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Inhibition of cellular esterases by the antitumour imidazotetrazines mitozolomide and temozolomide: demonstration by flow cytometry and conventional spectrofluorimetry

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Summary. Using flow cytometry and conventional spectrofluorimetry we have previously shown that chloroethylnitrosoureas (CNUs) can exhibit marked inhibition of cellular enzymes catalysing hydrolysis of fluorescein diacetate (FDA). More potent inhibition was seen for the carbamoylating CNUs, whereas alkylating agents were largely inactive. We now report results obtained with the developmental imidazotetrazines mitozolomide and temozolomide in comparison with BCNU, the novel alkylating agents clomesome and cyclodisone, and the active mitozolomide metabonate MCTIC. Inhibition of EMT6 mouse mammary-tumour esterases was seen for mitozolomide and temozolomide, and activity against purified porcine carboxylesterase was demonstrated. Flow cytometric analysis showed that inhibition occurred across the entire EMT6 cell population, with no evidence of a subpopulation resistant to enzyme inhibition. Inhibitory potency for the imidazotetrazines was much weaker than for BCNU. With EMT6 cells, I_{50} values from flow cytometry were 9.7×10^{-3} *M* and 1.5×10^{-3} *M* for mitozolomide and temozolomide compared with 3.7×10^{-4} *M* for BCNU. These were higher than the ID_{50} values for in vitro antitumour activity (MTT assay), 8.5×10^{-6} *M* in the case of mitozolomide and 1.2×10^{-5} *M* for BCNU, but similar to that of 5.6×10^{-4} *M* for the less toxic temozolomide. MCTIC and cyclodisone showed very low activity, but significant inhibition was seen for clomesome. The results are consistent with the view that the imidazotetrazines do not exhibit major carbamoylating ability, although significant effects are seen at cytotoxic concentrations of temozolomide. In addition, the potential for the generation of carbamoylating species at the enzyme active site cannot be ruled out.

Introduction

The imidazotetrazines mitozolomide and temozolomide are undergoing phase II trials in Europe. Interest in them has resulted from their novel structure and chemistry [23] as well as their encouraging antitumour activity in animal model systems [15, 24]. The antitumour effect of mitozolomide is thought to be caused by chloroethylation and cross-linking of DNA, as occurs with the CNUs [1, 8–10]. The overall shared mechanism of action of mitozolomide with CNUs is indicated by their cross-resistance in cell lines and in vivo tumours, including those demonstrated to exhibit relatively high levels of the DNA repair protein O^6-alkyl guanine DNA alkyltransferase (ATase) [8, 9, 15, 18, 29]. Some, albeit probably not complete, commonality of mechanism of action with these agents has also been proposed for temozolomide [13, 24]. Differences in DNA sequence specificity and reaction products between CNUs and mitozolomide have been demonstrated [11, 14], but the significance for antitumour selectivity remains unclear.

In addition to the spontaneous formation of chloroethylating species in aqueous solution, CNUs also form organic isocyanates that carbamoylate cellular proteins [1, 3]. Although carbamoylation does not appear to be a major requirement for antitumour activity, differences in the degree of resistance between carbamoylating and non-carbamoylating analogues in the Mer^+ vs Mer^- cell lines [7] as well as in those transfected with the bacterial ATase gene [2, 17–19] suggest that carbamoylation does play some role in cytotoxicity. Mitozolomide and temozolomide have a theoretical ability to form isocyanates and may do so under certain conditions [23, 24]. However, compared with BCNU, mitozolomide exhibited only modest inhibition of glutathione reductase, gamma-glutamyl transpeptidase and chymotrypsin [16]. Since these enzymes are sensitive to inhibition by carbamoylating agents, it was concluded that such species were not formed under physiological conditions. DNA damage by mitozolomide is thought to be mediated by the

Abbreviations. Drugs: ACNU, 1-(2-chloroethyl)-3-(4-amino-2-methylpyrimidine-5yl)methyl-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-2-nitrosourea; cis-2-OH CCNU, 1-(2-chloroethyl)-3-(*cis*-2-hydroxycyclohexyl)-1-nitrosourea; chlorozotocin, 1-(2-chloroethyl)-3-(D-2-glucopyranosyl)-1-nitrosourea; clomesome, 2-chloroethyl(methylsulphonyl)methanesulphonate; CNU, chloroethylnitrosourea; cyclodisone, 1,5,2,4-dioxadithiepane-2,2,4,4-tetroxide; MCTIC, 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide; mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one; MTIC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; temozolomide, 8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one. Others: ATase, O^6-alkylguanine DNA alkyltransferase; DMSO, dimethylsulphoxide; FDA, fluorescein diacetate; MTT, 3-4-5 dimethylthiazol-2-5 diphenyl tetrazolium bromide; PBS, phosphate-buffered saline

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chloroethylating metabonate MCTIC [8, 23], whereas temozolomide may spontaneously release MTIC, which is the demethylated and putative active metabolite of dacarbazine [24].

We have recently reported a novel flow cytoenzymological assay to measure inhibition of cellular esterases by CNUs and derived isocyanates [4, 5]. Using a wide range of derivatives, we showed a correlation between chemical carbamoylating potential and enzyme inhibition. Alkylating agents were largely inactive, suggesting the possibility that this technique may provide a measure of intracellular carbamoylation of a particular protein target. An important feature of the flow cytometric assay is the ability to identify heterogeneous subpopulations. This paper presents flow cytometric results obtained with mitozolomide, MCTIC and temozolomide. These were compared with the potent carbamoylating agent BCNU and the developmental non-carbamoylating agents clomesome and cyclodisone [9–13]. Selected agents were also tested against purified esterase. To ensure that the effects could be related to concentrations causing cytotoxicity, the in vitro antitumour effects of mitozolomide, temozolomide, chlorozotocin and BCNU were determined in EMT6 cells by MTT dye reduction assay [25].

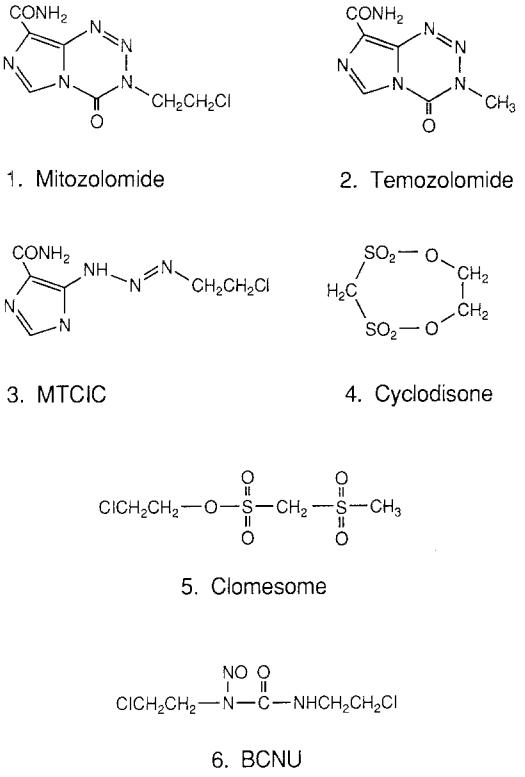

Fig. 1. Structures of mitozolomide, temozolomide, MCTIC, cyclodisone, clomesome and BCNU

Materials and methods

Cells. EMT6/CCVJAC, a mouse mammary-tumour cell line, is grown in monolayer culture [21]. After 2 days growth, cells were harvested in log phase by trypsinization. Single-cell suspensions (10^6 cells/ml) were prepared in Eagle's minimal essential medium with Earle's salts and 10% (new born) calf serum for flow cytometric assay, or in phosphate-buffered saline (Dulbecco "A", PBS; Sigma) in the case of conventional spectrofluorimetry.

Reagents. The fluorogenic esterase substrate FDA (Koch Light) was prepared as a stock solution in 0.012 *M* acetone (grade A). This was diluted with PBS immediately before use to give a concentration of 2 μ*M*. Partially purified porcine liver carboxylesterase (E.C.3.1.1.1.) was obtained as a suspension from Sigma; it was diluted to 1 μg/ml with PBS immediately before use.

Drug structures are shown in Fig. 1. BCNU and clomesome were obtained from the United States National Cancer Institute. Mitozolomide, temozolomide and MCTIC were kindly provided by the CRC Experimental Chemotherapy Research Group (Aston University, Birmingham, UK). Stock solutions of BCNU in ethanol (grade A) and of mitozolomide, MCTIC, clomesome and cyclodisone in DMSO were diluted with PBS immediately before their addition to cells, maintaining final solvent concentrations of 0.5% (v/v) for ethanol and 2.5% (v/v) for DMSO. Temozolomide was dissolved directly in PBS just prior to use.

Flow cytometry. All studies were, carried out with the Cambridge MRC dual-laser flow cytometer [26–28] using procedures previously described in detail [4–6]. Briefly, fluorescein produced by intracellular esterase hydrolysis of FDA was excited at 488 nm, giving rise to green/yellow fluorescence which was collected between 515 and 560 nm. The flow cytometer was set to trigger on 90° light scatter, an indirect measure of cell size, and to record green fluorescence together with time (from the computer clock). This was done for each of 50,000 cells continuously sampled over a 5-min reaction period at a constant flow rate of about 170 cells/s. All procedures were carried out at room temperature (20° – 22° C). Putative inhibitor solutions were added to cell samples (0.15 ml at 10^6 cells/ml in medium) 1 h before the reaction was initiated by the addition of FDA (2 μ*M*, 0.3 ml). Appropriate, untreated control samples were included at regular intervals during the experiment. Data were analysed as the medians of eight sequential fluorescence vs frequency histograms. Enzyme reaction progress curves were plotted (e.g. Fig. 3) and initial velocities were determined by least-squares linear regression analysis. Comparison of the initial velocities for control vs inhibited reactions enabled the calculation of the percentage of activity remaining at various inhibitor concentrations (e.g. Fig. 4). Values of I_{50} (the inhibitor concentration producing 50% inhibition) were generated from the dose-response data by probit analysis using the GLIM statistical programs of The Royal Statistical Society of Great Britain.

Conventional spectrofluorimetry. Parallel experiments were carried out with intact cells, cell sonicates and purified esterase using an MPF-4 spectrofluorimeter (Perkin-Elmer) with monochromators set at 490 and 520 nm for excitation and emission, respectively, as previously described in detail [4–6]. Inhibitor preincubations, enzyme reactions and data analysis were carried out essentially as outlined above. Prior to assay of enzyme activity

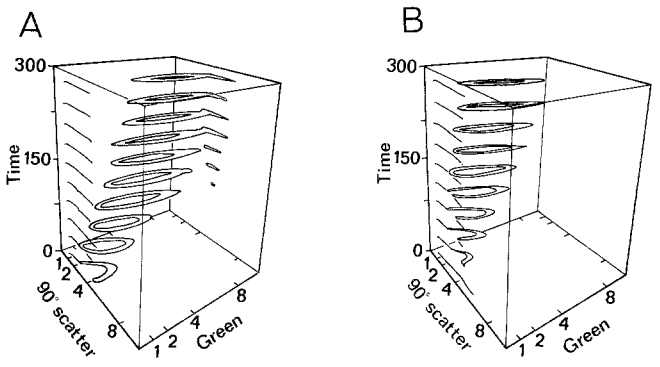

Fig. 2. Three-dimensional displays of green fluorescence vs time vs 90° light scatter for untreated log-phase EMT6 mouse mammary tumour cells (*panel A*) and those pretreated with $5 \times 10^{-3} M$ temozolomide for 1 h at 20° – 22°C (*panel B*). Green fluorescence is due to fluorescein released from FDA (1 μ*M*) by intracellular esterases, whereas 90° light scatter is related to cell size and shape. Both parameters are expressed in arbitrary units (channel number) and time is measured in seconds. Scales are linear. Results are from a typical experiment

agents were incubated at 20° – 22°C with cells (10^6/ml) or purified enzyme (0.5 μg/ml) for a period of 1 h.

In vitro cytotoxicity. The MTT assay [25] was used to assess the cytotoxicity of mitozolomide and temozolomide against EMT6 cells as compared with that of potent and weak carbamoylating agents BCNU and chlorozotocin, respectively. Optical density due to conversion of MTT to formazan by dehydrogenases of viable cells was measured. Cells (6×10^2) in medium (200 μl) were incubated in microwell plates for 3 days in the presence of test substances at 10^{-6}–3.3×10^{-3} *M*. MTT was then added (0.5 mg/ml) and, after a further 4 to 6-h incubation, the resulting optical density was compared with that of untreated controls using a Titertek Multiscan MCC/340 automated plate reader.

Results

Flow cytometry

At a concentration of 5×10^{-3} *M* for 1 h, both mitozolomide and temozolomide exhibited significant inhibition of esterases in cultured EMT6 mouse mammary-tumour cells. Figure 2 shows a three-dimensional display of green fluorescence vs time vs 90° light scatter for untreated control cells (panel A) compared with those pretreated for 1 h with 5×10^{-3} *M* temozolomide (panel B). It can be seen that for control cells the frequency contours traverse the three-dimensional data space, and inhibition is clearly demonstrated for the temozolomide-treated sample. No populational heterogeneity is indicated in either case; in particular, there is no evidence of a subpopulation resistant to esterase inhibition by temozolomide. In addition, neither the accumulation of fluorescein nor temozolomide treatment appeared to effect cell size, as indicated by the lack of change in 90° light scatter; this held true for all other compounds.

Figure 3 shows typical enzyme-reaction progress curves for the hydrolysis of FDA by esterases of intact EMT6 mouse mammary-tumour cells treated with mitozolomide, MCTIC and BCNU, as compared with vehicle controls.

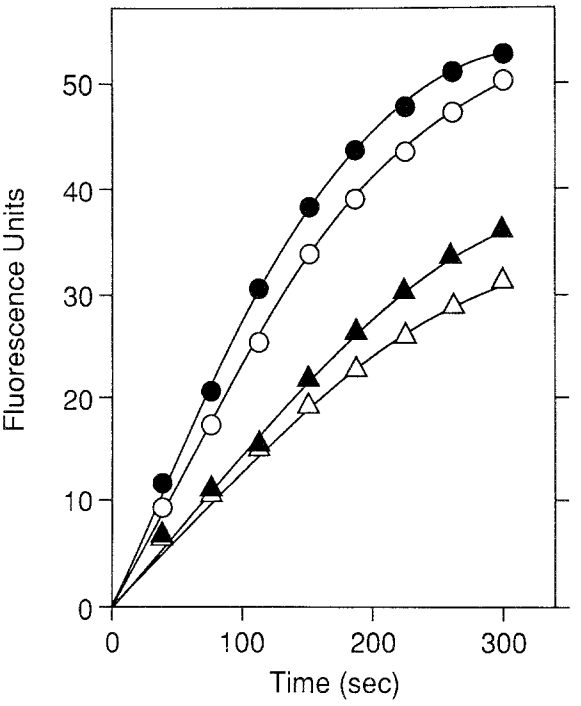

Fig. 3. Enzyme-reaction progress curves for the hydrolysis of FDA in control and inhibited samples of EMT6 cells. Agents were incubated with cells (10^6/ml) for 1 h prior to assay of esterase activity with 1 μ*M* FDA, all at 20° – 22°C. ●, control (2.5% DMSO or 0.5% ethanol); ○, 5×10^{-3} *M* MCTIC; ▲, $5 \times 10^{-3} M$ mitozolomide; △, 5×10^{-4} *M* BCNU. Results are from a typical experiment measured by flow cytometry. Each point represents the mean of duplicate values

There was no significant difference in the enzyme activity for cells exposed to 2.5% DMSO or 0.5% ethanol with respect to that seen in the absence of these drug vehicles.

A comparison of the inhibitory potency of the various agents is given in the form of dose-response curves (Fig. 4) and I_{50} values (Table 1). It can be seen that mitozolomide is about 26-fold less potent than BCNU, whereas temozolomide has only 4-fold lower potency. The percentage of esterase activity remaining at an inhibitor concentration of 5×10^{-3} *M* is also given in Table 1, since the I_{50} values for MCTIC and cyclodisone were not approached in the concentration range studied. Only 23% inhibition occurred with 5×10^{-3} *M* MCTIC, whereas 35%, 84% and 100% inhibition were seen at this concentration of mitozolomide, temozolomide and BCNU, respectively. At the same concentration, 31% inhibition was seen with clomesome, and only 13% with cyclodisone. In addition, various other alkylating agents such as mustine, melphalan, chlorozotocin, methyl methanesulphonate, cyclophosphamide and cisplatin also showed minimal effect [6].

Conventional spectrofluorimetry

The inhibitory activities of mitozolomide, temozolomide, MCTIC and BCNU were determined by spectrofluorimetry using intact EMT6 cells and cell sonicates (Table 1). For all drugs there was little difference in activity in the presence vs absence of an intact cell membrane. The results confirm the order of inhibitory potency found by

Table 1. Summary of the inhibitory effects of mitozolamide, temozolamide, MTCIC, BCNU, clamesame and cyclodisone on FDA hydrolysis measured by flow cytometry and conventional spectrofluorimetry

Drug	Flow cytometry:		Spectrofluorimetry:					
	Intact EMT6 cells		Intact EMT6 cells		EMT6 cell sonicate		Purified porcine liver carboxylesterase	
	I_{50}[a] (*M*)	% Activity remaining at 5×10^{-3} *m*	I_{50}[a] (*M*)	% Activity remaining at 5×10^{-3} *m*	I_{50}[a] (*M*)	% Activity remaining at 5×10^{-3} *m*	I_{50}[a] (*M*)	% Activity remaining at 5×10^{-3} *m*
Mitozolamide	9.7×10^{-3} (6.2×10^{-3}–1.5×10^{-2})	65	9.6×10^{-3} (3.1×10^{-3}–2.9×10^{-3})	63	9.5×10^{-3} (5.5×10^{-3}–1.6×10^{-2})	68	$<5 \times 10^{-3}$	14
MTCIC	$>5 \times 10^{-3}$	77	ND	ND	ND	ND	$>5 \times 10^{-3}$	89
Temozolamide	1.5×10^{-3} (1.0–2.1×10^{-3})	16	4.6×10^{-4} (3.6–6.0×10^{-4})	2	4.2×10^{-4} (3.2–5.6×10^{-4})	3	ND	ND
BCNU	3.7×10^{-4} (2.9–4.7×10^{-4})	0	5.0×10^{-5} (3.1–8.2×10^{-5})	0	9.2×10^{-5} (8.1×10^{-5}–1.1×10^{-4})	0	$<2 \times 10^{-6}$	0
Clomesome	$>5 \times 10^{-3}$	69	ND	ND	ND	ND	ND	ND
Cyclodisone	$>10^{-2}$	87	ND	ND	ND	ND	ND	ND

Agents were incubated with cells (5×10^6/ml) or enzyme (0.5 μg/ml) for a period of 1 h prior to assay of esterase activity at 1 μ*M* FDA, all at 20°–22° C. Results are mean values from at least two experiments

[a] 95% confidence limits are shown in parentheses

ND, not done

flow cytometry; for example, temozolomide was 9-fold less active than BCNU. Figure 5 shows the dose-response curves obtained for MCTIC, mitozolomide and BCNU inhibition of purified porcine liver carboxylesterase. MCTIC was almost inactive, whereas mitozolomide inhibited the esterase by 60% at a concentration of 10^{-3} *M*. At the same concentration the activity was totally inhibited by BCNU.

Chemosensitivity

Figure 6 shows the comparative cytotoxicities of mitozolomide, temozolomide, BCNU and chlorozotocin against EMT6 cells in vitro, as determined using the MTT tetrazolium dye-reduction assay. The respective ID_{50} values derived from these data are also shown. It can be seen that temozolomide is considerably less cytotoxic than mitozolomide.

Discussion

The object of this study was to investigate the ability of the developmental imidazotetrazines mitozolomide and temozolomide to inhibit the hydrolysis of FDA by esterases of EMT6 mouse mammary-tumour cells. Our previous studies with a wide range of CNUs have shown that those compounds known to be more strongly carbamoylating, as defined mainly by chemical assay, also produce a more potent inhibition of cellular esterases [6].

Results presented in this paper show that both mitozolomide and temozolomide can inhibit EMT6 mouse mammary-tumour esterases, as measured by both flow cytometry and conventional spectrofluorimetry. The results obtained with the two techniques were quantitatively similar. In addition, the use of flow cytometry enabled us to demonstrate inhibition across the entire population of log-phase EMT6 cells, with no evidence of a subpopulation resistant to enzyme inhibition. More complex and

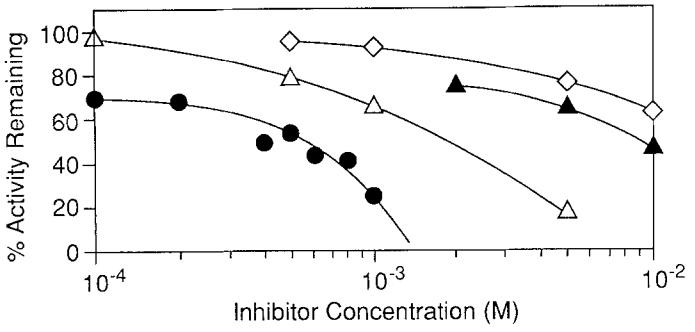

Fig. 4. Dose-response curves for the inhibitory effects of BCNU (●), temozolomide (△), mitozolomide (▲) and MCTIC (◇) on the hydrolysis of FDA by EMT6 cells measured by flow cytometry. Agents were incubated with cells (10^6/ml) for 1 h prior to assay of esterase activity with 1 μ*M* FDA, all at 20°–22°C. Each point represents the mean of 2–14 repeat experiments

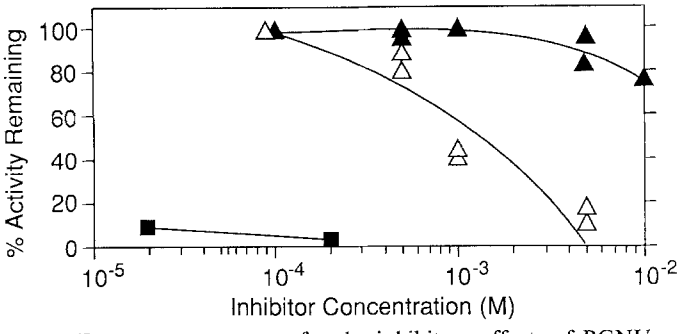

Fig. 5. Dose-response curves for the inhibitory effects of BCNU (■), mitozolomide (△) and MCTIC (▲) on the hydrolysis of FDA by purified porcine liver carboxylesterase. Agents were incubated with enzyme (0.5 μg/ml) for 1 h prior to assay of esterase activity by conventional spectrofluorimetry, all at 20°–22°C. For BCNU, each point represents the mean value of three repeat experiments; for mitozolomide and MCTIC, each point represents an individual experiment

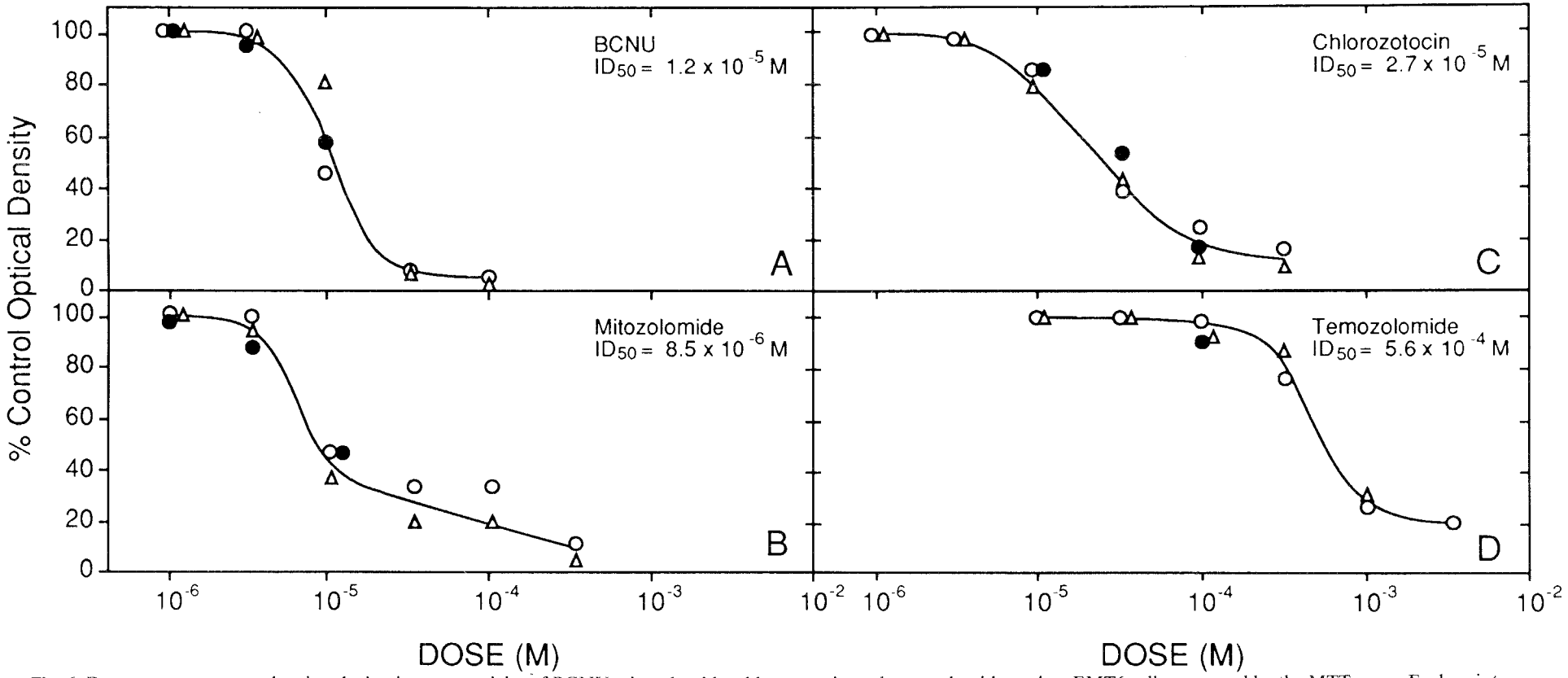

Fig. 6. Dose-response curves showing the in vitro cytotoxicity of BCNU mitozolomide, chlorozotocin and temozolomide against EMT6 cells measured by the MTT assay. Each point represents the mean of six replicates, and the different symbols indicate three independent experiments. ID_{50} values are also indicated

heterogeneous behaviour has recently been seen for mouse bone marrow (C. Dive et al., unpublished data).

Although clear inhibition of enzyme activity was seen for mitozolomide and temozolomide, they were less active than BCNU, especially mitozolomide. Similar inhibition in intact cells and sonicated preparations indicated that cellular influx of either drug does not appear to be a limiting factor. The chloroethylating metabonate of mitozolomide, MCTIC, was less inhibitory than mitozolomide as measured by flow cytometry, and this difference was even greater when determined by conventional spectrofluorimetry. Inhibition of purified porcine liver carboxylesterase was demonstrated for mitozolomide, and this was again greater than that for MCTIC but less than that for BCNU.

The I_{50} values for the two imidazotetrazines, as determined by flow cytometry, are broadly similar to those we obtained for *cis*-2-OH CCNU and ACNU [6]. The latter CNUs are believed to undergo intramolecular carbamoylation, resulting in only weak carbamoylation at cellular targets. Previous studies on the inhibition of the carbamoylating agent-sensitive enzymes chymotrypsin, glutathione reductase and gamma-glutamyl transpeptidase by mitozolomide suggest the absence of a carbamoylating species [16]. Although mitozolomide failed to inhibit purified chymotrypsin significantly at 1 m*M*, we observed about 60% inhibition of purified carboxylesterase at this drug concentration. Throughout the present studies, cells or purified enzyme were incubated with the various agents for the fixed exposure time of 1 h at 20° – 22° C. This was previously shown to give close to maximal inhibition by BCNU without compromising cell viability [5] and was subsequently used to screen a variety of CNUs, isocyanates and alkylating agents. Extensive breakdown of the imidazotetrazines and BCNU occurs under these incubation conditions [8, 22, 24].

The greater inhibitory effects of mitozolomide as opposed to MCTIC against esterases suggests that the parent drug itself or some other intermediate species may be responsible. It is possible that mitozolomide and temozolomide might be capable of generating a carbamoylating species under conditions occurring at or close to the active site of the enzyme. Although the activity is weak compared with that of more potent carbamoylating agents such as BCNU, it would nevertheless be interesting to elucidate the mechanism; at this stage, the possibility of carbamoylation cannot be ruled out. The likelihood that inhibition of cellular esterases is sensitive to carbamoylating but not alkylating agents was supported by our previous studies using a wide range of compounds [5, 6]. However, although MCTIC and cyclodisone showed very low activity, the inhibition obtained with clomesome, comparable with that for *cis*-2-OH CCNU, suggests that alternative mechanisms of inhibition may occur. However, it should be emphasized that the inhibition of esterase activity seen for temozolomide is considerably more potent than that for clomesome.

Using the MTT dye reduction assay, the concentrations required for in vitro cytotoxicity were determined for mitozolomide, temozolomide, chlorozotocin and BCNU, such that these could be related to those giving rise to enzyme inhibition. As expected from their Mer^- status, EMT6 cells were quite sensitive to these agents [20], but temozolomide (lacking the cross-linking chloroethyl group) exhibited lower potency [13, 24]. In view of the non-pharmacological concentrations required for inhibition of esterase activity by mitozolomide, it is unlikely that this is of major significance. However, concentrations of temozolomide causing enzyme inhibition and cytotoxicity were quite similar, and its pharmacological relevance cannot be ruled out. In addition, the possibility exists that other enzyme systems might be identified for which inhibitory potency may be much greater.

References

1. Ames MM, Powis G (1983) Pharmacokinetics of nitrosoureas. In: Ames MM, Powis G, Kovach JS, (eds) Pharmacokinetics of anticancer agents. Elsevier, Amsterdam
2. Brennand J, Margison GP (1986) Reduction of the toxicity and mutagenicity of alkylating agents in mammalian cells harboring the *E. coli* alkyltransferase gene. Proc Natl Acad Sci USA 83: 6292
3. Colvin M, Brundrett R (1981) Chemical decomposition of the chloroethylnitrosoureas. In: Nitrosoureas. Academic, New York, p 43
4. Dive C, Workman P, Watson JV (1987) Improved methodology for intracellular enzyme reaction and inhibition kinetics for flow cytometry. Cytometry 8: 552
5. Dive C, Workman P, Watson JV (1987) Novel dynamic flow cytoenzymological determination of intracellular esterase inhibition by BCNU and related analogues. Biochem Pharmacol 36: 3731
6. Dive C. Workman P, Watson JV (1988) Inhibition of intracellular esterases by antitumour chloroethylnitrosoureas: measurement by flow cytometry and correlation with molecular carbamoylating activity. Biochem Pharmacol 37: 3987
7. Erikson LC, Matthews BO, Ducore JM, Ewig RAG, Kohn KW (1980) DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumour nitrosoureas. Proc Natl Acad Sci USA 77: 467
8. Gibson NW, Hickman JA, Erikson LC (1984) DNA crosslinking and cytotoxicity in normal and transformed human cells treated in vitro with 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one. Cancer Res 44: 1772
9. Gibson NW, Erikson LC, Kohn KW (1985) DNA damage and differential cytotoxicity produced in human cells by 2-chloroethyl(methylsulphonyl)-methansesulphonate (NSC-338947), a new DNA chloroethylating agent. Cancer Res 45: 1674
10. Gibson NW, Mattes WB, Hartley JA (1985) Identification of specific DNA lesions induced by three classes of chloroethylating agents: chloroethylnitrosoureas, chloroethylmethanesulphonates, and chloroethylimidazotetrazines. Pharmacol Ther 31: 153
11. Gibson NW, Hartley JA, Strong JM, Kohn KW (1986) 2-Chloroethyl (methylsulphonyl)-methanesulphonate (NSC 338947), a more selective DNA alkylating agent than the chloroethylnitrosoureas. Cancer Res 46: 553
12. Gibson NW, Hartley JA, Kohn KW (1986) DNA reactivity and in vivo cytotoxicity of the novel antitumour agent 1,5,2,4-dioxadithiepane-2,2,4,4-tetroxide (NSC-348948) in human embryo cells. Cancer Res 46: 1679
13. Gibson NW, Hartley JA, La France RJ, Vaughan K (1986) Differential cytotoxicity and DNA-damaging effects produced in human cells of the Mer^+ and Mer^- phenotypes by a series of alkyltriazenylimidazoles. Carcinogenesis 7: 259
14. Hartley JA, Gibson NW, Kohn KW, Mattes WB (1986) DNA sequence selectivity of guanine-N7 alkylation by three antitumour chloroethylating agents. Cancer Res 46: 1943

15. Hickman JA, Stevens MFG, Gibson NW, Langdon SP, Fizames C, Lavelle F, Atassi G, Lunt E, Tilson RM (1985) Experimental antitumour activity against murine tumour systems of 8-carbamoyl-3-(2-chloroethyl)imidazo-[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (mitozolomide), a novel broad spectrum agent. Cancer Res 45: 3008
16. Horgan CMT, Tisdale MJ (1984) Antitumour imidazotetrazines: IV. An investigation into the mechanism of antitumour activity of a novel antitumour agent, mitozolomide (CCRG 81010, M and B 39565; NSC 353451). Biochem Pharmacol 33: 2185
17. Lindahl T (1987) Regulation and deficiencies in DNA repair. Br J Cancer 56: 91
18. Margison GP, Brennand J (1987) Functional expression of the *Escherichia coli* alkyltransferase gene in mammalian cells. J Cell Sci [Suppl] 6: 83
19. Morten JEN, Margison GP (1988) Increased O^6-alkylguanine alkyltransferase activity in chinese hamster V79 cells following selection with chloroethylating agents. Carcinogenesis 9: 45
20. Mulcahy RT (1986) Misonidazole-induced chemopotentiation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea toxicity in O^6-methylguanine-DNA methyltransferase proficient (Mer^+) and deficient (Mer^-) cell lines. Cancer Res 46: 2892
21. Rockwell SC, Kallman RF, Farjardo J (1972) Characteristics of a serially transplanted mouse mammary tumour and its tissue-culture-adapted derivative. J Natl Cancer Inst 49: 735
22. Slack J, Goddard C (1985) Antitumour imidazotetrazines: VII. Quantitative analysis of mitozolomide in biological fluids by high-performance liquid chromatography. J Chromatogr 337: 178
23. Stevens MFG, Hickman JA, Stone R, Gibson NW, Baig GU, Lunt E, Newton CG (1984) Antitumour imidazotetrazines: 1. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl)-imidazo-[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one, a novel broad spectrum antitumour agent. J Med Chem 27: 196
24. Stevens MFG, Hickman JA, Langdon SP, Chubb D, Vickers L, Stone R, Baig GU, Goddard C, Gibson NW, Slack JA, Newton C, Lunt E, Fizames C, Lavelle F (1987) Antitumour activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo-[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (CRC 81045: M and B 398310), a novel drug with potential as an alternative to dacarbazine. Cancer Res 47:5846
25. Twentyman PR, Luscombe M (1987) A study of some of the variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56: 279
26. Watson JV (1980) Enzyme reaction kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. Cytometry 1: 143
27. Watson JV (1981) Dual laser beam focussing for flow cytometry through a single crossed cylindrical lens pair. Cytometry 2: 14
28. Watson JV, Chambers SC, Workman P, Horsnell TS (1977) A flow cytometric method for measuring enzyme reaction kinetics in populations of single cells. FEBS Lett 81: 179
29. Workman P, Kooistra KL, Lee FYF, Donaldson J, Margison GP (1988) In vivo induced resistance to chloroethylating agents correlates with increased O^6-alkylguanine DNA alkyltransferase expression in a murine solid tumour. Br J Cancer 58: 233

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