Backbone and benzoyl mustard carrying moiety modifies DNA interactions of distamycin analogues

Alessandra Ciucci, Stefano Manzini, Paolo Lombardi and Federico Arcamone

Menarini Ricerche Sud, Via Tito Speri 10, 00040 Pomezia, Rome, Italy

Received September 22, 1995; Revised and Accepted December 1, 1995

ABSTRACT
Alkylating distamycin derivative FCE 24517 (I) is the prototype of a novel class of alkylating agents. In the present study we have investigated the effect of further chemical modifications introduced in the alkylating distamycin-derived molecule with the aim of improving their ability to bind DNA. The new compound, MEN 10710 (II), has a four pyrrolecarboxamide backbone linked at its N-terminus and through a butanamido residue to a 4-[bis(2-chloroethyl)amino]phenyl moiety. We have demonstrated that the presence of the flexible trimethylene chain confers to the novel distamycin derivative a peculiar mode of interaction with DNA as compared with I or melphalan. In fact, interstrand cross-links are detected in DNA samples treated even with low concentrations of II (being 200-fold more efficient than melphalan) but not with I. Similar results were obtained with a related compound of II containing a three pyrrole ring backbone. Compound II induces a conformational change in the DNA structure as deduced from the inhibition of T4 DNA ligase activity. In alkylation experiments, unlike melphalan, both I and II induce DNA breaks at bases closely located to AT-rich tracts, however II was more potent than I in producing greater amount of covalent adducts. These data suggest that the new compound shows a different and peculiar mechanism of interaction with DNA.

INTRODUCTION
Nitrogen mustard derivatives, such as melphalan and chlorambucil, still have a place in the chemotherapy of several types of leukemias and solid tumours (1). The biological activity of these typical alkylating agents is mediated through alkylation at N-7 of guanine and subsequent DNA interstrand cross-linking (2–4). In 1989, a novel class of distamycin analogues bearing a benzoyl mustard group have been described as new potential anticancer agents (5). The prototype of this novel class of alkylating agents, namely compound FCE 24517 (tallimustine, structure of Fig. 1), exerted relevant growth inhibitory activity in several murine and human experimental tumours (5–7) and is currently under clinical investigation. Modalities of DNA interaction of I are different when compared with typical alkylating agents: the compound exhibits a greater affinity for AT-rich sequences in the minor groove of DNA (7), coupled to a weak, albeit characteristic, alkylating activity. In particular, a preferential alkylation at N-3 of adenine, but not at N-7 of guanine, together with the absence of DNA cross-links has been reported (8). Further studies have indicated that compound I induces breaks at any bases in the proximity of AT regions (9). Compound I has also been described as an inhibitor of DNA ligase activity (10). Recently, a series of new compounds have been synthesized in our laboratories with the aim at evaluating the effect of the insertion of a flexible carbon chain between the oligo-pyrrolecarboxamido backbone and the alkylating moiety. Among them, MEN 10710 (II, Fig. 1) emerged as an interesting compound possessing, as compared with I and melphalan, (i) a greater cytotoxic activity against different tumour cell lines in vitro, (ii) a higher potency in the antitumour tests in vivo and (iii) a reduced myelotoxicity in an in vitro system (11). Compound II differs from I for two structural features: an additional residue of 4-amino-1-methylpyrrole-2-carboxylic acid in the backbone and the presence of a butanamido residue linking the alkylating moiety to the oligo-pyrrolecarboxamido backbone (Fig. 1). It appeared worthwhile investigating whether the mode of DNA interaction of II could be different as compared to typical (melphalan) and atypical alkylating agents such as I. With this aim, these compounds have been compared for their ability to induce cross-links and to exhibit specific alkylation sites as well as to interfere with DNA joining and relaxation (T4 ligase). Present findings indicate that II possesses a peculiar mode of DNA interaction, different from either that shown by I or by melphalan.

MATERIALS AND METHODS

Reagents
pBR322 DNA, T4 polynucleotide kinase, T4 ligase, HindIII, EcoRI and bacterial alkaline phosphatase were purchased from Boehringer. AMP, ATP, chloroquine phosphate, L-phenylalanine mustard (melphalan) were obtained from Sigma. A 226 bp EcoRI–Aval fragment, used in alkylation assay, was obtained by restriction enzyme digestion of a commercial plasmid (SureTrack footprinting kit, Pharmacia, Sweden). FCE 24517, MEN 10710 and MEN 10569 (III, Fig. 1) were synthesized in the Department of Chemistry of Menarini Ricerche S.p.A., by F. Animati, G. Giannini and C. Rossi. Their characterization and purity were assessed by comparing spectral data for FCE 24157 with those reported in the literature (5) and by NMR for new compounds.
Figure 1. Structural formulas of FCE24517 (I), MEN 10710 (II) and MEN 10569 (III).

II: δ 1.82 (2H, m), 2.25 (2H, t), 2.45 (2H, t), 2.64 (2H, t), 3.50 (2H, m), 3.70 (8H, s), 3.82 (3H, s), 3.85 (3H, s), 3.86 (3H, s), 3.87 (3H, s), 6.69 (2H, d), 6.90 (1H, d), 6.97 (1H, d), 7.04 (2H, d), 7.08 (2H, bs), 7.15 (1H, d), 7.18 (1H, d), 7.22 (2H, bs), 8.19 (1H, t), 8.57–8.95 (4H, bd), 9.76 (1H, bs), 9.90 (2H, bs), 9.95 (1H, bs);

III: δ 1.80 (2H, m), 2.22 (2H, t), 2.48 (2H, t), 2.60 (2H, t), 3.50 (2H, m), 3.70 (8H, s), 3.78–3.80 (9H, s), 6.65 (2H, d), 6.89 (1H, s), 6.92 (1H, s), 7.02 (1H, s), 7.03 (1H, s), 7.12 (1H, s), 7.15 (1H, s), 7.19 (1H, s), 8.19 (1H, t), 8.60–8.97 (4H, bd), 9.75 (1H, s), 9.85 (2H, s).

Agarose gel cross-link assay

Cross-link assay was carried out according to Hartley et al. (12). Briefly, pBR322 plasmid DNA was linearized by digestion with HindIII and dephosphorylated by treatment with bacterial alkaline phosphatase. The DNA was 5′-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP (5000 Ci/mmol, Amersham). Following precipitation and removal of unincorporated ATP, the DNA was resuspended in sterile double-distilled water at 1 mg/ml. Labeled DNA (~10 ng) was used for each experimental point. Reactions with drug were performed in 25 mM triethanolamine, 1 mM EDTA (pH 7.2) at 37°C for 2 h and terminated by the addition of an equal volume of stop solution (0.6 mM sodium acetate, 20 mM EDTA, 100 µg/ml tRNA). DNA was precipitated by the addition of 3 vol of 95% ethanol. Following centrifugation and supernatant removal, the DNA pellet was obtained by lyophilization. Samples were dissolved in 10 ml 6% sucrose, 0.04% bromophenol blue and loaded directly. Electrophoresis was performed on 20 cm long 0.8% submerged horizontal agarose gels at 40 V for 16 h. The gel and running buffer was 40 mM Tris, 20 mM acetic acid, 2 mM EDTA (pH 8.1). Gels were dried at 80°C onto one layer of Whatman 3MM paper on a Bio-Rad Model 583 gel drier connected to a vacuum. Autoradiography was performed with X-OMAT Kodak films for 4 h at ~70°C using DuPont-Cronex Lightening-plus intensifying screen. Sharper images were obtained by overnight exposure without the intensifying screen. Quantitation was achieved by autoradiograph microdensitometry.

Polynucleotide ligation assays

DNA joining activity was assayed by the method of plasmid DNA circularization (13). Linear pBR322 DNA was incubated with T4 DNA ligase in the following reaction buffer (20 ml): 66 mM Tris–HCl, pH 7.6, 6.6 mM MgCl2, 1 mM DTT, 0.7 mM ATP plus the drug at the desired concentration. After incubation at 15°C for 30 min, reactions were stopped by addition of EDTA (20 mM final concentration). Ligated samples were analysed on 1% agarose gels made with Tris–acetate–EDTA (TAE) buffer (40 mM Tris–acetate, 2 mM EDTA, 18 mM NaCl, final pH 8). The gel was then stained with ethidium bromide (1 mg/ml) for 30 min and destained with H2O for 15 min. DNA was visualized and the gel photographed on Polaroid 55 films, with the aid of an ultraviolet illuminator.

DNA topoisomerization

AMP-dependent DNA relaxation catalyzed by T4 ligase was assayed as previously described (14): the reaction mixture (20 ml) contained 20 mM Tris–HCl, pH 8.0, 3 mM MgCl2, 100 mg/ml BSA, 1 mM EDTA and 200 ng of naturally supercoiled pBR322 DNA, 1 mM AMP and 20 nM of T4 ligase. After 30 min at 30°C, reactions were stopped by adding 2 ml of a solution containing 1 mg/ml Bromophenol Blue, 50% (v/v) glycerol and 5 mg/ml sodium dodecyl sulfate and analysed on a 1.2% agarose gel.

Adenylation

Enzyme adenylation was evaluated by the reported technique (15) in which DNA ligase is incubated at 37°C in the presence of [35S]adenosine-5′-O-(1-thiotriphosphate) (400 Ci/mmol) in the same reaction mixture (10 ml) utilized for DNA ligation. The reaction was stopped by the addition of a solution (20 ml) containing 10 mM EDTA and 150 mg of BSA/ml; then 25 ml of the reaction mixture was spotted on to a Whatman GF/C filter; the filter was batch-washed with trichloroacetic acid, dried and counted for radioactivity.
RESULTS

Induction of DNA interstrand cross-linking

The presence of a cross-link between the two DNA strands prevents complete separation of the strands upon denaturation so that the cross-linked DNA reanneals in a neutral agarose gel to run as double stranded. Quantitation of the amount of double-stranded versus single-stranded therefore gives a measure of the extent of drug-induced cross-linking in a given DNA fragment (12). A gel autoradiograph is shown in Figure 2, illustrating the cross-link adducts induced by II but not by I. In fact no cross-linking was induced by I in the concentration range 0.5–200 µM (Figs 2 and 3). On the other hand, II was very efficient in inducing DNA cross-links starting at concentrations as low as 0.5 nM and reaching a maximal effect at 5–50 nM (Figs 2 and 3). As expected, also melphalan induced cross-linking although at higher concentrations (50 nM–1 µM). In order to better understand the reason for the different ability of the two drugs to form cross-links, we have analysed the activity of III, which differs from I only for the presence of the trimethylene chain (Fig. 1).

Interstrand cross-links are clearly formed by III at doses that are similar to those of II (Fig. 3). Compounds I, II and III showed a similar pattern of sequence preference for their alkylating action. In fact, alkylation occurred at tracts of DNA with sequences: 5′-AGAGATTGGAT-3′, 5′-ATATTGGCATT-3′, 5′-AAAATGGATT-3′, 5′-TTTGGACA-3′. The sites susceptible to attack by the active distamycin derivative were the residues preceded by (A/T)n, rich sequences, that were important for binding of the distamycin (15). In fact, the same DNA sequences were recognized by no alkylating distamycin derivatives in footprinting studies (16). The new derivatives II and III alkylated more efficiently than I (Fig. 4). Melphalan as most of the available alkylating agents which are used in cancer therapy (17–19), produced only breaks corresponding to guanine sites, indicating N-7 guanine alkylation (8) (data not shown).

Inhibition of T4 ligase-induced DNA joining

Montecucco et al. (10,13,20) have indicated that DNA intercalating agents or minor groove binders interfere with DNA ligase. In the present study, EcoRI-digested pBR322 DNA was the substrate and different, increasing amounts of the drugs were used. Inhibition of the ligation was deduced from a progressive reduction of the amount of ligated linear product. Quantitation was achieved by microdensitometry of the photograph and the percentage of T4 ligase activity was calculated. Both distamycin analogues I and II inhibited T4 ligase joining activity in a concentration-dependent manner (2–20 µM) (IC50 12 and 17 µM for II and I, respectively) (Fig. 5). Compound III showed an inhibitory effect that was similar to that exhibited by I (IC50 12 µM). By contrast, melphalan in the concentration range 0.5–400 µM was devoid of any inhibitory effect. Interestingly, both I and II (up to 200 µM) were ineffective against T4 DNA ligase-induced DNA adenylation (data not shown), suggesting that for inhibition of DNA joining the DNA and not the enzyme was their target.

Inhibition of AMP-dependent DNA topoisomerization

We have also investigated whether these drugs interfered with DNA topoisomerization, the so-called ‘reverse reaction’ of DNA ligation (21). In this experimental setting T4 DNA ligase can generate fully relaxed DNA molecules starting from naturally supercoiled plasmid DNA in an AMP-and Mg2+ dependent
Figure 4. Binding of alkylating agents at DNA fragment. A 226 bp EBNA-1/NF-1 fragment 5′-end labeled at AvaI end has been subjected either to sequencing reaction or to treatment with drugs. Lanes 1, 2 and 3 DNA treated with 250, 50 and 2.5 µM, respectively, of FCE 24517; lanes 4, 5 and 6 the treatment was with 250, 50 and 2.5 µM, respectively, of MEN 10569; lanes 8, 9 and 10 DNA treated with 250, 50 and 2.5 µM, respectively, of MEN 10710. Lane 7 was a G+A reaction of the same fragment. The incubation with alkylating agents was at room temperature for 5 h.

reaction (13,22). Unlike melphalan (1–400 µM), either I or II inhibited the reaction in the range 1–5 µM in a concentration-dependent manner. Reference I, however, showed slightly lower activity than II (Fig. 6).

DISCUSSION

The novel distamycin analogue MEN 10710 (II) shows strong cytotoxic activity against tumour cell lines and a remarkable antitumour activity in a variety of human tumour xenografts in immunodepressed mice (11). In this study, we have investigated the DNA interaction properties of II in an attempt to relate the marked biological effects with a specific molecular mechanism of action.

The results of the cross-linking assay suggest that the flexible trimethylene chain, present as a linker between the peptidic backbone and the bis(2-chloroethyl)aminophenyl moiety, gives rise to a novel class of distamycin related alkylating agents with improved ability to strongly bind DNA sequences as compared with FCE 24517 (I) type derivatives. The trimethylene chain interrupts the conjugation through the molecule resulting in a different charge distribution. The alkylating group becomes more electron-rich, providing most likely II with stronger affinity for double stranded DNA and, in essence, a higher alkylation rate as compared with I. In addition, the length and flexibility of the chain allows the formation of double strand cross-links making the new compound, from this standpoint, more similar to the classic agents like, for example, melphalan.

Recently, we have shown that modifications in the N-terminal residue of distamycin analogues have a consequence in their properties of competing with nuclear factors for the binding to their target DNA sequences (16,23). Most likely, this portion of the molecule is strongly implicated in the DNA recognition and
therefore in the corresponding interference with the specific DNA–protein interaction. In this study we have demonstrated the ability of the new compounds to form molecular complexes with DNA and to induce conformational changes in the DNA structure, through the inhibition of T4 ligase. In fact the inhibition of the DNA joining or topoisomerizing activity of the enzyme reflects the complexation of the molecules with the DNA structure, through the inhibition of T4 ligase. In fact the inhibition of DNA and to induce conformational changes in the DNA ability of the new compounds to form molecular complexes with DNA–protein interaction. In this study we have demonstrated the corresponding interference with the specific DNA–protein interaction as observed by Montecucco et al. for I (13,20).

The present work shows that structural variations of molecules of the distamycin type, as obtained by introducing selected reactive groups together with modifications of the backbone, can vary the DNA binding properties of these drugs and improve antitumour potency. The presence of the trimethylene spacer in II and III determines a higher affinity for double stranded DNA of these compounds in respect to I and is responsible for the formation of interstrand cross-links. This is particularly evident when the behaviour of III is compared with that of I, as the two compounds differ only in the presence of the said spacer in the former and not in the latter. On the other hand, the presence of an additional residue of 4-amino-1-methylpyrrole-2-carboxylic acid in II might be responsible for a greater sequence specificity. The latter point has already been proved in a physico-chemical study concerning the complexation of DNA oligomers with the corresponding derivatives with three and four of the said residues but not possessing the alkylating N-terminal groups (24).

In conclusion, compound II has peculiar mode of DNA interaction that differs either from FCE 24517 (induction of cross-links and either potency in DNA alkylation and T4 ligase inhibition) or from melphalan (different pattern of sequence preference and alkylation). This mode of DNA interaction could be of relevance to the interesting cytotoxic and antitumor properties exhibited by this compound.

ACKNOWLEDGEMENTS

This work was carried out in the frame of a joint project of A. Menarini, Industrie Farmaceutiche Riunite, Florence and of Bristol-Meyers Squibb Italia, Rome. It was supported by a grant of the Istituto Mobiliare Italiano (contract No. 53658).

REFERENCES