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UPTAKE, CYTOTOXICITY AND METABOLISM OF *m*-AZIDOPYRIMETHAMINE AND RELATED LIPOPHILIC ANTIFOLATES IN SV-K14 HUMAN KERATINOCYTES *IN VIVO*

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Abstract—The growth-inhibitory properties of a series of lipophilic diaminopyrimidine antifolates were evaluated in comparison with methotrexate (MTX) against SV-K14 human keratinocytes *in vitro* under folate-dependent and folate-independent conditions. Under folate-dependent conditions metoprine (DDMP) proved more cytotoxic than MTX, despite the greater inhibitory activity of the latter compound against mammalian dihydrofolate reductase (DHFR), possibly reflecting differences in cellular accumulation. The significantly lower activity of both compounds under folate-independent conditions indicated DHFR as the primary target. Pyrimethamine (PYM), *m*-azidopyrimethamine (MZP) and *m*-aminopyrimethamine (MAP), a metabolite of MZP, were approximately equiactive but less cytotoxic than MTX or DDMP. The unexpected activity of MAP, an inferior DHFR inhibitor, suggests differences in the mechanism of action or cellular transport of the drug, although the reduction of cytotoxicity observed under folate-independent conditions indicates folate metabolism as the cytotoxic locus. In contrast, the cytotoxicity of PYM or MZP was not reduced under folate-independent conditions implying an alternative mechanism of action. The uptake of 2-[^{14}C]pyrimethamine by SV-K14 keratinocytes was rapid with steady-state intracellular concentrations being observed after approximately 100 min, partition of drug into the plasma membrane preceding redistribution and extensive accumulation within the particulate cell components. The previously reported NADPH-dependent metabolism of MZP to MAP by murine liver microsome preparations was not observed with SV-K14 keratinocytes nor with murine skin homogenates in the present study.

Antifolates, which are inhibitors of the enzyme dihydrofolate reductase (DHFR§; EC 1.5.1.3), have an established role in the treatment of proliferative and infectious disease, and the many studies conducted in this therapeutic area form the subject of several comprehensive reviews [1]. Two general classes of DHFR inhibitor have evolved: the classical or folate-analogue type, so called because of a close structural resemblance to the natural substrate dihydrofolate, exemplified by MTX(1), and the small-molecule or lipophilic antifolates of which the diaminopyrimidine DDMP(2) is the prototype agent.

Chronic systemic administration of MTX for the treatment of cutaneous proliferative disease, in particular severe psoriasis, whilst highly effective is often accompanied by hepatotoxicity even at low doses and this must be weighed against disease severity [2]. Attempts to surmount this problem by administering MTX topically have been largely unsuccessful to date, inactivity being attributed to the polar nature of the molecule and although antipsoriatic activity has been achieved by formulating MTX in combination with penetration enhancers [3], the likelihood of systemic toxicity following prolonged application cannot be discounted. Studies addressing the possibility of utilizing lipophilic antifolates via the percutaneous route are few but in one multi-centre screening program the antimalarial antifolate PYM(3), but not DDMP, demonstrated significant topical antipsoriatic activity [4] suggesting a possible role for these compounds in the topical treatment of psoriasis and related skin disorders.

The novel diaminopyrimidine MZP(4) was developed in our laboratories in an attempt to circumvent one of the problems associated with existing lipophilic antifolates, notably that of cumulative toxicity emanating from the protracted biological half-life of these highly lipoidal compounds (DDMP $T_{\frac{1}{2}}$ = 10–15 days) [5]. MZP was designed using the 'soft drug' approach, i.e. exploiting the lipophilicity and susceptibility to metabolism of the azido substituent.

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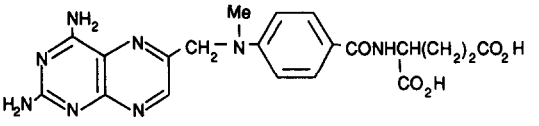
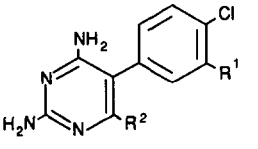
§ Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; DDMP, 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine, metoprine; PYM, 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine, pyrimethamine; MZP, 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine, *m*-azidopyrimethamine; MZPES, the corresponding ethanesulphonate salt of MZP; MAP, 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine, *m*-aminopyrimethamine; SV-K14, simian virus-transformed human neonate foreskin keratinocyte line; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's medium; BME, basal medium Eagle's; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline; FCS, foetal calf serum.

Thus, the azido group confers DHFR-inhibitory activity as required but undergoes metabolism to the corresponding arylamine MAP(5) which, being polar and a 50-fold weaker inhibitor of the target enzyme, is rapidly eliminated, thereby reducing the half-life ($T_{\frac{1}{2}}$ = 34 hr) and toxicity of the parent drug [6, 7]. Although MZP exhibited significant activity against a panel of murine tumours, including an MTX-resistant cell line *in vivo*, subsequent clinical evaluation of the drug as an antitumour agent via the systemic route proved disappointing [8]. One possible therapeutic application emanating from these studies centres on the use of MZP topically for the treatment of cutaneous disease. A hypothesis may be proposed whereby the cytotoxicity of the drug following percutaneous absorption is restricted to the proliferate layers of the skin as a consequence of cutaneous or systemic conversion to MAP, followed by the rapid elimination of MAP thereby precluding systemic toxicity.

The present study set out to investigate the potential therapeutic utility of lipophilic antifolates in the treatment of cutaneous disease by evaluating the *in vitro* growth-inhibitory activity of MZP, its putative metabolite MAP and the two established lipophilic antifolates DDMP, and PYM in comparison with that of MTX against a cell culture model of cutaneous proliferation, the SV-K14 cell-line. By modifying the composition of the culture media it was possible to conduct these studies under both folate-dependent and folate-independent conditions of cell growth. Folate-dependent conditions were achieved by growing cells in a culture medium containing minimal concentrations of hypoxanthine, thymidine and glycine (BME medium) which would otherwise provide an alternative source of these essential products of folate metabolism. Conversely, the use of a culture media supplemented with hypoxanthine, thymidine and glycine (DMEM/F12) enabled cell growth to occur independently of folate metabolism. The sub-cellular localization of ^{14}C-labelled PYM in this model system was also investigated. In addition, the metabolism of MZP by SV-K14 cells was compared with that of murine skin and liver homogenate preparations. The structures of the antifolates used in this study are shown in Fig. 1.



(1) MTX



(2) DDMP : R^1 = Cl : R^2 = Me

(3) PYM : R^1 = H : R^2 = Et

(4) MZP : R^1 = N_3 : R^2 = Et

(5) MAP : R^1 = NH_2 : R^2 = Et

Fig. 1. Structures of the antifolates investigated.

MATERIALS AND METHODS

Chemicals. MZP, MZPES and MAP were synthesized as described previously [7]. MTX and PYM were purchased from the Sigma Chemical Co. (Poole, U.K.) and DDMP was a kind gift from the Wellcome Foundation, Beckenham, U.K. 2-[^{14}C]-PYM (sp. act. 54 mCi/mmol) was purchased from Amersham International (Amersham, U.K.). Tissue culture media were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

Cell culture conditions and cytotoxicity studies. SV-K14 cells, a kind gift from Drs B. Lane and J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London) were grown to confluency in a 1:1 mixture of DMEM and F12 in a Falcon 3028 tissue culture flask (175 cm^2) at 37° under an atmosphere of 5% CO_2 in air, and used after 15–20 passages. Subcultivation was achieved as follows: after removal of medium by vacuum aspiration, cells were incubated (5–8 min) with 0.04% (w/v) trypsin and 1.0% (w/v) EDTA in PBS at 37°. After detaching the cells by gentle agitation, trypsinization was terminated by the addition of FCS (2 mL), cells were collected by centrifugation, resuspended in DMEM/F12 medium and counted using a haemocytometer. Stock solutions (10 mM) of the drugs were prepared in DMSO such that the final concentration of DMSO in the culture medium did not exceed 1%, a concentration known not to exert a cytotoxic effect on SV-K14 cells.

SV-K14 keratinocytes are anchorage-dependent and detach from the support matrix when dead. Thus the reduction in the number of attached cells following exposure to a drug may be quantified and related to drug potency. Cells were seeded (Falcon 3047 multiwell plate) at a density of 5×10^3 cells cm^{-3} in 495 μL of either DMEM/F12 (folate-independent) or BME (folate-dependent) culture medium. The necessary inclusion of 5% FCS in both media resulted in small, uncontrollable quantities of folate-metabolism products being present. As a consequence, total folate-dependency could not be assumed for the BME system, although the effects were minimized by utilizing the same batch and volume throughout. Following incubation (37°; 5% CO_2 in air) for 6 hr an aliquot (5 μL) of the drug solution, diluted to the required concentration with DMSO, was added to the appropriate well and the plates were incubated for a further 6 days with a change of drug solution and culture medium after 3 days. Each concentration of drug was evaluated in triplicate and control wells contained DMSO alone (5 μL). After 6 days the drug/culture medium was removed by aspiration, dead cells were removed by washing with PBS (2 × 1 mL) and those remaining were solubilized with 0.5 M sodium hydroxide

solution (0.5 mL). Final dissolution of cellular material was achieved by freezing the solutions at −80° and thawing to room temperature with agitation using a Titertek agitator (ICN Flow Laboratories, High Wycombe, U.K.). Cell extracts from replicate incubations were pooled and assayed for protein content as follows: the extract (0.2 mL) was acidified with 0.071 M hydrochloric acid (0.6 mL), protein assay dye reagent (0.2 mL) (Biorad Chemical Division, Richmond, CA, U.S.A.) was added and the protein content was determined spectrophotometrically at 595 nm [9] using bovine serum albumin as standard.

Protein assays conducted on SV-K14 cell extracts after incubation were utilized for the estimation of the number of viable cells present using a conversion factor of 400 pg protein/SV-K14 keratinocyte [10]. For control incubations of cells (in the absence of drug) the cell titre after 6 days was at least 10-fold that of the initial inoculum, thus demonstrating the viability of the cell-line. The reliability of the SV-K14 cell-line as a model for evaluating drug cytotoxicity was also demonstrated with the well known antipsoriatic drug anthralin which, when tested under conditions identical to those employed for the DHFR inhibitors, gave an ID_{50} value of 5.9 μM, correlating reasonably well with the published value of 7.0 μM [10]. The results obtained from the growth-inhibition studies were fitted by non-linear regression (MINSQ program, Micromath Scientific Software, Salt Lake City, UT, U.S.A.) to the following dose–response equation from which the dose of drug producing 50% inhibition of cell growth (ID_{50}), and the associated error could be estimated:

$$A_c = A_0/[1 + (C/ID_{50})]$$

where A_0 = absorbance at zero drug concentration; A_c = absorbance value from protein determination in presence of drug and C = drug concentration.

Metabolism studies: SV-K14 keratinocytes. Cells were seeded at a density of 12.5×10^3 cells cm^{-3} in DMEM/F12 medium (0.5 mL) in each well of a tissue culture flask and incubated as above for 24 hr. The medium was removed by aspiration and replaced with fresh medium (0.5 mL) containing 25 μM MZPES. The cells were incubated for a further 72 hr and at appropriate time intervals samples were taken as follows: ethanol (0.5 mL) was added to the well, the solution was transferred to an Eppendorf tube and protein was removed by centrifugation (3000 rpm, 5 min). MZPES content was determined by HPLC using a previously published method [11]. Control wells without cells were included to assess drug stability and extraction efficiency. The extraction efficiencies for MZP from the cell culture system and the cell-free growth medium were 91.8% and 90.4%, respectively, of the theoretical quantities added at time zero. All studies were conducted in triplicate.

Murine liver and skin homogenates. Two male NMR1 mice were killed by cervical dislocation and the hair was removed with an electric razor. The skins (5 g) and livers (3 g) were removed, transferred to ice-cold PBS, and finely macerated to give a final concentration of approximately 200 mg wet tissue/mL PBS. The crude homogenate preparations were pipetted (0.5 mL) into ice-cold test tubes containing an NADPH-generating system comprising glucose-6-phosphate (4 mg), $NADP^+$ (3.2 mg), 0.15 M $MgCl_2.6H_2O$ (0.4 mL), 0.5 M nicotinamide (0.4 mL) and 5 mM phosphate buffer pH 7.4 (3.45 mL). Control tubes contained phosphate buffer pH 7.4. The reaction was initiated by the addition of aqueous MZPES solution (0.35 mM, 0.25 mL) and after vortexing the tubes (five for each time point) were incubated at 37° for up to 60 min (liver) and 24 hr (skin). Samples were made alkaline (10 M NaOH, 0.5 mL), extracted with chlorobutane:dichloromethane (96:4, 5 mL) and the organic layer was separated by centrifugation (2500 *g*) for 5 min. Aliquots (4 mL) were taken, evaporated to dryness under N_2 at 40° (Tecam SC-3 Sample Concentrator with a DB-3 Dri-block), redissolved in methanol (2 mL) containing PYM (10 μM) and analysed by HPLC.

Hairless mouse skin homogenate. Three male MF1 hairless mice were killed and the skin dissected. After carefully removing subcutaneous tissue, the skin was macerated into small pieces, frozen rapidly in liquid N_2 and ground in a ceramic mortar. The powder was suspended in ice-cold PBS (100 mg tissue/mL), homogenized with a Teflon/glass homogenizer and the homogenate was centrifuged (9000 *g*) for 20 min. Solids were discarded and an aliquot (250 μL) of the supernatant was diluted 1 in 50 with phosphate buffer pH 7.4, and assayed for protein content as described for the cytotoxicity studies. The supernatant was diluted with phosphate buffer to a final concentration of 1 mg/mL and aliquots (4.9 mL) were pipetted into sample tubes and stirred at 37° with protection from light. All incubations were performed in triplicate and control tubes contained phosphate buffer only. The reaction was initiated by the addition of aqueous MZPES solution (2.5 mM, 0.1 mL). Samples (0.5 mL) were taken for up to 24 hr and analysed, following extraction, as described above using PYM as standard. Extraction efficiencies (mean ± SEM) from the murine skin homogenate were 98.0 ± 1.8 and 99.5 ± 0.7% of the theoretical quantities added at time zero for MZP and MAP, respectively.

Subcellular localization studies. SV-K14 cells were grown in five Falcon 3028 tissue culture flasks (175 cm^2) in DMEM/F12 medium to near confluency, whereupon the culture medium was removed and the cells were washed with PBS to remove serum. 2-[^{14}C]PYM (10 μM) in serum-free DMEM/F12 medium (10 mL) was added to each flask and the cells were incubated (37°; 5% CO_2) for up to 180 min. Cells were trypsinized as described above but without further addition of FCS. The distribution of 2-[^{14}C]PYM was determined after conducting a subcellular fractionation using the microbead density perturbation technique essentially as described by Schmidt *et al.* [12]. The following procedure was conducted at 4°: the cell pellet was resuspended in 5 mL of coating buffer (25 mM sodium acetate pH 5.0; 0.8 M sorbitol; 0.1 M NaCl) containing 100 μL of a 30% (w/v) solution of cationic silica microbeads (50 nm diameter; >2 g/cm^3; a kind gift from Dr B. Jacobson, Dept. of Biochemistry,

University of Massachusetts, Amherst, MA, U.S.A.) and the suspension was centrifuged (2000 rpm, 5 min). The supernatant was discarded, the cells resuspended in coating buffer (5 mL) containing 75 μg/mL polyacrylic acid (M_r 90,000) and the suspension was again centrifuged (2000 rpm, 5 min). The microbead-coated cells were washed once in 5 mL of hypotonic lysis buffer [5 mM Tris–HCl pH 7.5; 1 mM ethylene glycol-bis-(2-aminoethyl ether) *N,N'*-tetraacetic acid; 1 mM dithioerythritol; 1 μg/mL aprotinin] to remove traces of coating buffer, resuspended in lysis buffer (2 mL) and homogenized in a Dounce glass homogenizer until no nuclei were apparent by optical microscopy. The plasma membranes were separated by centrifugation (3000 rpm, 15 min) and the resulting supernatant was centrifuged (50,000 *g*, 30 min; Beckman L5-50B Ultracentrifuge). The plasma membrane pellet was washed in lysis buffer (5 mL), recentrifuged (3000 rpm, 15 min) and resuspended in 200 μL of lysis buffer. The supernatant emanating from the ultracentrifugation step was taken as the cytosoluble fraction and the pellet, which was resuspended in 200 μL lysis buffer, as the intracellular particulate fraction. Aliquots from each fraction were assayed spectrophotometrically for protein content as described above. Samples of the appropriate fraction were taken up into Pico-Fluor 30 scintillation cocktail (Packard Instruments, Downers Grove, IL, U.S.A.) and radioactivity was determined in an LKB Wallace 1211 Rackbeta liquid scintillation spectrometer with a Model 43 programmable controller. Total cellular uptake of radiolabelled drug was assessed by repeating the experiment with omission of the fractionation procedure, radioactivity being determined following trypsinization.

RESULTS

Dose–response curves for each drug under folate-dependent and folate-independent growth conditions are shown in Fig. 2, and the corresponding ID_{50} values are summarized in Table 1. Inhibitory activities of the compounds against partially purified rat liver DHFR are included in Table 1 for comparison. Inhibition of cell growth was observed for all of the compounds evaluated although the degree of growth inhibition varied considerably. Under folate-dependent conditions of cell growth, DDMP was the most potent compound followed by MTX. Weaker still were MZP, MAP and PYM, and these proved approximately equipotent. These results do not parallel the relative *in vitro* potencies of the compounds as inhibitors of DHFR, the putative target, where MTX replaces DDMP as the most active inhibitor and, perhaps more interestingly, MAP is a very weak inhibitor of the enzyme. The ID_{50} value of 6.8 ± 2.3 μM observed for MTX is comparable to a previously described value of 11.0 μM determined under essentially identical conditions against the SV-K14 cell-line [10], again providing evidence for the reproducibility of this cell-line.

In keeping with the expectation that DDMP and MTX exert their cytotoxic effects through inhibition of DHFR, the activity of these antifolates was significantly reduced under folate-independent conditions of growth. The reduced cytotoxicity of these antifolates in DMEM-F12 medium also indicates that folate-dependent growth conditions were achieved using the BME medium, despite the necessary addition of 5% FCS. The greater potency of DDMP in this model may reflect differences in the intracellular concentrations of the drugs, higher levels being achieved with the lipophilic antifolate. A similar situation was also observed with MAP, the ID_{50} increasing approximately 3-fold under folate-independent conditions, although the cytotoxic potency of the drug was unexpected in the light of its weak DHFR-inhibitory activity. The results obtained with MZP and PYM were also surprising in that cytotoxicity against the SV-K14 cells appeared to be enhanced under folate-independent growth conditions, the ID_{50} being significantly lowered in both cases. Moreover, both MZP and PYM exhibited cytotoxicity under folate-dependent conditions approximately equal to that observed for MAP, and these results are not consistent with the activities of the respective antifolates as DHFR inhibitors.

Incubation of MZP with mouse liver homogenate over 1 hr in the presence of an NADPH-generating system reduced the level of detectable MZP from 25 μg/mL to under 7 μg/mL. Conversely, in the absence of the co-factor-generating system, no metabolism was observed and these results are consistent with the findings of Kamali *et al.* [13]. No metabolism of MZP was observed in a monolayer of SV-K14 keratinocytes during a 72 hr incubation period and an identical result was obtained for the control experiment where the drug was incubated with the culture medium alone. Moreover, the cells were observed to be growing actively throughout the duration of the experiment as the final MZP concentration was sub-inhibitory and folate-independent conditions were employed, thus providing optimal conditions for metabolism of the drug to occur. No detectable metabolism of MZP was observed with the mouse skin homogenate preparation over the 24 hr incubation period, regardless of the presence of absence of a co-factor generating system, and an identical result was also obtained with the hairless mouse skin homogenate prepared by an alternative method. The use of a modified HPLC analytical method allowed the simultaneous detection of MZP and MAP in these experiments, but the formation of MAP was not evident. Control experiments demonstrated that MZP was stable to the buffer used in the homogenate preparations and that MAP was stable to both buffer and homogenate.

The uptake of 2-[^{14}C]PYM by SV-K14 cells was rapid following exposure to the drug, saturation being observed after approximately 1 hr as shown in Fig. 3. A final total intracellular concentration of approximately 0.625 nmol/mg was achieved. Cellular uptake was also confirmed by sampling the extracellular medium at appropriate time points during the incubations and approximately 25% of the applied 2-[^{14}C]PYM was taken up during the 180-min incubation time. Subcellular distribution of the drug between the plasma membrane, cytosol and particulate fraction (primarily mitochondria and

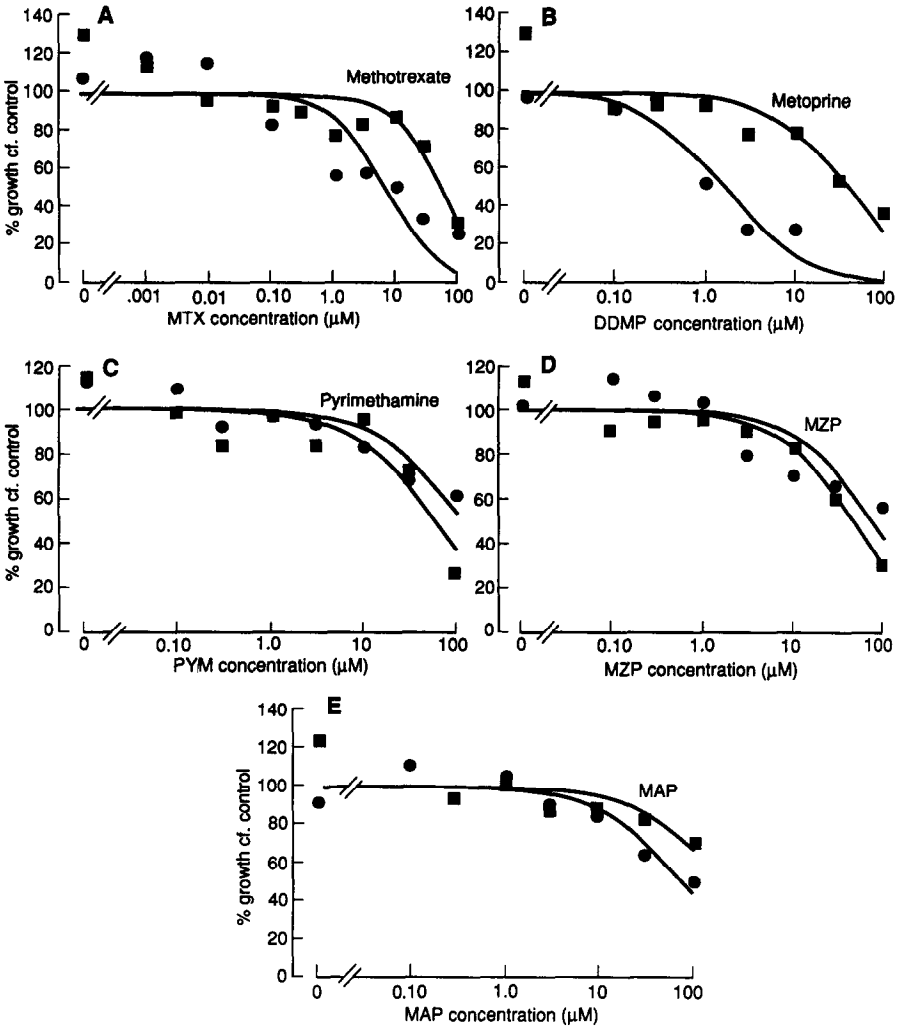


Fig. 2. Growth-inhibitory activity of MTX (A), DDMP (B), PYM (C), MZP (D) and MAP (E) against SV-K14 keratinocytes under folate dependent (●) and folate-independent (■) conditions. Values are the means of three determinations and standard deviations were ≤ 10% of the mean. See Materials and Methods for details of these computer-generated dose–response curves.

Table 1. Comparative *in vitro* cytotoxicity and enzyme-inhibitory activity of antifolates against SV-K14 keratinocytes and rat liver DHFR

	SV-K14 Cytotoxicity ID_{50} (μM)			DHFR-inhibitory activity*	
Compound	Folate-dependent (FD)	Folate-independent (FI)	Ratio (FI/FD)	IC_{50} (μM)†	K_i (nM)
MTX	6.80 ± 2.3	56.7 ± 15.6	8.3	0.002	0.005 ± 0.001‡
DDMP	1.54 ± 0.3	36.6 ± 9.8	23.8	0.10	0.12 ± 0.04
PYM	112.0 ± 22.6	60.6 ± 13.6	0.5	1.40	2.60 ± 0.31
MZP	74.6 ± 19.9	44.9 ± 6.0	0.6	1.30	1.60 ± 0.68
MAP	73.4 ± 12.4	195.4 ± 63.5	2.7	62.0	ND

* From Refs 6 and 7.
† The concentration of inhibitor required to reduce enzyme activity to 50% of control activity.
‡ 95% confidence limits.
Values are means ± SEM.

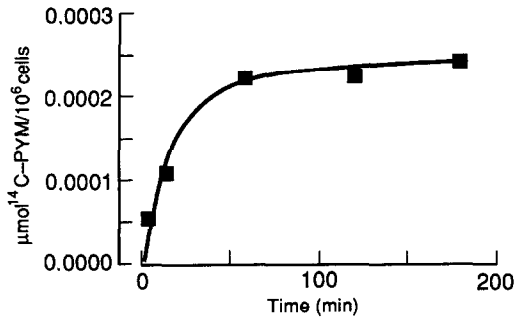


Fig. 3. Uptake of 2-[^{14}C]PYM by SV-K14 keratinocytes. Cells were exposed to 10 µM drug for up to 180 min. See Materials and Methods for additional details.

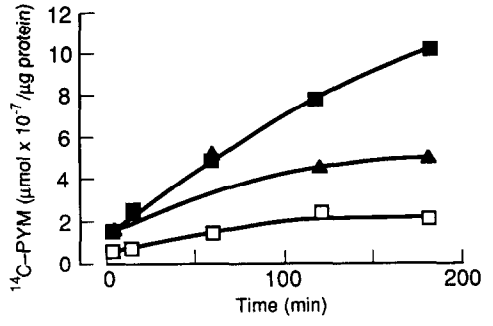


Fig. 4. Subcellular distribution of 2-[^{14}C]PYM within SV-K14 keratinocytes following incubation with 10 µM drug for up to 180 min. Key: (▲) plasma membrane, (□) cytosolic fraction and (■) particulate fraction.

lysosomes), following cellular uptake is illustrated in Fig. 4. Accumulation of radiolabelled material in the plasma membrane and cytosolic fraction appears to plateau after approximately 100 min, while accumulation of drug in the particulate fraction continued throughout the duration of the experiment (180 min).

DISCUSSION

Human cells in culture are of increasing importance as *in vitro* models for cutaneous proliferative disease. The SV-K14 cell line is hyperproliferative with an impaired propensity to undergo terminal differentiation [14] and consequently bears a functional similarity to the hyperproliferative germinative cells of psoriatic lesions. In the absence of a satisfactory *in vivo* model for psoriasis, the SV-K14 cell-line provides a useful primary screen for the evaluation of prospective anti-proliferative agents for the treatment of this disease.

The results obtained in this study demonstrate that SV-K14 keratinocytes are moderately sensitive to the antifolates evaluated but that the order of potency does not parallel that observed for inhibition of DHFR. Thus, under folate-dependent conditions, where cytotoxicity would be expected to result from inhibition of DHFR, the most potent compound was DDMP although this diaminopyrimidine is a weaker inhibitor of the enzyme (K_i = 0.12 ± 0.04 nM) than the folate analogue MTX (K_i = 0.005 ± 0.001 nM). Differential cytotoxicity of this nature between classical and lipophilic antifolate drugs is well established for several tumour cell-lines *in vitro* and *in vivo*, and indeed forms the basis of treating MTX-resistant tumours with lipophilic antifolates [15]. MTX is dependent upon an active transport process to gain ingress to cells and consequently resistance may occur through changes in the transporter protein leading to cellular exclusion of the drug. Since lipophilic antifolates enter cells, at least in part, by passive diffusion they are less subject to the vagaries of a transport system [16]. Thus, although other resistance mechanisms are known, it is conceivable that the observed lower sensitivity of the SV-K14 cell line to MTX may be transport-mediated.

As expected MZP and PYM proved approximately equitoxic to SV-K14 keratinocytes under folate-dependent conditions, reflecting the comparable DHFR-inhibitory activity of these structurally similar diaminopyrimidines. Although any extrapolation of results emanating from studies conducted *in vitro* to the clinical situation is tenuous, the demonstrated efficacy of PYM as a topical antipsoriatic agent [4] suggests that analogous studies with MZP may be warranted and we have recently reported preliminary investigations to this end [17]. The cytotoxicity observed for MAP, the putative inactive metabolite of MZP, is more difficult to explain. The aromatic amino substituent renders MAP more polar (log P = 1.25) than MZP (log P = 2.94), and it is an inferior DHFR inhibitor with a reduced ability to enter cells via passive diffusion. Surprisingly, MAP proved equitoxic with MZP and marginally more cytotoxic than PYM to SV-K14 keratinocytes in folate-dependent culture, suggesting a possible mechanism of action other than DHFR inhibition or, alternatively, that higher intracellular levels of MAP are achieved through a process other than passive diffusion.

Evidence that the unusual cytotoxicity profile of MAP involves a blockade of folate metabolism, if not necessarily DHFR inhibition, was obtained from studies conducted under folate-independent conditions where the culture medium adopted (DMEM/F12 + 5% FCS) contained the end products of folate metabolism (hypoxanthine, thymidine, and glycine). Clearly the cytotoxicity of MAP is lowered considerably and, in fact, the approximate 3-fold reduction in activity observed under folate-independent conditions is probably a conservative value since only a 25% inhibition of growth occurred at the maximum concentration of drug employed (100 µM), making accurate extrapolation to the ID_{50} value difficult. Unfortunately, the effect of higher concentrations could not be monitored due to the low solubility of drug (100 µM) in the growth media used.

The ID_{50} values for DDMP and MTX were substantially elevated under folate-independent conditions indicating reduced toxicity, and supporting the proposition that a blockade of folate metabolism,

presumably via inhibition of DHFR, is the locus of cytotoxicity for these antifolates. The reduced toxicity of MTX to SV-K14 keratinocytes under folate-independent conditions has also been reported previously [10]. Interestingly, the growth-inhibitory activity of MZP and PYM was not reduced under folate-independent conditions and, in contrast, both antifolates proved significantly more cytotoxic. The fact that the two drugs are cytotoxic regardless of the growth conditions employed suggests an additional target for these diaminopyrimidines not dependent upon folate metabolism. Compelling evidence for a second folate-independent target for lipophilic antifolates has been reported previously by Greco and Hakala [16] who examined the activity of a series of lipophilic diaminopyrimidines in comparison with MTX against several murine and human cell-lines *in vitro*. While the growth-inhibitory activity of MTX was fully reversible under folate-independent conditions, sensitivity to the diaminopyrimidines including DDMP and PYM remained, and a similar potency was observed regardless of inhibitor type. More recent biochemical studies with the novel lipophilic antifolate piritrexim also suggest that cytotoxicity cannot be entirely attributed to inhibition of DHFR [18]. The apparent absence of a folate-independent target for DDMP activity against SV-K14 keratinocytes in the present study remains to be explained but it is interesting that in contrast to PYM this drug also failed to demonstrate antipsoriatic activity in the clinic [4], suggesting the possibility that the clinical activity of PYM may emanate from cytotoxicity at the hitherto unidentified folate-independent target. Schwartz and Milstone [19] have demonstrated recently that keratinocytes efficiently salvage extracellular thymidine and that dipyridamole, an inhibitor of the salvage pathway, potentiates the cytotoxicity of MTX *in vitro*. The lack of antiproliferative activity exhibited by many topical antifolates in the clinic may, thus, possibly result from inherently high levels of thymidine in the skin, effectively providing folate-independent conditions *in vivo*, and one may conjecture that antifolates exhibiting activity do so via another mechanism.

Studies regarding the biotransformation of azides are few and the present investigation into the ability of keratinocytes to metabolize an azido-substituted drug is, to our knowledge, without precedent. We have demonstrated recently that aryl azides react readily with thiols to furnish the corresponding arylamine [11] and it was suggested that the analogous reaction with glutathione could conceivably provide a non-enzymatic route for the bioreduction of MZP. Clearly, in the present study the reductive biotransformation of MZP by actively growing SV-K14 keratinocytes was negligible, with greater than 90% of the parent drug being recovered unchanged following a 72 hr incubation with sub-inhibitory concentrations under folate-independent conditions. Diaminopyrimidine antifolates are predominantly metabolized via oxidative pathways *in vivo* [20] but no metabolism of the diaminopyrimidine ring of MZP was observed in this study.

Conversion of MZP to the corresponding amine metabolite MAP has been observed *in vitro* with a murine liver homogenate preparation [13] and *in vivo* in mice [21]. Although the *in vitro* bioreduction appeared to be predominantly enzyme-mediated requiring NADPH as cofactor, the nature of the enzyme system involved remains to be established. The results of our preliminary *in vitro* experiments with a murine liver homogenate preparation confirm these observations. In contrast, no metabolic conversion of MZP was detectable with the skin homogenate preparations even following protracted incubation times (> 24 hr). Thus, the results obtained imply that cutaneous metabolic inactivation of MZP is unlikely to occur following topical administration and that, following percutaneous absorption of the drug, bioinactivation processes necessary to minimize toxicity may depend upon systemic metabolism, principally within the liver.

Earlier studies concerning the cellular pharmacokinetics of lipophilic diaminopyrimidine antifolates *in vitro* have been conducted exclusively with tumour-derived cell lines and the present study appears to be the first to utilize a human keratinocyte model. In one previous investigation of note, the cellular uptake of 2-[^{14}C]PYM by several human carcinoma and murine sarcoma cell-lines was found to proceed rapidly via a partition process between the plasma membrane and extracellular medium initially, with subsequent redistribution into intracellular components; equilibrium intracellular concentrations some one to two orders of magnitude in excess of that in the extracellular medium were achieved [16]. The authors concluded that, although the diaminopyrimidine bound tightly to intracellular DHFR, extensive non-specific binding to intracellular components also occurred and constituted the major fraction of total cellular uptake. This sequence of partition of drug into the plasma membrane followed by redistribution and extensive accumulation within the particulate cell components was also observed in the present study, confirming that PYM is able to penetrate SV-K14 keratinocytes. Presumably the fraction of radiolabelled PYM observed in the cytosol of SV-K14 cells corresponds at least in part to drug associated with cytoplasmic DHFR. The unavailability of 2-[^{14}C]MZP has prevented analogous uptake studies being conducted with this antifolate. However, since the azido group of MZP appears to be resistant to metabolism by SV-K14 cells, the structural and physicochemical similarities between PYM and MZP (log P = 2.69 and 2.81, respectively) suggest that the two drugs should exhibit similar cellular pharmacokinetics. Thus, MZP may have therapeutic potential as a cutaneous antiproliferative agent either through DHFR inhibition or via an alternate target, and while metabolic inactivation within the skin appears unlikely, rapid hepatic metabolism following systemic absorption constitutes a viable mechanism to circumvent cumulative toxicity.

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