Acknowledgements—We thank Mr. E. Walsh, Mr. E. Nice and Mr. W. Down for skilled technical assistance. L.F.C. is grateful to the Medical Research Council for a grant. This investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and from the British Empire Cancer Campaign for Research.

Chester Beatty Research Institute,
Institute of Cancer Research,
Royal Cancer Hospital,
London S.W.3

E. BOYLAND
L. F. CHASSEAUD*

* Present address: Huntingdon Research Centre, Huntingdon, England.

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Biochemical Pharmacology, Vol. 19, pp. 1528–1533. Pergamon Press, 1970. Printed in Great Britain

Quinazoline antifolates as inhibitors of dihydrofolate reductase from human leukemia cells

(*Received* 15 *July* 1969; *accepted* 3 *October* 1969)

SIGNIFICANT antileukemic activity of a series of 2,4-diaminoquinazolines in L1210 mouse leukemia test systems has recently been reported by Hutchinson and Shimoyama.[1, 2] Several compounds of the series were found to be more effective in these test systems than was methotrexate (MTX), the dihydrofolate reductase inhibitor presently used clinically. *In vitro*, the 2,4-diaminoquinazolines were found to be potent inhibitors of the enzyme dihydrofolate reductase from mouse L1210 leukemia cells.[3]

The effectiveness of the 2,4-diaminoquinazolines antifolates as inhibitors of human dihydrofolate reductase has not yet been reported. The purpose of the present communication is to describe the activity of the 2,4-diaminoquinazoline antifolates as inhibitors of dihydrofolate reductase from human leukemia cells, and to present some additional studies concerning the mode of action of these compounds.

METHODS AND MATERIALS

Dihydrofolate reductase from human leukemia cells

Leukemic leukocytes were obtained by a dextran sedimentation method[4] from blood of patients with acute myelocytic or myelomonocytic leukemia, and were stored frozen until sufficient cells had been accumulated for enzyme purification. To the pooled human leukemia cells were added 4 vol. of 0·9% saline solution; the cell suspension was then lysed by freeze-thawing twice, and the resulting lysate centrifuged for 20 min at 27,000 *g* at 4°. The supernatant fraction was removed and brought to pH 6 with hydrochloric acid (0·1 N). Solid ammonium sulfate was then added with stirring, until a level of 45 per cent saturation was reached. The solution was stirred slowly for 20 min, and then centrifuged for 20 min at 27,000 *g*. The supernatant fraction was separated, the pH readjusted to 6 with hydrochloric acid (0·1 N), and additional ammonium sulfate added until a level of 90 per cent saturation was reached. The suspension was stirred slowly for 1 hr, and then centrifuged for 20 min at 27,000 *g* at 4°. The precipitate was redissolved in a small volume of water and subjected to gel filtration on Sephadex G-75 followed by chromatography on hydroxylapatite as previously described.[5] The specific activity of the final enzyme preparation was 73 μmoles dihydrofolate reduced per hour per milligram of protein. Protein concentration was determined by the method of Waddell.[6]

The purified enzyme was stored under toluene after the addition of bovine serum albumin, 1 mg/ml. Under these conditions, the enzyme solution could be stored at 4° for several months without significant loss of activity.

Enzyme assays

Dihydrofolate reductase activity was assayed as follows: cuvettes contained Tris-HCl buffer, pH 7·5, 100 μmoles; KCl, 150 μmoles; NADPH, 0·1 μmole; dihydrofolate, 0·01 μmole; and enzyme, in a total volume of 1 ml. The conversion of NADPH and dihydrofolate to NADP and tetrahydrofolate, respectively, was followed at 340 mμ, as previously described.[5] All assays were performed at 37°, using a Gilford multiple sample absorbance recorder. NADPH was obtained from the Sigma Chemical Co., and dihydrofolate was synthesized by Blakley's modification of the method of Futterman.[7]

To determine the inhibitory activity of the compounds to be tested, sufficient enzyme was used in the assay mixture to give a control rate of approximately 0·015 absorbance units per minute at 340 mμ. Inhibitors were preincubated with the assay mixture for 2 min, and the reaction started by the addition of dihydrofolate. Rates in the presence of several levels of inhibitor were determined, the data plotted, and the I_{50} levels obtained graphically. As an illustration of this method, the titration curve of human dihydrofolate reductase by MTX is shown in Fig. 1.

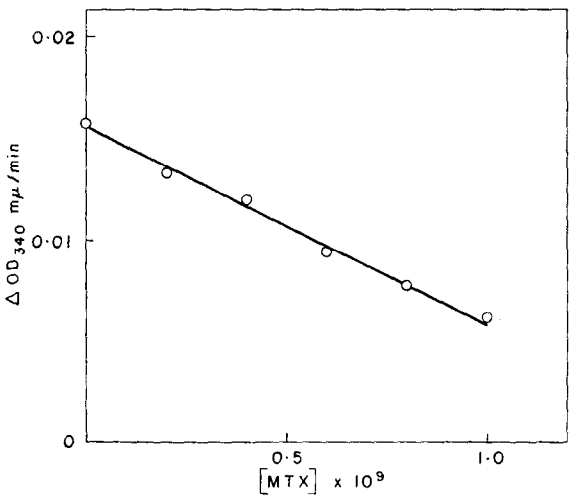


FIG. 1. Inhibition of human leukemia cell dihydrofolate reductase by MTX. Assay conditions are described in Methods.

Inhibition of the conversion of deoxyuridine to thymidylate

(1) *Mouse L1210 ascites tumor cells.* L1210 mouse ascites tumor cells were harvested 3 days after the intraperitoneal inoculation of 10^6 cells into male BDF_1 mice. At this time interval after inoculation, more than 95 per cent of the cells in a typical smear could be classified as neoplastic. Approximately 10^6 cells were suspended in 1 ml of Eagle's basal medium containing 10% horse serum. The cells were then preincubated at 37° for 30 min with the antifolate to be tested. Tritiated UdR* (3 μc) was added to each flask, and incubations carried out for time periods of 15, 30, 45 and 60 min. The reaction was terminated by the addition of 5 ml of cold 5% perchloric acid. After 3 washes with the latter, the DNA was hydrolyzed with hot perchloric acid. The incorporation of the radiolabel into DNA was determined by liquid scintillation counting and was plotted against time. The slope of the line was determined by linear regression analysis, and inhibition determined by comparison with the slope of the line obtained in the absence of inhibitor.[8] With each experiment, control parallel determinations of the rate of incorporation of ^{3}H-TdR† were carried out.

(2) *Human leukemia cells.* Blood samples were collected in heparinized tubes from patients with acute leukemia. The erythrocytes were allowed to settle by gravity and the leukocyte-rich plasma was harvested. The cells were collected by centrifugation and resuspended in Eagle's basal medium containing 10% horse serum. The cell suspensions were adjusted to contain 1 to 5 × 10^6 myeloblasts per milliliter of medium. Measurements of the incorporation of ^{3}H-TdR radioactivity into DNA were carried out as described above for L1210 mouse leukemia cells.

Inhibition of the uptake of MTX by L1210 mouse ascites tumor cells

L1210 mouse ascites tumor cells were harvested 7 days after the intraperitoneal inoculation of 10^6 cells into female BDF_1 mice. Approximately 4 × 10^7 cells were incubated with tritium-labeled MTX,‡ 2 × 10^{-7} M in 3 ml of Eagle's basal medium, without folic acid, to which 10% horse serum had been added. Incubations were carried out at 37° for 1 hr, with and without the 2,4-diaminoquinazoline antifolate to be tested, with aliquots being removed every 2 min for the first 10 min, and every 5 min thereafter. On removal from the incubation mixture, the cell suspensions were diluted with an equal volume of cold saline, and separated from the incubation medium by centrifugation through sucrose by the method of Fischer.[9] The cells were washed twice with cold saline, 0·9%, lysed by the addition of perchloric acid, 5%, and the radioactivity of the supernatant fraction determined by liquid scintillation counting.

2,4-*Diaminoquinazolines*

The 2,4-diaminoquinazolines used in the present studies were synthesized by Dr. John Davoll, Parke, Davis & Co., Middlesex, England, and were supplied by Drs. Florence White and H. B. Wood, Jr., Cancer Chemotherapy National Service Center, National Cancer Institute.

RESULTS

Inhibition of dihydrofolate reductase from human leukemia cells

The activity of 8 compounds of the 2,4-diaminoquinazoline series as inhibitors of dihydrofolate reductase from human leukemia cells is listed in Table 1. The most potent inhibitors in the series were aspartic acid, *N*-(*p*-[((2,4-diamino-5-chloro-6-quinazolinyl)methyl]amino)benzoyl)-,dihydrate, L- (CCNSC 529,861; "5-chloro-Q-asp") and glutamic acid, *N*-(*p*-[((2,4-diamino-6-quinazolinyl)-methyl]amino)benzoyl)-, L- (CCNSC 529,860): these compounds showed slightly greater activity than did MTX in the same system. Several other compounds of the series showed inhibitory activity of the same order of magnitude as that of the reference compound, MTX.

Inhibition of the conversion of UdR to dTMP

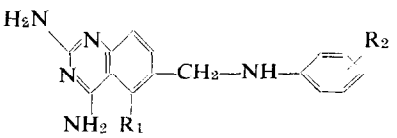
All the quinazoline antifolates tested were effective in inhibiting the incorporation of deoxyuridine radioactivity into the DNA of human and of mouse L1210 leukemia cells. The results of typical experiments are shown in Fig. 2 A to C; in these experiments, the inhibitory activity of the three 2,4-diaminoquinazolines examined was as great as, or greater than, that of the reference compound, MTX.

* Deoxyuridine-6-^{3}H, Schwarz; sp. act., 3·1 c/m-mole.

† (Methyl-^{3}H) thymidine, Schwarz; sp. act., 1·9 c/m-mole.

‡ Methotrexate-3′,5′-T, Amersham/Searle; sp. act., 3 c/m-mole.

TABLE 1. INHIBITION OF HUMAN LEUKEMIA CELL DIHYDROFOLATE REDUCTASE BY 2,4-DIAMINOQUINAZOLINES



CCNSC No.	R_1	R_2	I_{50}* (M)
105952	H	*p*-CONHCH(COOH)$_2$	$0{\cdot}8 \times 10^{-9}$
112846	H	*p*-CONHCH(COOH)CH_2COOH (L-)	$1{\cdot}2 \times 10^{-9}$
121346	H	*m*-CONHCH(COOH)CH_2CH_2COOH (L-)	$3{\cdot}8 \times 10^{-8}$
122761	Cl	*p*-CONHCH(COOH)CH_2CH_2COOH (L-)	$0{\cdot}8 \times 10^{-9}$
122870	CH_3	*p*-CONHCH(COOH)CH_2COOH (L-)	$1{\cdot}3 \times 10^{-9}$
529859	H	*p*-CONHCH(COOH)CH_2CH_2COOH (D-)	$2{\cdot}2 \times 10^{-9}$
529860	H	*p*-CONHCH(COOH)CH_2CH_2COOH (L-)	$0{\cdot}6 \times 10^{-9}$
529861	Cl	*p*-CONHCH(COOH)CH_2COOH (L-)	$0{\cdot}6 \times 10^{-9}$

* Concentration required for 50 per cent inhibition of human leukemia cell dihydrofolate reductase, under the assay conditions described in the text. The I_{50} value for MTX under these conditions is $0{\cdot}8 \times 10^{-9}$ M.

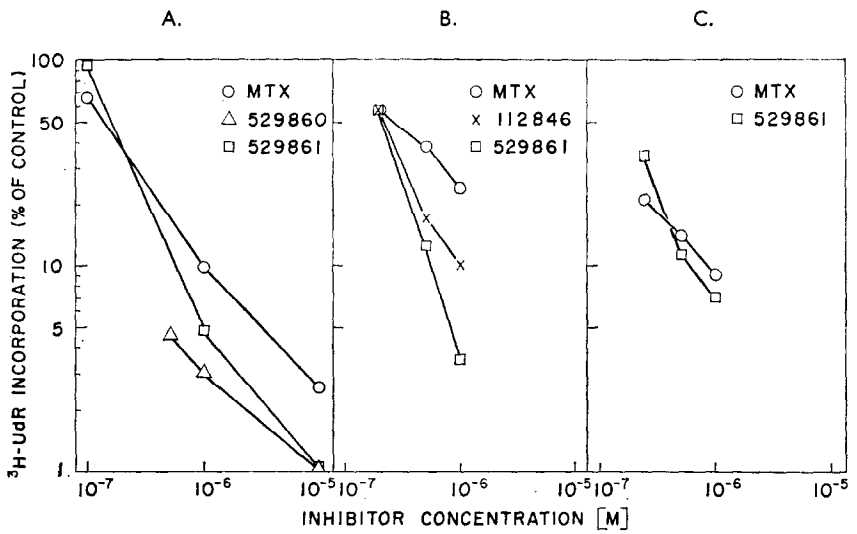


FIG. 2. Inhibition by 2,4-diaminoquinazolines and by MTX of incorporation of ^{3}H-deoxyuridine radioactivity into DNA of human and mouse L1210 leukemia cells. A (left): Uptake by mouse L1210 ascites tumor cells. B (center): Uptake by human leukemia cells obtained from patient A. S., female, age 43, with acute lymphocytic leukemia in relapse, who had previously received courses of treatment with MTX, prednisone, vincristine and chlorambucil. This patient was clinically resistant to MTX. C (right): Uptake by human leukemia cells obtained from patient M. R., female, age 76, with untreated acute myelocytic leukemia. Ordinate: Radioactivity incorporated, expressed as a percentage of control incorporation without inhibitor.

Inhibition of the uptake of MTX by mouse L1210 ascites tumor cells

Because of its unusually high activity in the two test systems described above, 5-chloro-Q-asp (CCNSC 529,861) was tested for its ability to inhibit the uptake of tritium-labeled MTX by mouse

L1210 leukemia cells. In the presence of 5-chloro-Q-asp at a concentration of 2×10^{-5} M, the rate of uptake of ^{3}H-MTX, 2×10^{-7} M, was significantly inhibited, being 28 per cent of the control rate in the absence of inhibitor.

DISCUSSION

The quinazoline antifolates examined were found to be potent inhibitors of dihydrofolate reductase from human leukemia cells. In these studies, the inhibition exerted by several members of the quinazoline series was of the same order of magnitude as, and, with two of the compounds, slightly greater than, that of MTX. Whether the relative activities of the 2,4-diaminoquinazolines and of MTX found in these studies would obtain over a wider range of assay conditions was not explored; it is known, however, that the degree of inhibition exerted by MTX and other antifolates varies significantly with the assay conditions used, particularly with the pH and with the degree of dilution of the assay mixture.[10] It should be noted, also, that because of the limited availability of leukemia cells from any one patient, these experiments were carried out with enzyme from pooled cells. We have never noted individual differences in the properties of human leukemia cell dihydrofolate reductase; if such differences exist, however, it is possible that in some patients, the relative sensitivity of the enzyme to inhibitors might differ from the average values given here.

The effectiveness of a folate antagonist in inhibiting purified dihydrofolate reductase *in vitro* does not necessarily correspond to its ability to inhibit the enzyme *in vivo*, since under physiologic conditions additional factors such as transport and metabolism may be of significance.[11] A useful aid in assessing the activity *in vivo* of a dihydrofolate reductase inhibitor is the measurement of ability to inhibit the incorporation of deoxyuridine radioactivity into DNA, since the latter process involves the conversion of dUMP to dTMP, a reaction requiring the coenzyme N^5,N^{10}-methylenetetrahydrofolate.[12] In the present studies, it was observed in some experiments (e.g., Fig. 2 A and B) that the ability of the 2,4-diaminoquinazolines to inhibit the incorporation of deoxyuridine when compared to the activity of MTX under the same experimental conditions appeared to be greater than would be anticipated from their relative ability to inhibit the enzyme dihydrofolate reductase (Table 1). This result can most readily be explained by postulating more rapid entry of the 2,4-diaminoquinazolines than of MTX into leukemia cells, although inhibition by the quinazolines at an additional site in thymidylate biosynthesis or tighter binding of the quinazolines than of MTX to dihydrofolate reductase under conditions *in vivo* could also account for these observations. The ability of 5-chloro-Q-asp (CCNSC 529,861) to inhibit the uptake of tritium-labeled MTX indicates that this member of the quinazoline series has appreciable affinity for the MTX transport system;[13, 14] the possible existence of additional modes of uptake for the 2,4-diaminoquinazoline antifolates has not been excluded, however.

The unusually high activity here reported of the 2,4-diaminoquinazoline antifolates as inhibitors of human leukemia cell dihydrofolate reductase, together with the effectiveness of these agents in experimental mouse tumor systems,[1, 2] indicates that continued biochemical and preclinical study of the mode of action and efficacy of these compounds as antitumor agents is warranted.

Acknowledgements—The able technical assistance of Mrs. Rachel Hillman, Mrs. Inger-Anne Skjeltorp and Mrs. Judy Uhoch is gratefully acknowledged. These studies were supported by Contract PH43-67-1193 with Chemotherapy, National Cancer Institute, National Institutes of Health.

Departments of Pharmacology and Medicine,
Yale University School of Medicine,
New Haven, Conn., U. S. A.

D. G. JOHNS
R. L. CAPIZZI
A. NAHAS
A. R. CASHMORE
J. R. BERTINO

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Biochemical Pharmacology, Vol. 19, pp. 1533–1535. Pergamon Press. 1970. Printed in Great Britain

Microsomal activation of cyclophosphamide *in vivo*

(*Received* 18 *November* 1969; *accepted* 2 *December* 1969)

IN STUDIES on the metabolism of anti-tumour agents a microsomal system has been developed which is capable of activating Endoxan (cyclophosphamide) *in vitro* to the levels of activation observed with this compound *in vivo*. Activation of Endoxan has previously been reported using liver slices, homogenates and microsomes[1–4] but the degree of activation was not defined.

Methods

Female Wistar rats, 6 weeks old and 220–250 g in weight were used in all experiments. The Walker ascites tumour was routinely passaged by intraperitoneal injection of 2×10^6 cells in saline containing penicillin and streptomycin. Solid tumours were obtained when required by the subcutaneous injection of 2×10^6 ascites cells in the inguinal region.

Rat liver microsomal fractions were prepared essentially by the method described by Grover and Sims.[5] Rat liver was homogenised in 5 volumes of 0·1 M phosphate buffer (pH 7·4) in a Teflon-glass homogenizer and centrifuged at 1000 *g* for 15 min at 5°. The supernatant was collected, spun at 21,000 *g* for 20 min at 2° and the resulting supernatant recentrifuged at 70,000 *g* for 1 hr at 2°. The microsomal pellet was resuspended in approximately the same volume of buffer and centrifuged again at 70,000 *g* for 1 hr at 2°. The successful preparation of microsomes non-toxic to Walker cells *in vitro*, depended on the speed with which the procedure was carried out and on maintenance of low temperature throughout. Preparations were stable for at least 24 hr if deep frozen.

Microsomes from 2·5 g of liver were suspended in 1 ml of saline containing 1·4 mg NADP, 10·4 mg glucose-6-phosphate, 2·1 μl glucose-6-phosphate dehydrogenase, specific activity 140 units/ml (all from Boehringer Corporation (London) Ltd) and 2·1 mg $MgCl_2$ and added to a suspension of tumour cells (about 10^7/ml) in 9 ml of 60% TC199/40% horse serum (Burroughs Wellcome No. 2) together with varying amounts of Endoxan dissolved in 0·1 ml of water. Control incubations consisted of 9 ml of tumour cell suspension mixed with either 1 ml of saline containing microsomes and co-factors or 1 ml of saline only. These mixtures were incubated for 2 hr at 37° with gentle agitation. The viability of the ascites cells in the mixtures was tested by injecting 10^7 cells into groups of five or six animals whose survival times were subsequently observed.