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# Stable Analogues of the Antitumour Agent Trimelamol Retain *In vitro* Cytotoxicity in Drug-sensitive and Resistant Rodent and Human Cell Lines

**H.M. Coley, M. Jarman, N. Brooks, T.J. Thornton and I.R. Judson**

**In spite of clinical activity in heavily-pretreated ovarian cancer, the antitumour *s*-triazine trimelamol [TM; tris(hydroxymethyl)-tris(methyl)melamine] had to be withdrawn from further clinical studies due to formulation difficulties related to instability. A synthetic programme has produced tris(hydroxymethyl) analogues containing electron-withdrawing groups in place of methyl-*tris*cyanomethyl CB 7669, *tris*trifluoroethyl CB 7639, CB 7529 and *tris*propargyl CB 7547, all showing markedly superior stability to TM. Chemosensitivity testing of analogues (MTT assay, continuous exposure) using a panel of rodent and human cell lines showed activity close to that of TM, e.g. for the CH1 human ovarian cancer cell line. $IC_{50}$ values were TM 23.4 μM, CB 7639 30.5 μM, CB 7529 29.5 μM, CB 7547 28.5 μM and CB 7669 27.3 μM. CB 7669 and CB 7639 required prolonged exposure (>12 h) in order to exhibit equivalent cytotoxicity to a 2-h exposure to TM. Thus, rather than administration as a single daily dose, the stable analogues may be more suited to prolonged infusion, which was suggested as being a more beneficial regimen in clinical trials with TM. In line with clinical observations indicating the efficacy of TM in platinum-refractory ovarian cancer, we saw no significant cross-resistance to TM or CB 7529 in a range of platinum-sensitive and acquired-resistant cell line pairs or in an alkylating-agent resistant cell line, despite TM's ability to crosslink DNA. Data obtained using cell lines with acquired resistance to TM, CB 7669 and formaldehyde (released in the breakdown of TM) suggest a pivotal role for formaldehyde and a more minor role for alkylating activity in the mechanism of action of the *N*-(hydroxymethyl)melamines *in vitro*. Further clinical trials of these compounds are eagerly awaited, and their usefulness as second-line chemotherapy for heavily pretreated ovarian cancer deserves further investigation.**

**Key words: trimelamol, stable analogues, *in vitro*, alkylating-agent/platinum resistance, ovarian cancer**
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## INTRODUCTION

HEXAMETHYLMELAMINE (HMM) is an *s*-triazine antitumour agent used in the treatment of ovarian cancer, and has been under clinical evaluation for a number of years. Clinical administration is complicated by the need to give HMM orally, owing to its low solubility. This may be a problem since the drug can cause significant nausea. A clinical study in ovarian cancer patients using HMM described wide interpatient variability in overall drug exposure, i.e. area under the concentration–time curve (AUC) [1] and other studies have indicated that bioavailability may be low and variable [2, 3]. HMM itself is relatively inactive *in vitro* and requires bioactivation via the P450 monooxygenase system to yield an *N*-hydroxymethylated intermediate [4–6]. *N*-Demethylation was shown to be a major metabolic pathway for the *N*-methylmelamines in rats and humans [7]. The possibility arises that the sometimes disappointing clinical activity seen with HMM may in part be due to poor bioactivation.

The water soluble compound trimelamol (TM) [$N^2$, $N^4$, $N^6$-tris(hydroxymethyl)]-$N^2$, $N^4$, $N^6$ trimethylmelamine, representing a bioactivated form of HMM, was developed for parenteral administration in an effort to overcome the aforementioned problems. The phase I clinical trial of TM showed promising activity in patients with platinum-refractory ovarian cancer [8]. The activity of a fractionated dose schedule in animal models and man, as demonstrated in the phase II clinical trial [9], suggested that the antitumour activity of TM is related to the AUC rather than to the peak plasma concentration of the drug and its breakdown products. Thus, prolonged infusion may be a better regimen for TM administration. Unfortunately, in both phase I and phase II clinical trials, scaling-up of the intravenous (i.v.) formulation of TM proved difficult due to the presence of insoluble particles in reconstituted lyophilised preparations. A heavy precipitate also developed if solutions were left standing for several hours, as had been previously

Correspondence to H.M. Coley.
H.M. Coley, M. Jarman, N. Brooks and I.R. Judson are at the Department of Drug Development, Institute of Cancer Research, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5NG, U.K.; and T.J. Thornton is at the Hafslund Nycomed Pharma AG, A-4021 Linz, Austria.
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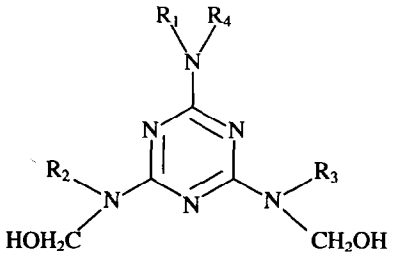


| | $R_1, R_2, R_3$ | $R_4$ |
|---|---|---|
| Trimelamol | $-CH_3$ | $-CH_2OH$ |
| CB 7639 | $-CH_2CF_3$ | $-CH_2OH$ |
| CB 7669 | $-CH_2CN$ | $-CH_2OH$ |
| CB 7547 | $-CH_2C\equiv CH$ | $-CH_2OH$ |
| CB 7527 | $-CH_2CF_3$ | $-CH_2O-CH_2OH$ |

**Figure 1. Structures of tris(hydroxymethyl) substituted analogues of trimelamol.**

observed by Rutty and colleagues [10]. This process was temperature and concentration dependent, suggesting polymerisation. The insoluble material has been identified by Jackson and colleagues [11] as a dimer of TM linked by a methylene bridge. In addition, TM has been shown to be relatively unstable in aqueous solution at room temperature with a half-life of approximately 200 min (at pH 7.4).

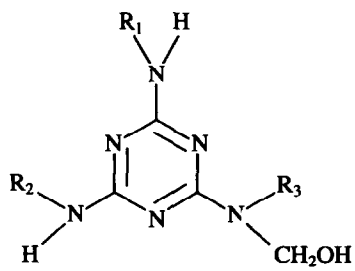
In order to address these problems, analogues of TM with markedly improved stability and showing a lack of dimer formation have been synthesised (Figure 1). The analogues contain ring stabilising electron-withdrawing groups trifluoroethyl (CB 7639, CB 7529), propargyl (CB 7547) or cyanomethyl (CB 7669) in place of methyl, for which extensive synthetic and stability data have been reported previously [12]. As well as the compounds containing three hydroxymethyl groups (as in TM), examples containing two (CB 7682, CB 7683) or one such moiety (CB 7518, CB 7541) have also been made (Figure 2). In addition, we have examined non-hydroxylated melamines including HMM (Figure 3). This series of compounds has assisted in our mechanistic and structure activity relationship studies.

We now describe *in vitro* cytotoxicity data obtained in a panel of human and murine cell lines using these TM analogues. In these studies, we have tried to address the efficacy of the methylmelamines as second-line chemotherapy against pretreated tumour groups, in particular platinum-refractory ovarian carcinoma. Hence, the present study has also included various drug-resistant cell line models to examine patterns of cross-resistance. The methylmelamines have been shown to covalently bind to DNA [13] and have been proposed to possess alkylating activity at the site of the exocyclic nitrogen, yielding an iminium species upon degradation [14]. Jackson and colleagues [11] demonstrated DNA crosslinking using isolated plasmid DNA, and Ross and associates [15] were able to measure DNA interstrand crosslinks in L1210 cells treated with TM using alkaline elution. An additional mechanism of action is suggested by the fact that TM releases formaldehyde during its breakdown which could represent a cytotoxic species in its own right. The generation of cell lines with acquired resistance to TM, CB 7669 and formaldehyde has helped towards our understanding of the mechanism of action of the *N*-(hydroxymethyl)melamines and data showing their cross-resistance profiles are presented.

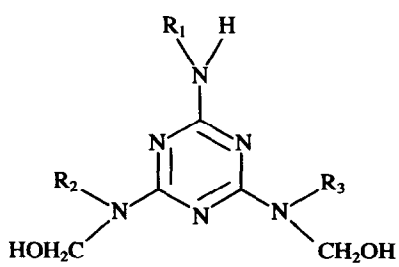
## MATERIALS AND METHODS

*Compounds*

The synthesis of tris and mono(hydroxymethyl) derivatives having electron-withdrawing substituents is described elsewhere [12]. The bis(hydroxymethyl) derivatives are prepared as follows.

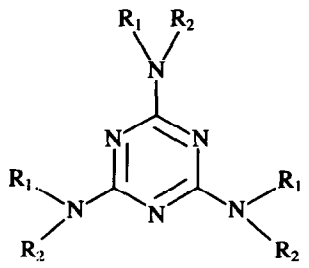


| | $R_1, R_3$ | $R_2$ |
|---|---|---|
| CB 7518 | $-CH_2CF_3$ | $-CH_2CF_3$ |
| CB 7541 | $-CH_2CF_3$ | $-CH_3$ |



| | $R_1, R_2, R_3$ |
|---|---|
| CB 7682 | $-CH_2CN$ |
| CB 7683 | $-CH_2CF_3$ |

**Figure 2. Structures of mono and bis(hydroxymethyl) substituted analogues of trimelamol.**



| | $R_1$ | $R_2$ |
|---|---|---|
| Hexamethylmelamine (HMM) | $-CH_3$ | $-CH_3$ |
| Trimethylmelamine (TriMM) | $-CH_3$ | -H |
| Tris(cyanomethyl) melamine (TCN) | $-CH_2C{\equiv}N$ | -H |

**Figure 3. Structures of non-hydroxymethylated melamine compounds.**

2,4-*Bis*[(*cyanomethyl*)(*hydroxymethyl*)*amino*]-6-[(*cyanomethyl*) *amino*]-1,3,5-*triazine* (*CB* 7682)

2,4,6-Tris[(cyanomethyl)amino]-1,3,5-triazine (1,2.432 g, 10 mmol) was added to 40% aqueous formaldehyde (50 ml) and the suspension was stirred at ambient temperature for 18 h. The now clear solution was extracted with ethyl acetate (150 ml, then 100 ml), and the white solid, obtained by treating the concentrated ethyl acetate extract with water, was dissolved in a minimum of acetone and applied to a column of silica gel (Merck, Dagenham, U.K.; Art. No. 7734), which was eluted with ethyl acetate to give successively the bis(hydroxymethyl) derivative (CB 7682) and the tris(hydroxymethyl) derivative (CB 7669). The products were isolated as white solids after addition of water. The yield of CB 7682 was 0.952 g (30%) and of CB 7669 (also a hemihydrate) 0.971 g (28%). Analytical data for CB 7682; $\delta_H$ ($Me_2SO$-$d_6$) 4.37 (s, 2H, $HNCH_2CN$), 4.64 (m, 4H, $H_2CNCH_2CN$), 5.14 (m, 4H, $CH_2OH$), 6.03 (br s, 2H, OH), 7.98 (br d, 1H, NH). Analytical $C_{11}H_{13}N_9O_2.O.5H_2O$ requires C, 42.31; H, 4.52; N, 40.37: found 42.63; H, 4.60; N, 39.64%.

2,4-*Bis*[(*hydroxymethyl*) (2,2,2-*trifluoroethyl*)*amino*]-6-(2,2,2-*trifluoroethyl*) *amino*-1,3,5-*triazine* (*CB* 7683)

A solution of 2-([hydroxymethoxy]methyl)(2,2,2-trifluoroethyl)amino-4,6-bis(hydroxymethyl) (2,2,2-trifluoroethyl) amino-1,3,5-triazine (CB 7529 500 mg, 1.02 mmol) in acetone (3 ml) and water (2 ml) was set aside at room temperature for 18 h. Acetone was removed under vacuum, and the organic materials were extracted with diethyl ether and separated on a column of silica gel which was eluted with diethyl ether to give successively the mono(hydroxymethyl) derivative (CB 7518, 23 mg), the title compound (144 mg, 33%), obtained as a white solid by trituration with ice-cold water, and the tris(hydroxymethyl) derivative (CB 7639, 111 mg). NMR spectrum: $\delta_H$ ($Me_2SO$-$d_6$) 4.09 (br q, 2H, $F_3CCH_2NH$), 4.41 (br q, 4H, $F_3CCH_2OH$), 5.06 (d, 4H, $CH_2OH$), 5.78 (br s, 2, OH), 7.80 (br s, 1, NH); $\delta_F$ −70.23, −70.03 (2 s, 3F, $F_3CCH_2NH$), −68.3 (s, 6F, $F_3CCH_2NHCH_2OH$).

*Cell lines*

Three rodent drug sensitive cell lines were included in the present study. The murine PC6 plasmacytoma grows as a suspension culture in Dulbecco's modified Eagle's medium supplemented with 20% donor horse serum (Flow Laboratories, Thame, U.K.), non-essential amino acids, 2 mM glutamine, 10 μg/ml insulin (Sigma, Poole), 0.5 μg/ml hydrocortisone (Sigma), 50 μg/ml gentamicin and 2.5 μg/ml amphotericin. The L1210 murine leukaemia line was grown as a suspension in RPMI 1640 medium supplemented with 10% donor horse serum, glutamine and antibiotics as described above. The Walker 256 carcinoma WS and the chorambucil-resistant subline WR [16] were cultured in suspension in Dulbecco's modified Eagle's medium supplemented with 10% donor horse serum, glutamine and antibiotics as described above.

The melamine-resistant cell lines WTM/R and W7669/R with acquired resistance to TM and CB 7669, respectively, were generated from the Walker WS (drug-sensitive) line. Cells were treated with increasing concentrations of the inducing agent in chronic exposure, starting with 20% of the chronic $IC_{50}$ dose. The resulting lines were maintained at 55 μM and 45 μM for WTM/R and W7669/R, respectively. The L1210 formaldehyde-resistant line was similarly developed, starting with an initial chronic treatment of L1210 cells to 20 μM formaldehyde. Stepwise increments up to 200 μM resulted in the L1210HCHOR line, which was maintained in continuous culture at this dose.

A panel of human ovarian tumour cell lines were used in the study. These included the LK1 cell line [17], derived from a patient with epithelial ovarian carcinoma who relapsed on treatment with cisplatin, but who developed stable disease following treatment with TM; the HX/62 line derived from a papillary cystadenocarcinoma [18]; and SKOV-3, an adenocarcinoma (obtained from the American Tissue Type Culture Collection). In addition, four human ovarian cancer cell lines together with their respective *in vitro*-derived platinum-resistant counterparts (cisplatin-resistant denoted by Cis/R; carboplatin-resistant denoted by Carbo/R) were examined. These comprised CH1 and CH1/CisR, a papillary cystadenocarcinoma [18, 19]; 41 M and 41 M/CisR, an adenocarcinoma [18, 19]; OVCAR-3 and OVCAR-3/CarboR, an adenocarcinoma [20]; A2780 and A2780DDP (acquired platinum-resistance), an adenocarcinoma cell line [21]. In addition to these cell line pairs, a human testicular non-seminomatous germ cell line, GCT27, together with its *in vitro*-derived cisplatin-resistant counterpart, GCT27CisR [22], and the HX155 human cervical cell line and its cisplatin-resistant counterpart, HX155/CisR [23], were also used in this study. The parental 41M, A2780, GCT27 and HX155 lines were all derived from previously untreated patients. All cell lines grew as monolayers in Dulbecco's modified Eagle's medium plus 10% heat inactivated fetal calf serum, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, 10 μg/ml insulin and 0.5 μg/ml hydrocortisone in an atmosphere of 5% $CO_2$, 95% air in a humidifying gassing incubator.

The human small cell lung cancer cell line NCI-H69, along with its *in vitro*-derived doxorubicin-resistant MDR counterpart, H69/LX4 [24], were kindly provided by Dr P.R. Twentyman (Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Cambridge, U.K.). These cell lines grow as floating aggregates in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum and antibiotics as described above. All tissue culture medium, serum and supplements were obtained

from Gibco Life Technologies (Paisley, U.K.) unless otherwise stated.

*Preparation of cytotoxic agents*

HMM, trimethylmelamine (TriMM), tris(cyanomethyl)melamine (TCN), TM and analogues were made up as stock solutions in dimethylsulphoxide (DMSO), frozen at −20°C in aliquots which were thawed immediately before use. Drug dilutions were made up in tissue culture medium for use in cytotoxicity assays. The concentration of DMSO never exceeded 0.5%, a concentration which did not cause significant inhibition of cell growth. Formaldehyde was diluted from a 38% w/v stock solution (Fisons, Loughborough, U.K.) in tissue culture medium immediately prior to use. Melphalan, chlorambucil (Sigma) and phosphoramide mustard (ASTA Medica AG, Frankfurt, Germany) were dissolved in a small amount of hydrochloric acid (1 N) followed by neutralisation with NaOH (1 N) and further dilution in sterile water to give a working solution, which was subsequently diluted in tissue culture medium immediately prior to use.

In vitro *cytotoxicity testing*

Cell suspensions were dispensed in 200-μl aliquots into 96-well tissue culture plates (Falcon Plastics, Cowley, U.K.) to give 4–5 × $10^3$ cells/ml for all the ovarian and testicular cell lines, 8 × $10^3$ and 1 × $10^4$/ml for the H69/P and H69/LX4 lines, respectively; and 1 × $10^3$ cells/ml for all the rodent cell lines. The plates were then incubated for 24 h in a gassing incubator at 37°C.

Cytotoxic compounds appropriately diluted in tissue culture medium were added in aliquots of 50 μl to produce the required final concentration. The dose range selected encompassed concentrations shown to produce a decrease in final cell number (as indicated by optical density) to less than 10% of that given by the control, drug-free cells. Cells were exposed for the appropriate time period, i.e. chronic or short exposure, whereby drug-containing medium was removed (at the appropriate time interval) and replaced by fresh drug-free tissue culture medium, according to the cell doubling times. This time period was estimated as that during which there was a 10–20-fold increase in cell number for untreated cells. MTT solution (Sigma; 5 mg/ml in phosphate-buffered saline) was then added to each well in a volume of 20 μl and the plates incubated for 4–5 h at 37°C. Plates containing cell lines in suspension were centrifuged at 2000 rpm for 5 min. The medium was then aspirated from the wells and 200 μl of DMSO (BDH, Poole, U.K.) were added to dissolve the crystalline formazan reaction product. The plates were agitated for 10 min and the absorbances then read on a Titertek Multiskan MCC Elisa plate reader (Flow Laboratories) 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.

*Clonogenic assay*

Cells were treated with the drug at a cell density of 2 × $10^5$/ml in 5-ml Falcon double action tubes. Immediately after the addition of the drug, the tubes were sealed, mixed by inversion and incubated at 37°C for 2 h. A 0.125% preparation of Noble agar (Difco, Detroit, Michigan, U.S.A.) in complete tissue culture medium was prepared and maintained at 42°C. Cells were diluted to give densities of $10^5$/ml, $10^4$/ml and $10^3$/ml. For each cell density, tubes containing agar were prepared in triplicate for each drug concentration. An assessment of colony counts was made at day 10.

## RESULTS

Note: data for Tables 1–7 relate to continuous (chronic) drug treatment.

*Cytotoxicities of tris(hydroxymethyl)melamines in rodent cell lines*

As shown in Table 1, the murine PC6 plasmacytoma cell line was sensitive to TM, with an $IC_{50}$ of 12.9 μM. All the analogues containing electron-withdrawing groups were relatively less cytotoxic to this cell line, with $IC_{50}$ values 1.5-fold to 2.7-fold greater than for TM. Of the panel tested, the L1210 cell line appeared the least sensitive to the tris(hydroxymethyl) melamines. CB 7669 and TM gave very similar $IC_{50}$ values, whereas CB 7639 and CB 7547 were somewhat less potent. The analogue CB 7529 is a prodrug form of CB 7639. Once in aqueous solution, CB 7529 readily forms the tris(hydroxymethyl) form (confirmed by HPLC analysis, data not shown). The $IC_{50}$ values obtained for CB 7639 and CB 7529 were virtually identical.

The Walker cell lines appeared to be most sensitive to this group of compounds. The $IC_{50}$ values obtained for the analogues were similar to those obtained for the parent compound TM. In addition, these results were interesting inasmuch as no cross-resistance to the tris(hydroxymethyl)melamines was demonstrated in the WR cell line with acquired resistance to chlorambucil and broad spectrum cross-resistance to several bifunctional alkylating agents [16].

*Cytotoxicities of tris(hydroxymethyl)melamines in human tumour cell lines*

The human ovarian cancer cell lines CH1, 41M, LK1, SKOV-3 and HX62 showed a similar level of sensitivity to TM and related analogues, as shown in Table 2. Again, $IC_{50}$ values for

*Table* 1. *Cytotoxicity data for tris(hydroxymethyl)-substituted melamine compounds in a range of rodent cell lines*

| | Compounds | | | | |
|---|---|---|---|---|---|
| Cell line | TM | CB 7639 | CB 7547 | CB 7669 | CB 7529 |
| PC6 | 12.9 (2.7) | 29.5 (4.5) | 35.2 (5.5) | 19.4 (2.0) | 29.8 (5.6) |
| L1210 | 25.6 (6.3) | 34.0 (2.6) | 35.6 (4.0) | 23.7 (1.1) | ND |
| Walker S | 9.4 (0.5) | 12.2 (1.0) | 14.1 (3.8) | 7.5 (0.4) | ND |
| Walker R | 9.6 (0.5) | 13.5 (2.8) | 12.3 (5.2) | ND | ND |

ND, not done. Figures in parentheses denote the standard deviation obtained for three or more replicate assay results. All results are expressed as $IC_{50}$ values (μM).

*Table 2. Cytotoxicity data ($IC_{50}$, μM) for tris(hydroxymethyl)-substituted melamine compounds in a range of human tumour cell lines*

| Cell line | Compound | | | |
|---|---|---|---|---|
| | TM | CB 7639 | CB 7547 | CB 7669 |
| CH1 | 23.4 (4.4) | 30.5 (0.4) | 28.5 (3.80) | 27.3 (2.6) |
| 41M | 24.1 (4.1) | 26.0 (3.2) | 31.1 (3.6) | 30.5 (2.4) |
| LK-1 | 28.1 (4.8) | 30.9 (3.1) | ND | 29.1 (2.5) |
| SKOV-3 | 29.1 (4.0) | 32.9 (3.4) | 29.2 (3.4) | ND |
| HX62 | 54.1 (4.1) | 62.8 (5.7) | 65.0 (5.0) | 68.4 (1.1) |
| H69/P | 8.5 (2.3) | 12.8 (3.2) | 13.1 (1.5) | 11.8 (2.8) |
| H69/LX4 | 10.4 (2.8) | ND | ND | ND |

Notes as for Table 1.

the analogues were slightly higher than those obtained with TM, with CB 7669 being the most potent of the series.

The H69/P human small cell lung cancer parental cell line emerged as the most sensitive cell line of the cell line panel under study, with an $IC_{50}$ of 8.5 μM for TM and a similar level of sensitivity for the analogues. The MDR cell line, H69/LX4, gave an $IC_{50}$ of 10.4 μM for TM suggesting that the tris(hydroxymethyl)melamines are not recognised by the P-glycoprotein-mediated drug-resistance mechanism.

*Cytotoxicities of tris(hydroxymethyl)melamines in a range of platinum-sensitive and -acquired-resistant cell line pairs*

For the CH1, 41M, A2780 human ovarian and the GCT27 human testicular cell lines, alongside their respective platinum-resistant counterparts, no cross-resistance was demonstrated when using TM or the analogues, as seen in Table 3. The mechanism of acquired platinum-resistance in the CH1CisR line has been shown to be due to increased DNA repair or tolerance to platinum adducts [19]. For the 41MCisR line, the resistance mechanism has been shown to be one of reduced cellular drug accumulation [19]. The A2780DDP line possesses increased cellular glutathione, increased tolerance to DNA–platinum adducts and a drug transport defect [21]. The GCT27CisR line shows an increased ability to remove platinum from DNA, but also contains increased cellular metallothionein and reduced platinum accumulation [22]. For the OVCAR-3 ovarian and the HX155 cervical cell line pairs, only a low level cross-resistance was seen with 2.2-fold resistance shown to TM (both cell line pairs) and an even lower level for the analogues. The resistance mechanism(s) underlying the OVCARCarboR line have not yet been established. The mechanism of acquired resistance in the HX155CisR line is multifactorial, comprising reduced cellular accumulation, elevated cellular glutathione and metallothionein levels resulting in reduced DNA–platination levels [23].

In conclusion, minimal or no cross-resistance was seen in a range of platinum-resistant cell lines with a range of tris(hydroxymethyl)melamines. These compounds, therefore, appear capable of circumventing multiple forms of acquired platinum-resistance.

*Cytotoxicities of bis- and mono(hydroxymethyl)melamines in human and rodent cell lines*

The mono(hydroxymethyl)-substituted analogues, CB 7518 and CB 7541, gave the highest $IC_{50}$ values observed in this series of compounds, as shown in Table 4. Compared with values for TM, $IC_{50}$ values were increased by 2.3- to 5-fold (L1210 and H69, respectively). In general, the values obtained for CB 7541 were somewhat higher than those obtained using CB 7518.

The bis(hydroxymethyl) substituted analogues CB 7682 [tris(cyanomethyl) substituted] and CB 7683 [tris(trifluoroethyl)

*Table 3. Cytotoxicity data ($IC_{50}$, μM) for tris(hydroxymethyl)-substituted melamine compounds in a range of platinum-sensitive and acquired-resistant human cell line pairs*

| Cell lines | TM | RF | CB 7529 | RF | CB 7547 | RF |
|---|---|---|---|---|---|---|
| CH1 | 23.4 (4.4) | | 29.5 (4.0) | | 28.5 (3.8) | |
| $CH1CisR^{6.5}$ | 26.6 (1.6) | 1.1 | 30.0 (2.4) | 1.0 | 26.6 (2.1) | 0.9 |
| 41M | 24.1 (4.1) | | 26.0 (3.2) | | 31.1 (3.6) | |
| $41MCisR^{4.7}$ | 28.8 (4.0) | 1.2 | 27.7 (2.6) | 1.1 | 30.6 (3.5) | 1.0 |
| OVCAR | 35.6 (9.6) | | 33.9 (0.8) | | 53.2 (12.9) | |
| $OVCAR\text{-}CarboR^{4.8}$ | 78.9 (4.6) | 2.2 | 62.1 (7.2) | 1.8 | 81.3 (1.9) | 1.5 |
| A2780 | 27.4 (0.8) | | 30.9 (2.2) | | 31.3 (1.8) | |
| $A2780DDP^{16.0}$ | 27.6 (0.7) | 1.0 | 28.4 (3.3) | 0.9 | 27.2 (3.8) | 0.9 |
| GCT27 | 24.3 (0.9) | | 25.8 (2.5) | | 53.9 (13.0) | |
| $GCT27CisR^{5.6}$ | 30.9 (1.4) | 1.3 | 23.7 (0.4) | 0.9 | 46.8 (4.7) | 0.9 |
| HX155 | 35.9 (7.8) | | 34.0 (10.0) | | | ND |
| $HX155CisR^{8.6}$ | 79.1 (16.9) | 2.2 | 36.5 (7.2) | 1.1 | | |

Notes as for Table 1. RF, resistance factor. Numbers in superscript denote levels of platinum resistance.

*Table 4. Cytotoxicity data ($IC_{50}$, μM) for mono and bis(hydroxymethyl)melamine compounds in human and rodent cell lines*

| Cell lines | Compound | | | |
|---|---|---|---|---|
| | CB 7518 | CB 7541 | CB 7682 | CB 7683 |
| CH1 | 69.2 (10.2) | ND | 33.9 (8.1) | 46.1 (13.3) |
| PC6 | 33.1 (1.1) | 71.4 (17.7) | 24.0 (7.1) | 28.9 (3.0) |
| H69 | 42.3 (3.5) | 32.2 (3.1) | 15.8 (9.4) | 32.2 (3.0) |
| L1210 | 66.8 (10.3) | 94.3 (18.0) | ND | ND |
| Walker S | 23.7 (2.7) | 27.5 (3.2) | ND | ND |

Notes as for Table 1.

substituted] gave $IC_{50}$ values that were generally higher than those obtained for their corresponding tris(hydroxymethyl) counterparts, i.e. CB 7669 and CB 7639, respectively. For the tris(cyanomethyl) series, $IC_{50}$ values were increased by 1.2–1.3-fold over those for CB 7669 in the CH1, H69 and PC6 cell lines studied.

The $IC_{50}$ values for the bis(hydroxymethyl) substituted analogue CB 7683 were either very similar (PC6) or 1.5- and 2.5-fold increased (CH1 and H69, respectively) over the results obtained for the corresponding tris(hydroxymethyl) substituted analogue CB 7639. Taking the tris(trifluoroethyl) substituted series as a whole, we observed a progressive loss in potency corresponding to a decreasing number of hydroxymethyl groups. This relationship is particularly clear for the CH1 cell line results, which is illustrated in Figure 4. In the H69 cell line, however, the bis(hydroxymethyl) and mono(hydroxymethyl) analogues gave identical results. The PC6 results were somewhat different as $IC_{50}$ values for the same series of trifluoroethyl substituted compounds were similar. In this cell line, we saw the largest overall increase in $IC_{50}$ values obtained for the stable analogue series compared with TM. It appears, therefore, that the extra stability conferred on these drug molecules by the presence of electron-withdrawing groups caused a reduction in potency in the PC6 cell line. However, only a marginal increase in $IC_{50}$ values was seen in all other cell lines studied using the stable analogues, with CB 7669 emerging as perhaps the most potent analogue of the group tested.

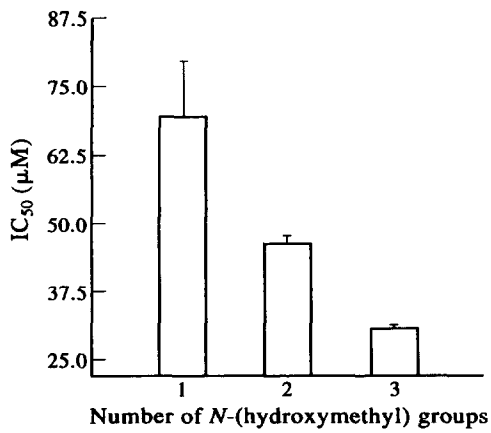



**Figure 4. Relationship between number of *N*-hydroxymethyl groups and cytotoxicity in the CH1 human ovarian cancer cell line for the tris-trifluoroethyl substituted series of analogues (CB 7639, CB 7683, CB 7518).**

*Cytotoxicities of non-hydroxylated melamine derivatives*

Table 5 shows cytotoxicity data for non-hydroxylated melamines. HMM was the most cytotoxic compound in the series. The H69 and Walker cell lines appeared to be the most sensitive in the series, whereas the PC6 line, previously shown to be intrinsically sensitive to TM, did not follow this pattern. Trimethylmelamine (TriMM) and tris(cyanomethyl)melamine (TCM) showed low potency throughout the series examined. It can, therefore, be concluded that the tris *N*-hydroxylated forms of TriMM and TCM, TM and CB 7669, respectively, are markedly more potent, pointing to the importance of the presence of *N*-(hydroxymethyl) groups in the cytotoxicity of these compounds.

*Drug-resistant cell lines*

The melamine-resistant cell lines WTM/R and W7669/R possessed very similar cross-resistant profiles, as shown in Table 6. Both cell lines possessed similar levels of cross-resistance to TM and to CB 7669. This provides evidence for there being a shared mechanism of action for these two compounds. Both cell lines are cross-resistant with formaldehyde, suggesting a role for local formaldehyde release in the cytotoxic action of the *N*-(hydroxymethyl)melamines. In line with the lack of cross-resistance we saw with the panel of platinum-resistant cell lines treated with melamines (Table 3), no cross-resistance to cisplatin was seen in either the WTM/R or W7669/R lines. However, approximately 3-fold resistance to melphalan was seen in both cell lines. In contrast, no cross-resistance to the cyclophosphamide active metabolite phosphoramide mustard was seen in either cell line. This is in marked contrast to the WR line which showed 10- and 20-fold levels of cross-resistance to melphalan and phosphoramide mustard, respectively (data not shown).

The L1210HCHOR line with 7-fold resistance to formaldehyde showed an approximate 4-fold resistance to TM as shown in Table 7. This is in line with results described above and

*Table 5. Cytotoxicity data ($IC_{50}$, μM) for non-hydroxylated melamine compounds in human and rodent cell lines*

| Cell line | HMM | TriMM | TCM |
|---|---|---|---|
| CH1 | 132 (4.3) | >1000 | >1000 |
| L1210 | 90.5 (5.8) | >1000 | ND |
| PC6 | 146 (24.4) | 563 (39.0) | >1000 |
| H69/P | 33.4 (5.1) | >1000 | >1000 |
| WS | 35.7 (4.9) | >1000 | ND |
| WR | 30.3 (7.5) | >1000 | ND |

Notes as for Table 1. For results of >1000 μM, $n = 3$.

*Table 6. Cytotoxicity data ($IC_{50}$, μM) showing cross-resistance profiles for WTM/R and W7669/R resistant cell lines*

| Compound | WS | WTM/R | RF | W7669/R | RF |
|---|---|---|---|---|---|
| TM | 9.5 (0.4) | 45.1 (5.5) | 4.7 | 35.5 (9.6) | 3.7 |
| Formaldehyde | 29.2 (4.2) | 128 (23.2) | 4.4 | 129.2 (11.0) | 4.4 |
| CB 7669 | 7.5 (0.4) | 49.7 (10.7) | 6.6 | 45.2 (15.1) | 6.0 |
| Melphalan | 2.6 (0.4) | 7.4 (2.1) | 2.8 | 7.3 (3.2) | 2.8 |
| Chlorambucil | 11.6 (4.9) | 18.3 (1.7) | 1.6 | 13.7 (2.5) | 1.2 |
| Phosphoramide mustard | 29.4 (6.5) | 32.0 (3.7) | 1.1 | 35.7 (8.4) | 1.2 |
| Cisplatin | 7.8 (3.8) | 5.0 (2.3) | 0.6 | 6.6 (2.9) | 0.9 |

Notes as for Table 1.

*Table 7. Cytotoxicity data ($IC_{50}$, μM) obtained for the L1210 formaldehyde-resistant line L1210HCHOR*

| Compound | $IC_{50}$ values (μM) L1210 | L1210HCHOR | Resistance factor |
|---|---|---|---|
| Formaldehyde | 41.1 (0.8) | 305 (20.0) | 7.5 |
| TM | 27.3 (3.5) | 106.0 (21.0) | 3.9 |
| CB 7646 | 34.3 (8.7) | 98.1 (29.0) | 2.9 |
| CB 7669 | 23.7 (1.1) | 80.3 (24.0) | 3.4 |
| CB 7639 | 34.0 (2.6) | 125 (24.0) | 3.7 |
| CB 7541 | 95.8 (1.3) | 296 (46.7) | 3.1 |
| Melphalan | 2.9 (1.8) | 4.3 (1.6) | 1.5 |

further underpins the proposed contribution of formaldehyde to the cytotoxicity of the *N*-(hydroxymethyl)melamines. Resistance factors to tris(hydroxymethyl)melamines were 3.4–3.9, those obtained for the bis(hydroxymethyl) analogue CB 7646 were 2.9. No cisplatin-resistance was noted.

*Cytotoxicity of trimelamol in the Walker sensitive and alkylating agent resistant lines using the clonogenic assay*

Figure 5 shows there to be a complete lack of cross-resistance to TM in the WR line as compared to the WS line. These data confirm the results shown in Table 1 which utilised the MTT assay.

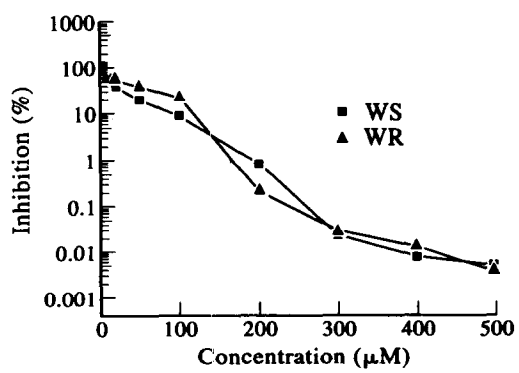



**Figure 5. Clonogenic assay of TM in the Walker WS and WR (alkylating agent resistant) cell lines. Plating efficiency 89%, both lines $IC_{50}$ values WS 32.8 μM (3.1); WR 48.4 μM (11.9). Standard deviation values for three replicate analyses are given in parentheses.**

*Varying exposure time of CH1 cells to TM, CB 7639 and CB 7669*

Figure 6 shows the effect of varying exposure time of TM, CB 7639 and CB 7669 at four different drug concentrations. Treatment with TM at 200 μM resulted in substantial cell killing at 1 h (73%). At 2 h, both the 200 and 100 μM doses caused significant cell killing with values of 74 and 50%, respectively. After 6 h exposure to 50, 100 and 200 μM TM, substantial cell killing was observed in each case (52, 88 and 90%, respectively).

The results were somewhat different for the more stable analogues CB 7669 and CB 7639. For all the concentrations tested, we observed no more than 18% cell killing with up to 2 h exposure with either drug. At 6 h, the levels of cell killing increased with values of 56 and 85% for CB 7669, and 63 and 72% for CB 7639 for the 100 and 200 μM doses, respectively (compared with data for TM, 88 and 90%, respectively).

We found that more than 12-h exposure was required in order for the stable analogues CB 7669 and CB 7639 to exert a similar level of cytotoxicity to that of TM. From these data, it appears that the increased stabilities conferred by the presence of the electron-withdrawing groups cyanomethyl and trifluoroethyl render the drug molecules slower to react. This may be an important factor in determining the method of drug administration (i.e. continuous versus bolus) necessary in order to produce optimal antitumour effect.

## DISCUSSION

The data obtained in the present study show that substituted analogues of TM retain *in vitro* activity in a wide range of human and rodent cell line models. Characteristically, we see a relatively small margin between the cytotoxicity for the most sensitive and that for the intrinsically resistant cell lines, i.e. approximately 5-fold variation in $IC_{50}$ values. The most sensitive cell lines appear to be the human small cell lung cancer cell line H69, a result which is in line with the clinical observations that drugs such as HMM may be effective agents against this tumour group [25]. The activity of TM has been demonstrated *in vivo* in human lung cancer xenograft models [4] and the PC6 plasmacytoma [10], as well as a variety of human ovarian cancer xenograft models (unpublished data). For the human ovarian cancer cell lines and the GCT27 testicular tumour cell line, the sensitivity to the *N*-(hydroxymethyl)melamines was broadly similar, with the majority of cell lines giving $IC_{50}$ values varying from 23.4 to 35.6 μM for TM, with slightly higher values for CB 7639, CB 7547 and CB 7669.

By addressing two important problems associated with the use of both HMM and TM, we have developed a group of analogues in an effort to facilitate further development of this

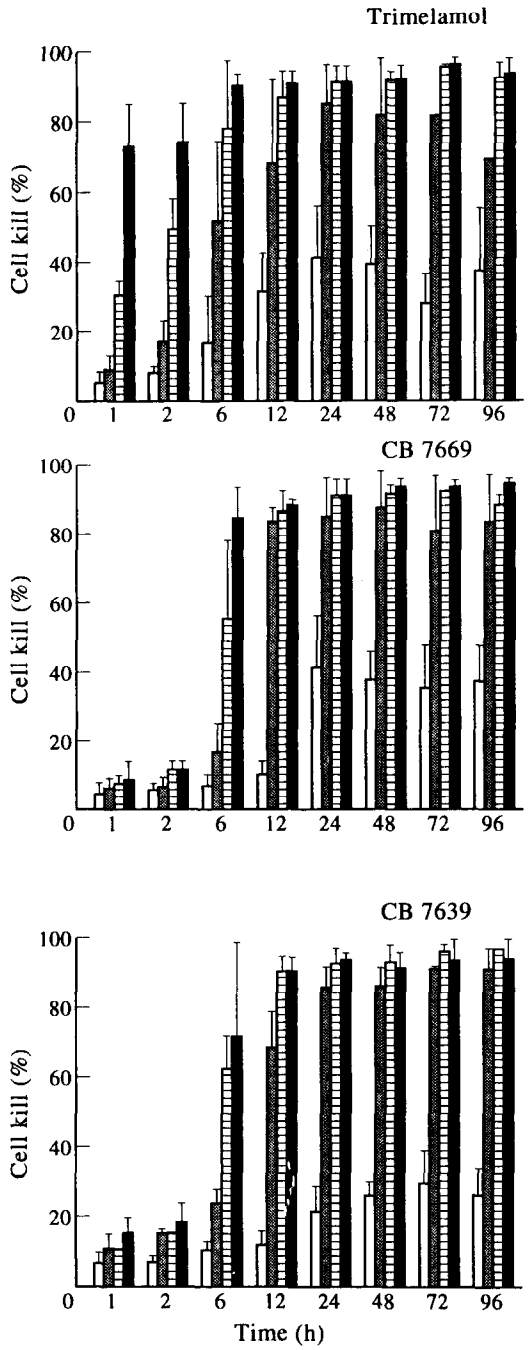



**Figure 6. Effect of exposure time on cytotoxicity seen for trimelamol and the stable analogues CB 7639 and CB 7669 seen in the human ovarian cancer cell line CH1. Error bars indicate the standard deviation obtained for three replicate sets of data. Key: □ 20 μM, □ 50 μM, □ 100 μM, ■ 200 μM.**

group of anticancer agents. Firstly, the wide interindividual variation in the (proposed) bioactivation of HMM [1] should be circumvented by the use of a hydroxymethylated analogue, e.g. TM. Secondly, the formulation difficulties encountered during the clinical trials of TM should be overcome by the use of more stable analogues suitable for prolonged infusion which do not lead to insoluble dimer formation, as has been previously described for the former [9, 11]. Indeed, CB 7669, CB 7547 and CB 7639 all show markedly superior stability in solution, with half-life values in 0.9% sodium chloride at room temperature of 5700 and 8090 min, respectively, compared with a value of 275 minutes for TM [12]. It was evident from data obtained in the present study that in stabilising the tris(hydroxymethyl) melamine molecule, there was a concomitant change in the time course of cytotoxicity. The cytotoxic activities of CB 7669 and CB 7639 only matched those of TM after a 12-h exposure *in vitro*. However, this enhanced stability could offer significant advantages over TM in terms of prolonged i.v. administration. Further studies are planned employing the use of osmotic mini pumps in murine human tumour xenograft models which will address the activity of these analogues in prolonged infusion regimens.

Our current studies continue to focus on the mechanism of action for the methylmelamines. A key issue concerning their proposed cytotoxic action is the tenet that metabolism to yield *N*-(hydroxymethyl) species is a prerequisite for antitumour activity. We have shown data suggesting that melamine compounds, containing three hydroxymethyl groups, are, in general, more cytotoxic than those containing one or two such moieties. This observation is in line with studies which point to the *N*-(hydroxymethyl) species as being pivotal in terms of antitumour activity. Certainly, *in vitro* systems incorporating HMM have shown markedly enhanced cytotoxicity by prior incubation with liver microsomes [4]. Our data indicate that HMM has markedly reduced cytotoxicity compared to TM. The variation in $IC_{50}$ seen throughout the cell line panel used (Table 5) could be due to intrinsic cellular levels of P450 monooxygenases to yield various *N*-(hydroxylated) forms of HMM. Metabolic activation and cytotoxicity studies have suggested that the carbinolamine intermediate formed during the initial HMM *N*-demethylation [leading to *N*-(hydroxymethyl) pentamethylmelamine] could lead to a reactive iminium species which covalently binds to macromolecules such as DNA [14]. Based on this hypothesis, the methylmelamines have been proposed to be alkylating agents. TM has been shown to behave as a DNA crosslinking agent using a plasmid DNA gel electrophoresis technique which quantitates crosslinked DNA following drug treatment [11], a result which has been confirmed by us [26]. We are currently exploring the use of this assay and also alkaline elution using whole cells in tissue culture to measure DNA interstrand crosslinks following exposure to the *N*-(hydroxymethyl)melamines.

Local formaldehyde release during the breakdown of *N*-(hydroxymethyl)melamines may contribute to cytotoxicity. Formaldehyde itself can give rise to the formation of DNA protein crosslinks [15], and TM has been shown to produce high levels of these lesions in treated cells [15, 27]. Formaldehyde-equivalents have been described in the plasma of mice treated with *N*-methylmelamines [28], and were subsequently confirmed to be formaldehyde precursors rather than formaldehyde itself. These precursors may represent mono- or multi-*N*-(hydroxymethyl) derivatives of *N*-methylmelamines. Hydroxymethyl groups might be transferred intact or as formaldehyde and sequestered by molecules such as glutathione. We are currently exploring the role of cellular glutathione in the modulation of the cytotoxicity of the *N*-(hydroxymethyl)melamines. Ross and associates [29] examined a number of HMM analogues, and found a correlation between their antitumour activity and the plasma levels of formaldehyde precursors they produced. However, these authors state that if this relationship was a quantitative correlation then the HMM analogue, pentamethylmelamine (PMM), which produced significantly higher peak levels and greater AUC values of formaldehyde precursors than the other analogues in their study, should be expected to be a better antitumour agent than HMM. This has not been borne

out in *in vivo* studies and, in fact, both HMM and PMM have similar therapeutic indices of the PC6 model [5]. However, Ross and colleagues [29] do go on to suggest that a plasma threshold of formaldehyde precursors has to be exceeded for an *N*-methylmelamine to exert a significant antitumour effect. Acquired resistance to formaldehyde in the L1210HCHOR line resulted in cross-resistance with TM. Along with the 4-fold cross-resistance to formaldehyde seen in the WTM/R and W7669/R lines, the role of formaldehyde release in the cytotoxic action of the *N*-(hydroxymethyl)melamines is highlighted. It would appear that, at least *in vitro*, compounds such as TM have at least a dual mechanism of action, i.e. that of local formaldehyde release producing cytotoxicity and perhaps to a lesser extent that of DNA alkylation.

Data presented herein demonstrate that a marked lack of cross-resistance to TM exists in a number of cell lines with a variety of drug-resistant phenotypes. The Walker 256 resistant variant line WR has been previously described [16] as possessing a broad cross-resistance with several bifunctional alkylating agents and possessing 27-fold resistance to its inducing agent chlorambucil. The mechanism underlying this form of drug-resistance has hitherto not been characterised, and there was shown to be little difference in the binding of a variety of agents to DNA or in the formation of DNA-interstrand crosslinks between the WS and WR lines [16]. In spite of the demonstration that TM can behave as a bifunctional alkylating agent, inferred by the demonstration of its ability to form DNA interstrand crosslinks, we were unable to demonstrate any cross-resistance to this agent in the WR line. However, the resistant lines WTM/R and W7669/R both showed modest cross-resistance to the classical alkylating agent melphalan. The major site of DNA adduct formation for this compound is established as being on the N-7 position of guanine. In addition, metabolites of the alkylating agent cyclophosphamide have been shown to form similar adducts. We were only able to see low levels of alkylating agent resistance, in line with the findings of Teicher and coworkers [30]. In addition, the same authors found that resistance to alkylating agents, such as BCNU in the Raji (Burkitt's lymphoma) cell line, does not necessarily result in cross-resistance to other alkylating agents, such as nitrogen mustard. Cross-resistance patterns in alkylating agent-resistant cell lines can often be complex and difficult to interpret. One explanation put forward is that as alkylating agents are mutagenic, these cell lines may be expected to show more heterogeneity than their parental counterparts [31].

Stable analogues of TM, such as CB 7669, may prove useful as second-line or 'salvage' chemotherapy in heavily pretreated ovarian cancer patients, as suggested by a number of studies using HMM (reviewed in [32]) and from the clinical trials involving TM [8, 9]. A recent report by Thigpen and associates [33] discusses second-line chemotherapy for recurrent carcinoma of the ovary. For the treatment of clinically resistant disease (defined as progression during or within 6 months of front-line platinum-based therapy), the authors cite paclitaxel, ifosfamide, and HMM as being effective agents. Our data suggest that TM is an effective agent against platinum-resistant tumours since we were unable to demonstrate any cross-resistance to the *N*-(hydroxymethyl)melamines, in a panel of platinum-resistant cell lines, with a variety of resistance phenotypes, including drug transport defects, altered glutathione metabolism and DNA repair. As well as providing a rationale for their use in heavily pretreated cancers, possibly including the use of classical dealkylating agents such as cyclophosphamide and also cisplatin, these data point to a possible alternative locus of action for the methylmelamines. The lack of cross-resistance to TM seen in the cell lines with acquired resistance to platinum and bifunctional alkylating agents is an important observation.

Along with paclitaxel and ifosfamide, the methylmelamines still show promise as agents for second-line chemotherapy of ovarian cancer. It is hoped that these studies will lead to the development of new agents for clinical study and that the lack of cross-resistance observed in preclinical models will be confirmed in man.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# *Viscum album* L. Extracts Reduce Sister Chromatid Exchanges in Cultured Peripheral Blood Mononuclear Cells

A. Büssing, T. Azhari, H. Ostendorp, A. Lehnert and K. Schweizer

**Increasing concentrations of *Viscum album* L. extracts were shown to significantly reduce sister chromatid exchange (SCE) frequency of phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) of healthy individuals. This decrease of SCE could not be explained either by changes in lymphocyte subpopulations, by cytostatic effects of the drug or by accelerated proliferation of PHA-stimulated PBMC. Currently, no other cells tested have shown this effect. One therapeutic effect of these anti-mutagenic drugs could be a stabilisation of mononuclear blood cell DNA.**

**Key words: sister chromatid exchange, SCE, mistletoe extracts, *Viscum album* L., DNA, anti-mutagenic, peripheral blood mononuclear cells**
*Eur J Cancer*, Vol. 30A, No. 12, pp. 1836–1841, 1994

## INTRODUCTION

IT IS generally agreed that *Viscum album* L. (mistletoe) preparations may play a beneficial role during and after conventional cancer therapy [1]. Explanations of these effects usually postulate an enhanced immune reactivity or immune reconstitution with reappearance of anti-tumour activity. Notwithstanding the crucial and controversally discussed role of the immune system in tumour surveillance and defence, the reconstitution of a therapeutically altered immune system may be of substantial benefit for the patient.