Biochemical Pharmacology, Vol. 42, No. 11, pp. 2091–2097, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

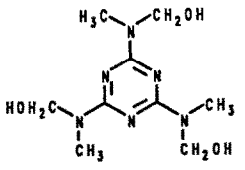
N^2,N^4,N^6-TRI(HYDROXYMETHYL)-N^2,N^4,N^6-TRIMETHYLMELAMINE (TRIMELAMOL) IS AN EFFICIENT DNA CROSS-LINKING AGENT *IN VITRO**

CLAIRE JACKSON,† JOHN A. HARTLEY,‡ TERENCE C. JENKINS,§ RUSSELL GODFREY,‖ RICHARD SAUNDERS‖ and DAVID E. THURSTON†¶

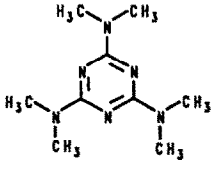
†School of Pharmacy and Biomedical Sciences, Portsmouth Polytechnic, King Henry Ist Street, Portsmouth, Hants. PO1 2DZ; ‡Department of Oncology, University College and Middlesex School of Medicine, 91 Riding House Street, London W1P 9BT; §CRC Biomolecular Structure Unit, Institute of Cancer Research, Sutton, Surrey SM2 5NG; ‖Cyanamid U.K. Ltd, 154 Fareham Road, Gosport, Hants. PO13 0AS, U.K.

(*Received* 24 *June* 1991; *accepted* 12 *August* 1991)

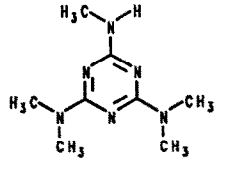
Abstract—An investigation of the mechanism of action of the antitumour agent trimelamol has established that it is an efficient interstrand DNA cross-linker *in vitro*, comparable to nitrogen mustards such as melphalan. Studies have shown that the cross-linking reaction is acid-catalysed but, unlike the nitrogen mustards, only partially reversible after treatment with piperidine. The bisalkylation (cross-linking) reaction appears to be concerted, and no "second arm" reaction has been detected. The results of thermal denaturation studies are consistent with general DNA binding, and suggest a preference for GC-rich sites. The acid-catalysed reaction of trimelamol with a model nucleophile (thiophenol) has also been investigated and an adduct resulting from displacement of the three carbinolamine functions has been isolated and characterized.



Trimelamol (1)



Hexamethylmelamine (2)



Pentamethylmelamine (3)

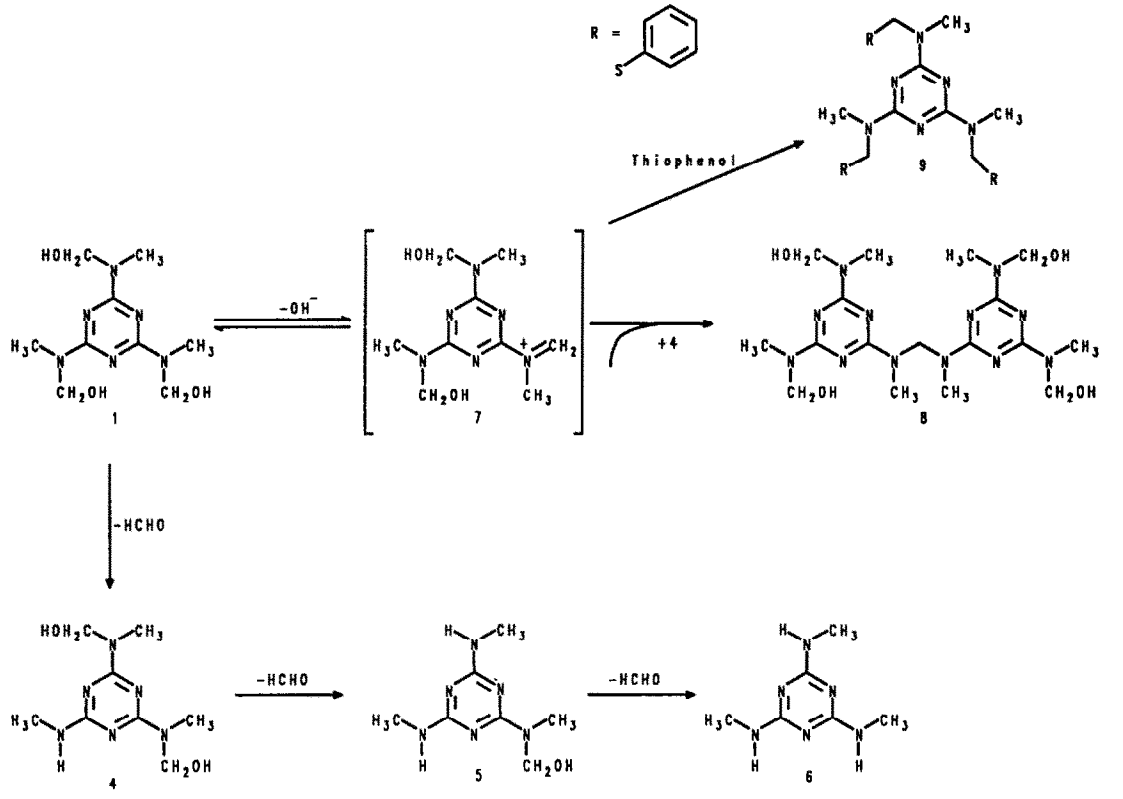
The antitumour agent N^2,N^4,N^6-tri(hydroxymethyl)-N^2,N^4,N^6-trimethylmelamine (trimelamol, 1**) was developed from hexamethylmelamine (**2**), a clinically useful antitumour agent that exhibits activity against a variety of human tumours [2]. Hexamethylmelamine is extensively metabolized via N-demethylation by hepatic microsomal preparations to hydroxymethyl derivatives [3]. Its efficacy is severely restricted by low aqueous solubility which precludes its administration as an injectable preparation. As a consequence, pentamethylmelamine (**3**), the more water-soluble monodemethylated analogue, was developed for formulations of this type. However, Phase-I clinical trials were disappointing, as pentamethylmelamine lacked significant antitumour activity [4] and was as emetic as hexamethylmelamine. Also, at higher doses pentamethylmelamine caused sedation and, in some cases, coma [5]. Pharmacokinetic studies in mice revealed that the drug was readily metabolized to *N*-hydroxymelamine metabolites and, in this species, antitumour activity was observed [6]. As the metabolic activation of pentamethylmelamine is known to be relatively inefficient in man, this may account for the poor clinical activity. Based on these results, a demethylated but fully activated analogue, trimelamol (**1**), was developed which in Phase-I clinical trials exhibited activity against refractory ovarian cancer with less emetic and neurotoxic effects than pentamethylmelamine [7]. A phase-II clinical trial of trimelamol has been reported recently [8].

The mode of action of trimelamol has not been established, although various mechanisms have been

* This work was presented in part at the 32nd Annual Meeting of the British Association for Cancer Research, Owen's Park, University of Manchester, U.K.; 15 April 1991 [1].

¶ Corresponding author: Dr David E. Thurston, Division of Medicinal Chemistry, School of Pharmacy and Biomedical Sciences, Portsmouth Polytechnic, Park Building, King Henry Ist Street, Portsmouth, Hampshire PO1 2DZ, U.K. Tel. 0705-842632/3/4 or 842628; FAX 0705-843335.

** Abbreviations: trimelamol, N^2,N^4,N^6-tri(hydroxymethyl)-N^2,N^4,N^6-trimethylmelamine; CT, calf thymus; ML, *Micrococcus lysodeikticus*; CP, *Clostridium perfringens*; T_m, thermal denaturation temperature.



Scheme 1. Chemical pathways of degradation of trimelamol in aqueous solution, and nucleophilic attack of thiophenol on the iminium species (7).

suggested for the parent compound and related analogues. These include function as either a pyrimidine antimetabolite [9] or as a source of cytotoxic formaldehyde [10]. Alkaline elution experiments [11] have also pointed towards a possible role in DNA interstrand cross-linking, although extensive DNA–protein cross-linking was also observed in the same experiments, consistent with the action of released formaldehyde. In addition, a ^{14}C-ring-labelled analogue, *N*-hydroxymethylpentamethylmelamine, has been shown to bind covalently to DNA [12].

While recently studying the degradation chemistry of trimelamol in aqueous solution, we observed two major degradation pathways [13]. The first involves the release of formaldehyde by the successive elimination of hydroxymethylene units (Scheme 1: **1**→**4**→**5**→**6**) [14], and the second involves the coupling of two parent molecules to form bis-(trimelamol) (**8**) via the highly reactive iminium ion intermediate (**7**) [13]. As these pathways are consistent with either the release of cytotoxic formaldehyde or alkylation of a nucleophilic species such as DNA, a study of the direct interaction of trimelamol with DNA was undertaken.

MATERIALS AND METHODS

Reagents

Trimelamol (Batch No. RM150) was supplied by Cyanamid U.K. pBR322 DNA and T4 polynucleotide kinase were purchased from Northumbria Biologicals Ltd; *Hind* III and bacterial alkaline phosphatase from BRL; acetonitrile and thiophenol from the Aldrich Chemical Co.; piperidine from Fisher; yeast transfer RNA from Boehringer-Mannheim; triethanolamine and hydrochloric acid from BDH; ultrapure urea, acrylamide, *N,N,N',N'*-tetramethylethylenediamine and ammonium persulphate from Bio-Rad; and [γ^{32}P]dATP (3200 Ci/mmol) from New England Nuclear. The three types of DNA used for thermal denaturation studies were purchased from the Sigma Chemical Co.

Cross-link assay

This assay was carried out according to the method of Hartley *et al.* [15]. Reaction of trimelamol with double-stranded, linearized, ^{32}P-end-labelled pBR322 DNA in a concentration range 0.5–500 μM was allowed to proceed in a triethanolamine/EDTA (25 mM:1 mM, pH 7.2) buffer at 37°. After 1 hr incubation, the reaction was stopped by the addition of an equal volume of sodium acetate/EDTA (0.6 M:20 mM) buffer and tRNA (100 μg/cm^3). The DNA was immediately precipitated by the addition of 95% ethanol (3 vol.), followed by freezing in a dry ice/ethanol bath. The resulting pellet, after centrifugation and removal of the supernatant, was dried by lyophilization. In a parallel experiment, end-labelled DNA from the same batch was exposed to formaldehyde (39% w/w aqueous HCHO, diluted to obtain a final concentration range of 1.5 to 1500 μM), and assayed in the same manner.

pH studies. Solutions containing the DNA

and increasing concentrations of trimelamol were incubated at 37° for 1 hr at pH 4.0 and 7.2, and then treated as in the previous experiment.

"Second-arm" reaction. In order to investigate whether the two alkylation events of the cross-linking process occur in a step-wise or concerted manner, an attempt was made to observe the second alkylation event (the "second-arm" reaction). Trimelamol (250 μM) was incubated for 15 min at 37° with end-labelled pBR322 DNA, and the reaction was stopped by the addition of sodium acetate to give a final concentration of 0.3 M. After precipitation of the DNA with 95% ethanol followed by centrifugation, the remaining unbound drug was removed in the supernatant and the pellet resuspended in triethanolamine buffer. Incubation of the DNA–trimelamol complex was continued at 37° and then assayed at various times as described above. The experiment was repeated at room temperature.

Cross-link reversibility. The labelled pBR322 DNA was treated with trimelamol (250 μM) and then incubated for 1 hr at 37°. After precipitation and centrifugation, the unbound drug was removed in the supernatant and the dried DNA pellets were resuspended in various buffers (pH 4.0, 7.2 and 10.0), incubated at 37° for 15 min and then assayed as above. One pellet was resuspended in 10% (w/w) aqueous piperidine, incubated at 90° for 15 min, and then frozen in a dry-ice/ethanol bath. The piperidine was removed by lyophilization.

Electrophoresis

Samples were redissolved in strand separation buffer (10 μL of 30% v/v dimethyl sulphoxide, 1 mM EDTA, 0.04% bromophenol blue, 0.04% xylene cylanol), heated to 90° for 2 min to ensure denaturation, and then chilled in an ice-water bath immediately prior to loading. Controls consisting of undenatured samples were dissolved directly in loading buffer (10 μL of 6% sucrose, 0.04% bromophenol blue) prior to loading. Electrophoresis was performed on 20 cm submerged agarose gels (0.8%) at 40 V for 16 hr. The gel and running buffer consisted of 40 mM Tris, 20 mM acetic acid and 2 mM EDTA (pH 8.1).

Autoradiography

Gels were dried onto Whatman 3 mm and DE81 filter papers on a Bio-Rad Model 583 gel drier at 80° *in vacuo*. Initial autoradiography was carried out with Hyperfilm MP (Amersham) for 4 hr at −70° using a Dupont-Cronex Lightening-Plus intensifying screen. Sharper images were obtained by overnight exposure without the intensifying screen.

Densitometry

Quantitation was achieved by microdensitometry of the autoradiograph using a LKB Ultrascan-XL laser densitometer. For each lane, the amount of single- and double-stranded DNA was determined and the percentage cross-linked (double-stranded) DNA calculated.

N7-Alkylguanine assay

The N7-assay was carried out as described by Mattes *et al.* [16].

Thermal denaturation studies

Trimelamol was subjected to DNA thermal denaturation (melting) experiments using calf thymus (CT) DNA [type-I, highly polymerized; 42% G + C], *Micrococcus lysodeikticus* (ML) DNA [GC-rich; 72% G + C] and *Clostridium perfringens* (CP) DNA [AT-rich; 30% G + C] which were used as supplied without further purification. CT DNA had $A_{260}/A_{280} = 1.9(3)$ and was satisfactorily free from protein. Molar extinction values at 260 nm, ε_{260} = 6600, 6000 and 6900 dm^3/mol/cm, respectively, were used for the DNA samples. Aqueous solutions of DNA were prepared in Millipore-purified water buffered at pH 7.00 ± 0.01, containing 10 mM sodium phosphate and 1 mM EDTA. No added salt or support electrolyte was used. Working solutions containing 100 μM DNA (as DNAp) alone and in the presence of varying concentrations of trimelamol (10–200 μM) were monitored at 260 nm using a Varian-Cary 219 spectrophotometer fitted with a Neslab ETP-3/RTE4 circulating water heating/cooling accessory. Heating was applied at 1°/min over the range 40–90° until thermal denaturation was complete, as judged from the increase in absorption. The absorbance versus temperature curves were analysed as described previously [17]. Thermal denaturation temperatures (T_m) were determined at a relative absorbance value of 0.50, and the mean ± SEM of at least four determinations was calculated for each sample. The change in T_m (ΔT_m) following interaction of the DNA with an added compound is given by

$$\Delta T_m = T_{m\text{DNA–drug}} - T_{m\text{DNA}},$$

and results are shown in Fig. 6. For DNA samples of different base-pair composition, trimelamol (50 μM) was reacted with CP or ML DNAs (100 μM DNAp) under the solution conditions described for CT DNA above. The results are described in the text.

Preparation of the tris(phenylthio) adduct (**9**) *of trimelamol*

A solution of trimelamol (0.5 g, 1.94 mM) in acetonitrile (500 mL) was treated with thiophenol (0.63 g, 5.72 mmol) and hydrochloric acid (1 mL, 11.5 M), and then stirred at room temperature for 12 hr until reaction was complete by TLC (Merck DC-Alufolien, Kieselgel 60F$_{254}$, BDH; 5% CH_3OH/$CHCl_3$; visualized with UV_{254} or I_2 vapour). The acetonitrile was removed *in vacuo*, and the residue (0.94 g, 91% crude yield) purified by column chromatography (silical gel, Merck grade 60, 230–400 mesh; $CHCl_3$) to afford 0.41 g (40% yield) of the tris(phenylthio) adduct (N^2,N^4,N^6-tri-(phenylthiomethyl)-N^2,N^4,N^6-trimethylmelamine, **9**) as a white powder, m.p. 89.8–90.3°. i.r. (nujol): 1540, 1480, 1440, 1395, 1275, 1230, 1120, 1050, 900, 855, 805, 745, 690 cm^{-1}; ^{1}H-NMR ($CDCl_3$; 60 MHz, Varian EM-360, Me_4Si, δ ppm): 2.94 (s, 9H, NCH_3), 5.00 (s, 6H, NCH_2S), 7.0–7.24 (m, 9H, H-3,4,5), 7.25–7.50 (m, 6H, H-2,6); ^{13}C-NMR ($CDCl_3$, Jeol GSX 270MHz FT, δ ppm): 33.6 (NCH_3), 55.1 ($-CH_2-$), 127.0, 128.7, 132.4 (Ar), 164.9 (triazine); MS (Jeol JMS-DX 303 GC–MS, EI mode, 70 eV, source 120–150°); *m/z* (relative intensity): 425 (M^+·–SPh, 100%), 381 (M^+·–$N(CH_3)CH_2SPh$,

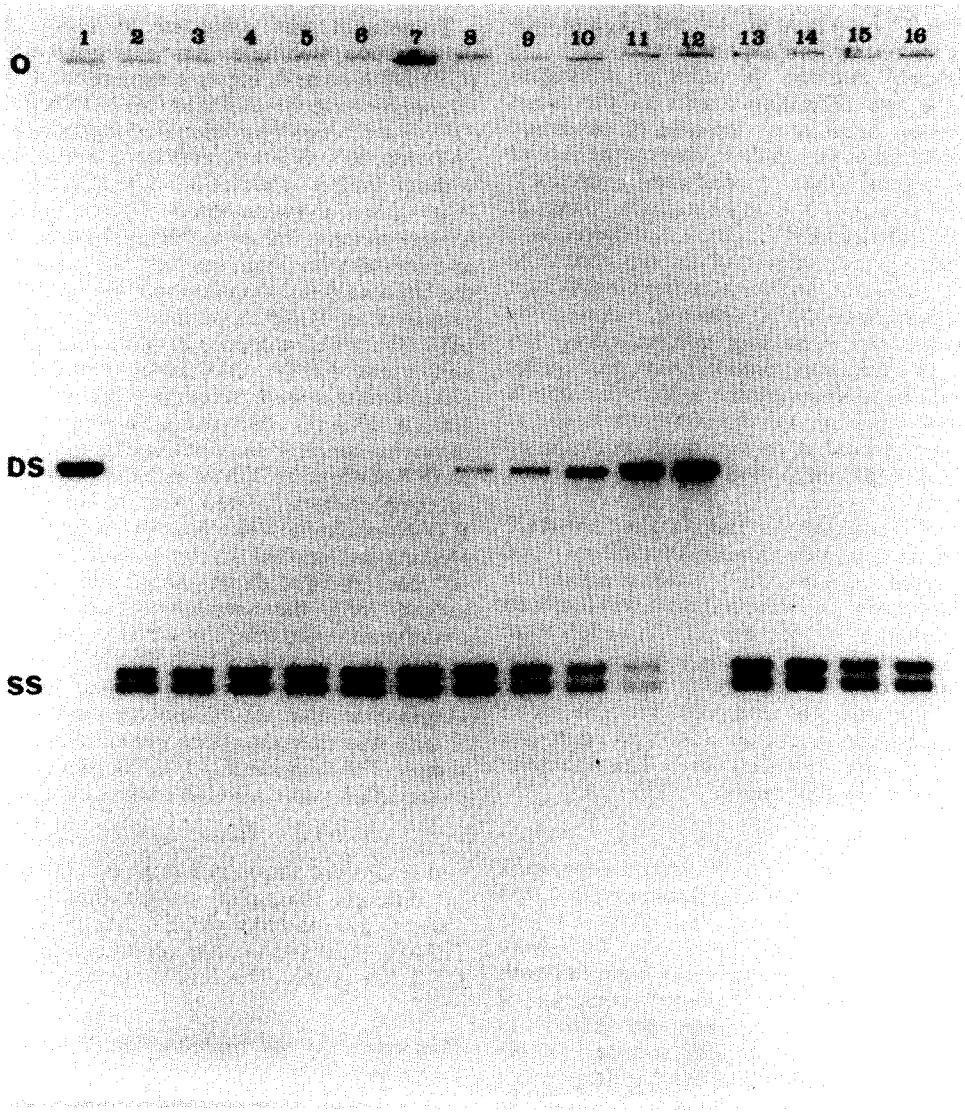


Fig. 1. Cross-linking gel autoradiograph showing the effect of increasing concentrations of trimelamol and formaldehyde on double-stranded, linearized ^{32}P-end-labelled pBR322 plasmid DNA. Lane 1: untreated double-stranded DNA; lane 2: untreated single-stranded DNA; lanes 3–12: trimelamol at 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0 and 500 μM; lanes 13–16: formaldehyde at 1.5, 15.0, 150.0 and 1500 μM; O = origin of gel, SS = single-stranded, DS = double stranded.

17%), 316 ($M^+\cdot$–[2 × SPh], 11%), 231 ($M^+\cdot$–[2 × –N(CH_3)CH_2SPh], 41%); High resolution MS: Calc. for $C_{21}H_{25}N_6S_2$ ($M^+\cdot$–SPh) m/z 425.1582; found, 425.1548.

RESULTS

Cross-linking of DNA by trimelamol was determined using a simple agarose gel electrophoresis assay based on the principal that, following complete denaturation of linear DNA, the presence of an interstrand cross-link results in renaturation to double-stranded DNA in a neutral gel [15]. The results in Fig. 1 indicate that trimelamol is an effective DNA cross-linker (lanes 3–12), whereas formaldehyde (lanes 13–16) has no effect in the 3:1 stoichiometric concentration range (1.5–1500 μM) likely to result from degradation of the trimelamol concentrations used in the experiment (0.5–500 μM). Quantitation of this data (Fig. 2) demonstrates that the percentage of double-stranded DNA increases with trimelamol concentration and, under the conditions employed, trimelamol cross-linking is measurable down to concentrations of 2.5 to 5.0 μM. This level of activity is similar to that for the nitrogen mustard alkylating agents. Figure 3 demonstrates the effect of pH on the cross-linking process. A significant increase in the rate of cross-linking by a factor of 10 is observed at the lower pH of 4.0 compared to the pH 7.0 control.

The results of the "second-arm" experiments (Fig. 4) indicate that no measurable "second-arm" reactions occur at either room temperature or 37°, suggesting that trimelamol forms both covalent

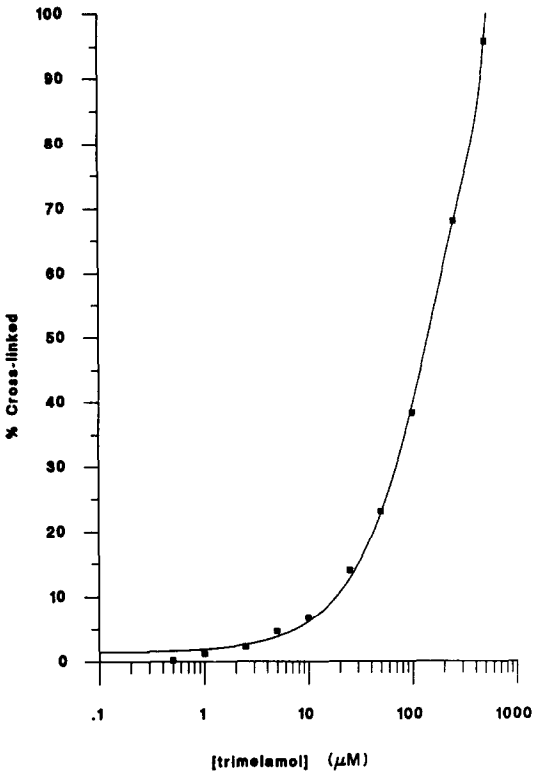


Fig. 2. Dose–response curve for cross-linking of pBR322 plasmid DNA by trimelamol in the 0.5–500 μM range.

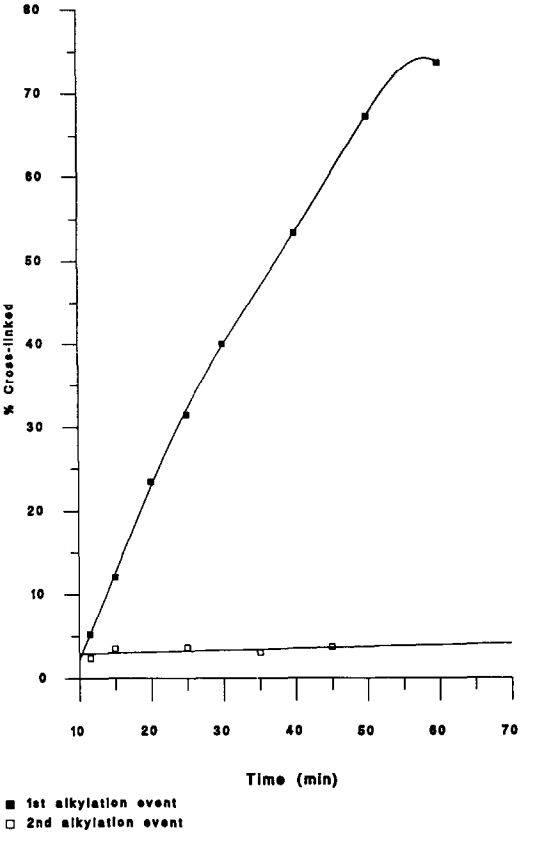


Fig. 4. Investigation of the "second-arm" reaction of trimelamol. (■) 1st Alkylation Event: time course of cross-linking of pBR322 DNA by 250 μM trimelamol; (□) 2nd Alkylation Event: time course after 15 min exposure of pBR322 DNA to 250 μM trimelamol followed by removal of non-bound trimelamol by precipitation of drug–DNA complex.

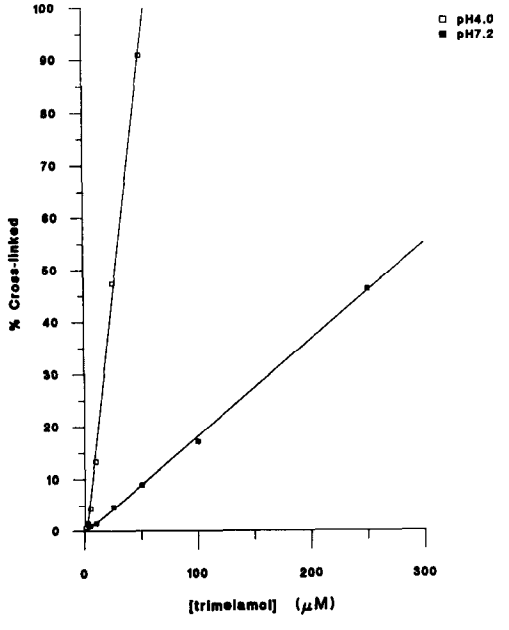


Fig. 3. Comparison of the dose–response of cross-linking pBR322 DNA by trimelamol (0.5–250 μM) at pH values 4.0 and 7.2.

bonds of the cross-link in rapid succession, if not simultaneously. A sequencing-based assay [16] designed to measure alkylation at the guanine-N7 position through treatment with hot piperidine did not reveal any reaction, even at concentrations of trimelamol known to give extensive DNA cross-linking. However, using the cross-linking assay [15] it was shown that trimelamol cross-links are not reversed by incubation for 15 min (37°) in either low (4.0) or high (10.0) pH conditions, although 50% reversibility does occur after treatment with piperidine at 90° for 15 min (Fig. 5).

Trimelamol was found to raise the T_m of DNA, consistent with general binding. Figure 6 shows the results of the effect of concentration of trimelamol on the T_m of CT DNA. A range of trimelamol:DNA molar ratios (0.1–2.0:1) was examined for a fixed DNA concentration (100 μM DNAp) at pH 7.0. Trimelamol was found to increase the T_m of CT DNA by a maximum ΔT_m of 1.4° at a drug:DNA ratio of 0.5:1, after which there was a progressive reduction in ΔT_m with further increase in concentration. To examine the effect of trimelamol on DNA samples of different base-pair composition, it was reacted with DNA from *Clostridium perfringens* (AT-rich; 30% [G + C]) and *Micrococcus lysodeikticus* (GC-rich; 72% [G + C]) using a fixed

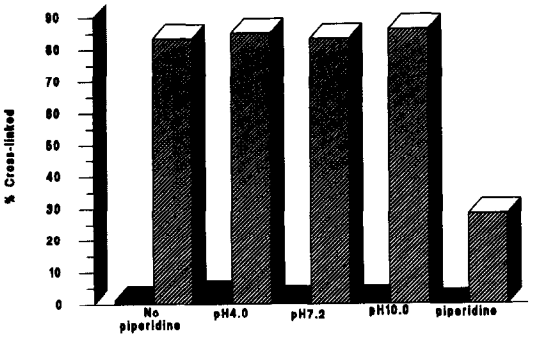


Fig. 5. Effect of pH (4.0, 7.2 and 10.0) and piperidine on the reversibility of cross-linking of pBR322 DNA by 250 μM trimelamol; light bars = treated DNA, dark bars = untreated control DNA.

have suggested that a reactive electrophilic iminium ion can be formed in aqueous solution. A chemical mechanism to rationalize its formation has been postulated based on the acid-catalysed elimination of water (Scheme 1; **1**→**7**). In support of formation of a reactive iminium ion (e.g. **7**) prior to DNA alkylation, it was found that reaction with DNA is enhanced at low pH (Fig. 3). Further evidence for formation of **7** was obtained through trapping experiments with the model nucleophile, thiophenol. Nucleophilic displacement occurs at all three reactive carbinolamine positions to form the tris(phenylthio) adduct **9**. However, no reaction occurs unless HCl is present in the mixture, suggesting that iminium ion formation is an essential prerequisite for reaction with a nucleophile.

The results of the assay used to investigate guanine N7 alkylation suggest that no aminal bonds are formed between trimelamol and the N7-position of

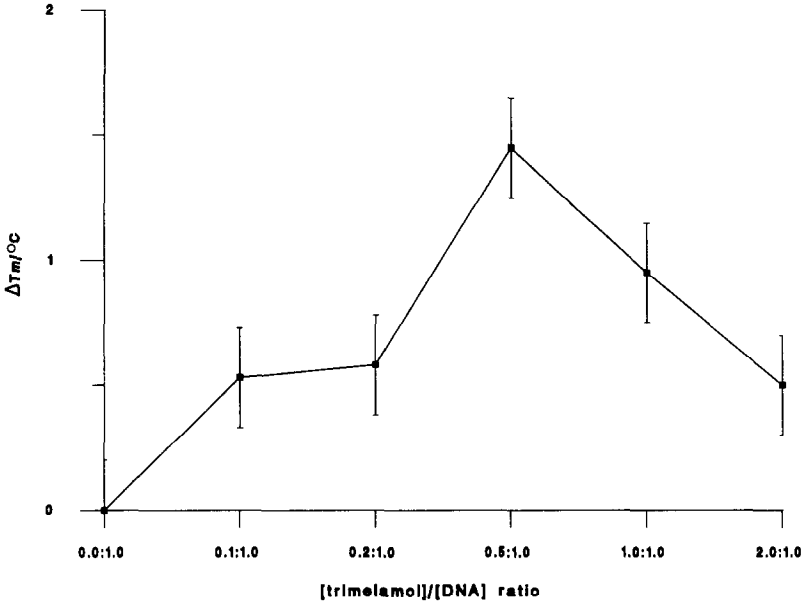


Fig. 6. Effect of increasing concentration of trimelamol (10.0, 20.0, 50.0, 100.0 and 200.0 μM) on the T_m of CT DNA (100 μM), expressed as molar ratios of drug:DNA.

drug:DNA molar ratio of 0.5:1 at pH 7.0. The determined T_m value for ML DNA was raised by $+0.23 \pm 0.08°$, although no significant change was observed ($-0.02 \pm 0.06°$) for CP DNA.

DISCUSSION

The results indicate that trimelamol is an efficient DNA interstrand cross-linker. Unlike a number of known chemical cross-linking agents that have slow "second-arm" reactions and for which there is an observable time period between the first and second alkylation events [15], bisalkylation by trimelamol appears to be either rapid or concerted, as no time dependence is observed. This result is comparable to that obtained for the nitrogen mustard mechlorethamine where the second-arm reaction is too fast to be measured, but differs from L-phenylalanine mustard (melphalan) which exhibits delayed interstrand cross-linking [15].

Previous degradation studies on trimelamol [13] guanine. This assay was based on the tendency of N7-alkylated guanine residues to cleave upon treatment with hot piperidine, resulting in strand breakage at the alkylation site [16]. However, aminal bonds formed between trimelamol and DNA could reverse under such conditions, leading to the release of drug without accompanying strand cleavage. An experiment designed to demonstrate reversibility of this type under similar conditions (Fig. 5), clearly indicates that approximately 50% reversal occurs after treatment with piperidine, but not at pH 4.0, 7.2 or 10.0 in the absence of piperidine. This suggests that guanine N7 alkylation cannot be excluded as a possible alkylation event based on the negative result in the N7-assay described above. The fact that reversal of DNA cross-linking does not occur at pH 4.0, 7.2 or 10.0 is also significant, as an aminal bond resulting from the alkylation of an amine such as guanine-NH_2, would be expected to reverse under conditions of low pH as, for example, in the case of the antitumour antibiotic anthramycin [18].

Trimelamol increases the T_m of CT DNA at drug:DNA molar ratios of up to 0.5:1, consistent with increased thermal stability of the helix due to favourable binding, alkylation and/or cross-linking. At higher drug:DNA ratios (i.e. >0.5:1) the stabilization afforded to CT DNA is reduced with increasing concentration of trimelamol (Fig. 6). One explanation for this effect is that formaldehyde (evolved during the degradation of trimelamol [13]) is known to decrease the T_m of DNA through the formation of transient Schiff's bases with the exocyclic guanine-NH_2 residues. This reduces hydrogen bonding interactions between base-pairs, leading to a reduction in thermal stability [19]. At trimelamol:DNA molar ratios of >0.5:1, it is possible that sufficient free formaldehyde is generated to compete with the T_m-increasing effect of trimelamol, with the result that a net decrease in melting temperature is observed.

The T_m values determined for ML and CP DNAs suggest that the drug has a weak preference for GC- rather than AT-rich DNA sites. However, the fact that neither of the bacterial DNAs are stabilized to the extent observed for CT DNA suggests that a more even distribution of GC and AT base-pairs may be important at the binding site(s), or that other structural factors may be involved.

CONCLUSION

Trimelamol (**1**) is an efficient DNA interstrand cross-linker. The reaction with DNA is acid-catalysed and the two alkylation events appear to occur simultaneously. Thermal denaturation studies suggest a weak preference for GC-binding sites, although an assay specific for guanine-N7 alkylation failed to demonstrate DNA cleavage at those sites upon treatment with hot piperidine. However, similar conditions were shown to cause significant reversal of cross-linking, suggesting that guanines cannot be discounted as possible binding sites, and that more than one type of functional group may be involved at one or more different binding sites. This is also illustrated by the thermal denaturation studies which suggest that a more even distribution of GC and AT base-pairs may be important at the binding site(s).

In general, these results support the alkaline elution studies of Ross *et al.* [11], and suggest that DNA interstrand cross-linking events may be associated with the antitumour activity of trimelamol. In addition, the observed enhancement of cross-linking efficiency at low pH could form the basis of a mechanism for selective tissue toxicity *in vivo*. Efforts are continuing to establish the preferred nature of the binding site(s) of trimelamol and to deduce the structure of the drug–DNA cross-linked adduct(s) formed.

REFERENCES

1. Jackson C, Hartley JA, Jenkins TC, Godfrey R, Saunders R and Thurston DE, N^2,N^4,N^6-Tri(hydroxymethyl)-N^2,N^4,N^6-trimethylmelamine (trimelamol) is an efficient DNA cross-linking agent. *Br J Cancer* Suppl XIII) **63**: 46, 1991.
2. Cumber AJ and Ross WCJ, Analogues of hexamethylmelamine; the anti-neoplastic activity of derivatives with enhanced water solubility. *Chem Biol Interact* **17**: 349–357, 1977.
3. Rutty CJ and Connors TA, *In vitro* studies with hexamethylmelamine. *Biochem Pharmacol* **26**: 2385–2391, 1977.
4. Muindi JRF, Newell DR, Smith IE and Harrap KR, Pentamethylmelamine (PMM): Phase I clinical and pharmacokinetic studies. *Br J Cancer* **47**: 27–33, 1983.
5. Casper ES, Gralla RJ, Lynch GR, Jones BR, Woodcock TM, Gordon C, Kelsen DP and Young CW, Phase I and pharmacological studies of pentamethylmelamine adminstered by 24-hour intravenous infusion. *Cancer Res* **41**: 1401–1406, 1981.
6. Rutty CJ, Newell DR, Muindi JRF and Harrap KR, The comparative pharmacokinetics of pentamethylmelamine in man, rat and mouse. *Cancer Chemother Pharmacol* **8**: 105–111, 1982.
7. Judson IR, Rutty CJ, Gumbrell L, Abel G, Harrap KR and Calvert AH, Phase I trial and pharmacokinetics of trimelamol (N^2,N^4,N^6-trihydroxymethyl-N^2,N^4,N^6-trimethylmelamine): a less neurotoxic analogue of PMM. *Prog Am Assoc Cancer Res* **27**: 174, 1986.
8. Judson IR, Calvert AH, Gore ME, Balmanno K, Gumbrell LA, Perren T and Wiltshaw E, Phase II trial of trimelamol in refractory ovarian cancer. *Br J Cancer* **663**: 311–313, 1991.
9. Borkovec AB and DeMilo AB, Insect chemosterilants. V. Derivatives of melamine. *J Med Chem* **10**: 457–461, 1967.
10. Rutty CJ and Abel G, *In vitro* cytotoxicity of the methylmelamines. *Chem Biol Interact* **29**: 235–246, 1980.
11. Ross WE, McMillan DR and Ross CF, Comparison of DNA damage by methylmelamines and formaldehyde. *J Natl Cancer Inst* **67**: 217–221, 1981.
12. Sanders ME and Ames MM, Synthesis of *N*-hydroxymethylpentamethylmelamine (ring ^{14}C), a cytotoxic alkylating metabolite of hexamethylmelamine *J Labelled Compounds Radiopharm* **21**: 893–898, 1984.
13. Jackson C, Crabb TA, Gibson M, Godfrey R, Saunders R and Thurston DE, Studies on the stability of trimelamol, a carbinolamine-containing antitumour drug. *J Pharm Sci* **80**: 245–251, 1991.
14. Rutty CJ, Judson IR, Abel G, Goddard PM, Newell DR and Harrap KR, Preclinical toxicology, pharmacokinetics and formulation of N^2,N^4,N^6-trihydroxymethyl-N^2,N^4,N^6-trimethylmelamine (Trimelamol), a water soluble cytotoxic *s*-triazine which does not require metabolic activation. *Cancer Chemother Pharmacol* **17**: 251–258, 1986.
15. Hartley JA, Berardini MD and Souhami RL, An agarose gel method for the determination of DNA interstrand cross-linking applicable to the measurement of the rate of total and second-arm cross-linking reactions. *Anal Biochem* **193**: 131–134, 1991.
16. Mattes WB, Hartley JA and Kohn KW, Mechanism of DNA strand breakage by piperidine at sites of *N*7-alkylguanines. *Biochim Biophys Acta* **868**: 71–76, 1986.
17. Jones GB, Davey CL, Jenkins TC, Kamal A, Kneale GG, Neidle S, Webster GD and Thurston DE, The non-covalent interaction of pyrrolo[2,1-*c*][1,4]-benzodiazepine-5,11-diones with DNA. *Anticancer Drug Design* **5**: 249–264, 1990.
18. Kohn KW and Spears CL, Reaction of anthramycin with deoxyribonucleic acid. *J Mol Biol* **51**: 551–572, 1970.
19. Friefelder D, *Molecular Biology*, 2nd Edn, p. 92. Jones and Bartlett Inc., Boston, U.S.A., 1987.