Induction of a Heat-stable Topoisomerase II-DNA Cleavable Complex by Nonintercalative Terpenoides, Terpentecin and Clerocidin

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ABSTRACT

Terpentecin and clerocidin, microbial terpenoides, have been known to be potent antitumor antibiotics. However, the critical biochemical target of these terpenoides has not been identified. Our present studies, using purified mammalian topoisomerase II, have shown that terpentecin and clerocidin induce topoisomerase II-mediated DNA cleavage in vitro with comparable potency to that of demethylepipodophyllotoxin ethyldene-β-D-glucoside. These terpenoides produced a similar DNA cleavage pattern which is distinctly different from those generated in the presence of the known topoisomerase poisons, demethylepipodophyllotoxin ethyldene-β-D-glucoside and 4'-(9-acridinylamino)methanesulfon-m-anisidide. Brief heating at 65°C, which abolishes completely the cleavable complex with demethylepipodophyllotoxin ethyldene-β-D-glucoside, of the reaction mixture containing these terpenoides resulted in slight reduction in DNA cleavage. Thus, different from other topoisomerase II-active antitumor agents, terpentecin and clerocidin induce formation of a cleavable complex which is stable for heat or salt treatments. The lack of significant DNA binding or intercalation activity of terpentecin and clerocidin suggests that topoisomerase II is a cellular target for these drugs.

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, thereby controlling the topological state of DNA (reviewed in Ref. 1). Topoisomerase II is involved in many processes of DNA metabolism, including replication, transcription, recombination, and chromosome segregation at mitosis. Recent studies have demonstrated that topoisomerase II is the primary cellular target for a number of clinically important antitumor agents with diverse and unrelated chemical structures (reviewed in Refs. 2 and 3). These drugs, referred to as topoisomerase II poisons, have been classified as intercalating agents (acridines, ellipticines, and anthracyclines) or nonintercalative epipodophyllotoxins (4-7). 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 good 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 epipodophyllotoxins (8-10). Thus, the identification of other new drugs which induce formation of the cleavable complex has been viewed as a promising approach to find clinically effective anticancer agents.

We have screened cultures of antinomycetes and fungi for their ability to induce topoisomerase II-mediated DNA cleavage in vitro. We found that flavonoids, such as genistein, orobol (11), and the antitumor antibiotic streptonigrin (12), are potent inducers of topoisomerase II-mediated DNA cleavage. In the course of screening, we have now found that diterpenoides, such as terpentecin (13) and clerocidin (14), show potent topoisomerase II-mediated DNA cleavage activity in vitro. These compounds have been known to have potent antitumor activities (13, 14); however, the critical biochemical target has not been identified.

In this report, we describe the nature of the cleavable complex induced by terpentecin and clerocidin and discuss the possible relation of topoisomerase II to their 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. (15) and partially purified with Bio-Rex70, hydroxyapatite, and P-11phosphocellulose column chromatography. Proteinase K was from Sigma Chemical Co. Supercoiled pUL402 DNA, which contains the scaffold-associated regions from the Drosophila histone gene cluster (16), was purified from Escherichia coli as described (17). Terpentecin was isolated from culture broth of Streptomyces sp. S-464 and was identified by spectroscopic analysis. Clerocidin was isolated from culture broth of Oidiodendron truncatum and was identified by comparing a standard sample which was a gift from Leo Pharmaceutical Co. m-AMSA and VP-16 were provided by Warner Lambert Co. and the National Cancer Institute, respectively. Stock solutions of these drugs were dissolved in 50 mM dimethyl sulfoxide and diluted in methanol containing 20% dimethyl sulfoxide before use.

Agarose Gel Assay for 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 of bovine serum albumin, 0.4 μg of pUL402 DNA, and calf thymus DNA topoisomerase II with or without drug were incubated at 37°C. After 30 min, reactions were terminated by the addition of 2 μl of a solution containing 5% SDS and 2.5 mg/ml of proteinase K. Following an additional incubation at 37°C for 30 min, the samples were electrophoresed through a 1.2% agarose gel in 89 mM Tris-borate (pH 8.3)-2 mM EDTA buffer containing 0.1% SDS. The concentration of topoisomerase II used in the DNA cleavage assay was at least 20 times higher than that required for full relaxation of pUL402 DNA in the relaxation assay. After 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. The 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. (5). Briefly, the relaxed form of DNA was prepared by treatment of pUL402 DNA with an excess amount of topoisomerase II 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 of bovine serum albumin. After phenol extraction and ethanol precipitation, relaxed pUL402 DNA was resuspended in Tris-EDTA buffer and was 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 of relaxed pUL402 DNA, excess topoisomerase I, and drugs. The

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2 The abbreviations used are: m-AMSA, 4'-(9-acridinylamino)methanesulfon-m-anisidide; VP-16, demethylepipodophyllotoxin ethyldiene-β-D-glucoside; VM-26, 4'-demethylepipodophyllotoxin thienyldiene-β-D-glucoside; SDS, sodium dodecyl sulfate.

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Results

Induction of Topoisomerase II-mediated DNA Cleavage by Terpentecin and Clerocidin. In the course of screening for microbial products with topoisomerase II-mediated DNA cleavage activity, we have found that two topoisomerase II-active compounds isolated from culture broth of Streptomyces sp. S-464 and O. truncatum are identical to terpentecin and clerocidin, respectively, which have closely related structures (Fig. 1). Different from known topoisomerase-active agents, these terpenoid antibiotics do not have an aromatic ring, and their characteristic structures are distinct from known antitumor drugs. Therefore we have investigated further the nature of topoisomerase-mediated DNA cleavage induced by terpentecin and clerocidin, using purified calf thymus topoisomerase II and pUL402 DNA.

Fig. 2 shows a photograph of agarose gel electrophoresis comparing the topoisomerase II-mediated DNA cleavage activities of terpentecin, clerocidin, and VP-16. As the concentration of drugs increased (terpentecin from 0.25 μM in Lane d to 250 μM in Lane h, clerocidin from 0.25 μM in Lane i to 250 μM in Lane m), the linear DNA progressively appeared. To obtain the quantitative data, the amount of linearized DNA was measured by scanning the gels with a densitometer. As shown in Fig. 3, terpentecin induced the topoisomerase II-mediated DNA cleavage with stronger potency than that of VP-16, and clerocidin showed a potency similar to that of VP-16. DNA breaks increased linearly as the amount of terpentecin or clerocidin increased to over 100 μM. Such has been the case only for the epipodophyllotoxins, VP-16 (etoposide) and VM-26 (teniposide). In the absence of topoisomerase II, terpentecin and VP-16 did not induce any changes in the supercoiled structure of pUL402 DNA (Fig. 4, Lanes e and h). The breaks induced by terpentecin (Fig. 4, Lane g) and clerocidin (data not shown) were detected only after the addition of SDS and proteinase K in a manner similar to that of VP-16 (Fig. 4, Lane j).

Fig. 2. Topoisomerase II-mediated DNA cleavage by terpentecin, clerocidin, and VP-16. Cleaveage of DNA was analyzed by the agarose gel assay described in "Materials and Methods." Lane a, covalently closed circular (CCC) and open circular (OC)-DNA control; Lane b, linear DNA control; Lane c, no drug; Lanes d to h, terpentecin; Lanes i to m, clerocidin; Lanes n to r, VP-16. Drug concentrations were the following: Lanes d, i, and n, 0.5 μM; Lanes e, j, and o, 2.5 μM; Lanes f, k, and p, 12.5 μM; Lanes g, l, and q, 50 μM; Lanes h, m, and r, 250 μM.

Fig. 3. Quantitative comparison of the topoisomerase II-mediated DNA cleavage induced by terpentecin, clerocidin, 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. Terpentecin, Clerocidin, VP-16.

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, resuspended in Tris-EDTA buffer, and analyzed by agarose gel electrophoresis as described above.

Cytoxic Activity. Cytotoxic activity of terpentecin and clerocidin on BALB 3T3/H-ras was determined by the method described by Takahashi et al. (18).
II-mediated DNA cleavage with terpentecin and clerocidin have a similar pattern which is distinctly different from those with VP-16 and m-AMSA (data not shown).

Stability of the Cleavable Complex. The formation of a cleavable complex with antitumor drugs has been shown to be reversible, and DNA cleavage is greatly reduced by dilution or increased salt concentration in a reaction mixture (4–7). Recently, Hsiang and Liu (20) demonstrated that brief exposure of the cleavage complex formed by VM-26 to an elevated temperature (e.g., 65°C) caused a rapid reversal of the cleavage reaction in both a purified system and cultured cells. To see whether the cleavable complex formed with terpentecin and clerocidin also has the property similar to that with VP-16 and VM-26, we tested the heat reversibility of topoisomerase II—mediated DNA cleavage activity induced by terpentecin and clerocidin.

As shown in Fig. 5, exposure to 65°C for 15 min of incubated reaction mixture with terpentecin (Lanes c to g) and clerocidin (Lanes h to l) prior to the addition of SDS and proteinase K could not suppress the DNA cleavage. In contrast, there was a great heat reversal of the DNA cleavage induced by VP-16, and linear DNA was not detectable after 5 min (lanes m to q). Addition of NaCl (0.5 M final concentration) to the reaction mixture showed a result similar to that of heat treatment (data not shown). These results indicate that the cleavable complex induced by terpentecin and clerocidin appears to be more stable than that of VP-16.

Terpentecin and Clerocidin Do Not Intercalate DNA. Most of the antitumor drugs which can induce cleavable complex formation are intercalative drugs, such as m-AMSA, Adriamycin, and ellipticine (4), and so we examined whether terpentecin and clerocidin can intercalate into plasmid DNA using a DNA-unwinding assay (5, 11). As shown in Fig. 6, terpentecin (Lanes b to e) and clerocidin (Lanes f to i) did not show the unwinding activity even at a concentration as high as 250 μM, in contrast to a typical intercalator m-AMSA which produced concentration-dependent alteration of topoisomerase I-mediated DNA relaxation (Lanes j to m). Therefore terpentecin and clerocidin can be categorized as nonintercalative drugs with the cleavable complex-inducing activity like VP-16 and VM-26.

Biological Activities and Ability to Induce Topoisomerase II—mediated DNA Cleavage of Terpentecin and Clerocidin. Structure-activity studies on topoisomerase poisons, which represent a homologous group with limited structural modifications, suggest that there is a good correlation between cytotoxicity and the ability of drugs to induce a cleavable complex (9, 10). In agreement with this possibility is the present finding that the level of cytotoxicity (50% inhibitory concentration) of terpentecin and clerocidin on BALB 3T3/H-ras cells is 0.03 and 0.28 μg/ml, respectively, which correlates well with their ability to induce topoisomerase II-mediated DNA cleavage activity (Fig. 3).

These results show that terpentecin and clerocidin represent a new class of nonintercalative antitumor agents which induce a stable DNA cleavable complex with topoisomerase II.

DISCUSSION

We have previously shown that a potent antitumor agent, streptonigrin, induces the topoisomerase II-mediated DNA cleavage. In this study, we present data showing that the antitumor antibiotics, terpentecin and clerocidin, have a strong cleavable complex-inducing activity which is comparable to that of m-AMSA or VP-16. These findings on streptonigrin, terpentecin, and clerocidin 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 new topoisomerase poisons.

To understand the nature of the interaction among drug, DNA, and topoisomerase II, we compared the effects of terpentecin and clerocidin on DNA and topoisomerase II with m-AMSA and VP-16. Both terpentecin and clerocidin are nonintercalative drugs as shown in Fig. 6. Topoisomerase II-mediated DNA cleavage activities of terpentecin, clerocidin, and VP-16 were dose dependent; terpentecin has stronger activity than does VP-16, and clerocidin has activity comparable to that of VP-16. In this study, we used pUL402 DNA which contains scaffold-associated regions from the Drosophila genome (16). Our other standard cleavage experiments using pBR 322 DNA have shown that the relative potency to induce DNA cleavage is of the same order: terpentecin > clerocidin = VP-16 (data not shown). As has been reported before, the patterns of cleavage products are very different in the presence of different classes of drugs. In agreement with this, terpentecin and clerocidin produced similar DNA fragments which were distinctly different from the fragments generated by m-AMSA or VP-16.
Several studies on the stability of the cleavable complex revealed that the cleavable complex induced by topoisomerase II is resistant to heat treatment; heat treatment has been known to be more effective than high-salt treatment. Terpentecin and clerocidin have an epoxide moiety that has been known to react with DNA and other molecules. It is possible that the epoxide moiety of terpentecin and clerocidin covalently modifies the cleavable complex with topoisomerase II and DNA and, thereby, the cleavable complex induced by terpentecin and clerocidin is resistant to the heat or salt treatment which can reverse the known cleavable complex induced by VP-16 or m-AMSA.

At present, little is known about how topoisomerase-active drugs form a cleavable complex between the enzyme and DNA. Apparently a nonintercalative drug has been preferred for studies on this point. Terpentecin and clerocidin do not intercalate into DNA and, moreover, the structure of these diterpenoids is very distinctive from known nonintercalative topoisomerase poisons, such as quinolones and VP-16, which had been suggested to bind weakly to DNA. The nature of terpentecin and clerocidin, especially their ability to form a stable cleavable complex, will provide a useful tool for future experimental approaches to understand the mechanism of cleavable complex formation.

Terpentecin and clerocidin have been shown to have a potent cytotoxic activity in vitro and antitumor activity against murine tumor models in addition to antimicrobial activity (13, 14). However, the critical biochemical target of these terpenoids has not been identified. Tamamura et al. (21) have reported that terpentecin has a bacterioidal effect on E. coli, but it showed only partial inhibition of ATP-dependent DNA synthesis in toluene-treated E. coli, and synthesis of other macromolecules was little affected (21). We would like to infer that the results reported by Tamamura et al. (22) are similar to the effect of nalidixic acid and novobiocin, antimicrobial drugs targeting DNA gyrase.

In this paper, we have reported that the ability of the terpentecin and clerocidin to induce topoisomerase II-mediated DNA cleavage shows a good correlation to their cytotoxicities. In murine lymphocytic leukemia p388 models, terpentecin has shown antitumor activity at a single i.p. dose of 3 to 0.7 mg/kg, whereas the effective dose of clerocidin has been reported to be 250 to 12.5 mg/kg (14). These optimum doses in tumor models in vivo are also compatible to the relative effectiveness of the drugs in inducing topoisomerase II-mediated DNA cleavage in vitro. Thus, the results reported here suggest that induction of cleavable complex formation and subsequent cellular damage can be a key mechanism in the antitumor activity of terpentecin and clerocidin.

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