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Differences in the topoisomerase I cleavage complexes formed by camptothecin and wakayin, a DNA-intercalating marine natural product

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Wakayin is a bispyrroloiminoquinone isolated from a *Clavelina* sp. ascidian by cytotoxicity directed fractionation. Like camptothecin, it has been found to inhibit the topoisomerase I catalyzed relaxation of supercoiled DNA. Wakayin enhanced cleavage complex formation at the same DNA sequences as camptothecin. Both compounds showed dose-related increases in cleavage complex formation, though wakayin's effect is attenuated at high concentrations. Wakayin is a strong DNA binder. Wakayin also differed from camptothecin in that its cleavage complexes were much less stable than those of camptothecin in 0.5 M NaCl. Again in contrast to camptothecin, wakayin stabilized cleavage complexes poorly, if at all, at 0°C.

Key words: Camptothecin, cleavage complex, DNA intercalator, wakayin, topoisomerase I.

Introduction

Phase I and II trials of camptothecin analogs have confirmed their potential as anticancer drugs by demonstrating unprecedented activity against a number of human cancers including non-small cell lung cancer.[1,2] Part of the reason for this exceptional activity is likely the unique intracellular target of the camptothecins, topoisomerase I.[3] Camptothecin stabilizes a critical intermediate step of topoisomerase I catalysis wherein the enzyme induces a single-strand break in the substrate DNA and is covalently bound to it.[4] This intermediate is called a cleavage, or 'cleavable', complex because digestion with proteinase yields substrate DNA containing a single-strand break.[5] Topoisomerase I has been the specific target of drug discovery efforts for almost 10 years but few cleavage complex forming inhibitors have been found.[6,7] The subject of this report is a marine natural product called wakayin that inhibits topoisomerase I by producing cleavage complexes that differ from camptothecin in many significant ways.

Wakayin is a bispyrroloiminoquinone that was isolated as a cytotoxic metabolite from a *Clavelina* sp. ascidian collected at Wakaya island in the central Fiji islands.[8] Structural characterization has shown it to be related to the pyrroloiminoquinone makaluvamines and to be moderately cytotoxic towards human colon tumor cells. The makaluvamines are potent topoisomerase II inhibitors.[9,10] Wakayin also appears to share this activity but has potent activity against topoisomerase I as well. Data illustrating differences between wakayin's and camptothecin's topoisomerase I cleavage complex formation are reported here.

Materials and methods

Drugs and chemical reagents

The isolation and chemical characterization of wakayin has been described.[8] Its purity was assessed by chromatographic and NMR analysis. Drug standards were purchased from Sigma (St Louis, MO) or were supplied to us by Dr LA Dethlefsen or Dr WG Harker (University of Utah), or Dr RA Kramer (Wyeth-Ayerst Research). Purified calf thymus topoisomerase I and pBR322, used in the DNA relaxation experiments, was purchased from Gibco/BRL (Bethesda, MD). Sequenase 2.0 and all sequencing supplies were purchased from US Biochemical (Cleveland, OH). Radioactive thymidine and deoxyadenosinetriphosphate were purchased from New England Nuclear (Beverly, MA). Sequencing primers were

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synthesized by the University of Utah, Utah Huntsman Cancer Center DNA, Peptide Synthesizing and Sequencing core facility. All other chemicals were purchased from Sigma or Baker Chemical.

Ethidium bromide displacement

The ability to displace ethidium bromide from DNA was used as a measure of intercalation, the K_s is defined as the concentration of wakayin needed to decrease DNA-bound ethidium bromide fluorescence by 50%. The experiment was carried out as described by McDonald *et al.*[11] using 5 μM ethidium bromide and 25 μg/ml calf thymus DNA. Fluorescence was quantified at 600 nm excitation wavelength.

Isolation of human topoisomerase I

Isolation of DNA topoisomerase I from human placenta was carried out by the method Holden *et al.*[12] The 67 000 kDa form of topoisomerase I used in these experiments had no detectable contaminants by coomassie blue staining.

Radiolabeling DNA for cleavage reactions

DNA (pUC 19) was radiolabeled for topoisomerase cleavage using Sequenase 2.0 obtained from US Biochemical and the following approach. This strategy was adapted to enable quantitative comparison between topoisomerase–DNA cleavage reactions containing varying concentrations of drug. To radiolabel enough DNA for 10 subsequent cleavage reactions, 10 μl pUC 19 DNA (1.5 μg) was aliquoted into a 0.5 ml microfuge tube and mixed with 10 μl H_2O and 5 μl 1 M NaOH. To this, 2.5 μl of primer (5 pmol) was added with 10 min incubation at 37°C, the mixture was then placed on ice. Then, 1 M HCl (5 μl) way added along with 5 μl of labeling reaction buffer (400 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and the mixture was incubated again at 37°C for 10 min and again placed on ice. Dithiothreitol (DTT, 2.5 μl of a 0.1 M stock) was added with 5 μl of labeling mix (1.5 μM dCTP, dGTP and dTTP) and 1.25 μl [^{32}P]dATP (10 mCi/ml) and 2.5 μl diluted Sequenase 2.0 (1 μl of Sequenase, 13 units, plus 1 μl of pyrophosphatase, 5 units and 6 μl of glycerol enzyme dilution buffer, 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 50% glycerol). This mixture was incubated for 5 min at room temperature. The reaction was chased with 25 μl chase mix (1.7 mM of each deoxynucleotide triphosphate in H_2O) and 7.5 μl of additional diluted Sequenase 2.0 for 6 min at 37°C.

The reaction was stopped by the addition of 40 μl each of phenol and chloroform:isoamyl alcohol (24:1) with mixing. The reaction was then centrifuged for 15 min at 15 000 r.p.m. Then 65 μl of the aqueous phase (80% of the volume) was transferred to a second microfuge tube to which 130 μl of ice cold ethanol and 2.5 μl of 5 M NaCl and 1 μl 1 M $MgCl_2$ were added and mixed. Radiolabeled DNA was precipitated by freezing on solid CO_2 for 20 min and centrifugation at 4°C, 15 000 r.p.m. for 20 min. Ethanol was aspirated, 130 μl of ice cold 70% ethanol was added with mixing, followed by freezing on solid CO_2 for 5 min and centrifugation at 4 °C, 15 000 r.p.m. for 5 min. Ethanol was again aspirated and the labeled DNA was allowed to dry in air.

Topoisomerase I cleavage of radiolabeled DNA

Radioactive DNA was dissolved in 115 μl TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Each cleavage reaction (sufficient DNA for three to four lanes upon electrophoresis) utilized 11.5 μl of the radiolabeled DNA solution. To each 11.5 μl, 1.5 μl of topoisomerase I reaction buffer was added (1 × buffer contains 40 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mm EDTA and 50 μg BSA/ml) followed by 1 μl of DMSO or drug dissolved in DMSO, and 1 μl pure topoisomerase I (50 ng protein) with mixing. The reaction was incubated for 30 min at 37°C, and stopped with 0.8 μl 10% SDS and 1 μl of proteinase K (4 mg/ml) and further incubated for 1 h at 37°C. Loading solution (5 μl) was added (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and the samples stored at − 20°C until electrophoresis.

Sequence determination of topoisomerase I–DNA cleavage sites

To precisely determine the nucleotide at which topoisomerase I–drug complexes cut DNA, it was necessary to cut the DNA template with the enzyme and to fill in the complementary strand up to the cut. This was required because topoisomerase I in the drug-stabilized cleavable complex is covalently attached to the 3′OH of the DNA substrate. In the

previous approach (*vide supra*) topoisomerase I cleavage reactions were stopped with SDS and proteinase. A few amino acids remain attached to the DNA fragment and cause it to migrate more slowly during electrophoresis. To determine precisely the nucleotide of cleavage, the DNA was first cut with topoisomerase I and then used as template for the synthesis of a complementary strand that ends at the site of the cut. In a 0.5 ml microfuge tube on ice, 1.2 μl of pUC 19 DNA (0.3 μg) was mixed with 5 μl topoisomerase reaction buffer and 2.5 μl of DMSO or DMSO containing drug. To this, 5 μl pure topoisomerase I (750 ng protein) was added with mixing. The reaction was then incubated for 30 min at 37°C. It was stopped by the addition of 2.7 μl 10% SDS and 3.3 μl of proteinase K (as above) and incubated for 1 h at 37°C. The mixture was then extracted with 60 μl phenol:chloroform (as above). Then, 45 μl of the supernatant (80% of the volume) was transferred to a new microfuge tube to which 90 μl of ice cold ethanol, 5.6 μl of 5 M NaCl and 1.4 μl of 1 M $MgCl_2$ were added. The DNA was precipitated for 20 min on solid CO_2 and centrifuged for 20 min at 15 000 r.p.m. at 4°C. The supernatant was aspirated and 200 μl of ice-cold 70% ethanol was added with mixing followed by 5 min incubation on solid CO_2 and 5 min centrifugation at 15 000 r.p.m. at 4°C. The supernatant was again aspirated and the DNA allowed to dry at room temperature.

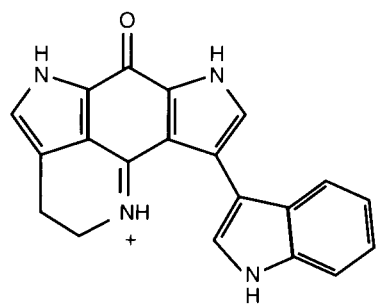
The recovered DNA (approxmately 240 ng) was redissolved in 10 μl TE. Of this, 4 μl (approximately 96 ng) was used as template. The annealing and labeling reactions are conducted as described under 'Radiolabeling of DNA for cleavage reactions' but using 1/10 the described reagent volumes. The DNA primer used in these experiments was custom synthesized to allow visualization of the drug-enhanced topoisomerase I cleavage sites determined by the 'Topoisomerase I cleavage of radiolabeled DNA' protocol, 88 bp from the closest of the cleavage sites under scrutiny. (In conducting the experiments described below, 0.3 μM final concentration of the custom primer was used, but it was added in the volume indicated above.)

DNA sequencing

DNA sequencing and polyacrylamide gel electrophoresis were performed as described in the Sequenase 'Quick-Denature' Plasmid Sequencing Kit using either the commercially supplied or custom synthesized primer.

Results

Wakayin (Figure 1) was previously tested for the ability to damage DNA in a panel of DNA repair-deficient Chinese hamster ovary (CHO) cell lines.[15] Wakayin showed enhanced cytotoxicity towards two CHO lines deficient in DNA strand break repair. These cell lines are super-sensitive to killing by topoisomerase cleavage complex forming drugs.[13] Wakayin was subsequently shown to inhibit topoisomerase I in a DNA unwinding assay using supercoiled pBR322 as substrate and commercially obtained topoisomerase (data not shown). Wakayin inhibited this catalytic activity at approximately 10 μM, compared to camptothecin which was active at approximately 2 μM. Control lanes showed that wakayin incubated with DNA in the absence of topoisomerase I did not cause DNA cleavage. It was observed, however, that supercoiled DNA in the presence of high concentrations (100 μM) of wakayin alone migrated more slowly during electrophoresis than untreated DNA. This suggested DNA unwinding due to wakayin intercalation. An ethidium bromide displacement assay[11] confirmed this activity. Wakayin is a strong DNA intercalator with a K_s of 20 μM for displacement of 50% ethidium bromide (5 μM) from DNA (25 μg/ml).



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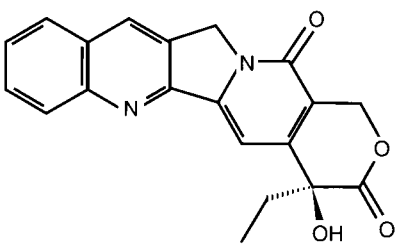

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Figure 1. Structures of wakayin (**1**) and camptothecin (**2**).

It was hypothesized that the DNA damage detected by the DNA repair-deficient CHO mutants was due to wakayin–topoisomerase cleavage complexes. Compounds that produce cleavage complexes stabilize covalently linked topoisomerase–DNA intermediates. Preliminary experiments using the KSDS[14] assay measures protein-linked DNA in lysates of treated cells. Both wakayin and camptothecin gave positive responses (data not shown) but the wakayin-induced protein-linked DNA was attenuated at higher drug concentrations (300 μM). A similar self-limiting effect was not seen with camptothecin, as had been shown by others.[15]

To confirm the formation of wakayin–topoisomerase I cleavage complexes, wakayin or camptothecin were incubated wtih pure human placental topoisomerase I and radiolabeled pUC 19 DNA (see Materials and methods). Figure 2(A) shows the two prominent camptothecin-enhanced cleavage sites that are used in this manuscript to illustrate wakayin and camptothecin cleavage complex behavior. Site 1 is at T 231 in pUC 19 DNA, site 2 at T 262 (the precise nucleotide of cleavage was determined as described below). Figure 2(B) is a larger section of a replicate experiment showing several sites of enhanced topoisomerase I cleavage that straddle the *Pvu*II cleavage site (base 308), and the adjacent sequenase generated primer-extension labeling artifact. Control lanes 6 and 10 containing labeled DNA incubated with either camptothecin or wakayin, respectively, show the labeling artifact (sequenase quit site) but the absence of the other bands generated by topoisomerase. The intensity of this labeling artifact was minimized by increasing the concentrations of deoxynucleotides used in the labeling reaction and by the addition of extra sequenase with the chase. A third major site (site 3) of drug-enhanced topoisomerase cleavage is apparent at T 341. Dose-dependent increases in cleavage complex formation were demonstrated at all three cleavage sites with wakayin as well (lanes 6–7). Consistent with literature reports,[16,17] camptothecin appeared primarily to enhance cleavage at sites that are normally subject to topoisomerase I attack. The topoisomerase I cleavage sites in the DNA sequence most susceptible to camptothecin enhancement were also the ones most susceptible to wakayin enhancement. The ability of wakayin to produce cleavage complexes was inhibited at higher concentrations of drug (e.g. 66 μM, data not shown). *Pvu*II-digested pUC 19 DNA was included as a marker; the fact that the DNA used in this lane had been radiolabeled more than a week previously is shown by minor bands due to the degradation of the DNA (lane 17).

Topoisomerase I in a cleavage complex is covalently bound to the 3′ phosphate of the cleaved DNA fragment. Even after proteinase digestion a few amino acids remain attached and cause the DNA fragments to migrate more slowly during electrophoresis. To determine the precise site of DNA cleavage by human topoisomerase I and drug it was necessary to cleave the pUC 19 DNA and then to determine the sequence of the cleavage site by synthesizing a complimentary strand of DNA up to the break. Thus, a fragment ending at an adenine therefore indicates thymine as the base on the 3′ side of the topoisomerase I-bound nucleotide (Figure 3). All three major sites of topoisomerase–drug cleavage shown in Figure 2 ended with 3′ T. Site 2 actually occurs at a doublet of Ts and drug enhanced cutting was occasionally detected at both of them. Figure 3 shows that the predominant cutting site appears to be after the first T of the pair, T 262. Thus, the DNA fragments with the 3′-hydroxyphosphate peptide attached migrated as if they were approximately three to four nucleotides longer. The sequences of seven other prominent wakayin and camptothecin enhanced cleavage sites were determined in other regions of the pUC 19 plasmid; with one exception they all contained a 3′ T at the cleavage site. Greater than 400 bp of pUC19 was analyzed overall and several different classes of topoisomerase I cutting sites were identified. These included sites where the topoisomerase cut but neither drug appeared to enhance the effect, the predominant cleavage sites where both drugs enhanced the effect and a few minor sites where one drug or the other alone appeared to enhance the effect. Site 1 shown in Figure 2 was one that displayed the greatest enhancement in the presence of drug.

Camptothecin does not intercalate DNA, it is thought to bind only the DNA–enzyme complex.[18] Wakayin, in contrast, is a DNA intercalator. It was hypothesized that the nature of the cleavage complexes produced by these two compounds must be significantly different. The data shown in Figure 4 confirm this hypothesis by showing that the wakayin-induced cleavage complexes close much more rapidly than those of camptothecin when moved to high salt (0.5 M NaCl).[19,20] This was true for all of the wakayin-enhanced cleavage sites examined.

Camptothecin is thought to stabilize cleavage complexes by inhibiting the religation step in topoisomerase I catalysis. The rate of enzyme-induced cleavage in normal substrate DNA is not

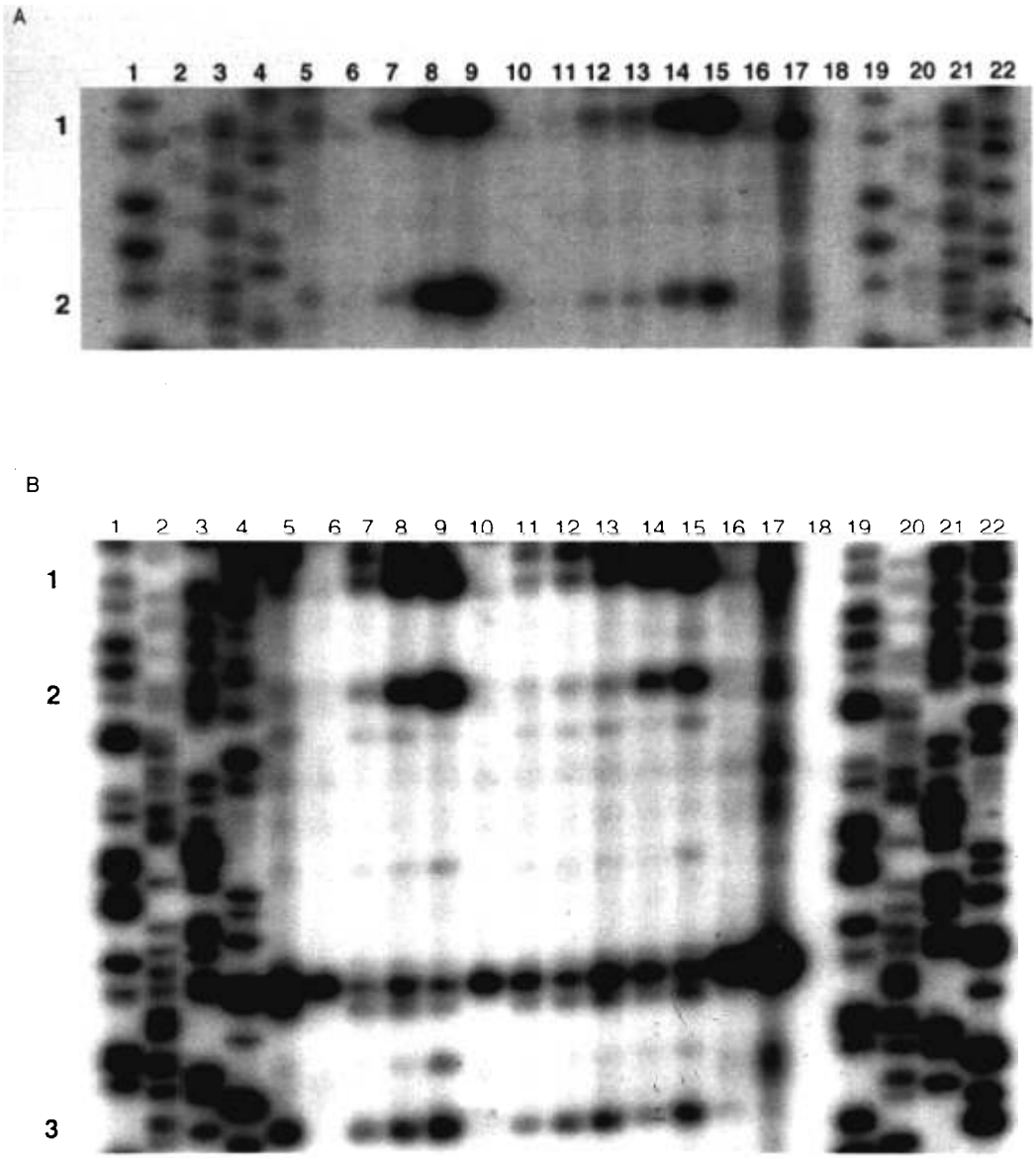

Figure 2. (A) Drug-enhanced cleavage of DNA. Lanes 1 and 19, 2 and 20, 3 and 21, and 4 and 22 are dideoxy-DNA sequencing reactions, A, C G and T respectively. Lane 5 shows labeled DNA incubated with topoisomerase I and DMSO with no added drug. Lane 6 shows labeled DNA incubated only with 67 nM camptothecin. Lanes 7, 8 and 9 show the DNA incubated with topoisomerase I and increasing concentrations of camptothecin, 6.7, 67 and 667 nM, respectively. Lane 10 shows labeled DNA incubated only with 667 nM wakayin. Lanes 11, 12, 13 and 14 show the DNA incubated with topoisomerase I and increasing concentrations of wakayin, 6.7, 67, 667 nM and 6.7 μM, respectively. Lanes 15 and 16 contain DNA cleaved in the presence of 667 nM wakayin with 5 $\times$ and 0.2 $\times$ (respectively) the concentration of topoisomerase I used in the other reactions. Lane 17 contains DNA digested with the restriction enzyme *Pvu*II as marker. Lane 18 was left blank so that the signal from the restriction enzyme cut DNA does not overwhelm the DNA sequence signal. Panel (B) is a repeat experiment showing a larger section of the gel. Sites 1, 2 and 3 are sites of drug-enhanced topoisomerase I cleavage. The *Pvu*II cutting site (pUC 19 base 308) and the Sequenase labeling artifact can be seen between sites 2 and 3.

thought to be affected by camptothecin. To determine if wakayin altered the rate of cleavage complex formation, experiments were performed at 0°C and the appearance of DNA cleavage was followed. Figure 5 shows a time-dependent increase in cleavage complex formation at sites 1 and 2 with camptothecin. Wakayin showed minimal cleavage complex formation only at the earliest time points,

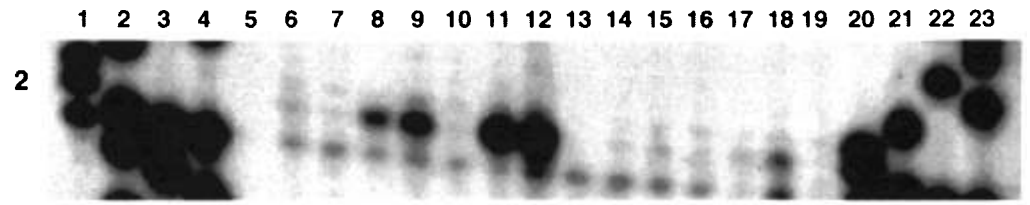

Figure 3. Drug-enhanced cleavage of DNA, second protocol. Lanes 1 and 20, 2 and 21, 3 and 22, and 4 and 23 are dideoxy-DNA sequencing reactions, A, C, G and T, respectively. Lane 5 was left blank. Lane 6 shows labeled DNA incubated with topoisomerase I and DMSO with no added drug. Lane 7 shows labeled DNA incubated only with 5 μM camptothecin. Lanes 8 and 9 show duplicate experiments in which the DNA incubated with topoisomerase I and 5 μM camptothecin. Lane 10 shows labeled DNA incubated only with 50 μM wakayin. Lanes 11 and 12 show duplicate experiments in which the DNA incubated with topoisomerase I and 50 μM wakayin. Lanes 13–18 are results obtained with discrete compounds. Lane 19 contains *Pvu*II cut DNA.

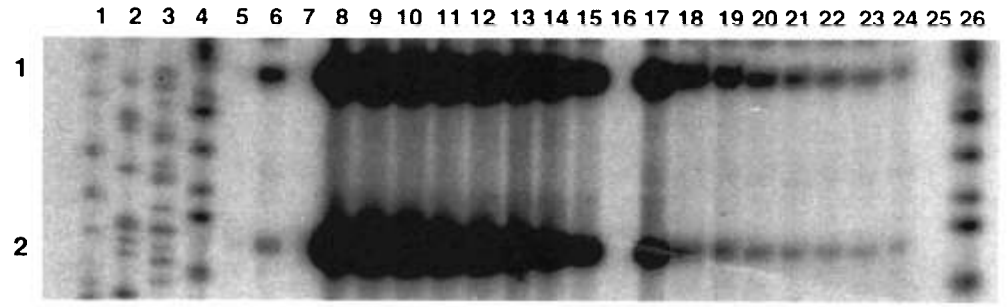

Figure 4. Effect of high salt on drug-enhanced cleavage of DNA. Lanes 1, 2, 3, and 4 and 26 are dideoxy-DNA sequencing reactions, A, C, G and T, respectively. Lane 5 was left blank. Lane 6 shows labeled DNA incubated with topoisomerase I and DMSO with no added drug. Lane 7 shows labeled DNA incubated only with 67 nM camptothecin. Lanes 8–15 are reactions that were incubated with topoisomerase I and 67 nM camptothecin and then moved to 0.5 M NaCl for 0, 15, 30, 45, 60, 90, 120 or 300 s. Lane 16 shows labeled DNA incubated only with 6.7 μM wakayin. Lanes 17–24 are reactions that were incubated with topoisomerase I and 6.7 μM wakayin and then moved to 0.5 M NaCl for 0, 15, 30, 45, 60, 90, 120 or 300 s. Lane 25 is a *Pvu*II marker lane.

time points when the reaction had been transiently warmed by manipulation and pipetting, with much less or no complex formation at the later time points. Clearly wakayin's ability to stabilize cleavage complexes is reduced at low temperature.

Discussion

In preliminary evaluation wakayin inhibited topoisomerase I catalyzed DNA unwinding of supercoiled pBR322 DNA *in vitro* and wakayin induced formation of protein-linked DNA in the KSDS assay. To prove that wakayin produced topoisomerase I cleavage complexes, a dose–response relationship was demonstrated for the production of DNA breaks in the presence of purified human topoisomerase I. The effect of wakayin was inhibited at higher drug concentrations (above 667 μM in this assay). Similar concentration-limited effects have been reported for other topoisomerase I poisons (e.g. H33342[21] and fagaronine[22]). Wakayin was compared to camptothecin and was found, predominantly, to enhance cleavage complex formation at the same DNA sequences. The 3′ nucleoside at almost all of the cleavage sites determined appeared to be a thymidine. Wakayin requires 10- to 100-fold the molar concentrations that camptothecin does to elicit similar DNA cleavage in this assay. Wakayin has a high affinity for DNA. The higher concentrations required of wakayin may result from its intercalation into the DNA substrates. 'Non-specific' intercalation

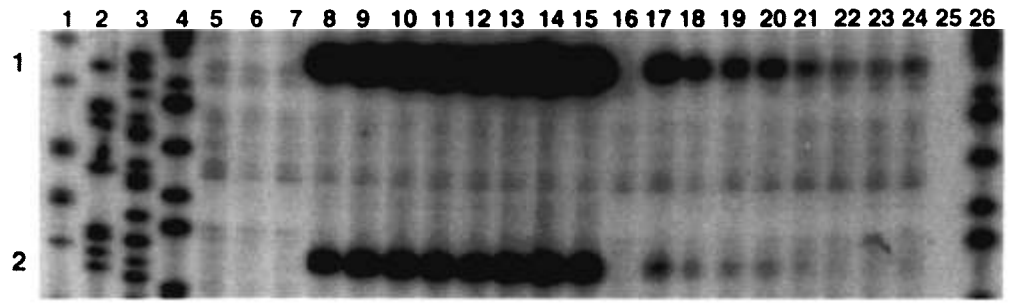

Figure 5. Drug-enhanced topoisomerase I cleavage of DNA at 0°C. Lanes 1, 2, 3, and 4 and 26 are dideoxy-DNA sequencing reactions, A, C, G and T, respectively. Lane 5 was left blank. Lane 6 shows labeled DNA incubated with topoisomerase I and DMSO with no added drug. Lane 7 shows labeled DNA incubated only with 67 nM camptothecin. Lanes 8–15 are reactions containing 67 nM camptothecin that were initiated with the addition of topoisomerase I and then stopped by the addition of SDS at 0, 15, 30, 45, 60, 90, 120 or 300 s. Lane 16 shows labeled DNA incubated only with 6.7 μM wakayin. Lanes 17–24 are reactions containing 6.7 μM wakayin that were initiated with the addition of topoisomerase I and then stopped by the addition of SDS at 0, 15, 30, 45, 60, 90, 120 or 300 s. Lane 25 is a *Pvu*II marker lane.

sites would have reduced the amount of wakayin bound at topoisomerase I cleavage sites.

Minor topoisomerase I cutting sites could be identified where camptothecin appeared to enhance cleavage complex formation and wakayin did not, or where wakayin enhanced cleavage complex formation and camptothecin did not. Still other topoisomerase I cleavage sites were observed where neither drug enhanced the cleavage. Quantitation of the intensity of the autoradiographic bands was not attempted, but it was obvious that the most intense bands were those produced by enzyme in the presence of drug and that these were at sites of cutting enhanced by both compounds.

Wakayin is believed to be a DNA intercalator (it caused unwinding of supercoiled DNA and displaced ethidium bromide from DNA). Therefore it was questioned whether the nature of the cleavage complex produced by wakayin could be the same as that produced by camptothecin. The stability of the wakayin cleavage complex was tested in conditions known to result in the closure of camptothecin cleavage complexes. The wakayin topoisomerase I cleavage complexes were found to close much more rapidly than those of camptothecin in 0.5 M NaCl. Camptothecin is thought to inhibit religation of topoisomerase I cleaved DNA.[6] The fact that approximately equal levels of wakayin-induced cleavage closed much more rapidly might indicate that wakayin acts to enhance DNA cleavage rather than to inhibit religation (such an effect has been hypothesized for quinolone compounds and topoisomerase II[23]). Displacement experiments performed in 0.5 M NaCl showed no significant decrease in wakayin's ability to displace ethidium bromide from DNA, compared to physiological salt concentrations. Therefore, the rapid closing of wakayin–topoisomerase I cleavage complexes in high salt is not likely due to disassociation of wakayin from DNA–topoisomerase complexes.

Differences between wakayin and camptothecin cleavage complexes were also shown by monitoring topoisomerase I cleavage complex formation at 0°C. Time-dependent formation of topoisomerase I cleavage complexes by camptothecin could be demonstrated under these conditions. In contrast, wakayin did not appear to induce significant cleavage complex formation at all. Only relatively small signals were detected for wakayin at the first time points. At these times the reaction mixture had been transiently warmed by removal from the ice bath, manipulation and mixing by repeated 'up and down' pipetting by the micropipet used to deliver and mix in the enzyme (chilled pipet tips were not used).

Conclusion

Wakayin is a marine natural product that is a DNA binding compound with the ability to induce topoisomerase I cleavage complexes. It differs structurally from the makaluvamines, also marine natural products, in that it possesses a second pyrrole ring and an indole substituent. The makaluvamines are DNA intercalating agents with potent topoisomerase II inhibitory and anticancer activity but they do not

possess appreciable topoisomerase I activity (unpublished data). Future comparison of wakayin to the makaluvamines may provide insight into the structural requirements for specific inhibition of topoisomerase I or II. Wakayin also provides a new structural class of topoisomerase I inhibitors with potential as anticancer agents. This potential remains to be explored.

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