not significantly influence the intracellular levels of PMEA, PMEAp, and PMEApp measured at 4 hr in K562/0 cells (Fig. 3). Conversely, in the presence of indomethacin there was an almost 3-fold higher amount of PMEA in K562/PMEA-1 cells at 4 hr (i.e., 407 ± 68 pmol/10^6 cells with indomethacin versus 153 ± 80 pmol/10^6 cells without indomethacin) (Fig. 3).

At 4 hr, the culture medium from K562/0 and K562/PMEA-1 cells contained 344 and 600 pmol PMEA/10^6 cells, respectively, in the absence of indomethacin, compared with 94 and 236 pmol PMEA/10^6 cells, respectively, in the presence of indomethacin. PMEAp and PMEApp could not be detected in the culture medium at 4 hr (data not shown). Thus, at 4 hr, in the absence of indomethacin 47% and 21% of total PMEA, and in the presence of indomethacin 79% and 64% of total PMEA was located intracellularly in K562/0 and K562/PMEA-1 cells, respectively.

Between 4 and 24 hr, the amount of PMEA in K562/0 cells decreased by 5-fold in the absence of indomethacin, and by <2-fold when indomethacin was present (Fig. 3). Strikingly, in K562/PMEA-1 cells incubated without indomethacin, only 0.13% of the intracellular PMEA level noted at 4 hr was detected at 24 hr (i.e., 0.2 pmol/10^6 cells). In contrast, in the presence of indomethacin, 23% of the amount of intracellular PMEA observed at 4 hr was still present at 24 hr in K562/PMEA-1 cells (i.e., 93 pmol/10^6 cells) (Fig. 3). The marked effect of indomethacin on PMEA accumulation in K562/PMEA-1 cells was also reflected in higher intracellular amounts of the active metabolite PMEApp. 10 pmol of PMEApp/10^6 K562/PMEA-1 cells were detected at 24 hr in the presence of indomethacin, compared with 0.4 pmol PMEApp/10^6 cells in the absence of indomethacin (Fig. 3).

The amounts of PMEA detected in the extracellular medium at 24 hr without indomethacin were 1272 and 768 pmol/10^6 cells for wild-type and mutant K562 cells, respectively, and 727 and 961 pmol/10^6 cells, respectively, in the

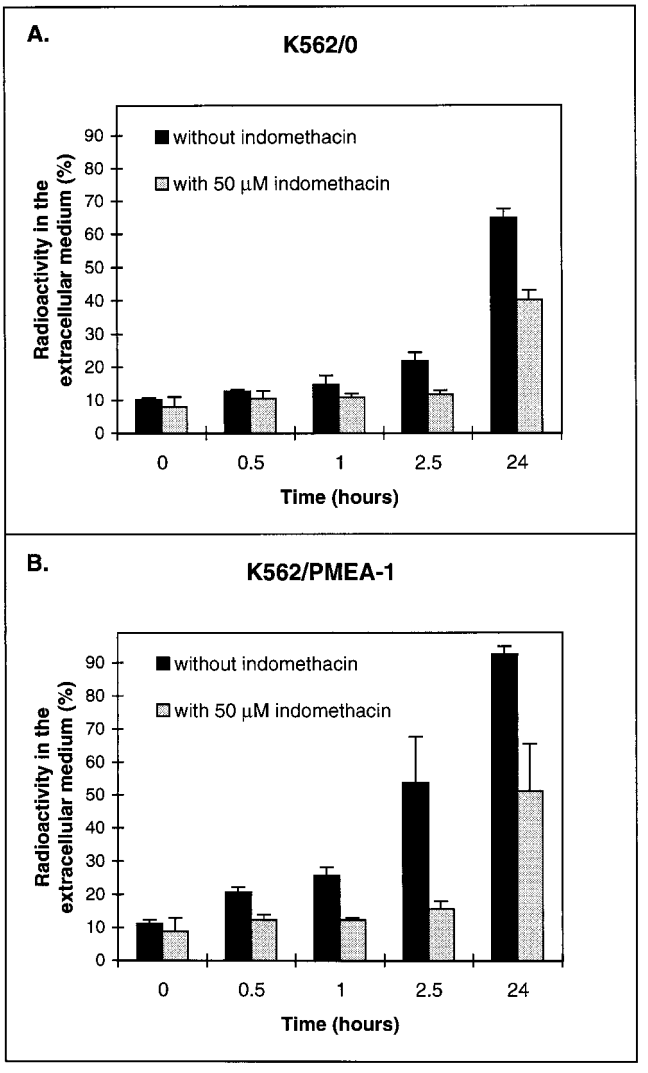

Fig. 2. Efflux of radioactivity from [8-^{3}H]bis(POM)-PMEA-loaded K562/0 and K562/PMEA-1 cells as a function of time in the presence or absence of 50 μM indomethacin. The total amounts of radiolabeled PMEA [i.e., bis(POM)-PMEA + mono(POM)-PMEA + PMEA + PMEAp + PMEApp] loaded in the cells during the preincubation with [8-^{3}H]bis(POM)-PMEA and measured at the zero time point were 190 ± 25 and 86 ± 5 pmol/10^6 cells for K562/0 and K562/PMEA-1 cells, respectively, in the absence of indomethacin, and 243 ± 21 and 279 ± 36 pmol/10^6 cells, respectively, in the presence of indomethacin. The data are expressed as the percentage of total radioactivity found in the extracellular medium. Each result represents the mean ± standard deviation of at least two independent experiments.

TABLE 3
Effect of indomethacin on the cytostatic activity of PMEA in wild-type and PMEA-resistant K562 cells
The IC_{50} values were determined after 3 days of incubation. The results represent the means ± standard deviation for three independent experiments.

Cell line	IC_{50} value of PMEA	
	Without indomethacin	With 50 μM indomethacin
	μM	
K562/0	32 ± 13	21 ± 9
K562/PMEA-1	4162 ± 872	331 ± 115

presence of indomethacin (data not shown). Thus, the percentage of total PMEA that was found intracellularly at 24 hr was 30% for K562/0 and 10% for K562/PMEA-1 cells incubated with indomethacin, compared with only 8.4% and 0.1%, respectively, in the absence of indomethacin.

Effect of azide treatment, temperature, cycloheximide, and actinomycin D on PMEA efflux from K562/0 and K562/PMEA-1 cells. The ATP-dependence of the PMEA secretory mechanism was investigated by measuring PMEA efflux from [8-^{3}H]bis(POM)-PMEA-loaded K562/0 and K562/PMEA-1 cells in the presence of the oxidative phosphorylation inhibitor sodium azide. Azide-treatment resulted in ATP depletion to 3–10% of control values within 15 min, as demonstrated by HPLC analysis (data not shown). The results in Table 4 clearly show the pronounced retardation of the PMEA efflux from ATP-depleted compared with control K562/0 and K562/PMEA-1 cells. After 3 hr in the absence of azide, 37% and 83% of the total radioactivity had appeared in the extracellular medium of wild-type and mutant K562 cells, respectively, compared with 23% and 25%, respectively, in the presence of azide (Table 4).

The effect of temperature on PMEA secretion was evaluated by monitoring the efflux of radioactivity from the [8-^{3}H]bis(POM)-PMEA-loaded K562/0 and K562/PMEA-1 cell cultures at 4° instead of 37°. Panels A and B of Fig. 4 show that the appearance of radioactivity in the extracellular medium proceeded markedly more slowly at 4° than at 37° for both wild-type and PMEA-resistant K562 cells.

To examine the role of RNA and protein synthesis in the PMEA secretory mechanism, the efflux experiment was performed in the presence of 100 μg/ml of the protein synthesis inhibitor cycloheximide or 200 ng/ml of the RNA synthesis inhibitor actinomycin D. As shown in Fig. 4, C and D, neither cycloheximide nor actinomycin D, applied at concentrations that effectively inhibit protein and RNA synthesis, respectively (data not shown), were able to inhibit PMEA efflux from wild-type and mutant K562 cells.

Efflux of adenine nucleotides and AZT metabolites from K562/0 and K562/PMEA-1 cells. To learn more about the specificity of the PMEA efflux mechanism, we also monitored the efflux of radioactivity from K562/0 and K562/PMEA-1 cells loaded with 1 μM [*methyl*-^{3}H]AZT. We found a very similar pattern for both wild-type and PMEA-resistant K562 cells: the percentages of radioactivity that had appeared in the extracellular medium at 30, 60, and 150 min and at 24 hr were 29, 43, 75, and 98%, respectively, for K562/0 cells and 27, 39, 78, and 98%, respectively, for K562/PMEA-1 cells. Also, the efflux of AZT-associated radioactivity from K562/PMEA-1 cells was not significantly altered in the presence of indomethacin (data not shown).

To find out whether the PMEA efflux pump also removes adenine nucleotides from the cells into the culture medium, K562/0 and K562/PMEA-1 cells were preincubated for 24 hr with 1 μM nonradioactive bis(POM)-PMEA to ensure optimal activity of the PMEA efflux pump, after which point [2,8-^{3}H]adenine was added to the cell cultures. After further incubation for 6 hr, the cell culture media were analyzed for the presence of radiolabeled AMP, ADP, and ATP. No detect-

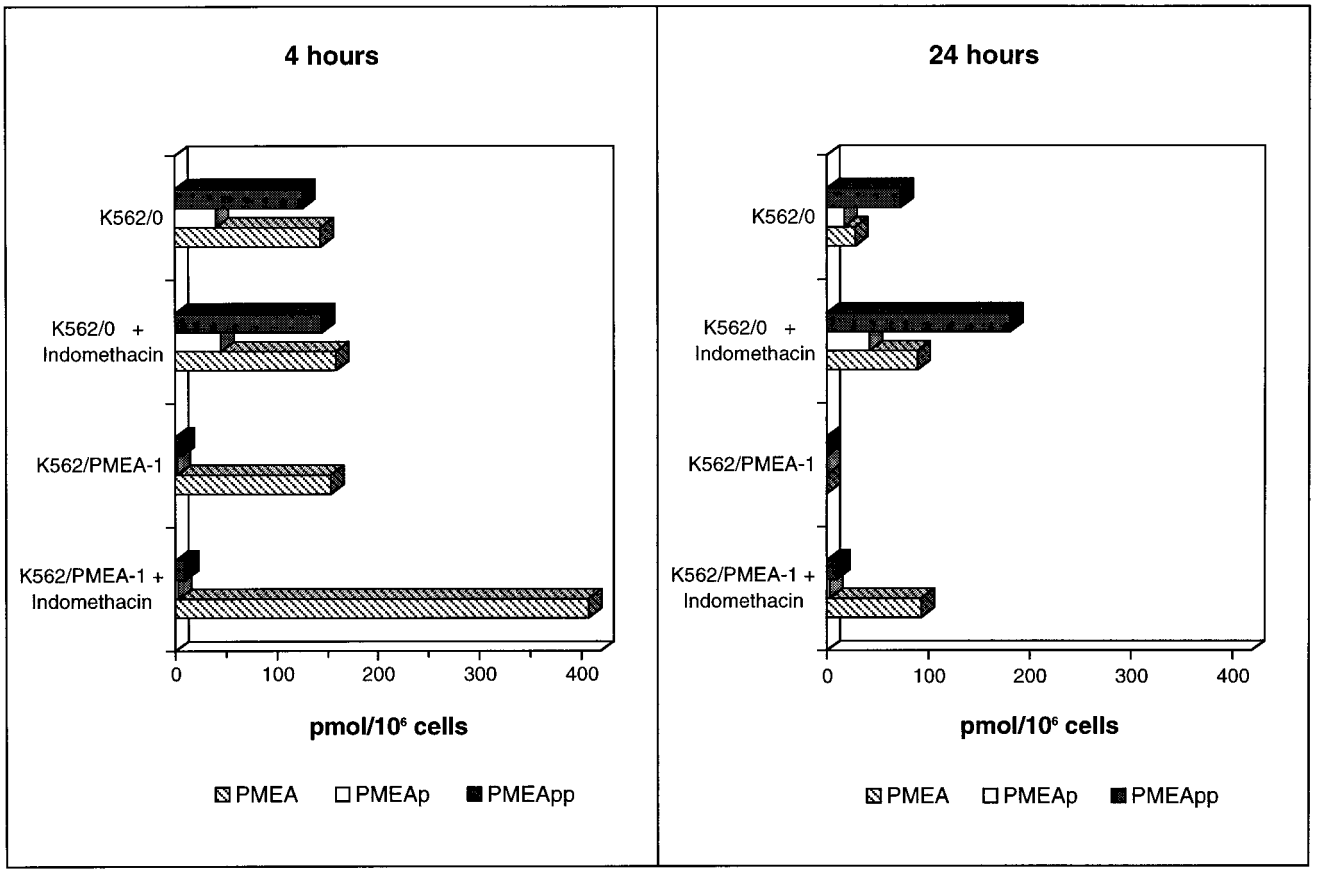

Fig. 3. Intracellular amounts of [^{3}H]PMEA, [^{3}H]PMEAp and [^{3}H]PMEApp at 4 and 24 hr in wild-type and PMEA-resistant K562 cells incubated with 1 μM [8-^{3}H]bis(POM)-PMEA in the presence or absence of 50 μM indomethacin. The data represent the means of two independent experiments.

able amounts of adenine nucleotides could be demonstrated in the extracellular medium of either wild-type or PMEA-resistant K562 cells (data not shown).

Detection of MRP via immunoblotting. In two independent experiments, staining of Western blots of K562/0 and K562/PMEA-1 protein extracts with mouse antihuman MRP as primary antibody did not reveal higher MRP expression in PMEA-resistant compared with wild-type K562 cells. On the contrary, the MRP expression level seemed to be slightly higher in K562/0 than in K562/PMEA-1 cells (data not shown).

Flow cytometric detection of P-gp and MRP. Wild-type and PMEA-resistant K562 cells were analyzed by flow cytometry after staining with a primary antibody against either P-gp or MRP and, subsequently, a fluorescein isothiocyanate-conjugated secondary antibody. Consistent with the observation of Fardel *et al.* (1995), P-gp expression was shown to be extremely low in K562/0 cells and was not significantly increased K562/PMEA-1 cells. Also, no overexpression of MRP in K562/PMEA-1 compared with K562/0 cells could be observed (data not shown).

Discussion

The development of tumor cell resistance to cytostatic drugs is a major obstacle to successful chemotherapy of many malignant diseases in humans. Moreover, cells selected for resistance to a single agent may show cross-resistance to a wide variety of structurally and functionally unrelated drugs. In many instances, this MDR is linked to the overexpression of P-gp, a membrane-bound ATP-dependent drug efflux pump encoded by the *MDR1* gene and physiologically present in various normal tissues (Gottesman and Pastan, 1988; Fardel *et al.*, 1995). Alternatively, tumor cells may acquire the MDR phenotype by amplification of the *MRP* gene (Zaman *et al.*, 1994; Lautier *et al.*, 1996). Hydrophobic, natural-product drugs, such as *Vinca* alkaloids, anthracyclines, actinomycin, taxol, colchicine, etc., are particularly susceptible to the MDR phenomenon. In contrast, resistance to water-soluble antimetabolites of purine and pyrimidine metabolism (e.g., 5-fluorouracil, gemcitabine, methotrexate, ara-C) is more frequently related to a biochemical alteration at the level of drug anabolism (Bhalla *et al.*, 1984) or at the site of interaction of the drug with its cellular target (Berger *et al.*, 1985) than to secretion of the drug. In this regard, the development of resistance of K562 cells to the nucleoside analogue PMEA seems to be most remarkable. Indeed, the present study revealed that the PMEA resistance of the mutant erythroleukemia K562/PMEA-1 cell line is based on enhanced PMEA secretion in combination with decreased activation of the compound to its biologically active form PMEApp.

As we have previously reported (Hatse *et al.*, 1996), K562/PMEA-1 cells cannot be efficiently loaded with radiolabeled PMEA. The lipophilic side chains of the diester form bis(POM)-PMEA markedly improve cellular uptake of the prodrug compared with free PMEA. Therefore, we have now used radiolabeled bis(POM)-PMEA for loading of the cells to better and more accurately compare PMEA metabolism in wild-type and PMEA-resistant K562 cells. We could demonstrate that entrance into K562/0 cells is 100-fold more efficient for bis(POM)-PMEA than for PMEA. Also, we have shown that the presence of free PMEA in the extracellular medium does not interfere with influx of radiolabeled bis(POM)-PMEA in K562/0 cells. This observation corroborates with the different uptake mechanisms of PMEA (active carrier-mediated transport, Palú *et al.*, 1991) and bis(POM)-PMEA (passive diffusion, Srinivas *et al.*, 1993). In addition, we have shown that the enzyme systems involved in PMEA metabolism in K562/0 cells are not saturated at extracellular PMEA concentrations up to 2.5 mM. This is in agreement with the earlier observation that PMEA uptake by human MT-4 cells and subsequent conversion to PMEAp and PMEApp are dose-dependent and proportional with the extracellular PMEA concentration (Balzarini *et al.*, 1991). Interestingly, a certain extracellular threshold concentration of PMEA must be exceeded to achieve optimal PMEA phosphorylation in K562/0 cells.

The comparative study of the intracellular levels of PMEA and its metabolites in PMEA-resistant versus wild-type K562 cells exposed to bis(POM)-PMEA disclosed two remarkable features of the K562/PMEA-1 cell line. First, PMEAp and PMEApp were formed to a 25- to 50-fold lesser extent in K562/PMEA-1 cells than in their PMEA-sensitive counterparts. The identity of the affected cellular enzyme(s) in K562/PMEA-1 cells, and thus the identity of the phosphorylating enzyme(s) of PMEA in K562 cells, remains to be clarified and is currently under investigation. Our earlier observation that PMEG, the guanine counterpart of PMEA, is equally cytostatic against wild-type and PMEA-resistant K562 cells (Hatse *et al.*, 1996) is consistent with the fact that PMEA and PMEG are phosphorylated by different cellular enzymes (Balzarini *et al.*, 1991; Ho *et al.*, 1991; Casara *et al.*, 1995; Robbins *et al.*, 1995b; Navé *et al.*, 1996).

Second, we found that the decline of intracellular PMEA and, consequently, PMEAp and PMEApp occurred considerably more rapidly in PMEA-resistant than in wild-type K562 cells. These observations point to increased drug secretion from K562/PMEA-1 compared with K562/0 cells. PMEA secretion was markedly retarded at lower temperature and in azide-treated (ATP-depleted) cells, indicating that an energy-consuming process is involved. Also, PMEA efflux from K562/PMEA-1 cells was independent of RNA and protein synthesis over a 28-hr time period, which suggests that the efflux pump is constitutively overexpressed in the mutant cells and is

TABLE 4

Effect of azide treatment on PMEA efflux from wild-type and PMEA-resistant K562 cells

The cells were loaded with 1 μM [8-^{3}H]bis(POM)-PMEA during 90 min in normal RPMI-based growth medium. After thorough washing at 4°, the cells were resuspended in drug-free normal growth medium (control) or in glucose-free medium containing 5% dialyzed serum, 10 mM azide, and 10 mM 2-deoxy-D-glucose. After 15 min, the measurements were started (zero time point). The total amounts of radiolabeled PMEA [bis(POM)-PMEA + mono(POM)-PMEA + PMEA + PMEAp + PMEApp] loaded in the cells, as measured at 15 min after resuspension of the cells in azide medium (i.e., at the zero time point) were 158 and 59 pmol/10^6 cells for K562/0 and K562/PMEA-1 cells, respectively. The data are expressed as the percentage of total radioactivity found in the extracellular medium.

Time (hr)	Percentage of radioactivity in the extracellular medium			
	K562/0		K562/PMEA-1	
	Control	Azide	Control	Azide
0	8	5	11	8
1	22	13	50	12
3	37	23	83	25
6	52	—	95	40

characterized by a turnover longer than 28 hr. Moreover, our finding that the K562/PMEA-1 cells can only be significantly loaded with PMEA at relatively high concentrations and not with low drug concentrations also points to a constitutive activity of the efflux pump, which is sufficient to guarantee the continuous efflux of small amounts of PMEA. At high

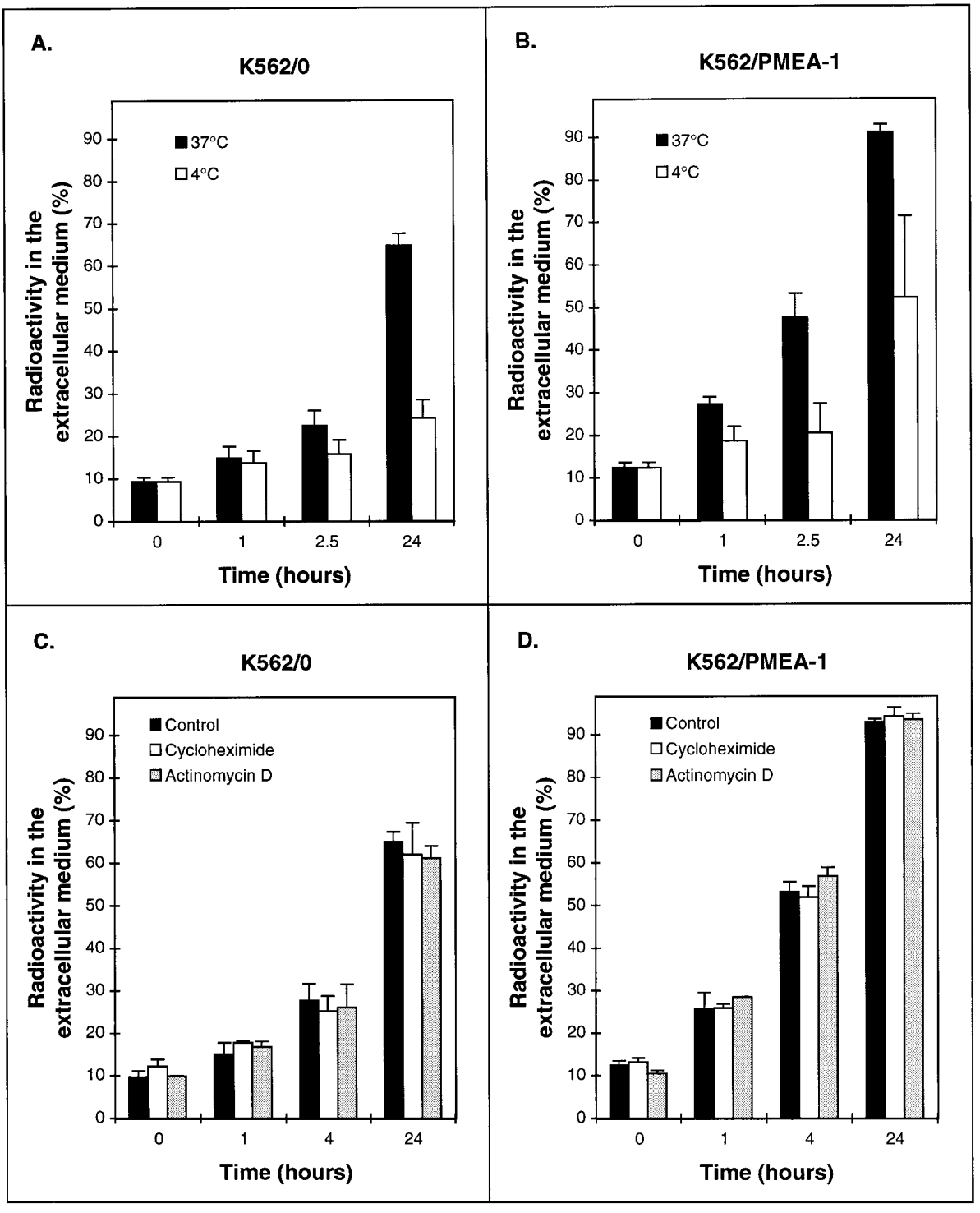

Fig. 4. A and B, Effect of temperature on the efflux of radioactivity from [8-^{3}H]bis(POM)-PMEA-loaded K562/0 and K562/PMEA-1 cells. At time zero, the total amounts of radiolabeled compound loaded in the cells were 190 ± 4 and 109 ± 37 pmol/10^6 cells for K562/0 and K562/PMEA-1 cells, respectively. C and D, Effect of inhibition of protein or RNA synthesis on the efflux of radioactivity from [8-^{3}H]bis(POM)-PMEA-loaded K562/0 and K562/PMEA-1 cells. The inhibitors cycloheximide (100 μg/ml) or actinomycin D (200 ng/ml) were already added during the preincubation step. At time zero, the total amounts of radiolabeled compound loaded in K562/0 cells were 200 ± 19, 267 ± 19 and 220 ± 17 pmol/10^6 cells for control, cycloheximide and actinomycin D, respectively; the corresponding values in K562/PMEA-1 cells were 109 ± 36, 155 ± 27 and 96 ± 35 pmol/10^6 cells, respectively. All the data are expressed as the percentage of total radioactivity found in the extracellular medium and represent the means ± standard deviation of two independent experiments.

drug concentrations, the efflux mechanism needs more time to remove PMEA from the cells.

The basis of the resistant phenotype in K562/PMEA-1 cells differs completely from that in the L1210/PMEA-1 cell line, a PMEA/PMEDAP-resistant variant of murine leukemia L1210 cells; the latter acquire their resistance from a defective PMEA influx (Balzarini *et al*., 1998). In contrast, the K562/PMEA-1 cell line, as well as the PMEA-resistant CEM cell line established by Robbins *et al*. (1995), show a combination of enhanced PMEA secretion and a PMEA phosphorylation defect. The impaired PMEA phosphorylation was attributed to a decreased mitochondrial AMP kinase activity in the PMEA-resistant CEM cell line (Robbins *et al*., 1995). However, unlike K562/PMEA-1 cells, the PMEA-resistant CEM variant proved highly cross-resistant to PMEDAP and partially cross-resistant to PMEG, the guanine counterpart of PMEA (Robbins *et al*., 1995), pointing to a different specificity pattern of the efflux pump.

In striking contrast to the MDR phenomenon, the transport system responsible for enhanced PMEA efflux was particularly specific, as illustrated by the observation that it did not recognize the phosphorylated metabolites of azidothymidine nor adenosine, in spite of the structural resemblance of PMEA to AMP (Fig. 1). Via immunoblotting and flow cytometry, using monoclonal antibodies against P-gp and MRP, we have excluded the overexpression of one of those multidrug efflux pumps as the genetic basis for the pronounced PMEA resistance of K562/PMEA-1 cells. In agreement with this, several of our earlier observations already argued against a MDR phenotype in K562/PMEA-1 cells (Hatse *et al*., 1996). First, colchicine, vinblastine, and rhodamine 123, which are usually included in the cross-resistance spectrum of MDR cell lines, remained equally cytostatic to PMEA-resistant and wild-type K562 cells. Second, PMEA resistance in K562/PMEA-1 cells was not reversed by verapamil and cyclosporin A, which are strong inhibitors of P-gp with a modest effect on MRP function (Twentyman and Versantvoort, 1996), nor by genistein, which abrogates MRP-mediated MDR (Zaman *et al*., 1994; Twentyman and Versantvoort, 1996). On the other hand, the experimental data presented here demonstrate that PMEA efflux is substantially inhibited by the organic anion-transport inhibitor indomethacin. This finding is in full agreement with the 10-fold reduced PMEA resistance of K562/PMEA-1 cells in the presence of indomethacin. Thus, like the recently described efflux mechanism of the fluorochrome 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein in epithelial cells (Collington *et al*., 1992), the enhanced PMEA secretion by K562/PMEA-1 cells displays a pharmacological profile different from P-gp- or MRP-mediated drug efflux and might be a manifestation of a novel ATP-dependent, indomethacin-sensitive xenobiotic secretory mechanism. Also, it is noteworthy that Annaert *et al*. (1998) have found that bis(POM)-PMEA is a substrate for a verapamil-sensitive, P-gp-like carrier mechanism in Caco-2 monolayers, whereas mono(POM)-PMEA and PMEA are transported by a distinct anion-specific efflux protein that is inhibited by indomethacin.

In conclusion, we have demonstrated a highly specific, indomethacin-sensitive efflux pump that contributes to the pronounced PMEA resistance of K562/PMEA-1 cells. In addition, we have shown that PMEA phosphorylation is substantially decreased in the PMEA-resistant K562 cells. This phosphorylation defect is currently subject to further investigation. The particularly specific character of the mechanisms that may lead to PMEA resistance, as demonstrated for PMEA-resistant K562 cells (data presented herein), CEM cells (Robbins *et al*., 1995), and L1210 cells (Balzarini *et al*., 1998), represents an important distinction between PMEA as a candidate antineoplastic drug and the large class of MDR-susceptible chemotherapeutic agents, which are removed from resistant tumor cells by broad-spectrum efflux mechanisms such as P-gp and MRP.

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