The Dihydropyridine Dexniguldipine Hydrochloride Inhibits Cleavage and Religation Reactions of Eukaryotic DNA Topoisomerase I†

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ABSTRACT: Dexniguldipine hydrochloride (B859-35, a dihydropyridine with antitumor and multidrug resistance-reverting activity) inhibits both the DNA cleavage and religation reactions of purified human DNA topoisomerase I at concentrations >1 μM, whereas at concentrations <1 μM it inhibits selectively the religation step and stabilizes the covalent topoisomerase I−DNA intermediate in a similar fashion as camptothecin. Inhibition of religation by camptothecin can be overcome by increasing the concentration of the DNA substrate in the religation reaction, indicating a competitive type of inhibition. In contrast, dexniguldipine hydrochloride decreases rate constants of topoisomerase I-mediated DNA religation independently of the concentration of the DNA substrate, suggesting a noncompetitive mechanism of inhibition, which is different from that of camptothecin.

Dexniguldipine hydrochloride (for structure see Figure 1) is the (−)-enantiomer of the potent L-type Ca^{2+} channel blocker niguldipine (Boer et al., 1989). It exhibits a 40-fold lower affinity for L-type channels than niguldipine. It has only minimal effects on blood pressure (Sanders et al., 1988) but a similar or better potency as most L-type channel blockers for inhibiting P-glycoprotein, the product of the multidrug resistance (*mdr*1) gene (Hofmann et al., 1995; Höllt et al., 1992; Reymann et al., 1993). It reverts multidrug resistance in a number of tumor cell lines (Hill & Hosking, 1994; Hofmann et al., 1991, 1992; Reymann et al., 1993) and has successfully been used as a chemosensitizer in the therapy of chronic myelogenous leukemia (Nüssler et al., 1994; Scheulen et al., 1993) and myeloma (Thaler et al., 1993). Moreover, dexniguldipine hydrochloride exhibits a remarkable antitumor activity of its own, which is directed against a variety of experimental tumors, most notably of neuroendocrine origin (Abdallah & Gietzen, 1990; Gietzen et al., 1990; Huebel et al., 1990; Schuller, 1992; Schuller et al., 1990, 1991, Schuller et al., 1994). It is assumed that inhibition of the Ca^{2+}/calmodulin/ protein kinase C-mediated stimulation of neuroendocrine cell proliferation contributes to this effect, because dexniguldipine hydrochloride has Ca^{2+}/calmodulin antagonistic (Gietzen et al., 1990) and protein kinase C inhibitory properties (Uberall et al., 1991). Dexniguldipine-HCL was recently also identified as an inhibitor of DNA replication in T-lymphoblastoid cells (Gekeler et al., 1994). Since certain protein kinase inhibitors can also inhibit type I DNA topoisomerases (Aflalo et al., 1994) and one of the results of topoisomerase I inhibition is replication fork arrest and breakage (Avemann et al., 1988; Cortes et al., 1993; Hsiang et al., 1989; Tsao et al., 1993), the latter observations led us to hypothesize that in effect inhibition of topoisomerase I may contribute to the antitumor potency of dexniguldipine hydrochloride. The present work investigates the interaction of dexniguldipine hydrochloride with the function of purified recombinant human DNA topoisomerase I.

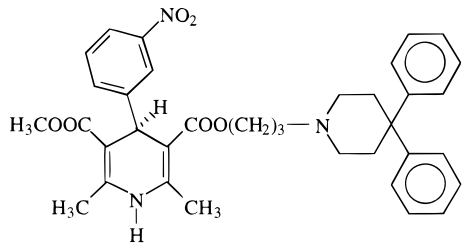

FIGURE 1: Structure of dexniguldipine hydrochloride (B8509-035), MW 609.73.

EXPERIMENTAL PROCEDURES

Materials

The mouse monoclonal antibody to human topoisomerase I was a gift of Dr. Igor Bronstein, Engelhard Institute, Moscow, Russia (Bronstein et al., 1992; Kudinov et al., 1992). Peroxidase-labeled goat anti-mouse IgG was purchased from Jackson Inc. Immunochemiluminescence detection reagents (ECL) were obtained from Amersham, Little Chelfore, U.K. Dexniguldipine hydrochloride (3-[(4,4-diphenyl-1-piperidinyl)propyl] 5-methyl (4*R*)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride); for structure see Figure 1) was obtained from Byk Gulden, Konstanz, Germany. It was dissolved at a final concentration of 10 mM in dimethyl sulfoxide and stored in glass vials at −20 °C in the dark. In order to avoid adsorption of the compound to plastic surfaces, all experiments involving dexniguldipine hydrochloride were carried out using glass vessels and glass capillary pipettes. For chromatography we used resins, columns, and an FPLC system of Pharmacia BioTech Inc., Uppsala, Sweden. Camptothecin and poly(ethylenimine) were obtained from

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Sigma, München, Germany. PMSF[1] was from Fluka, Neu-Ulm, Germany. All other reagents were of the highest degree of purity commercially available.

Methods

Enzyme Preparation. Human DNA-topoisomerase I was overexpressed in *Saccharomyces cerevisiae* as described in Bjornsti et al. (1989) and purified as described in Boege (1996), Boege et al. (1996), and Knudsen et al. (1996).

Measurement of topoisomerase I-mediated relaxation and nicking of pBR322 plasmid DNA and of noncovalent DNA binding followed published procedures (Boege, 1996; Boege et al., 1996). Briefly, 250 ng of pBR322 plasmid DNA was incubated at 37 °C for 30 min with 200 units of human topoisomerase I in the presence of camptothecin or dexniguldipine hydrochloride or both at the concentrations indicated. Controls were without drugs or without enzyme. Reactions were terminated by addition of 1% SDS, followed by digestion with proteinase K and agarose gel electrophoresis in the presence of ethidium bromide. For the study of noncovalent DNA binding of topoisomerase I by DNA mobility shift, SDS denaturation and proteinase K digestion were omitted. Fluorescence of ethidium bromide in the gels (excitation 302 nm, emission >600 nm) was documented by Polaroid photography.

Immuno-dot-blotting of DNA-linked topoisomerase I was carried out as described previously (Boege, 1996; Boege et al., 1996). Briefly, 400 units of human topoisomerase I was incubated with 3 μg of calf thymus DNA in the presence of 2 mM $MgCl_2$ in a final volume of 500 μL. Incubation at 37 °C for 30 min with and without drugs was terminated by addition of 0.2% SDS. Samples were passed through nitrocellulose filters using a 96-well vacuum dot-blotter (Schleicher & Schüll, Germany), followed by two washes (500 μL each) with 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl. Nitrocellulose filters were irradiated (254 nm, 2 min), dried at 20 °C for 12 h, and finally stained with mouse monoclonal antibody to human topoisomerase, peroxidase-linked goat anti-mouse IgG, and the ECL system.

Dissociation of Topoisomerase I−DNA Complexes. Human topoisomerase I (2000 units) was incubated with 12 μg of calf thymus DNA in the presence of 1 μM camptothecin or 3 nM dexniguldipine hydrochloride at 37 °C in a final volume of 100 μL. After 30 min, 4 mL of reaction buffer prewarmed to 37 °C was added. An aliquot (1 mL) of the diluted sample was immediately stopped by addition of 0.2% SDS. The rest of the diluted sample was further incubated at 37 °C and three more aliquots were stopped after 1, 5, and 10 min. Covalent topoisomerase I−DNA complexes in the aliquots were detected by immuno-dot-blotting and quantified by densitometry of the X-ray films using a video densitometer (Froebel, Wasserburg, Germany). Dissociation rate constants were calculated by computer-aided nonlinear regression analysis of these measurements.

Separate analysis of drug effects on topoisomerase I-mediated DNA cleavage and religation was carried out by a modification of the suicide substrate approach (Svejstrup et al., 1991) described in detail elsewhere (Boege et al., 1996) and schematically demonstrated in Figure 2. Briefly, a

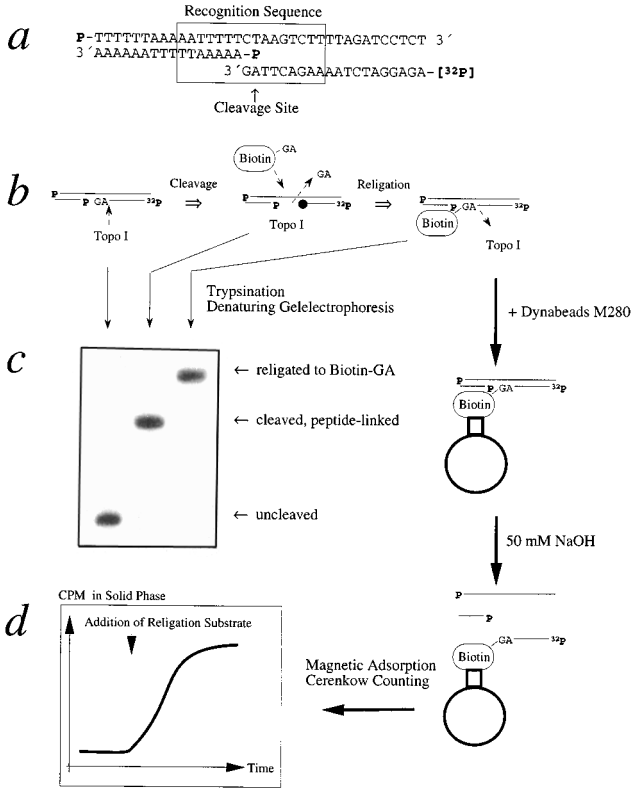

FIGURE 2: Schematic outline of the separate measurement of topoisomerase I-mediated DNA cleavage and religation: (a) Composition and labeling of the suicide substrate. Blocking of 5′-ends by nonradioactive phosphorylation is indicated by **P**; radioactive phosphorylation of 5′-ends is indicated by **[^{32}P]**. **(**b) Schematic drawing of suicidal cleavage by topoisomerase I and religation to 3′-biotinylated GA dinucleotide. (c) Electrophoresis of noncleaved (left), cleaved (middle), and religated substrate (right) in a 14% polyacrylamide gel under denaturing conditions. DNA bands in the gel were visualized by autoradiography. (d) Strategy for measuring religation by Cerenkow counting after specific adsorption of religated subtrates to magnetic beads coated with streptavidin.

synthetic partially double-stranded oligonucleotide containing a strong topoisomerase I cleavage site (Figure 2a) was suicidally cleaved by recombinant human DNA topoisomerase I. Subsequently, the religation reaction was initiated by addition of a 3′-biotinylated dinucleotide in the presence of 500 mM NaCl, preventing recleavage of the religated product (Figure 2b). Uncleaved, cleaved, and religated oligonucleotides were separated by denaturing polyacrylamide gel electrophoresis after trypsinization of the samples (Figure 2c). For a quantitative determination of the religation reaction, we used a modification of the solid-phase approach previously described (Alsner et al., 1991). The religation reaction was terminated by addition of NaCl to a final concentration of 1 M. After 2-fold dilution with binding buffer (10 mM Tris-HCl, 0.1 mM EDTA, and 1 M NaCl, pH 7.4) samples were incubated for 30 min at 37 °C with Dynabeads M-280 (Dynal, Oslo, Norway) containing 30 μg of covalently linked streptavidin. Subsequently, the beads were washed at room temperature, first with 3 × 100 μL of binding buffer and then with 2 × 100 μL of 50 mM NaOH. Radioactivity bound to the beads (e.g., the fraction of the cleaved oligonucleotide strand that had been religated to the biotinylated dinucleotide) was finally measured by Cerenkow counting (Figure 2d).

[1] Abbreviations: BisTrisPropane, 1,3-bis[tris(hydroxymethyl)methyl]-amino]propane; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

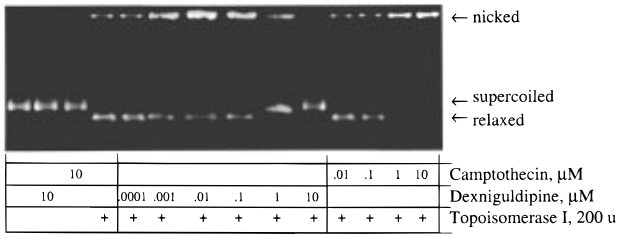

FIGURE 3: Drug-stimulated nicking of plasmid DNA by topoisomerase I: pBR322 plasmid DNA was reacted with purified recombinant human topoisomerase I in the presence of various concentrations of camptothecin (right section) or dexniguldipine hydrochloride (middle section). Controls (left section) were DNA alone, DNA incubated with maximal doses of dexniguldipine hydrochloride or camptothecin in the absence of enzyme, and DNA reacted with topoisomerase in the absence of drug.

Statistics. Where statistical analysis could not be applied to the data, representative examples of at least three independent experiments with similar outcomes are shown (Figures 3−8a). Quantitative determinations (Figures 8b and 9) were repeated three times by independent experiments. In each experiment, mean values of triplicate determinations were obtained for each data point. Mean values of three experiments are shown and standard errors of the mean are indicated by error bars.

RESULTS

Reversible Stabilization of the Covalent Topoisomerase I−DNA Intermediate. For the screening of drug effects directed at topoisomerase I, we used a strategy based on the alteration of the electrophoretic mobility of pBR322 plasmid DNA by the combined action of topoisomerase I and inhibiting drugs (Hecht et al., 1992). As demonstrated in Figure 3, the mobility of the naturally supercoiled closed circular double-stranded plasmid DNA increases, upon topoisomerase I-mediated relaxation, when electrophoresed with ethidium bromide (Figure 3, left section). In the presence of camptothecin, which binds to the covalent DNA−topoisomerase I intermediate and inhibits the religation half-reaction (Hertzberg et al., 1989), topoisomerase I introduces nicks into one of the DNA strands. The resulting open-circular plasmid migrates much more slowly than the closed circular plasmid in both its supercoiled and relaxed forms. Formation of the open circular plasmid form is clearly dependent on the presence of enzyme and camptothecin and is directly correlated to the concentration of the drug. At 1 μM and above the plasmid becomes completely converted to the open-circular form (Figure 3, right). Dexniguldipine hydrochloride, when assessed in this way (Figure 3, middle section), has a similar potency as camptothecin for stimulating topoisomerase I-mediated plasmid nicking but the dose−response relationship is bell-shaped with maximal formation of open circular DNA being observed at 10−100 nM concentrations.

In order to confirm that the generation of nicked plasmid DNA by the combined action of topoisomerase I and dexniguldipine hydrochloride shown in Figure 3 is indeed due to the stabilization of the covalent topoisomerase I−DNA catalytic intermediate, we made use of the selective binding of covalent topoisomerase I−DNA complexes to nitrocellulose filters in the presence of SDS (Hecht et al., 1992). As shown in Figure 4a (right), camptothecin increases filter binding of covalent DNA−topoisomerase I complexes in a

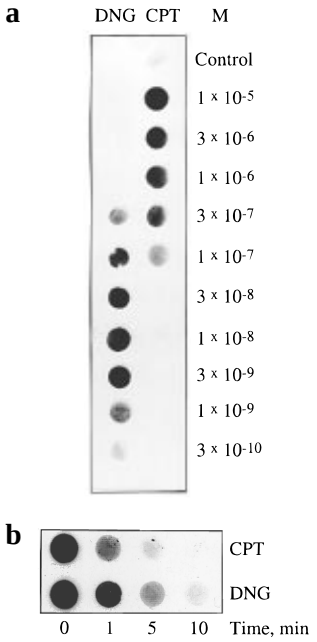

FIGURE 4: Detection of covalent topoisomerase I−DNA complexes by filter binding: (a) Purified recombinant human topoisomerase I was incubated with calf thymus DNA and various concentrations of camptothecin (right column) or dexniguldipine hydrochloride (left column). Controls were without drugs (line 1). Covalent topoisomerase I−DNA complexes were captured on nitrocellulose filters and visualized by immunostaining. (b) Reversibility of cleavage complexes: Complexes of calf thymus DNA and topoisomerase I were formed in the presence of 1 μM camptothecin (line 1) or 3 nM dexniguldipine hydrochloride (line 2) for 30 min at 37 °C. Subsequently, samples were diluted 40-fold and further incubated at 37 °C for the times indicated. Topoisomerase I−DNA complexes were determined by filter binding.

manner reciprocal to the concentration of the drug. In contrast, dexniguldipine hydrochloride enhances DNA linkages of the enzyme at concentrations between 3 and 100 nM but fails to do so at higher concentrations (Figure 4a, left). This is in good agreement with the data shown in Figure 3, confirming that both camptothecin and dexniguldipine hydrochloride are effectively stabilizing a covalent topoisomerase I−DNA complex.

Reversibility of the cleavage complexes stabilized by camptothecin or dexniguldipine hydrochloride was tested by diluting the drugs to ineffective concentrations subsequent to the cleavage reaction. Diluted samples were then incubated at 37 °C for various times and residual cleavage complexes were detected by filter binding and immunostaining of topoisomerase I (Figure 4b). A quantitative assessment (video densitometry of the dots) of three experiments identical to the one shown in Figure 4b and nonlinear regression of the data showed that dissociation kinetics of cleavage complexes stabilized by either drug were first-order. However, cleavage complexes stabilized by dexniguldipine hydrochloride dissociated at least 5 times more slowly (observed $k_{off} = 0.23 \pm 0.05$ min^{-1}) than those stabilized by camptothecin (observed $k_{off} = 1.38 \pm 0.4$ min^{-1}).

Inhibition of DNA Cleavage. At concentrations of 1 μM and higher, dexniguldipine hydrochloride becomes less potent in stabilizing the covalent DNA−topoisomerase I complex than at lower concentrations (Figures 3 and 4). On the contrary, at 1 μM concentrations and higher it inhibits topoisomerase I-mediated DNA relaxation (Figure 3) and at concentrations of 3 μM and higher it diminishes DNA

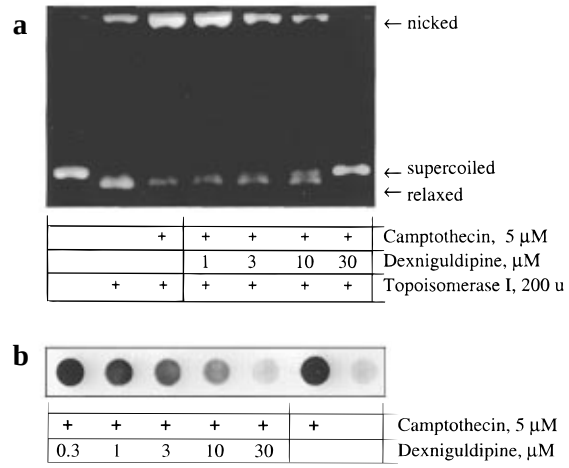

FIGURE 5: Camptothecin-antagonizing effect of dexniguldipine hydrochloride: (a) Inhibition of plasmid nicking: pBR322 plasmid DNA was reacted with purified recombinant human topoisomerase I in the presence of 5 μM camptothecin after preincubation with various concentrations of dexniguldipine hydrochloride (right section). Controls (left section) were DNA alone, DNA reacted with topoisomerase in the absence of drug, and DNA reacted with enzyme in the presence of 5 μM camptothecin but in the absence of dexniguldipine. Nicked, relaxed, and supercoiled plasmid forms were separated by agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide. (b) Inhibition of filter binding. Topoisomerase I was incubated with calf thymus DNA in the presence of camptothecin (5 μM) after preincubation with various concentrations of dexniguldipine hydrochloride (left section). Controls (right section) were without dexniguldipine hydrochloride or without both drugs. Upon lysis with SDS (0.2%), covalent topoisomerase I−DNA adducts were captured on nitrocellulose filters and visualized by immuno-dot-blotting.

cleavage stimulated by camptothecin (Figure 5a). The camptothecin-antagonizing effect of dexniguldipine hydrochloride can be seen even more clearly in Figure 5b, which shows that at concentrations of 10 μM and higher dexniguldipine hydrochloride antagonizes the formation of covalent topoisomerase I−DNA complexes induced by camptothecin in a dose-dependent manner. The data summarized in Figure 5 indicate that the drug at concentrations higher than 1 μM actually prevents DNA linkage of the enzyme, suggesting that at these concentrations it does affect not only DNA religation but also the DNA cleavage reaction of topoisomerase I. In order to confirm this notion, we studied drug effects on suicidal cleavage of a synthetic oligonucleotide substrate by topoisomerase I [for details see Figure 2 and Boege et al. (1996)]. Figure 6 summarizes the effects of camptothecin and dexniguldipine hydrochloride on the cleavage reaction. Camptothecin has no effect on suicidal cleavage of the oligonucleotide substrate by topoisomerase I even at concentrations as high as 10 μM. In contrast, dexniguldipine hydrochloride inhibits the suicidal cleavage reaction of topoisomerase I in a concentration-dependent manner, starting at 1 μM and being complete at 10 μM concentrations. Inhibition of the DNA cleavage reaction by dexniguldipine hydrochloride could be caused either by an inhibition of the DNA binding of topoisomerase I or by an inhibition of the subsequent cleavage reaction itself. In order to distinguish between these possibilities, we studied the effects of dexniguldipine hydrochloride on the noncovalent binding of topoisomerase I to pBR322 plasmid DNA by a DNA mobility shift assay previously established (Boege et al., 1996). As shown in Figure 7, topoisomerase I forms a complex with pBR322 DNA, which is immobile in native

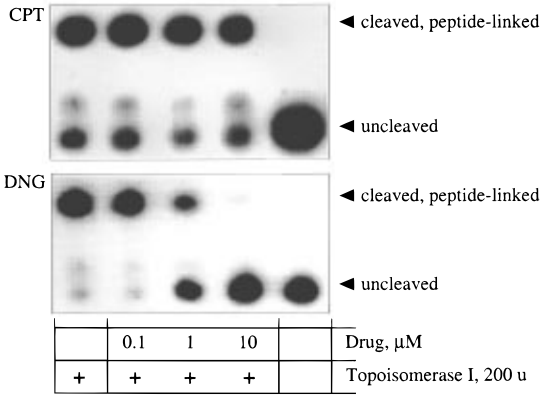

FIGURE 6: Inhibition of topoisomerase I-mediated DNA cleavage of an oligonucleotide substrate: Suicide substrate (cf. Figure 2a) was preincubated with the indicated concentrations of camptothecin (CPT) or dexniguldipine hydrochloride (DNG) for 10 min at 30 °C (middle section) and subsequently cleaved with purified human topoisomerase I. Controls were without enzyme (right section) or with enzyme but without drug (left section). Cleaved and uncleaved substrates were separated and visualized as explained in Figure 2c.

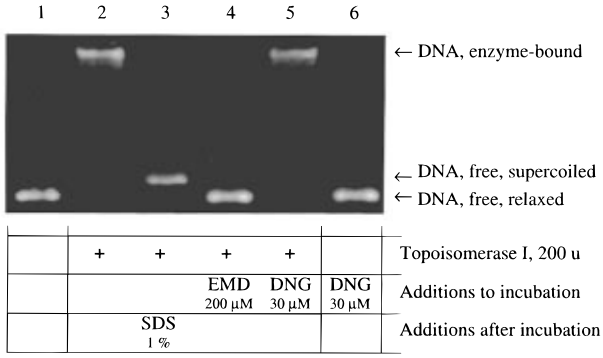

FIGURE 7: Effect of dexniguldipine hydrochloride on noncovalent DNA binding of topoisomerase I: pBR322 plasmid DNA and purified recombinant human topoisomerase I were incubated at 37 °C for 1 min. Samples were subsequently denatured with SDS (lane 3) or applied without further treatment (all other lanes) to agarose gel electrophoresis in the presence of ethidium bromide. Results obtained after preincubation of the enzyme with 200 μM EMD 50 689 or 30 μM dexniguldipine-HCL before addition of the DNA are shown in lanes 4 and 5, respectively. Lane 1 contains DNA alone. Lane 6 shows DNA preincubated with 30 μM dexniguldipine hydrochloride in the absence of topoisomerase I.

agarose electrophoresis, so that the plasmid DNA is retained in the application slot by the enzyme (compare Figure 7, lanes 1 and 2). The electrophoretically immobile topoisomerase I−DNA complex is noncovalent because denaturing of the enzyme with SDS prior to the electrophoresis releases the DNA from the immobile complex (Figure 7, lane 3). Formation of the electrophoretically immobile noncovalent topoisomerase I−DNA complex can be prevented by pretreating the enzyme with the synthetic flavonoid EMD 50 689 (Figure 7, lane 4), which has been shown to inhibit DNA binding of the enzyme (Boege et al., 1996). In contrast, pretreatment even with high concentrations (30 μM) of dexniguldipine hydrochloride does not disturb topoisomerase I-mediated retention of the plasmid in the application slot (Figure 7, lane 5). Since at these concentrations dexniguldipine hydrochloride *per se* does not interfere with electrophoretic mobility of the plasmid DNA (Figure 7, lane 6), the result shown in lane 5 of Figure 7 can only be interpreted in such a way that dexniguldipine hydrochloride, although inhibiting DNA cleavage by topoisomerase I, does not prevent noncovalent DNA binding of topoisomerase I

as does EMD 50 689. Thus, the inhibitory effect of dexniguldipine hydrochloride on topoisomerase I-mediated DNA cleavage must be directed at the cleavage reaction itself. From these observations it can also be concluded that dexniguldipine hydrochloride does not act by intercalating into the DNA. This is supported by the observation that dexniguldipine hydrochloride alone at concentrations up to 30 μM does not alter the electrophoretic mobility of pBR322 plasmid DNA and does not compete intercalation of ethidium bromide (Figure 7, lane 6), as would be expected from an intercalating drug.

Inhibition of DNA Religation. Religation of the [^{32}P]-labeled oligonucleotide to a 3′-biotinylated GA dinucleotide after suicidal cleavage by topoisomerase I and the effects of camptothecin or dexniguldipine hydrochloride on the religation reaction are demonstrated in Figures 8 and 9. Both substances inhibit topoisomerase I-catalyzed religation of the cleaved oligonucleotide suicide substrate, however, in different ways. Camptothecin blocks religation completely, whereas dexniguldipine hydrochloride slows down the reaction considerably but does not prevent the enzyme from completing religation eventually (Figure 8a). A quantitative assessment of the kinetics (Figure 8b) shows that in the absence of inhibitors the reaction is pseudo-first-order with an apparent rate constant of 0.14 ± 0.02 min^{-1} (mean ± SEM, n = 3). In the presence of 30 μM dexniguldipine hydrochloride the rate constant of religation decreases 10-fold to 0.012 ± 0.002 min^{-1} (mean ± SEM, n = 3), whereas with 30 μM camptothecin it becomes immeasurably small ($<10^{-6}$ min^{-1}). Measurements over longer time periods (not shown) suggest that inhibition by camptothecin under these conditions actually is more or less irreversible, limited only by the decomposition of the drug. One possible explanation of these differences would be that dexniguldipine hydrochloride decreases the catalytic religation activity of topoisomerase I, whereas camptothecin blocks the access of the religation substrate to the active site of the suicide complex. In order to test this hypothesis we studied religation with various concentrations of camptothecin and/or religation substrate. These experiments (Figure 9) show a steep inhibitory dose−response curve for camptothecin that becomes clearly shifted horizontally to higher concentrations upon increasing the religation substrate concentration in the assay (Figure 9a). Conversely, the concentration of religation substrate required for the religation reaction to take place is increased in the presence of camptothecin (Figure 9c). An exact determination of substrate binding and inhibition constants is not possible because Michaelis−Menthen assumptions do not apply due to the irreversibility of the religation reaction. However, the observations summarized in Figure 9a,c indicate that camptothecin and the religation substrate compete for the same or a closely related site of the topoisomerase I−DNA complex, which is involved in the religation reaction. This observation is in agreement with recent findings obtained with an alkylating camptothecin derivative, which show that the drug interacts with the enzyme−DNA interface and makes contact with a nucleotide on the 5′ end of the cleavage site (Pommier et al., 1995), approximately in the same position where the GA dinucleotide needs to associate with the suicide complex in order to be religated. Dexniguldipine hydrochloride exhibited a more shallow dose−response curve for inhibition of the religation reaction than camptothecin. Moreover, the curve

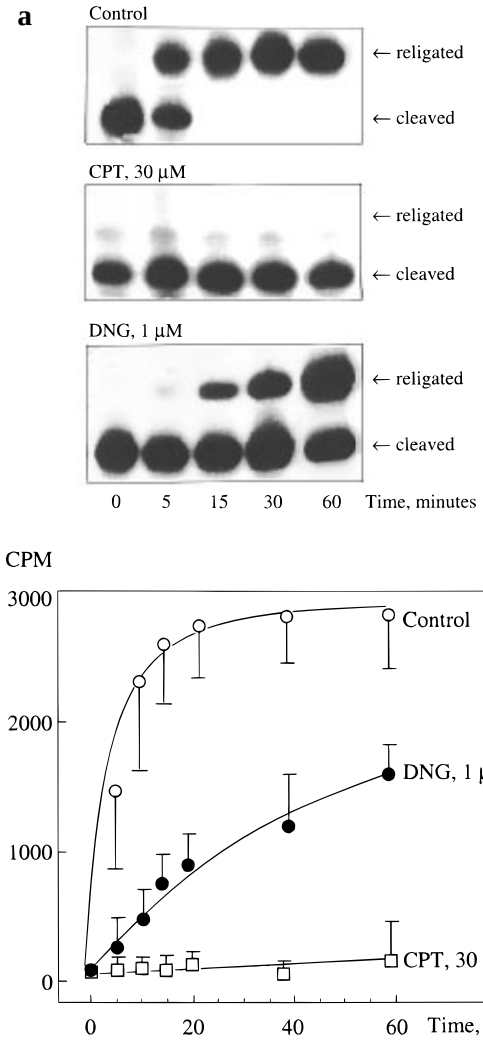

FIGURE 8: Religation kinetics of cleaved suicide oligonucleotide substrate to a biotinylated dinucleotide. (a) Oligonucleotide substrate (cf. Figure 2) was completely cleaved with purified recombinant human topoisomerase I. Cleavage complexes were incubated without drugs (top) or with a final concentration of 30 μM camptothecin (middle) or 1 μM dexniguldipine hydrochloride (bottom) for 10 min at 37 °C. Religation reaction was subsequently started by addition of 3′-biotinylated GA dinucleotide (cf. Figure 2b) and stopped after the times indicated. Cleaved and religated substrates were separated and visualized as explained in Figure 2c. (b) The amount of religated substrate was measured by adsorption to streptavidin coated magnetic particles and Cerenkow counting (cf. Figure 2d). Data points represent mean values of three independent experiments. The standard errors of the means are indicated by error bars. Solid lines were drawn by computer-aided nonlinear regression of the data. Apparent rate constants of pseudo-first-order religation kinetics (mean ± SEM, n = 3) were 0.14 ± 0.02 min^{-1} for the control, 0.012 ± 0.002 min^{-1} in the presence of 1 μM dexniguldipine hydrochloride, and $<10^{-6}$ min^{-1} in the presence of 30 μM camptothecin.

of dexniguldipine hydrochloride was not shifted upon increasing the concentration of the religation substrate by 10-fold (Figure 9b), as was the case with camptothecin (Figure 9a). On the contrary, at concentrations of the religation substrate ranging from 1 to 300 μM, dexniguldipine inhibited religation by a similar fraction of about 70% (Figure 9c). These data are in good agreement with the kinetic data shown in Figure 8b and suggest a noncompetitive type of inhibition for dexniguldipine hydrochloride.

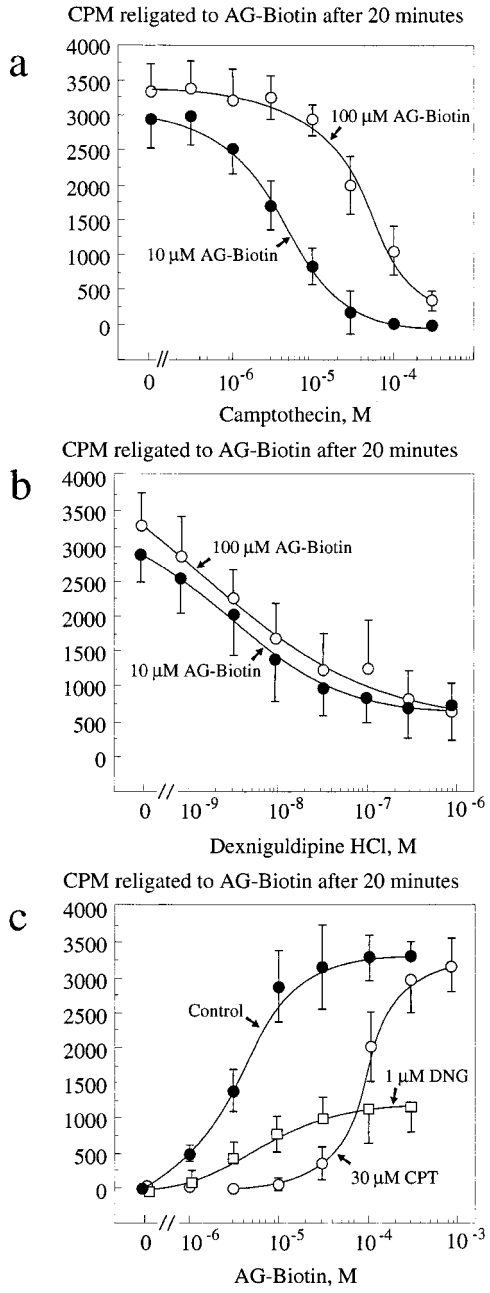

FIGURE 9: Dose-dependent effects of camptothecin and dexniguldipine hydrochloride on religation of cleaved suicide oligonucleotide substrate to a biotinylated dinucleotide. Cleavage complexes of topoisomerase I and oligonucleotide substrate (cf. Figure 2) were preincubated with various concentrations of camptothecin (a) or dexniguldipine (b) or without drugs for 10 min at 37 °C. Subsequently, religation to 10 (●) or 100 μM (○) of 3′-biotinylated GA dinucleotide for 20 min at 37 °C was measured by adsorption to avidin-coated magnetic beads and Cerenkow counting. (c) Cleaved enzyme−substrate complexes were preincubated with 30 μM camptothecin (○), 1 μM dexniguldipine hydrochloride (□), or without drugs (●) for 10 min at 37 °C. Religation at 37 °C was subsequently started by addition of 3′-biotinylated GA dinucleotide to final concentrations between 1 μM and 1 mM. The control was kept without religation substrate. The amount of religated substrate was determined after 20 min by adsorption to streptavidin-coated magnetic particles and Cerenkow counting (cf. Figure 2d). Nonspecific absorption of complexes to the beads in the absence of biotinylated dinucleotide (less than 100 cpm under the conditions given) was determined in each experiment and subtracted from the data. Data points represent mean values of three independent experiments. The standard errors of the means are indicated by error bars. Solid lines were drawn by computer-aided nonlinear regression of the data.

DISCUSSION

In this report we show that the dihydropyridine dexniguldipine hydrochloride inhibits both DNA cleavage and religation reactions catalyzed by eukaryotic topoisomerase I. With respect to the DNA religation reaction, its mode of inhibition is clearly different from that of camptothecin: It modulates the catalytic activity of the enzyme rather than competing with the binding of the DNA substrate to be religated (see Figures 8 and 9). It is interesting to note that dexniguldipine hydrochloride is also known to inhibit the binding of vinblastine to P-glycoprotein in a noncompetitive manner (Malkhandi et al., 1994). The inhibitory effect of dexniguldipine hydrochloride on topoisomerase I-catalyzed DNA cleavage could be due either to inhibition of the noncovalent binding of the enzyme to the DNA or to attenuation of the subsequent DNA cleavage reaction itself. As dexniguldipine hydrochloride does not block the DNA mobility shift observed in nondenaturing agarose gel electrophoresis upon binding of purified topoisomerase I to pBR322 plasmid DNA (Figure 7) and does not intercalate into the DNA under these conditions, it seems more likely that it inhibits the cleavage reaction itself and not the binding of the enzyme to the DNA. This notion is supported by previous studies also suggesting that dexniguldipine hydrochloride does not bind to DNA but to proteins (Hofmann et al., 1995; Uberall et al., 1991).

Dexniguldipine hydrochloride apparently is a catalytic inhibitor of eukaryotic topoisomerase I that attenuates both DNA cleavage and religation reactions by interacting with a site of the enzyme not identical to the DNA binding site. A similar mechanism of action has been reported of chebulagic acid (Hecht et al., 1992). The religation reaction appears to be more sensitive to the drug than the cleavage reaction. The maximally effective drug concentrations for the two inhibitory effects are at least 1 order of magnitude apart (>1 μM for cleavage inhibition and around 30 nM for religation inhibition). Thus, in catalytic assays where the drug simultaneously inhibits DNA cleavage and subsequent religation reactions, a bell-shaped dose−response relationship for the latter effect is obtained (Figures 3 and 4), because with increasing concentrations of the drug inhibition of the DNA cleavage reaction prevents formation of the covalent topoisomerase I−DNA catalytic intermediate. Taken together, these observation could point to an interaction of dexniguldipine hydrochloride with a high-affinity site of topoisomerase I responsible for inhibition of the DNA religation reaction and a low-affinity site responsible for inhibition of DNA cleavage, although this was not directly investigated.

On the basis of our data we cannot decide whether the inhibition of topoisomerase I by dexniguldipine hydrochloride actually contributes to the cytotoxicity of the drug, which is directed against a variety of experimental tumors (Abdallah & Gietzen, 1990; Gietzen et al., 1990; Huebel et al., 1990; Schuller, 1992; Schuller et al., 1990; 1991, 1994). We observed an immunoband depletion of DNA topoisomerase I upon treatment of human HL-60 leukemic cells with high doses of dexniguldipine hydrochloride (unpublished observation), indicating that the drug actually targets topoisomerase I in cells. However, dexniguldipine hydrochloride also has effects on several other cellular targets including the Ca^{2+}/calmodulin system (Gietzen et al., 1990) and protein kinase

C (Uberall et al., 1991). Recently, it has been shown that the drug abrogates DNA replication in T-lymphoblastoid cells (Gekeler et al., 1994). It could be imagined that the latter effect is mediated by inhibition of DNA topoisomerases I. But whether this actually is the sole mediator of the anticancer activity of dexniguldipine hydrochloride remains still to be elucidated.

The observation that a dihydropyridine has anti-topoisomerase I activity of a type different from camptothecin opens up some perspectives for drug development. In the past many thousands of dihydropyridine derivatives have been synthesized and at least 20 of those have been tested clinically . Thus, it should be easily possible to select a compound from that chemical library that has a more specific effect on eukaryotic topoisomerase I than dexniguldipine hydrochloride and might be suitable as an anticancer drug.

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