Induction of Mammalian DNA Topoisomerase II Dependent DNA Cleavage by Antitumor Antibiotic Streptonigrin

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ABSTRACT

Streptonigrin, a nonintercalative antitumor antibiotic, induced mammalian topoisomerase II dependent DNA cleavage in vitro. The cleavage activity of streptonigrin was comparable to that of demethylepipodophyllotoxin ethyldiene-D-glucoside at a low concentration (<10 μM) but one-third lower at a higher concentration (>250 μM). Exposure of a reaction mixture containing streptonigrin, DNA, and topoisomerase II to an elevated temperature (65°C) resulted in substantial reduction in DNA cleavage, suggesting that the mechanism of the topoisomerase II dependent DNA cleavage induced by streptonigrin was through the formation of a cleavable complex previously reported for topoisomerase II poisons such as 4'- (9-acridinylamino) methanesulfon-m-anisidide and epipodophyllotoxins.

INTRODUCTION

DNA topoisomerase II is a nuclear enzyme that alters DNA conformation through a concerted breaking and rejoining of both strands of the DNA backbone (reviewed in Ref. 1). Recent studies have demonstrated that topoisomerase II is the principal intracellular target for a number of clinically important antitumor agents with diverse and unrelated chemical structures (reviewed in Ref. 2). These drugs, referred to as topoisomerase II poisons, have been classified as intercalating agents (acridines, ellipticines, and anthracyclines) or nonintercalating epipodophyllotoxins (3–6). All these drugs trap the enzyme in an intermediary reversible complex with DNA, termed the "cleavable complex," which prevents the final rejoining step of the reaction and results in increased DNA strand cleavage. A correlation between the ability of a particular agent to induce cleavable complexes and its cytotoxicity has been reported in structure-activity studies on large numbers of acridine derivatives and epipodophyllotoxin congeners (7–9).

In order to identify new topoisomerase II poisons, we have screened cultures of actinomycetes and fungi for their ability to induce topoisomerase II dependent DNA cleavage in vitro. We found that flavonoids such as genistein and orobol are potent inducers of topoisomerase II dependent DNA cleavage (10) and have now found that a topoisomerase II active compound produced by Streptomyces sp. S-224 and S-502 is the antitumor antibiotic streptonigrin (Fig. 1). In this report, we describe the effect of streptonigrin on mammalian DNA topoisomerase II and discuss the possible relations to its cytotoxicity and antineoplastic activity.

MATERIALS AND METHODS

Enzymes, Nucleic Acids, and Chemicals. DNA topoisomerase II was isolated from calf thymus as described by Halligan et al. (11) and partially purified with Bio-Rex70, hydroxylapatite, and P-11 phosphocellulose column chromatography. Proteinase K was from Sigma Chemical Co. Supercoiled pBR322 DNA was purified from Escherichia coli as previously described (12). Streptonigrin and VP-162 were obtained from the National Cancer Institute. Stock solutions of these drugs were dissolved in dimethyl sulfoxide at 50 mM and were diluted in methanol containing 20% dimethyl sulfoxide before use.

Agarose Gel Assay for DNA Relaxation and Topoisomerase II Dependent DNA Cleavage. Reactions (20 μl) containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl2, 1 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 0.4 μg pBR322 DNA, and calf thymus DNA topoisomerase II with or without drug were incubated at 37°C for 60 min and analyzed by agarose gel electrophoresis as described below (DNA relaxation assay). For DNA cleavage, reactions were terminated by addition of 2 μl of a solution containing 5% SDS and 2.5 mg/ml proteinase K. Following an additional 60-min incubation at 37°C, the samples were electrophoresed through a 1.2% agarose gel. For DNA unwinding assay, each reaction mixture (20 μl each in the same reaction mixture as described above) contained 0.4 μg relaxed pBR322 DNA, excess topoisomerase I, and drugs. The concentration of topoisomerase I used in the unwinding assay was at least 10 times higher than that required for full relaxation of pBR322 DNA in the relaxation assay. Following electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination. To determine the amount of linear DNA produced, negatives were scanned by a Shimazu microdensitometer. Peak area of the Gaussian peak was measured and the percentage of linear DNA was calculated.

DNA Unwinding Measurement. DNA unwinding effects of intercalators were assayed according to the method described by Chen et al. (4). Briefly, relaxed form DNA was prepared by treatment of pBR322 DNA with an excess amount of topoisomerase I from Kato III cells in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 30 mg/ml bovine serum albumin. After phenol extraction and ethanol precipitation, relaxed pBR322 DNA was resuspended in Tris-EDTA buffer and used in additional experiments as substrate. For the unwinding assay, each reaction mixture (20 μl each in the same reaction mixture as described above) contained 0.4 μg relaxed pBR322 DNA, excess topoisomerase I, and drugs. The concentration of topoisomerase I used in the unwinding assay was at least 10 times higher than that required to generate full relaxation of any substrate we have used. After reaction for 60 min at 37°C, the DNA was ethanol precipitated and resuspended in Tris-EDTA buffer and analyzed by agarose gel electrophoresis as described above.

RESULTS

In the course of screening microbial products with topoisomerase II dependent DNA cleavage activity, we have found that a topoisomerase II active compound isolated from culture broth of Streptomyces sp. S-224 and S-502 was identical to streptonigrin. Streptonigrin was shown to inhibit the catalytic activity of purified calf thymus DNA topoisomerase II by relaxation of pBR322 DNA. VP-16 was included for comparison since it is a well established inhibitor of this enzyme (4). Streptonigrin inhibited the relaxation activity in a concentration dependent manner at 2.5–500 μM (Fig. 2, lanes d–h). VP-16 caused similar inhibition at higher concentrations (Fig. 2, lanes i–m). The concentration dependent DNA topoisomer shift produced by streptonigrin is not due to the reduction of topoisomerase II mediated DNA relaxation by removing DNA supercoiling.

2 The abbreviations used are: m-AMSA, 4'- (9-acridinylamino) methanesulfon- m-anisidide; VP-16, demethylepipodophyllotoxin ethyldiene-D-glucoside; VM-26, 4'-demethylepipodophyllotoxin thelyliden e-D-glucoside; SDS, sodium dodecyl sulfate; CCC-DNA, covalently closed circular DNA.
DNA CLEAVAGE INDUCTION BY STREPTONIGRIN

Fig. 1. Structure of streptonigrin.

because streptonigrin does not intercalate as described below. When DNA relaxation activity of calf thymus topoisomerase I was also assayed in the presence of streptonigrin at the same range of concentration, no inhibitory effect was observed (data not shown). These findings indicated that streptonigrin selectively inhibits the catalytic activity of mammalian topoisomerase II.

Several lines of evidence indicate that inhibitors of topoisomerase II stabilize the DNA-topoisomerase II cleavable complex, which upon exposure to denaturing agents results in the induction of DNA strand cleavage. Fig. 3 shows an agarose gel electrophoresis comparing the topoisomerase II dependent DNA cleavage activities of streptonigrin and VP-16. As the concentrations of streptonigrin increased (from 0.5 μM to 500 μM in lane i), the linear full length DNA progressively appeared. In the absence of the enzyme, streptonigrin did not induce any changes in the supercoiled structure of pBR322 DNA (data not shown). These results indicate that streptonigrin has comparable topoisomerase II dependent DNA cleavage activity to m-AMSA and VP-16 in vitro. To obtain quantitative data provided by streptonigrin and VP-16, the amount of linearized DNA was measured by scanning the gels with a densitometer. Low concentrations of streptonigrin (2.5–12.5 μM) induced the topoisomerase II dependent DNA cleavage with the same potency as VP-16 (Fig. 4). In contrast, at higher concentrations (50–500 μM), topoisomerase II dependent DNA cleavage activity of streptonigrin was lower than that of VP-16.

The formation of cleavable complex by antitumor drugs has been shown to be reversible, and DNA cleavage is greatly reduced by dilution or increased salt concentration in a reaction mixture (3–6). Recently, Hsiang et al. demonstrated that brief exposure of cleavable complex formed by VM-26 to an elevated temperature (e.g., 65°C) caused a rapid reversal of the cleavage reaction both in a purified system and in cultured cells (13). To determine whether streptonigrin also has a similar effect as that of VP-16 and VM-26 in stabilizing the cleavable complex, we tested the heat reversibility of topoisomerase II dependent DNA cleavage activity induced by streptonigrin. As shown in Fig. 5, brief exposure of incubated reaction mixture to 65°C prior to the addition of SDS and proteinase K resulted in the time dependent suppression of subsequent DNA cleavage. Within almost 1 min, the majority of DNA cleavage (linear form) induced by both streptonigrin and VP-16 was suppressed. These results suggest that the mechanism of the topoisomerase II dependent DNA cleavage induced by streptonigrin is through the cleavable complex formation as seen with VP-16 and the stability of its complex appears to be similar to that of VP-16.

Most of the antitumor drugs which can induce cleavable complex formation are intercalative drugs, such as m-AMSA, Adriamycin, and ellipticine (2), so we examined whether streptonigrin can intercalate plasmid DNA, using a DNA unwinding assay (4, 10). As shown in Fig. 6, streptonigrin did not show the unwinding activity even at a concentration as high as 500 μM, in contrast to a typical intercalator, m-AMSA, which produced concentration dependent alteration of topoisomerase I mediated DNA relaxation. This result is consistent with that of previous studies demonstrating that there is little tendency for streptonigrin to intercalate DNA (14, 15). Therefore streptonigrin can be categorized as a nonintercalative drug with cleavable complex inducing activity like VP-16 and VM-26.
DNA Cleavage Induction by Streptonigrin

Fig. 4. Quantitation of the induction of the topoisomerase II dependent DNA cleavage by streptonigrin and VP-16. The percentage of DNA linearized by topoisomerase II in the presence of the drugs was determined by scanning the gels with a densitometer.

Fig. 5. Heat reversal of drug induced topoisomerase II dependent DNA cleavage. DNA cleavage assays were done as described in “Materials and Methods.” A large reaction mixture (120 µl) containing streptonigrin and VP-16 (250 µM for each compound) were at 37°C for 30 min. The reaction mixture was then heated to 65°C and aliquots (20 µl) were withdrawn at various times after the heat treatment. SDS and proteinase K treatment were done as described in “Materials and Methods.” Lane a, CCC-DNA control (no enzyme, no drug); lane b, linear DNA control; lanes c–g, samples containing streptonigrin withdrawn at 0, 0.5, 1, 5, 15 min after heat treatment; lanes h–l, same as lanes c–g, respectively, except that VP-16 was used.

Fig. 6. Effects of streptonigrin and m-AMSA on DNA unwinding assay. Unwinding measurements were done as described in “Materials and Methods.” Lane a, CCC-DNA control; lane b, linear DNA control; lane c, no drug; lanes d–h, streptonigrin; lanes i–m, m-AMSA. Drug concentrations were: lanes d and i, 2.5 µM; lanes e and j, 12.5 µM; lanes f and k, 50 µM; lanes g and l, 250 µM; lanes h and m, 500 µM.

DISCUSSION

Recent studies have demonstrated that DNA topoisomerase II is an important cellular target for a number of antitumor drugs including both DNA intercalators (m-AMSA, ellipticine, and Adriamycin) and the nonintercalator epipodophyllotoxin derivatives (VP-16 and VM-26) (3–6, 16). Although these topoisomerase II inhibitors have been used to establish a relationship between drug induced cleavable complex formation and cytotoxicity (7–9), other new topoisomerase II targeting drugs with antitumor activity in vivo have not been reported yet. In this study, we have demonstrated that the antitumor antibiotic streptonigrin has a cleavable complex inducing activity similar to those of m-AMSA or VP-16.

To study the effect of streptonigrin on topoisomerase II activity, we compared it with the nonintercalative topoisomerase II poison VP-16, since streptonigrin is a nonintercalative drug (Fig. 6). The effects of streptonigrin and VP-16 on purified topoisomerase II relaxation activity was dose related (Fig. 2). More prominent topoisomer ladders produced by streptonigrin at low drug concentration (2.5–50 µM) indicated that streptonigrin has a higher inhibitory effect than VP-16 on the relaxation reaction catalyzed by topoisomerase II. In the case of topoisomerase II dependent DNA cleavage activity, which is considered to be important for antitumor activity of the drug, streptonigrin has almost comparable activity to that of VP-16 at low drug concentrations, but at high concentration, this activity tends to a plateau differing with a dose-dependent increase as observed with VP-16. Indeed, at 500 µM, streptonigrin induced about one-third of the topoisomerase II dependent DNA cleavage activity compared to that of VP-16. Equivalent stability of cleavable complex induced by these two drugs was supposed, since the reversibility of cleavable complex formation, which is one of the unique properties of topoisomerase II-DNA cleavable complex, is similar in both streptonigrin and VP-16. Several compounds like ethidium bromide and a-AMSA that do not induce cleavable complexes have been known to inhibit topoisomerase II activity by blocking topoisomerase II access to the template (3, 5), and it has been reported that cleavable complexes are actually reduced at high doses of some cleavable complex inducing drugs such as m-AMSA (17). Mizuno and Gilboe (14) showed that streptonigrin has at least two types of binding properties to DNA in vitro, one which is reversible by dialysis and the other which is irreversible. Therefore, the higher inhibitory effect of streptonigrin on relaxation activity is supposed to be induced by DNA structure alteration following drug-DNA binding rather than by stabilizing cleavable complex formation, while the limited DNA cleavage activity of streptonigrin at high concentration might be due to a secondary influence of the binding to DNA. In addition to the binding activity for DNA, the interaction between topoisomerase II and drug is also important to regulate formation of cleavable complex as pointed out with VP-16 and VM-26 (18). Further study will be required to reveal the detailed mechanisms by which streptonigrin induces cleavable complex formation as well as inhibition of topoisomerase II.

Streptonigrin has been shown to be a potent cytotoxic agent in vitro and possesses broad spectrum antitumor activity against a number of murine tumor models (19). Although the potential mechanism for its cellular toxicity has remained unclear, there is evidence that the lethal effect of streptonigrin is associated with DNA degradation. Streptonigrin inhibits mitosis upon addition to culture of human leukocytes and also causes extensive chromosomal breaks and other abnormalities (20). Many
researchers have speculated that the in vitro single-strand DNA cleavage activity of streptonigrin detected in situ reduction or in the presence of oxygen can be involved in the mechanism of cellular DNA degradation. However, the results reported here suggest that induction of cleavable complex formation and subsequent DNA damage may be a key mechanism in its cytotoxic effect. Recently, Eng et al. (21) showed that the repair deficient yeast strain with rad52 mutations is hypersensitive to the topoisomerase II poisons such as m-AMSA and ellipticine, and streptonigrin is also much more cytotoxic for rad52 mutants than for DNA repair proficient strains among cytotoxic drugs tested. This finding is consistent with our result in vitro indicating that streptonigrin may be classified as a new topoisomerase II poison.

We have previously shown that the isoflavonoids, genistein and orobol, are nonintercalative topoisomerase II poisons like VP-16 and VM-26 (10). In contrast to these isoflavonoids which have not shown any clear antitumor activity, streptonigrin has been known to be a potent antitumor agent in several murine models and also in clinical study. Our results on streptonigrin suggest that the further reevaluation of the mechanism of action of antitumor drugs should be important for understanding their antitumor activity as well as for designing of new topoisomerase poisons.

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