Biochemical Pharmacology, Vol. 34, No. 2, pp. 217–221, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

ANTITUMOUR IMIDAZOTETRAZINES—VIII

UPTAKE AND DECOMPOSITION OF A NOVEL ANTITUMOUR AGENT MITOZOLOMIDE (CCRG 81010; M and B 39565; NSC 353451) IN TLX5 MOUSE LYMPHOMA *IN VITRO*

CARMEL M. T. HORGAN and MICHAEL J. TISDALE

Cancer Research Campaign Experimental Chemotherapy Group, Department of Pharmacy, University of Aston, Birmingham B4 7ET, U.K.

(*Received* 8 *May* 1984; *accepted* 10 *August* 1984)

Abstract—The uptake and incorporation of 8-carbamoyl-3-(2-chloroethyl)(6-^{14}C-imidazo)[5,1-d]-1,2,3,5-tetrazin-4-(3H)-one (Mitozolomide) into TLX5 mouse lymphoma cells has been studied *in vitro*. Uptake was rapid, reaching a cell/medium distribution of approximately unity in 1 min at 37° and 10 min at 4°, directly proportional to drug concentration and was unaffected by metabolic inhibitors. These results are consistent with a simple diffusion mechanism. No difference in uptake was observed between drug sensitive and resistant TLX5 lymphoma cells.

Cellular radioactivity was found to be progressively accumulated into acid-insoluble material. Acid hydrolysis of this precipitate followed by hplc analysis of the DNA and RNA bases showed that the radioactivity was associated solely with adenine and guanine bases. Mitozolomide was unstable in tissue culture medium and over a 24 hr period about 80% of the drug was converted into 5-aminoimidazole-4-carboxamide (AIC). Non-radioactive AIC suppressed the incorporation of radioactivity into nucleic acids, but had no effects on the initial rate of uptake of mitozolomide into the cell. These results suggest that the radioactivity in nucleic acids arises as a result of salvage of AIC, formed by intracellular decomposition of mitozolomide.

8-Carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (Mitozolomide; CCRG 81010; M and B 39565; NSC 353451) (I) is a new antitumour agent, which exhibits essentially curative activity against a broad spectrum of murine tumours, L1210 and P388 leukaemia, TLX5 lymphoma, Lewis lung carcinoma, PC6A plasmacytoma, colon 38 carcinoma and the lung LX1 human tumour xenograft [1]. Mitozolomide undergoes decomposition in aqueous solution to yield a reactive species with potential antitumour activity. The rate of decomposition is pH dependent [2]. In phosphate buffer (pH 7.4) the half-life is 98 min at 28°; it is stable in 2N-sulphuric acid, but undergoes accelerated decomposition in 5% aqueous sodium carbonate to give the monochloroethyltriazene MCTIC$^+$ [2].* This agent has been suggested speculatively as being responsible for the antitumour activity of BCTIC [3] and is possibly also the mediator of the antitumour activity of mitozolomide, probably by chloroethylation of DNA [4, 5]. No evidence has been obtained for carbamoylation reactions by mitozolomide *in vitro* [6], which could occur through an alternative decomposition pathway.

The TLX5 lymphoma with induced resistance to triazenes (TLXRT) shows cross-resistance to mitozolomide suggesting a similar cytotoxic action [7]. One mechanism by which resistance may be acquired is through an impaired transport of the drug by resistant cells. This mechanism is usually confined to those drugs whose uptake is carrier mediated. Mitozolomide is a lipid soluble molecule and its transport may be expected to be by simple diffusion into the cell.

The present experiments using [6-^{14}C]mitozolomide have been performed to determine the mechanism of uptake of the drug by sensitive and resistant TLX5 lymphoma cells and to determine the intracellular fate of the radioactive label.

MATERIALS AND METHODS

5-(Methyl-^{3}H)thymidine (sp. act. 5Ci mmol^{-1}) [1,2-^{3}H] polethylene glycol (sp. act. 0.25 mCi/156.3 mg) ^{3}H water (sp. act. 0.09 mCi mmol^{-1}) and U-^{14}C-n-hexadecane (sp. act. 45 mCi mmol^{-1}) were purchased from Amersham International, Bucks. [6-^{14}C]Mitozolomide (sp. act. 3.41 mCi mmol^{-1}) was supplied by May and Baker Ltd. (Dagenham, U.K.). 5-Aminoimidazole-4-carboxamide was purchased from BDH Chemicals Ltd. (Poole, U.K.) and other reagents from Sigma Chemical Co. (Poole, Dorset, U.K.). Tissue culture medium was from Gibco Europe (Paisley, Scotland, U.K.)

Transport studies. TLX5 mouse lymphoma cells were passaged in the ascitic form in CBA/CA mice. For transport studies cells were removed in 0.9% NaCl and erythrocyte contamination was removed by washing with 0.016M Tris, HCl, pH 7.2, containing 7.5 g NH_4Cl/l [8]. Cells were resuspended in RPMI 1640 at a concentration of 2×10^7 cells/ml and

* Abbreviations: $^+$MCTIC, 5-[3-(2-chloroethyl)triazen-1-yl]-imidazole-4-carboxamide; BCTIC, 5-[3,3-bis(2-chloroethyl)-triazen-1-yl imidazole-4-carboxamide.

equilibrated for 10 min in a shaking water bath at 37° or in ice-water at 4°, under an atmosphere of 10% CO_2 in air. Uptake was initiated by the addition of mitozolomide (14.08 μCi/ml) in DMSO. At specified time points samples (200 μl in triplicate) were removed to an Eppendorf tube which contained 100 μl of a silicon oil:corn oil (10:3) mixture and 50 μl of 98% formic acid. After centrifugation (9000 *g*) for 1 min the tube was frozen in liquid nitrogen, cut at the oil/acid boundary and the radioactivity was determined in Fiso Fluor (Fisons Pharmaceutical, Loughborough, U.K.). The oil layer contained radioactivity from the extracellular solution and the acid layer contained the intracellular radioactivity. To establish the volume of the cells a 1 ml sample of cell suspension was treated with polyethylene glycol and a further sample with [^{3}H]-H_2O. Once equilibrium had been established the samples were treated as above.

Cell-medium distribution ratio. Cells were prepared as for transport studies and treated with a range of drug concentrations. Once equilibrium had been achieved (15 min) 200 μl portions were processed in triplicate as for transport studies. Cell volume was determined as above.

Incorporation of [6-^{14}C-imidazo] mitozolomide into acid precipitable material. TLX5 mouse lymphoma cells were suspended in RPMI 1640 medium containing 10% foetal calf serum at a concentration of 2×10^6 cells/ml in the presence of [6-^{14}C] mitozolomide (14.08 μCi/ml) at 37° under an atmosphere of 5% CO_2 in air. At appropriate times samples (1 ml) were removed, sedimented by centrifugation, washed with 0.9% NaCl and treated with 0.2 N $HClO_4$ (500 μl). The acid-insoluble material, after centrifugation, was taken up in Fiso-Fluor scintillation fluid (Fisons Pharmaceutical, Loughborough, U.K.) and the radioactivity was determined in a Packard, 'Tri-Carb' scintillation spectrometer. The ability of the cells to synthesize DNA was monitored by following the incorporation of 5-(methyl-^{3}H)thymidine as previously described [6]. The effect of 5-amino-imidazole-4-carboxamide on the initial uptake of mitozolomide and on the association of label with nucleic acids over a 4 hr period was also determined.

Determination of radioactivity incorporation into adenine and guanine bases. TLX5 mouse lymphoma cells at a concentration of 2×10^6 cells/ml were incubated in RPMI 1640 medium (50 ml) with mitozolomide 0.1408 μCi/ml for 5 hr. The cells were sedimented by centrifugation, washed with 0.9% NaCl and treated with 0.2 N $HClO_4$ (2 ml). The acid-insoluble material was washed twice with ice-cold distilled water and lyophilized. The lyophilized material was heated to 80° in 1 ml 1 N HCl for 1 hr. The resulting acid hydrolysate was separated by reverse phase chromatography on a 5 μm C_{18} analytical column eluted with 3–10% acetonitrile in phosphate buffer (25 mM KH_2PO_4 + 0.5 M diethylamine, pH 3.5) over 40 min at a flow rate of 0.5 ml/min. The method was based on that of Tong *et al.* [9]. The eluant was monitored with a Waters LC Spectrometer and a coincidence ESI Nuclear Analyser (Rotheroe and Mitchell Nuclear Division, Ruislip, U.K.). The eluant was collected and dissolved in Fiso-Fluor scintillation fluid for scintillation detection.

The decomposition of [6-^{14}C]mitozolomide and the production of [6^{14}C] AIC was determined by the hplc method of Slack *et al.* [10]. The eluant from the column was collected for quantitation.

RESULTS

A time course for the uptake of 4.4 mM [6-^{14}C]-mitozolomide into TLX5 mouse lymphoma cells sensitive (TLX5) and resistant (TLXRT) to the drug is shown in Fig. 1. Initial uptake is approximately linear and a steady-state level is reached within 1 min at 37° and 10 min at 4°. There is no significant difference between the equilibrium values attained in sensitive and resistant cell lines indicating that at least *in vitro* differential uptake does not contribute to resistance. The cell/medium drug distribution ratio remains constant at approximately 1.3 for 60 min. Evidence that uptake is nonsaturable comes from the linear relationship between the initial rate of cellular uptake of the drug and the extracellular concentration (Fig. 2) and the observation that the cell/medium distribution ratio remains essentially constant at extracellular mitozolomide concentrations ranging from 0.1 to 2.0 mM.

The effect of metabolic inhibitors (dinitrophenol, sodium cyanide, sodium fluoride and oligomycin) on the transport of mitozolomide is shown in Table 1. There is no effect on either transport into the cell or on the cell/medium distribution ratio, showing no active accumulation of the drug. These results provide strong evidence that influx of intact mitozolomide is by a simple diffusion mechanism.

Cellular radioactivity slowly becomes associated with macromolecules. The kinetics of incorporation

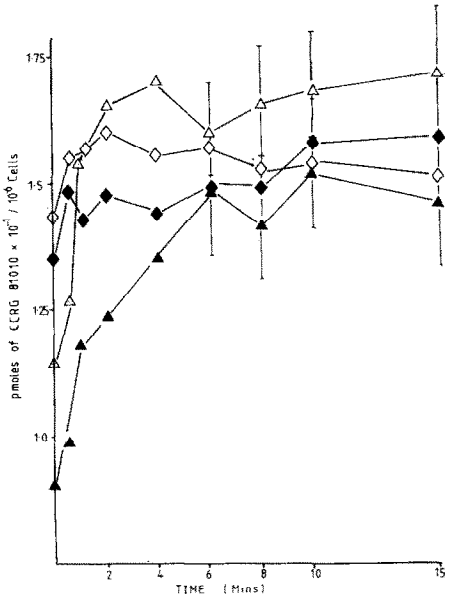

Fig. 1. Uptake of mitozolomide into TLXS (closed symbols) and TLXRT (open symbols) mouse ascites cells at 37° (◆ ◇) and 4° (▼ ▽).

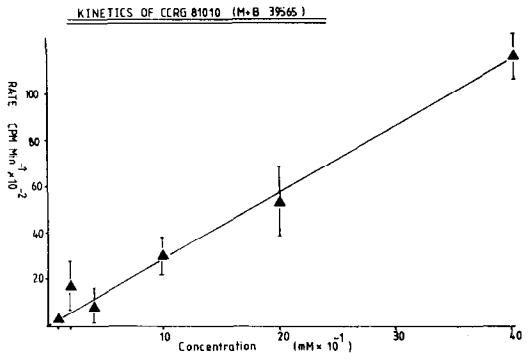

Fig. 2. Relationship between the initial rate of uptake and the extracellular concentration of mitozolomide in TLX5 cells.

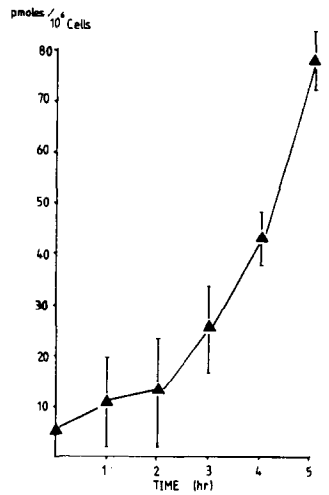

Fig. 3. Incorporation of [6-^{14}C]mitozolomide into acid-insoluble material.

(Fig. 3) show an initial lag period of 2 hr followed by an exponential incorporation over the next 3 hr. Incorporation was only monitored for 5 hr owing to loss of cell viability after this time, as measured by (methyl ^{3}H)thymidine incorporation into acid-insoluble material. The rate of incorporation of [6-^{14}C]mitozolomide into acid-insoluble material, calculated after the time-lag is 21 pmole/hr/10^6 cells. Mitozolomide is unstable in aqueous solution and undergoes decomposition to yield 5-amino-imidazole-4-carboxamide (AIC) [2]. The half-life for this decomposition in RPMI 1640 tissue culture medium (pH 7.2) is 104 min. Over a 24 hr period about 80% of the mitozolomide in the medium decomposes to AIC (Fig. 4) which is then available for *de novo* purine synthesis.

The radioactivity associated with acid-insoluble material is found to be incorporated solely into nucleic acids. Using hplc analysis of the hydrolysed nucleic acids and UV and radioactivity detectors in series the radioactivity appears coincident with UV peaks identifiable as adenine and guanine (Fig. 5). All of the radioactivity associated with the acid-insoluble material is found solely in adenine and guanine bases, presumably as a result of salvage of AIC.

If this were correct it would be expected that co-addition of AIC and [6-^{14}C]mitozolomide to TLX5 mouse lymphoma cells would decrease the incorporation of radioactivity into nucleic acids. This was found to be the case (Fig. 6). Incubation of TLX5 cells with AIC and [6-^{14}C]mitozolomide for 4 hr produces approximately the same decrease in incorporation of radiolabel as an equivalent experiment in which radioactive and non-labelled mitozolomide are co-administered (Fig. 6). Also AIC does not compete with the initial uptake of mitozolomide into TLX5 cells.

Table 1. Effect of metabolic inhibitors, on the uptake of mitozolomide

	CPM/10^6 cells	
Temperature	37°	4°
Control	1453	1474
1 mM DNP	1491	1552
1 mM NaCN	1358	1402
20 mM NaF	1436	1456
0.1 mM Oligomycin	1561	1456

DISCUSSION

The present experiments provide evidence that the uptake of mitozolomide into sensitive and resistant TLX5 lymphoma cells *in vitro* occurs by a passive diffusion process. The drug is not accumulated against a concentration gradient and is unaffected by the presence of metabolic inhibitors. No difference is observed in the initial rate of transport or in the saturation levels attained between drug sensitive and resistant cells and therefore differences in sensitivity must reflect intracellular reactions.

Mitozolomide undergoes decomposition in aque-

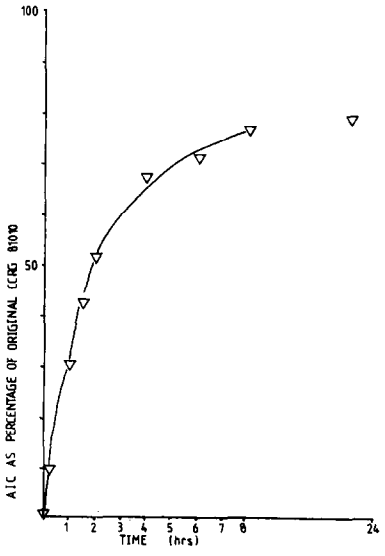

Fig. 4. Production of [^{14}C] AIC form [6-^{14}C]mitozolomide in RPMI 1640 tissue culture medium at 37°.

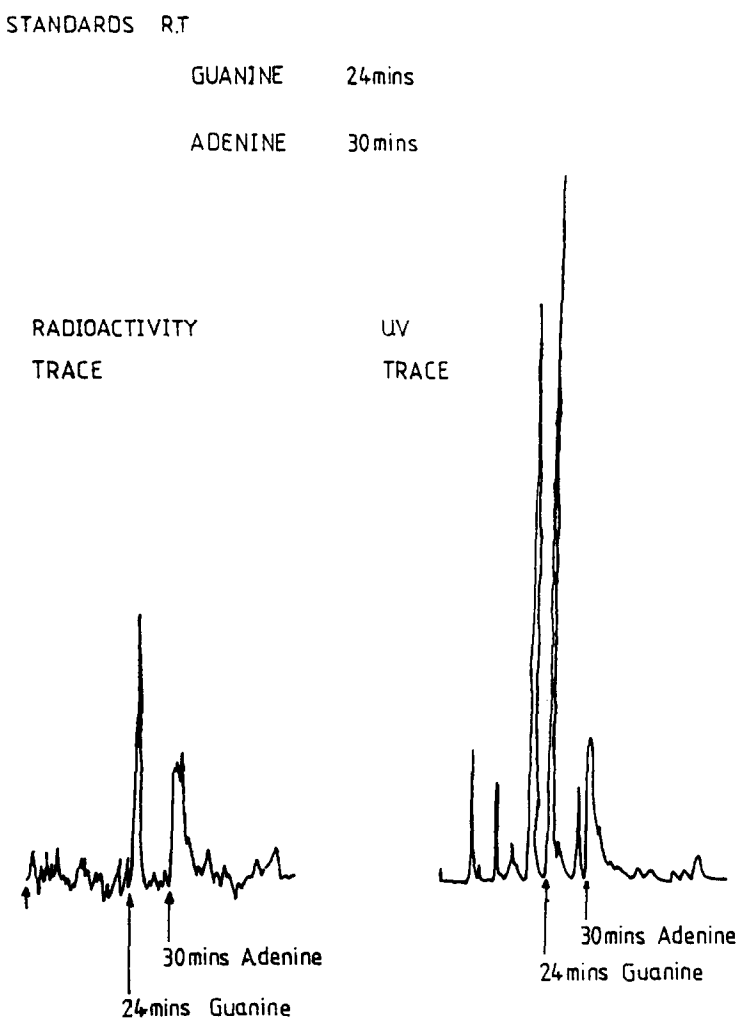

Fig. 5. Hplc traces of hydrolysed nucleic acids from TLX5 cells treated with [6-^{14}C]mitozolomide using u.v. and radioactive detectors.

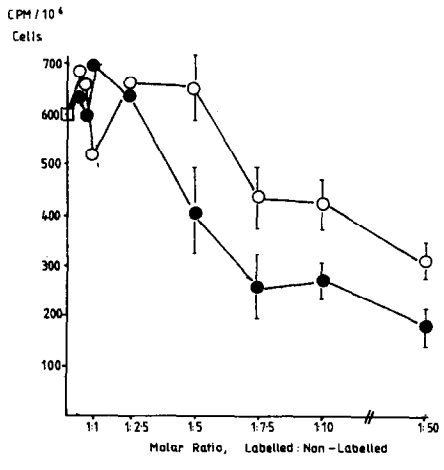

Fig. 6. Effect of mitozolomide (●) and AIC (○) on the incorporation of [6-^{24}C imidazo] mitozolomide into acid-insoluble material.

ous solution to give the monochloroethyltriazene MCTIC (IV) (Fig. 7) both mitozolomide and MCTIC produce DNA interstrand cross-links and this has been suggested as being responsible for their cytotoxic effects [4, 5]. The initial reaction in the formation of DNA interstrand cross-links is the formation of chloroethyl-DNA adducts, as occurs in cells treated with chloroethylnitrosoureas [9]. In the present studies the radioactive label is in the imidazole ring of mitozolomide and two possible interactions with DNA could take place: (1) Covalent binding of the intact molecule of MCTIC to DNA bases. (2) Incorporation of AIC into the purines of nucleic acids via the ribotide formed as a result of salvage by adenine phosphoribosyl transferase (APRT) (Fig. 8) [11, 12].

The cellular radioactivity associated with the acid-insolubule material has been shown to be incorporated into nucleic acids. Acid hydrolysis of the isolated nucleic acids followed by hplc analysis of the individual bases showed radioactivity from [6-^{14}C]-

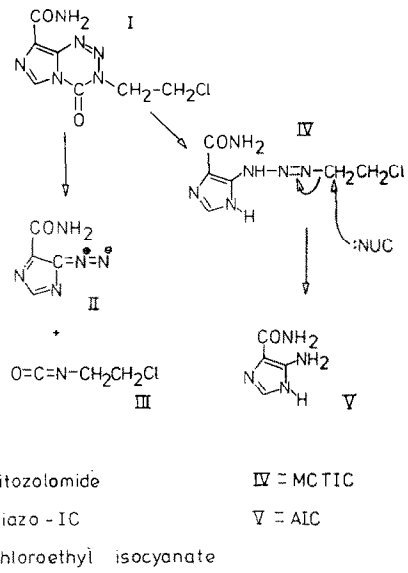

Fig. 7. Decomposition of mitozolomide (I) in aqueous solutions.

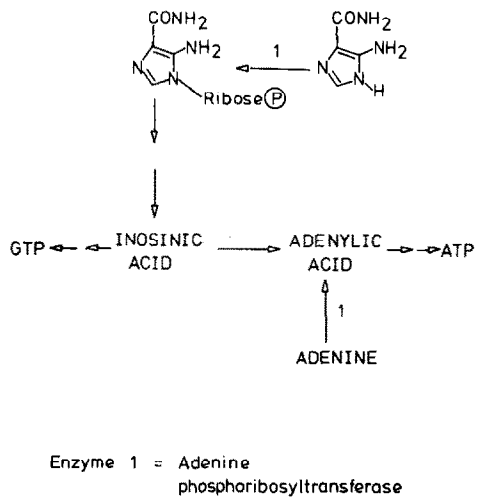

Fig. 8. Pathway of salvage of AIC.

mitozolomide to be associated solely with adenine and guanine and not with any modified bases. This shows that incorporation of radiolabel into macromolecules does not arise as a result of a covalent adduct between mitozolomide and nucleic acids.

The lag-period for incorporation of radioactivity into nucleic acids arises for the time required for decomposition of mitozolomide into AIC (Fig. 4). The half-life for decomposition in medium RPMI 1640, pH 7.2 at 37° is 104 min. Thus it is not until 2 hr after the addition of mitozolomide that the cells are exposed to sufficient amounts of [2-^{14}C]AIC for the incorporation into nucleic acids to be detectable. Unlabelled AIC competes with [6-^{14}C]mitozolomide for incorporation of radioactivity into nucleic acids thus confirming the *de novo* pathway of incorporation.

In view of the instability of mitozolomide in aqueous solutions at alkaline pH, care must be exercised in following the *in vivo* fate of labelled mitozolomide. The above studies provide confirmatory evidence for the route of breakdown of the molecule in the cell and suggest that mitozolomide is a transport form of the highly reactive triazene MCTIC.

Acknowledgements—This work has bee supported by a grant from the Cancer Research Campaign. Carmel Horgan gratefully acknowledges a studentship from the Science and Engineering Research Council.

REFERENCES

1. J. A. Hickman, N. W. Gibson, R. Stone, M. F. G. Stevens, F. Lavelle and C. Fizames, *Proc. 13th Int. Canc. Congr.* **551**, (1982).
2. M. F. G. Stevens, J. A. Hickman, R. Stone, N. W. Gibson, G. U. Baig, E. Lunt and C. J. Newton, *J. med. Chem.* **27**, 196 (1984).
3. Y. F. Shealy, C. A. O'Dell and C. A. Krauth, *J. pharm. Sci.* **64**, 177 (1975).
4. N. W. Gibson, L. C. Erickson and J. A. Hickman, *Cancer Res.* **44**, 1767 (1984).
5. N. W. Gibson, J. A. Hickman and L. C. Erickson, *Cancer Res.* **44**, 1772 (1984).
6. C. M. T. Horgan and M. J. Tisdale, *Biochem. Pharmac.* **33**, 2185 (1984).
7. N. W. Gibson, PhD Thesis, University of Aston (1982).
8. W. Boyle, *Transplantation* **6**, 761 (1968).
9. W. P. Tong, K. W. Kohn and D. B. Ludlum, *Cancer Res.* **42**, 4460 (1982).
10. J. A. Slack, M. F. G. Stevens, C. Goddard and A. Khan, *Proc. Am. Assoc. Cancer Res.* 1152 (1983).
11. M. P. Schlman and J. M. Buchanan, *J. biol. Chem.* **196**, 513 (1952).
12. G. M. Cowzelman Jr., H. G. Mandel and P. K. Smith, *J. biol. Chem.* **201**, 329 (1953).