Poisoning of Topoisomerase I by an Antitumor Indolocarbazole Drug: Stabilization of Topoisomerase I-DNA Covalent Complexes and Specific Inhibition of the Protein Kinase Activity

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Abstract

We have investigated the mechanism of topoisomerase I inhibition by an indolocarbazole derivative, R-3. The compound is cytotoxic to P388 leukemia cells, but not to P388CPT5 camptothecin-resistant cells having a deficient topoisomerase I. R-3 can behave as both a specific topoisomerase I inhibitor trapping the cleavable complexes and as a nonspecific inhibitor of a DNA-processing enzyme acting via DNA binding. In addition, the drug is a potent inhibitor of the kinase activity of topoisomerase I. Unlike camptothecin, R-3 completely inhibits the phosphorylation of S2F/ASF, a member of the SR protein family, in the absence of DNA. The inhibitory effect is also observed using mutant enzyme Y723F that lacks DNA cleavage/religation activity but does not affect phosphorylase activity, indicating, therefore, that R-3 acts independently at both DNA cleavage and protein kinase sites. R-3 is the only compound known thus far that interferes specifically with the kinase activity of topoisomerase I and not with other kinases, such as protein kinase C and the cdc2 kinase. The study reinforces the view that topoisomerase I is a dual enzyme with a DNA cleavage site juxtaposed to a functionally independent kinase site and shows for the first time that indolocarbazole drugs can inhibit both the DNA cleavage/religation and kinase activities of the enzyme.

Introduction

The DNA-topoisomerase I covalent complex is the nuclear target for a number of anticancer agents derived from the plant alkaloid camptothecin (1) and for indolocarbazole derivatives such as the antitumor agent NB-506, which is undergoing Phase I clinical trials (2–5). Over the last 2 years, we have developed different series of indolocarbazole compounds structurally related to NB-506 and the related antibiotic rebeccamycin with the aim of elucidating the structure-activity relationships (6–8). Among the molecules developed thus far, the analogue R-3 (Fig. 1) was found to be a potent DNA-intercalating topoisomerase I inhibitor (9). Indolocarbazole derivatives such as NB-506 and R-3 bear a structural analogy with the specific PKC1 inhibitor staurosporine. Neither NB-506 nor R-3 have significant effect on PKC (R-3 is about 10,000 times less potent than staurosporine on PKC; Ref. 6), but it is possible that they affect other kinases and, in particular, the phosphorylation activity of topoisomerase I, which has been recently discovered (10). To answer this question, and to comprehend further the mechanism of action of R-3, we have investigated the effect of the compound on wild type topoisomerase I that acts essentially as a DNA relaxing enzyme and the mutant enzyme Y723F that acts selectively as a phosphotransferase.

In the present study, we show that the antitumor indolocarbazole compound R-3 inhibits both the DNA cleavage/ligation and kinase activities of topoisomerase I. This is the first ligand known thus far that can inhibit the kinase activity of topoisomerase I in the absence of DNA. The results open new perspectives for the design of topoisomerase I-targeted antitumor agents.

Materials and Methods

Drugs. Camptothecin was purchased from Sigma Chemical Co. (La Verpillière, France). The synthesis of the indolocarbazole R-3 has been reported (6). Drugs were dissolved in DMSO at 3 mM and then further diluted with water. Fresh dilutions were prepared immediately before use. The final DMSO concentration never exceeded 0.3% (v/v), and under which conditions DMSO (also present in the controls) is known not to affect the topoisomerase I activity.

Cell Cultures and Growth Inhibition Assay. The P388 murine leukemia cell line was obtained from the tumor bank of the National Cancer Institute (Bethesda, MD). The P388CPT5 cell line resistant to camptothecin was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage (11). Both cell lines were grown in RPMI 1640 containing 0.01 mM 2-mercaptoethanol, 10 mM ‐glutamine, 10% (v/v) FCS, 100 IU/ml penicillin, 2 μg/ml streptomycin, 50 μg/ml gentamicin, and 50 μg/ml nystatin at 37°C in a humidified atmosphere containing 5% CO₂. Experiments were carried out with exponentially growing cells, as described previously (11). Briefly, 2× 10⁶ P388 or P388CPT5 cells were seeded in 96-well microculture plates with various drug concentrations (semi-log dilutions). After a 96-h incubation at 37°C, cells were incubated with 0.02% neutral red for 16 h, then washed and lysed with 1% SDS. The incorporation of the dye, which reflects cellular growth and viability, was evaluated by measuring the absorbance at 540 nm for all wells using a titertek multiwell spectrophotometer. Each point was done in quadruplicate, and all compounds were evaluated in parallel on the two cell lines.

Recombinant Proteins. Experiments were performed with either human topoisomerase I from TopoGen, Inc. (Columbus, OH) or with recombinant topoisomerase I proteins expressed in baculovirus. The procedures for the expression and purification of the wild type and mutant Y723F topoisomerase I proteins and the S2F/ASF recombinant protein have been reported recently (10).

DNA Relaxation Experiments. Supercoiled pKmp27 DNA (0.5 μg) was incubated with 6 units of topoisomerase I at 37°C for 1 h in relaxation buffer [50 mM Tris (pH 7.8), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 1 mM EDTA] in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/ml. DNA samples were then added to the electrophoresis dye mixture (3 μl) and electrophoresed in a 1% agarose gel containing ethidium bromide at room temperature for 4 h. Gels were washed and photographed under UV light.

Sequencing of Topoisomerase I-mediated DNA Cleavage Sites. The 160-bp tyrT DNA fragment was prepared by 3′-[32P]-end labeling of the DNA.
the reaction was initiated by the addition of 2 ng of recombinant SF2/ASF protein, 1100 ng of either wild type or mutant recombinant topoisomerase I proteins, 300 used to collect data. under vacuum at 80°C. A Molecular Dynamics 425E PhosphorImager was 10% acetic acid for 10 min, transferred to Whatman 3 MM paper, and dried

denaturing conditions (8 M urea). After electrophoresis, gels were soaked in 
and then resuspended in 5 l of compound R-3, or DMSO in a final volume of 20
3
m
l of drug solution at the desired concentration (5–25 µg/ml). After a 10-min incubation to ensure equilibration, the reaction was initiated by the addition of 2 µl (40 units) of purified topoisomerase I. Samples were incubated for 60 min at 37°C before adding SDS to 0.25% and proteinase K to 250

m
l of (4X) loading buffer and applied to a 12% SDS-polyacrylamide gel. After electrophoresis, gels were soaked in

l of formamide-Tris-borate EDTA loading buffer, denatured at 90°C for 4 min, then chilled in ice for 4 min before loading onto the sequencing gel. DNA cleavage products were resolved by PAGE under denaturing conditions (8 M urea). After electrophoresis, gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3 MM paper, and dried under vacuum at 80°C. A Molecular Dynamics 425E PhosphorImager was used to collect data.

Kinase Assays. The reaction mixtures for protein kinase activity contained 100 ng of either wild type or mutant recombinant topoisomerase I proteins, 300 ng of recombinant SF2/ASF protein, 1 µM ATP, 3 µCi [γ-32P]ATP (3000 Ci/mmol), and 1 µl of compound R-3 or DMSO in a final volume of 20 µl of buffer A [50 mM Hepes (pH 7.0), 10 mM MgCl2, 3 mM MnCl2, 50 mM KCl, and 0.5 mM DTT]. The samples were incubated at 30°C for 30 min and were then mixed with 6 µl of (4X) loading buffer and applied to a 12% SDS-polyacrylamide gel. Radioactivity incorporated into SF2/ASF on the dried gel was detected by autoradiography and by a Molecular Dynamics imaging analyzer. The relative amount of [γ-32P]ATP incorporated in each assay was quantitated by densitometry scanning of the gel using ImageQuant software version 3.22.

Results and Discussion

Before investigating the in vitro effects of the test compound R-3, we showed that topoisomerase I is involved in the cytotoxicity of this compound. P388 and P388CPT5 murine leukemia cell lines sensitive and resistant to camptothecin, respectively, were used. The resistance of the P388CPT5 cells has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the top1 gene of these cells (11). Under the experimental conditions used (4 days continuous exposure), R-3 proved to be very toxic to P388 cells, whereas it exhibited little or no effect on the growth of P388CPT5 cells (Fig. 2). With the sensitive P388 cells, IC50 values of 0.018 and 0.28 µM were measured for camptothecin and R-3, respectively.

Although R-3 is significantly less cytotoxic than camptothecin with this cell line, there is no doubt that topoisomerase I is a cellular target for the indolocarbazole derivative. P388CPT5 cells exhibit marked resistance to camptothecin (83-fold) and are cross-resistant to R-3 (the relative resistance index is >28).

To evidence the effect of the test drug on the DNA relaxation activity of topoisomerase I, we first resorted to a conventional DNA relaxation assay using supercoiled plasmid DNA analyzed on agarose gels. Negatively supercoiled plasmid pKMp27 was incubated with topoisomerase I in the presence of increasing concentration of R-3, ranging from 10–150 µg/ml. The DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved in a 1% agarose gel containing ethidium bromide. As shown in Fig. 3A, supercoiled DNA is fully relaxed by topoisomerase I in the absence of drug (compare Lanes DNA and Topo I). The relaxed DNA migrates faster than the supercoiled plasmid on an agarose gel containing ethidium bromide (because of DNA unwinding effects). In the presence of R-3, the intensity of the band corresponding to the nicked form of DNA has increased significantly. This effect, observed with camptothecin, reflects the stabilization of topoisomerase I-DNA cleavable complexes. The shift in the mobility of supercoiled plasmid (form I) observed with increasing concentrations of R-3 is attributed to an inhibition of the catalytic activity of the enzyme, rather than from a decrease in plasmid DNA-linking number due to intercalation, because no such effect is observed in the absence of the enzyme (data not shown). R-3 can behave both as a specific topoisomerase I inhibitor trapping the cleavable complexes and as a nonspecific inhibitor of a DNA-processing enzyme acting via DNA binding.

R-3 is a sequence-selective intercalating agent, whereas camptothecin does not appreciably bind to DNA in the absence of topoisomerase I (9, 12). However, the two drugs apparently interfere similarly with the DNA-relaxing activity of topoisomerase I. It is, therefore, likely that despite their profound structural differences and their distinct DNA-binding properties, camptothecin and the indolocarbazole drug interact with topoisomerase I in a similar fashion or, at least, recognize identical structural elements of the topoisomerase I-DNA covalent complex. The compound R-3 is not simply a mimic of camptothecin. Differences do exist in terms of sequence-specificity of topoisomerase
I-mediated DNA cleavage. To examine further the activity of the compound, the topoisomerase cleavage sites induced by R-3 and camptothecin were mapped using a 160-bp DNA fragment. The EcoRI-AvaI restriction fragment from plasmid pKMp27 was uniquely end-labeled at the 3'-end of the lower strand at the EcoRI site and used as a substrate for the topoisomerase I cleavage reaction. The cleavage products were analyzed on a sequencing polyacrylamide gel (Fig. 3B). Here again, it can be seen unambiguously that R-3 and camptothecin strongly promote DNA cleavage by topoisomerase I. Although in both cases the cleavage occurs principally at sites having a T and a G on the 5’ and 3’ sides of the cleaved bond, respectively (see Ref. 9 for a statistical analysis of the cleavage specificity), it is clear that the cutting can sometimes occur at different nucleotide positions. For example, R-3 but not camptothecin promotes the cleavage at positions 41 and 49, corresponding to the sequences ACTG↓GTTG and AAAA↓ATGA, respectively. Conversely, cleavage at position 56 (CGTT↓GAGA) is specific to camptothecin.

Then we examined the effect of the indolocarbazole on the kinase activity of topoisomerase I. Recent studies have demonstrated that in addition to its primary action at the DNA level, topoisomerase I can act as a specific SR protein kinase (Ref. 10 and for review Ref. 13). This prompted us to examine whether indolocarbazole drugs such as R-3 (which is a close cousin of staurosporine but has no significant effect on PKC) can interfere with the kinase activity of topoisomerase I. Kinase activity was detected by its ability to phosphorylate bacterially expressed recombinant SF2/ASF, a member of the SR protein family (14), with [γ-32P]ATP. The results in Fig. 4 show that the phosphorylation of SF2/ASF by topoisomerase I is markedly inhibited in the presence of the indolocarbazole compound. A concentration of 1–2 μM suffices to reduce the kinase activity of topoisomerase I by 50%, whereas a drug concentration of 0.2 μM is required to stabilize topoisomerase-DNA cleavable complexes (data not shown). Therefore, the compound is about 10 times

Fig. 3. A, effect of increasing concentrations of R-3 on the relaxation of plasmid DNA by topoisomerase I. Native supercoiled pKMp27 DNA (0.5 μg; Lane DNA) was incubated with topoisomerase I in the absence or presence of drug at the indicated concentration (μg/ml). DNA samples were separated by electrophoresis on an agarose gel containing ethidium bromide. Nc, nicked; Rel, relaxed; Sc, supercoiled. B, phosphorimage comparing the susceptibility of the 160-bp tyrT fragment to cutting by topoisomerase I in the presence of camptothecin and R-3. The 3'-end-labeled fragment (Lane DNA) was incubated in the absence (Lane Topo I) or presence of the indolocarbazole derivative at 5, 10, and 25 μg/ml or camptothecin at 5 μg/ml. Topoisomerase I cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel, as described in “Materials and Methods.” Numbers at the right of the gel show the nucleotide positions, determined with reference to the guanine track (G).

Fig. 4. Inhibition of topoisomerase I kinase activity by R-3. The kinase reactions were performed with the wild type (WT) and the mutant Y723F enzymes as described (“Materials and Methods”), with increasing concentration of the drug: 1, 5, 10, 20, and 50 μM (Lanes 1–5 or 8–12, respectively). Cil refers to the control reaction with DMSO alone in place of R-3. The labeled SF2/ASF was analyzed on a 12% SDS-polyacrylamide gel and revealed either by autoradiography (A) or quantitated by phosphor imaging (B). The position of SF2/ASF was localized by staining the gel with coomassie blue.
more potent at promoting the DNA cleavage than to inhibit the kinase reactions of topoisomerase I. Inhibition of top I/kinase activity was also observed with camptothecin (10) but required the presence of DNA. No inhibition was observed with camptothecin in the absence of DNA even when using a concentration as high as 500 μM. In sharp contrast, the strong inhibitory effect of R-3 does not require DNA, implying that its action on the kinase activity is independent of topoisomerase I-DNA complex covalent linkages. To confirm this, we mutated the tyrosine at position 723, which serves to form a covalent bond with the 3′ phosphate of the cleaved strand during the relaxation reaction (15), and examined the ability of R-3 to inhibit Topo I/kinase. The replacement of the tyrosine residue of topoisomerase I at position 723 with a phenylalanine residue prevents the covalent reaction with DNA but not the phosphorylation of SF2/ASF (16). As expected, the Y→F substitution affects neither the kinase activity of topoisomerase I, nor the sensitivity of the enzyme to the indolocarbazole. R-3 inhibits the phosphorylation of ASF/SF2 by the wild type enzyme and the mutant Y723F with the same efficiency (Fig. 4). This confirms that the two effects of R-3 at the DNA cleavage site and the protein kinase site proceed independently.

The kinase activity of topoisomerase I analyzed here with the baculovirus enzyme can also be detected with a bacterially expressed enzyme providing that the full-length protein is being used. The N-terminal domain of topoisomerase I is a major element of the interaction with the splicing factor (17). Recent studies have revealed that both the COOH- and amino-terminal domains of topoisomerase I contribute to the kinase activity (16, 17).

It is important to mention that R-3 interferes specifically with the kinase activity of topoisomerase I and not with other kinases. As previously indicated, the compound exerts very little effects on PKC. In addition, the compound does not affect the phosphorylation of SR proteins by the cdc2 kinase in vitro (data not shown). Cellular studies with the cdc2-mutant FT210 cell line have confirmed that unlike rebeccamycin, indolocarbazoles related to R-3 do not inhibit the phosphorylation of histones via a cdc2 kinase-dependent pathway and hence do not inhibit, or to a very limited extent, the condensation of chromosomes. Thus far, as we are aware, topoisomerase I is the only kinase inhibited by R-3.

Conclusion. The results presented above confirm that the indolocarbazole compound R-3 is a specific inhibitor of topoisomerase I, capable of promoting the formation of DNA-enzyme covalent complexes, and reveal that this intercalating compound can also behave as a nonspecific inhibitor preventing the enzyme from binding to DNA. The action of the drug is comparable with that of camptothecins, and it is possible that camptothecins affect transcriptional repression by a mechanism independent of transcriptional repression. This suggests that topoisomerase I can regulate transcription by a mechanism independent of transcriptional repression.

The major discovery of the present study is that, unlike camptothecin, R-3 is capable of inhibiting specifically the SR protein kinase activity of topoisomerase I in the absence of DNA. The experiments with the Y723F mutant enzyme indicate that the action at the topoisomerase I kinase site is independent of the DNA cleavage/ligation activity. These observations distinguish indolocarbazoles from the camptothecins and raise implication for future design of topoisomerase I poisons. Topoisomerase I must be viewed as a dual enzyme with a DNA cleavage site juxtaposed to a functionally independent kinase site. This unusual DNA-binding protein also possesses a nuclear binding site (18) and interacts with RNA polymerase I, TBP, p53, and HMG proteins (19–21). In the future, it may prove beneficial to design drugs targeting selectively the kinase site without interfering with the DNA relaxation activity of the enzyme. Such compounds might turn out to be potent topoisomerase inhibitors endowed with interesting and possibly selective anti-tumor properties with a different pharmacological profile than the camptothecins. The results reported here encourage us to believe that the indolocarbazole approach has the potential to yield important developments in the search for new and better anticancer drugs. Ongoing drug design will seek to explore this hypothesis.

Acknowledgments

We thank D. Sevère for technical assistance and H. Goulaut for critical reading of the manuscript.

References


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