0026-895X/99/061049-05$3.00/0

MOLECULAR PHARMACOLOGY, **55:**1049–1053 (1999).

Mechanism of Inhibition of a Poxvirus Topoisomerase by the Marine Natural Product Sansalvamide A

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Received December 22, 1998; accepted March 17, 1999

ABSTRACT

At present no antiviral agents are available for treatment of infection by the pathogenic poxvirus molluscum contagiosum virus (MCV). Here we report the identification and characterization of an inhibitor active against the virus-encoded type-1 topoisomerase, an enzyme likely to be required for MCV replication. We screened a library of marine extracts and natural products from microorganisms using MCV topoisomerase assays in vitro. The cyclic depsipeptide sansalvamide A was found to inhibit topoisomerase-catalyzed DNA relaxation. Sansalvamide A was inactive against two other DNA-modifying enzymes tested as a counterscreen. Assays of discrete steps in the topoisomerase reaction cycle revealed that sansalvamide A inhibited DNA binding and thereby covalent complex formation, but not resealing of a DNA nick in a preformed covalent complex. Sansalvamide A also inhibits DNA binding by the isolated catalytic domain, thereby specifying the part of the protein sensitive to sansalvamide A. These data specify the mechanism by which sansalvamide A inhibits MCV topoisomerase. Cyclic depsipeptides related to sansalvamide A represent a potentially promising chemical family for development of anti-MCV agents.

Here we present a study of a marine fungal product, sansalvamide A, which inhibits the topoisomerase enzyme of the pathogenic poxvirus molluscum contagiosum virus (MCV). MCV infection in healthy people causes only small papules that are easily treated. In AIDS patients, however, MCV causes severe lesions that are essentially untreatable. Cells near the surface of lesions become many times larger than normal, forming papules that become filled with a granular mass called "molluscum bodies". Untreated papules in healthy people usually disappear spontaneously within several months, but in AIDS patients dense crops can persist, disfiguring infected patients. In HIV-infected people, rates of infection may be as high as 5 to 18% and as many as 33% of AIDS patients with CD4+ counts of less that 100 cells/mm^3 may be infected (Petersen and Gerstoft, 1992; Porter et al., 1992; Schwartz and Myskowski, 1992; Gottlieb and Myskowski, 1994).

Among the MCV genes identified in the recently completed primary sequence was one encoding a putative type I topoisomerase (Senkevich et al., 1996). Type I topoisomerases catalyze the formation of transient nicks in DNA that permit DNA relaxation (Gupta et al., 1995). MCV and the other poxviruses all encode topoisomerases, and in the case of vaccinia, it has been shown that replication requires topoisomerase function (Shuman et al., 1989).

This work was supported by Grant R97-SAL-088 from the University of California University-wide AIDS Research Program (F.D.B.) and a grant from the Pendelton Foundation for imaging facilities, and by the National Institutes of Health National Cancer Institute Grant CA 44848 (W.F.). F.D.B. is a scholar of the Leukemia Society of America.

MCV topoisomerase, like that of other poxviruses, is highly sequence-specific (Hwang et al., 1998; Y.H., A. Burgin and F.B., in press). Poxvirus topoisomerases bind to the sequence 5′-(C/T)CCTT-3′ and cleave DNA just 3′ of the last T, forming a covalent phosphotyrosine linkage. After DNA relaxation, the single strand DNA break is resealed by transesterification with the adjacent 5′ hydroxyl, releasing the enzyme from the relaxed DNA product. Topoisomerase activity has been implicated as important for DNA replication, repair, transcription, and other biological processes (Wang, 1996).

We have carried out a survey of crude extracts and purified secondary metabolites from marine bacteria and fungi in an effort to identify useful inhibitors of MCV topoisomerase (Jensen and Fenical, 1994; Davidson, 1995). Here we report that sansalvamide A, a cyclic depsipeptide produced by a marine fungus *Fusarium* species, inhibits MCV topoisomerase in vitro. Assays of different steps in the topoisomerase catalytic cycle reveal that sansalvamide A inhibits DNA binding, but not strand religation. Sansalvamide A also inhibited the activity of the enzyme catalytic domain, beginning to specify the inhibitor site of action. This represents the first identification of a new inhibitor isolated by primary screening against MCV topoisomerase in vitro.

Materials and Methods

Purification of MCV Topoisomerase. MCV topoisomerase (MCV-TOP) was purified using nickel-chelating sepharose as described (Hwang et al., 1998). MCV-TOP (82–323), the catalytic do-

ABBREVIATIONS: MCV, molluscum contagiosum virus; sub a, oligonucleotide substrate a; MCV-TOP, MCV topoisomerase.

main, was purified using chromatography on nickel-chelating sepharose and carboxy-methyl-sepharose (Y.H., M. Park, W. Fisher and F.D.B., submitted).

MCV Topoisomerase Activity Assays. Standard conditions for assaying relaxation, DNA binding, covalent complex formation, and religation activities were 200 mM potassium glutamate, 20 mM Tris-Cl (pH 8.0), 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM EDTA. To test inhibition of MCV topoisomerase, sansalvamide A was added to the enzyme and preincubated for 5 min and then the reaction was started with the addition of substrate. The reaction mixtures contained a 10% (v/v) final concentration of dimethyl sulfoxide.

Relaxation assays using pUC19 DNA were carried out as described (Hwang et al., 1998). For the DNA binding assays, oligonucleotide substrate a (sub a) was 5′-end labeled with ^{32}P, added to MCV topoisomerase enzyme previously incubated with sansalvamide A, and incubated for 5 min at 37°C. The reaction mixtures were separated on 5% polyacrylamide gels, visualized by autoradiography, and the radioactivity was quantitated by a PhosphorImager. Sub a was a duplex DNA of 5′-TCCGTGTCGCCCTTATTCCCTTTTTGCGGCATTTTGCCT-3′ and 5′-GAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGG-3′. The covalent complex formation assay using sub a was carried out as described (Hwang et al., 1998).

For religation assays, a suicide substrate derived from sub a DNA was used. In this substrate (sequence 5′-TCCGTGTCGCCCTTATTCC-3′ and 5′-GAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGG-3′), the short duplex extension 3′ of the 5′-CCCTT-3′ sequence can be dissociated upon covalent complex formation, trapping the covalent complex but permitting religation to an added complementary DNA strand. Covalent complex formation was followed by religation induced by addition of 50-fold excess of a 15-mer single strand DNA (5′-ATTCCCTTTTTGCGG-3′). Covalent complex reaction mixtures were transferred to individual tubes, followed by addition of sansalvamide A, incubated for 5 min at room temperature, and then religation reaction was started by addition of a 15-mer single strand DNA to generate a product containing a 29-mer DNA. After incubation at 37°C for 5 min, formamide was added to 50% (v/v), the samples were denatured for 5 min at 95°C, and then the aliquots of samples were analyzed on DNA sequencing type gels. The extent of inhibition of religation was visualized by autoradiography and quantitated by PhosphorImager.

Sansalvamide A. Purification and structure determination for sansalvamide A (Fig. 1), produced by a marine fungus *Fusarium* species collected off the Bahamas, is described in (G. Belofsky and W. Fenical, submitted). The compound was assayed using NMR and thin-layer chromatography methods.

Results

Screening Compounds from Marine Sources for Inhibitory Activity. To identify inhibitors of MCV topoisomerase, 460 candidate extracts and purified compounds from marine microorganisms were screened. The investigation focused on secondary metabolites from marine fungi and bacteria, because compounds of interest could be readily obtained as needed by culturing the appropriate microorganisms (Jensen and Fenical, 1994). Assays in vitro were carried out initially in the presence of high concentrations of compound or extract in an effort to identify potential inhibitors. Extracts or compounds that selectively inhibited MCV topoisomerase were then titrated to determine the concentration sufficient for 50 percent inhibition (IC_{50}).

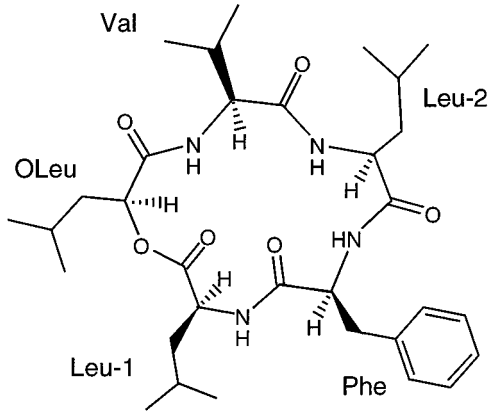

Fig. 1. Structure of sansalvamide A.

Inhibition of DNA Relaxation by Sansalvamide A. Type I topoisomerases carry out relaxation of DNA by first binding to duplex DNA, cleaving one strand to generate a enzyme-DNA covalent complex, relaxing the DNA, and subsequently resealing the nick by religation of the cleaved strand (Fig. 2).

To test the effect of sansalvamide A on DNA relaxation by MCV topoisomerase, the enzyme was incubated with supercoiled plasmid substrate, reaction mixtures were separated on agarose gels by electrophoresis, and DNA was visualized by staining with ethidium bromide. DNA relaxation results in a reduction in mobility of the relaxed DNA because the relaxed DNA form is less compact than the supercoil. Conversion of the supercoiled plasmid substrate DNA to relaxed circular DNA was measured after 5 min of incubation at 37°C in the presence of different concentrations of sansalvamide A. As shown in Fig. 3, sansalvamide A inhibited DNA relaxation by MCV topoisomerase in a concentration-dependent fashion. Quantitation of multiple tests yielded an IC_{50} of 124 μM.

Specificity of Inhibition by Sansalvamide A. As a counterscreen, inhibition of HIV-1 integrase was tested. HIV-1 integrase directs the cleavage of the termini of the HIV-1 cDNA and the subsequent covalent integration of the cleaved DNA ends into target DNA (for review, see Hansen et al., 1998). No inhibition of integrase was detectable (IC_{50} > 850 μM, data not shown).

In another counterscreen, inhibition of DNA cleavage by the restriction enzyme *Hin*dIII was tested. The IC_{50} was determined to be >400 μM (data not shown).

Effect of Sansalvamide A on DNA Binding. To investigate the mechanism of inhibition of MCV topoisomerase by sansalvamide A, we assayed inhibition of DNA binding, covalent complex formation, and religation separately.

To test the effect of sansalvamide A on DNA binding, 39-mer duplex DNA matching the highly active sub a sequence was used as substrate. MCV topoisomerase binds and produces a covalent complex at sites containing the sequence 5′-(C/T)CCTT-3′. The sub a substrate used for these tests contains the 5′-CCCTT-3′ embedded in optimal flanking sequences (Hwang et al., 1998; Y.H., A. Burgin and F.B., in press). MCV topoisomerase was mixed with end-labeled sub

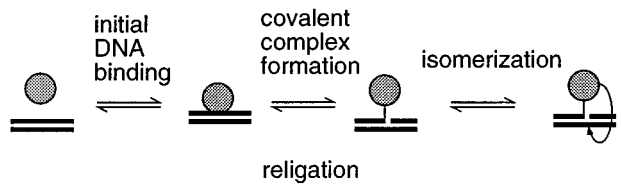

Fig. 2. Diagram of the mechanism of type I topoisomerases such as that encoded by MCV. Rotation as drawn in the "isomerization" step is hypothetical.

a DNA and the reaction mixture was separated by electrophoresis on native polyacrylamide gels. Protein-DNA complexes were visualized as slower migrating bands. Exposure of topoisomerase to sansalvamide A before addition of sub a caused a concentration-dependent decrease in the extent of complex formation (Fig. 4 lanes 3–7). The IC_{50} for inhibition of DNA binding was approximately 80 uM.

Effect of Sansalvamide A on Covalent Complex Formation. The effect of sansalvamide A on covalent complex formation was tested using sub a as substrate. Sub a was end-labeled on the 5′-end of the scissile DNA strand, then mixed with MCV topoisomerase and incubated at 37°C for 5 min. Covalent protein-DNA complex formation was assayed by SDS-polyacrylamide gel electrophoresis and autoradiography. The covalent complex was visualized as a labeled species migrating more slowly than the substrate DNA that exhibited the molecular weight expected of the topoisomerase linked to the covalently bound DNA. Exposure of topoisomerase to sansalvamide A before addition of the sub a substrate caused a concentration-dependent decrease in the extent of covalent complex formation (Fig. 5, lanes 3–7) with an IC_{50} of approximately 110 μM.

Effect of Sansalvamide A on Religation of the Covalent Complex. The effect of sansalvamide A on the religation activity of MCV topoisomerase was also tested. The covalent complex was trapped using a suicide substrate, which contains a short (5 base pairs) DNA segment 3′ of the 5′-CCCTT-3′ sequence. Upon covalent complex formation, the resulting 5-base strand will be released and lost by diffusion (Fig. 6A). To monitor religation, a labeled 15-base sequence was added that is complementary to the single stranded DNA in the covalent complex. Religation of the labeled input DNA was detected by the formation of a radioactive 29-mer product (Fig. 6B).

Religation product was detected after addition of the labeled 15-mer single strand DNA to the covalent complex (arrow marked “29”, Fig. 6B, lane 3). Quantitative measurement showed that 70% of the suicide substrate was converted into covalent complex (compare Fig. 6B, lanes 1–2 and data not shown). Exposure of topoisomerase-DNA covalent complex to sansalvamide A before addition of the 15-mer single stranded DNA had no effect on the accumula-

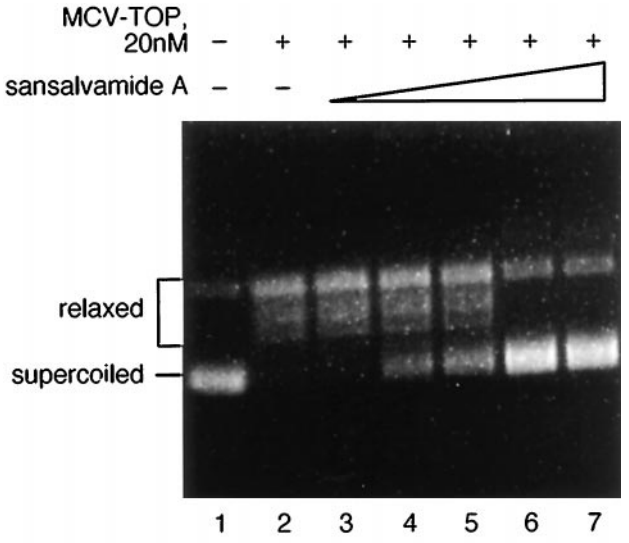

Fig. 3. Inhibition of DNA relaxation by sansalvamide A. Control reactions were performed without enzyme (lane 1) or without sansalvamide A (lane 2). Concentrations of sansalvamide A added were 10 μM (lane 3), 30 μM (lane 4), 100 μM (lane 5), 200 μM (lane 6), and 300 μM (lane 7).

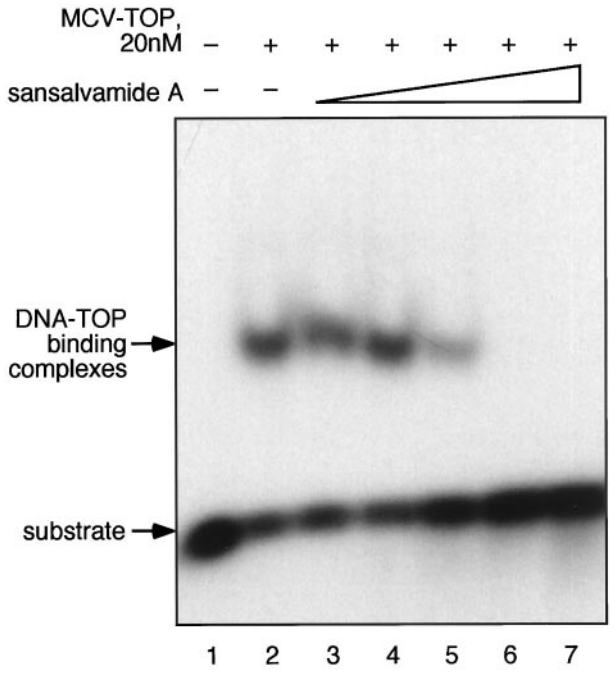

Fig. 4. Inhibition of DNA binding by sansalvamide A. Control reactions were performed without enzyme (lane 1) or without sansalvamide A (lane 2). Concentrations of sansalvamide A added were 10 μM (lane 3), 30 μM (lane 4), 100 μM (lane 5), 200 μM (lane 6), and 300 μM (lane 7). Enzyme-DNA complexes are indicated by the arrow.

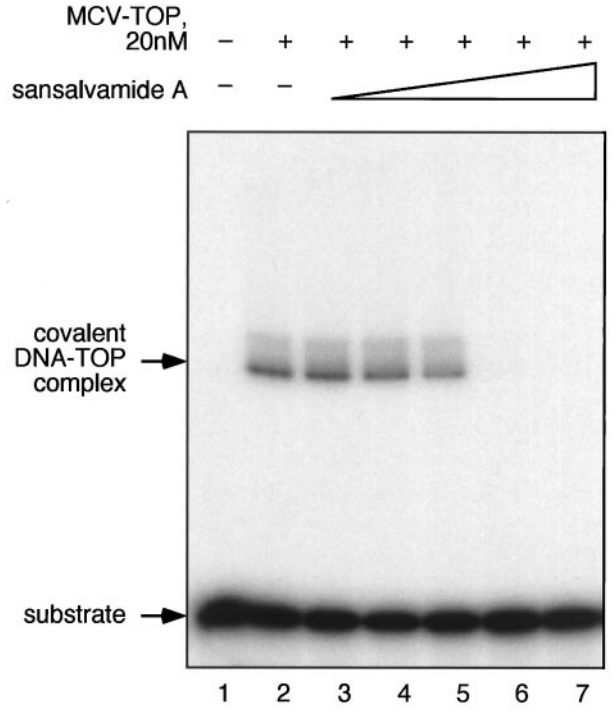

Fig. 5. Inhibition of covalent complex formation by sansalvamide A. Control reactions were performed without enzyme (lane 1) or without sansalvamide A (lane 2). Concentrations of sansalvamide A added were 10 μM (lane 3), 30 μM (lane 4), 100 μM (lane 5), 200 μM (lane 6), and 300 μM (lane 7).

tion of religated products over the concentration range tested (Fig. 6B, lanes 4–7).

Inhibition of DNA Binding by the Catalytic Domain of MCV Topoisomerase. Residues 82 to 323 of MCV topoisomerase comprise a flexible linker and the carboxyl terminal catalytic domain of the enzyme (Y.H., M. Park, W. Fisher and F.D.B., submitted). To examine the part of the enzyme affected by sansalvamide A, MCV-TOP (82–323), the purified linker and catalytic domain, was tested in reactions containing sansalvamide A. Titration of sansalvamide A into reactions containing 100 nM MCV-TOP (82–323) and labeled sub a DNA revealed inhibition of DNA binding (Fig. 7). The IC_{50} was indistinguishable from that seen with the full length enzyme. Note that covalent complex formation by MCV-TOP (82–323) is slower than with the full enzyme, so most of the complex seen in this experiment is the noncovalently bound form. Separate analysis of inhibition of covalent complex formation revealed inhibition paralleling the inhibition of DNA binding (data not shown). Evidently sansalvamide A is also capable of inhibiting function of the catalytic domain by blocking DNA binding, specifying the site of action of the compound.

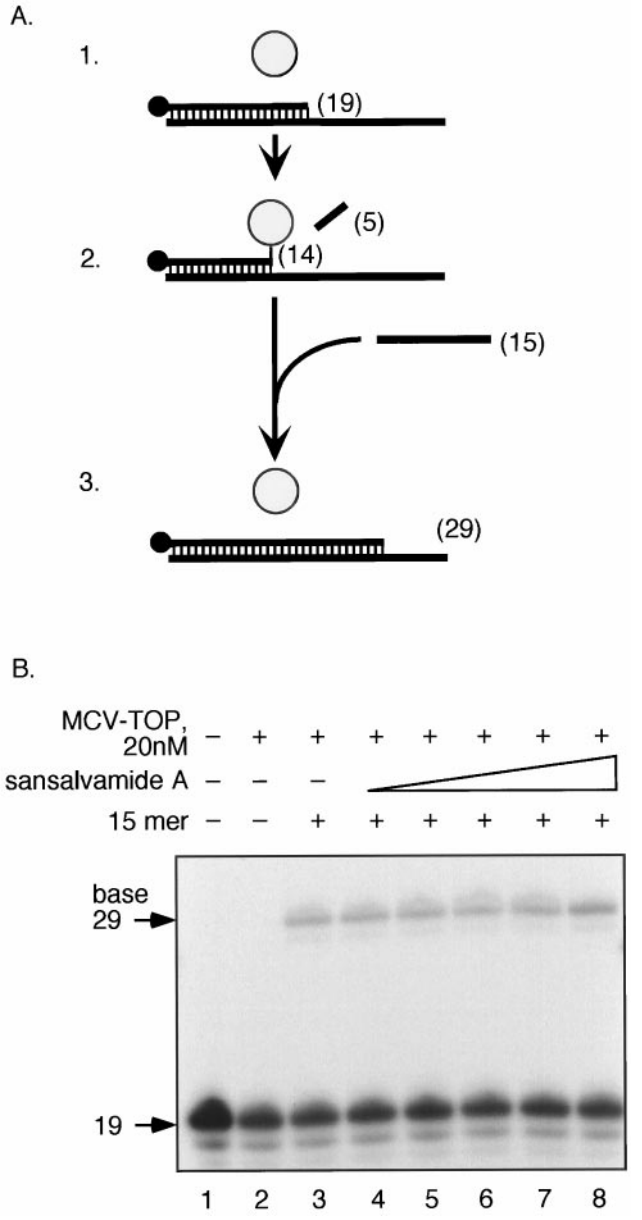

Fig. 6. Effect of sansalvamide A on DNA strand religation. A, diagram of the religation reaction. The suicide substrate labeled on 5′ end of the top strand (indicated by the ball) was incubated with MCV topoisomerase (step 1). Covalent complex formation resulted in releasing the short duplex extension 3′ of the 5′-CCCTT-3′ sequence (step 2), thereby trapping the covalent complex. This allowed the religation reaction to be carried out after addition of the 15-mer donor DNA to covalently linked topoisomerase. Religation generates a product containing a radiolabeled 29-mer DNA strand (step 3). MCV topoisomerase protein is indicated by the shaded circle. The number of bases in single stranded DNAs are indicated in parentheses. B, lack of Inhibition of DNA strand religation by sansalvamide A. Control religation reactions were performed without enzyme (lane 1), with enzyme (lane 2), or with enzyme and 15-mer single strand DNA (lane 3). Concentrations of sansalvamide A added were 10 μM (lane 4), 30 μM (lane 5), 100 μM (lane 6), 200 μM (lane 7), and 300 μM (lane 8).

Discussion

Here we describe the identification of the marine depsipeptide sansalvamide A as an inhibitor of MCV topoisomerase. Sansalvamide A inhibited the initial DNA binding by MCV topoisomerase and, consequently, formation of covalent protein-DNA complexes, but did not inhibit religation by the covalent protein-DNA intermediate. This work provides a starting point for possible development of depsipeptide inhibitors for treating MCV infection.

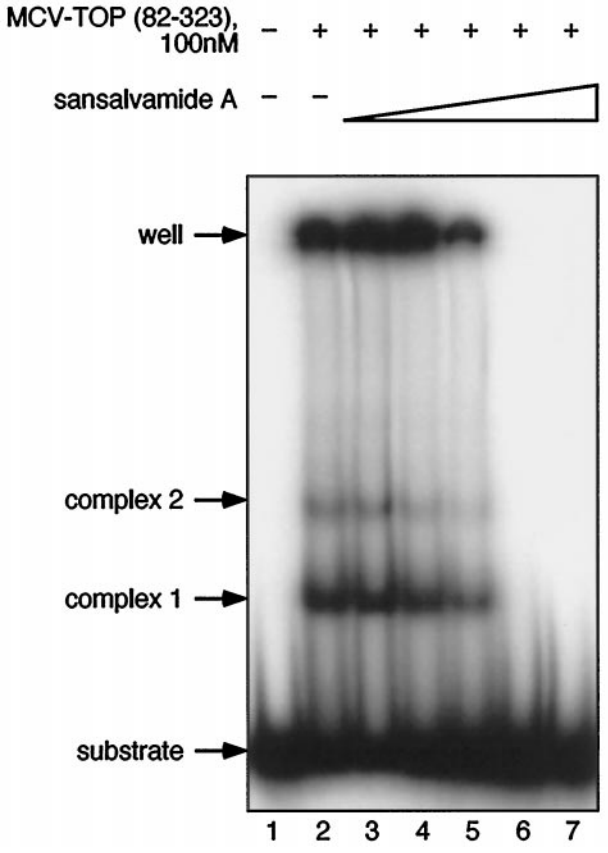

Fig. 7. Inhibition of DNA binding of the catalytic domain (MCV-TOP 32–323) by sansalvamide A. Control reactions were performed without enzyme (lane 1) or without sansalvamide A (lane 2). Concentrations of sansalvamide A added were 10 μM (lane 3), 30 μM (lane 4), 100 μM (lane 5), 200 μM (lane 6), and 300 μM (lane 7). DNA binding complexes are indicated by the arrows.

Cyclic peptides such as sansalvamide A are a potent class of naturally occurring bioactive molecules. The immunosuppressant drug cyclosporin is a cyclic peptide secondary metabolite produced by the fungus *Cylindrocarpon lucidum* (Borel et al., 1976). Bacitracin and polymyxin, also cyclic peptides of microbial origin, are in use as topical antibiotic agents (Strohl, 1997). Marine invertebrates are also prolific producers of bioactive cyclic peptides, including the antiviral and cytotoxic molecule didemnin B (Rinehart et al., 1988), the thrombin inhibitor cyclotheonamide A (Fusetani et al., 1990), and patellamide and lissoclinamide cytotoxins (Ireland et al., 1982). Recently, several potent bioactive molecules have also been isolated from combinatorial libraries of cyclic peptides (Eichler et al., 1995; Giebel et al., 1995).

Cyclic depsipeptides have several advantages as inhibitors. Cyclic depsipeptides by definition contain one or more amino acids replaced by a hydroxy acid, forming at least one ester bond in the core ring structure. These compounds lack charges at the peptide amino and carboxyl termini and lacking zwitterionic character are more lipophilic and membrane-permeable. Oral bioavailability is increased by faster membrane absorption in the digestive tract (Amidon and Lee, 1994) and cyclic peptides have greater half-lives in vivo than the cognate linear peptides (Blackburn and Van Breemen, 1993; Pauletti et al., 1996). The cyclic nature of these compounds also restricts bond rotation, creating more rigid three dimensional structures. This conformational constraint can result in greater binding affinity and selectivity for protein ligands (Giebel et al., 1995). Even slight changes in the core ring structure of molecules such as cyclosporin and didemnin B can greatly reduce their biological activities, emphasizing the specificity of binding (Wenger, 1986; Sakai et al., 1996).

The mechanism by which sansalvamide A inhibits DNA binding has not been fully clarified. It seems unlikely that sansalvamide A binds indiscriminately to the substrate DNA, because it did not inhibit HIV-1 integrase or *Hin*dIII. Sansalvamide A did inhibit catalysis by an isolated domain of MCV topoisomerase containing the catalytic center, implying action at least in part against this protein domain and potentially the active site. It has not been possible to study the target of sansalvamide A in vivo due to the toxicity of the compound to cells. If more potent or less toxic derivatives of sansalvamide A can be identified, it may be possible to isolate a poxvirus insensitive to sansalvamide A and map the target of action by identifying the location of viral drug escape mutants.

Acknowledgments

We thank members of the Fenical and Bushman laboratories for suggestions and comments and Allison Bocksruker for artwork.

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