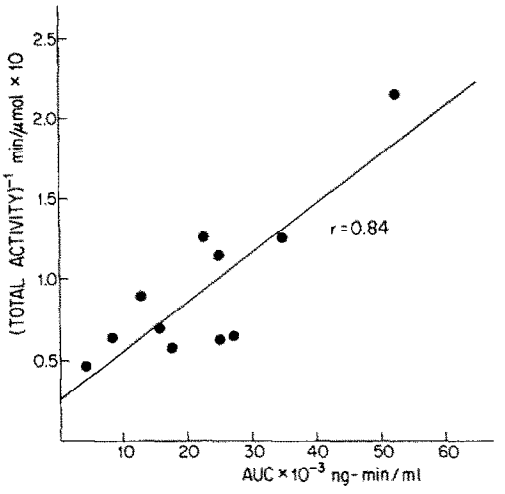

Fig. 2. Relationship between *in vitro* organic nitrate reductase activity and area under the plasma nitroglycerin concentration vs time curve in eleven rats.

nitroglycerin systemic availability and *in vitro* enzyme activity.

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REFERENCES

1. P. Needleman (Ed.), *Organic Nitrates*, Ch. 5. Springer, New York (1975).
2. P. Needleman, S. Lang and E. M. Johnson, Jr., *J. Pharmac. exp. Ther.* **181**, 489 (1972).
3. M. Gibaldi and S. Feldman, *Eur. J. Pharmac.* **19**, 323 (1972).
4. M. E. Davidov and W. J. Mroczek, *Angiology* **28**, 181 (1977).
5. T. Winsor and H. J. Berger, *Am. Heart J.* **90**, 611 (1975).
6. P. S. K. Yap, E. F. McNiff and H-L. Fung, *J. pharm. Sci.* **67**, 582 (1977).
7. H. P. Blumenthal, H-L. Fung, E. F. McNiff and S. K. Yap, *Br. J. clin. Pharmac.* **4**, 241 (1977).
8. P. S. K. Yap and H-L. Fung, *J. pharm. Sci.* **67**, 584 (1978).
9. S. T. Horhota and H-L. Fung, *J. pharm. Sci.* **67**, 1345 (1978).
10. P. Needleman and F. E. Hunter, Jr., *Molec. Pharmac.* **1**, 77 (1965).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. S. Lang, E. M. Johnson and P. Needleman, *Biochem. Pharmac.* **21**, 422 (1971).
13. E. Johnson, A. B. Harkey, D. J. Blehm and P. Needleman, *J. Pharmac. exp. Ther.* **182**, 56 (1972).
14. G. Reach, H. Nakane, Y. Nakane, C. Auzan and P. Corvol, *Steroids* **30**, 621 (1977).
15. W. R. Taylor, J. S. Forrester, P. Magnusson, T. Takano, K. Chatterjee and H. J. C. Swan, *Am. J. Cardiol.* **38**, 469 (1976).
16. M. Gibaldi and D. Perrier, *Pharmacokinetics*, p. 146. Marcel Dekker, New York (1975).
17. J. R. Hodgson and C. C. Lee, *Toxic. appl. Pharmac.* **34**, 449 (1975).
18. F. J. DiCarlo, M. C. Crew, L. J. Haynes, M. D. Melgar and R. L. Gala, *Biochem. Pharmac.* **17**, 2179 (1968).

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Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts

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A major clinical problem in cancer patients treated with methotrexate (MTX) is temporary remission followed by renewed tumor growth and lack of response to further MTX therapy. According to current views [1, 2], this type of 'acquired' resistance, as opposed to 'intrinsic' resistance wherein little or no response occurs even at the start of therapy, is due to the survival of a few initially resistant cells which can repopulate the tumor within a relatively short time. The rate of appearance of clinically resistant disease depends, among other things, on the number of such refractory cells and on their cytokinetic properties.

Although several other factors have been suggested as possible causes of MTX resistance in experimental animal systems [2], the principal relevant phenomena in humans are believed to be (1) the ability of tumor cells to synthesize increased levels of dihydrofolate reductase after exposure to MTX [3, 4] ('enzyme resistance'), and (2) the loss of the ability of tumor cells to take up the drug in quantities sufficient to inhibit the enzyme [5–7] ('transport resistance'). It may be possible for both phenomena to coexist in the same host, though their onset need not necessarily occur at the same time or to the same degree [7, 8]. There is evidence which indicates that in human cell lines transport resistance is more likely to occur than enzyme resistance [7], in contrast to murine systems in which enzyme resistance seems to be favored and stable transport-resistant mutants are difficult to maintain [8].

Uptake of MTX into human tumor cells can occur via either of two pathways. In the presence of relatively low concentrations (i.e. in the micromolar range), uptake in many cells has been demonstrated to occur by way of the same saturable, carrier-mediated and energy-dependent transport route as is utilized by naturally occurring reduced folates [9–11]. At millimolar levels, which more closely approximate conditions prevailing *in vivo* during high-dose MTX therapy, a large part of the intracellular MTX concentration results from passive diffusion [6, 12]. The two highly polar, negatively charged COOH groups in the side-

Table 1. Biochemical comparison of CEM and CEM/MTX human leukemic lymphoblasts

Cells	Dihydrofolate reductase activity [^{3}H]MTX assay*	[^{3}H]Folate assay†	MTX uptake One-hr uptake‡	Initial uptake velocity§
CCRF-CEM	14	3.8	1.9	0.060
CEM/MTX	18	3.4	0.32	0.013
Ratio‖	1.3	0.89	0.17	0.22

* Expressed in pmoles of MTX bound/10^8 cells; assay method of Kamen *et al.* [29].
† Expressed in pmoles of folic acid reduced/10 min/10^8 cells; assay method of Rothenberg [30].
‡ Expressed in pmoles of MTX taken up/60 min/10^7 cells; $[MTX]_{ex} = 0.5$ μM (Fig. 1).
§ Initial rate of uptake is expressed as pmoles of MTX/min/10^7 cells.
‖ (CEM/MTX)/(CEM).

chain of MTX would be expected to have a strongly retardant effect on the rate of passive diffusion of this molecule through the cell membrane.

When cells become transport-resistant, they have a diminished capacity to take up MTX by the carrier-mediated route, but not by passive diffusion. Theoretically, at least, it ought to be possible to kill transport-resistant cells merely by increasing the extracellular concentration of drug. Unfortunately this cannot always be translated into clinical practice, inasmuch as high serum levels of MTX may be toxic to the bone marrow, epithelial mucosa and other normal proliferative tissues of the host [13].

One approach to this problem which has met with success in the clinic is to give high doses of MTX with carefully timed administration of a rescue agent such as leucovorin [14–16], thymidine [17, 18] or carboxypeptidase G_1 [19]. A second approach has been to use antifolates that can penetrate cells more readily by passive diffusion, such as 2,4-diamino-5-(3′, 4′-dichlorophenyl)-6-methylpyrimidine (DDMP) [20] or triazinate (TZT, 'Baker's antifol') [21]. The later agents lack a negatively charged glutamate side-chain and are, therefore, regarded as 'small-molecule' antifolates rather than 'classical' antifolates.

A third approach has been to modify the glutamate side-chain of MTX so as to decrease the polar character of the molecule and improve membrane penetration [22, 23]. This has given rise to several different classes of lipid-soluble 'hybrid antifolates', which may be defined as compounds whose overall structure retains most of the essential features of classical antifolates (i.e. the 2,4-diaminopteridine ring, bridge region and *para*-aminobenzoate moiety), but whose uptake into cells is expected to resemble more closely that of small-molecule antifolates. Among the compounds of this type that have been studied in this laboratory have been diester [24] and bis(amide) [25] derivatives. Diesters of MTX have also been investigated independently by Johns *et al.* [26, 27].

In this paper we report the finding that a human tumor cell line which is transport-resistant to MTX retains its sensitivity to lipophilic monoester and diester derivatives of MTX, as well as to small-molecule antifolates such as DDMP.

Human lymphoblasts (CCRF-CEM line) [28] were grown in serial culture in the presence of increasing concentrations of MTX until a 120-fold resistant subline (CEM/MTX) was obtained. This subline appears to be a stable mutant which maintains its resistant properties even in the absence of drug; details concerning the isolation of CEM/MTX cells will appear separately.* The amount of dihydrofolate reductase in the sensitive as well as resistant lines was determined by measuring the extent of complex formation with [^{3}H]-MTX (competitive ligand-binding assay) [29] and the functional activity was determined by measuring the conversion of [^{3}H] folate to [^{3}H]tetrahydrofolate [30]. As shown in Table 1, the amount of [^{3}H]folic acid that can be reduced by 1×10^8 cells per unit time is not significantly different in the two cell lines. Moreover, since binding of [^{3}H]MTX to dihydrofolate reductase is approximately stoichiometric, the amount of enzyme in the two cell lines must be very similar. Taken together, these results indicate that the 120-fold difference in sensitivity to MTX between CCRF-CEM and CEM/MTX cells cannot be accounted for on the basis of increased dihydrofolate reductase activity, and that a more likely explanation is one involving impaired drug uptake.

Figure 1 shows a comparison of [^{3}H]MTX uptake into CCRF-CEM and CEM/MTX cells at an external drug concentration of 0.5 μM. This concentration was selected to conform to the 'low dose' component of [^{3}H]MTX uptake observed previously in CCRF-CEM cells by other investigators [6]. Both the initial rate of drug uptake and the intracellular drug level at 1 hr (approaching equilibrium) are reduced significantly in the CEM/MTX cell relative to the parent line at this concentration. In Table 1 it can be seen that there is about a 5-fold reduction in both the 1 hr uptake and the initial uptake velocity in the resistant cell. Although the uptake experiments reported in this paper were performed only at a single extracellular MTX concentration of 0.5 μM, i.e. approximately one-tenth the reported K_m for uptake of 6 μM [6], studies at higher concentrations (up to 200 μM), as well as comparative kinetic analyses of drug influx and efflux in the sensitive and resistant lines, indicate that, when the extracellular MTX concentration is varied from 0.5 μM to as high as 200 μM, both the initial uptake velocity and the 1 hr uptake in the resistant line are significantly lower than in the sensitive line.†

With regard to the apparent discrepancy between the 5-fold difference in uptake and the 120-fold difference in cytotoxicity for the CCRF-CEM and CEM/MTX cells, it should be noted that cytotoxicities were compared at the ID_{50} dose, whereas uptakes were measured at an extracellular drug concentration (0.5 μM) corresponding approximately to an ID_{90} dose. This was necessitated by the fact that, at lower doses, the uptake of [^{3}H]MTX into the transport-deficient CEM/MTX cells was less than could be measured with any degree of accuracy. Moreover, it should be noted that uptake experiments were conducted over a 1 hr period in Earle's balanced salt solution supplemented only with amino acids (see Fig. 1), whereas cytotoxicities were compared after 48 hr of incubation in Eagle's minimal essential medium (MEM) to which 10% whole fetal calf serum had been added (thereby supplying reduced folates to the cells) [28]. Thus, it should be emphasized that, although these experiments provide a basis for making a qualitative correlation between drug sensitivity and drug uptake in the two cell lines, an absolute quantitative correlation is not necessarily to be expected.

* H. Lazarus and G. C. Yuan, manuscript in preparation.
† P. W. Rossow, L. Mangini and E. J. Modest, manuscript in preparation.

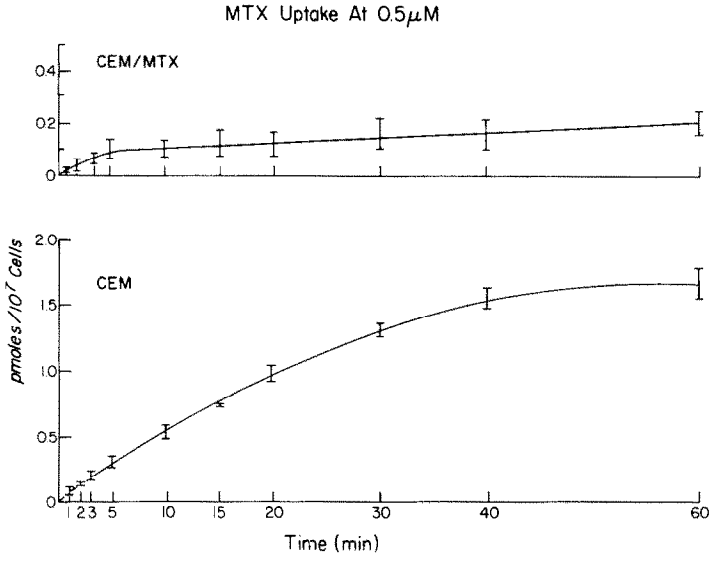

Fig. 1. Time course of MTX uptake by CCRF-CEM and CEM/MTX cells. CCRF-CEM lymphoblasts were maintained in continuous culture as described previously [28]. Prior to use, exponentially growing cells were harvested by centrifugation and washed twice with phosphate buffered saline (PBS). The cells were resuspended in Earle's balanced salt solution supplemented with essential amino acids at approximately 1×10^7 cells/ml and incubated for 1 hr at 37° under a 5% CO_2 atmosphere. Uptake was initiated by the addition of an appropriate amount of [^{3}H]MTX. At selected time intervals, 1 ml aliquots were removed and pipetted into 10 ml of ice-cold PBS to stop further drug uptake. The cells were washed twice with 10 ml of ice-cold PBS and the final cell pellet was lysed with 1 ml of 0.1 M Tris (pH 9.0) and 1% sodium dodecylsulfate. The lysate was counted by liquid scintillation spectrometry with a counting efficiency of 50 per cent. Non-specific binding, as measured by drug uptake at 4°, is equal to the extrapolated drug uptake at zero time. The data shown in Fig. 1 are corrected for this non-specific binding, and are the average range of three experiments performed on different days.

Growth inhibition assays employing MTX and several lipophilic antifolates were carried out against CCRF-CEM and CEM/MTX cells, as described previously [28]. Table 2 presents ID_{50} values for MTX, the di-*n*-butyl ester (DBMTX) [24], the γ-mono-*n*-butyl ester (γ-MBMTX) [31], and the small-molecule antifolates DDMP and 1,3-diamino-9-chlorobenzo[*f*]quinazoline (PY490). The last compound is a tricyclic DDMP analog of fixed planar geometry [32]. Also given in Table 2 are the ratios of ID_{50} values for each drug against the two cell lines. These ratios may be viewed as a measure of cross-resistance. It can be seen that the diester DBMTX compares very favourably with the small-molecule antifolates DDMP and PY490 in being toxic to CEM/MTX cells at a dose that is only one-third the ID_{50} against the parent line. The monoester γ-MBMTX does not show this apparent collateral sensitivity, though it is still equitoxic to both cells. Thus, blocking one or both COOH groups in MTX by esterification appears to be an effective means of overcoming resistance in CEM/MTX cells.

Although the exact mechanism of cell-kill by DBMTX and other MTX diesters has not yet been fully elucidated]33, 34], preliminary evidence suggests that the diesters probably act, in part, as passively diffusing carriers, or 'prodrugs', which can be converted into α- and γ-monoesters and ultimately MTX itself via intracellular esterases [24, 26, 27]. While it is true that γ-MBMTX has been shown to be approximately as potent as MTX in inhibiting dihydrofolate reductase [31], a number of factors other than dihydrofolate reductase inhibition have to be considered in respect to the mode of action of the diester in intact cells. For example, because of their very high lipophilicity, DBMTX and other MTX diesters might bind avidly to the membranes of lipid-rich subcellular particles so as to create a long-lasting intracellular depot. Gradual release of the diester from such a depot, followed by cleavage of the ester groups by intracellular esterases, might provide a mechanism for maintaining a cytotoxic level inside the cell for a longer period than is possible with MTX alone.

The biochemical basis for the observed 'collateral sen-

Table 2. Cytotoxicity of lipophilic antifolates in CEM and CEM/MTX human leukemic lymphoblasts

	ID_{50} (μM)				
Cells	MTX	γ-MBMTX	DBMTX	DDMP	PY490
CEM	0.029	1.7	0.069	0.13	0.029
CEM/MTX	3.4	1.6.	0.012	0.030	0.008
Ratio*	120	0.94	0.30	0.23	0.28

* (CEM/MTX)/(CEM).

sitivity' of CEM/MTX cells to DBMTX and small-molecule antifolates has not been determined, but one possibility is that these cells are less efficient than the parent line in taking up reduced folates which are present in Eagle's MEM medium that has been supplemented with whole serum. Another possibility is that the plasma membrane of CEM/MTX cells has undergone some structural alteration that renders it more permeable to lipid-soluble compounds while at the same time diminishing its capacity for active transport.

The ability of DBMTX to kill transport-resistant cells as effectively as DDMP is of potential clinical interest in view of the fact that the latter has been shown to produce headaches, confusion and other manifestations of neurotoxicity [35]. These undesirable side-effects of DDMP may be due, in part, to the long tissue half-life of the drug [36]. A more specific biochemical cause may be that DDMP is a very potent inhibitor of the enzyme histamine *N*-methyltransferase [37], which helps to regulate histamine levels in the brain. In contrast to DDMP and a number of other small-molecule antifolates that have been tested against this enzyme *in vitro*, side-chain esters and amides of MTX exhibit little or no inhibitory activity below 1 mM (D. S. Duch and C. A. Nichol, personal communication). The use of these 'hybrid' antifolates may, therefore, be viewed as an attractive alternative to small-molecule antifolates in the treatment of transport-resistant tumors. Further biochemical and pharmacologic studies with monoesters and diesters of MTX are in progress in this laboratory as a prelude to possible clinical trial of the optimal MTX ester.

In summary, human leukemic lymphoblasts (CCRF-CEM) were grown in serial culture in the presence of increasing concentrations of MTX until a 120-fold resistant subline (CEM/MTX) was obtained, which exhibited normal levels of dihydrofolate reductase activity but a markedly decreased rate of uptake of the drug. Lipophilic derivatives of MTX such as DBMTX and γ-MBMTX inhibited the growth of CEM/MTX cells and the parent line to the same degree as did the small-molecule antifolates DDMP and PY490. The importance of this study is that lipophilic antifolates are equal to or superior to MTX in inhibiting a human malignant cell that appears to be solely membrane-resistant to MTX. The results support the concept that MTX-resistant human tumors in which carrier-mediated transport of MTX across the cell membrane is impaired may be responsive to chemotherapy with lipophilic antifolates that can enter cells by passive diffusion. Compounds such as DBMTX and γ-MBMTX may be viewed as 'hybrid antifolates' whose overall structure retains most of the structures of classical antifolates, but whose mode of uptake into cells more closely resembles that of small-molecule antifolates.

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REFERENCES

1. R. T. Skeel and C. A. Linquist, in *Cancer: A Comprehensive Treatise* (Ed. F. F. Becker) Vol. 5, pp. 113–124. Plenum Press, New York (1977).
2. R. W. Brockman, in *Antineoplastic and Immunosuppressive Agents* (Eds. A. C. Sartorelli and D. G. Johns), Vol. 1, pp. 358–367. Springer, New York (1974).
3. P. L. Chello, C. A. McQueen, L. M. DeAngelis and J. R. Bertino, *Cancer Res.* **36**, 2442 (1976).
4. J. R. Bertino, W. L. Sawicki, A. R. Cashmore, E. C. Cadman and R. T. Skeel, *Cancer Treat. Rep.* **61**, 667 (1977).
5. R. A. Bender, *Cancer Chemother. Rep.* (Pt. 3) **6**, 73 (1975).
6. R. D. Warren, A. P. Nichols and R. A. Bender, *Cancer Res.* **38**, 668 (1978).
7. D. Niethammer and R. C. Jackson, *Eur. J. Cancer* **11**, 845 (1975).
8. R. C. Jackson, D. Niethammer and F. M. Huennekens, *Cancer Biochem. Biophys.* **1**, 151 (1975).
9. A. Nahas, P. F. Nixon and J. R. Bertino, *Cancer Res.* **32**, 1416 (1972).
10. I. D. Goldman, *Cancer Chemother. Rep.* (Pt. 3) **6**, 63 (1975).
11. F. M. Sirotnak and R. C. Donsbach, *Cancer Res.* **36**, 1151 (1976).
12. M. T. Hakala, in *Antineoplastic and Immunosuppressive Agents* (Eds. A. C. Sartorelli and D. G. Johns), Vol. 1, pp. 240–269. Springer, New York (1974).
13. S. W. Pitman, L. M. Parker, M. H. N. Tattersall, N. Jaffe and E. Frei, III, *Cancer Chemother. Rep.* (Pt. 3) **6**, 43 (1975).
14. M. Levitt, M. B. Mosher, R. C. DeConti, L. R. Farber, R. T. Skeel, J. C. Marsh, M. S. Mitchell, R. J. Papac, E. D. Thomas and J. R. Bertino, *Cancer Res.* **33**, 1729 (1973).
15. I. Djerassi, *Cancer Chemother. Rep.* (Pt. 3) **6**, 3 (1975).
16. E. Frei, III, N. Jaffe, M. H. N. Tattersall, S. Pitman and L. Parker, *New Engl. J. Med.* **292**, 846 (1975).
17. W. D. Ensminger and E. Frei, III, *Cancer Res.* **37**, 1857 (1977).
18. S. B. Howell, W. D. Ensminger, A. W. Krishan and E. Frei, III, *Cancer Res.* **38**, 325 (1978).
19. H. T. Abelson, W. D. Ensminger, A. Rosowsky and J. Uren, *Cancer Treat. Rep.* **62**, 1549 (1978).
20. L. A. Price, J. H. Goldie and B. T. Hill, *Br. med. J.* **2**, 20 (1975).
21. R. T. Skeel, A. R. Cashmore, W. L. Sawicki and J. R. Bertino, *Cancer Res.* **36**, 48 (1976).
22. M. Chaykovsky, A. Rosowsky and E. J. Modest, *J. heterocyclic Chem.* **10**, 425 (1973).
23. M. Chaykovsky, A. Rosowsky, N. Papathanasopoulos, K. K. N. Chen, E. J. Modest, R. L. Kisliuk and Y. Gaumont, *J. med. Chem.* **17**, 1212 (1974).
24. A. Rosowsky, *J. med. Chem.* **16**, 1190 (1973).
25. A. Rosowsky, W. D. Ensminger and C-S. Yu, *J. med. Chem.* **20**, 925 (1977).
26. D. G. Johns, D. Farquhar, B. A. Chabner, M. K. Wolpert and R. H. Adamson, *Experientia* **29**, 1104 (1973).
27. D. G. Johns, D. Farquhar, M. K. Wolpert, B. A. Chabner and T. L. Loo, *Drug Metab. Dispos.* **1**, 580 (1973).
28. G. E. Foley and H. Lazarus, *Biochem. Pharmac.* **16**, 659 (1967).
29. B. A. Kamen, P. L. Takach, R. Vatev and J. D. Caston, *Analyt. Biochem.* **70**, 54 (1976).
30. S. P. Rothenberg, *Analyt. Biochem.* **16**, 176 (1966).
31. A. Rosowsky, G. P. Beardsley, W. D. Ensminger, H. Lazarus and C. S. Yu, *J. med. Chem.* **21**, 380 (1978).
32. A. Rosowsky, K. K. N. Chen, M. E. Nadel, N. Papathanasopoulos and E. J. Modest, *J. heterocyclic Chem.* **9**, 275 (1971).
33. G. A. Curt, J. S. Tobias, R. A. Kramer, A. Rosowsky, L. M. Parker and H. M. N. Tattersall, *Biochem. Pharmac.* **25**, 1943 (1976).
34. G. P. Beardsley, A. Rosowsky, R. P. McCaffrey and H. T. Abelson, *Biochem. Pharmac.* **28**, 3075 (1979).

35. D. S. Miller, R. W. Rundles, C. A. Nichol, J. L. Wooley and C. W. Sigel, *Proc. Am. Soc. clin. Oncol.* **17**, 263 (1976).
36. C. A. Nichol, J. C. Cavallito, J. L. Wooley and C. W. Sigel, *Cancer Treat. Rep.* **61**, 559 (1977).
37. D. S. Duch, S. W. Bowers and C. A. Nichol, *Biochem. Pharmac.* **27**, 1507 (1978).

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Inhibition of tubulin polymerization by nitrosourea-derived isocyanates

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Antitumor active chloroethylnitrosoureas are chemically unstable and upon degradation yield reactive intermediates capable of alkylation by the 2-chloroethyl moiety and of carbamylation by the isocyanate moiety [1]. Alkylation of certain proteins by bis-1,3-(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) has been shown to occur non-selectively, compared to highly specific carbamylation by an active site-directed mechanism [2,3].

Preliminary studies in this laboratory have shown that treatment of L5178Y cells with certain 2-chloroethyl-nitrosoureas during growth in culture resulted in a decreased mitotic index. We have explored the question of whether drug-microtubule interactions may participate to some extent in antitumor activity. Several 2-chloroethyl-nitrosoureas that yield varying amounts of isocyanate upon degradation were studied for their effects upon the polymerization of isolated tubulin. Inhibition of polymerization was observed, and the stoichiometry of the inhibition was related to the nitrosourea-derived isocyanates.

Tubulin was isolated from freshly slaughtered cow or pig brain, using 0.1 M Pipes buffer (pH 6.9) and 1 mM guanosine triphosphate, according to the procedure of Borisy *et al.* [4]. Two polymerization cycles were performed, with the final pellet stored at −80° and used within 1 week. All protein concentrations were determined using the assay of Lowry *et al.* [5]. CCNU, BCNU, methyl CCNU (MeCCNU), chlorozotocin (2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose) (all supplied by the National Cancer Institute, Bethesda, MD), cyclohexyl isocyanate (CHI, supplied by the Eastern Kodak Co., Rochester, NY), *cis*-2-hydroxy CCNU (*cis*-2-OH CCNU) [6], and *trans*-4-hydroxy CCNU (*trans*-4-OH CCNU) [6] were freshly dissolved in acetone before adding to the tubulin. Acetone only was added to tubulin solutions for control experiments.

Thawed tubulin was depolymerized in 0.1 M Pipes + 1 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether) *N,N'* tetra acetic acid] + 5×10^{-4} M $MgCl_2$ (pH 6.9). After centrifuging at 100,000 *g*, 4°, for 30 min, the pellet was discarded and GTP (10^{-3} M) was added to the supernatant fraction containing 2 or 3 mg/ml tubulin. The tubulin was then incubated at 15° for 2 hr with or without nitrosourea. Following this incubation, the method of Gaskin *et al.* [7] was used to monitor the extent of tubulin polymerization. By this procedure, the increase in turbidity at 350 nm was measured at 37°, using a Gilford model 220 spectrophotometer equipped with a thermostated sample changer and a chart recorder. The effect of CHI on tubulin polymerization was measured by the above method after incubation with the drug for 5 min at room temperature.

For electron microscopy, samples were removed from the polymerizing tubulin preparations. A drop was placed on a formvar-coated grid for 1 min and then stained for 1 min with six drops of 1% uranyl acetate before blotting dry. The grids were observed in a Phillips EM300.

1 - (2 - Chloroethyl) - 3 - ([1 - ^{14}C]cyclohexyl) - 1 - nitrosourea (134 nmoles) reacted with purified microtubule protein (27 nmoles) in 0.1 M Tris–HCl (pH 7.5) for 4 hr at room temperature. After overnight dialysis [5 M urea in 0.1 M Tris–HCl (pH 7.5) followed by water], the sample was incubated at 37° for 2 hr in 8 M urea, 0.1% sodium dodecylsulfate (SDS), and 0.5% mercaptoethanol. The tubulin was separated by gel electrophoresis, as described by Eipper [8]. The gels were cut, digested overnight with protosol, and counted in toluene 2,5-diphenyloxazole (PPO) fluor on a Beckman liquid scintillation counter LS-230. A similar gel was stained with Coomassie and scanned on a Gilford scanner on a Beckman DU spectrophotometer.

Within 8–10 min after placing the tubulin dimers at 37° to initiate polymerization, maximum turbidity was reached. This level was maintained for at least 30 min at 37°, similar to that seen by Gaskin *et al.* [7]. Normal tubules were observed in the electron microscope from samples removed at 6 and 30 min. Thus, turbidity correlated with polymerization of tubulin.

The CCNU and BCNU inhibition of maximum polymerization, as shown in Table 1, was dose dependent. MeCCNU and *trans*-4-OH CCNU at 5×10^{-7} moles behaved similarly, inhibiting 55 and 62 percent of control,

Table 1. Inhibition of polymerization

	Absolute amount (nmoles)	Percent inhibition	Standard deviation	Number of experiments
CCNU	250	26	13	4
	500	40	17	5
	750	70	11	3
BCNU	250	34	17	3
	500	51	12	4
	750	77	2	3