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STIMULATION BY PTERIDINES OF THE UPTAKE OF AMETHOPTERIN BY HUMAN LYMPHOCYTES*

DAVID KESSEL, VIVIENNE BOTTERILL and THOMAS C. HALL

Laboratories of Pharmacology, Children's Cancer Research Foundation and Departments of Pathology and Medicine, Harvard Medical School, Boston, Mass., U.S.A.

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Abstract—Triamterene (2,4,7-triamino-6-phenylpteridine) and certain other pteridines stimulate the uptake of amethopterin by human small lymphocytes, apparently by removing a barrier to amethopterin transport. This stimulation did not extend appreciably to other cell types or to other lymphocyte transport systems tested.

TRIAMTERENE (2,4,7-triamino-6-phenylpteridine, Dyrenium) is an effective diuretic in rat and dog[1] and in man.[2] The compound inhibits sodium resorption and potassium secretion in the kidney distal tubule.[3] The ability of triamterene to inhibit the enzyme dihydrofolate reductase was attributed to the 2,4-diaminopteridine structure of the compound;[4] compounds containing this structure are generally found to be tightly bound to the enzyme.[5]

During studies on amethopterin transport by human leukoeytes *in vitro*, we observed an unexpected stimulation of uptake after addition of triamterene to the medium.[6, 7] Such a stimulation is of potential value, since impaired uptake of amethopterin was associated with resistance to this drug in human[8] and animal[9, 10] leukemias.

METHODS

Human leukocytes were isolated from whole blood by the method of Fallon *et al.*[11] Small lymphocytes were obtained from normal leukocyte preparations by differential centrifugation;[12] the resulting cell preparations were freed from contaminating granulocytes on columns of siliconized glass beads,[13] if necessary. Final suspensions used contained 90–100 per cent small lymphocytes. Preparations consisting mainly (90–100 per cent) of small lymphocytes were obtained from patients with chronic lymphocytic leukemia. Granulocytes were collected after differential centrifugation of normal leukocyte suspensions;[12] the bottom layer of cells consisted of 85–90 per cent granulocytes. All cell preparations were examined microscopically, after staining, to determine relative proportions of each cell type present. No cell preparation was used unless at least the purity criteria described above were achieved.

For transport studies, cells were suspended in 10 vol. of medium which contained 62 mM TES† buffer[14] at pH 7·2, 65 mM NaCl, 15 mM KCl and 8 mM $CaCl_2$; this medium was found to permit optimal uptake of model amino acids by human leukocytes.[15] In some cases, human serum was added to this medium (25 per cent by

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† TES = *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

volume) to promote cell stability during long incubations. The volume of the cell suspension used for incubations was 150 μl. The concentration of amethopterin in the medium was 0·2 μM, a level comparable to that found in blood during low dose clinical therapy.[16] Uptake of cycloleucine, adenine, uracil, inulin and sulfate were measured from 0·1 mM concentrations. Cell suspensions were brought to the specified temperature, usually 37°, and labeled compounds together with other agents were added in a total volume of 10 μl or less. After incubation for 1–30 min with gentle shaking, the cells were collected by centrifugation, washed by resuspension in medium* and again collected by centrifugation; labeled compounds were extracted by addition of 250 μl of 0·01 N acetic acid. The resulting suspensions were heated at 60° for 10 min, cell debris was removed by centrifugation and 200 μl of the supernatant fluid was used for determination of radioactivity by liquid scintillation techniques. All centrifugations described above were carried out in a Microchemical Specialties Co. model 5500 centrifuge for 30 sec at 300 *g*.

Binding of pteridines to serum proteins was determined by ultrafiltration. For this purpose, 1-ml samples of serum were mixed with 10-μl portions of 1 mg/ml solutions of pteridines. The mixtures were placed in dialysis tubing and sequential samples of 20–30 μl of ultrafiltrate were obtained by centrifugation of the closed bags in graduated centrifuge tubes. Determination of concentrations of pteridines inside the dialysis bags and in the ultrafiltrate was made with an Aminco-Bowman spectrophotofluorometer, with an excitation wavelength of 360 mμ. Light emission at 460 mμ was measured.

Amethopterin-3′,5′-^{3}H (2-9 c/m-mole) was purchased from the Nuclear Chicago Corp. Triamterene (nonradioactive and ^{14}C-labeled) and other pteridines were provided by Dr. Alfred Maass of Smith, Kline & French Laboratories, Philadelphia, Pa. Other labeled drugs were purchased from New England Nuclear Corp., Boston, Mass. Human serum was purchased from Grand Island Biological Co., Grand Island, N.Y. and was provided by the Children's Cancer Research Foundation.

RESULTS

Effect of pteridines on uptake of amethopterin by normal leukocyte types. Triamterene and certain analogs (Fig. 1) stimulated uptake of amethopterin by normal small lymphocytes and, to a much more limited extent, by normal granulocytes (Table 1). In the absence of added pteridines, uptake of amethopterin during a 15-min incubation at 37° was 6 $\pm$ 1 mμmole/kg for small lymphocytes and 33 $\pm$ 4 mμmole/kg for granulocytes.† Triamterene and amethopterin are both 2,4-diaminopteridines and, like the other 2,4-diaminopteridines listed here, they inhibit‡ dihydrofolate reductase from rat liver.[18] However, it is noteworthy that compound 6 of Table 1 stimulated amethopterin uptake by lymphocytes, but is not a 2,4-diaminopteridine and did not inhibit rat liver dihydrofolate reductase.

Addition of serum to incubation mixtures abolished the stimulation of amethopterin by triamterene (Table 2). Ultrafiltration studies showed extensive capacity for binding

* This wash was usually carried out at 0°, but the results were not altered if the wash and incubation temperatures were the same.

† These figures represent average values ($\pm$ S.D.) of measurements on 20 cell preparations from different normal donors. The greater ability of granulocytes to take up amethopterin has been noted before.[17]

‡ D. Roberts, unpublished results. All of the 2,4-diaminopteridines of Table 1 inhibited the rat liver enzyme; $K_i = 10^{-7}$ to 10^{-8} M.

of triamterene to serum proteins. An even greater capacity for binding of 2,4-diamino-6,7-diphenylpteridine (DDP) to serum proteins was observed, but the stimulation of amethopterin uptake by DDP was not significantly altered by addition of serum to cell suspensions. Stimulation of amethopterin uptake by biopterin was found, although

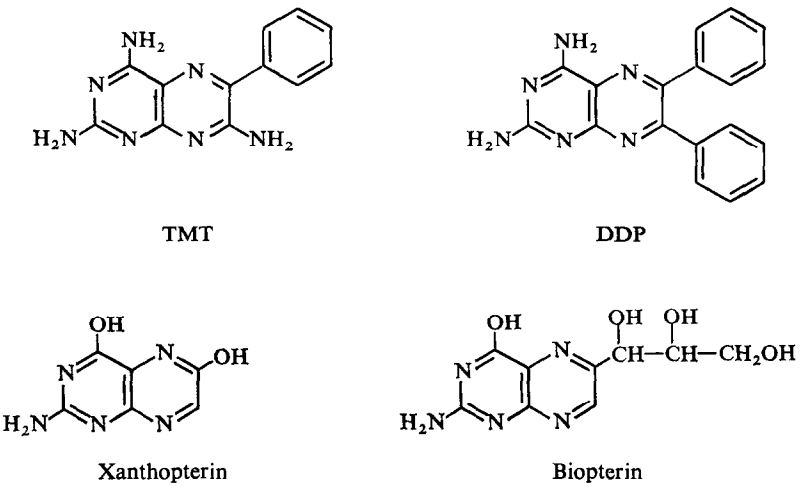

FIG. 1. Molecular structures of triamterene (TMT), 2,4-diamino-6,7-diphenylpteridine (DDP), biopterin and xanthopterin.

TABLE 1. PTERIDINE STIMULATION OF AMETHOPTERIN UPTAKE BY NORMAL HUMAN LEUKOCYTES*

Compound tested	Stimulation of amethopterin uptake† Granulocytes (%)	Lymphocytes (%)
1. 2,4,7-Triamino-6-phenylpteridine‡	+ 0	+ 75
2. 2,4,7-Triaminopteridine	+ 0	+ 12
3. Biopterin	+ 0	+ 90
4. 2,4-Diamino-6,7-diphenylpteridine	+ 23	+ 300
5. 2,6,7-Triphenyl-4-aminopteridine	+ 0	+ 30
6. 4,7-Diamino-2,6-diphenylpteridine	+ 5	+ 70
7. Xanthopterin	+ 0	+ 2
8. 4,6,7-Triamino-2-phenylpteridine	+ 0	+ 24
9. 2,4-Diamino-6,7-dimethylpteridine	+ 0	+ 0
10. 4,7-Diamino-2-phenylpteridine	+ 0	+ 25

* Cells were incubated for 15 min at 37° in medium containing 0·2 μM labeled amethopterin and, in replicate tubes, 2×10^{-4} M pteridine.

† Data represent values obtained with typical cell preparations. Data on 20 other samples yielded values that did not differ from these by more than ± 10 per cent.

‡ Triamterene.

the latter compound was not significantly bound to serum proteins. We concluded that the relative serum-binding ability of the pteridines was not related to the stimulation phenomenon.

TABLE 2. EFFECT OF SERUM ON PTERIDINE STIMULATION OF AMETHOPTERIN UPTAKE BY NORMAL SMALL LYMPHOCYTES*

Pteridine tested†	Amethopterin uptake‡ (mμmole/kg cells) + Serum	— Serum	Serum§ binding (%)
Triamterene	5 ± 1·2	10·5 ± 2·0	70 ± 6
2,4-Diamino-6,7-diphenylpteridine	15 ± 2·4	18·2 ± 2·7	89 ± 5
Biopterin	9·1 ± 1·5	9·4 ± 1·6	2 ± 1·4
Controls	5 ± 1	6 ± 1	

* Cells were incubated for 15 min at 37° in medium containing 0·2 μM labeled methotrexate. Human serum (25 per cent by volume) was present where specified.

† Pteridines (2×10^{-4} M) were added at the start of the incubations.

‡ Average values ± S.D. for 20 samples.

§ Average values ± S.D. for 5 samples.

When graded amounts of triamterene or DDP were added to suspensions of normal small lymphocytes, stimulation of amethopterin uptake was observed only when the pteridine level reached about $1·6 \times 10^{-4}$ M (Fig. 2).

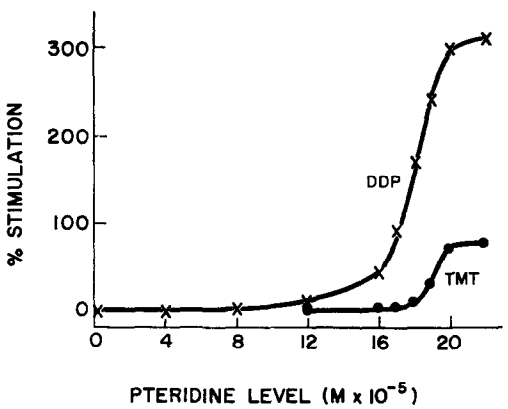

FIG. 2. Effect of addition of graded amounts of triamterene (TMT) and of 2,4-diamino-6,7-diphenylpteridine (DDP) on uptake of amethopterin (0·2 μM) by a typical preparation of normal small lymphocytes. Incubations were for 15 min at 37°.

Effect of pteridines on uptake of amethopterin by leukemic lymphocytes. Our studies on stimulation of amethopterin uptake with cells isolated from 14 patients with chronic lymphocytic leukemia are summarized in Table 3. Cell preparations used contained 90–100 per cent small lymphocytes. The stimulation of amethopterin uptake caused by triamterene varied widely; DDP was found effective in some cell types refractory to triamterene.

Some patients (J.A., M.C. and L.W. of Table 3) had sufficiently high leukocyte counts so that pure preparations of cells could be obtained by centrifugation of whole blood samples without the need for osomotic lysis or treatment on columns of

glass beads. Samples of these cells were subjected to the lysis and column treatments, and it was found that neither procedure altered the results shown in Table 3.

Uptake of ^{14}C-triamterene by small lymphocytes. Transport and subsequent loss of labeled triamterene by normal small lymphocytes was found to be temperature-sensitive (Fig. 3). Uptake of the drug was only slightly decreased upon addition of serum to the medium (25 per cent by volume). The apparent drug distribution ratio of

TABLE 3. PTERIDINE STIMULATION OF AMETHOPTERIN UPTAKE BY SMALL LYMPHOCYTES

Source of cells*	Amethopterin uptake† (mμmole/kg cells)	Stimulation by pteridines‡	
		TMT (%)	DDP (%)
Normals	6 ± 1·0	75 ± 35	200 ± 45
J.A.	5·8	0	300
W.B.	10·5	0	400
M.C.	13·2	10	
P.C.	9·0	0	300
R.D.	6·2	40	
A.H.	5·0	200	
C.M.	7·5	80	
A.N.	8·5	200	
C.S.	8·6	0	
N.S.	7·5	300	
R.S.	6·2	70	
A.T.	7·8	0	
B.W.	5·0	400	
L.W.	4·0	0	300

* Normals = normal small lymphocytes isolated from donors. Data shown represent average ± S.D. of 20 samples from different donors. Initials refer to different patients with chronic lymphocytic leukemia from whom small lymphocytes were obtained. Data here represent the average of 3 determinations on single samples; these did not differ by more than ± 10 per cent.

† Cells were incubated with 0·2 μM amethopterin for 15 min at 37°

‡ 0% = No stimulation. Pteridines were added at 2×10^{-4} M under conditions specified in the preceding footnote. TMT = 2,4,7-triamino-6-phenylpteridine (triamterene); DDP = 2,4-diamino-6,7-diphenylpteridine.

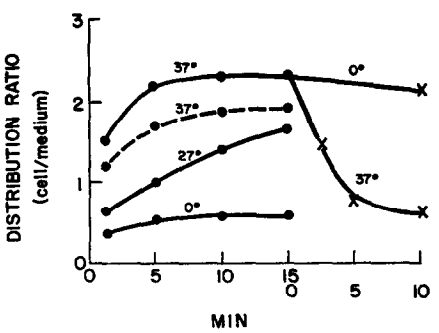

FIG. 3. Uptake and loss of ^{14}C-triamterene by a typical preparation of normal small lymphocytes from medium containing 2×10^{-4} M of the compound; ●——● = serum absent, ●- - - -● = serum present (25 per cent by volume), ×——× = subsequent loss of drug from previously-loaded cells.

2 attained may not represent "active transport," since electrophoretic measurements showed that triamterene has a net positive charge at pH 7·0. The rate of loss of triamterene from previously loaded cells was found to be temperature-dependent; drug loss was much more rapid at 37° than at 0°.

Effects of pteridines on other transport systems of human leukocytes. The pteridines tested were found to be relatively specific in their action. Uptake of at least five other labeled compounds by lymphocytes or granulocytes was not affected by addition of triamterene, DDP, biopterin or xanthopterin. Distribution ratios achieved after 15-min incubations of small lymphocytes at 37° in medium containing 0·1 mM cycloleucine, uracil, adenine, inulin or sulfate were, respectively, 2·5, 0·8, 0·7, 0·02 and 0·01. These values did not significantly vary when granulocytes were employed, and addition of 2×10^{-4} M triamterene, DDP, biopterin or xanthopterin to either small lymphocyte or granulocyte suspensions did not affect the distribution ratios. These data suggest that the pteridines do not cause a general increase in cellular permeability.

The effect of triamterene and certain of its analogs on amethopterin uptake appears to be limited to human lymphocytes. Uptake of amethopterin by human lymphoblasts or myeloblasts *in vitro*, under conditions described here, was not affected by addition of 2×10^{-4} M triamterene or DDP to incubation media. In other studies, using mouse leukemia cells with a relatively low capacity for amethopterin uptake,[9] we observed no stimulation of amethopterin uptake by triamterene or DDP. The cell lines tested were P329, P1534J$_a$, L1210, L1210/MTX and the Ehrlich ascites carcinoma. Methods used for cell isolation and incubation are given in reference 9.

DISCUSSION

Certain pteridines were found to stimulate the uptake of amethopterin *in vitro* by human small lymphocytes isolated from normal donors or from patients with chronic lymphocytic leukemia. Amethopterin uptake by normal granulocytes, by human lymphoblasts or myeloblasts, or by five mouse leukemia cell lines tested was not much affected by pteridines tested. It is noteworthy that facilitated uptake of amethopterin could be demonstrated by all normal and leukemic human leukocyte types except small lymphocytes.* Facilitated uptake of amethopterin could also be shown in mouse leukemia cells.[19] We conclude that triamterene and DDP act by removing barriers to uptake of amethopterin which are unique to small lymphocytes.

The stimulation observed was not limited to 2,4-diaminopteridines and was unrelated to the relative affinity of the pteridines for serum proteins. Serum binding of pteridines was generally enhanced by the presence of aromatic substituents on the pteridine ring system. The ability of serum to reverse the stimulation of amethopterin uptake by triamterene is probably related to serum–pteridine interactions, since a decrease in the triamterane level to below 2×10^{-4} M greatly decreased the effect of the diuretic on amethopterin uptake. But another pteridine, DDP, seemed less sensitive to addition of serum to the incubation mixture, although DDP is extensively serum-bound. DDP was also able to stimulate amethopterin uptake in some chronic lymphocytic leukemia cell types which were insensitive to triamterene (see Table 3).

These observations illustrate the potential use of appropriate agents for alteration of drug permeability by mammalian cells. Therapeutic applications of the present study appear limited, since uptake of amethopterin could only be stimulated in one cell

* D. Kessel, D. Roberts and T. C. Hall, unpublished observations.

type, small lymphocytes. In addition, the stimulation by triamterene was reversed by serum. Another agent, however, was unaffected by serum *in vitro* and might therefore be representative of a class of compounds effective *in vitro*.

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