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THE EFFECTS OF A BENZOIC ACID MUSTARD DERIVATIVE OF DISTAMYCIN A (FCE 24517) AND RELATED MINOR GROOVE-BINDING DISTAMYCIN ANALOGUES ON THE ACTIVITY OF MAJOR GROOVE-BINDING ALKYLATING AGENTS

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Abstract—The distamycin derivative FCE 24517 is a potent antitumour agent. Efforts have been made towards elucidating the mechanism of action of this compound which, to date, have highlighted a high level of DNA sequence specificity for covalent adduct formation. Compared to classical alkylating agents, FCE 24517 forms very few covalent adducts with double-stranded DNA, and thus appears to be a weak alkylating agent. Examination of the effects of this minor groove-binding agent on the major groove of DNA have been facilitated by use of the alkylating agents mustine, melphalan, dabis maleate, quinacrine mustard and uracil mustard. Pretreatment of plasmid DNA with FCE 24517 followed by reaction with any one of these mustards gave rise to a marked abolition of N-7 guanine adduct formation, as assayed by Maxam and Gilbert sequencing gels. The smallest effect was seen for quinacrine mustard, whereas total abolition of N-7 guanine alkylation was seen with uracil mustard. Using related structural analogues of FCE 24517 in the same experiments, it was not possible to reproduce this effect, with very few exceptions. Some effects were seen when using distamycin A itself in these experiments, although these were marginal compared to those seen for FCE 24517. We were able to extrapolate our findings to a whole cell system using murine L-1210 leukaemia cells. Alkaline elution experiments showed that treatment of cultured cells with FCE 24517 followed by either mustine or uracil mustard caused a marked inhibition of DNA interstrand crosslink formation, compared to treatment with mustine or uracil mustard alone. The present study has demonstrated the marked effect that FCE 24517 has upon the reactivity of double-stranded DNA, an effect possibly separate from that of its alkylating function. This study has highlighted the complex nature of the DNA interactive compound FCE 24517 which possesses potent and broad spectrum antitumour activity.

The distamycin derivative FCE 24517 is currently in Phase I clinical trial in Europe. The mechanism of action for this compound has been the subject of recent research [1, 2]. Although it possesses a benzoyl mustard group attached to the pyrrolamide chain (the latter structural characteristics pertaining to the distamycin portion of the molecule), FCE 24517 does not appear to behave as a typical alkylating agent.

Previous studies have indicated that FCE 24517 and some related analogues [3, ‖] share some characteristics with the antitumour antibiotic CC-1065 [4–6]. Selective DNA interaction has been reported for these compounds with a high level of sequence selectivity for adenine adduct formation. However, it remains to be proven whether these DNA–drug adducts are the main cytotoxic species contributing to the potent antitumour activity seen for these agents.

Physico-chemical studies using the DNA minor groove binding agents netropsin and distamycin have indicated that structural characteristics of AT (A = adenine, T = thymine) base pairs may prove to be of importance for agents that rely to a large extent on stabilizing van der Waal's contacts and hydrophobic binding for non-covalent binding stabilization [7]. For compounds like CC-1065 the initial non-covalent complexes may be expected to form preferentially within the narrower sterically more accessible AT rich regions in the minor groove. Several effects on local DNA integrity have been reported due to the binding of CC-1065 [8] and, although such information is not available, some similarities may exist for FCE 24517. The activity of CC-1065 has been proposed to be derived from the over-stabilization of the double-stranded DNA helix, inhibition of normal unwinding and strand separation required for DNA synthesis [8]. Unlike classical alkylating agents CC-1065 itself is incapable of forming N-7 guanine adducts.

In order to provide more information on the mode of action for FCE 24517 the present study was designed to look at effects on the major groove of B-DNA. This was facilitated by examination of the combined drug treatment of naked plasmid DNA with FCE 24517, followed by treatment with a major groove-binding alkylating agent. The resulting effects were visualized by examination of autoradiographs

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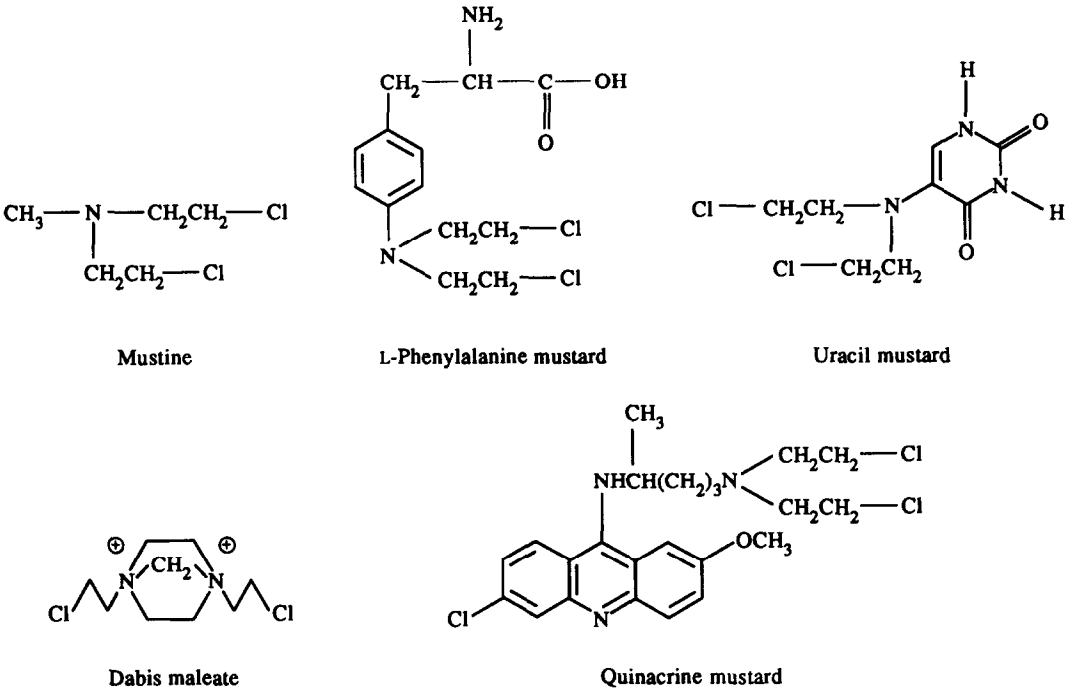

Fig. 1. Structures for the major groove-binding alkylating agents used in the study.

produced from Maxam and Gilbert sequencing gels, to demonstrate patterns of alkylation for DNA residues. In order to confirm these results further experiments were carried out on *in vitro* cultured cells using the alkaline elution assay for the detection of DNA interstrand crosslinks to assess interaction between the major and minor groove-binding agents.

MATERIALS AND METHODS

Drugs. The distamicin compounds distamycin A, FCE 24517, FCE 25853, FCE 26766, FCE 26767 and FCE 26761 were kindly provided by Dr N. Mongelli, Farmitalia-Carlo Erba, Milan, Italy.

The nitrogen mustard compounds mustine (mechlorethamine), melphalan and uracil mustard were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Dabis maleate was kindly supplied by the New Drug Development Office (Amsterdam, The Netherlands). Quinacrine mustard was purchased from Fluka Chemicals AG (CH-9470 Buchs, Switzerland).

The structures for these compounds are shown in Figs 1 and 2.

Detection of N-7 guanine adduct formation using Maxam and Gilbert sequencing gels. Plasmid SV40 or pBr322 DNA (Boehringer Mannheim, Milan, Italy) was digested with the restriction enzymes indicated in the figure legends e.g. *Eco*R1, *Bam*H1 etc., dephosphorylated with calf intestinal alkaline phosphatase and 5′ end-labelled with T4 polynucleotide kinase (BRL, Life Technologies) and [γ-^{32}P]ATP (Amersham International, Amersham, U.K.) according to the method described [9]. A second digestion with a different enzyme resulted in two fragments with only one end-labelled.

Labelled DNA (at 20,000 cpm/sample) was preincubated with either distamycin or FCE analogues (at 20 μM) for 30 min at room temperature and subsequently reacted with the appropriate major groove-binding agent at pH 7.2 using 25 mM ethanolamine–1 mM EDTA for 1 hr. Following precipitation and washing, the DNA was treated with 1 M piperidine for 15 min at 90° to produce breaks at alkylated N-7 guanine sites. Drug concentrations used are indicated in the figure legends.

To check for any interaction between the distamycins and the alkylating agents, the former was reacted with the DNA, as above. The DNA was then rapidly washed in 95% ethanol three times with centrifugation at 4°. The major groove-binding agent was then added at the appropriate concentration at pH 7.2 using 25 mM ethanolamine–1 mM EDTA, as before, and processed as above.

Maxam and Gilbert sequencing reactions for guanine (G) were prepared by incubating labelled DNA with a 0.02% solution of dimethyl sulphate (Aldrich Chemicals, Gillingham, U.K.) for 1 hr at room temperature. Adenine and guanine residues (A and G) were reacted with 1 M formic acid for 7 min at room temperature. All reactions were stopped by the alkylation precipitation solution (0.3 M sodium acetate pH 7.0, 0.1 mM EDTA pH 8.0 and 100 μg/mL yeast tRNA). The DNA was then recovered by precipitation with 3 vol. of 95% ethanol and treated with piperidine as before.

Processed DNA was then lyophilized, resuspended

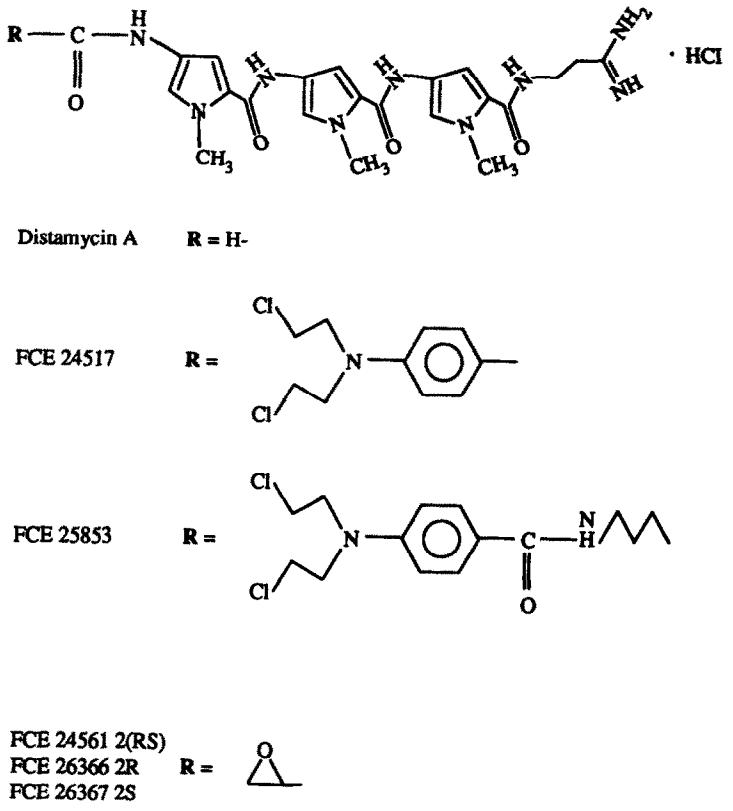

Fig. 2. Structures for the distamycin compounds used in the study.

in formamide loading buffer, heated to 90° for 1 min and then chilled prior to loading on to the gel.

DNA fragments were separated on 0.4 mm 6% polyacrylamide denaturing gel containing 7 M urea, incorporating a tris–boric acid EDTA buffer system. Gels were then transferred to Whatman 3M filter paper and dried on a Biorad gel dryer for 90 min at 80° under vacuum.

All autoradiographs were exposed to Kodak X-OMAT AR X-ray film at −70° for 24 hr with intensifying screens followed by a 6-day incubation at −70° without screens.

Cell culture and conditions. The L-1210 cell line was grown up in medium (Gibco, Grand Island Biological, NY, U.S.A.), supplemented with 10% foetal calf serum (PAA laborgesellshaft, Gallenkuchen, Austria), supplemented with 2 mM L-glutamine (Gibco Europe, Glasgow, U.K.) and 1% 2-mercaptoethanol. Cultures were grown up in 5% gassing CO_2 incubators.

Alkaline elution assay for DNA interstrand crosslinks. L-1210 cells were labelled for 24 hr using a medium supplemented with 0.05–0.1 μCi/mL [^{3}H]-thymidine (sp. act. 20 ci/mmol, Amersham) and 10^{-3} M unlabelled thymidine. Post-labelling 16–18 hr chasing in medium without [^{3}H]thymidine was carried out before drug treatment. Alkaline elution assays were carried out as described previously [10]. Cell suspensions were divided into four treatment groups: control (no drug treatment), nitrogen mustard treatment only, FCE 24517 treatment only and combined nitrogen mustard and FCE 24517 treatment. For treatment with FCE 24517 cell suspensions were exposed to a 0.01 μM dose for 30 min, followed by a 1 hr treatment with mustine 9 μM or uracil mustard 5 μM for the appropriate treatment groups. Cell suspensions for each group (assayed in triplicate) were immediately placed on ice. Cells were irradiated at ice temperature with 450 rads of X-rays (with a control set of samples set aside without X-ray exposure, to check the exposure was adequate).

Following irradiation, cells were deposited on 0.8 μm pore size, 25 mm diameter polycarbonate filters (Nucleopore, Milan, Italy) and lysed with 5 mL of 2% sodium dodecyl sulphate, 0.02 M Na EDTA, 0.1 M glycine pH 10. After connecting the outlet of the filter holders to the pumping system, 2 mL of proteinase K dissolved in the lysis solution (0.5 mg/mL) were added to a reservoir over the polycarbonate filters and pumped for approximately 2 mL/hr. DNA was eluted from the filter by pumping the elution buffer (0.02 M EDTA, 1% SDS solution adjusted to pH 12.2 with tetrapropylammonium hydroxide (Fluka). Fractions were collected at 3 hr intervals with fractions and filters processed as described previously [10]. Elution profiles were normalized by computation to compensate for differences in flow rates between individual funnels. Interstrand crosslinks were expressed by the crosslink index and given by the following formula:

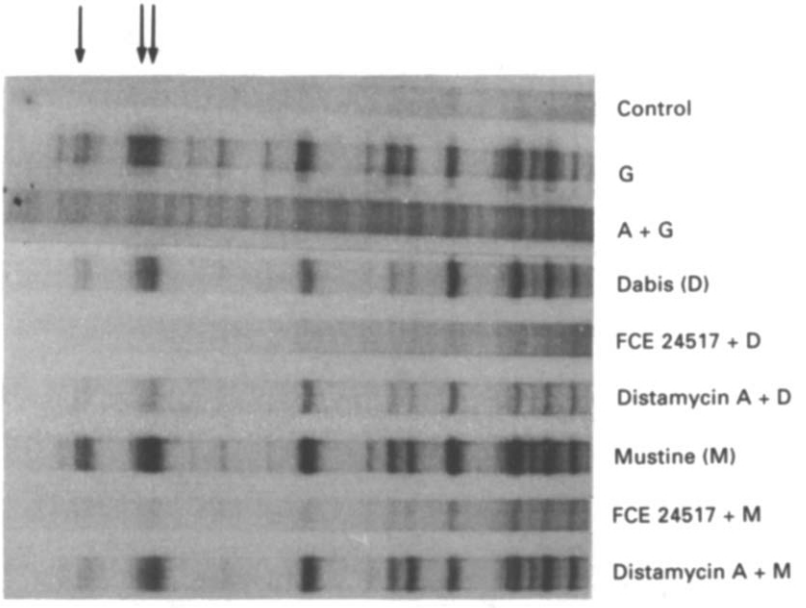

Fig. 3. Autoradiograph of Maxam and Gilbert sequencing gel showing the SV40 *Eco*R1/*Bam*H1 762 bp fragment. DNA was treated as described in Materials and Methods. Dabis was used at 100 μM and mustine at 20 μM. FCE 24517 and distamycin A were used at 20 μM.

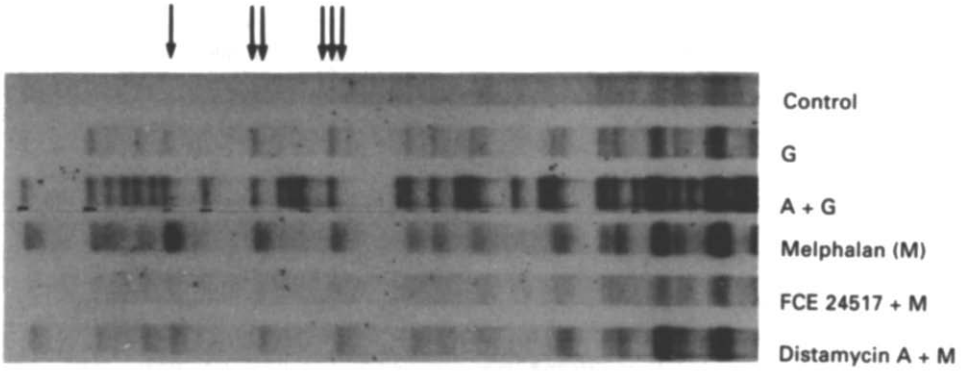

Fig. 4. Autoradiograph of Maxam and Gilbert sequencing gel showing the SV40 *Eco*R1/*Bam*H1 4474 bp fragment. Melphalan was used at 50 μM and uracil mustard at 10 μM. Distamycin concentrations were as in Fig. 3.

$$\text{crosslink index} = [(\sqrt{1 - R_0/1 - R_1})] - 1 \times 450$$

where R_0 and R_1 are the normalized cpm for ^3H obtained for the fourth elution fraction [10]. The factor of 450 expresses the results in terms of rad-equivalent units.

RESULTS

Effects of minor groove-binding agents on the ability of major groove-binding agents to form guanine N-7 adducts

Figure 3 shows that there is a dramatic effect when DNA is first incubated with FCE 24517 followed by incubation with dabis maleate. A total abolition of guanine N-7 adduct formation can be seen. In the case of distamycin A a weaker inhibition of alkylation of the sequences 5′GGT3′ (1779–1781), as indicated by (↑) and 5′GGGG3′ (1785–1788), indicated by (↑ ↑) can be seen, for this particular fragment. A marked effect is seen if DNA is co-incubated with FCE 24517 and mustine although sites of alkylation have not been completely abolished. In the case of distamycin A, a marginal effect is seen where only occasional alkylation sites appear weaker.

Figure 4 shows a marked effect is produced when FCE 24517 and melphalan treatments are combined. In particular, for the fragment shown inhibition of alkylation for 5′TGT3′ (1653–1650 bottom strand), indicated by (↑), 5′TGC3′ (1635–33), indicated as

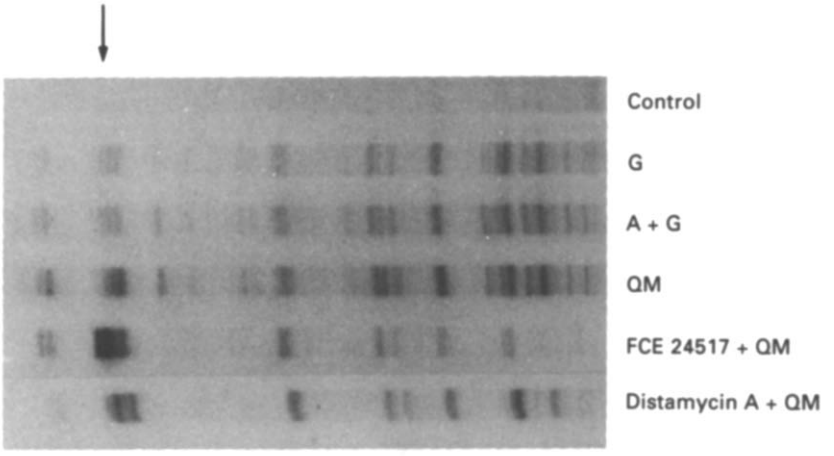

Fig. 5. Autoradiographs of Maxam and Gilbert sequencing gel showing the SV40 *Eco*R1/*Bam*H1 762 bp fragment. Quinacrine mustard (QM) was used at 100 nM and both distamycin compounds were used at 20 μM.

(↑ ↑) and 5′AGC3′ (1630–1628), indicated by (↑ ↑ ↑) can be seen. A moderate inhibition of N-7 guanine alkylations was seen in the case of distamycin A with melphalan. Again this was not so marked as in the case of the FCE 24517 combination, as judged by the relative intensities of the sites of alkylation seen on the autoradiograph.

Figure 5 demonstrates the activity of FCE 24517 and distamycin on the alkylation pattern produced by quinacrine mustard. Here we can see that the alkylation is altered—but not abolished by incubation with quinacrine mustard. Interestingly, the major effect was seen for the FCE 24517 combination, whilst very much smaller changes were seen for distamycin A. On the particular fragment used in this instance, the 5′GGGG3′, indicated by (↑) appears to be more heavily alkylated whereas other sites appear to be fainter.

Figure 6 illustrates the effects of minor groove-binding distamycin analogues on the formation of N-7 guanine adducts in the presence of uracil mustard. In this case, two analogues FCE 24517 and FCE 25853 appear to have the most marked effects on the alkylation patterns where total abolition is seen. A small effect is seen for distamycin A with uracil mustard where an overall reduction in intensity is seen. For the other analogues no effects are seen. The lanes indicating "wash" serve to illustrate that removal of the distamycin compound prior to incubation with the major groove-binding mustard gives the same results as those obtained without the wash step. This effect was also shown to hold true for the quinacrine mustard/FCE 24517 combination (data not shown).

It is important to note that it has previously been described that FCE 24517 forms very few alkylations at sites of adenine residues on plasmid DNA [2]. In fact, for the DNA fragments examined in the present study we were only able to detect a maximum of two to three alkylations along the entire plasmid sequence (manuscript in preparation).

To summarize this series of results, the minor groove-binding agent FCE 24517 had a marked effect on the ability of major groove-binding alkylating agents to form guanine N-7 adducts. Marked effects were seen using the compounds melphalan, dabis maleate, mustine and uracil mustard. Smaller effects were seen for quinacrine mustard in combination with FCE 24517. The cytotoxic agents FCE 24517 and FCE 25853 were both capable of disrupting the alkylating action of uracil mustard, whilst marginal effects were seen for the other compounds. It may be of significance that both compounds contain benzoyl groups. The other biologically active analogue FCE 26766 appeared incapable of producing the same effects as the related analogue FCE 24517. Distamycin A itself was shown to have a modest effect on the formation of guanine N-7 adducts in those cases where marked effects were seen for FCE 24517. Hence, whilst the effect of altering the DNA major groove is a property seen chiefly for the most potent cytotoxic compound FCE 24517, the relatively inactive distamycin A appears also to possess the ability to cause the same effects albeit to a much smaller degree.

Effects of minor groove-binding agents on the ability of alkylating agents to form DNA interstrand crosslinks

Table 1 shows the effects of FCE 24517 on DNA interstrand crosslink formation due to the presence of FCE 24517 seen in L-1210 cells. Results are calculated to give a crosslink frequency index [10].

It can be seen that both mustine and uracil mustard have a reduced ability to form DNA crosslinks due to treatment with FCE 24517. The greatest effect was seen for the inhibition of crosslink formation by mustine. The crosslink frequencies given by the combined treatment of mustine and FCE 24517 represent approximately only 25% of the frequencies seen for the treatment with mustine alone. A smaller but significant effect was also seen for uracil mustard

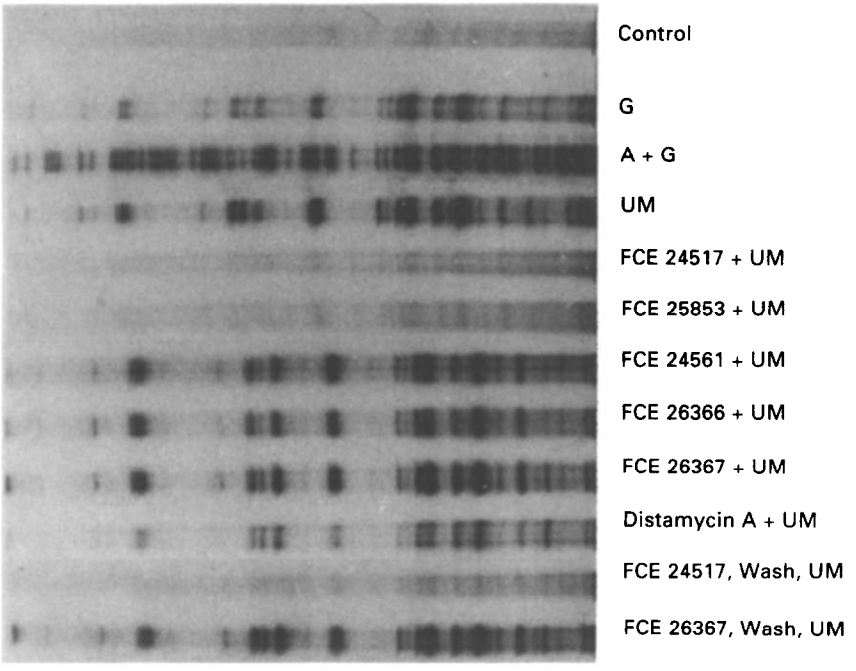

Fig. 6. Autoradiograph of Maxam and Gilbert sequencing gel showing the SV40 *Eco*R1/*Bam*H1 762 bp fragment. Uracil mustard (UM) was used at 10 μM and all distamycins compounds were used at 20 μM.

Table 1. Influence of FCE 24517 or distamycin A pretreatment on mustine or uracil mustard induced DNA interstrand crosslinks in mouse L-1210 leukaemia cells

Drug treatment	DNA interstrand crosslinks (rad-equivalents) Expt. 1	Expt. 2
Mustine (9 μM)	221 ± 20	298 ± 99
FCE 24517 (0.01 μM)	ND	ND
Mustin (9 μM) + FCE 24517 (0.01 μM)	58 ± 12*	67 ± 27*
Uracil mustard (5 μM)	266 ± 17	174 ± 18
FCE 24517 (0.01 μM)	ND	
Uracil mustard (5 μM) + FCE 24517 (0.01 μM)	111 ± 26*	75 ± 33*
Uracil mustard (5 μM)	104 ± 21	—
Distamycin A (10 μM)	ND	—
Uracil mustard (5 μM) + distamycin A (10 μM)	114 ± 15	—

Results are taken from individual experiments and each represents the mean of three separate data points ± SD.
ND, not detectable, ≤25 rad equivalent units.
* $P < 0.01$ as assessed by Student's *t*-test.

where the combined treatment gave approximately 40% of the crosslink frequencies seen for uracil mustard alone.

Hence, the results obtained earlier on isolated DNA could predict the effects seen by this series of experiments where data obtained from whole cell cultures was in line with that seen for plasmid DNA.

DISCUSSION

Although a possible mechanism of action for FCE 24517 has been proposed as being one of very selective adenine adduct formation, the present study indicates that this and similar compounds can produce marked changes in the chemical reactivity of the major groove of DNA. Initial studies showed how effectively and strongly FCE 24517 binds to naked double-stranded DNA. By studying the effects of this minor groove-binding agent on major groove-binding agents in *in vitro* experiments using L-1210 cells these effects were to some extent confirmed.

A pronounced effect of FCE 24517 treatment was the abolition of DNA guanine adduct formation for major groove-binding agents, as evidenced by use of the Maxam and Gilbert sequencing methodology (Figs 3–6). The effect of removal of FCE 24517, washing and subsequent treatment with nitrogen mustards was just as pronounced as that seen without prior removal of FCE 24517. This eliminates any possibility that the effects seen are a result of drug–drug interactions.

The N-7 position of guanine is a common site for DNA adduct formation as it is the nucleophilic site in DNA with the greatest negative molecular electrostatic potential. The nitrogen mustard compounds under study here are all capable of N-7 guanine adduct formation, according to the cleavage method used [11]. In these experiments the most striking results were seen for uracil mustard and the most modest effect was seen for quinacrine mustard. It is difficult to say why there should be a variation in the effects seen for the various alkylating agents but there are known to be differences in DNA

sequence preference amongst these compounds [12]. In addition, quinacrine mustard differs from the other compounds in the study in that it has an intercalating function.

Distamycin A and netropsin have been shown to bind to DNA very strongly by interacting with the minor groove through hydrogen bonding mainly with O-2 thymine and N-3 adenine [13]. Interestingly, the hydrogen bonding is not necessary for the binding to or sequence preference for the minor groove of B-DNA. The binding is thought to be more one due to steric good fit and stabilization due to a favourable negative potential in the AT rich regions of the minor groove. It can only be presumed at this stage that FCE 24157 binds to B-DNA in a similar tight fashion as distamycin A and netropsin.

The present study has shown the effect of a DNA minor groove-binding agent on the chemistry of the DNA major groove, as demonstrated by the combined drug studies. A study by Said and Shank [14] described the binding of more than one carcinogen/cytotoxic agent on DNA as being a complex process. The authors describe pretreatment of DNA with photoactivated aflatoxin B1 (capable of N-7 guanine formation) as being able to block the subsequent reaction of *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU) by possible modification of methylation sites. It is unclear from the present study, however, exactly how the distamycin compounds alter the major groove of DNA to give rise to the effects seen throughout the study. FCE 24517 has previously been shown to form adenine adducts with very specific sequences of DNA but no guanine adduct formation can be demonstrated [2, *].

We were able to confirm our findings in a whole cell system as well as on isolated plasmid DNA. This was highlighted by the significant inhibitory effect of FCE 24517 on DNA interstrand crosslink formation using mustine or uracil mustard. Previously, only a low level of covalent binding of FCE 24517 to DNA has been demonstrated using L-1210 cells, whereas that for distamycin A in the same experiments was undetectable [2]. Some experiments were designed to evaluate whether FCE 24517 antagonized the cytoxic effects of nitrogen mustards in L-1210 cells. However, due to the cytotoxic potency of FCE 24517 it is difficult to evaluate if concomitant treatment with this compound inhibits the cytotoxic effect of nitrogen mustards. Nevertheless, using very low drug concentrations causing less than 20% cytotoxicity an antagonist effect was seen with a melphalan and FCE 24517 combination (data not shown).

In spite of this correlation between these sets of data there still remains the possibility for there being a number of mechanisms operating in our experimental system which have not been considered. Nevertheless, the results from the present study highlight the marked effect that minor groove-binding agents, particularly FCE 24517, have on the DNA helix. Our findings have suggested that very precise structural requirements are necessary to produce this effect, as closely related structural analogues of FCE 24517 were unable to do so in most instances. That for the large part those particular analogues were either biologically inactive or less potent than FCE 24517 may be of significance in terms of antitumour activity. Evidence has been provided which indicates FCE 24517 as being highly reactive with DNA, despite being a weak alkylating agent. In addition, although much of the data obtained by this study provides indirect evidence for this, it is clear that drugs used in combination may not always be expected to give an additive effect.

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