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DNA–DNA INTERSTRAND CROSSLINKING BY DIMETHANESULPHONIC ACID ESTERS

CORRELATION WITH CYTOTOXICITY AND ANTITUMOUR ACTIVITY IN THE YOSHIDA LYMPHOSARCOMA MODEL AND RELATIONSHIP TO CHAIN LENGTH

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Abstract—Members of the homologous series of nine antitumour dimethanesulphonic acid esters, with the exception of ethylene dimethanesulphonate (EDMS), were found to cause DNA–DNA interstrand crosslinking in cells derived from the transplantable rodent Yoshida lymphosarcoma. The ability of the series to induce interstrand crosslinks was compared with *in vitro* cytotoxicity and *in vivo* antitumour activity and is discussed in relation to their chain length and ability to span critical target distances in DNA.

Members of the homologous series of dimethanesulphonic acid esters of general formula: $H_3C{\cdot}SO_2{\cdot}O{\cdot}(CH_2)_n{\cdot}O{\cdot}SO_2{\cdot}CH_3$ ($n = 1$–10) have been shown to be active against a number of proliferating cell systems in experimental animals. With the exception of $n = 1$, the compounds have previously been tested for their antitumour activity against the Walker 256 rodent carcinoma [1], their selective neutrophil depressing activity [2] and their immunosuppressive activity [3]. In each case, optimal biological activity was observed with the 4-carbon chain member of the series, 1,4-dimethanesulphonyloxybutane (Myleran®, or busulphan). Later studies were carried out [4, 5] on the effects of the entire series including $n = 1$ on bone marrow elements and again busulphan ($n = 4$) demonstrated the maximum biological effect. Thus, on the basis of these antitumour and haemosuppressive properties in rats busulphan was subjected to clinical trials against chronic myeloid leukaemia [6] for which it is still one of the drugs of choice today. Maximum biological activity of this series was found also to parallel the ether/water solubility ratios which was greater for busulphan than for the other members of the series and suggested that the differential activities of the latter could be due to the pharmacokinetic distribution of drug to the target organs [3]. Ethylene dimethanesulphonate ($n = 2$) is ineffective in depressing the haemopoietic system in rats [2] but has potent antispermatogenic properties [7] in mice and rats, which has been attributed to a drug-induced inhibition of testosterone synthesis [8]. The mechanism of action of busulphan and related esters of dimethanesulphonic acid is still unclear. An immobile 'spot' on a paper chromatogram of the reaction products of [^{14}C] busulphan and calf thymus DNA [9] was thought to represent the formation of 1,4-di-(guanin-7-yl) butane, although verification using a marker compound was not undertaken at the time. Recently, however, this product has been identified by reaction of busulphan with native DNA [10]. Previous attempts to identify interstrand crosslinking after busulphan using classical physicochemical techniques, such as resistance to thermal denaturation of DNA [11, 12], caesium chloride gradients [13], and dispersion of DNA in high salt concentrations [14] failed, which may have been due to their insensitivity and also the low reactivity of busulphan with isolated DNA (estimated to be as little at 0.7% [9]). Recently, however, we have used the more sensitive technique of alkaline elution to identify the presence of a proteinase-resistant filter retention in Yoshida lymphosarcoma cells after busulphan [15] as well as methylene dimethanesulphonate (MDMS, $n = 1$) [16] which we concluded to be due to DNA–DNA interstrand crosslinking.

In this present study, the ability of the entire series of dimethanesulphonates ($n = 1$–9) to induce DNA–DNA interstrand and DNA–protein crosslinks is examined and compared with their *in vitro* and *in vivo* activity using the Yoshida lymphosarcoma model.

MATERIALS AND METHODS

Chemicals. Busulphan was synthesized according

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Abbreviations: LD_{50}, Dose producing 50% cell or animal death; MDMS, methylene dimethanesulphonate; EDMS, ethylene dimethanesulphonate; PDMS, propylene dimethanesulphonate; hexa-DMS, hexane dimethanesulphonate; hepta-DMS, heptane dimethanesulphonate; octa-DMS, octane dimethanesulphonate; nona-DMS, nonane dimethanesulphonate.

to the method of Timmis [17] by the reaction of 1,4-butanediol and methanesulphonyl chloride. The other esters were synthesized according to the method of Emmons and Ferris [18] by the reaction of the appropriate dibromoalkane with silver methanesulphonate in acetonitrile solvent. The products were recrystallized from hot ethanol and their purity was verified by melting point and i.r. spectral absorption measurments.

Cell culture. The Yoshida lymphosarcoma cells used were obtained as previously described [19]. All cells were grown in Fischer's medium (Flow Laboratories, Irving, U.K.) supplemented with horse serum (20% v/v; Gibco Bio-cult, Glasgow, U.K.) in the presence of penicillin G (1000 U/ml) and streptomycin (60 U/ml). Cells were incubated in an atmosphere of 5% CO_2:95% air and were subcultured while in exponential growth at 5×10^4 cells/ml. All cells were routinely screened for mycoplasmal contamination using Hoechst 33258 and found to be free. To determine survival, cells were treated with each member of the series dissolved in a non-cytotoxic concentration of dimethylsulphoxide (DMSO) for 1 hr (with the exception of MDMS, where treatment was limited to 30 min., since the half-life of hydrolysis at 37° is 22 min [20]). Drug containing medium was removed after centrifugation (150 *g*; 5 min; 37°) prior to resuspension of the cell pellet in fresh medium at 37°. Cell survival was estimated using the method of back-extrapolation of growth-curves [21].

Antitumour studies. Outbred female rats bearing Yoshida tumours were treated on day 5 after routine tumour transplantation using a single intraperitoneal injection of the appropriate dimethanesulphonate finely suspended in arachis oil. The tumour vol. was measured daily using calipers, until no discernible nodule could be detected, or the tumour volume had exceeded 20 cm^3 whereupon the animal was sacrificed. Animals were allowed food and water *ad lib.* and kept in conditions of diurnal lighting.

Alkaline elution of DNA. The technique used was as previously described [16], using [^{3}H]-labelled internal standard cells to compensate for differences in flow rates between replicate filters and including proteinase K (0.5 mg/ml; Sigma Chemical Co.) in the lysis solution (flow stopped for 1 hr at room temperature) to distinguish between retention due to DNA–DNA interstrand crosslinks and that of DNA–protein crosslinks.

RESULTS

Cell survival

Cell survival curves for the nine dimethanesulphonate esters used were obtained. Both ethylene dimethanesulphonate (EDMS, $n = 2$) and propylene dimethanesulphonate (PDMS, $n = 3$) are comparatively non-cytotoxic even at concentrations bordering the limits of solubility in culture medium (>1000 μM). Concentrations of agent producing 50% cell kill (LD_{50}) in Yoshida cells are given in Table 1. Maximum cytotoxicity against the YS cells was shown by hexane dimethanesulphonate ($n = 6$; $LD_{50} = 1.5$ μM) which was over 20 times more cytotoxic than busulphan ($n = 4$; $LD_{50} = 30.8$ μM).

Antitumour activity

The antitumour activity of members of the series was expressed as a therapeutic index (I/F) where I (tumour inhibition index) is the ratio of the tumour vols. of treated and control animals at 9 days after routine transplantation, and F is the ratio of antitumour dose given to the LD_{50} in rats. Table 2 shows antitumour data for the YS tumour line. The maximum therapeutic index was observed after octane dimethanesulphonate (3.17) which was more than twice as effective as busulphan (1.14) as an antitumour agent based on these criteria. Low therapeutic indices were seen after EDMS (0.64) and PDMS (0.29) which paralleled the lack of *in vitro* cytotoxicity.

DNA crosslinking

Crosslinks were assayed in YS cells after a single 250 μM dose of each member of the series dissolved in DMSO. With the exception of MDMS (30 min), the treatment time was for 1 hr followed by removal of drug-containing medium (as above) and resuspending the cells in fresh medium (37°). It is to be noted that this dose level is supralethal for all drugs used to this cell line, in order to produce measurable levels of crosslinking. It is, therefore, necessary to assume that these levels of DNA–DNA crosslinking

Table 1. LD_{50} data for YS cells treated with members of the dimethanesulphonate series

Member of series	No. of methylene groups	Mol. wt	Alkylating Range (Å)	LD_{50} (μM)
MDMS	1	204	2.2	5.0
EDMS	2	218	2.4–3.5	>1000
PDMS	3	232	1.8–4.7	>1000
Busulphan	4	246	0.6–6.0	30.8
Penta-DMS	5	260	0.0–7.1	4.5
Hexa-DMS	6	274	0.0–8.5	1.5
Hepta-DMS	7	288	0.0–9.8	3.5
Octa-DMS	8	302	0.0–10.8	1.75
Nona-DMS	9	316	0.0–12.3	41.1

The measurements of alkylating range were determined from Dreiding® models and represent the maximum and minimum stretch of these molecules.

Table 2. Antitumour data for YS tumour line treated intraperitoneally in arachis oil with members of the dimethanesulphonate series

Member	LD_{50}* (mg/kg)	Dose† (mg/kg)	F‡	I§	Therapeutic index (I/F)
1	30	10	0.33	0.93	2.78
2	280	50	0.18	0.12	0.64
3	70	50	0.71	0.21	0.29
4	20	15	0.75	0.85	1.14
5	28	12	0.43	0.95	2.20
6	70	40	0.57	0.89	1.56
7	120	50	0.42	0.88	2.11
8	180	50	0.28	0.88	3.17
9	200	150	0.75	0.94	1.25

* Values from Dunn and Elson [4].
† Dose of antitumour compound administered intraperitoneally in arachis oil.
‡ Fraction of LD_{50} given as antitumour dose.
§ Tumour inhibition index (derived as in text).

will be much higher but proportional to those that could be measured if the technique were capable of demonstrating this. However, the prime object was to determine if the structural differences in this series of alkylating agents resulted in qualitative differences in the type and extent of DNA–DNA and DNA–protein interaction and, thus, measurements were made at 4 hr after termination of treatment, but at a relatively toxic level in order to optimise the crosslinking with minimal effect of any repair systems present [15]. Inclusion of proteinase K in the lysis solution of one half of the filters used allowed for a distinction to be made between DNA–DNA and DNA–protein crosslinks. Pooled relative retention values (the retention of irradiated cells relative to drug pretreated and irradiated cells at 50% retention of the internal standard—N.B. inverted in error in text only in previous paper 15) from three individual experiments are presented in Table 3. A comparative histogram of relative retention of DNA for each member of the series at constant molarity (250 μM) is shown in Fig. 1. With the exception of EDMS all of the dimethanesulphonates studied caused a proteinase-resistant filter retention which indicated the presence of DNA–DNA interstrand crosslinks. In addition, unambiguous proteinase-sensitive retention was observed for members 1 and 7–9 of the series, denoting the presence of DNA-protein crosslinks. The agent causing maximum DNA–DNA interstrand crosslinking was hexane dimethanesulphonate (hexa-DMS) with a frequency of 0.26 ± 0.06 crosslinks per 10^9 a.m.u. A barely significant level of DNA-protein crosslinks was observed after this agent. The frequency of interstrand crosslinks induced by hexa-DMS was six times greater than for busulphan. With the exception of MDMS, DNA interstrand crosslinking was seen to increase with increasing carbon chain length, reaching a maximum at $n = 6$ and then decreasing to nonane dimethanesulphonate (nona-DMS, $n = 9$). The lack of DNA–DNA interstrand crosslinking after EDMS contrasts with the results obtained using BCNU previously reported [22], where a dimethylene link was proposed.

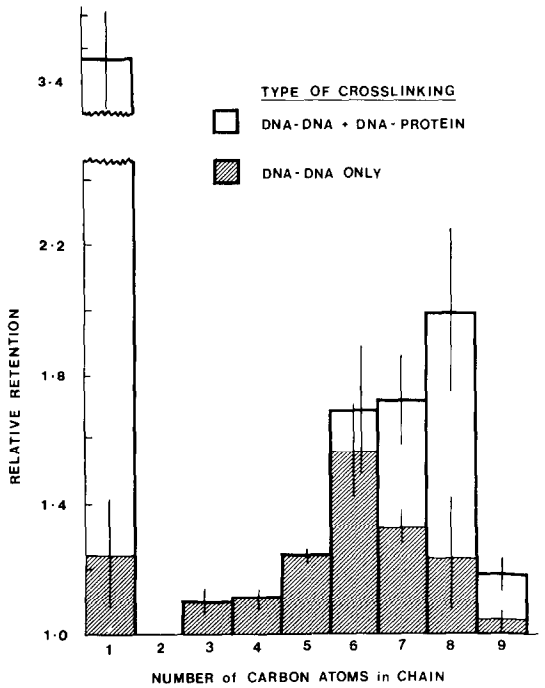

Fig. 1. Relative retention values for YS cells after 250 μM concentrations of members of the dimethanesulphonate series with or without proteinase K (0.5 mg/ml; 1 hr). Each value is the mean ±S.D. of triplicate determinations.

The relationship between DNA interstrand crosslinking and cytotoxicity was investigated by comparing LD_{50} values from the cell survival experiments (Table 1) with the frequency of interstrand crosslinks induced 4 hr after a single 250 μM dose of each member of the homologous series (from Table 3). Values of S.D. for the survival data were included in the analysis. A graph of $1/LD_{50}$ against the frequency of interstrand crosslinks is shown in Fig. 2. Some evidence for a correlation ($r = 0.810$) between cytotoxicity and the ability to induce DNA–DNA interstrand crosslinks under the conditions we employed was observed. However, octane dimethanesulphonate (octa-DMS, $n = 8$) appeared to diverge from this relationship and suggested that it possessed a greater degree of cytotoxicity than its ability to induced DNA–DNA interstrand crosslinks would imply.

DISCUSSION

The relative ability of members of the homologous series of dimethanesulphonates to induce DNA–DNA interstrand crosslinks was investigated and found to vary according to the number of carbon atoms in the alkylating chain. This observation suggests that the frequency of crosslink formation by each member of the series will depend upon the ability of the alkylating moiety to span critical nucleophile distances within the DNA. Using Dreiding® stereo-models, it was possible to measure range of distances separating the two alkylating groups (formed after the loss of both methane-sulphonate moieties) for each member of the series (Table 1).

Table 3. Relative retention and crosslink frequency for YS cells 4 hr after treatment with 250 μM concentrations of each dimethanesulphonate

Member	No. of carbon atoms in chain	Mean relative retention (± S.D.)		Crosslinks/10^9 a.m.u.	
		−PK	+PK	−PK	+PK
MDMS	1	3.49 ± 0.55	1.25 ± 0.16	1.01 ± 0.19	0.13 ± 0.09
EDMS	2	0.85 ± 0.11	0.89 ± 0.07	None	None
PDMS	3	1.03 ± 0.03	1.09 ± 0.05	0.02 ± 0.01	0.05 ± 0.03
Busulphan	4	1.12 ± 0.07	1.07 ± 0.10	0.06 ± 0.03	0.04 ± 0.01
Penta-DMS	5	1.14 ± 0.01	1.24 ± 0.05	0.07 ± 0.01	0.11 ± 0.02
Hexa-DMS	6	1.69 ± 0.10	1.56 ± 0.14	0.33 ± 0.04	0.26 ± 0.06
Hepta-DMS	7	1.72 ± 0.14	1.33 ± 0.05	0.32 ± 0.06	0.15 ± 0.03
Octa-DMS	8	2.00 ± 0.26	1.23 ± 0.18	0.38 ± 0.09	0.11 ± 0.07
Nona-DMS	9	1.17 ± 0.06	1.04 ± 0.04	0.09 ± 0.03	0.02 ± 0.02

Values represent the mean ± S.D. of three individual determinations.

Busulphan, with a maximum extended configuration of 6.0 Å, would be unable to span the distance between N7 atoms of adjacent guanosine nucleotides on opposite DNA strands (a narrow groove distance of 8.0 Å, from Dreiding model, B form DNA) and, therefore, it is unlikely that the crosslinked adduct isolated by Tong and Ludlum [10] was due to interstrand crosslinks. However, the distance between the N7 atoms of adjacent guanosine residues on the *same* DNA strand is only 4.7 Å, suggesting that busulphan may be capable of producing an *intra*-strand crosslink. To explain the busulphan-induced *inter*strand crosslinking observed by alkaline elution, a crosslink between topographically closer nucleophilic centres, such as guanosine (O_6) and cytidine (N_4) (a distance of 5.6 Å in the wide groove) could, however, occur. Hexa-DMS, with a maximum extended configuration of 8.5 Å would be ideally suited to span the interstrand N7 distances. MDMS, with an alkylating chain length of only 2.2 Å would be far too small to span many of the nucleophile distances listed. However, the distance between the atoms involved in hydrogen bonding is approximately 2.3 Å and it is possible, therefore, that MDMS may be able to substitute for a hydrogen bond between the DNA strands. The local denaturation of DNA reported after treatment of calf-thymus DNA [23] with MDMS, may be a consequence of such an interaction.

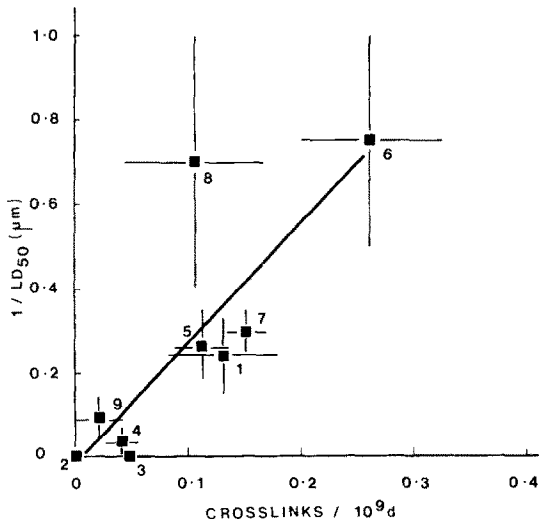

Fig. 2. Relationship between DNA–DNA interstrand crosslink frequency (calculated as in ref. [16]) and in the reciprocal of $1/LD_{50}$ in YS cells (from Table 1) for the dimethanesulphonate series.

The ability of members of the dimethanesulphonate series to span only selected target nucleophile distances, coupled with the availability and reactivity of these sites, could be an important factor in determining the relative ability of these esters to crosslink DNA.

With the exception of octa-DMS, a good correlation was obtained between *in vitro* cytotoxicity and the ability of the series to induce interstrand crosslinks. The fact that octa-DMS was nearly as cytotoxic to YS cells as hexa-DMS, yet induced only half as many crosslinks at an equimolar concentration, suggests either that (a) the nature of the interstrand crosslink may be an important factor in determining cytotoxicity or (b) that the high level of DNA–protein crosslinking may in the case of this compound, be contributary to its lethal action.

The correlation between antitumour activity of the series *in vivo* and their ability to form interstrand crosslinks was less clear. In this study the peak of activity occurred with octa-DMS. The difference, however, may be related to the differences in pharmacokinetics within the series and at this stage it would not be justified to make such a comparison.

The DNA–protein crosslinking observed for members 1 and 7–9 of the series were not as closely related to the cytotoxicity as the DNA–DNA interstrand crosslinking would appear to be. Previous studies with MDMS [16] have suggested that the DNA–protein crosslinking induced by this agent could be related entirely to the action of formaldehyde produced on hydrolysis. At the same time it was demonstrated that at concentrations far exceeding those of MDMS, formaldehyde exerted little or no cytotoxicity towards the YS line, suggesting that these particular DNA–protein crosslinks may not be cytotoxic.

In conclusion, members of the homologous series of dimethanesulphonic acid esters, with the exception of EDMS and possibly 1,3-propane

dimethanesulphonate, were able to form DNA–DNA interstrand crosslinks in Yoshida lymphosarcoma cells. In most cases, the magnitude of interstrand crosslinking could be related to *in vitro* cytotoxicity and was thought to reflect the ability of these agents to span critical nucleophilic distances in Yoshida DNA.

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