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Inhibitory Effects of Prostaglandin A_1 on Membrane Transport of Folates Mediated by Both the Reduced Folate Carrier and ATP-Driven Exporters

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ABSTRACT. Studies are reported that describe the multifaceted inhibitory effects of prostaglandin A_1 (PGA_1) on processes that govern the transport of folates across the plasma membrane of Chinese hamster ovary (CHO) cells: the reduced folate carrier, RFC1, and ATP-dependent exporters. PGA_1 was a noncompetitive inhibitor of MTX influx mediated by RFC1 with a K_i of ~21 μM. The onset of inhibition was virtually instantaneous, not reversible, and appeared to require the incorporation of PGA_1 into the lipid membrane; surface adsorption alone was insufficient for inhibition of RFC1 transport activity. In contrast, the effect of PGA_1 on folic acid transport was small (~20% inhibition of total influx), consistent with the observation that the major portion of folic acid transport in CHO cells is mediated by a low pH mechanism distinct from RFC1. PGA_1 was also a potent inhibitor of the ATP-driven efflux of both MTX and folic acid. At a concentration of 7 μM PGA_1, the efflux rate constants for these folates were depressed by ~70 and ~50%, respectively. The net effects of PGA_1 on the bidirectional folate fluxes translated into marked alterations in net transport. The addition of 7 μM PGA_1 to cells at steady state with 1 μM MTX produced a rapid onset of net uptake and the achievement of an ~3-fold increase in the steady-state free MTX level as compared with untreated CHO cells. The addition of 7 μM PGA_1 to cells at steady state with l μM folic acid produced an ~5-fold increase in the free folate level. These studies establish PGA_1 as a potent inhibitor of both the reduced folate carrier and ATP-driven folate exporter(s). The noncompetitive nature of the inhibition of RFC1 is unique among anionic compounds, which are usually competitive inhibitors of the carrier. BIOCHEM PHARMACOL **58**;8:1321–1327, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. prostaglandin A_1; reduced folate carrier; (anti)folate transport; efflux transporters

Folate cofactors play a key role in biological processes as the sole one-carbon donors required for the synthesis of purine and pyrimidines and as determinants of the methylation state of nucleic acids through the synthesis of methionine [1, 2]. Mammalian cells cannot synthesize folates and must rely on their absorption from exogenous sources and subsequent cellular uptake [1, 2]. There are two dominant routes of transport of folate compounds in mammalian systems grown *in vitro* in usual levels of folic acid. The RFC1§ has the characteristics of a facilitative process that generates uphill transport of folates into cells through an exchange with organic anions that are concentrated within the intracellular compartment [3, 4]. This system has a high affinity for MTX and reduced folates (1–5 μM) and a very low affinity for folic acid (~200–400 μM) [3, 5], and is opposed by exit pumps directly linked to ATP hydrolysis [3, 6–8]. The transmembrane folate gradient is determined by the net effect of these two independent processes. When the exit pump is blocked by energy inhibitors, uphill folate transport is enhanced; when the exporter is energized, as with glucose, the transmembrane folate gradient is depressed [3, 9–11].

A variety of agents have been employed as inhibitors of RFC1-mediated folate transport. These include folate-based specific inhibitors such as the *N*-hydroxysuccinamide ester of MTX, which irreversibly blocks RFC1 activity [12]. RFC1 is inhibited by a variety of structurally unrelated inorganic and organic anions [3, 4, 14]; indeed, virtually any negatively charged species will inhibit influx mediated by this transporter if present at a sufficiently high concentration. These include, among others, probenecid, and the anion exchange stilbenedisulfonate inhibitor 4-acetamido-4′-isothiocyanatostilbene-2′, 2′-disulfonic acid [4, 13, 16]. Other compounds, also structurally unrelated to folates, are more potent inhibitors of MTX influx, such as CI-920, with a K_i of 30–60 μM [17], BSP, with a K_i of 2 μM, and

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§ *Abbreviations*: RFC1, reduced folate carrier; PGA_1, prostaglandin A_1; MTX, methotrexate; TMQ, trimetrexate; BSP, bromosulfophthalein; DNP-SG, 2,4-dinitrophenol-*S*-glutathione; HBS, HEPES-buffered saline; and CHO, Chinese hamster ovary.

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5′-cholesteryl-phosphorothioate oligodeoxynucleotides of varying polymer length, with K_i values as low as 0.2 μM [18].

Prostaglandins and related compounds play ubiquitous and important physiological, pathophysiological, and therapeutic roles in humans. Prostaglandins such as PGA_1 are charged organic anions at physiological pH and traverse cell membranes poorly [19]. There is evidence, based upon studies with inside-out membrane vesicles from L1210 leukemia cells, that there is an energy-driven folate exporter exceptionally sensitive to PGA_1 [7]. In the present paper we have demonstrated that PGA_1 is a potent inhibitor of both RFC1 and ATP-dependent folate exporters in intact CHO cells and that the impact of this agent on transmembrane folate gradients depends upon the net effect of the relative inhibition of these two independent processes.

MATERIALS AND METHODS

Chemicals

[3′, 5′, 7′, 9-^{3}H]Folic acid and [3′, 5′, 7′-^{3}H]MTX were obtained from Amersham. Radiolabeled and unlabeled folic acid and MTX were purified prior to use by high performance liquid chromatography [20]. Folic acid and MTX were obtained from the Sigma Chemical Co. TMQ was purchased from Warner-Lambert, Parke-Davis. PGA_1 was obtained from the Cayman Chemical Co. Stock solutions of PGA_1 (20 mM) were prepared in ethanol and stored at −20°. In all transport experiments the final concentration of ethanol did not exceed 0.5%, a level that had no effect on folate transport in CHO cells.

Cell Cultures

Parental CHO AA8 cells were maintained in monolayer or suspension culture conditions in RPMI-1640 medium containing 2.3 μM folic acid (HyClone), supplemented with 5% dialyzed fetal bovine serum (Gemini Bio-Laboratories Inc.), 1 mM sodium pyruvic acid (Mediatech), 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Folic Acid and MTX Transport

Transport measurements were performed as before with some modifications [21]. Exponentially growing cells from spinner flask suspension cultures were collected by centrifugation (750 *g* for 2 min), and washed three times with ice-cold HBS (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM glucose). Cells were resuspended to a density of 2–6 × 10^7 cells/mL in 1 mM pyruvate-containing HBS. TMQ (5 μM) was always present in studies with folic acid to ensure complete blockade of folic acid reduction by dihydrofolate reductase [8].

Following 20 min of incubation at 37°, uptake was initiated by the addition of [^{3}H]folic acid or [^{3}H]MTX; portions of the cell suspension (0.5 to 1.0 mL) were drawn at given times, and uptake was terminated by injection into 10 mL of ice-cold HBS. After centrifugation (750 *g* for 2 min) at 4°, cells were resuspended in ice-cold HBS, washed twice in the same buffer, and processed for determination of intracellular radiolabel with normalization to dry weight [21]. Efflux measurements were performed by first loading cells with 1 μM [^{3}H]folic acid or 1–2 μM [^{3}H]MTX for 30 min at 37°. Then, a portion of the cells was removed for determination of the intracellular folate level. The remaining radiolabeled cells were sedimented by centrifugation (as above) and resuspended in a large volume of pre-warmed HBS, following which portions of cell suspension were drawn at given times and processed as detailed above for cellular radiolabel. The free MTX level was the difference between total MTX and the level of MTX remaining in cells after efflux for 30–60 min, a time sufficient to allow for the loss of all free drug. All intracellular folic acid was unmetabolized and was considered to be exchangeable in the presence of TMQ [8]. Data are expressed as means ± SEM.

RESULTS

Effects of PGA_1 on Influx of Folates

PGA_1 produced a concentration-dependent inhibition of [^{3}H]MTX influx. At an extracellular MTX level of 1 μM, the IC_{50} for PGA_1 was 10 ± 2 μM, and the maximum inhibitory effect was achieved at drug concentrations in excess of 25 μM (Fig. 1A). The component of MTX influx not inhibitable by PGA_1 was 21.1 ± 2.3% of the rate in control cells of 0.331 ± 0.069 nmol/g dry weight/min (based upon three experiments). In contrast, 25 μM PGA_1 had only a marginal inhibitory effect (~19%) on folic acid influx, with an uninhibitable component of 81.3 ± 1.8% of the total flux of 59.1 ± 29.6 pmol/g dry weight/min (based upon three experiments as in Fig. 1B). The very low level of inhibition of folic acid influx by PGA_1 was consistent with the observation that folic acid uptake in these cells is mediated largely by mechanisms other than RFC1, in particular, a folate transporter that operates optimally at low pH [22]. The basis for the component of MTX influx not inhibitable by PGA_1 is not clear but also must be related to an additional transport route.

The inhibitory effect of PGA_1 on MTX influx was essentially instantaneous. The degree of MTX influx inhibition upon simultaneous addition of 25 μM PGA_1 and 1 μM [^{3}H]MTX, or when PGA_1 was added 15 min prior to MTX, was the same. The effects of PGA_1 were not reversible. When cells were incubated with 25 μM PGA_1 for 15 min, then washed and exposed to MTX in PGA_1-free buffer, suppression of influx was as potent as in cells continuously exposed to PGA_1. This did not occur, however, when cells were preincubated with PGA_1 at 0° (Fig. 2), indicating that inhibition required incorporation of

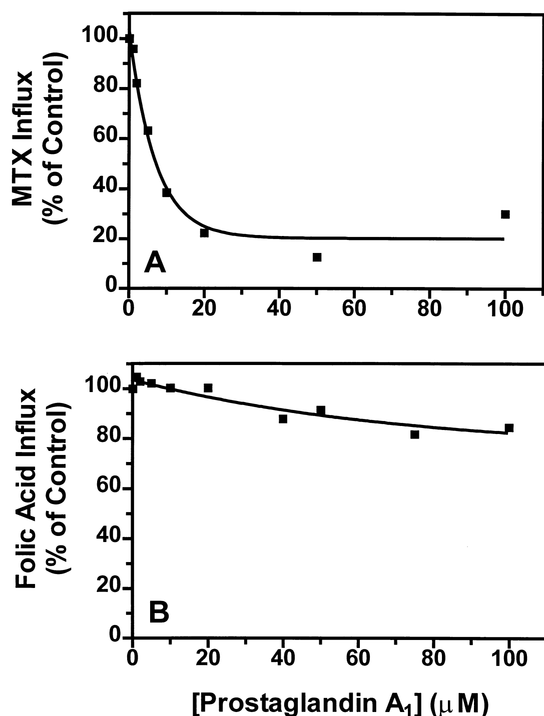

FIG. 1. Effects of PGA$_1$ on the influx of MTX (A) and folic acid (B) in parental CHO AA8 cells. Initial rates of uptake were determined over 2–3 min after the addition of 1 μM folate. Cells exposed to 1 μM [^{3}H]MTX (A) or 1 μM [^{3}H]folic acid (B) but no PGA$_1$ served as the 100% influx value, from which the percentage of folate transport at each PGA$_1$ concentration was determined. In all folic acid influx experiments, 5 μM TMQ was included to block folic acid reduction by dihydrofolate reductase. The data in each panel are representative of three experiments.

PGA$_1$ into the cell membrane; surface adsorption, alone, was not sufficient to suppress RFC1 activity.

The kinetics of PGA$_1$ inhibition of MTX influx is illustrated by the Dixon plot of Fig. 3. Inhibition was noncompetitive, with a K_i of 21 ± 4 μM (based upon four experiments). Hence, the data indicated that PGA$_1$ bound to a site in RFC1 distinct from the binding site for folates, resulting in inhibition of carrier function.

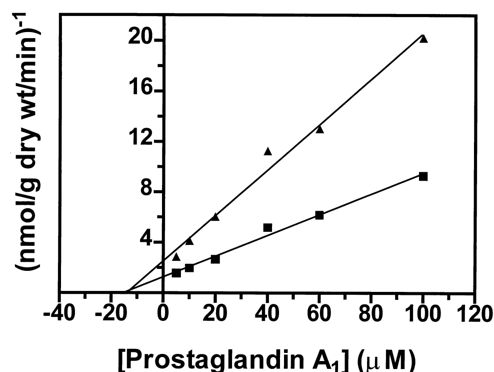

FIG. 3. Dixon analysis for the determination of the PGA$_1$ K_i based on inhibition of MTX influx. Initial rates of MTX uptake were determined in the presence of 5–100 μM PGA$_1$ using [^{3}H]MTX substrate concentrations of 1 μM (triangles) and 2.5 μM (squares). The inhibition constant (K_i) was calculated from the interception point of the lines extrapolated perpendicularly to the abcissa. These data are representative of four separate experiments, in which the mean K_i value for noncompetitive inhibition of MTX influx by PGA$_1$ was 21 ± 4 μM (SEM).

Effects of PGA$_1$ on MTX and Folic Acid Efflux

Whereas MTX influx was mediated almost exclusively by RFC1, efflux is mediated by both RFC1 and one or more energy-dependent exporters. As indicated in Fig. 4A, 7 μM PGA$_1$ suppressed the efflux rate constant for the free intracellular MTX component by ~70%, to 0.057 ± 0.012 min^{-1} as compared with 0.208 ± 0.073 min^{-1} in control cells (based upon three experiments). As indicated in Fig. 4B, 7 μM PGA$_1$ inhibited the efflux rate constant for folic acid by ~50%, to 0.0551 ± 0.001 min^{-1} as compared with the control value of 0.111 ± 0.008 min^{-1} (based upon

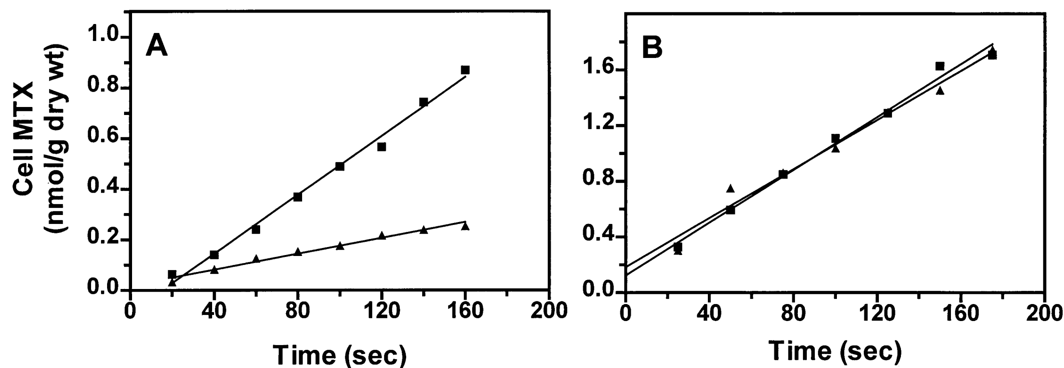

FIG. 2. Reversibility and temperature-dependence of PGA$_1$ inhibition of MTX influx. (A) AA8 cells were incubated with 50 μM PGA$_1$ at 37° for 15 min, then washed in HBS at 4° and resuspended in PGA$_1$-free buffer at 37°. Influx of 1 μM MTX was measured (triangles) and compared with MTX influx in control cells subjected to the same procedures but in the absence of PGA$_1$ (squares). (B) Cells were incubated with 30 μM PGA$_1$ at 0° for 15 min, washed with PGA$_1$-free HBS at 4°, and resuspended at 37°; then, influx of 1 μM MTX was monitored (squares). Control cells were subjected to the same procedures in the absence of PGA$_1$ (triangles). The data are representative of three experiments.

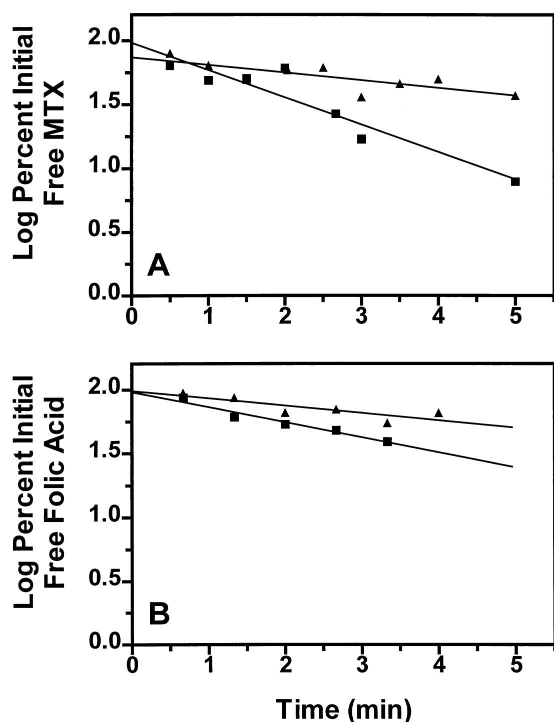

FIG. 4. Effect of PGA_1 on the efflux of MTX (A) or folic acid (B). AA8 cells were loaded in the presence of 1–2 μM [^{3}H]MTX or 1 μM [^{3}H]folic acid for 30 min at 37°, then washed at 0° and resuspended in folate-free HBS at 37° either with 7 μM PGA_1 (triangles) or without PGA_1 (squares); finally, efflux of radiolabel was monitored. Efflux is plotted as the log of the percentage of initial free folate in the presence or absence of PGA_1 as a function of time. TMQ (5 μM) was included during folic acid loading and efflux. Each panel is representative of three experiments.

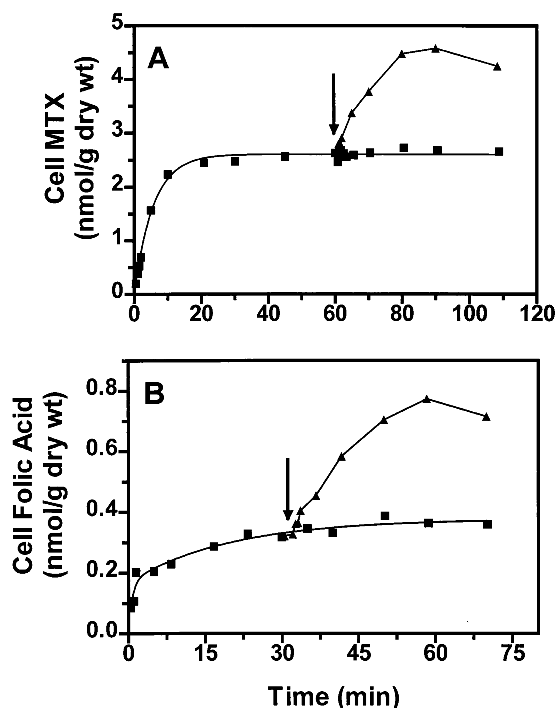

FIG. 5. Effect of PGA_1, added when cells were at steady state with folates, on net uptake of MTX and folic acid. Following a 1-h incubation with 1 μM [^{3}H]MTX (A), or a 40-min incubation with 1 μM [^{3}H]folic acid (B) (squares), PGA_1 was added (arrow) to achieve a final concentration of 7 μM, and cell folate was monitored (triangles) for an additional 30–45 min. In the folic acid transport study, 5 μM TMQ was present during the entire experiment to block metabolism to reduced derivatives. The data in panels A and B are each representative of three experiments.

three experiments). Hence, PGA1 was a potent inhibitor of the efflux of both folates.

Effects of PGA_1 on MTX and Folic Acid Transmembrane Gradients

PGA_1 resulted in suppression of the bidirectional fluxes of folates; however, the change was not symmetrical and was determined by the relative effects on carrier versus the ATP-driven export pump(s). As illustrated in Fig. 5A, when cells were brought to steady state with 1 μM MTX, following which 7 μM PGA_1 was added, there was a very rapid onset of net MTX uptake, and an ~3-fold increase in the free intracellular MTX level was achieved (based upon the difference between total and bound MTX). With folic acid, as illustrated in Fig. 5B, there was also a rapid initiation of net uptake upon the addition of PGA_1 and the peak folic acid level achieved was substantially increased.

The composite effects of PGA_1 on the overall net uptake process are illustrated in Fig. 6 under conditions in which inhibitor was added prior to the radiolabeled folate. Net uptake of folic acid was increased rapidly because there was little suppression of folic acid influx by 7 μM PGA_1 (Fig. 6A). By 50 min the net folic acid level in the PGA_1-treated cells markedly exceeded that of control cells. The pattern was different for MTX; at 7 μM, PGA_1 inhibited the initial uptake rate of MTX by approximately 50%, but net uptake ultimately exceeded that of control cells due to the concurrent, and more potent, inhibition of the export pump (Fig. 6B). The greater effect on net folic acid uptake in comparison with MTX uptake was expected, since inhibition of folic acid efflux by PGA_1 was unopposed by suppression of influx, as was the case for MTX.

DISCUSSION

PGA_1 is an agent that is structurally unrelated to the folates but is a potent inhibitor of two distinct processes that mediate the membrane transport of these compounds, RFC1 and one or more energy-driven exporters. The inhibitory effect of PGA_1 on RFC1 was confirmed by potent inhibition of MTX influx. Consistent with a selective effect of PGA_1 on RFC1 was its weak inhibitory effect on folic acid influx, which is mediated largely by another route that has a low pH optimum and accounts for a major component of folic acid transport at physiological pH; as reported recently, PGA_1 does not inhibit this process [22].

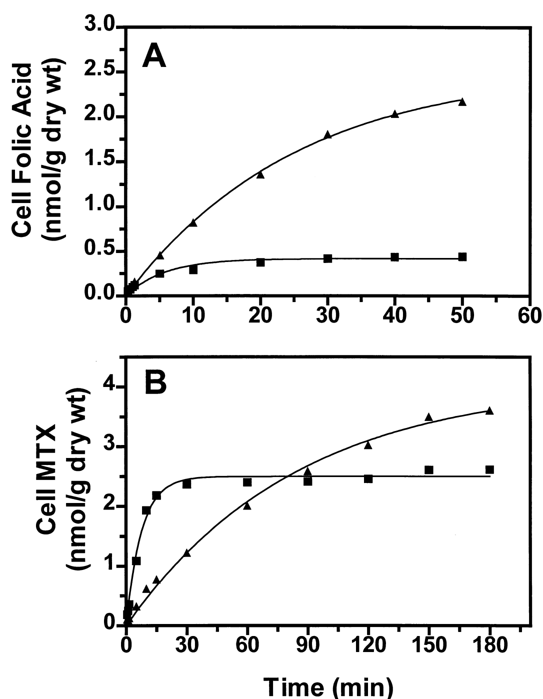

FIG. 6. Effect of PGA_1 on the net transport of folic acid (A) or MTX (B). Following a 3-min preincubation with (triangles) or without 7 μM PGA_1 (squares), [^{3}H]folic acid or [^{3}H]MTX was added to achieve a final concentration of 1 μM, and net uptake was monitored for 50 min or 3 hr, respectively. The data in panels A and B are representative of three and four experiments, respectively.

The basis for the component of MTX influx not inhibitable by PGA_1 is not clear. Such a component, although of a smaller magnitude, was observed in the Pyr^{R100} cells. This may represent, to some extent, transport mediated by the low pH route or some other process in CHO cells not as yet identified.

PGA_1 is an amphipathic compound with a cyclopentane ring and two partially unsaturated side chains, one of which terminates in a negatively charged carboxyl group. As with many other anionic compounds, prostaglandins do not cross biological membranes unless a prostaglandin transporter is expressed [23]. The onset of PGA_1 inhibition of MTX influx was very rapid and did not occur when cells were exposed transiently to this agent at 0° before the addition of MTX. The effect of PGA_1 on influx was persistent even when it was removed from the transport buffer. These observations suggest that PGA_1 was incorporated into, and was retained within, the cell membrane, consistent with its high lipid solubility (log $P = 2.7$) [24], and that its presence within this lipid bilayer compartment was sufficient to achieve inhibition of RFC1. Adsorption of PGA_1 to the surface of the cell membrane, alone, is insufficient to inhibit influx. It is likely that the hydrophobic cyclopentane ring is incorporated into the lipid bilayer, while the negatively charged carboxyl moiety protrudes from the cell membrane into the extracellular milieu where it presumably interacts with the carrier.

While influx of MTX is mediated virtually exclusively by RFC1, efflux of folates has been proposed to be mediated by carrier and at least two other energy-dependent unidirectional processes in L1210 cells [7, 8, 13, 16, 25]. The major exporter accounts for ~70% of MTX efflux in that system, exports cholic acid, and is inhibited by a variety of agents including PGA_1, indomethacin, BSP, and ethacrynic acid [25]. A second route has been proposed in L1210 cells that does not transport cholic acid but is inhibited by 4-biphenylacetic acid, indoprofen, and flurbiprofen. This latter route is also sensitive to PGA_1, but to a lesser extent [26]. Recent studies with inside-out vesicles isolated from murine L1210 leukemia cells demonstrate two ATP-driven efflux exporters that can be discriminated by their high ($K_m = 0.6$ μM) and low ($K_m = 450$ μM) affinity for DNP-SG [7]. PGA_1 blocks efflux of DNP-SG in inverted vesicles from L1210 cells, consistent with the inhibition of these GS-X pumps [7]. In early studies with a variety of cell lines including WI-38 human fibroblasts, C-6 rat glioma cells, as well as avian erythrocytes, PGA_1 (at 30 μM) also was found to be a potent inhibitor of the energy-dependent efflux of cyclic AMP [27]. Further, PGA_1 undergoes glutathione conjugation in at least some mammalian cells and, therefore, would be a substrate for GS-X pumps. While the inhibitory effects of PGA_1 may be related to a direct interaction with the folate exporter, it is also possible that PGA_1 is taken up into these cells via a specific independent transporter [23], following which it is converted to its glutathione conjugate, which, in turn, inhibits folate efflux via an energy-dependent exporter.

Recently, we characterized a CHO cell line, Pyr^{R100}, which lost folate exporter function under pyrimethamine selective pressure [8]. These cells accumulate high levels of folic acid and MTX due to a marked reduction in their energy-dependent efflux. Interestingly, the rate constant for MTX efflux in Pyr^{R100} cells (0.04 min^{-1}) [8] is similar to the rate constant for MTX efflux in parent CHO cells treated with PGA_1 (~0.06 min^{-1}) determined in this study, and also similar to the rate constant for MTX efflux in energy-deprived L1210 cells (0.036 min^{-1}) [9, 28], consistent with the loss of PGA_1-sensitive exporter function in Pyr^{R100} cells. PGA_1 markedly augments net folic acid and MTX uptake in parental CHO AA8 cells, also consistent with inhibition of the exporter. Augmentation of net uptake is more profound for folic acid since there is no inhibitory effect of PGA_1 on influx; hence, the block of folic acid efflux is unopposed.

The observations reported in this paper, along with other studies, must be interpreted within the context of reports that have established a superfamily of ABC exporters in a variety of mammalian cells. These include the Multidrug Resistance Proteins (MRP1) [29, 30] and the highly homologous liver canalicular Multispecific Organic Anion Transporter (cMOAT or MRP2) and ATP-driven GS-X pumps that transport various anionic conjugates of glutathione [31–35]. Recently, the liver canalicular cMOAT has been shown to export MTX into the bile [36]. Taken

together, the data suggest that the ATP-driven exporter(s) for folates in CHO cells is, or is closely related to, the MRP1 and/or MRP2 (cMOAT) family of ABC transporters and that PGA_1 blocks one or both of these processes. Indeed, in a recent study, transfection of MRP1 and MRP2 in human ovarian carcinoma cells decreases net cellular accumulation of MTX and results in drug resistance [37].

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