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Comparative studies of DNA cross-linking reactions following methylene dimethanesulphonate and its hydrolytic product, formaldehyde

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Summary. The technique of alkaline elution was employed to study the interactions of methylene dimethane sulphonate (MDMS) and formaldehyde (HCHO) with DNA from Yoshida lymphosarcoma cells treated with these agents. MDMS and HCHO produced a proteinase sensitive filter retention which indicated the presence of DNA–protein cross-links. MDMS also produced some proteinase K-resistant filter retention which was believed to indicate DNA–interstrand cross-linking, whilst only single-strand breaks could be detected following treatment with HCHO. Co-incubation with semicarbazide prevented all DNA–protein cross-links induced by MDMS and HCHO as well as single-strand breaks, most obvious following HCHO treatment. Semicarbazide also reduced HCHO-induced cytotoxicity in the Yoshida lymphosarcoma cell line, while no significant alteration in MDMS-induced cytotoxicity was observed. These results suggest that HCHO-induced DNA–protein cross-links and single-strand breaks do not contribute to MDMS-induced cytotoxicity, and therefore the small but significant level of MDMS-induced DNA–interstrand cross-links is the most likely cytotoxic lesion of this agent.

Introduction

Methylene dimethane sulphonate (MDMS) is the first member of a series of straight-chain dimethane sulphonic acid esters having the general formula
$H_3C\text{-}SO_2\text{-}O\text{-}(CH_2)_n\text{-}O\text{-}SO_2\text{-}CH_3$
Butane 1,4-dimethanesulphonate (busulphan) commenced clinical trials for the treatment of chronic myeloid leukaemia [12, 13] and is still routinely used in the treatment of this disease. MDMS, however, was found to possess marked antitumour activity against the rodent Yoshida lymphosarcoma [9] and is currently undergoing clinical trials.

The mechanism of action of the dimethane sulphonic acid esters is still unclear; however, the formation of 1,4-di (guanin-7-yl) butane on reaction of busulphan and calf thymus DNA has been suggested [5], although verification using an appropriate marker compound was not undertaken at the time. More recently, by reaction of busulphan with native DNA [24] 1,4-di (guanin-7-yl) butane has been identified.

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Attempts to identify DNA–interstrand cross-linking following busulphan treatment using physicochemical techniques such as resistance to thermal denaturation [19], caesium chloride gradients [25] and dispersion of DNA in high salt concentrations [1] have failed. This may have been due to the insensitivity of such techniques and also the low reactivity of busulphan with isolated DNA.

The more sensitive technique of alkaline elution developed by Kohn et al. [18] was employed by us to identify any DNA–interstrand cross-linking. A proteinase K-resistant filter retention in Yoshida lymphosarcoma (YS) cells following both busulphan [3] and MDMS [2] treatment was believed to represent DNA–interstrand cross-linking.

MDMS is, however, rapidly hydrolysed ($t_{1/2}$ = 22 min at 37 °C) [11] to release formaldehyde (HCHO). HCHO alone is known to be cytotoxic [22], and reacts with DNA in cultured mammalian cells, forming DNA–protein cross-links and single-strand breaks [8, 16, 21] and covalent base modifications [20]. These lesions may be important in the mutagenic [14] and carcinogenic [17] activity of HCHO. It has also been suggested [15] that HCHO, through an inhibition of normal cellular DNA repair processes, could act to potentiate the effects of DNA damage induced by itself or by other DNA – damaging agents. Apart from a direct action, therefore HCHO could contribute to MDMS-induced cytotoxicity by inhibiting normal DNA repair processes. The possible contribution of HCHO to MDMS-induced cytotoxicity could be studied by inactivating the HCHO released by the drug and thus distinguishing the effects of the parent molecule from those of its hydrolytic product. Semicarbazide inactivates HCHO by direct formation of non-toxic semicarbazone and has been shown to prevent HCHO-induced [22] and methylmelamine-induced [22, 23] cytotoxicity in the L1210 cell line.

In the present study, semicarbazide has been employed to correlate the role of DNA cross-linking reactions following MDMS and HCHO treatment with the cytotoxicity of the two agents in the YS cell line.

Materials and methods

Chemicals. MDMS was prepared by the method of Emmons and Ferris [7]. HCHO was purchased from Evans Medical Ltd. (Liverpool, UK) as a 36% (w/v) solution. Dimethyl sulphoxide (DMSO) was obtained from Sigma Chemical Company Ltd. (Dorset, UK) and purified by

passage through an alumina column. Semicarbazide hydrochloride (Gold Label) was purchased from Aldrich Chemical Company Ltd. (Dorset, UK). All other chemicals were of analytical grade or the highest purity available.

Alkaline elution studies. The technique employed was essentially the same as previously described [3]. However, in the present studies irradiated cells were layered onto polycarbonate filters (Nucleopore Comp., California, USA).

Exponentially growing YS cells were template-labelled with (methyl-^{14}C-) thymidine overnight and then treated with semicarbazide (10 m*M*) 1 h prior to the administration of MDMS (250 μ*M*) or HCHO (250 μ*M*); 4 h later the drugs were removed in the usual manner. Drug-treated ^{14}C-labelled cells were mixed with ^{3}H-labelled untreated cells prior to gamma irradiation (5 Gy, 4 °C) from a ^{137}Cs source. ^{3}H-Labelled untreated YS cells were employed as internal standard cells to compensate for differences in flow rates between filters. Proteinase K (0.5 mg/ml, Sigma Chemical Co., Dorset, UK) was included in the lysis solution to distinguish between filter retention due to DNA–DNA interstrand cross-links and that of DNA–protein cross-links.

Cell culture. The YS cell line was derived as previously described [10]. Cells were routinely incubated at 37 °C in the dark in an atmosphere of 95% air, 5% CO_2 in Fischer's medium (Gibco-Biocult Ltd.) and supplemented with 20% (v/v) horse serum (Gibco-Biocult Ltd.), 1.4 m*M* *L*-glutamine (Flow Laboratories), penicillin G (500 IU/ml), streptomycin sulphate (80 mg/l) and bicarbonate (0.11% w/v), giving a final pH of 7.5. All cells were routinely screened for mycoplasma using Hoechst 33258 [6] and found to be free of contamination.

Exponentially growing cells (1×10^5 cells/ml) were treated with drug or vehicle (controls) in triplicate for 1 h, unless otherwise stated. Drug-containing medium was removed after centrifugation (400 *g*, 5 min, 37 °C) prior to resuspension of the cell pellet in fresh medium. Samples (0.5 ml) were removed at 0, 24, 48, and 72 h post treatment and counted using an electronic Coulter Counter (Coulter Electronics, Luton, Beds., UK) previously calibrated using YS cells. Cells were diluted when necessary to maintain exponential growth ($< 3 \times 10^5$ cells/ml) over 24 h. Cytotoxicity was assessed as a percentage of control growth over 72 h post treatment and an unpaired Student's t-test was applied to the data at the 72-h time point. The doubling time of the parental YS cell line used in these studies was 18.8 h.

MDMS was dissolved in DMSO and used immediately. The final non-toxic concentration of DMSO in cell incubate was 1% (v/v). HCHO and semicarbazide were made up in phosphate-buffered saline pH 7.3. Semicarbazide hydrochloride was neutralized with sodium hydroxide to pH 7.5. Semicarbazide and HCHO were filter-sterilized through a 0.22-μm filter before use. In semicarbazide protection experiments, cells were pretreated for 60 min and left in situ throughout MDMS and HCHO treatments. In semicarbazide toxicity studies, cells were treated for 2 h before removal of the drug.

Results

Growth inhibition studies

MDMS produced a time- and dose-dependent decrease in cell growth (Fig. 1). The lowest dose employed (0.5 μ*M*) did not significantly inhibit cell growth ($P > 0.5$). However, doses above 0.5 μ*M* all produced significant reductions in cell growth (2.5 μ*M*, $P < 0.01$ to 30 μ*M*, $P < 0.001$). The ID_{50} (drug concentration causing 50% inhibition of growth compared with control cells) for MDMS at the 72-h time point was estimated as 5 μ*M* (51.7% ±3.0% control growth). This value is in accordance with previous studies [4]. HCHO was found to be approximately 50 times less cytotoxic to the Yoshida lymphosarcoma cell line than MDMS (Fig. 1). The ID_{50} for HCHO in these studies was found to be approximately 250 μ*M* (44% ±3% control growth). Semicarbazide was not found to be significantly toxic to YS cells up to doses of 10 m*M* (Fig. 1). Above this dose, however, a time- and dose-dependent decrease in cell growth was observed, with a significant ($P < 0.001$) inhibition of growth at 50 m*M* semicarbazide.

Semicarbazide prevented HCHO-induced cytotoxicity such that at the 72-h time point, cells treated with HCHO (250 μ*M*) in the presence of semicarbazide (86% ±3%) had percentage growth similar to that of the control cells (91%

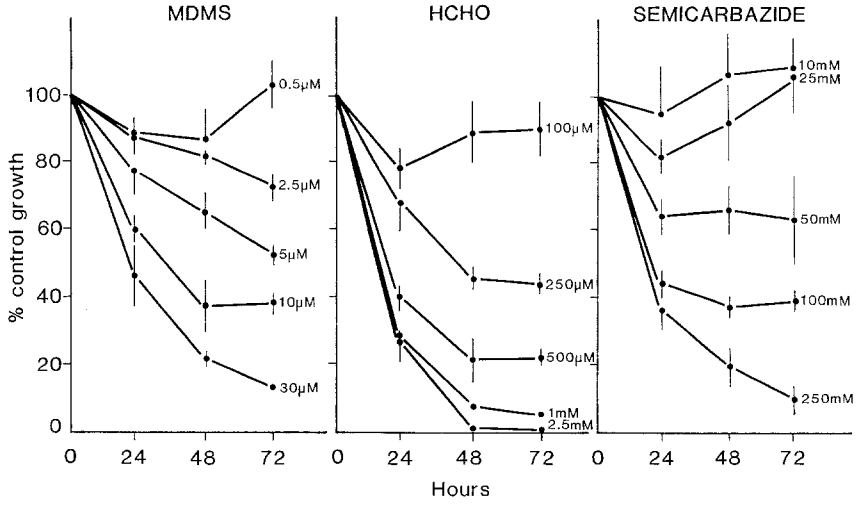

Fig. 1. Growth inhibition data of YS cells in Fisher's medium with 20% horse serum over 72 h. Data for MDMS, HCHO (1 h treatment) and semicarbazide (2 h treatment) alone are given. Mean and standard deviation of triplicate determinations shown at each data point

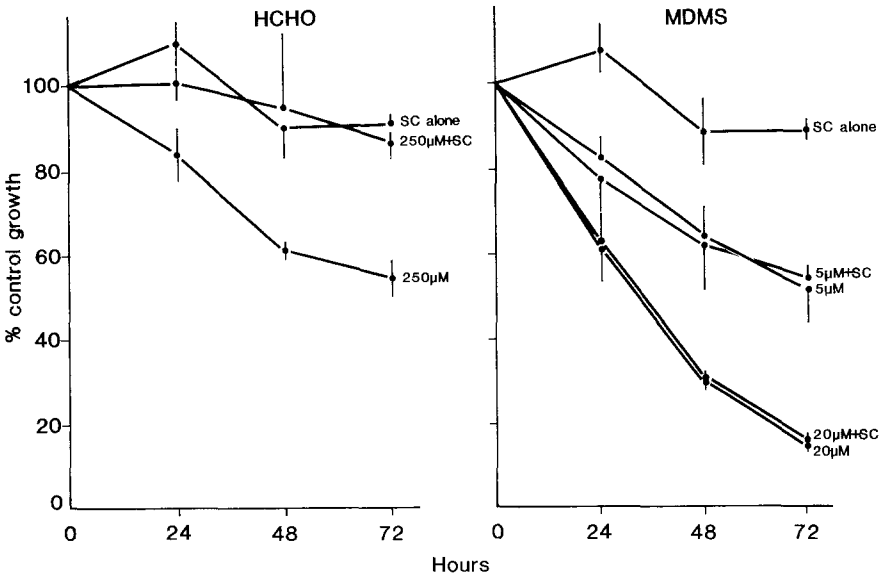

Fig. 2. Growth inhibition of YS cells over 72 h following treatment with HCHO and MDMS alone, at the treatment levels indicated, and in the pressence of semicarbazide (*SC*; 10 m*M*). Mean and standard deviation of triplicate determinations shown at each data point

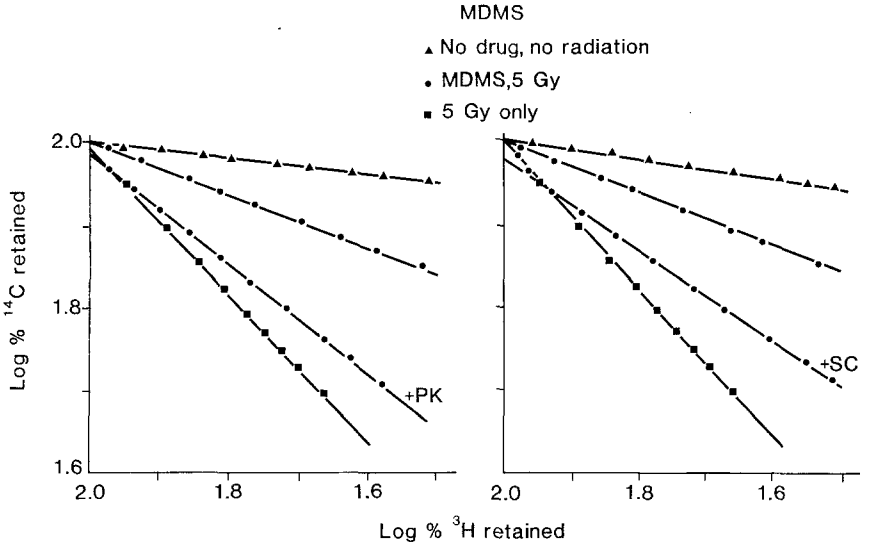

Fig. 3. Alkaline elution profile of YS cell DNA after treatment with MDMS (250 μ*M*, 4 h). Results of two separate experiments are shown, except for the treatment with proteinase K (*PK*; 0.5 mg/ml/1 h) or semicarbazide (*SC*; 10 m*M*, 5 h total treatment) as shown

Table 1. Effect of semicarbazide (SC) on the elution of YS cell DNA

Sample	Percent ^{14}C retained on filter	
	No proteinase	Proteinase
Unirradiated	83.8 (1.0)	84.5 (2.6)
Unirradiated + SC	83.4 (3.0)	82.2 (1.9)
Irradiated	57.0 (1.4)	55.3 (2.7)
Irradiated + SC	58.1 (0.4)	54.0 (1.2)

Data expressed as mean values (SD) of triplicate determinations

±2%). In a parallel experiment, no significant change in the cytotoxicity of two doses of MDMS (5 μ*M* and 20 μ*M*) was observed in the presence of semicarbazide (Fig. 2).

Alkaline elution studies

The sensitive technique of alkaline elution was employed to study the effect of MDMS and HCHO on DNA derived from YS cells.

The filters employed in these studies retain 83.8% ± 1.0% of unirradiated YS cell ^{14}C-DNA at 50% elution of the internal standard (Table 1). The retention of the DNA from these cells is not affected by proteinase K or semicarbazide treatment. Elution of ^{14}C-DNA increases when cells are gamma-irradiated (5 Gy) such that only 57% ± 1.4% of ^{14}C-DNA is retained on the filter at 50% elution of the internal standard. Combinations of proteinase K and semicarbazide treatments show a slight, non-significant reduction in ^{14}C-filter retention, suggesting that proteinase K and semicarbazide do not significantly alter the elution profile of YS cell DNA.

MDMS treatment produces a marked filter retention of irradiated ^{14}C-DNA (86.7% ± 5.6%) similar to that observed with unirradiated cell DNA (88.6% ± 2.8%). However, treatment with proteinase K reverses most of this filter retention (Fig. 3), suggesting the presence of DNA – protein cross-links. A similar result is obtained by treatment of the cells with semicarbazide, which inactivates any HCHO produced on hydrolysis of MDMS (Fig. 3). However, a certain fraction of ^{14}C-filter retention is resistant to proteinase K and semicarbazide treatments. This resistant fraction was attributed to MDMS-induced DNA – interstrand cross-linking (Fig. 5) and is in agreement with the results of Bedford and Fox [4].

Table 2. Effect of semicarbazide (SC) on the alkaline elution characteristics of YS cells treated with MDMS and HCHO

Sample	Relative retention		Cross-links per 10^9 daltons	
	−PK	+PK	−PK	+PK
MDMS	3.49 (0.94)	1.24 (0.15)	1.03 (0.30)	0.13 (0.07)
MDMS + SC	1.19 (0.15)	1.27 (0.04)	0.10 (0.08)	0.14 (0.02)
HCHO	3.10 (0.94)	0.72 (0.10)	0.90 (0.33)	0[a]
HCHO + SC	0.95 (0.11)	1.04 (0.04)	0.01 (0.01)	0.02 (0.02)

Values in parentheses are SD. PK, proteinase K

[a] Single-strand breaks were observed at a level of 0.55 (0.23) per 10^9 daltons

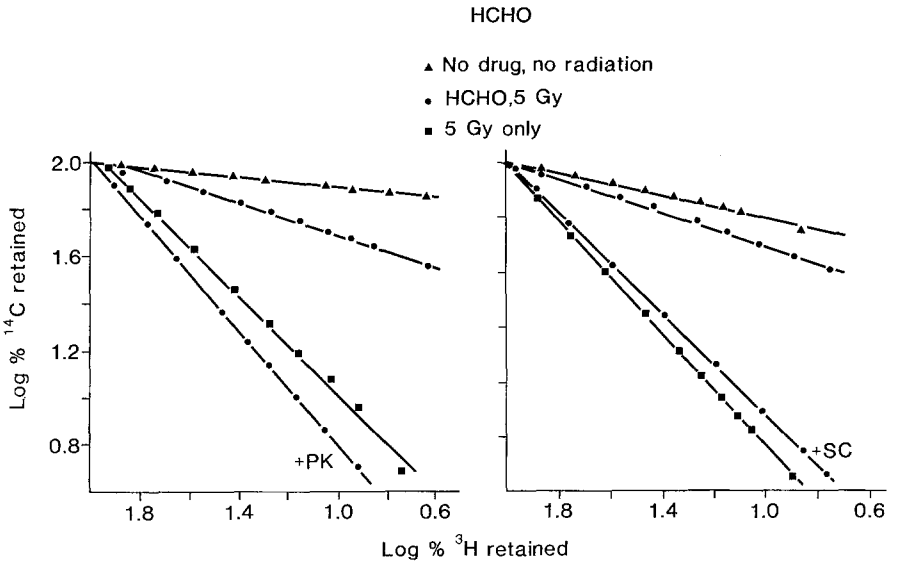

Fig. 4. Conditions as shown in Fig. 3 except that HCHO (250 μ*M*, 4 h) was used rather than MDMS

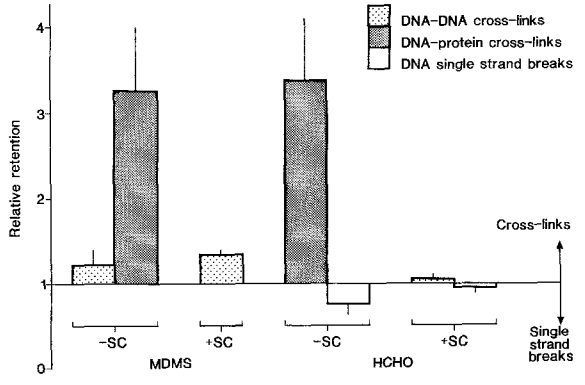

Fig. 5. Effect of semicarbazide (*SC*; 10 m*M*, 5 h) on MDMS and HCHO (250 μ*M*, 4 h)-induced DNA cross-links in the YS cell line. Mean and standard deviation of four determinations shown at each bar. Relative retention is as defined in [3]

In a parallel experiment, HCHO was shown to induce proteinase K-sensitive filter retention (Fig. 4) attributable to DNA – protein cross-links. Treatment with proteinase K removes DNA – protein cross-links and, in this technique, unmasks some DNA single-strand breaks (Table 2, Fig. 5) induced by HCHO treatment, Semicarbazide treatment, however, inactivates sufficient HCHO present to prevent HCHO-induced DNA – protein cross-links as well as the DNA single-strand breaks that would otherwise have been seen on treatment with proteinase K (Figs. 4, 5).

Discussion

Both MDMS and HCHO have been shown to produce DNA – protein cross-links, which are preventable by concomitant treatment with semicarbazide. This result would thus imply that this lesion is the result of HCHO action in both cases. However, semicarbazide does not prevent all MDMS-induced filter retention in the alkaline elution studies. This proteinase K-resistant filter retention, believed to be due to DNA – interstrand cross-links, is unaffected by the level of semicarbazide used, and thus represents the activity of the parent drug and not its hydrolytic product, HCHO.

Proteinase K, in removing HCHO-induced DNA – protein cross-links, unmasks a small number of single-strand breaks. No statistically significant increase in DNA – interstrand cross-links, expected on removal of the HCHO component of single-strand break formation, could be observed after MDMS treatment in the presence of semicarbazide. However, to analyse this effect in a more quantitative way, many more measurements would be required to satisfy the statistical analysis, or an alternative, more sensitive system would have to be devised.

Thus, semicarbazide has been shown to prevent HCHO-induced DNA – protein cross-links and single-strand break formation, but not the DNA – interstrand cross-links produced by MDMS. Furthermore, semicarbazide can be shown to prevent the cytotoxicity of HCHO, but does not modify the cytotoxicity of MDMS. Thus, the small level of DNA – interstrand cross-links produced by

MDMS represents a strong candidate for the direct cytotoxic lesion of this drug, if the DNA proves to be the primary origin of the cytotoxic effect. HCHO-induced DNA – protein cross-links and single-strand breaks are therefore unimportant DNA lesions at cytotoxic concentrations of MDMS. In order to achieve comparable levels of toxicity to MDMS, it is necessary to increase the concentrations of HCHO to some 50-fold that expected on complete hydrolysis of MDMS. This difference in effectiveness may be related to an efficient repair process available within the cell for HCHO-induced DNA lesions, in agreement with observations made by other workers [16].

In conclusion, the DNA – interstrand cross-links frequency for MDMS in these studies remains similar to that observed in the presence of semicarbazide (Fig. 5), suggesting that MDMS is responsible for DNA – interstrand cross-linking, while HCHO, its hydrolytic product, is responsible for the extensive DNA – protein cross-links and, by inference, the DNA single-strand breaks observed following MDMS treatment. Since semicarbazide does not affect MDMS-induced cytotoxicity, the most likely candidate for the cytotoxic effectiveness of the drug is the induction of DNA-interstrand cross-links.

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