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Differential reversal of lipophilic antifolate resistance in mammalian cells with modulators of the multidrug resistance phenotype

Yehuda G Assaraf and Mario J Borgnia

Department of Biology, Technion—Israel Institute of Technology, Haifa 32000, Israel. Tel: (+972) 4 293744; Fax: (+972) 4 225153.

Chinese hamster ovary (CHO) T19 cells express a stable P-glycoprotein (P-170)-dependent multidrug resistance (MDR) phenotype and display a 24- to 29-fold cross-resistance to the lipophilic antifolates piritrexim (PTX) and trimetrexate (TMTX). We have examined the ability of various modulators of the MDR phenotype to sensitize T19 cells to TMTX and PTX in a clonogenic assay. An almost complete reversal of TMTX resistance in T19 cells was achieved with several modulators of the MDR phenotype whereas only a partial sensitization of T19 cells to PTX was obtained with the most potent modulator. In an attempt to explore the apparent P-170-independent locus of protection against PTX, resistant T19 sublines were isolated after stepwise selection with PTX and TMTX. Thus, T19 cells made resistant to PTX displayed a dramatic decreaase in P-170 mRNA levels despite the maintenance of the parental T19 MDR gene amplification, whereas T19 cells selected for TMTX resistance exhibited a further increase in P-170 mRNA levels. Hence, the modulation experiments together with the established lipophilic antifolate-resistant T19 variants suggest that although T19 cells possess a P-170-dependent MDR phenotype and display a similar cross-resistance to TMTX and PTX, the protective pathway need not be necessarily via P-170. Rather, a pathway appears to exist that protects T19 MDR cells from the cytotoxicity of PTX without requiring a P-170 function.

Key words: Dihydrofolate reductase, lipophilic antifolates, modulators, multidrug resistance, P-glycoprotein.

Introduction

Combination chemotherapy plays a key role in the treatment of various human cancers.[1] Methotrexate (MTX), the 4-amino analog of folic acid, is an integral component of chemotherapy currently used in the cytotoxic treatment of a variety of human malignancies. MTX, which binds dihydrofolate reductase (DHFR) with a high affinity, restricts the availability of tetrahydrofolate, a key cofactor that serves as a one-carbon donor in a variety of biosynthetic reactions that result in the formation of purines, thymidylate and glycine. Unfortunately, as with various chemotherapeutic agents, the pre-existence and/or acquisition of antitumor drug resistance including MTX remains a major obstacle toward curative cancer chemotherapy. Among the well established mechanisms of resistance to MTX are qualitative and/or quantitative alterations in the carrier-mediated transport system of MTX,[2,3] alterations in the affinity of DHFR for MTX[4,5] or alterations in the cellular retention of MTX polyglutamates.[6–9] It was shown that these modes of antifolate-resistance that are mechanistically distinct could simultaneously coexist within single MTX-resistant animal cells.[2,4,6,7,9–11]

In one attempt to overcome well-defined mechanisms of MTX-resistance that result from transport alterations as well as from deficient formation of MTX polyglutamates, lipophilic analogs of MTX including piritrexim[12] (PTX) and trimetrexate[13] (TMTX) have been introduced. PTX and TMTX are devoid of the glutamate residue present in MTX and contain two or three methoxy groups, respectively, which render them highly lipophilic, thereby enabling their accumulation in mammalian cells by simple and/or a facilitated diffusion process.[12–14] PTX and TMTX are currently undergoing phase II clinical testing, as they have initially shown activity against MTX-resistant mammalian cells as well as against infections with parasitic protozoa including *Toxoplasma gondii*[15] and *Pneumocystis carinii*.[16]

Unfortunately, mammalian cells acquire resistance also to TMTX and PTX by a variety of mechanisms including: (i) Amplification of DHFR and subsequent overproduction of the enzyme.[17] (ii) multidrug resistance (MDR) gene amplification, subsequent overexpression of P-170 and exhibition of a typical MDR phenotype.[17,18] (iii) Coexistent

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Correspondence to YG Assaraf

amplification of the DHFR and MDR genes in single mammalian cells selected for TMTX resistance.[17] (iv) A yet undefined lipophilic antifolate resistance pathway that is independent of quantitative and qualitative alterations in DHFR and that is distinct of the MDR phenotype.[19] (v) A novel route of lipophilic antifolate resistance that involves a profoundly reduced accumulation of lipid-soluble antifolates including 2,4-diaminopyrimidines, while maintaining wild-type sensitivity to MTX.[20] Thus, as mammalian cells with a P-170-dependent MDR phenotype display a similar level of cross-resistance both to TMTX and PTX, only TMTX could select for MDR gene amplification and an MDR phenotype,[17] we performed this study in an attempt to determine whether the resistance of MDR cells to TMTX and PTX is solely achieved via a P-170-dependent pathway. Toward this end we have, on the one hand, employed a variety of well-established modulators of the MDR phenotype and found that while TMTX-resistance in MDR cells could be almost fully reversed by efficient modulators such as reserpine, resistance to PTX could be, in the best case, only partially overcome. On the other hand, T19 MDR cells challenged with increased cytotoxic pressure with TMTX showed a further P-170 overexpression. In contrast, MDR cells further selected with PTX, displayed a dramatic decrease in P-170 despite the maintenance of the original amplified MDR genes. Hence, these results suggest that a P-170 independent pathway(s) of resistance exists which protects T19 MDR cells from the cytotoxic effect exerted by PTX.

Materials and methods

Drugs

MTX was from Abic (Netanya, Israel). TMTX glucoronate was a gift from Warner-Lambert/Parke-Davis (Ann Arbor, MI), whereas PTX isethionate and metoprine were kindly provided by Burroughs Wellcome (Research Triangle Park, NC). Reserpine, verapamil hydrochloride, quinidine hydrochloride, quinine hydrochloride, dipyridamole, Tween 80, pyridoxal 5-phosphate and L-ornithine hydrochloride were from Sigma (St Louis, MO).

Cell cultures

A clonal isolate of wild-type Chinese hamster ovary (CHO) AA8 cells was maintained under monolayer conditions in α-minimal essential medium (α-MEM; Biological Industries, Beth Haemek, Israel) containing 5% dialyzed fetal calf serum (Beth Haemek), 2 mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (Sigma). T19 is a clonal derivative isolated by single dose exposure of parental AA8 cells to 25 nM TMTX[19] and exhibited a typical MDR phenotype.[21] T19 cells were grown in TMTX-free medium for over 1600 cell doublings after which their MDR gene copy number was determined in order to examine whether they maintained the authentic MDR gene amplification. Wild-type AA8 and T19 MDR cells were passaged biweekly using a standard trypsinization procedure.

Drug selection protocols

In order to establish antifolate-resistant and pleiotropic drug-resistant variants, T19 cells (5×10^4–10^5/25 cm^2 tissue culture flask) were treated with various cytotoxic drugs in a multiple step selection protocol. Selection was initiated at the LD_{50} of each cytotoxic agent (unless otherwise stated). For antifolate selection T19 cells growing in α-MEM medium containing 5% dialyzed fetal calf serum were initially selected with 30, 50 and 100 nM of MTX, TMTX and PTX, respectively, and selection was terminated at 5, 10 and 50 μM of the respective antifolates. For pleiotropic drug selection, T19 cells were selected with an initial concentration of 80 nM vinblastine and 2.5 μM colchicine; selection was terminated at 0.5 μM vinblastine and 10 μM colchicine. Following growth at 37°C in a humidified atmosphere of 5% CO_2 and whenever monolayer cells reached midconfluency, they were detached by trypsinization, counted and replated at the initial density and challenged with a 25–100% increment in the cytotoxic drug concentration. These selections yielded several variants including MTX^{R5}, $TMTX^{R10}$, PTX^{R50}, $Vin^{R0.5}$ and Col^{R10}. Drug-resistant variants were considered established only after growth for many passages in drug-containing medium and after showing no evidence of cell death as monitored by Trypan blue exclusion.

Cytotoxicity assays and colony scoring

To assess cellular sensitivity to the various antifolates parental AA8 and MDR T19 cells were grown for six or more doublings under non-

selective conditions (i.e. lacking TMTX). Then, 10^3 cells were seeded in 6 cm Petri dishes (in duplicates) in growth medium (5 ml/dish) lacking or containing various concentrations of lipophilic or hydrophilic antifolates. Then, following 5–12 days of incubation at 37°C, plates containing visible colonies (colony of 50 or more cells) were washed once with phosphate buffered saline (5 ml/dish), fixed with methanol and stained with crystal violet. Colonies were scored with the aid of a computer-controlled image scanner (Dest PC Scan 3000). Groups of four plates were placed on the scanner, a white paper (Whatman 3MM Chr) was used to obtain the appropriate background for the colonies immobilized onto the plates. Plates were then scanned and the images were stored as binary files. Resolution values between 250 and 300 dots per squared inch were used. The image files were processed in a PC compatible computer using a program designed to identify the plates, count the number of colonies present and to report this number for each plate. The LD_{50} for each experiment was determined from the reported data.

Modulation experiments

To examine the ability of various modulators to sensitize T19 MDR cells to different antifolates we have included non-cytotoxic concentrations of these modulators in clonogenic assays. Thus, the cytotoxicity of the various modulators themselves to parental AA8 and T19 cells was first examined. Then, non-toxic micromolar concentrations were employed in experiments aimed to reverse the resistance of T19 MDR cells to various antifolates. The potentiation index is defined here as the ratio of the 50% lethal dose values in the absence and presence of a modulator, for a given antifolate using a defined MDR cell line. The consequent relative potency values were thus obtained by determination of the modulator concentration that yields 50% potentiation by lipophilic antifolate cytotoxicity in MDR T19 cells and by an arbitrary attribution of a relative potency value of 1.0 to Tween 80. Relative potency values proved useful in evaluating and comparing the efficiency of reversal of lipophilic antifolate cytotoxicity in MDR T19 cells on a molar basis.

Determination of MDR and DHFR gene copy numbers and mRNA levels

High molecular weight genomic DNA and cytoplasmic RNA were prepared from nuclear and cytoplasmic fractions, respectively, isolated from 10^7–4×10^7 cells by a single step centrifugation on a sucrose cushion.[22] Hamster MDR gene copy number was determined by Southern blot analysis of *Eco*RI-digested DNA using a ^{32}P-oligolabeled[23] 0.66kb *Eco*RI insert of the CHO MDR cDNA clone pCHP-1.[24] Steady-state levels of P-170 mRNA were determined by Northern blot analysis using this probe. DHFR gene copy number and mRNA levels were detected using a 0.93 *Pst*I insert of a hamster DHFR cDNA clone, MQ19-97.[25] The cDNA probes were purified by electrophoresis on low-melting agarose gel (Sigma) prior to random hexamer priming.[23]

Results

CHO T19 cells were originally isolated by a single dose exposure of wild-type AA8 cells to 25 nM TMTX[20] (about 4-fold the LD_{50}). These cells were grown in the absence of TMTX for 1600 cell doublings; we have therefore re-examined the MDR gene copy number and subsequent P-170 mRNA levels in T19 cells. Scanning densitometry of the autoradiograms obtained by Southern and Northern blot analyses revealed that T19 cells that were grown under non-selective conditions, possessed 17 copies of the MDR gene (Figure 1A) and overexpressed 55-fold more P-170 mRNA relative to parental AA8 cells (Figure 1B). Thus, these results show that T19 cells maintain a stable MDR gene amplification even after a long-term growth under non-selective conditions. Furthermore, these results are in accord with our recent findings that T19 cells displayed a 16- to 84-fold resistance to a variety of anticancer drugs of the MDR group.[21] This stable MDR phenotype presents an important advantage as T19 cells could be grown continuously in antifolate-free medium thus rendering modulation experiments using clonogenic assays more accurate. We have therefore extended the cytotoxicity experiments and assessed the clonogenic survival of parental AA8 and T19 cells in the presence of various lipophilic and hydrophilic antifolate concentrations (Figure 2). The mean ($n = 3$) LD_{50} values of TMTX, PTX, metoprine and MTX for T19 cells were approximately 69, 113, 34 and 31 nM, whereas those for AA8 cells were 2.4, 4.6, 13 and 25 nM, respectively. Thus, relative to parental AA8 cells, T19 MDR cells exhibited 29-, 24- and 2.6-fold resistance to TMTX, PTX and metoprine, while maintaining sensitivity to the parent hydrophilic antifolate MTX. Thus, mamma-

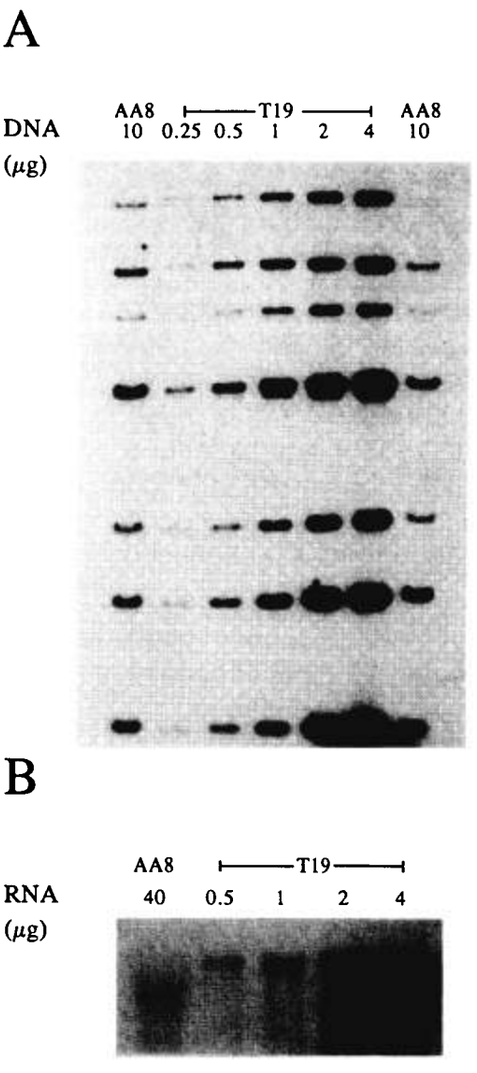

Figure 1. Autoradiograms of Southern and Northern blots containing DNA and RNA from wild-type AA8 cells and their MDR T19 subline probed with a hamster MDR cDNA sequence. (A) Genomic DNA extracted from AA8 and MDR T19 cells was digested with *Eco*RI, fractionated on 0.8% agarose gels and transferred to Zetabind filter membrane. (B) Cytoplasmic RNA isolated from wild-type AA8 cells as well as from T19 MDR cells was fractionated on a 1.5% agarose–formaldehyde gel and transferred to a Zetabind membrane. Blots were then hybridized with a ^{32}P-oligolabeled CHO MDR cDNA probe (0.66 kb *Eco*RI insert of pCHP-1). Hybridizations were carried out at 42°C for 48 h in 50% formamide, 5 × saline–sodium phosphate–EDTA (SSPE), 1% SDS and 1 × Denhardt's (1 × = 20 mg polyvinylpyrolidone-360/20 mg Ficoll/20 mg bovine serum albumin/100 ml of distilled water). High stringency washes at 65°C for 1 h in a solution of 0.1 × SSPE/0.1% SDS were performed. Following autoradiography, scanning densitometry was performed on linear film exposures in order to determine MDR gene copy number and P-170 mRNA levels.

lian cells with a typical MDR phenotype display a similar level of significant cross-resistance to TMTX and PTX and a modest resistance to the 2,4-diaminopyrimidine metoprine, yet only the former could select for MDR gene amplification.[17,19] In an attempt to understand this discrepancy between the apparent participation of PTX in an established MDR phenotype and its inability to provoke a P-170-dependent MDR phenotype, we have questioned here whether the resistance of T19 MDR cells to TMTX and PTX is mediated solely via a P-170-dependent pathway. Thus, in one approach to resolve this discrepancy, we thought to reverse the cross-resistance to TMTX and PTX in T19 MDR cells with well-established modulators of the MDR phenotype. These modulators are believed to function as competitive inhibitors of the P-170 efflux transporter.[26–29] Thus, in order to work with subcytotoxic concentrations of modulators, we have first examined the sensitivity of parental AA8 cells and their MDR T19 cells to a wide range of known modulators of the MDR phenotype. Table 1 shows that T19 MDR cells maintained wild-type sensitivity to most of the modulators whereas displaying to a few of them either slightly increased sensitivity or a modest resistance. Thus, we have selected a number of modulators that could be used at concentrations of 5 μM or above without exerting a cytotoxic effect on either parental AA8 or T19 MDR cells. Thus, the cytotoxicity of TMTX and PTX was examined in the presence of non-toxic concentrations of reserpine, verapamil and quinidine. A dose-dependent sensitization of T19 MDR

Table 1. Sensitivity of wild-type AA8 cells and their subline T19 to various modulators of the MDR phenotype

Modulator	LD_{50}(μM)		T19/AA8
	AA8	T19	
Cefoperazone	>300	>300	1
Chloroquine	23	34	1.5
Cremophor EL	221	362	1.6
Dipyridamole	12.2	11.2	0.92
Perhexiline maleate	4.5	3.6	0.80
Progesterone	40	16.5	0.41
Quinidine	99.4	97.6	0.98
Quinine	60.4	283	4.7
Reserpine	13	13	1
Trifluoperazine	5	2.1	0.42
Tween 80	182	298	1.64
Verapamil	70	52.8	0.75

LD_{50} values represent the means of at least two experiments.

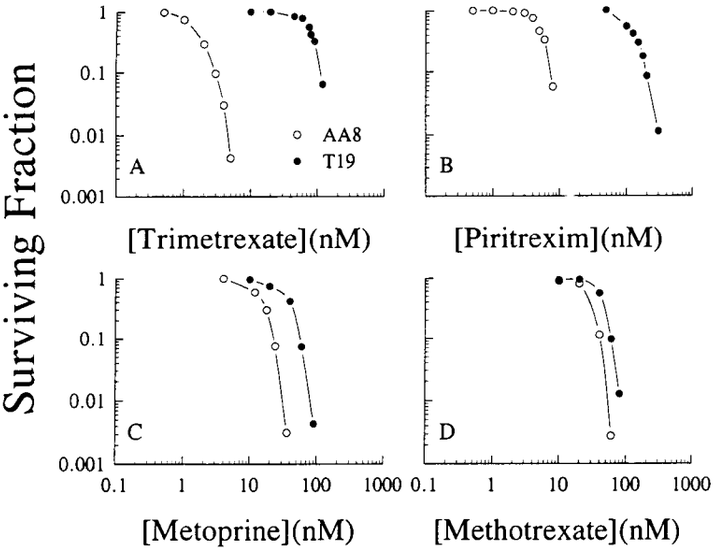

Figure 2. Cytotoxicity of lipophilic and hydrophilic antifolates to parental AA8 cells and their MDR T19 subline. Cells (10^3) were exposed to various concentrations of TMTX (A), PTX (B), metoprine (C) and MTX (D) after which the clonal surviving frequency was determined as given in details under Materials and methods. Open and closed circles represent AA8 and T19 MDR cells, respectively.

cells to TMTX was obtained with these potentiators (Figure 3A–C). In contrast, although a dose-dependent potentiation of PTX cytotoxicity was obtained with these modulators, the maximal reversal achieved with the most efficient modulator, reserpine, was only partial and did not exceed 40% (Figure 3D–F). Thus, while an almost complete sensitization of T19 MDR cells to TMTX was achieved with some of the modulators of the MDR phenotype, resistance to PTX could be in the best case only partially overcome. Although T19 cells are similarly cross-resistant to TMTX and PTX, they display a distinct pattern of sensitization to these related lipophilic antifolates with modulators of the MDR phenotype. This suggests that a major component of PTX-resistance in T19 MDR cells is apparently P-170-independent.

We have extended the lipophilic antifolate reversal analysis to additional agents that have been shown to display variable degrees of sensitization of MDR cells to pleiotropic anticancer drugs. Thus, as quinidine is the dextrostereoisomer of the parent cinchona alkaloid quinine, the latter was also examined in its ability to sensitize T19 MDR cells to TMTX and PTX. Quinine showed a dose-dependent sensitization of T19 MDR cells to TMTX, yet without achieving a complete reversal (Figure 4A). In contrast, none of the resistance to PTX in T19 MDR cells could be reversed by quinine (Figure 4D). Additionally, dipyridamole (Figure 4B and E), an inhibitor of nucleoside membrane transport[30] and Tween 80 (Figure 4C and F), a non-ionic detergent, both of which are modulators of the MDR phenotype showed a poor modulation effect of both TMTX and PTX resistance in T19 MDR cells. Cefoperazone, chloroquine and cremophor EL failed to yield any sensitization effect with TMTX or PTX on T19 MDR cells (data not shown). Thus, consistent with the above reversal data, modulators such as quinine that sensitizes MDR cells to pleiotropic drugs also sensitize T19 MDR cells to TMTX but fail to yield any potentiation effect of PTX cytotoxicity in T19 MDR cells. The sensitization of wild-type AA8 cells to TMTX and PTX with various modulators was minimal (up to 3-fold) and was dose-independent at the concentrations used (Figures 3 and 4A–F).

We have also examined the effects of various modulators on the modest resistance (2.6-fold) of T19 MDR cells to metoprine, the prototype of lipophilic antifolates, and on the cytotoxicity of MTX to AA8 and T19 MDR cells. Interestingly, rather than sensitizing cells, these modulators conferred upon parental AA8 and T19 MDR cells a similar level of protection against metoprine cytotoxicity (Figure 5A). These modulators either

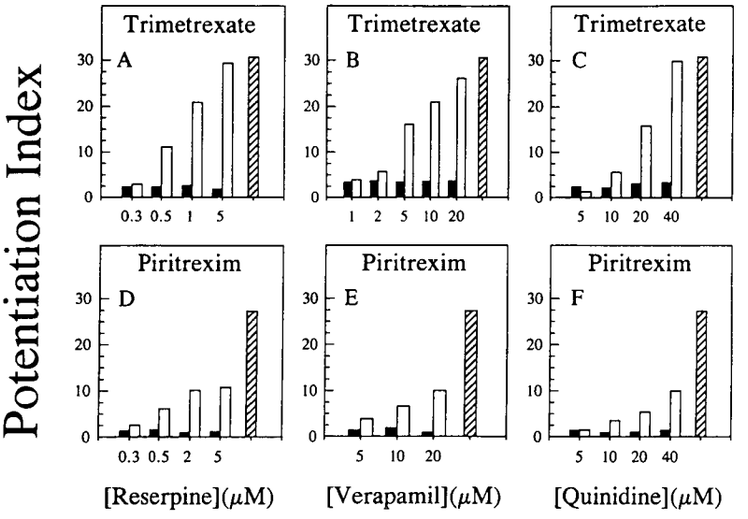

Figure 3. Sensitization of AA8 and T19 MDR cells to TMTX and PTX using selected modulators of the MDR phenotype. Parental AA8 cells and their T19 MDR subline were exposed to various concentrations of TMTX (A–C) or PTX (D–F) in the absence or presence of various concentrations of the MDR modulators reserpine (A,D), verapamil (B–E) and quinidine (C,F). The potentiation indices depicted represent the ratio of the LD_{50} of the antifolate in the absence of modulator divided by the LD_{50} in the presence of the modulator (see Materials and methods for details). Closed symbols represent AA8, open symbols denote T19 MDR cells, whereas the semi-filled symbols describe the degree of T19 resistance to TMTX (A–C) or PTX (D–F), i.e. representing the theoretical maximal reversal achievable.

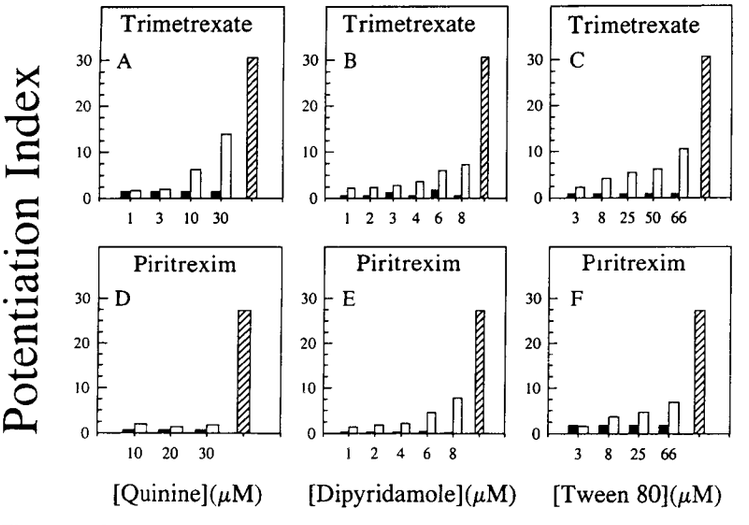

Figure 4. Sensitization of AA8 and T19 MDR cells to TMTX and PTX using different agents that can modulate the MDR phenotype. Wild-type AA8 and MDR T19 cells were treated with various TMTX (A–C) or PTX (D–F) concentrations in the absence or presence of various concentrations of the potentiators quinine (A and D), dipyridamole (B and E) and Tween 80 (C and F), after which the clonal survival fractions were determined in a colony formation assay. The symbols are those described in the legend of Figure 3.

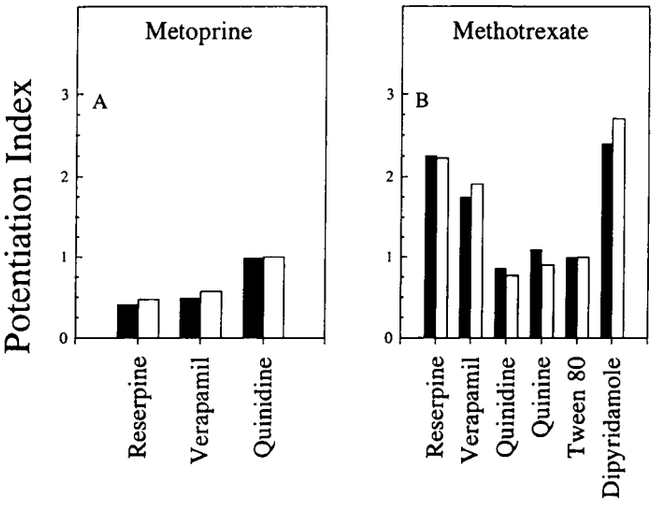

Figure 5. Effect of various modulators of the MDR phenotype on the cytotoxicity of metoprine and MTX to AA8 and T19 MDR cells. Parental AA8 cells and their T19 MDR subline were treated with various metoprine (A) and MTX (B) concentrations in the absence or presence of various compounds that increase anticancer drug cytotoxicity. Then, the clonal surviving fractions were determined and the extent of sensitization (i.e. potentiation) for each modulator was determined relative to cells that were not treated with modulators.

had no effect or a poor sensitization effect of both AA8 and T19 cells to MTX (Figure 5B). Thus, the potentiation of T19 cells to antifolates is specific to lipophilic analogs of MTX; this sensitization does not extend to MTX or simple structure lipophilic antifolates such as metoprine that does not participate in the MDR phenomenon.[20]

In order to provide a measure for the potency of the various potentiators studied in reversing the resistance of T19 MDR cells to TMTX and PTX, the concentration that yields 30% sensitization was derived for each modulator from the curves of potentiation indices versus modulator concentration. Thus, a histogram was plotted in which the least potent modulator, Tween 80, was given an arbitrary value of 1.0; thus, on a molar basis, the relative potency order of sensitization of T19 MDR cells to TMTX was reserpine > verapamil > dipyridamole > quinidine > quinine > Tween 80 (132 > 23 > 7 > 4.3 > 3.8 > 1), whereas that to PTX was reserpine > dipyridamole > verapamil > quinidine > Tween 80 (78 > 6.4 > 5.4 > 2 > 1) (Figure 6A and B).

The present findings from the modulation experiments suggested that a major component of PTX-resistance but not of TMTX-resistance that exists in T19 MDR cells appears to be independent of the P-170 pathway. Thus, in order to provide further evidence to the existence in T19 cells of such a P-170-independent component of PTX-resistance, T19 MDR cells were selected in gradually increasing concentrations of the lipophilic antifolates PTX, TMTX, the parent hydrophilic antifolate MTX as well as the pleiotropic antimitotic agent vinblastine. Northern blot analysis was performed with cytoplasmic RNA derived from the different variants and the steady-state mRNA levels of P-170 (Figure 7A) and DHFR (Figure 7B) were determined by scanning densitometric analysis of linear film exposures (Table 2). T19 MDR cells displayed a 55-fold over-expression of P-170 mRNA relative to the barely detectable level of P-170 in parental AA8 cells (Figure 7A). However, in MTX^{R5}, P-170 mRNA levels decreased by 2.4-fold and DHFR mRNA levels simultaneously increased to 21-fold (Figure 7B), relative to T19 cells. The PTX^{R20} subline of T19 cells maintained similar levels of P-170 mRNA while concomitantly overexpressing 46-fold more DHFR mRNA (Figure 7B). However, in the more antifolate resistant PTX^{R50} variant, P-170 mRNA levels decreased dramatically (Figure 7A) and were 5-fold lower than in parental T19 cells (Figure 7A). Simultaneously, DHFR mRNA levels in PTX^{R50}

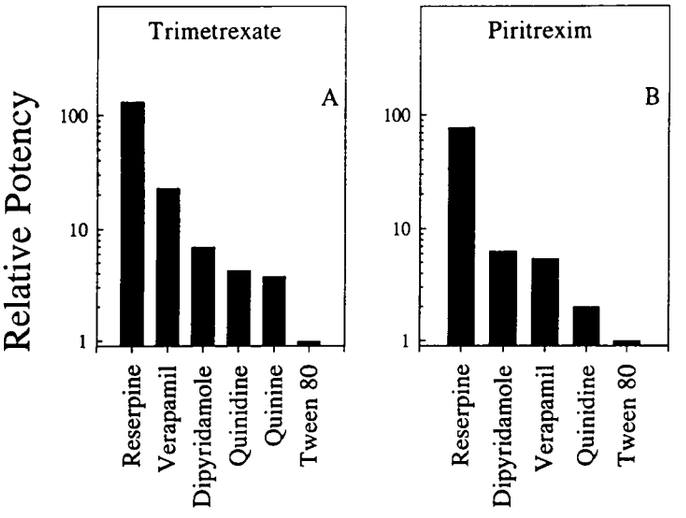

Figure 6. Histogram of the relative potency of reversal of TMTX and PTX resistance in T19 MDR cells for the various modulators. Based on the clonogenic cytotoxicity experiments in the absence or presence of the various modulators the concentration of a modulator that yields 30% reversal of TMTX (A) or PTX (B) resistance in T19 cells was derived. Then, Tween 80, the least active modulator was given a relative molar potency value of 1.0 to which all other modulators' potencies were related. Thus, this histogram that is constructed on a modulator molar basis yields a relative potency order for the different modulators used with TMTX and PTX cytotoxicity experiments.

cells further increased to 61-fold relative to parental T19 cells or AA8 cells (Figure 7B). In contrast, $TMTX^{R5}$ cells overexpressed 50% more P-170 mRNA than parental T19 cells and had a 12-fold elevation in DHFR mRNA relative to parental T19 cells (Figure 7B). Expectedly, $Vin^{R0.5}$, a vinblastine-resistant T19 derivative, displayed a 4-fold increase in P-170 mRNA levels relative to parental T19 cells (Figure 7A). $Vin^{R0.5}$ cells, however, maintained an identical expression of DHFR mRNA as compared with parental T19, as well as wild-type AA8 cells (Figure 7B). Ethidium bromide staining of the formaldehyde–agarose gels confirmed that similar amounts of cytoplasmic RNA were being analyzed (Figure 7C).

We have also documented the MDR (Figure 8A) and DHFR (Figure 8B) gene copy numbers in the different T19 sublines (Table 2). The 17-fold MDR gene amplification present in parental T19 cells was maintained in MTX^{R5}, $TMTX^{R5}$, PTX^{R20}, Col^{R10} and $Vin^{R0.5}$ relative to the single MDR gene copy present in wild-type AA8 cells (Figure 8A). In contrast, $TMTX^{R10}$ contained approximately 40-fold MDR gene amplification relative to parental T19 cells (Figure 8A). Figure 8(B) is a reprobing of the Figure 8(A) blot with a CHO DHFR cDNA

Table 2. MDR, DHFR gene copy number and mRNA levels in different drug-resistant variants

Gene/transcript	Cell line								
	AA8	T19	MTX^{R5}	PTX^{R20}	PTX^{R50}	$TMTX^{R5}$	$TMTX^{R10}$	$Vin^{R0.5}$	Col^{R10}
pgp	1	17	35	30	20	25	40	19	24
P-170 mRNA	1	55	23	60	11	84	ND	224	ND
dhfr	1	1	11	34	34	7	7	1	1
DHFR mRNA	1	1	21	46	61	12	ND	1	1

Values which are presented relative to wild-type AA8 cells were obtained by scanning densitometry of linear exposures of the blots.

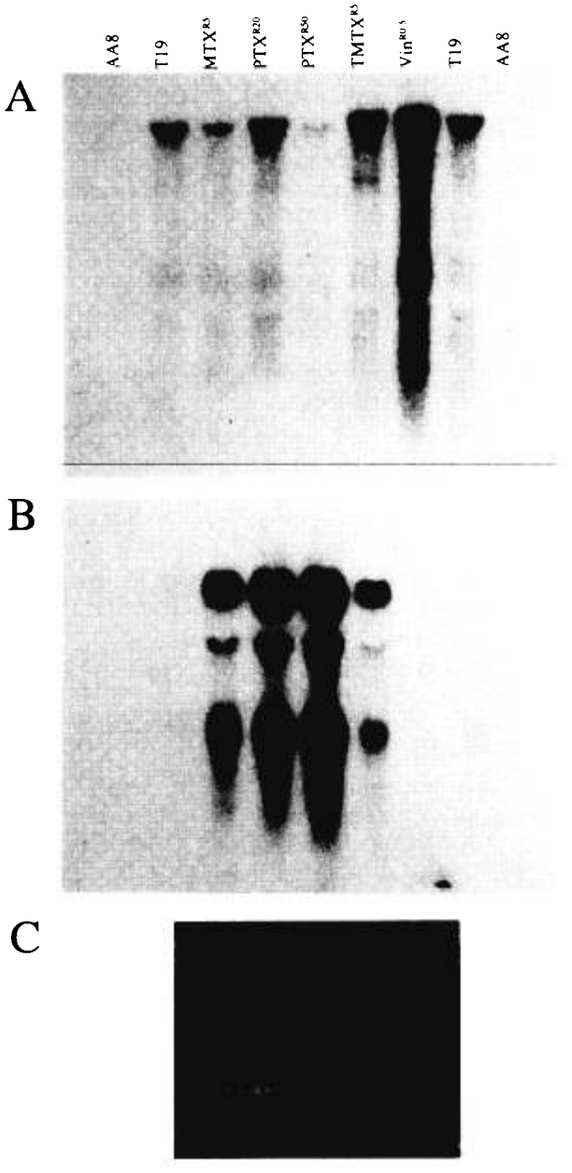

Figure 7. Autoradiogram of Northern blots containing RNA from parental AA8 cells, T19 MDR cells and from different AA8 resistant variants probed with hamster MDR and DHFR cDNA sequences. Cytoplasmic RNA (30 μg/lane) isolated from wild-type AA8 cells, from T19 MDR cells as well as from various T19 sublines made resistant to different antifolates and pleiotropic drugs was fractionated on a 1.5% agarose–formaldehyde gel, transferred to a Zetabind membrane. Following hybridization to a ^{32}P-oligolabeled MDR cDNA probe (A) the blot was then reprobed (after removing the MDR probe by a 10 min wash in a boiling solution of 0.1 × SSPE/0.1% SDS) with a radiolabeled hamster DHFR cDNA sequence (0.93 kb *Pst*I insert of MQ19-97; Panel B). An ethidium bromide staining of the formaldehyde-agarose gel is shown to indicate that near identical amounts of RNA were being analyzed (C).

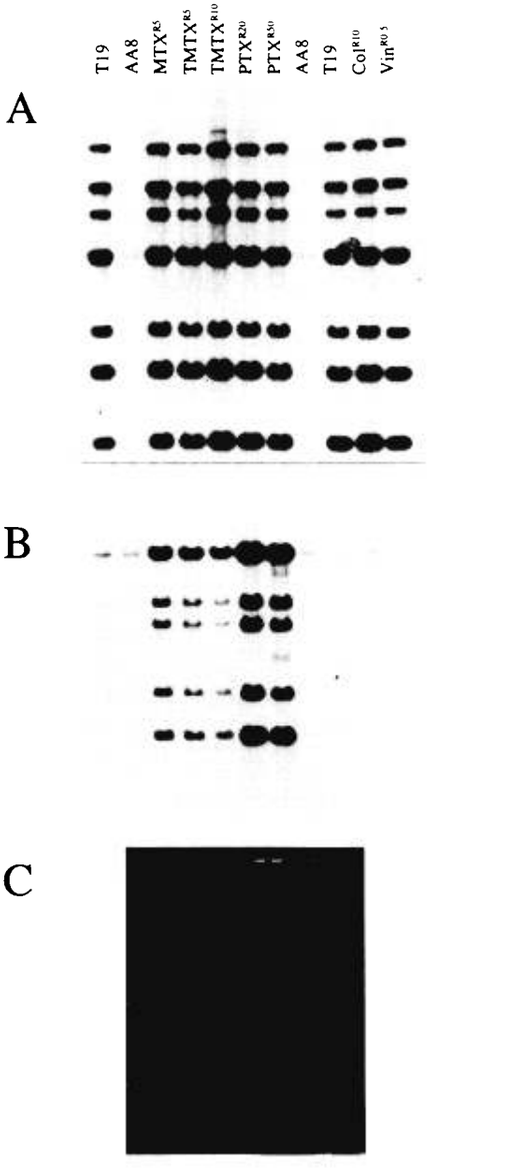

Figure 8. Autoradiograms of Southern blots containing DNA from AA8, T19 MDR cells as well as from their different antifolate resistant variants probed with hamster MDR and DHFR cDNA sequences. Genomic DNA (10 μg/lane) extracted from AA8 and T19 cells as well as from the various drug-resistant sublines was digested with *Eco*RI, fractionated on 0.8% agarose gels and transferred to Zetabind filter membrane. Following hybridization to a ^{32}P-labeled MDR cDNA sequence (A), the probe was removed and the DNA immobilized onto the blot was hybridized to a CHO DHFR cDNA sequence (B). An ethidium bromide staining is given to show that comparable amounts of DNA were being analyzed (C).

sequence; expectedly, T19 cells as well as Col^{R10} and $Vin^{R0.5}$ cells maintained the single copy of the DHFR gene present in wild-type AA8 cells (Figure 8B), whereas MTX^{R5}, $TMTX^{R5}$, $TMTX^{R10}$, PTX^{R20} and PTX^{R50} cells contained approximately 11-, 7-, 7-, 34- and 34-fold DHFR gene amplification (Table 2). Ethidium bromide staining of the agarose gels confirmed that comparable amounts of genomic DNA were being analyzed (Figure 8C). Thus, when T19 cells that stably overexpress P-170 mRNA are challenged with increasingly cytotoxic concentrations of TMTX, P-170 expression further increases as a result of an increased amplification of the MDR genes; this was accompanied also by DHFR mRNA elevation. In contrast, although increased cytotoxic PTX pressure imposed, T19 cells maintained the original 17 copies of the MDR gene, it surprisingly brought about a dramatic decrease in P-170 mRNA expression; this was accompanied by a marked overproduction of DHFR mRNA as a result of a prominent DHFR gene amplification (Table 2). Furthermore and as expected in control selections where a classic MDR drug such as vinblastine and colchicine were applied upon stepwise selection on T19 MDR cell cultures, a marked increase in P-170 mRNA level was observed. These results agree with, and complement the modulation experiments thereby suggesting that while CHO T19 cells can protect themselves from the cytotoxic effect of TMTX mainly by P-170 and partially by DHFR overproduction, a major component in the protection of T19 MDR cells against PTX is independent of P-170 expression and distinct of the MDR phenotype. In conclusion, the modulation experiments together with the established antifolate-resistant variants suggest that although CHO T19 cells possess a classic P-170-dependent MDR phenotype and display a similar level of cross-resistance to TMTX and PTX, the protective pathway need not be necessarily via P-170 activity. Rather, a pathway appears to exist in T19 cells that protects T19 MDR cells from the cytotoxicity of PTX without requiring P-170 function.

Discussion and conclusions

We undertook this study in order to determine whether cells with a classic P-170 overexpression-dependent MDR phenotype could support a significant cross-resistance to a given pleiotropic anticancer drug via pathway(s) that are presumably distinct of P-170. Thus, we have used CHO T19 cells that stably display P-170 overproduction,[19] and consequently, a typical MDR phenotype of resistance to a variety of multiple hydrophobic anticancer drugs[21] including a comparable cross-resistance to the lipophilic antifolates, TMTX and PTX, while maintaining sensitivity to the parent hydrophilic antifolate, MTX. The rationale behind the examination of the mechanisms of protection against TMTX and PTX in MDR cells was that while selection of parental CHO AA8 cells in gradually increasing concentrations of TMTX yielded MDR gene amplification,[17] PTX, a structurally related lipid-soluble antifolate failed to do so.[17] Yet, surprisingly, T19 cells[19] that express a typical and stable P-170-dependent MDR phenotype[21] displayed 24- to 29-fold resistance to PTX and TMTX (Figure 2). Hence, the question provoked by these findings was whether resistance of T19 MDR cells to PTX is solely mediated via P-170 or via additional P-170-independent pathway(s) that could protect T19 MDR cells from the cytotoxic effect of PTX. Toward this end we have taken two approaches. The first approach involves an attempt to reverse the resistance to TMTX and PTX in T19 cells with various modulators of the MDR phenotype. These modulators, including reserpine, verapamil and quinidine, are thought to function as competitive inhibitors of the mammalian P-170 efflux transporter.[26–29] Resistance to TMTX was almost fully reversed by classical modulators of the MDR phenotype whereas PTX resistance was, in the best case, only partially reversed. One way to explain this differential modulation is to postulate the existence of a second mechanism involved in PTX resistance in T19 cells. This mechanism should not be present in wild-type AA8 cells as they are sensitive to PTX. Consequently, it should be involved in TMTX resistance as well, since T19 cells have been derived from wild-type AA8 cells by a single step selection to this drug.[19] Another potential explanation could involve a non-modulatable P-170-dependent pathway that may preferably mediate PTX resistance.

Another experimental approach was also taken; T19 MDR cells were further challenged with a gradually increasing lipophilic antifolate pressure of TMTX and PTX in an attempt to uncover the most efficient protective pathways. Further selection of T19 cells to TMTX resulted in an increased P-170 expression along with a slight elevation in DHFR levels. Conversely, selection of T19 cells to PTX resulted in a major rise in DHFR expression along with a dramatic decrease in P-170 mRNA levels, despite the maintenance of the original 17-fold

MDR gene amplification. These results suggest that while P-170 overexpression is a good means of protection against TMTX, it seems markedly less efficient in protecting cells from the cytotoxic effect of PTX.

Thus, experiments assessing the modulation of PTX resistance suggest the coexistence of a second mechanism having a minor contribution to TMTX resistance whereas bearing a major contribution to PTX resistance. The poor protection conferred by P-170 with the inability of PTX to elicit MDR gene amplification in CHO cells seem to rule out a possible non-modulatable P-170-dependent pathway as the mechanism of cross-resistance. These results imply that the phenomenon of cross-resistance of cells with a classic P-170 overexpression-dependent MDR phenotype to a certain hydrophilic anticancer drug does not mean that P-170 is necessarily associated and/or required for the resistance; rather, alternative mechanism(s) of defense and/or resistance could coexist that may protect MDR cells from the cytotoxic action of the anticancer drug. Thus, mammalian cells can protect themselves from the cytotoxic effect of lipophilic antifolates via one or more of the following mechanisms. (i) Amplification of DHFR and consequent overproduction of the enzyme.[17] (ii) MDR gene amplification, overexpression of P-170 and subsequent exhibition of a typical MDR phenotype.[17,19] (iii) Reduced accumulation of these lipophilic MTX analogs alone[14,31] or also of 2,4-diaminopyrimidines.[20] (iv) A yet undefined mechanism of lipophilic antifolate resistance pathway that is neither involved in quantitative and qualitative alterations in DHFR nor with the MDR phenotype.[19]

The present work extends on our previous studies that focused on mechanisms of lipophilic antifolate resistance and their relation to the MDR phenotype.[17–20,32] TMTX but not PTX appears to fulfill a number of critical requirements that render it a classic pleiotropic drug that participates in the MDR phenotype. (i) Established CHO cells with a typical P-170-dependent MDR phenotype display a significant cross-resistance both to TMTX and PTX (Figure 2). (ii) However, upon selection of parental AA8 cells to TMTX and PTX only TMTX yields MDR gene amplification, P-170 overexpression and a subsequent MDR phenotype.[17] (iii) Further TMTX stepwise selection of T19 MDR cells yields an increased expression of P-170, whereas selection of T19 MDR cells with PTX results in a dramatic decrease in P-170 expression of the amplified MDR genes along with a high overexpression of DHFR (Figures 7 and 8). (iv) Single step selection of parental AA8 cells to TMTX yields MDR gene amplification in a number of clonal isolates,[19] whereas single step selection with PTX fails to do so.[32,33] (v) Potent modulators (e.g. reserpine) of the MDR phenotype that are thought to function as competitive inhibitors of the P-170 efflux transporter,[26–29] fully reverse TMTX resistance in T19 MDR cells (Figures 3 and 4). In contrast, these modulators either completely fail or, in the best case, only partially sensitize T19 MDR cells to PTX.

We note here with a great deal of surprise the dramatic decrease in P-170 mRNA levels in PTX^{R50} cells, despite the maintenance of the original 17-fold MDR gene amplification of their parental T19 MDR cells; this was accomplished by a major overproduction of DHFR mRNA. Thus, regardless of whether the exact mechanism responsible for the markedly reduced P-170 mRNA levels involves decreased transcriptional activity *per se* and/or decreased P-170 mRNA stability, the selection with high PTX concentrations has resulted in PTX^{R50} cells in which P-170 expression is an inferior mechanism of protection, whereas DHFR overproduction is an efficient one against the cytotoxic effect of PTX. One possible explanation for this phenomenon could be that under such stringent conditions of antifolate (i.e. PTX) selection and of severe restriction in the availability of purine nucleotides, cell survival may require the dedication of these valuable nucleotides for expression of the most crucial genes, some of which confer efficient protection against PTX, whereas the genes with the inferior cell protection ability to be down regulated. Furthermore, it is possible that if the amplified MDR sequences in PTX^{R50} cells are indeed undergoing a transcriptional down regulation, then it could be an early stage of the future complete transcriptional shut-off of the 'useless' amplified MDR sequences that could be a subject for deletion and loss.

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