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# Inhibition of Eukaryotic DNA Topoisomerase I and II Activities by Indoloquinolinedione Derivatives

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**SUMMARY**

With the aim of obtaining new inhibitors of topoisomerases, we have evaluated various heterocyclic quinone derivatives for their ability to induce topoisomerase I (Topo I)- or Topo II-associated DNA breaks, using P388 cell nuclear extract. Several compounds belonging to the indolo[3,2-*c*]quinoline-1,4-dione series have been shown to possess DNA-cleavage activity. Further analysis using purified Topo I and II preparations has indicated that the members of the series stimulate cleavable complex formation of both Topo I and II. 3-Methoxy-11*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-*c*]quinoline-1,4-dione (AzaIQD), one of the most active members of the series, stimulates cleavable complex formation and inhibits the catalytic activities of both eukaryotic Topo I and II, with, however, less potency than camptothecin and etoposide. Topo I cleavage site patterns for AzaIQD and camptothecin were found to be nearly identical, with, however, some differences in cleavage site intensities. Use of filter binding assays also indicates that AzaIQD is at least 10 times more potent against Topo I than against Topo II. Structure-activity relationships of indoloquinolinedione derivatives have been established and have shown that Topo I and II inhibitions are strongly linked, with a dose-selective preference towards Topo I. AzaIQD does not display detectable DNA-unwinding properties. AzaIQD induces a preferential cytotoxicity for the yeast strain JN2-134 bearing the human *top1* gene under the control of the *GAL1* promoter, indicating that Topo I inhibition is responsible for the yeast cytotoxicity. These data indicate that AzaIQD and its structural analogs represent a new distinct class of eukaryotic Topo I and II inhibitors.

DNA topoisomerases are nuclear enzymes that interconvert topological isomers of DNA by breaking and resealing phosphodiester bonds (for review, see Refs. 1 and 2). Two types of topoisomerases (Topo I and Topo II) have been described in mammalian cells. Topo I modifies the DNA linking number in steps of one, whereas Topo II modifies the DNA linking number in steps of two. Both enzymes are able to relax the supercoiled form of a circular closed double-stranded DNA molecule. In addition, Topo II performs specific reactions that necessitate a double-strand breakage of the DNA molecule (decatenation or unknotting) but is dependent upon the presence of an energetic cofactor such as ATP. Biological functions of topoisomerases are tightly associated with DNA metabolism, because Topo I activity is found to be enriched in the nucleolus and is associated with RNA polymerase I (3). Furthermore, Topo II is localized in the nuclear matrix of mitotic chromosomes (4). Topo I and II activities are tightly associated with highly transcribed genes (5, 6), and it has been suggested that these enzymes are involved in modulating the torsional waves generated by transcription (for a review, see Ref. 7).

A number of anticancer drugs have been described that specifically inhibit Topo II (for a review, see Ref. 8). Their antitumor activity is related to the formation of protein-concealed DNA strand breaks, resulting in the stabilization by the drug of an intermediary complex of the Topo II reaction. Two classes of drugs have been described, i.e., intercalating agents such as acridines, anthracyclines, or ellipticines and nonintercalating derivatives such as epipodophyllotoxines. Also, camptothecin, a specific nonintercalating inhibitor of Topo I, has been described and displays strong antitumor activity (9). Similar to Topo II-related antitumor drugs, camptothecin induces the formation of an intermediary complex of the Topo I reaction.

A special case is observed with actinomycin D, because this agent is able to inhibit both Topo I and II (10, 11). These properties could be related to its structure and its specific interaction with DNA (12).

In the present study, we have examined a new series of heterocyclic quinone derivatives for their ability to induce Topo I- and Topo II-associated DNA breaks. We report that 1) several indolo[3,2-*c*]quinoline-1,4-dione derivatives stimulate cleavable complex formation and inhibit both eukaryotic Topo

**ABBREVIATIONS:** Topo I, DNA topoisomerase I; Topo II, DNA topoisomerase II; AzaIQD, 3-methoxy-11*H*-pyrido[3′,4′:4,5]pyrrolo[3,2-*c*]quinoline-1,4-dione; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; mAMSA, 4′-(9-aeridinylamino)-methanesulfone-*m*-anisidide.

I and II activities, 2) structure-activity relationships of Topo I and II inhibition are closely linked, with a preferential effect of the compounds on Topo I, 3) AzaIQD does not display DNA-unwinding properties, and 4) AzaIQD induces a preferential cytotoxicity in the yeast strain JN2-134, bearing the human *top1* gene (21). These data indicate that indolo[3,2-*c*]quinoline-1,4-dione derivatives are a new series of Topo I and II inhibitors and that Topo I inhibition might be one of the features responsible for the observed *in vitro* cytotoxic properties.

## Materials and Methods

**Drugs and drug treatments.** Table 1 lists the quinone derivatives tested. Compounds were prepared as previously described (compounds **6**, **53**, and **5**, Ref. 13; compounds **9**, **12**, **8**, and **11**, Ref. 14; compounds **18**, and **22**, Ref. 15; compounds **32**, **34**, **37**, and **51**, Ref. 16; and compounds **2**, **3**, **27**, **15**, **30**, and **24**, Ref. 17. Camptothecin base (lactone form) was purchased from Sigma and etoposide from Sandoz laboratories. All drugs were dissolved in dimethyl sulfoxide at 10 mM concentrations and further diluted in water or in water/dimethyl sulfoxide to obtain the desired concentration.

**Topoisomerases preparations.** Nuclear extracts were prepared from P388 cells in exponential phase of growth. Approximately $10^8$ cells were harvested, washed three times in 1× phosphate-buffered saline (10 mM $Na_2HPO_4$, 1.7 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and then resuspended in 4 ml of nucleus buffer (5 mM $KH_2/K_2HPO_4$, pH 7.0, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF) and kept in ice for 30 min. Cellular suspensions were gently homogenized and cellular membranes were disrupted with a Dounce apparatus. Nuclear suspensions were centrifuged for 15 min at 1700 × *g*, and pellets were resuspended in 2 ml of buffer containing 1 mM $KH_2/K_2HPO_4$, pH 6.5, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 1 mM PMSF, and 10% glycerol. Nuclei were then centrifuged for 15 min at 1700 × *g*, and pellets were resuspended in 1 ml of extraction buffer (0.35 M NaCl, 5 mM $KH_2/K_2HPO_4$, pH 7.0, 2 mM $MgCl_2$, 1 mM PMSF, 10% glycerol), for 1 hr at 4°. Nuclei and DNA were pelleted at 15,000 × *g* for 15 min, and nuclear extracts were stored at −20° for 1 month.

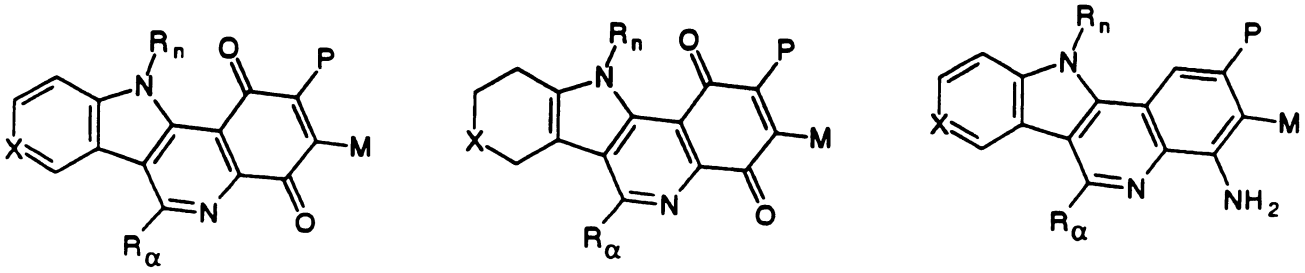
Purification of Topo I and II was performed using the nuclear extract preparation, with $10^9$ cells as starting material, by two successive columns of phosphocellulose and hydroxylapatite, as described previously (18). Human Topo I was also prepared from the cell line Calc18 (5) and calf thymus Topo I was purchased from GIBCO-BRL.

**Labeling procedure for pBR322 DNA.** $^{32}$P-end-labeled pBR322 DNA was prepared as follows. The DNA was linearized with *Eco*RI restriction endonuclease, and its termini were labeled with [$\alpha$-$^{32}$P] dATP and Klenow polymerase (Boeringher). The 3′-end-labeled DNA was then cut with *Hind*III restriction endonuclease, generating 4333-base pair and 33-base pair fragments of $^{32}$P-3′-end-labeled DNA.

TABLE 1

**Structure-activity relationships of indoloquinolinedione derivatives for Topo I and II inhibition**

Topo I and II inhibition was determined by the cleavage assay, using P388 Topo I or II (see Materials and Methods). Comparison of the inhibitory potency of the compounds was made by inspection of the autoradiogram. Compared with the enzyme control without drug, cleavage is noted, ++, +, +/−, and −, respectively, when it is stimulated at 10 μM, stimulated at 100 μM, slightly stimulated at 100 μM, and undetectable at 100 μM. Me, methyl.




| General structure | Compound number | Substituent | | | | | Inhibition | |
|---|---|---|---|---|---|---|---|---|
| | | X | M | P | $R\alpha$ | $Rn$ | Topo I | Topo II |
| A | **3** | CH | H | H | Me | H | − | − |
| | **27** | CH | H | H | Me | Me | − | − |
| | **15** | CH | H | Me | Me | H | + | ND |
| | **30** | CH | H | Me | Me | Me | + | ND |
| | **18** | CH | H | OMe | Me | H | + | ND |
| | **22** | CH | H | N⁁ | Me | H | + | +/− |
| | **24** | CH | H | N NMe | Me | H | +/− | ND |
| | **6** | CH | OMe | H | H | H | ++ | + |
| | **9** | CH | OMe | H | Me | H | + | +/− |
| | **12** | CH | N⁁ | H | Me | H | + | +/− |
| | **32 (AzaIQD)** | N | OMe | H | H | H | ++ | + |
| | **34** | N | OMe | H | Me | H | + | ND |
| | **37** | N | N NMe | H | H | H | +/− | + |
| B | **2** | $CH_2$ | H | H | Me | H | − | − |
| | **5** | $CH_2$ | OMe | H | H | H | +/− | − |
| | **8** | $CH_2$ | OMe | H | Me | H | − | − |
| | **11** | $CH_2$ | N⁁ | H | Me | H | − | +/− |
| C | **51** | N | OMe | H | H | H | + | ND |
| | **53** | CH | OMe | H | H | H | +/− | − |

[a] ND, not determined.

**Topoisomerase reactions.** Cleavage reactions performed with nuclear extract, Topo I, or Topo II were assembled using the same reaction mixture, containing 20 mM Tris·HCl, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20,000 dpm of 3′-end-labeled pBR322 DNA, and 10–20 units of enzyme preparations, in a final volume of 20 μl. ATP (1 mM) was added to all except the Topo I reactions. In the case of drug treatment, the compound was added to the reaction mixture on ice just before the addition of enzyme preparation. Reactions were incubated at 37° for 10 min and terminated by the addition of 2 μl of 2.5% SDS, 2.5 mg/ml proteinase K. Samples were further incubated for a period of 30 min at 50° and were loaded on a 1% agarose gel in Tris-borate-EDTA (30 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) buffer with 0.1% SDS. For Topo I or nuclear extract, DNA samples were denatured by the addition of 10 μl of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green, just before gel loading, whereas Topo II reaction samples were not denatured. Gels were dried and autoradiographed for 1 or 2 days (Hyperfilms MP; Amersham).

Inhibition by drugs of the Topo II catalytic decatenation reaction was performed in the presence of 0.25 μg of *Trypanosoma cruzi* kinetoplast DNA (a gift from Dr. G. Riou, Institut Gustave Roussy, Villejuif, France), and inhibition of the Topo I catalytic relaxation reaction was performed with 0.25 μg of supercoiled pBR322 DNA, with 2-fold serial dilutions of calf thymus Topo I (GIBCO-BRL), as previously described (18). After 30 min of incubation at 37°, the reactions were stopped by the addition of 5 μl of 50 mM EDTA, 30% (w/v) sucrose, 0.1% bromophenol blue, and samples were electrophoresed in 1% or 2% agarose gels for relaxation and decatenation, respectively.

**Unwinding experiments.** The assay was a modification of the method previously described by Pommier *et al.* (19). The reaction was essentially like the catalytic reaction for relaxation of Topo I, except that excess calf thymus Topo I (32 units; GIBCO-BRL) was added to the reaction mixture. Drug was added to supercoiled pBR322 DNA for 15 min at 37° before the addition of Topo I. The reaction was stopped by the addition of 5 μl of 50 mM EDTA, 30% (w/v) sucrose, 0.1% bromophenol blue. Samples were electrophoresed in 0.8% agarose gels containing 15 μM chloroquine, in order to analyze the topoisomer distribution. After extensive washing using distilled water, gels were stained by ethidium bromide.

**Covalent DNA/topoisomerase binding assay.** Reactions performed as described for the cleavage assay were incubated for 10 min at 37° and terminated by the addition of 2 μl of 10% SDS, followed by 250 μl of buffer (prewarmed to 37°) containing 10 mM Tris·HCl, pH 7.5, 20 μg/ml bovine serum albumin, 1% SDS, and 20 μg/ml salmon sperm DNA. Topoisomerase-DNA complexes were then precipitated with KCl and filtered on GF/C glass fiber disks, using the procedure described by Trask *et al.* (20). Radioactivity on the disks was determined by liquid scintillation counting. Results are expressed as a multiple of the binding value measured with the enzyme alone.

**Cytotoxicity assay with yeast strain JN2-134, bearing the human *top1* gene.** Yeast strain JN2-134, which contains one copy of the complete human *top1* cDNA inserted in a YCP50 vector under the dependence of the *GAL1* promotor, was a generous gift from Dr. J. C. Wang (Harvard University, Cambridge, MA) (21). Yeast cells we grown in liquid minimal medium (yeast nitrogen base) supplement with 2% glucose and essential amino acids but without uracil (21 Untransformed JN2-134 cells were grown in the same medium contai ing uracil. For the differential cytotoxic assay, an overnight preculture of cells was separately diluted 1/300 in minimal medium supplemented with 2% glucose or with 2% galactose. Diluted cells (180 μl) were replated on 96-well microtiter plates, and drugs to be tested were added in a volume of 20 μl. Microplaques were incubated at 30°, with gentle rotation for 36 hr. Yeast growth was determined by absorbance measurement at 620 nm with a multiplaque photometer (Titertek). Each point was measured in quadruplicate, and results (mean) are expressed relative to the value obtained under glucose-repressed conditions without drug (defined as 1).

## Results

**Stimulation of Topo I- and Topo II-associated DNA breaks by indoloquinolinedione derivatives.** A 0.35 M salt extract from P388 nuclei, which is enriched in Topo I and II, was used in a first-line test for screening of new topoisomerase inhibitors. This test is based on the ability of such compounds to generate a stabilized covalent complex between topoisomerase(s) and DNA (also termed a cleavable complex), which can be revealed as a double- or single-strand DNA break under denaturing conditions. In our experimental procedures, a strand-denaturating treatment of DNA before gel electrophoresis allows us to detect both Topo I and Topo II cleavable complexes as a single-strand DNA break. A typical experiment is illustrated in Fig. 1; camptothecin (Fig. 1, *lane 4*) and etoposide (Fig. 1; *lane 3*), specific inhibitors of Topo I and II, respectively, generate a lower molecular mass form of a 3′-end-labeled pBR322 DNA (compared with the untreated P388 nuclear extract in Fig. 1, *lane 2*). The same effect is observed, in a dose-dependent manner, when AzaIQD (compound **32**; Table 1) is added (Fig. 1, *lanes 6* and *7*). The DNA cleavage production is, however, not detected when 100 μM drug is incubated with DNA in the absence of the P388 nuclear extract (Fig. 1, *lane 8*) or when 10% dimethyl sulfoxide is added with the nuclear extract (Fig. 1, *lane 5*). These results suggest that these derivatives interfere with a nuclear protein that is probably a topoisomerase. In order to determine which topoisomerase is involved, the same type of experiment was performed using purified preparations of each enzyme. Cleavage activity was detected in the presence of various sources of eukaryotic Topo I, such as calf thymus, human Calc18 cells, or murine P388 cells. Results for 19 derivatives tested are summarized in Table 1. Further studies were performed on one of the two most potent derivatives, AzaIQD, compared with the Topo I inhibitor camptothecin. As shown in Fig. 2, AzaIQD induces a dose-dependent cleavage stimulation in the presence of human Topo I (Fig. 2, *lanes 7–10*), which is detectable at a concentration of

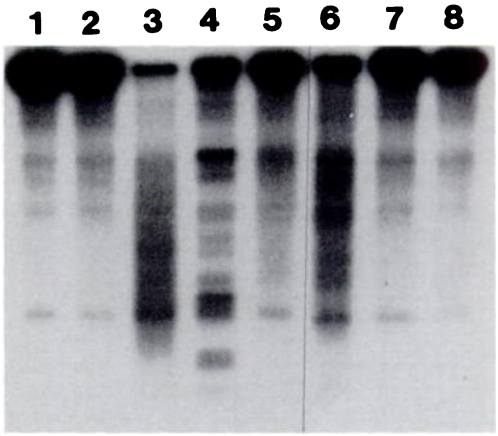



**Fig. 1.** Drug-induced DNA cleavage in the presence of P388 nuclear extract. Cleavage reactions were carried out as described in Materials and Methods, in the presence of P388 nuclear extract, 3′-end-labeled pBR322 DNA, and various drugs. Single-strand DNA fragments were analyzed by agarose gel electrophoresis after alkali denaturation. *Lane 1*, control pBR322 DNA; *lane 2*, DNA and P388 nuclear extract, without drug; *lane 3*, same as *lane 2*, with 100 μM etoposide; *lane 4*, same as *lane 2*, with 100 μM camptothecin; *lane 5*, same as *lane 2*, with 10% dimethyl sulfoxide; *lanes 6* and *7*, same as *lane 2*, with 100 and 10 μM AzaIQD, respectively; *lane 8*, DNA and 100 μM AzaIQD without nuclear extract.

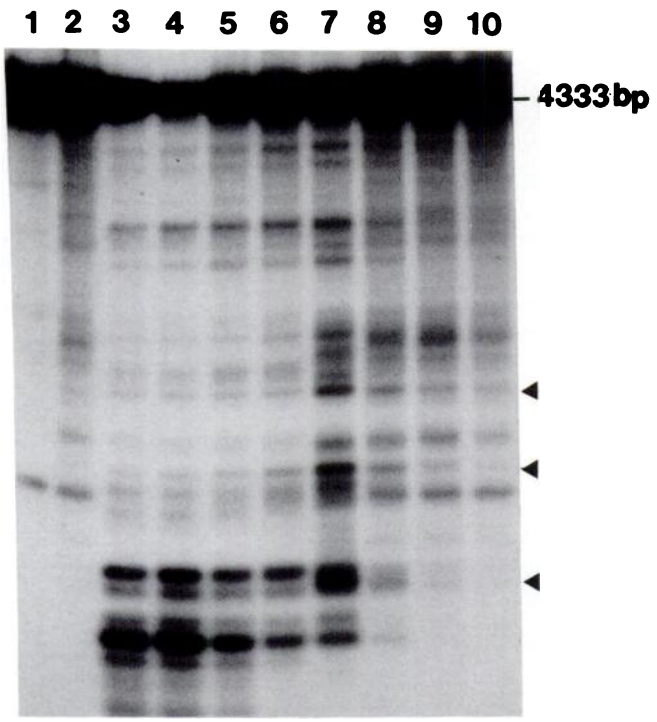



**Fig. 2.** Effects of camptothecin and AzaIQD on Topo I-mediated cleavage of pBR322 DNA. Cleavage reactions were carried out as described in Materials and Methods, using Calc18 human Topo I, 3′-end-labeled pBR322 DNA, and drugs at various concentrations. Single-strand DNA fragments were analyzed by agarose gel electrophoresis after alkali denaturation. *Lane 1*, control pBR322 DNA; *lane 2*, DNA and Calc18 Topo I, without drug; *lanes 3–6*, same as *lane 2*, with 100, 30, 10, and 3 μM camptothecin, respectively; *lanes 7–10*, same as *lane 2*, with 100, 30, 10, and 3 μM AzaIQD, respectively. *Arrowheads on the right*, Cleavage sites markedly stimulated by AzaIQD.

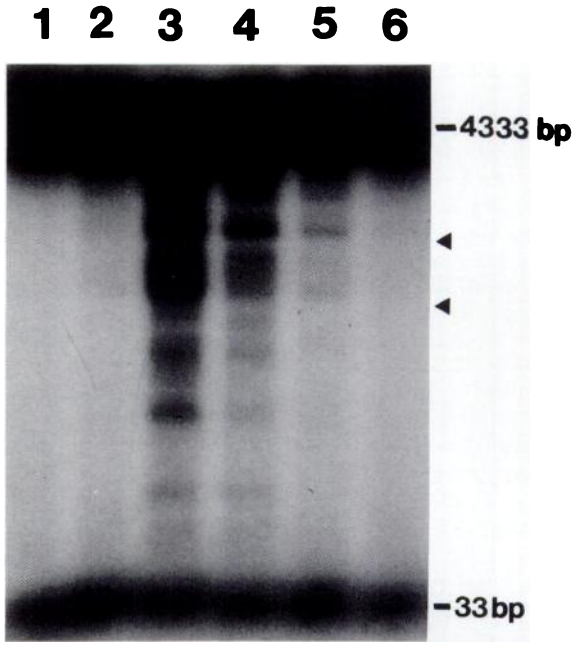



**Fig. 3.** Effects of etoposide and AzaIQD on Topo II-mediated cleavage of pBR322 DNA. Cleavage reactions were carried out as described in Materials and Methods, using P388 Topo II, 3′-end-labeled pBR322 DNA, and drugs at various concentrations. Double-strand DNA fragments were analyzed by agarose gel electrophoresis. *Lane 1*, control pBR322 DNA; *lane 2*, DNA and P388 Topo II, without drug; *lanes 3* and *4*, same as *lane 2*, with 100 and 10 μM etoposide, respectively; *lanes 5* and *6*, same as *lane 2*, with 100 and 10 μM AzaIQD, respectively. *Arrowheads* on the right, cleavage sites stimulated by AzaIQD and etoposide.

10 μM (Fig. 2, *lane 9*). Compared with camptothecin (Fig. 2, *lanes 3–6*), AzaIQD at 100 μM (Fig. 2, *lane 7*) induces approximately the same amount of cleavage as does 3 μM camptothecin (Fig. 2, *lane 6*), as judged by the appearance of DNA cleavage bands and the decrease of the 4.3-kilobase pBR322 DNA band. Qualitatively, AzaIQD primarily enhances the formation of Topo I cleavage sites generated in the absence of drug, corresponding to the "natural cleavage" of the enzyme (Fig. 2, *lane 2*). In addition, AzaIQD stimulates other cleavage sites not observed with the enzyme alone, as does camptothecin, which is in agreement with previous results (9). The cleavage patterns of AzaIQD and camptothecin are nearly identical. However, in the experiment presented in Fig. 2, some differences in the intensity of several cleavage sites, between AzaIQD and camptothecin, are observed (Fig. 2, *arrowheads*). Additional experiments have indicated that these differences are not obvious and that a more careful examination is needed. These data clearly indicate that AzaIQD induces the specific stabilization of the cleavable complex between Topo I and DNA.

Double-strand DNA breakage induced by Topo II was also studied with 13 indoloquinolinedione derivatives. The same methodology was applied, except that DNA was not denaturated before gel electrophoresis, allowing us to reveal Topo II-related double-strand DNA breaks. Some of these compounds induce the cleavable complex trapping of Topo II (see Table 1 and Fig. 3). However, the induction of DNA cleavage by indoloquinolinediones, as illustrated with AzaIQD (Fig. 3, *lanes 5* and *6*), is rather modest, compared with the effect of etoposide (Fig. 3, *lanes 3* and *4*), but significant (compared with the enzymatic control; Fig. 3, *lane 2*). Cleavage stimulation is not observed at a concentration of 10 μM for AzaIQD (Fig. 3, *lane 6*) or for the other Topo II-positive indoloquinolinedione derivatives (data not shown). Qualitatively, no striking difference of the cleavage site pattern could be shown, because the major cleavage sites stimulated by AzaIQD are also strongly stimulated by etoposide (Fig. 3, *arrowheads*). These results indicate that indoloquinolinediones also show some inhibitory properties towards purified Topo II.

**Structure-activity relationships of indoloquinolinedione derivatives for Topo I and II cleavable complex stimulation.** The Topo I cleavable complex stimulation was determined with 19 derivatives and is presented in Table 1. Comparison of three groups of quinones (compounds **3, 15, 30, 18, 22**, and **24**; compounds **3, 9**, and **12**; and compounds **32** and **37**) indicates that 1) when the quinonic nucleus is unsubstituted in *m*- and *p*-positions, there is no Topo I inhibition; 2) introduction of at least one substituent affords active derivatives; 3) the *N*-methylpiperidyl group is less interesting; and 4) the position of the substituent on the quinonic nucleus has no effect (compare compounds **18** with **9** and **22** with **12**). Two other structural modifications are unimportant for the Topo I activity, i.e., methylation of the indolic nitrogen (compare compounds **3** with **27** and **15** with **30**) and introduction of a nitrogen on the benzene nucleus (compare compounds **6** with **32** and **9** with **34**).

On the other hand, saturation of the benzene nucleus affords inactive or less active compounds (quinones **2, 5, 8**, and **11**). The presence of a methyl group in *Rα*-position is very unfavor-

able (compare compounds **6** with **9** and **32** with **34**). Aromaticity of the molecule without steric hindrance, therefore, seems to be the most important structural factor for inhibition activities. This point of view is corroborated by the fact that methoxypyrrolo[3,2-*c*]quinoline-6,9-dione without (13) or with (14) a methyl group in the *Rα*-position, i.e., three-nuclei quinones analogs of compounds **6** and **9**, are inactive (data not shown). With the aim of knowing the role of the quinonic function in inhibition activities, amines **51** and **53**, precursors of quinones **32** and **6**, respectively, were tested. The presence of the quinonic moiety seems to be important, because the two quinones are more active on Topo I than are the amines. The most active derivatives for inhibiting eukaryotic Topo I activities are compounds **32** and **6**, methoxyquinones annellated to a three-ring aromatic unsubstituted nucleus.

Topo II cleavable complex stimulation was also examined with 13 quinonic compounds (Table 1). Data indicate that activity on Topo II is closely linked with that on Topo I with, however, a generally lower activity towards Topo II. Exceptions were noted with compound **11**, which is inactive on Topo I but active on Topo II, and with compound **37**, which is more potent against Topo II. In addition, compounds **32** and **6** are two of the most potent against Topo II.

**Effect of AzaIQD on the DNA-binding activity of Topo I and II.** A more precise quantification of the effect of AzaIQD on the cleavable complex stimulation of both Topo I and II was performed using a filter binding assay. This method allows us to determine the amount of radioactive DNA retained on a filter when a protein is covalently bound (20). Results presented in Table 2 indicate that AzaIQD stimulates the retention of DNA in the presence of purified Topo I or II. This experiment also indicates that Topo I or II binding stimulation induced by AzaIQD is 2–3 times less than that detected for either camptothecin or etoposide at a similar concentration. In terms of concentration, AzaIQD is 30 and 100 times less efficient than camptothecin and etoposide, respectively, on Topo I and II.

TABLE 2

**Effect of AzaIQD on Topo I and II binding to pBR322 DNA**

Mean values of duplicate determinations are expressed as a multiple of the binding measured with the enzyme alone, defined as 1. Lowest and highest values are presented in parentheses. Reaction mixtures (0.02 ml) contained 10 units of P388 Topo I or II and 20,000 dpm (about 25 ng) of $^{32}$P-3′-end-labeled pBR322 DNA, with different concentrations of the drugs, and were incubated for 10 min at 37°. Covalent complex formation was assayed by filter binding, as described in Materials and Methods (20).

| Reaction conditions | Relative covalent binding |
|---|---|
| Topo I control | 1.0 (0.91–1.09) |
| +AzaIQD | |
| 1 μM | 2.3 (2.20–2.40) |
| 10 μM | 3.0 (2.67–3.33) |
| 100 μM | 4.4 (4.35–4.45) |
| +Camptothecin | |
| 0.3 μM | 3.6 (3.31–3.89) |
| 3 μM | 6.4 (6.08–6.72) |
| 30 μM | 9.6 (9.10–10.1) |
| Topo II control | 1.0 (0.75–1.24) |
| +AzaIQD | |
| 1 μM | 1.6 (1.16–2.04) |
| 10 μM | 1.8 (1.60–2.00) |
| 100 μM | 2.9 (2.15–3.65) |
| +Etoposide | |
| 0.1 μM | 1.7 (1.60–1.80) |
| 1 μM | 2.5 (2.30–2.70) |
| 10 μM | 5.0 (3.35–6.65) |

These results are in good agreement with the cleavage data (Figs. 2 and 3).

**Inhibition of the catalytic relaxation reaction of Topo I and unwinding effect of AzaIQD.** The effect of AzaIQD on the catalytic activity of calf thymus DNA Topo I was studied using the relaxation of supercoiled pBR322 DNA, under conditions specific for Topo I (no ATP). Serial 2-fold dilutions of Topo I were used to monitor the catalytic activity of the enzyme (Fig. 4A, *lanes 2–6*). In the presence of AzaIQD (100 μM), the relaxation reaction is markedly inhibited, as evidenced by the decreased conversion of the supercoiled DNA in topoisomers or relaxed DNAs (Fig. 4A, *lanes 7–11*). The relaxation inhibition by AzaIQD is dependent on the enzyme concentration, because higher Topo I concentrations are able to reverse the inhibition process (Fig. 4A, *lane 7*). This result suggests that AzaIQD interferes with Topo I rather than with DNA, and this is consistent with a mechanism of inhibition mediated by cleavable complex stabilization. AzaIQD displays a weaker inhibitory effect than camptothecin (Fig. 4A, *lanes 12–16*), because similar relaxation inhibition is obtained with camptothecin (Fig. 4A, *lane 12*) at a 4-fold higher Topo I amount than with AzaIQD (Fig. 4A, *lane 9*).

In order to examine whether AzaIQD could unwind DNA and, therefore, interact nonspecifically with the catalytic reaction of Topo I, we performed the same relaxation experiments with an excess of Topo I (32 units), and samples were analyzed in chloroquine-containing agarose gels, in order to examine the DNA-topoisomer distribution (Fig. 4B). At this enzyme concentration, the inhibitory properties of AzaIQD on relaxation are abolished and the assay corresponds to an indirect unwinding assay (19). AzaIQD, at concentrations ranging from 10 to

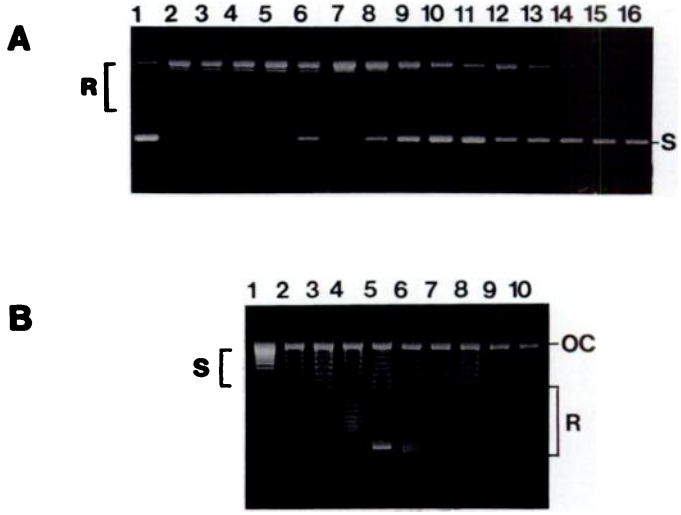



**Fig. 4.** Inhibition of Topo I relaxation and unwinding effect of AzaIQD. Relaxation assay (A) was done in the presence of 2-fold serial dilutions of calf thymus Topo I and either 100 μM AzaIQD or camptothecin, as described in Materials and Methods. *Lane 1*, supercoiled pBR322 DNA, 0.25 μg; *lanes 2–6*, control Topo I at 16, 8, 4, 2, and 1 units, respectively; *lanes 7–11*, same as *lanes 2–6*, with 100 μM AzaIQD; *lanes 12–16*, same as *lane 2–6*, with 100 μM camptothecin. Unwinding assay (B) was done in the presence of excess Topo I (32 units) and electrophoresed in 15 μM chloroquine-containing 0.8% agarose gels, as described in Materials and Methods. *Lane 1*, supercoiled pBR322 DNA, 0.25 μg; *lane 2*, control Topo I, 32 units; *lanes 3–5*, same as *lane 2*, with 1, 10, and 100 μM mAMSA, respectively; *lanes 6–10*, same as *lane 2*, with 10, 25, 50, 100, and 300 μM AzaIQD, respectively. *R*, relaxed DNA and topoisomers; *S*, supercoiled form I DNA; *OC*, open closed or nicked circular DNA.

300 μM (Fig. 4B, *lanes 6–10*), does not modify the topoisomer distribution of relaxed DNA. In contrast, the weak intercalator mAMSA induces a detectable modification of the topoisomer distribution at a concentration equal to 10 μM (Fig. 4B, *lane 4*). These data indicate that AzaIQD does not display detectable unwinding properties at concentrations up to 300 μM.

**Inhibition of the catalytic decatenation reaction of Topo II by AzaIQD.** The decatenating activity of Topo II on the kinetoplast DNA network is a specific double-strand DNA-passing activity of Topo II that Topo I is unable to perform. To determine whether AzaIQD can inhibit the strand-passing catalytic activity of DNA Topo II, we assessed the decatenating activity of purified P388 Topo II in the presence of AzaIQD. As shown in Fig. 5, AzaIQD inhibits 50% of the release of minicircles from the network at a concentration of 100 μM (Fig. 5, *lane 4*), corresponding to the concentration that stimulated cleavable complex formation (Fig. 3, *lane 5*).

**Cytotoxicity of AzaIQD on a yeast strain expressing the human Topo I.** AzaIQD is a compound that displays *in vitro* cytotoxic properties towards the L1210 leukemia cell line ($IC_{50}$ = 0.3 μM) (16) and the Calc18 human mammary adenocarcinoma cell line ($IC_{50}$ = 3 μM) (results not shown). We further examined whether Topo I inhibition could be related to the mechanism of cell killing by AzaIQD, using a permeable yeast strain expressing the human Topo I gene under the control of the *GAL1* promotor (21). When yeast cells are grown in the presence of glucose, which represses the *GAL1* promotor of the *top1* construction, Topo I is not synthesized and AzaIQD does not inhibit yeast growth (Table 3). However, when yeast cells are grown in the presence of galactose, which induces the expression of human Topo I, AzaIQD induces a concentration-dependent enhanced cytotoxic activity. The control experiment, using the untransformed JN2-134 strain, indicates that AzaIQD does not display cytotoxic properties for this strain under glucose or galactose conditions (Table 3). This result demonstrates that the cytotoxicity of AzaIQD in yeast is due to Topo I inhibition. As has already been described, camptothecin displays a strong preferential cytotoxicity in this model but not in the untransformed strain (21) (Table 3). In contrast, actinomycin D, which was also reported to inhibit Topo I activity (11), shows the same cytotoxicity for yeast in the presence of either glucose or galactose (Table 3).

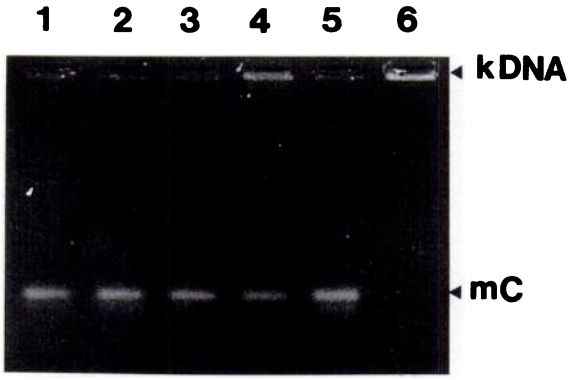



**Fig. 5.** Inhibition of Topo II decatenation by AzaIQD. Decatenation assay was done in the presence of 2 units of P388 Topo II, as described in Materials and Methods. *Lane 6*, control *T. cruzi* kinetoplast DNA (*kDNA*), 0.25 μg; *lane 5*, control Topo II; *lanes 1–4*, same as *lane 5*, with 1, 10, 100, and 200 μM AzaIQD, respectively. *mC*, minicircles.

TABLE 3

**Effects of AzaIQD, camptothecin, or actinomycin D on *YCPGAL1*-human *Top1*-transformed and untransformed JN2-134 yeast cells**

Yeast strain JN2-134 cells bearing the human *top1* gene (21) or untransformed cells were grown in minimal medium supplemented with glucose or galactose, in the presence of various drug concentrations, for 36 hr at 30° (see Materials and Methods). Results are the mean value of quadruplicates and are expressed relative to JN2-134-human *top1* cells in glucose conditions (defined as 1). Standard deviation values are between 0.001 and 0.08.

| Drug | Relative number of cells | | | |
|---|---|---|---|---|
| | JN2-134-human *top1* | | JN2-134 untransformed | |
| | GLU[a] | GAL[b] | GLU | GAL |
| μM | | | | |
| Control[c] | 1.00 | 0.78 | 0.98 | 0.81 |
| Camptothecin | | | | |
| 0.01 | 1.02 | 0.67 | 1.04 | 0.82 |
| 0.03 | 0.99 | 0.09 | 0.98 | 0.76 |
| 0.1 | 1.02 | 0.02 | 1.05 | 0.79 |
| 0.3 | 0.92 | 0.01 | 0.95 | 0.75 |
| AzaIQD | | | | |
| 1 | 1.01 | 0.79 | 0.99 | 0.77 |
| 10 | 1.02 | 0.60 | 0.97 | 0.78 |
| 25 | 0.97 | 0.10 | 1.01 | 0.81 |
| Actinomycin D | | | | |
| 0.1 | 0.90 | 0.75 | 0.87 | 0.73 |
| 1 | 0.60 | 0.55 | 0.63 | 0.48 |
| 10 | 0.01 | 0.11 | 0.03 | 0.07 |
| Control[c] | 1.00 | 0.82 | 1.03 | 0.84 |
| AzaIQD | | | | |
| 10 | 0.99 | 0.62 | 1.03 | 0.85 |
| 20 | 1.02 | 0.33 | 1.05 | 0.83 |
| 30 | 0.96 | 0.12 | 0.98 | 0.77 |
| 60 | 0.92 | 0.03 | 0.89 | 0.71 |
| 100 | 0.82 | 0.02 | 0.86 | 0.60 |

[a] GLU, glucose-repressed.
[b] GAL, galactose-induced.
[c] Two independent experiments.

## Discussion

In the present study, we have demonstrated that different heterocyclic quinones belonging to the series of indolo[3,2-*c*] quinoline-1,4-diones are able to interact specifically with Topo I or II. Compounds **32** (AzaIQD) and **6** are the most potent derivatives tested and correspond to methoxyquinones annellated to a three-ring aromatic unsubstituted nucleus (Table 1). Our demonstration is based on *in vitro* experiments showing that these compounds are able to stabilize an intermediary complex of the catalytic reaction of topoisomerization, using either purified eukaryotic Topo I or II. As has previously been shown for other Topo I or II inhibitors, the stabilization of topoisomerases onto DNA, as an intermediary complex, corresponds to blockage of the DNA religation step by the drug, leading to covalent linking of a topoisomerase polypeptide to one end of a broken DNA strand (8, 9). The DNA break is concealed by the topoisomerase polypeptide, and a protein-denaturing treatment leads *in vitro* to the formation of single- or double-strand DNA breaks, respectively, for Topo I or II (Figs. 1, 2, and 3). No DNA cleavage was detected in the absence of the enzyme preparation, indicating that DNA cleavage is mediated specifically by topoisomerases. The enzyme covalently bound to DNA was also detected by a filter binding assay (Table 2); AzaIQD stimulates the Topo I- or Topo II-induced retention of labeled DNA on a filter, with, however, an efficiency lower than that of the reference compounds camptothecin and etoposide. This cleavable complex stabilization leads

to a catalytic inactivation of Topo I, as measured by a specific assay of ATP-independent relaxation (Fig. 4). A partial catalytic inactivation of Topo II was observed in the specific assay of ATP-dependent decatenation (Fig. 5). As regards the mechanism of inhibition of such enzymes (Topo I and II), a complete inhibition of the catalytic reaction would be achieved when all topoisomerase molecules are trapped by the drug onto DNA. This occurs for AzaIQD, camptothecin, and etoposide at concentrations higher than those used in the cleavage assay, which is performed with an excess of enzyme, thereby allowing a stoichiometric detection of the cleavable complex (9, 22).

In order to determine whether the topoisomerase inhibition could be due to some effect of the drug on DNA, we have performed an indirect unwinding assay in the presence of excess Topo I (19). No DNA-unwinding activity was detected for AzaIQD at a concentration of 300 $\mu$M. Because the inhibition by AzaIQD of the Topo I relaxation reaction was reversed in the presence of increased amounts of Topo I, these data might suggest that AzaIQD interacts directly with the enzyme, rather than with DNA. Under our assay conditions, in agreement with previous results (19), the weak intercalator mAMSA displays detectable unwinding activity at 10 $\mu$M. These data indicate that AzaIQD is at least 30 times less potent than mAMSA, but the possibility cannot be excluded that AzaIQD might display some DNA-binding properties in a nonintercalating mode that are undetectable by the methodology used. For some derivatives of the series that are inactive in cleavable complex stimulation (compounds **2** and **3**), we have observed an inhibition of the natural cleavage of topoisomerases at high drug concentration (100 $\mu$M) (results not shown). This effect, which is not observed for AzaIQD, might reflect some interaction of these molecules with the DNA. Such cleavage inhibition has been already described for intercalators (23) or molecules displaying high affinity for DNA, such as distamycin (24). Numerous classes of Topo II inhibitors are DNA-intercalating agents, and a misalignment model that explains the interaction of these agents with the cleavable complex has been recently proposed (8). Unlike these agents, etoposide and camptothecin do not exhibit intercalation properties, and it was suggested that they might directly interact with a specific site located on the enzyme (8).

Interestingly, and by comparison with other known topoisomerase inhibitors, indoloquinolinedione derivatives could affect both Topo I and II, with a dose-effect preference for Topo I. Such ubiquitous action has only been described before for actinomycin D, which is a strong DNA intercalator (10–12). However, actinomycin D was reported to be a potent inhibitor of RNA polymerase (25), and it is not well known whether topoisomerase inhibition plays a major role in its cytotoxic action. The effect of actinomycin D was studied on the yeast strain JN2-134, bearing the *Gal1*-inducible human *top1* gene, but we did not detect any difference in cytotoxicity between the induced and uninduced conditions. These data suggest that either 1) Topo I is not the principal target of actinomycin D or 2) effects of actinomycin D on RNA polymerase have inhibited the expression of the human *top1* gene under the galactose conditions. In contrast, AzaIQD displays a preferential cytotoxicity for the yeast strain expressing the human *top1* gene. This result indicates that Topo I inhibition is the feature responsible for the cytotoxic action of AzaIQD in yeast. However, a comparison with the effect of camptothecin on the yeast strain reveals an important discrepancy with the *in vitro* effects of AzaIQD. We found that camptothecin is about 1000 times more potent (in concentration) than AzaIQD in the yeast system. A possible explanation could lie in the fact that the yeast system has demultiplicated the difference of potency of these two compounds, because cytotoxicity related to cleavable complex formation is thought to be a secondary process (8, 26, 27). It is also possible that the lesions induced by AzaIQD are more labile and rapidly removed in the yeast system, in contrast to those induced by camptothecin. On the other hand, the possibility cannot be excluded that AzaIQD does not penetrate the yeast cell very well. Further analysis of these compounds in other cellular models, such as resistant cell lines, will be necessary to determine the exact role of Topo I and II inhibition in the cytotoxic activity of the compounds in tumor cell lines.

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