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INHIBITION OF INTRACELLULAR ESTERASES BY ANTITUMOUR CHLOROETHYLNITROSOUREAS

MEASUREMENT BY FLOW CYTOMETRY AND CORRELATION WITH MOLECULAR CARBAMOYLATION ACTIVITY

CAROLINE DIVE, PAUL WORKMAN* and JAMES V. WATSON
MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge, U.K.

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Abstract—Antitumour chloroethylnitrosoureas (Cnus) decompose in physiological conditions yielding alkylating species and organic isocyanates. While antitumour activity is mainly attributed to the alkylation of DNA, carbamoylation of intracellular proteins by isocyanates may also have pharmacological and toxicological relevance. We previously reported a novel dynamic flow cytoenzymological assay for esterase inhibition in intact murine cells by BCNU and related isocyanates, and proposed that this might form the basis of an assay for intracellular carbamoylation. We have now examined a wide range of Cnus, isocyanates, and alkylating agents for their ability to inhibit cellular esterases. BCNU, CCNU, their derived isocyanates, and the 4-OH metabolites of CCNU exhibited potent inhibitory activity (I_{50} values 5.5×10^{-5}–7.3×10^{-4} M). Chlorozotocin and GANU were relatively inactive ($I_{50} \gg 10^{-2}$ M). ACNU, TCNU and the 2-OH metabolites of CCNU exhibited intermediate activity (I_{50} values 1.1×10^{-3}–2.3×10^{-2} M). Compounds not able to form isocyanates were essentially inactive. Poor membrane permeability was also implicated in the weak activity of chlorozotocin and GANU. There was overall a good correlation between esterase inhibition and chemical carbamoylating activity, but some particular differences were identified. We concluded that flow cytoenzymological assay appears to have the potential to provide useful measurement of intracellular protein carbamoylation by existing Cnus and novel derivatives, and also offers the advantage of cell subpopulation identification for *in vivo* evaluation of these agents.

Antitumour chloroethylnitrosoureas (Cnus)† are widely used in cancer chemotherapy, but have on the whole proved disappointing in clinical use when compared with their impressive broad spectrum of activity in rodent tumour systems [1, 2]. New derivatives continue to be developed, principally with the aim of improving specificity or reducing dose-limiting myelosuppression [3]. Cnus undergo spontaneous breakdown to various metabonates in aqueous solution [4]. Antitumour activity is attributed to alkylation of DNA, and particularly to DNA cross-links formed via chloroethylation [5]. As well as alkylating fragments, organic isocyanates are produced by Cnu decomposition [6]. The Cnu-derived isocyanates are able to carbamoylate intracellular proteins [7, 8]. The latter reaction is thought not to be directly contributory to antitumour activity, since Cnus without apparent carbamoylating activity retain antitumour activity *in vitro* [9] and *in vivo* [10, 11]. It has been considered that carbamoylation may also lead to unwanted toxicity, including myelosuppression, thus reducing antitumour selectivity [12]. On the other hand, carbamoylating activity may enhance cytotoxicity by inhibition of the repair of alkylated DNA [13]. Other proposed roles for carbamoylation include inhibition of macromolecular synthesis [14] and RNA processing [15], enhancement of radiation cytotoxicity [13], and inactivation of various enzymes [12, 16–21].

We have previously reported a novel, dynamic flow cytoenzymological procedure to measure intracellular esterase inhibition by BCNU and related isocyanates [22]. BCNU and its metabonate chloroethylisocyanate (CEI) exhibited closely similar inhibitory potencies, and *n*-butyl isocyanate (*n*-BI) was also active. The non-carbamoylating, pure alkylating agents, methyl methanesulphonate (MMS), melphalan and nitrogen mustard, failed to show significant inhibition. Potent BCNU inhibition of purified porcine liver carboxylesterase was also demonstrated. In view of these results and the known

* To whom correspondence should be addressed.

† Abbreviations used: Cnu, chloroethylnitrosourea; CNU, 2-chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; *trans*-4-OH CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; *cis*-4-OH CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea; *trans*-2-OH CCNU, 1-(2-chloroethyl)-3-(*trans*-2-hydroxycyclohexyl)-1-nitrosourea; *cis*-2-OH CCNU, 1-(2-chloroethyl)-3-(*cis*-2-hydroxycyclohexyl)-1-nitrosourea; CEI, chloroethyl isocyanate; CHI, cyclohexyl isocyanate; ACNU, 1-(4-amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea; TCNU, 1-(2-chloroethyl)-3-[2-(dimethylaminosulphonyl)-1-ethyl]-1-nitrosourea; chlorozotocin, [2-3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose; GANU, 1-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose; GANU, 1-(2-chloroethyl)-3-(D-glucopyranosyl)-1-nitrosourea; KOCN, potassium cyanate; MMS, methyl methanesulphonate; clomesome, 2-chloroethyl-(methylsulphonyl)-methanesulphonate; cyclodisone, 1,5,2,4-dioxadithiepane-2,2,4,4-tetraoxide.

sensitivity of serine hydrolases to inactivation by carbamoylation [16, 20], we proposed that inhibition of cellular esterases was a result of carbamoylation of enzyme molecules. We further suggested that flow cytometric analysis of esterase activity might provide the basis for the assay of protein carbamoylation potential of Cnus and isocyanate molecules in intact cells. This would have the advantage over existing chemical assays for carbamoylation [23, 24] of greater biological relevance, since the reaction is monitored in whole cells. In addition, the use of flow cytometry allows identification of multiple heterogeneous populations with important potential for *in vivo* applications.

The object of the present series of experiments was to evaluate further the possibility of using flow cytoenzymological analysis of esterase activity to measure intracellular protein carbamoylation by examining selected Cnus, isocyanates and related compounds, together with additional pure alkylating agents, for their inhibitory effects on esterases of intact EMT6 mouse mammary tumour cells. In order to assess the effects of drug access, we have also carried out complementary studies on intact cells, sonicates and purified esterase. The results showed a good overall correlation between esterase inhibition and chemical carbamoylating activity, but some interesting differences were identified.

MATERIALS AND METHODS

Cells. The EMT6/CaVJAC cell line is a tissue-culture adapted derivative of the EMT6 mouse mammary tumour [25]. Details of cell culture and preparation of cell suspensions are given elsewhere [22, 26]. In brief, cells were grown in Eagle's Minimal Essential medium with Earle's salts, supplemented with glutamine, antibiotics and 20% new born calf serum (all Gibco (Paisley, U.K.)). The latter was heat treated at 65° for 30 min. Cell monolayers were harvested by trypsinisation in log phase after 2 days of growth and the appropriate cell cycle distribution confirmed by flow cytometric analysis using ethidium bromide and Triton-X-100 [27, 28]. Single cell suspensions (10^6 cells/ml) were washed and resuspended in medium for flow cytometry. For conventional spectrofluorimetry, cells were further washed and resuspended in phosphate-buffered saline (Dulbecco "A" PBS, Gibco).

Porcine liver carboxylesterase [*EC* 3.1.1.1.]. This partially purified esterase was obtained from Sigma (Poole, U.K.) as a suspension in 3.2 M ammonium sulphate solution, pH 8, containing 10.71 mg protein/ml. It was stored in the dark at 4° and diluted with PBS immediately prior to analysis to give a solution containing 1 μg protein per ml.

Substrate. Fluorescein dilacetate (FDA, Koch Light) was dissolved in grade A acetone to give a stock solution at 0.012 M. It was prepared fresh each week and stored at 4° in the dark. The solution was diluted in PBS immediately before use to obtain a concentration of 2 μM.

Drugs. BCNU, CCNU, MeCCNU, ACNU, CNU, GANU, chlorozotocin, clomesome, cyclodisone and hydroxy metabolites of CCNU were obtained from the US National Cancer Institute (Bethesda, U.S.A.). TCNU was provided by Leo (Aylesbury, U.K.). Potassium cyanate (KOCN), methyl methane-sulphonate (MMS), and methyl, ethyl, *n*-propyl and *n*-butyl isocyanate (MI, EI, *n*-PI, *n*-BI respectively) were obtained from Aldrich (Gillingham, U.K.). Cyclohexyl isocyanate (CHI) and chloroethyl isocyanate (CEI) were obtained from Sigma. MMS was obtained from Aldrich, nitrogen mustard from Boots (Nottingham, U.K.), and melphalan was supplied by The Chester Beatty Institute (London, U.K.).

BCNU, CCNU, MeCCNU, TCNU, CNU and the hydroxy metabolites of CCNU were dissolved in ethanol to give a stock concentration 100 times that of the highest test concentration required. ACNU, GANU, chlorozotocin, clomesome and cyclodisone were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions 20 times that of the highest test concentration required. The alkyl isocyanates were diluted in acetone to 0.02 M. The above stock solutions were made freshly each week and stored in the dark at −70°, except for the alkyl isocyanates which were freshly prepared for each individual experiment. Stock solutions were diluted in PBS immediately before addition to cells, so that the solvent concentrations were maintained at 1, 2 and 5% v/v for ethanol, acetone and DMSO respectively. For each experiment, immediately before use, a stock solution of melphalan was prepared in ethanolic HCl (2% v/v 0.1 M HCl in ethanol); this was then diluted with PBS such that the solvent concentration was 2% v/v. KOCN, MMS and nitrogen mustard were dissolved directly in PBS, again just prior to addition to cells. The various drug vehicles were shown to have minimal effect on the enzyme reaction at the concentrations used.

Flow cytometry. The Cambridge MRC dual laser flow cytometer was designed and built in this laboratory [29, 30]. Full details of the instrumentation and data acquisition and processing are given elsewhere [26]. In brief, fluorescein, produced intracellularly by esterase-catalysed hydrolysis of FDA, was excited at 488 nm and the green/yellow fluorescence emitted from each individual cell was recorded. Other parameters simultaneously recorded were time, measured directly by the computer clock, and forward and right angle scatter.

Aliquots of cell suspensions (150 μl, 10^6 cells/ml) in Eppendorf tubes (1 ml) were gassed with 5% CO_2/air. Drug or appropriate vehicle solution (150 μl) was added 1 hr prior to analysis, then the tubes were regassed and held at room temperature (20–22°). To start the enzyme reaction 300 μl of 2 μM FDA were added and the reactants quickly mixed. Thus the final FDA substrate concentration was 1 μM. After a preset 20 sec dead time the reaction mixture was pumped into the flow cell and sampled continuously at room temperature throughout the 5 min reaction time. During this period 50,000 cells were analysed at a flow rate of about 166 cells/sec. Control samples were run at least every half hour to confirm cellular esterase stability throughout each experiment. Non-viable cells and debris were excluded from the analysis by electronic gating based on light scatter characteristics (Watson, in preparation).

In-house computer programs were used to display

data as 2-dimensional frequency contour maps of fluorescence versus time. Data were analysed by determination of the medians of eight sequential fluorescence versus time histograms. These values were plotted against time to generate enzyme reaction progress curves, from which reaction velocities were determined by linear regression analysis of the linear phase. Two replicate test samples were included at each concentration to obtain a mean value for initial velocity, and the appropriate control sample values were also averaged. By comparison of reaction velocities for test and appropriate control samples, the % esterase activity remaining was determined. For the more potent esterase inhibitors results are presented as I_{50} values (the drug concentration required to produce 50% esterase inhibition) and these were determined with 95% confidence limits by probit analysis using the GLIM statistical programs of The Royal Statistical Society of London. The % activity remaining after exposure to a concentration of 10^{-3} M drug is reported in all cases.

Conventional spectrofluorimetry. Studies were carried out with EMT6 cells and their sonicates, together with purified porcine carboxylesterase. An MPF-4 spectrofluorimeter (Perkin–Elmer (Norwalk, U.S.A.)) was used with monochromators set at 490 nm for excitation and 520 nm for emission. The band width was 4 nm, the chart speed 20 mm/min, the range 5 mV, and the sensitivity setting 3–100. A more detailed account of the method used is given elsewhere [22, 26]. Briefly, cells (10^6/ml), sonicates (equivalent to 10^6/ml) or pure esterase (1 μg protein/ml) were suspended in PBS (200 μl). These preparations were held on ice and brought to room temperature (20–22°) 16 min prior to drug addition. Drug or appropriate vehicle solutions (200 μl) were added to the cells, sonicates or pure esterase samples for a 1 hr preincubation period. The enzyme reaction was then started by addition of 300 μl of the preincubation mixture to 300 μl FDA (2 μM) with rapid mixing. Thus as for flow cytometry, the final FDA substrate concentration was 1 μM. The reaction was continuously monitored for 8 min at room temperature. Stability of the enzyme and substrate was confirmed by the inclusion of control samples interspaced at regular intervals between test samples. The controls contained the appropriate concentration of drug solvent, which in all cases had no significant effect on enzyme activity. Initial reaction velocities were obtained from the linear gradient of the progress curves in the first min of the reaction, and results processed as described for flow cytometry.

RESULTS

Flow cytometry

Figure 1 shows typical esterase reaction progress curves obtained for control EMT6 mouse tumour cells and those treated with BCNU, *cis*-2-OH CCNU and chlorozotocin. As previously described [22, 26] an initial lag phase is followed by a linear increase in reaction rate up to at least 4 min. Per cent inhibition was determined from the linear phase, and Fig. 2 illustrates the dose–response curves obtained

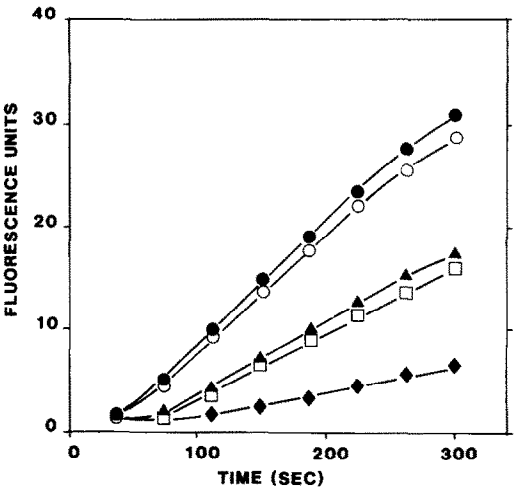

Fig. 1. Typical enzyme reaction progress curves for the hydrolysis of FDA (1 μM) by EMT6 mouse mammary tumour cells in the presence and absence of inhibitors as measured by flow cytometry: ●, control, 0.5% ethanol; ○, control, 2.5% DMSO; ▲, BCNU 10^{-4} M; □, chlorozotocin 10^{-2} M; ◆, *cis*-2-OH CCNU 10^{-2} M.

for a range of Cnus and isocyanates, with BCNU included as a reference standard throughout. Panel A compares CCNU, BCNU and their respective isocyanate metabonates, the latter being the more potent inhibitors. Panel B demonstrates the differences in inhibitory potency between various hydroxy metabolites of CCNU. The *cis*- and *trans*-4-hydroxy metabolites exhibited inhibitory potency similar to BCNU (and CCNU see Panel A), whereas the corresponding 2-hydroxy metabolites showed only modest activity. Panel C shows the dose–response data for the more recently developed Cnus, ACNU and TCNU, both of which are seen to be less inhibitory than BCNU. The glucose analogues chlorozotocin and GANU exhibited minimal effect on cellular esterases relative to BCNU (Panel D).

Table 1 summarises the inhibitory potency of Cnus and related compounds for inhibition of 1 μM FDA hydrolysis within intact EMT6 mouse mammary tumour cells measured by flow cytometry. The results may be summarized as follows. Greatest inhibitory potency was seen with the Cnus, BCNU, CCNU, their respective isocyanate metabonates CEI and CHI, together with the 4-hydroxy metabolites of CCNU (I_{50} values from 5.5×10^{-5} to 7.3×10^{-4} M). MeCCNU exhibited similar activity to BCNU and CCNU up to the highest concentration which could be examined for solubility reasons, 5×10^{-4} M, at which the activity remaining was 77% (mean value of three repeat experiments). The glucose Cnus chlorozotocin and GANU were only weakly active, with I_{50} values $>10^{-2}$, at which concentration they exhibited little or no inhibitory activity. This was also true for the pure alkylating agents MMS, nitrogen mustard, melphalan, clomesome and cyclodisone, and to a lesser extent for KOCN and CNU. Intermediate between these two groups were TCNU, ACNU and the 2-hydroxy metabolites of CCNU (I_{50} values from 1.1×10^{-3} to 2.3×10^{-2} M).

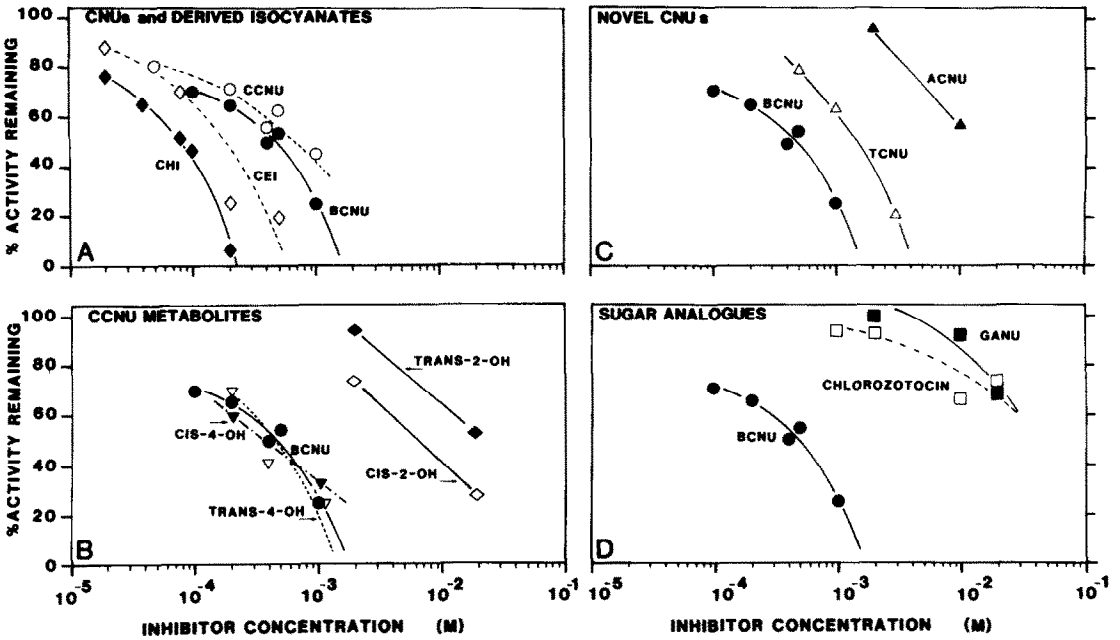

Fig. 2. Dose–response curves for the inhibitory effect of Cnus, isocyanate metabonates, and CCNU metabolites on the hydrolysis of FDA (1 μM) by EMT6 mouse mammary cells as measured by flow cytometry. Panel A: ●, BCNU; ○, CCNU; ◇, CEI; ◆, CHI. Panel B: ●, BCNU; ▼, *cis*-4-OH CCNU; ▽, *trans*-4-OH CCNU; ◇, *cis*-2-OH CCNU; ◆, *trans*-2-OH CCNU. Panel C: ●, BCNU; △, TCNU; ▲, ACNU. Panel D: ●, BCNU; □, chlorozotocin; ■, GANU. Each point is an average value of 2–8 independent experiments, and of 14 experiments for BCNU.

Conventional spectrofluorimetry

Values of I_{50} for inhibition of FDA hydrolysis by Cnus and isocyanates in intact mouse tumour cells and cell sonicates are given in Table 2. Generally speaking, inhibitory potency was little affected by the absence of the cell membrane with two major exceptions, namely chlorozotocin and GANU. For these, I_{50} values were about tenfold higher in the

Table 1. Inhibitory potency of CNUs and related compounds for inhibition of FDA (1 μM) hydrolysis within intact EMT6 mouse mammary tumour cells measured by flow cytometry*

Drug	I_{50} (M)†	% Esterase activity remaining (10^{-3} M drug)
CHI	5.5×10^{-5} ($4.4–6.6 \times 10^{-5}$)	0
CEI	1.0×10^{-4} (7.8×10^{-5}–1.3×10^{-4})	0
Trans-4-OH CCNU	3.5×10^{-4} ($2.1–5.9 \times 10^{-4}$)	25
Cis-4-OH CCNU	3.7×10^{-4} ($2.1–6.3 \times 10^{-4}$)	33
BCNU	3.7×10^{-4} ($2.9–4.7 \times 10^{-4}$)	27
CCNU	7.3×10^{-4} (3.2×10^{-4}–1.7×10^{-3})	45
TCNU	1.1×10^{-3} (9.4×10^{-4}–1.3×10^{-3})	63
Cis-2-OH CCNU	6.6×10^{-3} (3.7×10^{-3}–1.2×10^{-2})	88‡
ACNU	1.2×10^{-2} (8.5×10^{-3}–1.7×10^{-2})	100‡
Trans-2-OH CCNU	2.3×10^{-2} (8.5×10^{-3}–6.1×10^{-2})	100‡
KOCN	$>10^{-3}$	73
CNU	$\sim 5 \times 10^{-3}$ M	78
Clomesome	$>10^{-3}$	86
MMS	$>10^{-3}$	90
Melphalan	$>10^{-3}$	93
Chlorozotocin	$>10^{-2}$	93
Cyclodisone	$>10^{-2}$	94
GANU	$>10^{-2}$	100
Nitrogen mustard	$>10^{-2}$	100

* Results shown are means of 2–14 repeat experiments with two replicate concentrations per experiment. Values for BCNU and CEI are calculated from data including those cited in Ref. 22; those for MMS and melphalan are reproduced directly.

† 95% confidence limits in parentheses.

‡ Interpolated values from dose–response data.

Table 2. I_{50} values for FDA hydrolysis (1 μM) by intact EMT6 mouse mammary tumour cells and cell sonicates measured by conventional spectrofluorimetry*

Drug	I_{50} (M)†	
	Intact cells	Sonicates
CEI	1.0×10^{-5} (5.8×10^{-6}–1.7×10^{-5})	1.6×10^{-5} (7.7×10^{-6}–3.3×10^{-5})
CHI	1.1×10^{-5} (7.4×10^{-6}–1.7×10^{-5})	2.6×10^{-5} (1.8–3.5×10^{-5})
CCNU	3.8×10^{-5} (2.1–6.8×10^{-5})	8.3×10^{-5} (6.5×10^{-5}–1.1×10^{-4})
BCNU	5.0×10^{-5} (3.1–8.2×10^{-5})	9.2×10^{-5} (8.1×10^{-5}–1.1×10^{-4})
TCNU	1.8×10^{-4} (1.1–2.8×10^{-4})	9.9×10^{-5} (6.7×10^{-5}–1.5×10^{-4})
ACNU	7.3×10^{-3} (4.0×10^{-3}–1.3×10^{-2})	3.2×10^{-3} (1.0–9.9×10^{-3})
Chlorozotozin	~1.5×10^{-2}	2.4×10^{-3} (1.4–4.3×10^{-3})
GANU	~1.5×10^{-2}	2.4×10^{-3} (1.4–4.3×10^{-3})

* Results shown are means of 3–8 repeat experiments with two replicate samples per experiment. Values for BCNU and CEI are calculated from data including those cited in Ref. 22.

† 95% confidence limits in parentheses.

cells compared to the sonicates, suggesting relatively poor membrane permeability of these polar agents (or their metabonates) as compared to the more lipophilic derivatives.

The structure–activity relationships for esterase inhibition which are apparent from analysis by conventional spectrofluorimetry are very similar to those described previously for flow cytometric evaluation (compare Tables 1 and 2). Thus BCNU, CCNU and their derived isocyanates were highly active and chlorozotocin and GANU were weakly effective, with TCNU and ACNU intermediate.

Figure 3 shows the dose–response curves obtained with a small series of simple *n*-alkyl isocyanates for esterases in intact EMT6 mouse tumour cells (A) and purified porcine carboxylesterase (B). In both

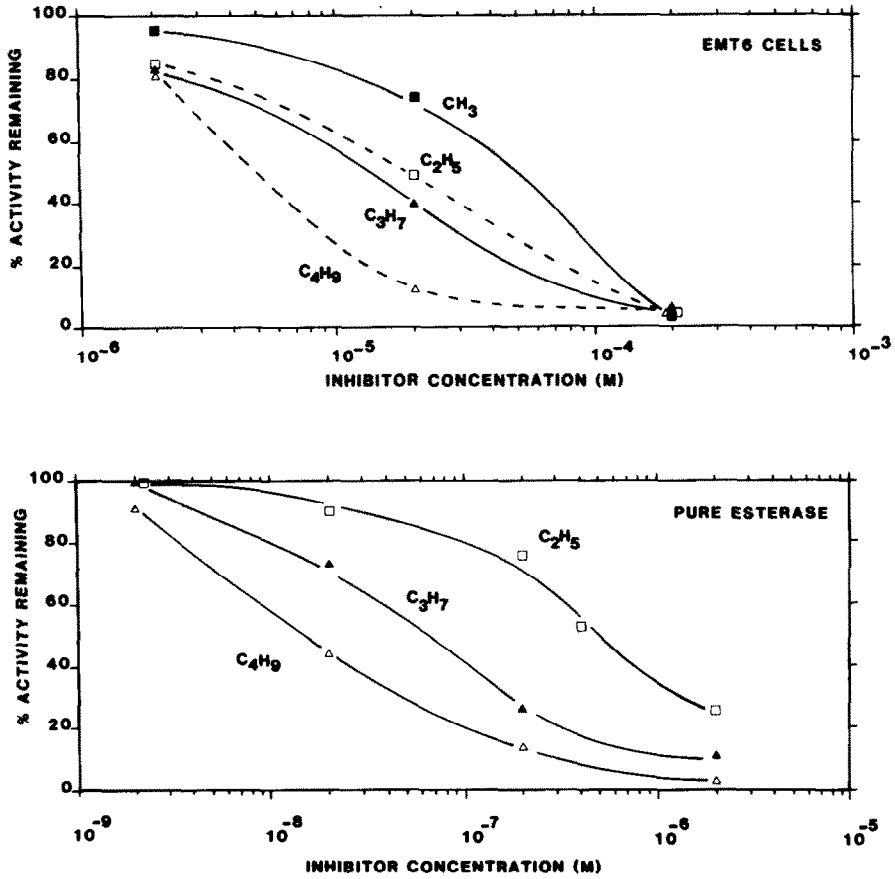

Fig. 3. Dose–response curves for the inhibitory effect of *n*-alkyl isocyanates on the hydrolysis of FDA (1 μM) by (A) EMT6 mouse mammary tumour cells, measured by flow cytometry and (B) purified porcine liver carboxylesterase, measured by conventional spectrofluorimetry: ■, MI; □, EI; ▲, *n*-PI; △, *n*-BI. Each point is the average value of 2–8 independent experiments.

systems a structure–activity relationship exists, where increasing the carbon chain length of the isocyanate results in enhanced inhibitory potency. For *n*-BI the I_{50} was 5×10^{-6} M in intact cells, somewhat lower than for CEI and CHI (Table 2).

DISCUSSION

We have previously reported inhibition by BCNU, its derived isocyanate CEI and the related isocyanate *n*-BI on esterases of intact and sonicated EMT6 mouse mammary tumour cells, intact H69 human small cell lung cancer cells, and purified porcine liver carboxylesterase [EC 3.1.1.1] [22]. These findings, together with the lack of activity of pure alkylating agents unable to form isocyanate metabonates (including the novel developmental agents clomesome and cyclodisone [31, 32]), and the known sensitivity of serine hydrolases to inactivation by carbamoylation [16, 20], suggested that the inhibitory effect of BCNU is due to the carbamoylation of cellular esterase molecules by the derived isocyanate CEI. We described a novel dynamic flow cytoenzymological technique to determine the extent of esterase inhibition, and proposed that this may provide a method to determine the intracellular carbamoylation potential of Cnus. The advantage of using intact, viable cells over cell-free systems lies in the fact that access to intracellular enzyme is included as a contributory factor. In addition, the use of flow cytometry allows multiple heterogeneous populations to be identified, a potentially important factor for *in vivo* applications.

To examine further the potential of the flow cytoenzymological technique to measure intracellular protein carbamoylation we examined a selected range of Cnus, isocyanates and related compounds, together with some additional alkylating agents, for inhibitory activity in this assay. For comparative purposes, and in addition to establish the contribution of membrane permeability to the overall inhibitory activity, complementary studies were carried out with intact cells, cell sonicates and purified esterase.

The inhibitory potency of the Cnus studied varied considerably. Potency appeared to reflect both intrinsic inhibitory potential and the ease with which the inhibitor penetrates the cell membrane. The Cnus divided into three main groups according to their inhibitory activity. BCNU, CCNU, their isocyanate metabonates CEI and CHI, and the 4-hydroxy metabolites of CCNU (the major metabolites in man [33]) are highly potent inhibitors with I_{50} values in the range 5.5×10^{-5}–7.3×10^{-4} M. As seen for BCNU previously, there was little difference in inhibitory activity for whole cells compared to sonicates, thus membrane permeability is not limited for these agents. Chlorozotocin and GANU were comparatively inactive with I_{50} values $>10^{-4}$ M. For chlorozotocin this can be attributed in part to its intrinsically weak ability to generate isocyanate species as a result of intramolecular carbamoylation [1, 34]. Intramolecular carbamoylation does not appear to occur with GANU, since it exhibits similar carbamoylating activity to BCNU [12]. However, both GANU and chlorozotocin were found to exhibit weak inhibition of purified glutathione reductase, indicative of poor ability to carbamoylate the active site of this enzyme [12]. In addition to reduced protein carbamoylating potential, the present results suggest that impaired membrane permeability of the polar Cnus is also important, as demonstrated by their markedly reduced inhibitory activity in intact cells compared to sonicates. The comparatively poor uptake of chlorozotocin and GANU is almost certainly a result of the polar character conferred by the glucose substituent. Propensity to undergo intramolecular carbamoylation may also account for the reduced inhibitory potency of the 2-hydroxy metabolites of CCNU, as compared with the 4-hydroxy derivatives which do not undergo this reaction to any appreciable extent [33].

CNU and KOCN were also found to exhibit weak inhibitory activity. Both generate cyanate and isocyanate in aqueous solution, and show similar chemical carbamoylation potential to CCNU [35, 36]. The absence of significant effects of CNU on RNA processing and DNA repair has been attributed to the possibility of poor cell uptake due to the charged nature of the carbamoylating species [36]. Our observation of weak inhibition of esterase activity by CNU in both homogenates and intact cells (I_{50} value $>10^{-3}$) suggests that the carbamoylating species derived from CNU and KOCN also have minimal potential for reaction with cellular esterases.

The results described above are clearly consistent with the hypothesis that inhibition of cellular esterases by Cnus ocurs through carbamoylation of the enzyme molecules. That alkylating species are not involved is illustrated by the weak activity of CNU, chlorozotocin, GANU and the 2-hydroxy CCNU metabolites, which all exhibit potent alkylating activity [9, 33, 35, 37]. This was also demonstrated by the lack of inhibition by the diverse range of pure alkylating agents chemically incapable of generating isocyanates, viz. the aromatic and aliphatic nitrogen mustards, melphalan and nitrogen mustard respectively; the methane sulphonate MMS (methylating agent); the chloroethylethanesulphonate clomesome (chloroethylating agent); and the novel cyclic compound cyclodisone.

Two Cnus showing inhibitory activity intermediate between those of the highly active Cnus and the weakly active chlorozotocin and GANU were the pyridine derivative ACNU and the taurine-based TCNU. We confirm the recent result (using a glutathione reductase inhibition assay) that TCNU exhibits carbamoylating activity between BCNU and chlorozotocin [38]. According to the scenario that the antitumour activity of the Cnus is mainly a function of their crosslinking of DNA through chloroethylation, while carbamoylation may lead to unwanted toxicity [1, 36], ACNU and TCNU may represent an improvement over conventional Cnus.

We extended our previous studies with *n*-BI [22] to show that in a small series of *n*-alkyl isocyanates, inhibitory potency increased with increasing chain length, greatest activity being seen with the butyl analogue. This was true for both EMT6 cell sonicates and purified porcine esterase.

It is appropriate to compare the results of the

intracellular esterase inhibition with previous assays for carbamoylation. This is not always straightforward, as previous studies have often reported effects at a given concentration, rather than the concentration required for a given effect [9, 35, 36, 39]. Overall there was a good correlation between inhibition of cellular esterase activity and chemical carbamoylation potential, as determined by reaction with amino groups of lysine or 5′-amino-5′-deoxythymidine. There were, however, two important discrepancies, in addition to that of CNU discussed above. GANU appeared to be strongly carbamoylating using the lysine method [9, 12, 39], whereas it exhibited weak esterase inhibition almost identical to that of its structural isomer chlorozotocin. In addition ACNU, was inactive on chemical analysis [39], whereas it exhibited intermediate activity by esterase inhibition. It is important to note, however, that our results for cellular esterase inhibition for these two compounds are much more closely similar to those for inhibition of purified glutathione reductase [12]. Thus our results confirm the view [40] that carbamoylation of critical protein targets is not always well predicted by chemical carbamoylation potential.

In summary, we feel that the evidence presented in this paper further supports the view that inhibition of esterases in intact cells by Cnus is a measure of their protein carbamoylating ability. The flow cytoenzymological assay may prove a useful aid to study of the molecular and cellular pharmacology of established and novel Cnus and related compounds under development. A particularly attractive feature of this technique for *in vivo* use is the potential for measurement of intracellular carbamoylation in multiple subpopulations of heterogeneous samples, and our recent unpublished results suggest that this can be carried out successfully with bone marrow specimens. In addition, the facility for multiparametric analysis will allow additional biochemical properties to be studied simultaneously with esterase inhibition on an individual cell basis.

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