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# Inhibition of DNA topoisomerases I and II, and growth inhibition of human cancer cell lines by a marine microalgal polysaccharide

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**Abstract**

We have previously reported purification of an extracellular polysaccharide GA3P, D-galactan sulfate associated with L-(+)-lactic acid, produced by a toxic marine microalga Dinoflagellate *Gymnodinium* sp. $A_3$ (GA3), and induction thereby of apoptosis on human myeloid leukemia K562 cells. In the present report, we show that the GA3P is a potent inhibitor of DNA topoisomerase (topo) I and topo II, irrespective of the presence or absence of the lactate group. Dextran sulfate also showed similar level of inhibition of topo I and topo II. We also demonstrated that, unlike camptothecin (CPT) or teniposide (VM-26), the inhibition of topo I or topo II by the polysaccharide does not involve accumulation of DNA-topo I/II cleavable complexes, clearly showing that they are not topo poisons but catalytic inhibitors with dual activity. Furthermore, the polysaccharide, when added to the reaction mixture with CPT or VM-26, inhibited stabilization of cleavable complex induced by the latter compounds. In addition, when added to the reaction mixture after the formation of the cleavable complexes by topo poisons, CPT for topo I and VM-26 for topo II, either GA3P or dextran sulfate diminished the amount of the complexes already accumulated, i.e. reversal of the reaction. These results suggest that the polysaccharides bind to the enzymes with high affinities, and that, as for topo I/II inhibition, the GA3P shares a common mechanism with dextran sulfate. As examined *in vitro* with a human cancer cell line panel, GA3P exhibited significant cytotoxicity against a variety of cancer cells. These findings show that the polysaccharide GA3P would prove to be a potential anticancer chemotherapeutic agent with dual activity of topo I and topo II catalytic inhibition.


*Keywords:* DNA topoisomerase; Inhibitor; Polysaccharide; Marine microalga; Cytotoxicity; Anticancer agent

## 1. Introduction

An abnormal microalgal blooming, known as a red tide because of the pink-red coloration of the sea, results in the death of cultured fish in surrounding areas. The OKU-1 strain of a marine microalga, *Gymnodinium* sp. $A_3$ (GA3), originally isolated from a water sample of the Seto Inland Sea, when grown in sea water, produces an extracellular acidic polysaccharide GA3P, which is a D-galactan sulfate associated with L-(+)-lactic acid [1]. This polysaccharide was previously reported to show strong cytotoxicity to several human leukemic cell lines, as shown by induction of apoptosis [2]. In the course of testing the biological activities of the GA3P, we found that this polysaccharide is a potent inhibitor of both topo I (EC 5.99.1.2) and topo II (EC 5.99.1.3).

Topos are nuclear enzymes that regulate DNA topology. There are two classes of the enzymes, class I and class II, which differ in their functions and mechanisms of action. Topo I acts by making a transient break in one DNA strand allowing the DNA helix to swivel and release torsional strain, changing the linking number by steps of one. Topo II makes transient breaks in both strands of one DNA molecule allowing the passage of another DNA duplex through the gap, changing the linking number by steps of two.

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*Abbreviations:* topo, DNA topoisomerase; DTT, dithiothreitol; CPT, camptothecin; kDNA, kinetoplast DNA.

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These enzymes are crucial for cellular genetic processes, such as DNA replication, transcription, recombination, and chromosome segregation at mitosis [3–8].

It has long been accepted that topos are valuable targets for cancer chemotherapeutic agents [6,9]. Several classes of topo inhibitors have been introduced into cancer clinics as potent anticancer drugs, including CPTs inhibiting topo I [5], anthracyclines, epipodophyllotoxins, aminoacridines, and ellipticines targeting topo II [9]. These agents have activity in both hematologic and solid malignancies. The activity of these agents is thought to result from stabilization of DNA-topo cleavable complexes, intermediates in the catalytic cycle of the enzymes [6,9–11], resulting ultimately in apoptosis. A number of new topo inhibitors have recently been reported that do not stabilize the cleavable complexes. Thus, two general mechanistic classes of topo inhibitors especially for topo II have recently been described [12]: (1) classical topo "poisons" that stabilize the cleavable complexes and stimulate single- or double-stranded DNA cleavage, such as CPT and its derivatives, indolocarbazoles for topo I, and TAS-103 [13] for topo I and topo II, and (2) catalytic inhibitors that prevent catalytic cycle of the enzymes at steps other than cleavage intermediates, such as aclarubicin [14], intoplicine [15], and F11782 [16]. Some of these compounds are dual inhibitors of topo I and topo II. Merbarone [17,18] and dioxopiperazines are catalytic inhibitors of topo II [12,19,20]. Dioxopiperazines are studied extensively and are shown to inhibit the re-opening of the closed clamp formed by the enzyme around the DNA by inhibiting ATPase activity of the enzyme, thus sequestering the enzyme within the cell [12,21–23].

In this paper, we showed (1) that the GA3P is a potent catalytic inhibitor of both topo I and topo II; (2) that dextran sulfate, another sulfated polysaccharide, was quite similar to GA3P in the properties shown above, suggesting that GA3P shares a common mechanism of action with dextran sulfate; (3) that GA3P exhibited moderate growth inhibitory activity against various human cancer cell lines (HCCs), though the mechanism of inhibition would possibly be different from that of topo I or topo II inhibition, since no growth inhibition was observed with dextran sulfate.

## 2. Materials and methods

### 2.1. Materials

GA3 OKU-1 strain of microalgae [1] isolated from a water sample of the Seto Inland Sea, produced GA3 polysaccharide (GA3P), which is a D-galactan sulfate associated with L-(+)-lactic acid (Fig. 1), when grown in a sea water medium. The GA3P used in this study was purified according to the procedures described previously [1]. In this study, we tested two independent preparations of GA3P having high (>80%) and low (<20%) lactic acid content (GA3Pl+ and GA3Pl−, respectively) determined as described previously [1]. CPT was provided by Yakult Honsha Co, and VM-26 was provided by Bristol-Myers-Squib Co. Dextran sulfate (MW 500,000), L-lactic acid, and other chemicals were purchased from Wako Pure Chemical Industries Ltd.

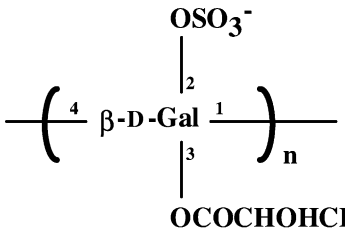


Fig. 1. Structure of GA3P. β-D-Gal, β-D-galactopyranose unit. The numbers indicate the position on the galactopyranose ring.

#### 2.1.1. Topo I inhibition assay

Mouse topo I was purified from Ehrlich ascites tumor cells [24] with modifications as described earlier [25]. One unit of the enzyme was defined as minimal amount of activity required to relax 0.2 μg of the supercoiled pT2GN DNA under the conditions used. Topo I activity was determined by relaxation of supercoiled pT2GN plasmid DNA, essentially as described previously [24,26]. The reaction mixture in 20 μL contained 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol, 30 μg/mL BSA, 1 μL of test compound diluted with $H_2O$ (for polysaccharides) or DMSO (for CPT), 1 U of topo I, and 0.2 μg of the supercoiled pT2GN plasmid DNA. The reaction mixtures were incubated at 37° for 15 min, and the reaction was terminated by addition of 4 μL of dye solution consisting of 0.1% bromophenol blue, 4.5% SDS, and 45% glycerol. The mixtures were applied to 0.8% agarose gel and electrophoresed. The gel was stained with ethidium bromide and photographed under UV light. $IC_{50}$ values were determined by densitometry of the photographs.

#### 2.1.2. Topo II inhibition assay

Recombinant human topo IIα was purified as described previously [25]. One unit of the enzyme was defined as the minimal amount of activity required to decatenate 0.2 μg of catenated kinetoplast DNA (kDNA) under the following conditions. Inhibitory activity of test compounds on topo II activity was evaluated by detecting the conversion of catenated kDNA to monomer minicircles essentially as described previously [25]. In brief, the reaction mixtures in 20 μL containing 0.2 μg of catenated kDNA, 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, 0.5 mM DTT, 30 μg/mL BSA, 10% glycerol, 1 U of topo IIα, and 1 μL of varied concentrations of test compounds in $H_2O$ (for polysaccharides) or DMSO (for ICRF-193) were incubated at 37° for 15 min. ICRF-193 was used as a positive control. The reaction was

terminated by the addition of 4 μL of dye/SDS stop solution consisting of 0.1% bromophenol blue, 4.5% SDS, and 45% glycerol, then analyzed by electrophoresis on 0.8% agarose gels, and the extent of decatenation was evaluated as described for topo I.

*2.1.3. Topo I- and topo II-mediated DNA cleavage assay*

Topo I-mediated cleavable complex formation was carried out for polysaccharides essentially as described elsewhere [25]. Reaction mixtures in 20 μL contained 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 30 μg/mL BSA, 10 U of topo I, 0.2 μg of supercoiled pT2GN plasmid DNA, and 1 μL of solution of polysaccharides or CPT as a positive control. For every reaction the final concentration of DMSO was made 5%. Reaction mixtures were incubated for 15 min at 37°. In experiments of drug combination, 1 μL each of a test compound and CPT were simultaneously added prior to the reaction, or the two compounds were sequentially added, one added before the first incubation at 37° for 15 min followed by further 30-min incubation after the addition of the other, as denoted in the legend for Fig. 4. Then, 2.5 μL of 10% SDS and 2 μL of 20 mg/mL Proteinase K were added and the reaction mixtures were digested at 37° for 1 hr. Proteins and drugs were removed by extraction with phenol–chloroform–isoamyl alcohol mixture (25:24:1). Aqueous phases were collected and mixed with 4 μL of the dye/SDS stop solution and analyzed by electrophoresis on 0.6% agarose gels in the presence of 0.1 μg/mL ethidium bromide, a condition giving a good resolution of four forms of structural isomers of DNA, i.e. supercoiled circular form F I, relaxed circular form F Ir (with no nicks), nicked circular form F II, and linear form F III DNAs. Topo II-mediated DNA cleavage assay was carried out as described above for topo I with the following changes: reaction mixture contained in addition 10 mM $MgCl_2$, 1 mM ATP, 4 U of recombinant human topo IIα in place of topo I, and VM-26 in place of CPT.

*2.1.4. HCC panel analysis*

The growth inhibitory activities of GA3P (GA3Pl+) was evaluated using HCC panels consisting of 38 cancer cell lines, established in Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo [27,28].

## 3. Results

### 3.1. Inhibition of topo I activity by GA3P in vitro

We tested GA3Pl+, i.e. a GA3P preparation with high lactate content (>80%), and GA3Pl−, i.e. that with low lactate content (<20%), for inhibition of topo I by DNA relaxation assay. As shown in Fig. 2, the GA3Ps, with or without lactate group, showed potent inhibitory activity on topo I activity with $IC_{50}$ of 0.017 μg/mL for GA3Pl+ and 0.015 μg/mL for GA3Pl−. Lactic acid did not inhibit the enzyme ($IC_{50} > 10$ μg/mL). Another sulfated polysaccharide, dextran sulfate, also showed potent inhibition of topo I with $IC_{50}$ of 0.006 μg/mL.

### 3.2. Inhibition of topo II activity by GA3P in vitro

Next, we tested GA3Pl+ and GA3Pl− for inhibition of topo II by kDNA decatenation assay. As shown in Fig. 3, both of them showed potent inhibitory activity on topo II with $IC_{50}$ of 0.048 μg/mL for GA3Pl+ and 0.052 μg/mL for GA3Pl−. Lactic acid did not inhibit the enzyme ($IC_{50} > 10$ μg/mL). Dextran sulfate also showed potent inhibition of topo II as well with $IC_{50}$ of 0.024 μg/mL.

### 3.3. Inhibition of topo I and topo II by GA3P independent of DNA strand breaks

Topo inhibitors are classified according to whether they induce accumulation of topo-dependent DNA strand breaks as ‘‘cleavable complexes’’ or not, reflecting the

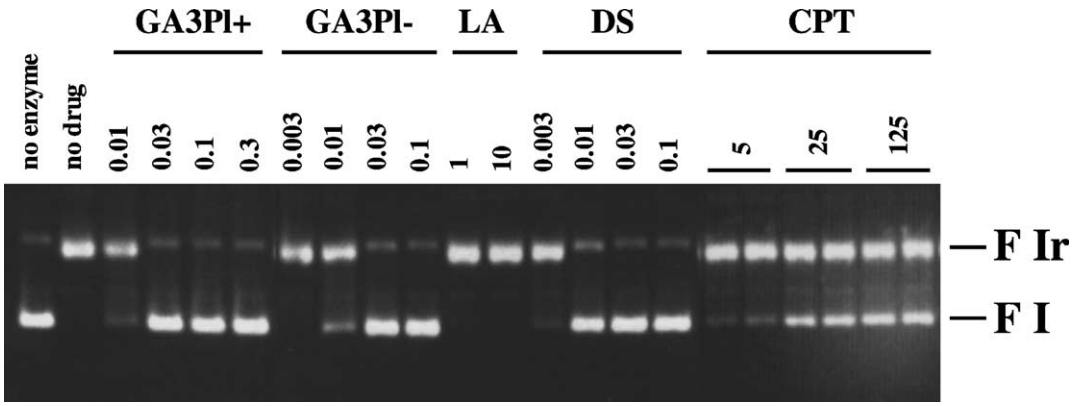


Fig. 2. Inhibition of topo I by GA3P. Supercoiled pT2GN plasmid DNA was treated with purified topo I enzyme in the presence of GA3Pl+, GA3Pl−, lactic acid (LA), dextran sulfate (DS), or CPT as described in Section 2. No enzyme was included in the reaction for ‘‘no enzyme’’, and no test compounds were added for ‘‘no drug’’. The indicated compounds were included in the reactions as test compounds. The concentrations are expressed in μM for CPT and in μg/mL for the others. F I, supercoiled circular form I DNA; F Ir, relaxed circular form Ir DNA.

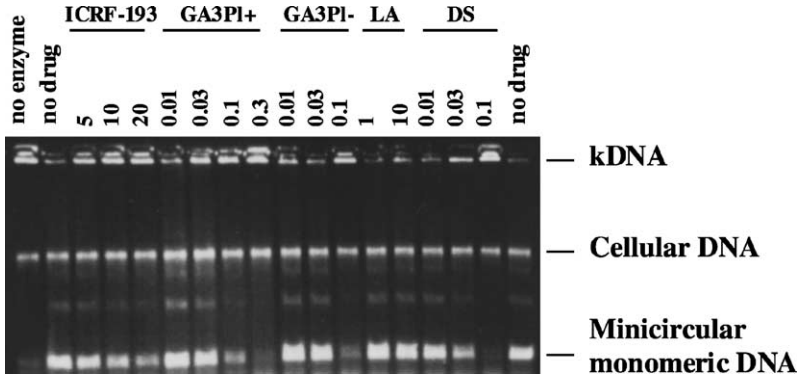


Fig. 3. Inhibition of topo II by GA3P. Decatenation of kDNA by topo II was performed as described in Section 2. No enzyme was included in the reaction for "no enzyme", and no test compounds were added for "no drug". The indicated compounds were included in the reactions as test compounds. The concentrations are expressed in μM for VM-26 and in μg/mL for the others. LA, L-lactic acid; DS, dextran sulfate; kDNA, kinetoplast DNA.

mechanism of inhibition. We examined whether the GA3P (GA3Pl+) induce accumulation of cleavable complexes in each of topo I and topo II inhibition. The reaction by topo I or topo II in the presence of the test compound was terminated by addition of 1% SDS, and digested with Proteinase K to convert the cleavable complexes into linear or nicked circular forms of DNA, which were then separated by electrophoresis in the presence of ethidium bromide.

In inhibition test of topo I (Fig. 4A), the GA3P itself showed no accumulation of cleavable complexes despite the complete inhibition of relaxation, while a topo I poison CPT stabilized the complex. When added simultaneously with CPT, accumulation of the complexes was completely inhibited, as evidenced by disappearance of F II DNA and accumulation of F I DNA. When the polysaccharide was added after addition of CPT, F II DNA was still missing, demonstrating that the CPT-induced DNA-topo I cleavable complex was fully reversed. Dextran sulfate showed apparently the same effect as the GA3P: dextran sulfate added simultaneously with CPT completely inhibited the accumulation of cleavable complexes and, when added after the formation of cleavable complexes, almost complete reversion of the reaction was observed.

In inhibition test of topo II (Fig. 4B), the GA3P itself showed no accumulation of cleavable complexes, again demonstrating that GA3P is a catalytic inhibitor. When the polysaccharide was added simultaneously with a topo II poison VM-26, accumulation of the complexes was inhibited, as evidenced by a large decrease in the amount of F II

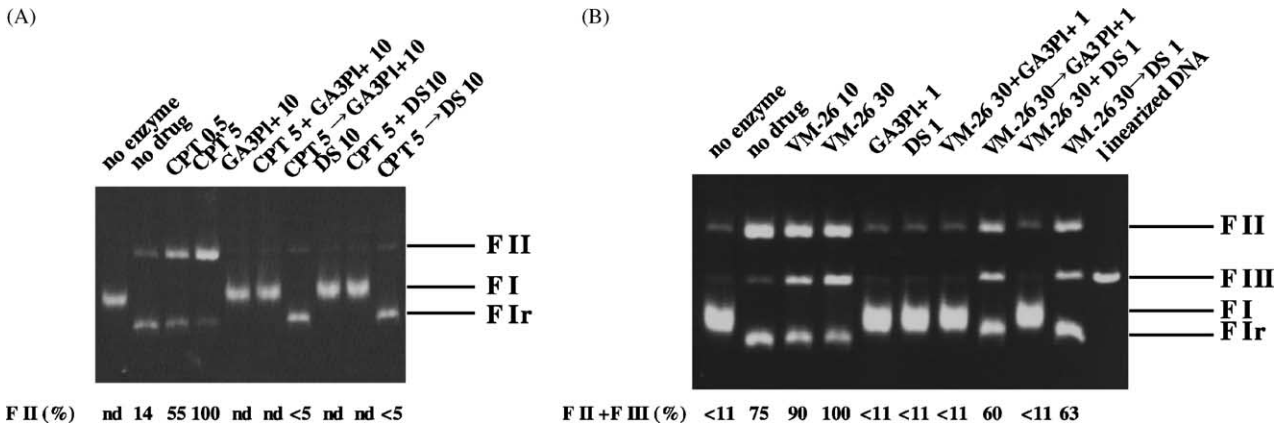


Fig. 4. Inhibition by GA3P (GA3Pl+) of topo I- or topo II-mediated DNA cleavage induced by CPT or VM-26, respectively. Reaction mixtures containing F I plasmid DNA as substrate, 10 U of topo I (A) or 4 U of topo IIα (B) were incubated in the presence of test compounds as described in Section 2. After incubation reaction mixtures were digested with Proteinase K/SDS and cleavable complexes in the reaction mixtures were converted to linear or nicked circular DNAs. The products were electrophoresed on 0.6% agarose gels in the presence of 0.1 μg/mL ethidium bromide enabling a good resolution of four forms of DNA: supercoiled circular form F I, relaxed circular form F Ir (with no nicks), nicked circular form F II, and linear form F III. (A) Topo I-mediated DNA cleavage. No enzyme was included in the reaction for "no enzyme", and no test compounds were added for "no drug". The indicated compounds were included in the reactions as test compounds, the concentrations being expressed in μM for CPT and in μg/mL for the others. The symbol "+" means simultaneous additions of the two compounds, and the arrows mean sequential addition of the two, as described in Section 2. Extent of cleavable complex formation was expressed as the percentage of F II DNA formed in the presence of 5 μM CPT ("CPT 5") set at 100% and shown under the panel. (B) Topo II-mediated DNA cleavage. Reactions and symbols were as in panel A. The concentrations are expressed in μM for VM-26 and in μg/mL for the others. Extent of cleavable complex formation was expressed as the percentage of F II and F III formed in the presence of 30 μM VM-26 ("VM-26 30") set at 100% and shown under the panel.

and F III DNAs and concurrent increase of F I DNA. When the polysaccharide was added after addition of VM-26, i.e. after accumulation of the complexes in the presence of VM-26 (linear F III and nicked circular form F II DNAs shown in the lane "VM-26 30"), they slightly decreased (down to 60% of the control), indicating that the GA3P is capable of partially reversing DNA-topo II cleavable complexes that were already formed by VM-26. Again, dextran sulfate showed apparently similar effect as the GA3P: dextran sulfate added simultaneously with VM-26 completely inhibited the accumulation of cleavable complexes, while, when added after the formation of cleavable complexes, slight reversion of the reaction was observed.

To summarize, these experiments showed the following observations: (1) the GA3P is not a topo poison but a catalytic inhibitor of both topo I and topo II; (2) the GA3P is capable of reversing the cleavable complexes of topo I and topo II which are already formed by potent topo poisons to various extents; (3) in all of these properties, the effect of dextran sulfate on topoisomerases is apparently the same as that of GA3P.

### 3.4. Growth inhibition of HCCs by GA3P

Growth inhibitory activity of GA3P (GA3Pl+) was investigated on an HCC panel of Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, consisting of 38 cell lines [27,28]. The GA3P showed moderate inhibitory activity, with $GI_{50}$ of 0.67−11 μg/mL in various cancer cell lines and MG-MID (the logarithmic average of $IC_{50}$ of the cell lines expressed in μg/mL for polymeric GA3P[3]) of −5.46 (Table 1). In contrast, no inhibition was observed with dextran sulfate at 100 μg/mL in HCT-116 colon and St-4 stomach cancer cells (data not shown).

Table 1
Growth inhibition of various human cancer cell lines by GA3 polysaccharide[a]

| Origin of cancer | Cell line | $GI_{50}$[b] (μg/mL) |
|---|---|---|
| Breast | HBC-4 | 5.2 |
| | BSY-1 | 0.67 |
| | HBC-5 | 6.2 |
| | MCF-7 | 2.9 |
| | MDA-MB-231 | 1.5 |
| Central nervous system | U251 | 2.0 |
| | SF-268 | 4.7 |
| | SF-295 | 2.7 |
| | SF-539 | 1.8 |
| | SNB-75 | 1.5 |
| | SNB-78 | 2.4 |
| Colon | HCC2998 | 2.3 |
| | KM-12 | 3.7 |
| | HT-29 | 3.6 |
| | WiDr | 3.1 |
| | HCT-15 | 3.4 |
| | HCT-116 | 3.7 |
| Lung | NCI-H23 | 2.8 |
| | NCI-H226 | 2.2 |
| | NCI-H522 | 1.3 |
| | NCI-H460 | 3.8 |
| | A549 | 11 |
| | DMS273 | 2.0 |
| | DMS114 | 2.7 |
| Melanoma | LOX-IMVI | 6.3 |
| Ovary | OVCAR-3 | 2.2 |
| | OVCAR-4 | 3.2 |
| | OVCAR-5 | 6.8 |
| | OVCAR-8 | 4.1 |
| | SK-OV-3 | 8.1 |
| Kidney | RXF-631L | 9.1 |
| | ACHN | 8.3 |
| Stomach | St-4 | 8.4 |
| | MKN1 | 3.0 |
| | MKN7 | 5.9 |
| | MKN28 | 7.0 |
| | MKN45 | 2.9 |
| | MKN74 | 4.6 |
| MG-MID[c] | | −5.46 |

[a] Determined by a human cancer cell line panel of Cancer Chemotherapy Center, Japanese Foundation for Cancer Research [28].
[b] 50% growth inhibitory concentration.
[c] Mean logarithm of $GI_{50}$ (expressed in g/mL) through the cell line panel.

## 4. Discussion

We have shown in the present report that GA3P is a potent catalytic inhibitor of both topo I and topo II, irrespective of the presence or absence of lactate group. As revealed in Figs. 2–4, all the observed inhibitory effects of the GA3P on topo I and topo II seem very similar to that of dextran sulfate. We have previously reported that heparin, which is normally sulfated to a higher degree than other natural glycosaminoglycans, is a potent inhibitor of topo I in a catalytic inhibition manner, and that other glycosaminoglycans, including heparan sulfate, chondroitin sulfate, dermatan, sulfate and dextran, are devoid of the activity [29,30]. However, they acquire potent inhibitory activity when chemically sulfated to a certain extent, and the acquisition of the inhibitory activities thereof required in addition a certain degree of polymerization [30]. Thus, the mechanism underlying the inhibitory activity of the GA3P against topo I and topo II may be the same as that of sulfated polysaccharides in general.

From similarity in structure of sulfated polysaccharides to DNA as the anionic polymer, it seemed possible that the GA3P and dextran sulfate inhibit any enzymes that bind to and work on DNA by some mechanism involving this property. So, we tested the inhibition of T4 DNA ligase by monitoring self-ligation of a linearized plasmid, as described previously [31]. The result showed that both polysaccharides, GA3P and dextran sulfate, exerted strong inhibition at 0.03 μg/mL, a concentration close to the $IC_{50}$ against topo, suggesting a general inhibition against enzymes working on DNA (data not shown). Hence, we

hypothesize that the sulfated polysaccharides having enough extent of sulfation and polymerization would bind to positively charged DNA-binding locus of the enzymes and inhibit its binding to DNA three-dimensionally or electrostatically. The enzymes examined, i.e. mammarian topo I, mammarian topo II, and virus ligase are, in fact, thought to bind to DNA with positively charged loci [32–34]. Sulfates are intrinsically stronger acids than phosphates, and hence sulfated polysaccharides would bind non-specifically to the DNA-interacting enzymes with higher electrostatic affinity than DNA. Although the binding by each single sulfate group should be weaker than the specific binding by DNA, multivalent nature of sulfated polysaccharides might enable the molecules to cover the DNA-binding locus of the enzymes, excluding the substrate DNA from the reaction.

In topo I- and topo II-mediated DNA cleavage assay (Fig. 4), the GA3P was shown to diminish even previously accumulated cleavable complexes induced by topo poisons to various extents, reflecting high affinity of the polysaccharide to the enzymes relative to the topo poisons CPT or VM-26. This property of the polysaccharide, along with the potent topo inhibitory activities, makes it an interesting model compound for the mechanistic study of chemotherapeutic agents on topo.

The GA3P was shown to have moderate growth inhibitory activity against a wide variety of cancer cell lines with MG-MID of −5.5, as compared to the standard topo inhibitors, CPT (topo I poison, MG-MID being −7.0), etoposide (topo II poison, MG-MID being −5.2), or ICRF-193 (topo II catalytic inhibitor, MG-MID being −5.9)[3] [25]. On the other hand, dextran sulfate did not show any significant growth inhibition. Thus, the mechanism underlying growth inhibition of tumor cells by GA3P should be distinct from that of topo I or topo II inhibition from the following reasons. In addition to the finding that dextran sulfate, sharing a common mode of action on topo I and topo II with GA3P, is devoid of growth inhibitory activity,[4] it seems unlikely that the extracellular polysaccharide with high molecular weight is incorporated into the nucleus and directly inhibits topos. Thus, it is reasonable to assume that some receptor(s) at cell surface respond to the ligand GA3P to trigger cell death signals. Further investigation is necessary to understand the mechanism of cell growth inhibition by GA3P.

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[3] The $IC_{50}$ to calculate the MG-MID is normally expressed in molar concentration. Hence, comparison of an MG-MID calculated on g/mL basis with normal one makes approximate significance.

[4] Unpublished results.

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