Biochemical Pharmacology, Vol. 24, pp. 535–538. Pergamon Press, 1975. Printed in Great Britain.

SHORT COMMUNICATIONS

Studies on the transport and distribution of diaminopyrimidines in L5178Y lymphoblasts in cell culture

(*Received* 7 *June* 1974; *accepted* 26 *August* 1974)

Diaminopyrimidines kill tumour cells in tissue culture [1, 2], animals [3–5] and man [6–8]. Methodichlorophen (2-4-diamino-5-(3′-4′-dichlorophenyl)-6-methylpyrimidine) (DDMP) is the most effective analog in some experimental systems [3, 4]. The antitumour effect of these agents has been ascribed to their ability to inhibit the enzyme dihydrofolate reductase [9–11]. However, since they are much less effective inhibitors of this enzyme than in methotrexate (MTX) [2], it is possible that their cytocidal effect may be associated with some other loci or mechanisms of action. The way in which antifolates (particularly MTX) penetrate cells has been widely studied [12–14]. However, little is known of the mechanism of translocation of diaminopyrimidines across the tumour cell membrane. Recently, it has been suggested that MTX and pyrimethamine (5-*p*-chlorophenyl)2,4-diamino-6-ethyl pyrimidine (PRM) may be transported through different sites, and that they do not compete for uptake [2]. The present study reports preliminary results on the transport of PRM and DDMP into L5178Y lymphoblasts.

Chemicals and reagents. [^{14}C]PRM (sp. act., 14·7 mCi/mmole) and [^{14}C]DDMP (sp. act., 13·8 mCi/mmole), both radioisotopically labelled in the 2-position, were checked for radioactive purity and kindly provided for these studies by Dr. C. A. Nichol, The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, N.C., U.S.A. Burroughs Wellcome Co. also provided gifts of non-radioactive PRM and DDMP. MTX and folinic acid, as calcium leucovorin, were obtained from Lederle Products Ltd., Montreal, Quebec, Canada. [^{14}C]inulin carboxylic acid (sp. act., 8·6 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, England, as was radioactive methotrexate 3′5′T sodium salt (sp. act., 250 mCi/mmole).

Cell cultures. L5178Y lymphoblasts were grown in suspension culture as described previously [2].

Transport studies. L5178Y cells selected from culture in logarithmic growth were used. Experimental design and analytical techniques have been described in detail previously [2, 15]. The following modifications were made. A cell concentration of 2×10^6 cells/ml in Hank's balanced salt solution, pH 7·4, without added serum, was employed. ^{14}C-labelled diaminopyrimidines were added to aliquots of these cell suspensions resulting in a final drug concentration of 5×10^{-5} M and a radioisotope concentration of 0·625 μCi/ml. The concentration for MTX was 5×10^{-5} M (1 μCi/ml). Radioactivity was determined using Aquasol (NEN, Dorval, Quebec, Canada) scintillant in a Nuclear Chicago scintillation counter Unilux II model 6853 at 63 per cent efficiency for ^{14}C.

For measurement of drug uptake at 4°, the cell suspensions were precooled in ice for 15 min prior to drug addition.

The rate of efflux of diaminopyrimidines from L5178Y cells at 37° was determined by pre-incubating cells with drug for 30 min. The cells were then pelleted by centrifugation at 350 *g* for 2 min at room temperature, washed free of ^{14}C-labelled drug not associated with the cells, and finally resuspended to the same volume in fresh Hank's solution without drug at 37°. Aliquots were removed at later time intervals and their radioactive content was determined.

The intracellular water content was estimated as the difference between the total water content of the cell pellet and the extracellular water, as determined by incubation with

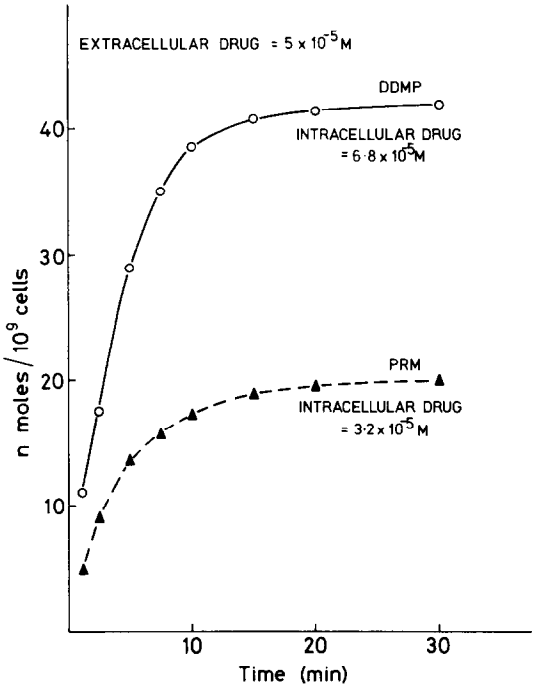

Fig. 1. Rates of uptake of ^{14}C-labelled PRM and DDMP expressed in nmoles of drug/10^9 cells as a function of time (min) of incubation at 37° and 4°. Each figure represents the mean of eight determinations. Overall scatter not in excess of 10 per cent.

Table 1. A comparison of the "steady-state" levels of PRM, DDMP and MTX achieved in L5178Y lymphoblasts during influx in the presence of 5×10^{-5} M drug and after efflux in drug-free medium from "preloaded cells"*

Drug	Influx "steady state" intracellular drug concn† (M)	Efflux "steady state" intracellular drug concn† (M)
DDMP	$6{\cdot}8 \times 10^{-5}$	$1{\cdot}9 \times 10^{-5}$
PRM	$3{\cdot}2 \times 10^{-5}$	$0{\cdot}75 \times 10^{-5}$
MTX	$0{\cdot}6 \times 10^{-5}$	$0{\cdot}08 \times 10^{-5}$

* 2×10^6 Cells/ml were incubated in the presence of drug for 30 min at 37° to achieve a "steady state" intracellular concentration. The cells were then re-incubated in the presence of fresh drug-free medium at 37° for a further 30-min period, after which the level of drug/cell was calculated.

† The intracellular drug concentration values were calculated using measurements of intracellular free space, determined as described in Methods. The intracellular water content was $6{\cdot}1 \pm 0{\cdot}2 \times 10^{-7}$ mg (S.D. over six estimations).

Each figure represents the mean value of eight estimations.

Overall scatter was not greater than 10 per cent.

[^{14}C]inulin, according to the method of Goldman *et al.* [16].

Influx of diaminopyrimidines. The rates of uptake of ^{14}C-labelled PRM and DDMP are shown in Fig. 1. The pattern of influx is similar for both drugs. Initially there is a rapid association of the drugs with the cells (within 1 min) which is not temperature dependent. This is followed by a period during which the amount of the drugs within the cells increases linearly as a function of time. This increase is temperature dependent, being reduced at 4°. Subsequently a steady state concentration is reached (after 20 min at 37°). The more lipophilic derivative, DDMP, is taken up over the linear portion of the curve at a faster rate (6·8 nmoles/10^9 cells/min) than the parent compound PRM (3·2 nmoles/10^9 cells/min). The final extent of uptake by the cells was greater for DDMP than PRM. The time course of uptake of these diaminopyrimidines follows three phases, similar to those described previously for MTX transport into L1210 mouse leukaemia cells [16]. However, the size of the rapidly associating fraction of diaminopyrimidines is much greater than that reported for MTX. This finding may be related to the more lipophilic nature of diaminopyrimidines. DDMP is taken up more rapidly and accumulated to a greater extent than PRM by these L5178Y cells. This is in agreement with previous studies which showed a significantly greater concentration of DDMP than PRM throughout the whole brain of animals.*

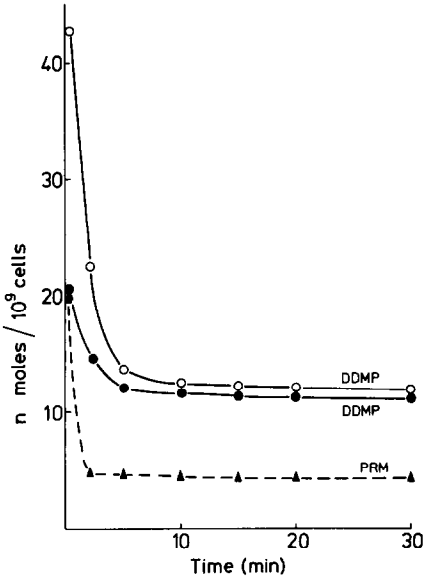

Fig. 2. Rate of efflux of ^{14}C-labelled PRM and DDMP from "preloaded" cells expressed in nmoles of drug/10^9 cells as a function of time of reincubation in drug-free medium at 37°. Each figure represents the mean of six determinations. Overall scatter not in excess of 15 per cent.

Table 2. Kinetic properties of the transport of PRM and DDMP by L5178Y lymphoblasts*

Drug	Concn. range (10^{-5} M)	K_m†	V_{max}‡
PRM	1–10	0·49 ± 26%	18·1 ± 18·1%
DDMP	1–10	0·131 ± 8%	18·4 ± 6%

* The data were derived by calculation of enzyme Michaelis–Menten parameters with standard errors by the statistical method of Wilkinson [17]. The data presented represent the mean values from triplicate estimations and duplicate experiments.

† The x intercept of the Lineweaver–Burk plot $= -1/K_m$. K_m is in millimolar concentration.

‡ The y intercept of the Lineweaver-Burk plot $= 1/V_{max}$. V_{max} is expressed as nmoles/min/10^9 cells.

* S. Simmonds, personal communication (1973).

Efflux of diaminopyrimidines. L5178Y lymphoblasts were preloaded with either PRM (20 nmoles/10^9 cells) or DDMP (to approximately 43 and 21 nmoles/10^9 cells) by incubation for 30 min at 37°. Aliquots of cell suspension were removed at the times indicated in Fig. 2, which illustrates the rate and extent of efflux of these drugs from the cells. There was a rapid efflux of both agents, but the final steady state level of drug was achieved more quickly for PRM (within 2 min) than for DDMP (by 5 min). The final amount of drug associated with the cells was higher (× 2 approx) for DDMP than for PRM. These differences in efflux characteristics between PRM and DDMP were still noted when the cells were preloaded with each drug to comparable concentrations (approx 20 nmoles/10^9 cells).

It is interesting (see Table 1) that the final steady state internal levels of both PRM and DDMP both during influx and after efflux, following initial exposure to an extracellular drug concentration of 5×10^{-5} M was greatly in excess of the levels achieved after exposure to 5×10^{-5} M MTX under comparable experimental conditions. These results suggest that, under these conditions, the intracellular concentrations of diaminopyrimidines may be considerably greater than those required to inhibit the enzyme dihydrofolate reductase.

Kinetics of diaminopyrimidine uptake. For these kinetic studies involving determinations of the initial velocity of drug uptake, the reactions were terminated after a 5-min incubation at 37°, when the rate of influx was approximately linear (Fig. 1). Uptake of both PRM and DDMP approximated Michaelis Menten kinetics for drug concentrations from 1 to 10×10^{-5} M. The kinetic parameters are described in Table 2.

The Michaelis constant (K_m) for uptake of PRM (0·49 mM) is approximately four times greater than that for DDMP, indicating a lower degree of saturability. The maximal levels of velocity of uptake achievable for both drugs were comparable, being of the order of 18 nmoles/min/10^9 cells.

Competition between various folic analogues for the transport of diaminopyrimidines. To establish whether these two diaminopyrimidines, PRM and DDMP, were transported by the same mechanisms, we investigated the possibility that they might compete with each other for entry into the cells. The effect of equimolar unlabelled diaminopyrimidines on the influx of ^{14}C-labelled materials was obtained following incubations at 37° for 30 min (by which time the "steady state" level has been reached; see Fig. 1). The uptake of PRM and DDMP in the presence of MTX or folinic acid (5-formyl tetrahydrofolate) was also measured. The results (Table 3) show that the presence of equimolar PRM did not influence the influx of [^{14}C]DDMP or vice versa. Similarly, MTX at a concentration equimolar to that of either of the diaminopyrimidines was without a significant effect on the influx into cells. However, the addition of equimolar folinic acid markedly reduced the uptake of both PRM and DDMP. This inhibition was more noticeable with PRM than DDMP.

The addition of equimolar folinic acid to cells "preloaded" with the diaminopyrimidines did not alter either the initial rate or final extent of efflux of these agents.

The failure to demonstrate competition for uptake between DDMP, PRM and/or MTX, raises the possibility that each of these agents may be transported into the cells via different sites. However, the significant degree of competition between diaminopyrimidines and folinic acid may suggest that these substances have a common transport mechanism, which is not shared by MTX. This observed inhibition of uptake of diaminopyrimidines by folinic acid may be of importance in the designing of clinical schedules using these agents, and may perhaps explain the previously noted ready reversibility of the effects of these drugs by the addition of reduced folates [6].

Table 3. Effect of various folate derivatives on the extent of uptake of PRM and DDMP by L5178Y cells after 30-min incubation at 37°*

	Concn. (M)	Extent of uptake (%)† PRM‡	DDMP‡
PRM	5×10^{-5}	—	98
DDMP	5×10^{-5}	92	—
MTX	5×10^{-5}	89	85
Folinic acid	5×10^{-6}	56	76
	10^{-5}	27	48
	5×10^{-5}		25

* Incubations were carried out in Hank's balanced salt solution using 2×10^6 cells/ml as described in Methods.

† The extent of uptake in the absence of added folate compound = 100%.

‡ The drug concentration was 5×10^{-5} M.

Acknowledgements—J. H. Goldie wishes to acknowledge the support of the Ontario Cancer and Treatment Research Foundation, Canada. The authors are grateful to Drs. R. C. Jackson and L. I. Hart for their valuable help in carrying out the statistical evaluation of the kinetic data.

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REFERENCES

1. B. T. Hill, J. H. Goldie and L. A. Price, *Br. J. Cancer* **28,** 263 (1973).
2. J. H. Goldie, M. E. Furness and L. A. Price, *Eur. J. Cancer* **9,** 709 (1973).
3. J. H. Burchenal, S. K. Goetchius, C. C. Stock and G. H. Hitchings, *Cancer Res.* **12,** 251 (1952).
4. D. A. Clarke, S. M. Buckley, S. S. Sternberg, C. C. Stock, C. P. Rhoads and G. M. Hitchings, *Cancer Res.* **12,** 255 (1952).
5. K. Sugiura, *Cancer Res.* **13,** 431 (1953).
6. L. M. Murphy, R. R. Ellison, D. A. Karnofsky and J. H. Burchenal, *J. clin. Invest.* **33,** 1388 (1954).
7. D. R. Stickney, W. S. Simmons, R. L. Deangelis, R. W. Rundles and C. A. Nichol, *Proc. Am. Ass. Cancer Res.* **14,** 52 (1973).

8. G. F. Geils, C. W. Scott Jr., C. M. Baugh and C. E. Butterworth, *Blood* **38,** 131 (1971).
9. C. A. Nichol, *Adv. Enzyme Regulat.* **6,** 316 (1968).
10. G. H. Hitchings, E. A. Falco, G. B. Elion, S. Singer, G. B. Waring, D. J. Hutchison and J. H. Burchenal, *Archs Biochem. Biophys.* **40,** 479 (1952).
11. G. H. Hitchings, E. A. Falco, H. Vanderwerff, P. B. Russell and G. B. Elion, *J. biol. Chem.* **199,** 43 (1952).
12. I. D. Goldman, *J. biol. Chem.* **244,** 3779 (1969).
13. D. Kessel and T. C. Hall, *Cancer Res.* **27,** 1539 (1967).
14. E. M. Nadel and J. Greenberg, *Cancer Res.* **13,** 865 (1953).
15. K. R. Harrap, B. T. Hill, M. E. Furness and L. I. Hart, *Ann. N.Y. Acad. Sci.* **186,** 312 (1971).
16. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, *J. biol. Chem.* **243,** 5007 (1968).
17. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).

Biochemical Pharmacology, Vol. 24, pp. 538–540. Pergamon Press, 1975. Printed in Great Britain.

A possible role for cyclic AMP in the regulation of histamine secretion and the action of cromoglycate

(*Received* 8 *August* 1974; *accepted* 30 *August* 1974)

The secretion of histamine from mast cells and basophil leucocytes is inhibited by the application of substances (adenylate cyclase activators, phosphodiesterase inhibitors and cyclic AMP analogues) which lead to the accumulation of intracellular cyclic AMP [1–7] and it has been shown that the antiallergic drug, cromoglycate inhibits cyclic AMP phosphodiesterase from several tissues [8].

Application of a specific antigen to sensitized mast cells triggers the secretion of histamine in a calcium-dependent manner [9, 10]. The secretion of histamine is also accompanied by an increase in calcium uptake by the mast cells, and cells made selectively permeable to calcium by treatment with the divalent cation carrier substance (ionophore), A 23187, release histamine when subsequently provided with calcium [11]. It has been suggested that the antigen-antibody reaction on the mast cell membrane opens calcium gates in the membrane [12], and the response of the mast cells to calcium following antigen stimulation, decreases rapidly with time; indicating that the gates do not remain open. The response of the cells to calcium has almost completely disappeared 4 min after antigen stimulation [13]. It has therefore been proposed that the control of histamine secretion following the antigen-antibody stimulus is brought about by time-dependent changes in the calcium permeability of the mast cell membrane. It must be pointed out, however, that whereas histamine secretion is complete in 1 min, the decay of the response to calcium following antigen stimulation is not complete before 4 min although it is substantially manifest after 1 min. Supporting the idea that the decay in the response to calcium is related to the control of histamine secretion is the observation that phosphatidyl serine prolongs the release process and slows the decay of the calcium response [13, 14]. In view of these observations, it was of interest to examine the role of cyclic AMP in relation to the action of calcium in rat peritoneal mast cells.

The source of the cells together with the methods of sensitizing them and of inducing and measuring histamine release from them have been described [10, 15]. Dibutyryl cyclic AMP (Sigma) produced a graded inhibition of antigen-evoked histamine release as the concentration was raised in the range from 10 μmoles/l to 10 m-moles/l (Fig. 1).

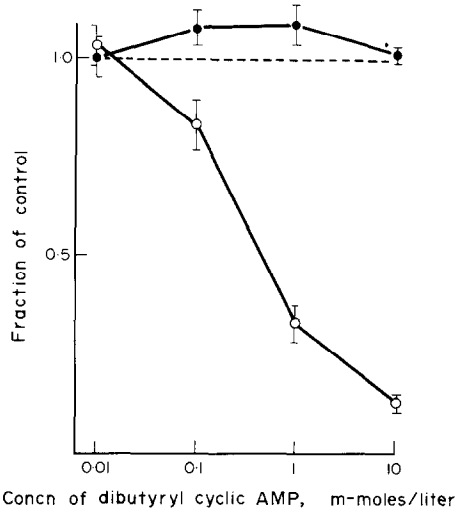

Fig. 1. Concentration–response relationship for the action of dibutyryl cyclic AMP on histamine release induced by antigen, 10 μg/ml (○) and A 23187, 5 μmoles/l (●) from the same cell population. Response is expressed as a fraction of control histamine release from cells suspended in a medium containing calcium, 1 m-mole/l and no dibutyryl cyclic AMP. Control histamine releases were: 22 ± 5 per cent (mean ± S.D.) of total for antigen and 77 ± 4 per cent of total for A 23187. Cells with or without dibutyryl cyclic AMP were preincubated for 30 min at 37° before the addition of the histamine releasing agent. Each point is the mean from four experiments and the vertical bars represent S.E.M.