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Inhibition of topoisomerases by fredericamycin A*

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Summary. Fredericamycin is an antibiotic product of *Streptomyces griseus* that exhibits modest antitumor activity in vivo and in vitro. Because of its unique structure and the absence of a clearly defined mechanism of action, we examined the effects of this compound on L1210 cells in culture as well as on several enzymes that bind to DNA. Fredericamycin exhibits an IC_{50} of 4.4 μM toward L1210 cells, and its cytotoxicity is a function of the time of exposure as well as drug dose. No DNA breakage was observed in L1210 cells or isolated nuclei following exposure to highly lethal concentrations of fredericamycin. As a first step toward understanding its mechanism of action, we examined the effect of fredericamycin on several enzymes involved in DNA metabolism. The catalytic activity of both DNA topoisomerases I and II were totally inhibited by fredericamycin concentrations of 4.4 and 7.4 μM, respectively. Fredericamycin blocked etoposide-stimulated DNA cleavage by topoisomerase II both in vitro and in isolated nuclei. In addition, the drug inhibits DNA polymerase a in vitro, exhibiting an IC_{50} of 93 μM. These diverse actions of fredericamycin do not enable us to draw conclusions regarding its mechanism of antitumor effect but clearly identify it as a compound of pharmacologic interest.

Introduction

Fredericamycin A, a product of *Streptomyces griseus*, exhibits antibacterial, antifungal, and antitumor activity [14]. It is a drug of somewhat unusual structure (Fig. 1) and physicochemical properties [5, 12]. Preliminary studies indicate that it does not bind to DNA, but its mechanism of action has not been clearly identified [14]. Hilton and Misra [5] have suggested that the drug may act by spontaneously forming on oxidized free radical, with subsequent electron transfer to molecular oxygen; however, biological evidence supporting this hypothesis has not been published. Because of areas of structural planarity within the molecule, to some extent resembling DNA-intercalating types of drugs, we also investigated the effects of fredericamycin on L1210 cells in culture and examined the drug for its ability to inhibit certain enzymes involved in DNA metabolism. Our results suggest that the drug has potent inhibitory activity against DNA topoisomerases I and II and DNA polymerase α. Furthermore, they suggest that notwithstanding fredericamycin's potential as an antitumor agent, it will provide a useful molecular basis for future syntheses directed at DNA enzymes.

Materials and methods

Mouse leukemia L1210 cells were grown in RPMI 1630 plus 20% fetal calf serum as previously described [16]. All studies were carried out in logarithmically growing cells, and cytotoxicity was determined by a soft agar clonogenic assay [16]. Fredericamycin was kindly supplied by Dr. Ross Kelly, Boston University (Boston, Mass) and the National Cancer Institute. A stock solution of fredericamycin (5 mg/ml) was prepared in dimethylsulfoxide (DMSO). We used only the basic (blue) form of the drug. For cytotoxicity and DNA breakage assays, cells were treated for 1 h with various concentrations of fredericamycin, and controls received appropriate amounts of DMSO. Etoposide (VP-16) was a gift of Bristol laboratories and was also dissolved in DMSO. DNA topoisomerase I was obtained from Bethesda Research Laboratories (Gaithersburg, Md). DNA topoisomerase II from Ehrlich ascites cells was purified to homogeneity using an FPLC-based procedure; this method has been described elsehwere in detail [2]. Calf thymus DNA polymerase alpha was obtained from Pharmacia (Piscataway, NJ).

Both the high- and low-sensitivity alkaline elution methods were used to assess DNA breakage in whole cells and isolated nuclei [9]. Nuclei from L1210 cells were prepared for drug treatment and subsequent alkaline elution using the method of Filipski et al. [4]. Proteinase k was used in the cell-lysis step of all experiments.

Topoisomerase I activity was assayed by relaxation of Form I pUC18 plasmid DNA. The reaction mixture contained Buffer A (50 mM TRIS, 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetate (EDTA), 30 μg/ml bovine serum albumin), 1 μg Form I pUC18 DNA, and 1 IU topoisomerase I in a final volume of 20 μl. The reaction was carried out at 30° C for 30 min and stopped by the addition of 5 μl 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 50% glycerol. Electrophoresis of DNA was carried out in 1% agarose for 2 h at 100 V in a TRIS borate EDTA

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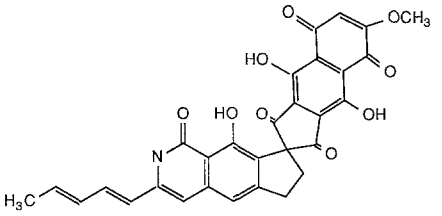

Fig. 1. Structure of fredericamycin A

buffer (pH 8.0). Catalytic activity of topoisomerase II was assayed by the removal of knots from phage P4 DNA [10]. The reaction mixture contained the same buffer as in the topoisomerase I assay, with the addition of 1 m*M* ATP. The DNA substrate used was 0.4 μg knotted phage P4 DNA. The minimal amount of topoisomerase II required to unknot this DNA completely in 30 min at 37° C was used; the final reaction volume was 20 μl. The reaction was stopped by the addition of 2% SDS, 0.1% bromophenol blue, and 50% glycerol, and electrophoresis of DNA was carried out in 1% agarose in a TRIS-borate-EDTA buffer (pH 8.0) for 2 h at 100 V. The K-SDS precipitation assay described by Liu et al. [11] was used to assay drug-induced DNA cleavage by pure topoisomerase II. Briefly 50 ng uniquely 3' end-labelled pUC18 DNA along with various concentrations of etoposide and/or fredericamycin were incubated with approximately 25 ng pure topoisomerase II in 50 μl Buffer A. The reaction was stopped by the addition of 2% SDS at denaturing pH and protein was precipitated by the addition of 0.25 *M* KCl. DNA that is covalently attached to topoisomerase II at the 5' end was recovered in the precipitate.

Calf thymus polymerase alpha was diluted [in 60 m*M* potassium phosphate (pH 7.6), 500 m*M* KCl, 50 m*M* β-mercaptoethanol, 50% glycerol] to 2 IU/ml and stored in 100-μl aliquots at −70° C until used, when it was diluted to the desired concentration for assay. Poly [dT] and oligo $(A)_{12-18}$ were annealed in 5:1 ratio by weight in 1 ml H_2O to an A_{260} activity of approximateley 20 and were stored at −20° C. The assay was started by the addition of 0.053 IU DNA polymerase a to the assay mixture, which contained: 50 m*M* TRIS (pH 7.8) at 37° C; 400 μg/ml bovine serum albumin; 1.0 m*M* DTT, 6 m*M* $MgCl_2$; 30 m*M* KCl; 7.2 μg/ml template; and 100 μ*M* [^{3}H]dATP (1.3 m*M*, 246 mCi/m*M*). The total volume of the assay was 50 μl. After incubation at 37° C for 60 min, the assay was quenched by the addition of 10% trichloroacetic acid/0.1 *M* sodium pyrophosphate. The samples were filtered through Millipore filters (HAWP, 0.45 μm) and washed three times with 5.0 ml trichloroacetic acid/pyrophosphate solution and once with 10.0 ml cold absolute ethanol. The filters were air-dried and transferred to scintillation vials with 10 ml aqueous counting scintillant. Radioactivity was measured by scintillation spectrometry on a Beckman LS 3150P liquid scintillation system. Inhibition studies were carried out such that organic solvents added (DMSO, ethanol) were less than 2% of the total volume. There was no loss of measured DNA polymerase α activity at this concentration.

Results

The cytotoxicity of fredericamycin towards L1210 cells was established using a soft agar clonogenic assay. A 1-h exposure to the drug inhibited clonogenicity in a dose-dependent fashion (Fig. 2). The approximate IC_{50} for this period of treatment is 4.4 μ*M*. The cytotoxicity of fredericamycin increased with the duration of drug treatment, up to 5 h (data not shown).

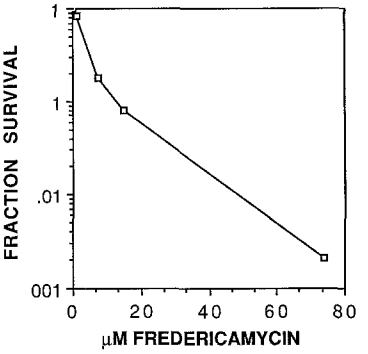

Fig. 2. Cytotoxicity of fredericamycin toward L1210 cells. Logarithmically growing cells were exposed to various concentrations of fredericamycin for 1 h and then seeded in soft agar. Values are normalized to survival of the nontreated cells

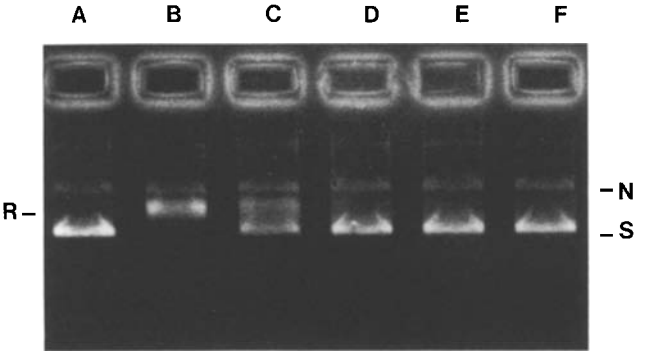

Fig. 3. Inhibition of topoisomerase I by fredericamycin: 1 μg supercoiled pUC18 DNA was incubated with 1 IU calf thymus topoisomerase I in the presence of various drug concentrations for 30 min at 30° C. DNA was electrophoresed in 1% agarose for 2 h at 100 V. The supercoiled (*S*), relaxed (*R*), and nicked (*N*) forms of DNA are noted in the margin. *Lane A*, DNA plus enzyme; *lane B*, DNA alone; *lanes C-F* contain DNa, enzyme, and 1.5, 4.4, 15, or 30 μ*M* fredericamycin, respectively. The drug alone had no effect on the mobility of DNA

The structure of fredericamycin exhibits certain similarities to that of DNA intercalating agents. The latter cause protein-associated DNA single- and double-strand breaks in mammalian cells by altering the activity of DNA topoisomerase II [14]. Furthermore, other investigators [5] have suggested that fredericamycin may generate oxygen radicals that might be expected to cause DNA breakage by a mechanism independent of topoisomerase II. It was thus of interest to determine whether fredericamycin caused strand breaks in L1210 cells at cytotoxic drug concentrations. Cells were exposed to fredericamycin (0–150 μ*M*) and then assayed by the high-sensitivity (0–900 rads) alkaline elution method for DNA breaks; no such breaks were observed (data not shown).

To further explore potential mechanisms for the cytotoxicity of fredericamycin, we examined its effect on the activity of several DNA enzymes required for cell growth. DNA topoisomerase I relaxes closed, circular, superheli-

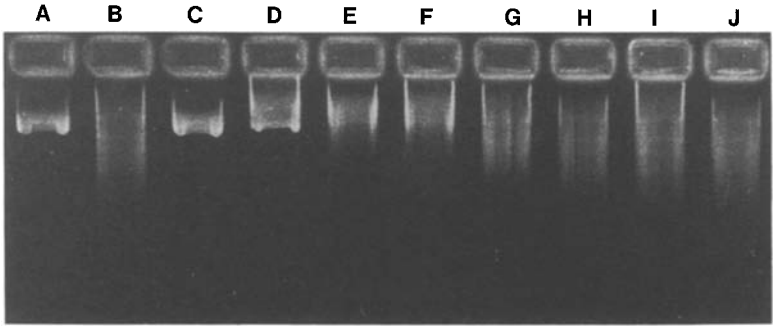

Fig. 4. Inhibition of topoisomerase II unknotting activity by fredericamycin. A reaction mixture containing 0.4 μg knotted phage P4 DNA, 1 m*M* ATP, purified topoisomerase II from Ehrlich ascites cells, and various amounts of drug was incubated for 30 min at 37° C. The DNA was electrophoresed in 1% agarose for 2 h at 100 V. *Lane A*, DNA plus topoisomerase II from Ehrlich ascites cells; *Lane B*, DNA alone; *lanes C-J* contain DNA, enzyme, and 1.5, 4.4, 7.4, 10.4, 15, 22, 30, and 37 μ*M* fredericamycin, respectively

cal DNA by a single-stranded nicking-resealing mechanism. Its activity can be assayed as a function of the conversion of closed, circular, superhelical plasmid DNA into the relaxed Form IV. As shown in Fig. 3, fredericamycin completely blocks the relaxation activity of DNA topoisomerase I at a concentration of 4.4 μ*M*. The drug alone (0.3–60 μ*M*) had no effect on DNA electrophoretic mobility.

The second class of enzymes capable of relaxing superhelical DNA are the type II topoisomerases. These enzymes create double-stranded breaks and form a reaction intermediate by covalently attaching to the 5' end of the break termini. They can also reversibly decatenate DNA and remove knots from double-stranded, closed, circular DNA. We used this latter assay to determine whether fredericamycin affects the enzymatic activity of DNA topoisomerase II isolated from Ehrlich ascites tumor cells. The results are shown in Fig. 4. Knotted phage-P4 DNA exists in a series of knotted topoisomers and therefore runs as a smear when electrophoresed in agarose. On removal of the knots by topoisomerase II, the DNA is resolved into a single monomeric, closed, circular, relaxed form represented by a discrete band in the gel.

Fredericamycin inhibits the unknotting activity of topoisomerase II in a dose-dependent fashion, with complete inhibition observed at 7.4 μ*M*. The ability of fredericamycin to inhibit the unknotting activity of DNA topoisomerase II and yet not induce DNA cleavage by the enzyme is distinct from that of most DNA-intercalating agents (ethidium bromide is an exception) and raised the question as to whether the drug might alter the topoisomerase II-mediated cleavage of DNA induced by the epipodophyllotoxin etoposide (VP-16). The K-SDS assay was used to test this hypothesis in vitro. As shown in Fig. 5, incubation of radiolabelled DNA with pure enzyme alone results in only 1.5% of the available counts being precipitated in K-SDS. However, when 100 μ*M* etoposide is present, precipitation of the DNA is markedly increased as a result of trapping of the DNA protein complex following DNA cleavage. The addition of fredericamycin inhibits the etoposide-stimulated cleavage of DNA by topoisomerase II in a dose-dependent fashion. It is noteworthy that fredericamycin alone did not stimulate DNA cleavage by topoisomerase II at any concentration. Indeed, the data suggest that the drug may reduce cleavage to a level below that exhibited by enzyme alone.

Having ascertained that fredericamycin could block topoisomerase II-mediated DNA cleavage in vitro, we wished to confirm this observation in a more biologically relevant model. To obviate difficulties of interpretation that might be posed by the presence of plasma membrane,

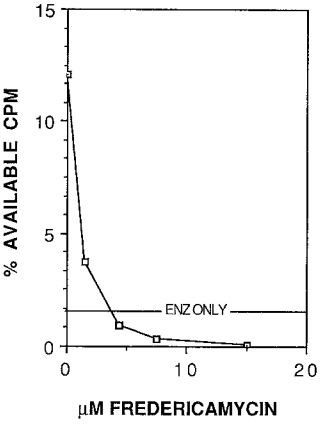

Fig. 5. Inhibition of etoposide-stimulated cleavage of DNA by topoisomerase II. Uniquely 3' end-labelled pUC18 DNA was incubated with 25 ng topoisomerase II for 60 min at 37° C in the presence of 100 μ*M* etoposide plus various concentrations of fredericamycin. For reference purposes, the effect of enzyme alone is represented by the *horizontal line*

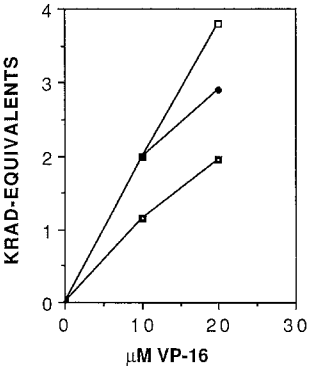

Fig. 6. Inhibition of etoposide-mediated DNA breakage in isolated L1210 nuclei. Nuclei were treated for 1 h with 10 or 20 μ*M* etoposide in the presence of 0 (□), 15 (◆), or 74 (□) μ*M* fredericamycin. DNA strand breakage was then assayed by the low-sensitivity alkaline elution technique. Fredericamycin alone induced no breaks

we isolated nuclei from mouse L1210 cells and assayed DNA cleavage induced by etoposide using the low-sensitivity (0–10,000 rads) alkaline elution technique (Fig. 6). As expected, VP-16 caused a dose-dependent increase in DNA single-strand breaks, with a maximum of 3,800 rad-equivalent breaks observed at a concentration of 20 μ*M*. Concurrent exposure to 15 μ*M* fredericamycin slightly reduced DNA cleavage, and when this was increased to 74 μ*M*, we observed a reduction in strand breakage by

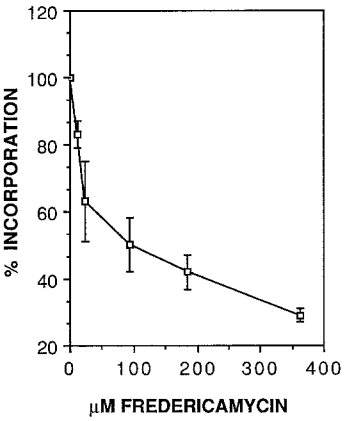

Fig. 7. Inhibition of DNA polymerase a by fredericamycin. Polymerase activity was assayed by its ability to incorporate [^{3}H]-dATP into a model substrate consisting of poly[dT] and oligo $(A)_{12-18}$ annealed in 5:1 ratio by weight. The reaction was carried out in the presence of various concentrations of fredericamycin

VP-16 of approximately 50%. To rule out DNA cross-linking by fredericamycin, which might artifactually reduce apparent DNA breakage by VP-16, L1210 cells were radiated (3,000 rads) on ice after a 1-h exposure to fredericamycin. No reduction in elution rate was observed; therefore, a cross-linking effect was excluded. Thus, fredericamycin appears to be capable of inhibiting topoisomerase-mediated DNA cleavage both in vivo and in vitro.

Inhibition of the activity of both DNA topoisomerases I and II suggested the possibility that fredericamycin may exhibit inhibitory properties toward other enzymes that bind to DNA. One such enzyme is DNA polymerase α, and its activity can be followed as the incorporation of radiolabelled nucleotide triphosphate into an oligonucleotide DNA fragment continuing a primer and template region. As shown in Fig. 7, fredericamycin causes a dose-dependent inhibition of the polymerase activity of this enzyme, with an IC_{50} of 93 μ*M*. The potent alpha polymerase inhibitor aphidicolin was used to standardize this experiment, with an IC_{50} of 7.2 μ*M*.

Discussion

The results of our work confirm the cytotoxicity of fredericamycin and indicate that it has potent inhibitory activity toward several mammalian enzymes that act in part by binding to DNA. Unfortunately, it is not possible to conclude from the available data, either our own or that of others, which of the many effects of fredericamycin are responsible for its cytotoxicity.

Warnick-Pickle et al. [15] first described the antibacterial, antifungal, and antitumor activities of fredericamycin A, observing in vitro activity against KB, L1210, P388, and human glioblastoma cells in culture. They reported that the drug exhibited potency similar to that of Adriamycin and actinomycin D; activity was also observed in the human stem-cell assay. Antitumor activity in vivo was exhibited toward P388 leukemia and the CD8F murine mammary tumor; marginal activity was observed against B16 melanoma. Further development of the drug has been hampered by its difficult synthesis and poor solubility. However, the crystalline structure of the compound is known [13], and a total synthesis was recently reported by Kelly et al. [8].

The ability to inhibit topoisomerases I and II and DNA polymerase alpha has not previously been reported for any single drug known to us. Distamycin, a compound that binds to the DNA minor groove, has recently been reported to block DNA cleavage by both topoisomerases I and II [3, 12]. This presumably occurs because the presence of the drug blocks access of the enzymes to DNA. Additional drugs of interest are fostriecin and merbarone, which are not inhibitory towards topoisomerase I but inhibit topoisomerase II catalytic activity without inducing DNA cleavage [1, 7]. Fostriecin is of particular interest since it shares some structural features with fredericamycin A. The most obvious explanation for fredericamycin's effect is binding of the drug to DNA, thereby interfering with the enzyme-substrate interaction. Warnick-Pickle et al. [15] could not demonstrate an interaction of fredericamycin with exogenous DNA in spectral difference studies. No metachromatic shift in the absorption spectrum of the drug was observed in the presence of DNA, nor was the hyperchromic shift in the DNA spectrum characteristic of DNA-binding agents seen in the presence of drug. Furthermore, the antibacterial activity of the drug was not affected by the presence of exogenous DNA, nor was the drug mutagenic in the Ames assay, with or without S9 activation. These studies certainly do not rule out DNA binding but would make it seem less likely to serve as a mechanism for the inhibitory effects of the drug. A possibility not experimentally addressed by us or others is that the drug exhibits structural similarity to the area of DNA to which the enzymes bind sufficient that it may compete for enzyme-binding sites with substrate DNA. Further work is required to explore this and other possibilities.

The diverse inhibitory activities of fredericamycin make it impossible to draw conclusions about its mechanism of cytotoxicity. In P388 leukemia cells in culture, fredericamycin was found to inihibit protein synthesis to a greater extent than that of RNA [15]; DNA synthesis was not affected at the concentrations of drug used (0.15 and 0.37 μ*M*). To elucidate a potential mechanism for fredericamycin A, Hilton and Misra [5] examined the drug by nuclear magnetic and electron paramagnetic resonance. They found that it underwent a change in color from red to blue on exposure to oxygen and that this was accompanied by loss of resonance from the quininoid half of the molecule. The basis for this appears to be a 1-electron oxidation of the quininoid group, with transfer to a molecular oxygen. Since free radicals of both oxidized drugs and reduced oxygen would be expected to be biologically toxic, these authors surmised that this would account for the mechanism of fredericamycin's antitumor effect. However, Hilton and Misra provide no biological evidence in support of this hypothesis. Other agents that have antitumor activity based on the generation of oxygen radicals, such as radiation and bleomycin, cause extensive DNA breakage, a phenomenon not associated with fredericamycin. However, we cannot rule out the possibility that free radicals of short duration might be generated in the cytoplasm or mitochondria of cells and would therefore not be expected to cause DNA breakage.

Our results suggest additional possibilities for the antitumor effect of fredericamycin. The potency of this compound with respect to its inhibition of topoisomerase I and

II and DNA polymerase alpha is considerable. For instance, the inhibition of topoisomerase I relaxation activity by 125 μM camptothecin is incomplete, and the complete inhibition of topoisomerase II unknotting activity by teniposide requires a concentration of approximately 125 μM [6]. Although fredericamycin is an order of magnitude less potent in its inhibition of DNA polymerase α than aphidicolin, there are few other structures that exhibit even this degree of inhibitory activity. If the drug exerts such diverse inhibitory effects intracellularly, it is possible that a single mechanism of action may not account for its cytotoxicity. In any event, the drug represents an interesting lead compound for future synthetic efforts. Structure-activity relationship studies of fredericamycin congeners should be both mechanistically and therapeutically productive.

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