Inhibition of Topoisomerase I Activity by Tyrophostin Derivatives, Protein Tyrosine Kinase Blockers: Mechanism of Action

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ABSTRACT

DNA topoisomerase I (topo I) is a member of a group of essential nuclear enzymes which control and modify the topological state of DNA and is recognized as the target for anticancer drugs. During the course of the catalytic activity of topo I, a covalent bond is formed between a tyrosine group at the active site of the enzyme and a 3’ phosphate group along the DNA backbone. This chemical reaction resembles the protein kinase-mediated tyrosine phosphorylation process. We assumed, therefore, that tyrophostins, potent and selective blockers of protein tyrosine kinases, might affect topo I activity. We found that three derivatives of tyrophostins (AG-555, AG-18, and AG-213) that inhibited topo I activity in an in vitro assay, AG-555 was the most active. Examination of the mechanism by which these compounds act as topo I inhibitors revealed that AG-555 blocked the binding of this enzyme to the DNA due to its interaction with the topo I enzyme. We showed that its mode of action differed from that observed for camptothecin, a known topo I inhibitor. However, AG-555 did not affect the activity of other major DNA binding enzymes (i.e., DNA ligase, DNA polymerase I, and reverse transcriptase). This study suggests that tyrophostins may serve as a new class of topo I inhibitors, and these results also present additional explanations for their antiproliferative effect.

INTRODUCTION

DNA topoisomerases are essential nuclear enzymes that function to resolve topological problems in DNA such as overwinding, underwinding, and catenation, which normally occur during replication, transcription, and other DNA-associated processes (1–3). In mammalian cells, two major topoisomerases, type I (topo I) and II (topo II), have been identified, which function by forming enzymes-bridge strand breaks that act as transient gates for the passage of other DNA strands (1–3). Topo I is virtually present in all somatic cells and is a M, 100,000 polypeptide (4–6) encoded by a single copy gene (7–8). It is the target for the anticancer drug CPT and its derivatives (9–11). Two of the agents that target this enzyme, topotecan and CPT-11, appear to be active against a broad range of human tumors (reviewed in Refs. 3 and 11). The involvement of this enzyme in most of the DNA transactions suggests that it may serve as a preferred target for developing new potent anticancer drugs. During the course of its catalytic activity, a covalent bond is formed between a tyrosine group at the active site of topo I and a 3’ phosphate group along the DNA backbone (12–15). This chemical reaction resembles the tyrosine phosphorylation process; therefore, it was interesting to investigate whether PTK antagonists may inhibit DNA topo I activity. It was demonstrated that genistein, which blocks EGF receptor kinase, can also block topo II (16).

Tyrophostins are synthetic compounds which are potent and selective PTK blockers. They can serve as antiproliferative agents and molecular tools to investigate signal transduction pathways of PTKs (17–19). We examined the effect of three different tyrophostin derivatives (AG-555, AG-18, and AG-213) on topo I activity in vitro and found that these compounds inhibited the activity of purified and unpurified topo I in an in vitro assay. Moreover, we also investigated the mechanism by which these tyrophostins inhibit topo I activity, and our results suggest that they prevent the binding of topo I to DNA by interacting with the enzyme and preventing the covalent bond formation between the tyrosine residue in the topo I active site and the phosphate moiety in the DNA backbone. We also showed that the mode of topo I inhibition by tyrophostin differs from that described for CPT.

MATERIALS AND METHODS

Compounds

Tyrophostin derivatives AG-555, AG-18, and AG-213 were received from Dr. A. Levitzky, the Hebrew University, Jerusalem. Stock solutions of the drugs, 10 mM or 100 mM in 100% DMSO, were stored at −20°C and diluted in DMSO before being added to the reaction mixture. CPT was obtained from the Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, National Cancer Institute. Stock solutions of the drug at 10 mM in DMSO were stored in aliquots at −20°C.

DNA Plasmids

Supercoiled pUC19 plasmid was obtained from the Fermentas Company. pHOT1 plasmid, a supercoiled pUC12 plasmid containing the high affinity topo I cleavage site and useful as a topo I cleavage substrate, was obtained from Topogen, Inc.

Enzymes

Purified calf thymus topo I was purchased from Topogen, Inc., and T4 DNA ligase was obtained from New England Biolabs.

Preparation of Nuclear Extract

Nuclear extract from NIH/3T3 cells was prepared as described by Auer et al. (20) except that 1 mM phenylmethylsulfonyl fluoride was added to the extract. Total protein concentration was determined using the Bio-Rad kit, and 4 μg of nuclear proteins were added to the topo I reaction mixture.

Topo I Assay

Purified calf thymus topo I (5 units) was added to a topo I reaction mixture containing, at a final volume of 25 μl, 20 mM Tris-HCl (pH 8.1), 1 mM dithiothreitol, 20 mM KCl, 20 μg/ml bovine serum albumin, and 0.5 μg supercoiled DNA plasmid. Where indicated, tyrphostins or CPT were added. Following incubation at 37°C for 30 min, the reaction was terminated by adding 5 μl of stopping buffer [final concentration; 1% sodium dodecyl sulfate, 15% glycerol, 0.5% bromophenol blue, and 50 mM EDTA (pH 8)]. The reaction products were analyzed by electrophoresis on 1% agarose gel using a TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 62 mM EDTA) at 1 V/cm, stained by ethidium bromide (1 μg/ml), and photographed using a short wavelength UV lamp.

Agarose Gel Shift Assay

DNA Labeling and Purification. α32P-labeled pUC19 fragments were prepared using the Random Primers DNA labeling system (BRL). To separate the labeled DNA from the free dCTP (α32P) nucleotide, we purified the DNA...
The reaction products were analyzed by electrophoresis on 1% agarose gel (4 Tris-HCl (pH 8)–1 mM EDTA).

**Gel Shift Assay.** Ten units of topo I protein were added to the topo I reaction mixture containing 10 ng (6 × 10^5 cpm) of labeled pUC19 fragments. The reaction products were analyzed by electrophoresis on 1% agarose gel (4 V/cm) in a TBE buffer. The gel was exposed to autoradiography as described by Sambrook et al. (21) for unfixed wet polyacrylamide gel. We found that it was also possible to use this method for agarose gel.

**DNA-Enzyme Cleavable Complex Assay**

Topo I protein (17 units) was added to the topo I reaction mixture containing 10 ng (6 × 10^5 cpm) labeled pUC19 fragments, which were purified from free-labeled nucleotides and from the enzymes used for DNA labeling as described above. The assay was performed in the presence or absence of CPT or tyrphostins. Following incubation for 1 h at 37°C, the samples were boiled for 2 min, and P1 nuclease digestion was performed as described previously (22, 23). A 0.1 M (final concentration) of sodium acetate (pH 5.6) and 30 units of P1 nuclease (BRL) were added for 2 h at 37°C. Where indicated, 400 µg/ml of proteinase K were added, and the reaction was further incubated for 1 h at 50°C. This reaction was stopped by 5 µl of protein sample buffer [0.5 M Tris-HCl (pH 6.8), 4% SDS, 0.1% bromophenol blue, 20% glycerol, and 4% (v/v) β-mercaptoethanol]. The reaction products were boiled for 10 min and analyzed on a 10% sodium dodecyl sulfates-polyacrylamide gel; the proteins were then transferred to a nitrocellulose membrane (24). Autoradiography was performed using X-ray film.

**T4 DNA Ligase Assay**

Purified T4 DNA ligase (200 units) was added to a specific reaction mixture containing 20 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 5 mM dithiothreitol, 50 µg/ml bovine serum albumin, and 5 µg of bacteriophage A HindIII DNA fragments (which served as the substrate for the enzyme) with the addition of various concentrations of tyrophostin AG-555. Following incubation at 16°C for 1 h, the reaction products were analyzed by electrophoresis on a 1% agarose gel using a TBE buffer. The gel was stained with ethidium bromide (1 µg/ml) and photographed using a short wavelength UV lamp.

**RT Assay**

RT activity was measured as described previously (25), and where indicated, various doses of AG-555 were added to the RT reaction mixture.

**DNA Polymerase I Activity**

DNA polymerase I activity was measured using the Random Primers DNA labeling system (BRL) and the pUC19 DNA plasmid as substrate. The labeled DNA was purified from the free-labeled nucleotides using the nick column (Pharmacia), and radioactivity was assayed by a liquid scintillation counter.

**RESULTS**

**Tyrophostin Derivatives Inhibit Topo I Activity.** Three different tyrophostin derivatives designated AG-555, AG-18, and AG-213 (for structural formula, see Fig. 1) were examined for their effect on the activity of purified calf thymus DNA topo I in an in vitro assay. Increasing concentrations of tyrophostins, added to a specific topo I reaction mixture, significantly inhibited the relaxation of the supercoiled DNA plasmid by topo I (Fig. 2). Since tyrophostins were dissolved in DMSO, an equal amount of this DMSO (4%) was added to the topo I reaction mixture; no effect on topo I activity was observed (data not shown). Densitometric analysis of the results shown in Fig. 2 was performed, and the data revealed that, at concentrations of 100 and 500 µM, AG-555 inhibited 89 and 100% of topo I activity, respectively, while at a concentration of 1 mM, AG-18 and AG-213 inhibited only 8 and 18%. These results which show that AG-555 is a more potent inhibitor of topo I activity, compared to AG-18 and AG-213, suggest that the observed inhibition is influenced by stereospecific requirements. Similar results were obtained when a nuclear extract prepared from NIH/3T3 cells was used as a source of the enzyme (Fig. 3), indicating that tyrophostins inhibit the activity of both purified and unpurified topo I.

**Tyrophostins Inhibit Topo I Activity by Mechanisms Other than Camptothecin.** CPT is currently the most potent and specific topo I inhibitor. It inhibits topo I activity by stabilizing the DNA-enzyme complex in which topo I is covalently bound to a 3' phosphate group of the DNA and thus prevents the ligation reaction catalyzed by topo
Tyrphostin AG-555 or CPT were added to the topo I reaction mixture, whether the mode of inhibition of topo I activity by tyrphostins is similar to that observed in the presence of CPT, we analyzed their ability to introduce single-stranded DNA breaks using a specific supercoiled DNA plasmid (pHOT1), which includes the high-affinity topo I cleavage site, and is useful as a topo I cleavage substrate. Tyrphostin AG-555 or CPT were added to the topo I reaction mixture, and we examined their effect on the relaxation of the supercoiled pHOT I plasmid by purified calf thymus topo I. In the presence of CPT, the linear form (II) and the nicked relaxed form (I) of the DNA plasmid were observed (Fig. 4, Lane 3) which are the result of DNA strand breaks. However, in the presence of tyrphostin AG-555, most of the supercoiled DNA molecules were not relaxed by topo I, and the linear or nicked relaxed pattern of the plasmid was not observed (Fig. 4, Lane 4). These results suggest that tyrphostin AG-555 inhibits the activity of topo I by direct binding to the enzyme.

Tyrphostin AG-555 Does Not Inhibit the Activity of Other DNA-binding Enzymes. Since topoisomerase I is a DNA-binding enzyme, the question then arises whether AG-555 may as well inhibit the activity of other such enzymes. To address this question, we measured topo I activity in the presence of constant amounts of enzyme and tyrphostin and increasing concentrations of DNA or vice versa; topo I activity was measured in the presence of a constant amount of DNA and tyrphostin and increasing amounts of enzyme. The results show that only by increasing the amount of the topo I enzyme is it possible to overcome the inhibitory effect of tyrphostin AG-555 (Fig. 7 compare a to b). These results suggest that tyrphostin AG-555 inhibits the activity of topo I by direct binding to the enzyme.

Tyrphostins Inhibit the Binding of Topo I to DNA. Since tyrphostin AG-555 inhibits the relaxation of supercoiled DNA by topo I, it could interfere with the binding of the enzyme to DNA. To examine this possibility, we used the electrophoretic agarose shift assay. pUC19 DNA was labeled by dCTP (α32P) using the random primers DNA labeling technique, and the labeled DNA (purified from the free-labeled nucleotides and from the enzymes used for the labeling process) was added to the topo I buffer containing purified enzyme in the presence or absence of tyrphostin. The results depicted in Fig. 5 demonstrate a significant decrease in the mobility of the DNA in the presence of topo I, which is the outcome of a complex formed between the enzyme and the DNA (Fig. 5, compare Lane 5 to Lane 6).

However, with the addition of AG-555, no gel retardation was observed, indicating that tyrphostin inhibits the binding of topo I to the DNA (Fig. 5, compare Lane 5 to Lanes 3 and 4). Moreover, tyrphostin AG-555 alone did not affect the migration of the DNA (Fig. 5, Lanes 1 and 2). To substantiate these results, we examined the ability of tyrphostin to inhibit the formation of a covalent bond between the enzyme and the phosphate moiety of the DNA backbone by using the DNA-enzyme cleavable complex assay in which topo I formed a covalent bond with a 32P-labeled phosphate moiety from the DNA backbone. When this complex was treated with P1 nuclease, the labeled phosphate remained attached to the enzyme. Analysis of the reaction products on polyacrylamide gel electrophoresis following autoradiography revealed a labeled M, 100,000 topo I polypeptide (Fig. 6, Lane 6) which disappeared following proteinase K treatment (Fig. 6, Lane 5). The assay was performed in the presence or absence of CPT or tyrphostin, and since CPT stabilized the DNA-enzyme cleavable complex, a more intensely labeled band was observed (Fig. 6, Lane 4), but when tyrphostin was used, the topo I protein was not labeled (Fig. 6, Lane 3). These results are compatible with the data obtained with the agarose shift assay, implying that tyrphostins inhibit the binding of topo I to the DNA, and their mode of action differs from that described for CPT.

Tyrphostin AG-555 Binds to the Topo I Enzyme. Since tyrphostin AG-555 inhibits the binding of topo I to the DNA, it is possible that it acts by binding to the DNA, thus interfering with the binding of the enzyme to the DNA, or by binding to the enzyme, and thus preventing its interaction with the DNA. In order to determine which of the above possibilities does occur, we performed two classical biochemical competition-based assays. Topo I activity was measured in the presence of constant amounts of enzyme and tyrphostin and increasing concentrations of DNA or vice versa; topo I activity was measured in the presence of a constant amount of DNA and tyrphostin and increasing amounts of enzyme. The results show that only by increasing the amount of the topo I enzyme is it possible to overcome the inhibitory effect of tyrphostin AG-555 (Fig. 7 compare a to b). These results suggest that tyrphostin AG-555 inhibits the activity of topo I by direct binding to the enzyme.

Fig. 4. Comparison between the effect of tyrphostins and CPT on topo I activity. Purified topo I (5 units) was added to a reaction mixture containing supercoiled pHOT1 DNA plasmid (0.2 µg Lane 2). Four hundred µm of CPT (Lane 3) or 100 µm of AG-555 (Lane 4) were added to the reaction mixture, and the samples were analyzed on agarose gel electrophoresis. Lane 1, pHOT1 plasmid DNA. S, supercoiled; R, relaxed; L, linear forms of the plasmid DNA.

Fig. 5. The effect of tyrphostin AG-555 on the binding of topo I to the DNA (agarose gel shift analysis). Purified calf thymus topo I (10 units) was added to a reaction mixture containing 32P-labeled pUC19 DNA (10 ng, lane 3). AG-555 in concentrations of 500 or 100 µm were added to the samples in the presence (lanes 5 and 4) or absence (lanes 1 and 2) of topo I. Lane 6, labeled pUC19 DNA plasmid.

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examined its effect on the activity of DNA ligase, DNA polymerase I, and Mo-MuLV reverse transcriptase. The results shown in Fig. 8, Lane 2, demonstrate the ligation of λHindIII DNA fragments by purified DNA ligase, but the addition of increasing concentrations of tyrphostin to the ligation assay did not affect this process (Fig. 8, compare Lane 2 to Lanes 3–5). DNA polymerase I activity was measured using the random primers DNA labeling kit and the pUC19 plasmid as a substrate. Reverse transcriptase activity was determined using the purified Moloney murine leukemia virus RT enzyme and polyadenylated oligodeoxythymidylate as substrate. Various doses of tyrphostin added to the reaction assay of these enzymes had no effect, suggesting that tyrphostin AG-555 specifically inhibits topo I activity and does not influence the activity of other major DNA-binding enzymes (Table 1).

**DISCUSSION**

Tyrphostins are synthetic compounds specifically designed to inhibit the activity of PTKs. They have been examined in many biological systems and are successful antiproliferative agents (18-19). It was shown previously that genistein, which blocks the EGF receptor tyrosine kinase (28), also blocks topo II (16). In our present work, we demonstrated that tyrphostin derivatives (AG-555, AG-18, and AG-213) inhibit the activity of both purified and unpurified topo I in vitro, and AG-555 was significantly more potent than AG-18 and AG-213. Experiments were performed to identify the mechanism by which these tyrphostins inhibit topo I, and we found that these compounds inhibit the binding of topo I to DNA, probably due to their interaction with the topo I enzyme and not with the DNA. It is possible that the interaction between AG-555 and topo I causes conformational and structural changes in the enzyme, which prevent its ability to bind to the DNA.

In accordance with the chemical structure of tyrphostin (derived from the benzylidene malononitrile nucleus), which resembles the phenolic moiety of tyrosine, it is unlikely that tyrphostin interacts with the active site of topo I which contains a tyrosine residue, but this assumption needs to be explored. Moreover, the inhibition of the topo I enzyme appears to be specific for this protein, since tyrphostin...
AG-555 failed to affect the activity of other DNA-binding enzymes such as DNA polymerase I, DNA ligase, and Mo-MuLV reverse transcriptase.

This indicates that the inhibition is based on stereospecificity and is not due to simple nonspecific intercalation to the enzyme. These results also support our findings that tyrophostins interact with the topo I enzyme and not with the DNA, since interaction with the DNA would possibly affect the activities of the aforementioned DNA-binding enzymes. The mechanism by which tyrophostin inhibits topo I activity differs from the mode of action of CPT. CPT stabilizes the complex of DNA-enzyme and prevents its ligation activity; thus, it introduces single-stranded DNA breaks, while the inhibition of topo I by tyrophostin did not cause single-stranded DNA breaks. In addition, unlike CPT, in the presence of tyrophostin, the enzyme does not form a covalent bond between the tyrosine residue in its active site and the phosphate moiety from the DNA backbone.

Therefore, tyrophostin AG-555 represents a new class of topo I inhibitors with a different mechanism of action from the known topo I antagonists. The concentrations of tyrophostin AG-555 needed for 89 or 100% inhibition of topo I activity in an in vitro assay were 100 μM or 500 μM, respectively, while we have previously shown that the 50% cytotoxic concentration of this drug examined on NIH/3T3 cells was 210 μM (29). It is well known that the inhibitory doses of topo I inhibitors with a different mechanism of action from the known topo I antagonist. The concentrations of tyrophostin derivatives (i.e., AG-213) which are potent blocker of EGF receptor transcriptase.

REFERENCES

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