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THE ROLE OF THE *N*-(HYDROXYMETHYL)MELAMINES AS ANTITUMOUR AGENTS: MECHANISM OF ACTION STUDIES

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Abstract—The hexamethylmelamine analogue trimelamol (*tris*-hydroxymethyl[trimethyl]melamine) and its equicytotoxic stable analogues CB 7547, CB 7639 and CB 7669 have been used to clarify the mechanism of action for the *N*-(hydroxymethyl)melamines as antitumour agents. Two main mechanisms have been proposed and explored: (i) formation of a reactive iminium species forming covalent adducts with DNA; and (ii) local formaldehyde release leading to cytotoxic damage. ^{32}P-postlabelling and thermal denaturation experiments showed these compounds to be interactive with cytosine and guanine. Trimelamol gave rise to DNA-interstrand crosslinks in naked plasmid DNA and in cultured cell lines, whereas the analogues failed to do so under a variety of experimental conditions. Along with our observations that cell lines with acquired resistance to the *N*-(hydroxymethyl)melamines showed no significant cross-resistance to classical bifunctional alkylating agents, DNA crosslinking may play only a minor role in their mechanism of action. In cultured cell lines treatment with formaldehyde, trimelamol and CB 7639 gave rise to high levels of DNA–protein crosslinks with a gradual disappearance over a 24 hr period. Along with our earlier observation that resistance to trimelamol coincides with cross-resistance to formaldehyde, we conclude that formaldehyde-release may be an important factor in their cytotoxicity. Further, the cytotoxicity of trimelamol or formaldehyde towards human ovarian cancer cells was not influenced by glutathione depletion. As the precise mechanism of action for the *N*-(hydroxymethyl)melamines is apparently not shared by many commonly used anticancer agents, this may confer their broad-spectrum activity versus heavily pretreated tumours.

Key words: *N*-(hydroxymethyl)melamines; mechanism of action; DNA alkylation; formaldehyde release

The *s*-triazine HMM (Fig. 1) is used in the treatment of advanced ovarian, lung and breast cancer. However, after more than 25 years since its first use, it is still under evaluation as an antitumour agent. Whilst a number of clinical studies have provided insight into the pharmacokinetics of orally administered HMM and have indicated activity in patients with resistance to classical bifunctional alkylating agents (reviewed in Ref. 1), mechanistic studies have provided limited insight into its mode of action.

The mechanism by which HMM exerts its antitumour effect has been shown to involve microsomal activation yielding *N*-(hydroxymethyl), or carbinolamine groups [2], resulting in a possible monofunctional alkylating agent by formation of a reactive iminium species (Fig. 1). Certainly metabolic activation studies have suggested that the carbinolamine species formed during HMM demethylation leads to a covalently bound DNA–drug adduct [3]. With multiple *N*-demethylations it is possible that these oxidative processes make bifunctional alkylation possible. In addition, HMM closely resembles TEM and it has therefore been proposed to be a putative alkylating agent on this basis.

TM contains three carbinolamine moieties, thus representing a bioactivated form of HMM. It was developed as a water-soluble cytotoxic agent for i.v. administration and circumvents the need for bioactivation, which has been shown to be variable in HMM treated patients [4]. Clinical trials of TM showed promising activity versus advanced ovarian cancer, particularly platinum-refractory disease [5, 6], but formulation difficulties, due to its inherent instability, halted further clinical progress. A synthetic analogue programme was subsequently initiated which has resulted in stable analogue forms of TM containing methyl groups replaced by electron-withdrawing groups [7], structures of which are depicted in Fig. 1. All analogue forms have been shown to retain *in vitro* cytotoxicity as compared

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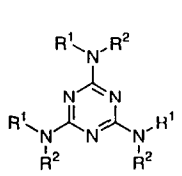
‖ Abbreviations: HMM, hexamethylmelamine; TEM, triethylenemelamine; TM, trimelamol; HCHO, formaldehyde; ISC, interstrand crosslinks; DPC, DNA protein cross-links; CT-DNA, calf thymus DNA; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethylsulphoxide; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; G, guanine; C, cytosine; A, adenine; T, thymine; TAE, Tris/acetic acid/EDTA; SDS, sodium dodecylsulphate; PI, propidium iodide; BSO, D,L-buthionine-*S*,*R*-sulphoxime; GSH, glutathione; 3′dGMP, 2′-deoxyguanosine-3′-monophosphate; TLC thin-layer chromatography; T_m, thermal denaturation temperatures; NBP, nitrobenzylpyridine.



	R^1	R^2
hexamethylmelamine	$-CH_3$	$-CH_3$
trimelamol	$-CH_3$	$-CH_2OH$
CB 7547	$-CH_2C{\equiv}CH$	$-CH_2OH$
CB 7639	$-CH_2CF_3$	$-CH_2OH$
CB 7669	$-CH_2CN$	$-CH_2OH$

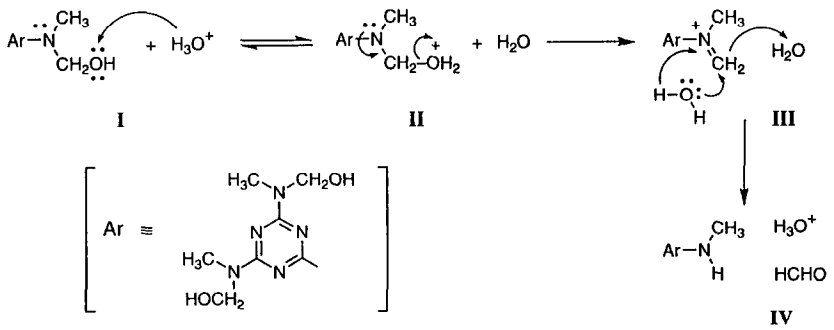


Fig. 1. Structures of melamine compounds with schematic diagram of their proposed mode of action.

with TM [8]. Our previous studies have indicated an almost complete lack of cross-resistance to TM and analogues in cell lines with acquired resistance to platinum [8], in line with clinical observations with TM and HMM. Despite the observation that TM possesses bifunctional alkylating activity [9], a broad-spectrum lack of cross-resistance to TM has been seen in a number of cell lines, including some with resistance to bifunctional alkylating agents [8].

In addition to alkylating activity, formaldehyde release may play a role in the mechanism of action of the *N*-(hydroxymethyl)melamines. As shown in Fig. 1, with the breakdown of each hydroxymethyl group a molecule of formaldehyde is released. Formaldehyde could represent a cytotoxic species in its own right as it is a highly reactive molecule. Indeed a cell line with acquired resistance to TM possesses cross-resistance with formaldehyde [8]. Hence, a dual mode of action for the *N*-hydroxymethylated melamines has been suggested. Using TM and the stable analogues we have carried out a number of mechanistic studies in an effort to clarify further the role of the *N*-(hydroxymethyl)-melamines as cytotoxic agents.

MATERIALS AND METHODS

All chemical reagents were supplied by BDH Chemicals (Poole, Dorset, U.K.) unless stated otherwise.

Cell lines and tissue culture

All tissue culture materials were obtained from Gibco Life Technologies (Paisley, U.K.) unless stated otherwise. The L1210 murine leukaemia line was grown as a suspension in RPMI 1640 medium supplemented with 10% donor horse serum (Flow Laboratories, Rickmansworth, U.K.), 2 mM glutamine, 50 μg/mL gentamycin and 2.5 μg/mL amphotericin.

Experiments also used two human ovarian cancer cell lines, the CH1 [9] and SKOV-3 line (obtained from the American Tissue Type Culture Collection). Both cell lines grow as monolayer cultures in Dulbecco's modified Eagle's medium plus 10% heat-inactivated foetal calf serum, glutamine and antibiotics as before with the addition of 10 μg/mL insulin and 0.5 μg/mL hydrocortisone (both from Sigma Chemicals, Poole, Dorset, U.K.). Cells were reduced to a single cell suspension following treatment of confluent layers with trypsin in EDTA followed by passing through a 19 gauge needle. All cultures were maintained at 37° in an atmosphere of 5% CO_2, 95% air in a humidifying incubator.

Drug preparation

The preparation of TM used in the present study was as used for the clinical trial, being synthesized by Warner Lambert (Ann Arbor, MI, U.S.A.) [5]. The analogues CB 7669, CB 7547 and CB 7639 were synthesized as previously described [7, 8]. All melamine compounds were dissolved in DMSO and stored as 50 mM stock solutions at −20°. Melphalan (Sigma Chemicals, Poole, Dorset, U.K.) was freshly prepared in 0.1 M HCl and further dissolved in sterile distilled water (with addition of 0.1 N NaOH to obtain neutral pH) immediately prior to use. CCNU (Sigma Chemicals, Poole, Dorset, U.K.) was dissolved in sterile distilled water immediately prior to use.

Cytotoxicity testing using the MTT assay

Cell suspensions were dispensed in 200 μL aliquots into 96-well tissue culture plates to 4–5 × 10^3 cells/

mL for the ovarian lines and 1×10^3 cells/mL for the L1210 line. Plates were then incubated for 24 hr in a humidifying gassing incubator in an atmosphere of 5% CO_2, 95% air. Cytotoxic agents were diluted in tissue culture medium and added in the appropriate volume to give the desired final concentration. The total incubation period selected for the cell lines used was chosen as the time required for control (i.e. non-drug-treated) cells to undergo 10–20 doublings, a period corresponding to 72 hr for the L1210 line and 96 hr for the ovarian cell lines. After this time period MTT solution (Sigma Chemicals, Poole, Dorset, U.K.; 5 mg/mL in PBS) was added to each well in a volume of 20 μL and the plates then incubated for 4–5 hr at 37°. The medium was then aspirated from the wells and 200 μL of DMSO added to dissolve the crystalline MTT-formazan reaction product. The plates were agitated for 10 min and the absorbances read on a Titertek Multiskan MCC ELISA plate reader (Flow Laboratories, Rickmansworth, U.K.) at a wavelength of 540 nm and a reference wavelength of 690 nm. Absorbance values obtained were expressed as a fraction of those obtained for control wells. In all experiments three to six replicate wells were used for each drug concentration, experiments being carried out on at least three separate occasions.

^{32}P-postlabelling

3′dGMP (Sigma Chemicals, Poole, Dorset, U.K.) in aqueous solution (1 mg/mL) was incubated at 37° overnight with 2 μmol of each melamine compound (initially dissolved in DMSO as a stock solution of 50 mM). Four micrograms of each sample was labelled with carrier-free [^{32}P]ATP (50 μCi; ICN Radiochemicals, Thame, Oxon, U.K.) and six units of T4 polynucleotide kinase (Life Technologies, Paisley, U.K.) at 37° for 30 min. The reaction was terminated by the addition of apyrase [10]. Resolution of ^{32}P-labelled adducts was performed on PEI-cellulose TLC sheets (10 × 10 cm) using the following solvent systems. (i) for CB 7639 and control (DMSO solvent only): D1 1.7 M sodium phosphate, pH 6.0; D2 3.5 M lithium formate, 8.5 M urea, pH 3.5; D3 0.8 M lithium chloride, 0.5 M Tris–HCl, 8.5 M urea, pH 8.0; D1 1.7 M sodium phosphate, pH 6.0. (ii) For TM, CB 7547, and CB 7669: D1 and D4, as above; D2 30% of above, D3 75% of above. The relative extents of reaction of the compounds with dGMP was determined by quantitation of the radioactivity in the adduct spots located by autoradiography [10].

Thermal denaturation studies

TM and analogues were subjected to DNA thermal denaturation (melting) experiments using calf thymus DNA (CT-DNA; type 1, highly polymerized; 42% G + C. CT-DNA was used as a fixed concentration of 100 μM (according to phosphate content). Aqueous solutions of DNA were prepared in Millipore-purified water buffered at pH 7.00 (± 0.02) containing 10 mM sodium phosphate and 1 mM EDTA. Working solutions containing DNA and varying concentrations of melamine compounds (50 μM or 0–200 μM for TM) were monitored at 260 nm using a Shimadzu UV-2101 PC spectrophotometer fitted with a Shimadzu SPR-8 programmed temperature controller. Heating was applied at 1°/min over the range 45–95°, with temperature values sampled at 10 sec intervals. Denaturation profiles were analysed as described previously [11, 12]. T_m values were determined as the mean ± SEM from three determinations for each sample, and were corrected for the effects of co-solvent DMSO upon the DNA. This solvent correction was not applied in the earlier study with TM [9]. Alterations in the T_m are given by the following formula:

$$\Delta T_m = T_m \text{ (drug–DNA)} - T_m \text{ DNA}$$

and the results are shown in Table 1.

Crosslink assay

This assay was carried out according to the method of Hartley *et al.* [13]. Using *Eco*R1 (Life Technologies, Paisley, U.K.) cut pBR322 plasmid DNA (Boehringer, Lewes, Sussex, U.K.) was dephosphorylated (calf intestinal alkaline phosphatase, Life Technologies) and 5′ end labelled as in the post labelling experiments. Each data point corresponded to approximately 10,000–30,000 cpm, which approximates to 10 ng of labelled material. Reactions with drug were performed in 25 mM triethanolamine, 1 mM EDTA, pH 7.2, at 37°. After the appropriate time interval incubations were terminated by addition of an equal volume of 'stop' solution (0.6 M sodium acetate, 20 mM EDTA, 100 μg/mL tRNA) and the DNA was precipitated, washed and dried by lyophilization. For the agarose gel electrophoresis (using a TAE system) samples were dissolved in strand separation buffer (30% DMSO, 1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cyanol), heat denatured at 90° for 2 min, and chilled immediately in an ice-bath prior to loading. Control undenatured samples were dissolved in 6% sucrose, 0.04% bromophenol blue and loaded directly. Autoradiography of dried gels was performed with Kodak XOMAT film for 4 hr at −70° using Cronex Lightening-Plus intensifying screens. Quantitation of crosslinked DNA was performed on the Bio-Rad 620 microdensitometer using the 1D analyst software package (Biorad Laboratories Ltd, Hemel Hempstead, Herts, U.K.). The percentage of crosslinked DNA (double-stranded) was calculated with respect to the total DNA loaded for each lane.

DNA filter elution to measure ISC

This procedure was first described by Kohn *et al.* [14]. Cells were seeded overnight in 25 cm^2 tissue culture flasks at a density of 2×10^5/mL. Cells in the logarithmic phase were divided into two groups and labelled either with 0.03 μCi/mL ^{14}C-(methyl)-thymidine (sp. activity 1.85–2.2 Bq/mmol, 50–60 mCi/mmol; Amersham, U.K.), or with 0.5 μCi/mL of ^{3}H-(methyl)-thymidine (sp. activity 37–370 Bq/mmol, 1–10 mCi/mmol; Amersham, U.K.) both for 24 hr at 37°. Radioactive medium was replaced with fresh complete medium and the cells were incubated for 1 hr at 37° with subsequent drug treatments. The treatment medium was then removed and replaced with drug-free medium and

Table 1. Effect upon thermal denaturation of calf thymus DNA

Compound	ΔT_M (°)						
	10 μM	20 μM	50 μM	100 μM	200 μM	400 μM	500 μM
Trimelamol	0.71 (0.16)	0.90 (0.18)	1.79 (0.17)	1.57 (0.19)	1.98 (0.21)	2.14 (0.26)	2.01 (0.31)
CB 7639	—	—	1.24 (0.18)				
CB 7547	0.49 (0.15)	0.73 (0.18)	1.42 (0.14)	1.25 (0.22)	1.63 (0.18)	1.65 (0.25)	1.60 (0.28)
CB 7669	—	—	1.07 (0.19)				

incubated for the appropriate time period (1–24 hr). Treated cells in suspension were then irradiated on ice with 5 Gy and 1 Gy, respectively, from a ^{60}Co source. Cells were loaded onto 0.2 μm polycarbonate filters (Costar, High Wycombe, Herts, U.K.) and lysed with a glycine/EDTA buffer (pH 10) and proteinase K. DNA was eluted from the filters with 0.1 M tetrapropylammonium hydroxide at pH 12.2. The fractions were collected overnight and subjected to liquid scintillation counting (Hionic Fluor; Canberra Packard, Pangbourne, U.K.). DNA-ISC were calculated using the following formula:

crosslink frequency expressed as Rad equivalents
$$[(1 - R_o/1 - R)^{1/2} - 1] \times P_b$$

where R and R_o are the fractions of ^{14}C-labelled DNA for treated versus control cells remaining on the filter when 50% of ^{3}H-labelled DNA is retained on the filter, and P_b is the radiation frequency in Rads. In the experiments unirradiated control cells were included to control for sufficient irradiation.

DPC assay

The method used was as previously described [15]. Cells were seeded overnight in 25 cm^2 tissue culture flasks at a density of 2×10^5 cells/mL and then labelled with 0.5 μCi/mL ^{3}H-thymidine for 24 hr. The medium was replaced and 2 hr later either (a) 250 μM of the appropriate melamine compound or melphalan, or (b) 250 μM formaldehyde for 1.5 hr was added in serum-free medium. Following drug treatment the cells were then subjected to a process of lysis and final resuspension of the resulting SDS-K^+ precipitate. The amount of radiolabelled DNA present in the pellet was determined by liquid scintillation counting as for DNA-ISC measurements. The DNA–protein coefficient was determined as ratio of the DPM on the SDS-precipitable DNA in the treated cells to that of the DPM of the SDS-precipitable DNA in the control sample, i.e.

DNA–protein coefficient =
DPM for treated cells/DPM for control cells

Flow cytometric analyses

All measurements were made on Ortho Cytofluorgraf 50H flow cytometers linked to an Ortho 2150 computer system. The system was equipped with an argon-ion laser tuned to give 488 nm light and either a second argon-ion laser tuned to output at 360 nm or a krypton laser tuned to 352 nm. To measure a DNA histogram, ethanol-fixed cells were incubated with PI (10 μg/mL) and ribonuclease (100 μg/mL). In the cytometer, cells were excited by laser light at 488 nm and the red fluorescence of the DNA–PI complexes recorded following the methods previously described [16].

In vitro *modulation of trimelamol cytotoxicity*

The effect of inactivation of alkyltransferase activity by O^6-benzylguanine. The procedure was based on that described by Dolan *et al.* [17]. These experiments used the human ovarian cancer cell line CH1 which was seeded in single cell suspension into 96-well plates, as described above. Following 24 hr incubation cells were exposed to 10 μM O^6-benzylguanine (kindly provided by Dr Robert Moschel, Frederick Cancer Research and Development Center, Frederick, U.S.A.) for 1 hr at 37°. This was followed by a 2 hr incubation with increasing concentrations of TM. The medium was then removed and replaced with drug-free medium and then incubated for a further 96 hr. Assessment of cell viability at the end of the incubation period was carried out using the MTT assay. The nitrosourea CCNU (Sigma Laboratories, Poole, Dorset, U.K.) was used in a similar fashion to control for alkyltransferase inactivation, as previously described [17].

Modulation of cytotoxicity by co-incubation with novobiocin. L1210 cells were seeded in 96-well plates to give a cell density of 1×10^3 cells/well. Following 24 hr incubation at 37° in a gassing humidifying incubator, novobiocin (Sigma Chemicals, Poole, Dorset, U.K.) made up in aqueous solution and filter sterilized was added to give a final concentration of 500 μg/mL. After 1 hr either TM or CCNU was added in a similar manner as before for a 2 hr incubation. Plates were then spun at 2000 rpm for 5 min to pellet the cells and the drug containing medium then removed and replaced with fresh medium. The plates were incubated for 72 hr and then the MTT assay carried out as above.

Effect of cellular glutathione depletion. The effect of glutathione depletion on cellular response to TM, formaldehyde and melphalan was assessed in two human ovarian cancer cell lines, the CH1 and SKOV-3. The method used was according to that described by Mistry *et al.* [18]. Cells were seeded in 96-well plates and left for 24 hr as before. Cells were then incubated in fresh medium containing 12.5 μM or 50 μM BSO for CH1 and SKOV-3, respectively, or controls with vehicle alone for a further 24 hr in order to deplete cellular glutathione levels. This was followed by a 2 hr incubation in medium containing

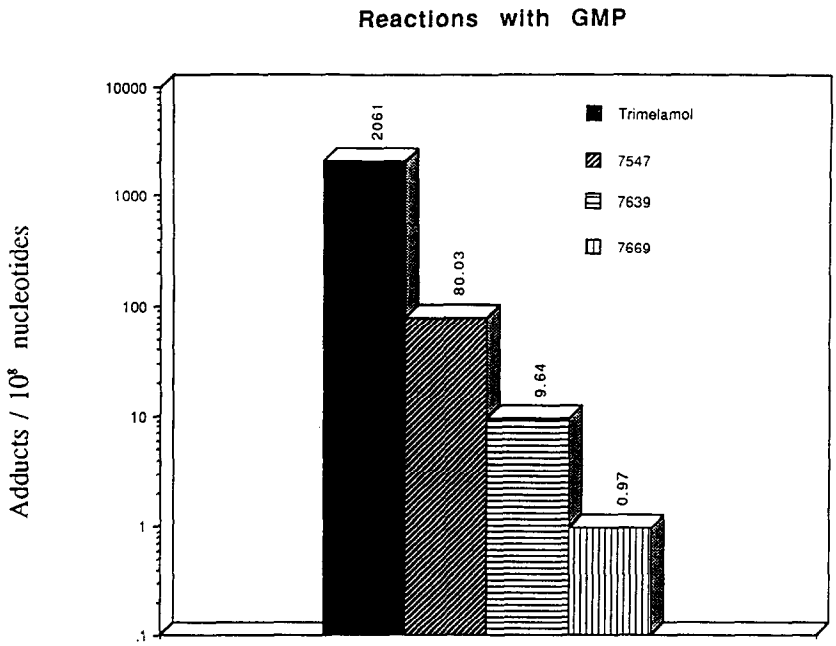


Fig. 2. Relative levels of reactivity of *N*-(hydroxymethyl)melamine compounds with 3'GMP, determined by ^{32}P-postlabelling analysis. Results were reproducible in two to five experiments with each compound.

appropriate concentrations of TM, formaldehyde or melphalan. At the end of this period fresh medium was applied and cell survival assessed 96 hr later, as described above using the MTT assay.

RESULTS

^{32}P-postlabelling

Figure 2 shows the levels of reactivity of the various melamine compounds with 3'-GMP. It is clear that TM was the most reactive compound tested. The levels of reaction shown in Fig. 2 may be underestimated because the efficiency of ^{32}P-labelling cannot be determined without synthetic standards. Nevertheless, it is reasonable to assume similar labelling efficiencies with all four compounds because of their closely similar structures and the results provide valid information on the relative reactivities of the compounds. The ranking order of reactivity for these compounds, i.e. TM > CB 7547 > CB 7639 > CB 7669, has no bearing on their relative cytotoxicity, e.g. in the Walker WS cell line IC_{50} values were as follows: TM 9.4 μM, CB 7639 12.2 μM, CB 7547 14.1 μM, CB 7669 7.5 μM [8].

Thermal denaturation studies with calf thymus DNA

All compounds effected stabilization of the thermal helix–coil transition for double-stranded CT-DNA, as previously reported for TM [9], and as indicated in Table 1. Further, the *N*-hydroxymethyl derivatives showed a differential effect upon the high-temperature portion of the melting curve, suggesting that the focus for interaction involves G/C sites rather than A/T-rich stretches of the DNA duplex. In the case of TM, progressive increase in T_m is observed in the 0–50 μM concentration range, giving maximal effect at 50 μM and a plateau effect at higher drug concentrations. This observation contrasts with an earlier report [9] and reflects our use of an appropriate T_m correction for the presence of the DNA-destabilizing DMSO co-solvent. Analogous concentration-dependent behaviour was determined for CB 7547. The ranking order obtained for the drug-induced T_m values using CT-DNA (100 μM) and each compound (50 μM) is TM > CB 7547 > CB 7639 > CB 7669.

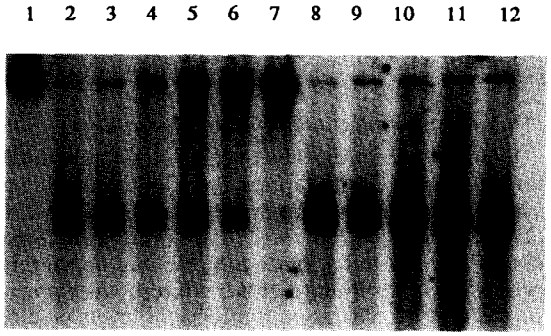


Fig. 3. Autoradiograph of cross-linking gel. Lane 1: undenatured control; lane 2: denatured double stranded DNA control; lanes 3–7: 10, 20, 50, 100 and 200 μM trimelamol, respectively; lanes 8–12: 10, 20, 50, 100 and 200 μM CB 7639, respectively.

DNA crosslinking using isolated plasmid DNA

Figure 3 shows an autoradiograph obtained from

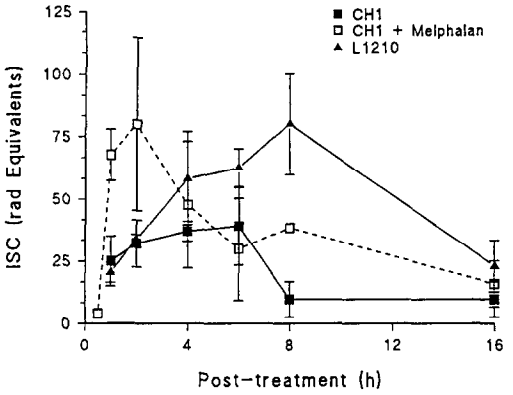


Fig. 4. Graph showing levels of DNA-interstrand crosslink (ISC) formation in CH1 and L-1210 cells following treatment with either melphalan (50 μM; CH1 only) or trimelamol (250 μM), over a time course of 16 hr post-treatment. Data are shown with error bars (SD) for three or more replicate assays.

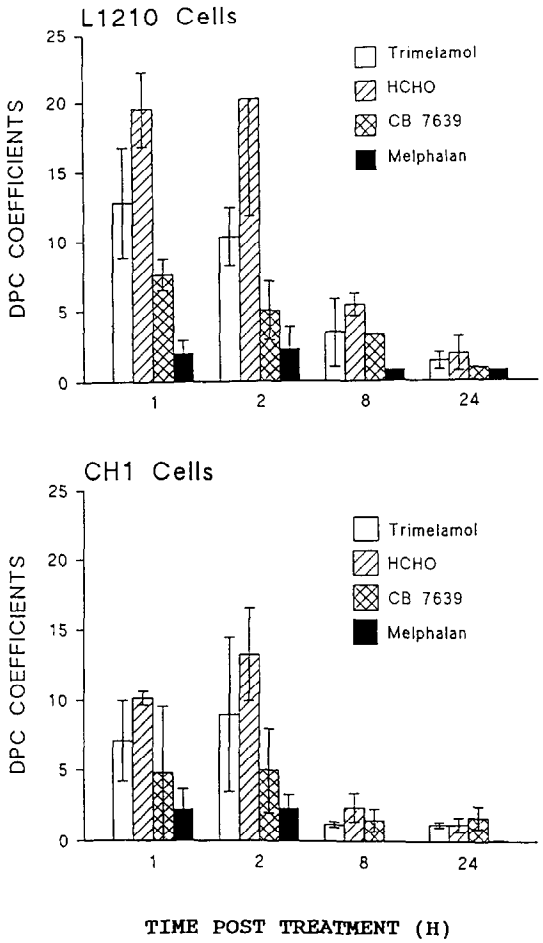


Fig. 5. Graph showing levels of DNA–protein crosslink (DPC) formation in the murine L1210 cell and CH1 cell lines following cytotoxic treatment (250 μM) over a time course of 24 hr post-treatment.

the crosslink gel based assay method. Crosslinking of double-stranded DNA was complete at 100 and 200 μM TM, whereas there was no crosslinking demonstrated for CB 7669 at any concentration. Subsequent experiments using CB 7547 and CB 7639 have failed to demonstrate any crosslinking using prolonged incubation times (up to 24 hr) or altering the pH of the incubation buffer to pH 4.0 (data not shown).

DNA crosslinking using alkaline filter elution

Figure 4 shows the level of DNA ISC obtained for the human CH1 ovarian cancer line, following a 1 hr treatment with either melphalan (50 μM) or TM (250 μM). Below 250 μM TM we were unable to obtain ISC above background levels and hence this dose was used as it represents the threshold at which we were able to measure appreciable levels of ISC formation. From Fig. 4 we can see that the peak of ISC formation in CH1 treated with TM approximates to 40 ISC Rad equivalents at 6 hr, whilst the treatment with melphalan led to a peak at 2 hr of approximately 78 ISC Rad equivalents. The pattern of ISC formation in L1210 cells treated with TM gave rise to a gradual rise up to 8 hr. Between 8–16 hr ISC levels declined to background levels in all experiments. Thus, TM appears to be an efficient cross-linking agent. Experiments using the stabilized analogues CB 7547 or CB 7639 were unable to demonstrate DNA cross linking in treated cells, even under conditions employing prolonged incubation times (24 hr) and elevated drug concentrations (500 μM; data not shown).

DNA–protein crosslinking

Figure 5 shows levels of DPC formation in the L1210 and CH1 cell lines, respectively, following acute treatments with cytotoxic agents TM, formaldehyde, CB 7639, CB 7669 and melphalan. In L1210 cells up to 2 hr post-treatment a 250 μM dose of formaldehyde gave rise to a DPC coefficient of approximately 20; similar levels of this lesion were apparent for a 250 μM dose of either TM, CB 7639 or CB 7669. At 8 hr all drug-treated cells gave DPC coefficients of < 3. Throughout all experiments melphalan treatments resulted in low levels of DPC coefficients (< 3). For CH1 cells a similar pattern was seen although levels of DPC measured were in general lower. In formaldehyde-treated cells DPC coefficients were closer to those seen for TM (approx. 7–12 coefficients). Levels of DPC measured following treatment of CH1 cells with melphalan were very similar to those seen for L1210 cells. The disappearance of the DPC formation followed a similar time course for both cell lines.

Flow cytometric analyses

Figure 6 shows DNA histograms obtained from cells which were continually exposed to TM and analogues in an overnight incubation. Whereas a G_2M block was seen for cells treated with 25 μM TM (G_1 11.7%; S 56.0%; G_2 32.4%; versus control G_1 30.0%; S 61%; G_2 9.0%), 50 μM continual

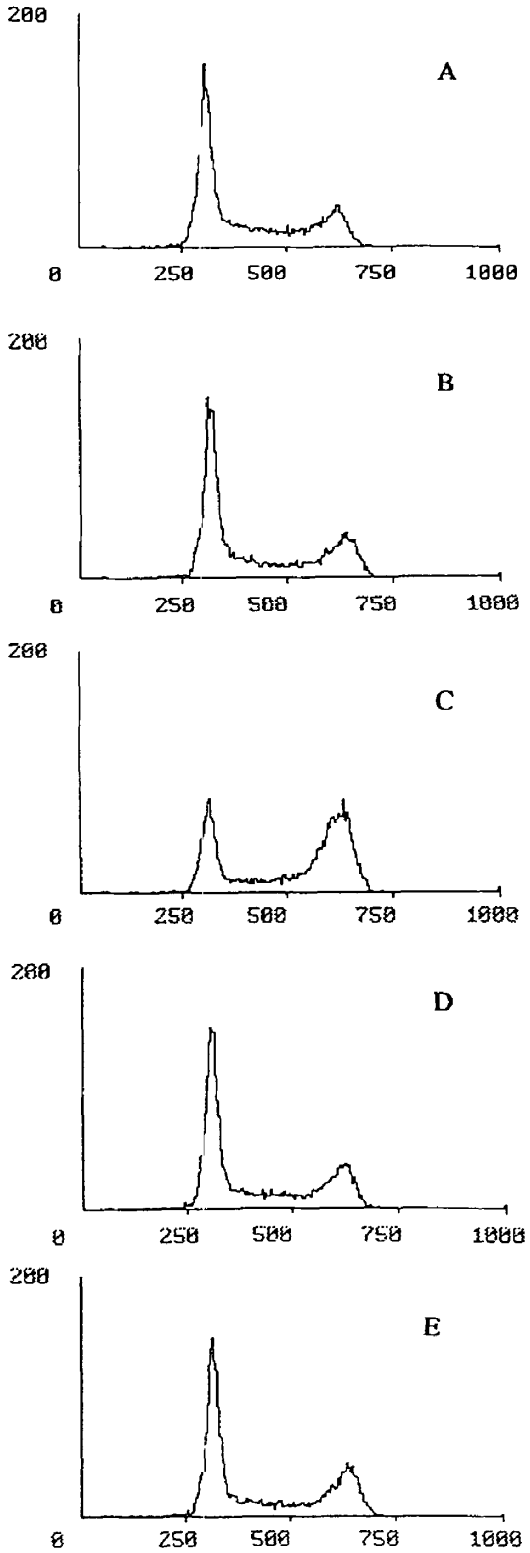


Fig. 6. Diagram of DNA histograms obtained from flow cytometric analysis of CH1 cells treated with TM and analogues in chronic exposure, with an overnight (> 14 hr exposure). A, control untreated cells; B, treatment with CB 7669 50 μM; C, treatment with TM 25 μM; D, treatment with CB 7639 50 μM; and E, treatment with CB 7547 50 μM. Histograms show cell number (y) versus DNA content (x).

exposure of the analogues in the same experiments did not bring about any cell cycle perturbations (CB 7547: G_1 26.2%; S 58.5%; G_2 15.3%; CB 7639: G_1 25.6%; S 60.3%; G_2 14.1%; CB 7669: G_1 29.3%; S 59.2%; G_2 11.5%). These concentrations were chosen as they brought about a similar level of cell killing in the duration of exposure used [8]. Initial experiments which employed acute exposures followed by examination of cell cycle perturbations showed that a G_2M blockade was achieved 10 hr following treatment of cells with TM whereas nothing was observed in those cells treated with the analogues. However, in order to compensate for the prolonged exposure necessary for the stabilized analogues to exert their cell killing effects we adopted the approach of incubating cells with the stable analogues and TM for 16 hr (overnight). In addition, subsequent experiments using 24–48 hr incubations gave essentially the same results.

In vitro *modulation of trimelamol cytotoxicity*

Effects of treatment with O^6*-benzylguanine.* Results are shown in Table 2. Treatment of CH1 cells with O^6-benzylguanine resulted in a 4–5-fold potentiation of CCNU cytotoxicity. No potentiation of TM cytotoxicity was seen following similar pretreatment of cells with O^6-benzylguanine. This provides evidence for a lack of adduct formation between TM and the O^6 position of guanine, an adduct associated with chloroethylnitrosourea cytotoxicity.

Effects of treatment with novobiocin. The cytotoxicity of CCNU was enhanced by cotreatment with novobiocin in the L1210 cell line, as shown in Table 2. There was an approximate 40% decrease in IC_{50}, consistent with an enhancement of cytotoxicity. The effect of co-incubation of TM with novobiocin was even more marked, showing a 54% decrease in IC_{50}. Hence, novobiocin is an effective modulator of CCNU and TM cytotoxicity.

Depletion of cellular GSH. The CH1 and the SKOV-3 cell lines have previously been used to assess the role of GSH in sensitivity to platinum-based anticancer agents [18]. The CH1 line represents a cell line with a relatively low level of cellular GSH, whereas SKOV-3 cells are rich in cellular GSH [18]. In both these cell lines depletion of cellular GSH had no effect on the cytotoxicity of TM or formaldehyde, as shown in Table 3. In contrast, BSO pretreatment resulted in a marked enhancement in the cytotoxicity of the alkylating agent melphalan. For the CH1 cell line a 2-fold enhancement in melphalan cytotoxicity was seen whereas in the SKOV-3 line with intrinsically high levels of GSH, a 5-fold enhancement in cytotoxicity was seen. In conclusion, modulation of cellular GSH metabolism had no effect on cellular sensitivity to TM or formaldehyde in the two human ovarian cancer cell lines studied.

DISCUSSION

The antitumour agent TM and the stabilized analogues appear to act as DNA interactive compounds but do not behave in the same way as other widely used chemotherapeutic agents (e.g. cisplatin and nitrogen mustards). We have shown

Table 2. Effect of modulation by either O^6-benzylguanine (BG) or novobiocin on the cytotoxicity of TM, compared with CCNU

	Drug treatment			Drug treatment		
Cell line	CCNU	+ O^6-BG	+ Novobiocin	TM	+ O^6-BG	+ Novobiocin
L-1210	6.9 (1.7)	—	4.1 (1.9)	79.1 (18.9)	—	36.5 (11.9)
CH1	21.3 (3.0)	4.7 (0.5)	—	75.8 (4.7)	73.5 (4.2)	—

Data shown are IC_{50} values (μM) obtained with the standard deviation for >3 replicate analyses given in parentheses.

Table 3. Effects of glutathione depletion (using BSO) on the cytotoxicity of trimelamol, formaldehyde and melphalan in the CH1 and SKOV-3 cell lines

	Trimelamol		Formaldehyde		Melphalan	
Cell line	Control	+ BSO	Control	+ BSO	Control	+ BSO
CH1	107 (35)	104 (35)	338 (79)	333 (82)	5.3 (2.5)	2.4 (1.2)
SKOV-3	176 (20.7)	177 (24.9)	683 (48.6)	638 (78.6)	68.8 (5.0)	13.9 (6.5)

Results shown are the mean of >3 replicate analyses; the standard deviation is indicated in parentheses.

that TM is capable of forming DNA-ISC in both naked plasmid DNA and whole cell DNA. Interestingly, analogues CB 7639 and CB 7669 with similar *in vitro* cytotoxicity to TM are incapable of forming DNA-ISC. Our data obtained for TM is in some agreement with earlier findings [9] describing the formation of DNA-ISC following treatment with TM. A subsequent report [19] emphasized the role of DNA-ISC in the mechanism of action of analogues of TM where one -$N(CH_3)CH_2OH$ function has been replaced with groups of varying structures (not disclosed). A comparison of *in vitro* cytotoxicity with ability to crosslink plasmid DNA showed a positive correlation. However, the authors pointed out that although one of the analogue series possessed similar crosslinking ability to melphalan on a molar basis, it was some 5-fold less cytotoxic *in vitro*. This observation, along with the findings of our present study tends to suggest that the role of crosslinking is unclear with regard to the mechanism of action of the *N*-(hydroxymethyl)melamines. Indeed, in an earlier study [20] HMM failed to crosslink DNA in L1210 cells in the presence of an activating system.

We have also obtained other lines of evidence which are not supportive of the role of DNA cross-linking as a major mechanism of action for the *N*-(hydroxymethyl)melamines. Like HMM, TM fails to give a positive result with the NBP test [21; data not shown] used to screen compounds for alkylating activity (both mono- and bi-functional). In addition, the drug-resistant variant cell lines WTM/R and W7669/R do not show cross-resistance with phosphoramide mustard, chlorambucil or cisplatin, showing only a small degree of cross-resistance to melphalan [8]. This is inconsistent with an alkylating agent-resistant phenotype. ^{32}P-Postlabelling studies and thermal stability studies have both indicated varying levels of reactivity with nucleotides and DNA for the melamine compounds used in the present study. Whilst none of these techniques are able to characterize the type of DNA adduct formed by the *N*-(hydroxymethyl)melamines, we can exclude the O^6-position of guanine as a possible reactive site. As pointed out previously [9], the N^7-position of guanine, commonly associated with nitrogen mustard–DNA interaction, cannot be ruled out as being the site of adduct formation for the *N*-(hydroxymethyl)melamines, despite the finding of a negative piperidine cleavage result. It may be that the conditions of alkaline pH and thermal denaturation give rise to unstable DNA adducts formed by this group of compounds.

Cell cycle perturbation at the G_2M boundary was a consistent finding for TM in the CH1 cell line and a number of other cell lines (data not shown), characteristically not emerging until 10–12 hr post-treatment. However, following a variety of treatment protocols we were unable to see any cell cycle perturbations following treatment of cells with either CB 7639 or CB 7669. These data would imply that the cell cycle block we see following the treatment of cells with TM reflects its ability to form DNA-ISC, as we failed to see any cell cycle blocks with the analogues with no apparent bifunctional alkylating ability. Other cytotoxic agents giving rise to cell cycle perturbations at the G_2M boundary include crosslinking agents such as nitrogen mustard and cisplatin [22]. However, our data show there to be no significant cross-resistance to either of these groups of agents in the melamine-resistant lines WTM/R or W776/R. Furthermore, it is important to note that even though the W7669/R line was

selected for resistance by treatment with CB 7669 (which is *not* a crosslinking agent) it possesses an almost identical resistance profile to that seen for the WTM/R line, which was generated by exposure to TM, which *is* a crosslinking agent [8].

We have shown that cells treated with formaldehyde, TM or CB 7639 form high levels of DPCs. It has previously been shown [23] that formaldehyde produced extensive DPC formation in L1210 cells, at similar concentrations to those used in the present study. In addition, the repair of these adducts was shown to occur over many hours, as we have also demonstrated (Fig. 5). The degradation of the stable analogue CB 7639 has been shown to be much slower than TM under the same conditions (e.g. half-life for TM 350 min, CB 7639 3000 min at room temperature in 5% dextrose solution) [7]. Consequently, CB 7639 is likely to release formaldehyde at a slower rate than the more unstable TM. This could explain the overall reduction in the peak of DPC formation seen for the CH1 and L1210 cells treated with CB 7639 as opposed to TM at an equivalent dose. The persistence of this lesion in treated cells could constitute an important component of the cytotoxicity of the *N*-(hydroxymethyl)melamines. Like TM, the topoisomerase II inhibitors give rise to high levels of DPC in treated cells [24].

Elevations in cellular GSH levels have been shown to be important in acquired and intrinsic resistance to alkylating agents and many other chemotherapeutic agents [18, 25, 26]. The role of GSH in the cytotoxicity of the *N*-(hydroxymethyl)melamines using the GSH-depleting agent BSO in human ovarian cancer cell lines appears negligible. Together with the observation that the levels of cellular GSH are similar in the WS parental and drug-resistant variant lines WTM/R and W7669/R, GSH cannot be considered an important factor in the modulation of sensitivity to the methylmelamines.

Previous *in vitro* [27, 28] and *in vivo* [29] studies have shown that novobiocin enhances the sensitivity of tumour cells to the effects of DNA alkylating agents. The mechanism by which this is brought about has been shown to be due to inhibition of DNA repair of alkylating agent adducts with agents such as BCNU and nitrogen mustard. We were able to see a marked enhancement in TM cytotoxicity in L1210 cells following pretreatment with novobiocin. The effect was of a similar magnitude to that seen for CCNU in the same experiments. These data provide evidence that DNA is an important locus of action for the *N*-(hydroxymethyl)melamines.

The methylmelamines are clinically active antitumour agents. Data obtained in the present study have implicated a novel mechanism of action for the *N*-(hydroxymethyl)melamines, which does not appear to overlap with those associated with commonly used antitumour agents. The nature of the mechanism of action of the *N*-(hydroxymethyl)melamines may therefore be pivotal in conferring its broad spectrum efficacy against numerous cell line models and, importantly, its activity against heavily pretreated tumours [5].

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