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A CARBOLINE DERIVATIVE AS A NOVEL MAMMALIAN DNA TOPOISOMERASE II TARGETING AGENT

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Abstract—The DNA intercalating, ellipticine analog drug, 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline, is able to stabilize *in vitro* the topoisomerase II–DNA cleavable complex and to induce DNA breaks in BPV I episome in rat fibroblasts. Cytotoxicity studies with DC3F cells resistant to ellipticine strongly suggest that topoisomerase II is a cellular target involved in the mechanism of cytotoxic action of this carboline derivative.

DNA topoisomerase II is the cellular target for DNA intercalating antitumor drugs with various structures such as [4′-(9-acridinylamino)methansulfon-*m*-anisidide] (*m*-AMSA§), anthracycline and ellipticine derivatives [1]. All these compounds appear to interfere with the rejoining step of the DNA strand breakage–rejoining reaction catalysed by DNA topoisomerase II by trapping an enzyme–DNA covalent intermediate in the reaction pathway. This covalent complex termed "cleavable complex", which can be revealed by addition of a strong protein denaturant, triggers a series of cellular responses leading ultimately to cell death [1]. Thus, the cytotoxicity of these antitumor drugs is not the result of the inhibition of the catalytic activity of DNA topoisomerase II, but is rather linked to the cleavable complex stabilization.

A large number of ellipticine derivatives have been synthesized, and their antitumor activity has been studied [2]. 2-Methyl-9-hydroxy-ellipticine exhibited a broad spectrum of *in vitro* antitumor activities in a human tumor-cloning system [3] and a marked activity against breast cancer in a phase II clinical trial [4]. The synthesis of 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline (DiMIQ) was described recently [5]. This compound, a benzo-iso-α-carboline derivative, displaying a structure remarkably close to that of ellipticine, was shown to have significant antineoplastic effects on three experimental tumors: P388, L1210 leukemia and B16 melanoma [5]. This led us to investigate the interaction of DiMIQ with DNA and with DNA topoisomerase II from calf thymus. *In vitro* and *in vivo* studies reported in this paper suggest that the cytotoxicity of DiMIQ is mediated by DNA topoisomerase II. The benzo-iso-α-carboline series therefore appears to be a promising source of potential new antitumor drugs.

MATERIALS AND METHODS

Drugs and chemicals. DiMIQ was synthesized as described previously [5] and its identity checked by mass spectrometry. *m*-AMSA (NSC 249992) was provided by Dr Alain Gouyette (Villejuif, France). Stock solutions (10 mM) in dimethyl sulfoxide (DMSO) (Sigma, St Quentin-Fallavier, France) were stored at −20° and diluted in water before use. The final concentration of DMSO in cell culture media never exceeded 0.2% (v/v).

DNA and enzymes. pSP65 DNA (3005 bp) was obtained from *Escherichia coli* strain HB 101 as described [6]. DNA topoisomerase II was purified from calf thymus according to a published procedure [7]. Low molecular mass DNA was obtained by ultrasonic treatment of calf thymus DNA as described [8]. Single strand regions were removed by *Neurospora crassa* endonuclease (Boehringer Mannheim, Germany) digestion for 10 min.

DNA–drug interaction in vitro. Spectrophotometric measurements, carried out using an Uvikon 860 spectrophotometer manufactured by Kontron Instruments AG (Zurich, Switzerland), allowed a determination of the number of drug molecules bound per DNA nucleotide (*r*). The binding parameters were obtained by determining the best fit of the equation of McGhee and von Hippel [9] with the data.

The length increase of low molecular mass DNA and the unwinding of closed circular DNA from bacteriophage PM2 were analysed using a capillary viscosimeter as described previously [8]. DNA unwinding upon drug binding was also measured by agarose gel electrophoresis after relaxation by calf thymus topoisomerase I of circular pSP65 DNA in the presence of the drug at various concentrations. After incubation for 15 min at 37° in a medium containing 0.1 M NaCl, 50 mM 4-morpholine-

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§ Abbreviations: DiMIQ, 5,11-dimethyl-5*H*-indolo[2,3-*b*]quinoline; *m*-AMSA, 4′-(9-acridinylamino)methansulfon-*m*-anisidide; DMSO, dimethyl sulfoxide; BPV I, bovine papilloma virus type I; 9-OH-E, 9-hydroxy-ellipticine.

ethanesulfonic acid pH 6.5 and 14.4 μg/mL pSP65 DNA, the reaction was stopped by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). After addition of 4 μL of loading buffer to 15 μL of aqueous phase, samples were analysed by electrophoresis at 20° in 1% agarose gels containing 1.2 μg/mL chloroquine. The gels were stained with ethidium bromide (0.5 μg/mL) and photographed under UV light. The films were analysed with a Chromoscan 3 densitometer (Joyce-Loebl, Gateshead, U.K.) and the medians of the topoisomer distributions were calculated for each drug concentration as described [10].

In vitro *topoisomerase II assay*. Cleavable complex was formed by incubation at 37° for 15 min of 100 ng pSP65 DNA with 200 ng topoisomerase II in 15 μL of 60 mM KCl; 7.5 mM $MgCl_2$; 0.5 mM Na_3-EDTA; 0.5 mM dithiothreitol; 20 mM Tris–HCl pH 7; 0.1 mg/mL bovine serum albumin and drug at indicated concentration. After addition of 2 μL of 3% sodium dodecylsulfate and 0.75 mg/mL proteinase K (Boehringer Mannheim), the mixture was incubated at 55° for 45 min and loaded on a 1.2% agarose gel. Electrophoresis was carried out in the presence of 0.45 μg/mL ethidium bromide in Tris-borate-EDTA buffer. DNA bands were photographed under UV light using Ilford FP4 film and their intensity was measured with the Chromoscan 3 densitometer.

In vivo *topoisomerase II assay*. Rat fibroblasts transformed by wild type I bovine papilloma virus genome (BPV I) were a generous gift from Prof. François Cuzin [11]. They maintained in Eagle's minimum essential medium (Gibco, Uxbridge, U.K.), supplemented with 10% fetal calf serum and penicillin–streptomycin. Ten centimeter diameter Petri dishes containing cells in the exponential growth phase (about 6 million cells) were incubated for 1 hr at drug concentrations ranging from 0.1 to 50 μM as indicated. Cell lysis, extraction and purification of DNA were carried out as described previously [12]. DNA was loaded on a 0.8% agarose gel in Tris-borate-EDTA buffer and after electrophoresis blotted on a positively charged nylon membrane (Hybond N^+ from Amersham, U.K.) and hybridized with a DNA probe synthesized with a specific oligonucleotide primer (made on a 381A DNA synthesizer, Applied Biosystems) and [α-^{32}P]-dATP (3×10^3 Ci/mmol, Amersham).

Cytotoxicity. Chinese hamster lung cell line DC3F and the topoisomerase II inhibitor-resistant subline DC3F/9-hydroxy-ellipticine (9-OH-E) [13], were grown in the same medium as above. Determination of ID_{50} was performed by incubating the cells (10^3 DC3F and 4×10^3 DC3F/9-OH-E cells/well) in 16 mm wells of a 24-well dish for 72 hr in the presence of increasing drug concentrations. The cells were then counted with a Coulter Counter (Coultronic).

RESULTS

DNA intercalation of DiMIQ

The DiMIQ molecule can be either protonated or uncharged depending on the pH of the medium (Fig. 1). Each of these forms has a distinct UV and visible absorption spectrum and by pH titration of a 17 μM solution of DiMIQ in water followed by absorption spectrophotometry at 280 nm, a pK_a value of 7.45 at 25° was determined. Therefore, the interaction of DiMIQ with DNA was studied at pH 5.0 when more than 99.5% of the drug is protonated and its solubility is favored.

Figure 2A shows the absorption spectrum of DiMIQ in the presence of DNA at various concentrations showing a bathochromic and an hyperchromic effect accompanying the binding of the drug to DNA. A clear isobestic point at 349 nm is indicative of a single mode of binding. Spectrophotometric measurements of DNA–DiMIQ interaction were carried out at 340 nm (Fig. 2B), and the data were analysed according to McGhee and von Hippel [9]. The binding parameters were found to be $2.3 \times 10^5\ M^{-1}$ for the binding constant and 4.96 for the number of nucleotides/binding site. To determine whether DiMIQ binds to DNA by intercalation as ellipticine derivatives do, the effect of the drug on the length of short DNA fragments was studied by viscosimetry (Fig. 3). The data show that log ($[n]/[n]_0$) varies linearly as a function of log ($1 + 2r$) with a slope equal to 2.43, a typical value for a DNA intercalating compound [8] (parameter definitions are indicated in the legend to Fig. 3). To determine the DNA unwinding angle upon DiMIQ binding, circular pSP65 DNA was relaxed by DNA topoisomerase I in the presence of the drug at various concentrations. After dissociation of the complex by phenol–chloroform/isoamyl alcohol extraction, the change in the DNA linking number induced upon drug binding was determined by agarose gel electrophoresis. From the slope of the straight line shown in Fig. 4, an unwinding of 7.5° per DiMIQ molecule bound was deduced. This value was confirmed by viscosimetric titration with DiMIQ

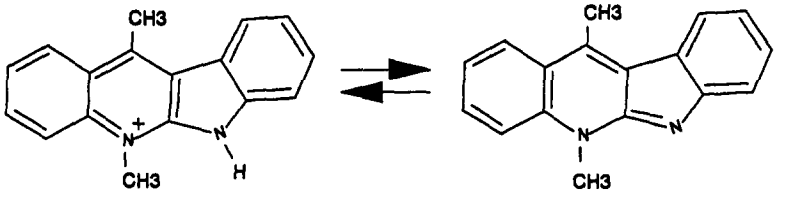

Fig. 1. Structure of protonated and uncharged forms of DiMIQ.

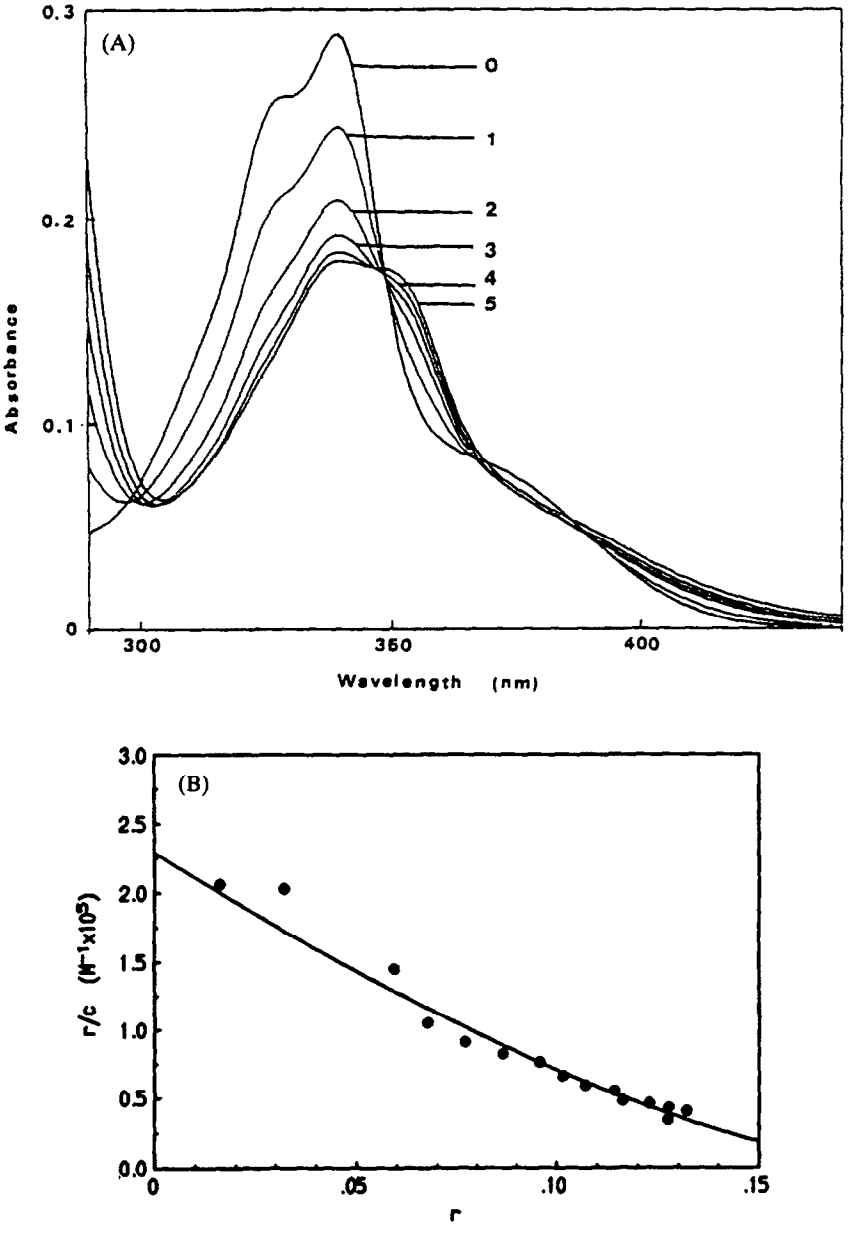

Fig. 2. Spectrophotometric study of the interaction of DiMIQ with calf thymus DNA. (A) UV absorption spectrum of DiMIQ in the presence of DNA in 1 cm pathlength cuvettes. DiMIQ at a concentration of 15 μM was mixed with calf thymus DNA at various concentrations in a buffer containing 0.1 M NaCl, 50 mM sodium acetate pH 5.0. The drug/nucleotide molar ratio was: 1 (1.24); 2 (0.62); 3 (0.41); 4 (0.31); 5 (0.25); and 0 is the spectrum obtained in the absence of DNA. (B) DiMIQ–DNA binding isotherm. Measurements were carried out at 25° in a 5 cm pathlength cuvette at a wavelength of 340 nm in the same medium as in Fig. 2A. The initial DNA concentration was 20 μM and DiMIQ concentration varied from 0 to 6.2 μM. Free (C) and bound DiMIQ concentration were calculated using a molar extinction coefficient of 1.86×10^4 and 1.23×10^4 M^{-1} cm^{-1} for the free and bound forms, respectively. r is the molar ratio bound DiMIQ/nucleotide.

of closed circular DNA from phage PM2 (data not shown).

Cleavable complex stabilization by DiMIQ in vitro

By analogy with ellipticine which acts by trapping the topoisomerase II–DNA cleavable complex [14], the ability of DiMIQ to interact with this enzyme was tested *in vitro* using purified calf thymus DNA topoisomerase II and circular pSP65 DNA as substrate. Double strand cleavage of the circular DNA plasmid generates linear form (form III)

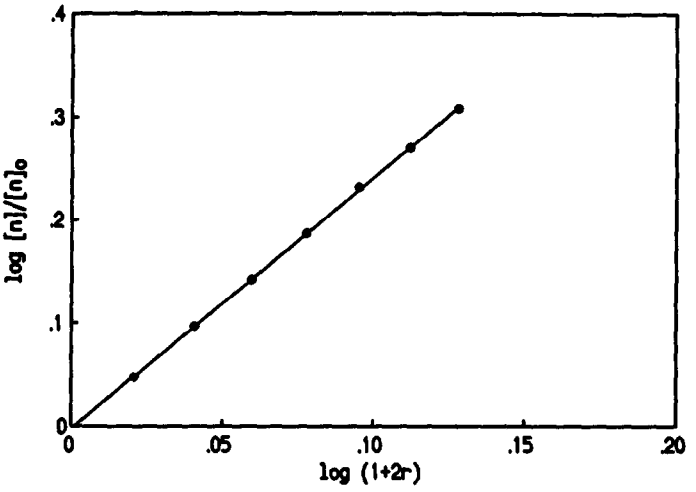

Fig. 3. Viscosimetric analysis of the effect of DiMIQ on the length of sonicated DNA. Measurements were carried out at 25° in a buffer containing 0.1 M NaCl, 50 mM sodium acetate pH 5.0. The initial DNA concentration was 8.49×10^{-4} M and the DiMIQ concentration varied from 0 to 1.36×10^{-4} M. DiMIQ was added as a 2.0 mM solution containing 20% DMSO, and solvent viscosity was corrected for the effect of DMSO added. $[n]_0$ is the free DNA intrinsic viscosity and [n] the complex intrinsic viscosity. r was calculated with the assumption that the drug was quantatively bound to the DNA.

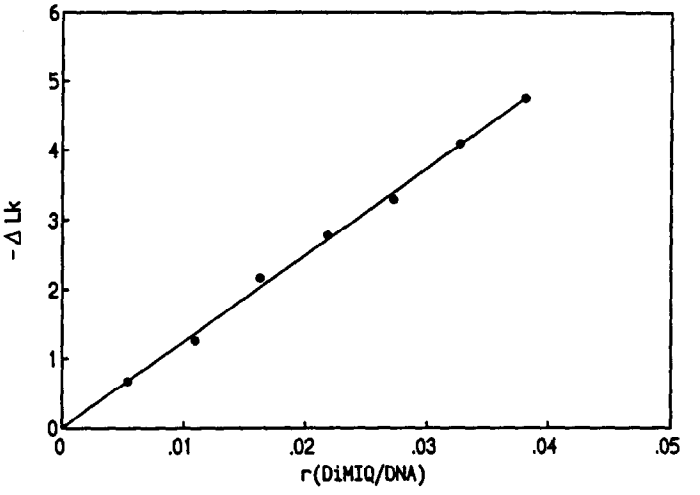

Fig. 4. Unwinding of pSP65 DNA by DiMIQ. The change in DNA linking number produced by topoisomerase I in the presence of DiMIQ at different concentrations was determined by agarose gel electrophoresis as described in Materials and Methods.

which can be easily quantified after agarose gel electrophoresis. Since a pK_a value of 7.45 was determined for DiMIQ, the topoisomerase II-mediated DNA cleavage was studied at two different pHs. At pH 8.0, when the slightly water-soluble uncharged form is predominant, a weak stimulation of DNA cleavage by DiMIQ was observed (not shown). At pH 7.0, when the quinolinium form is predominant, DiMIQ stimulated efficiently DNA cleavage (Fig. 5). In the presence of 50 μM *m*-AMSA as a control, a 14-fold increase in the amount of form III was observed relative to the amount of form III obtained in the absence of drug. DiMIQ, at the optimum concentration of 5 μM, induced an 8-fold increase in the amount of form III and a smaller but significant degree of cleavage was observed at the other drug concentrations tested (Fig. 5). Bell-shaped dose–response curves are

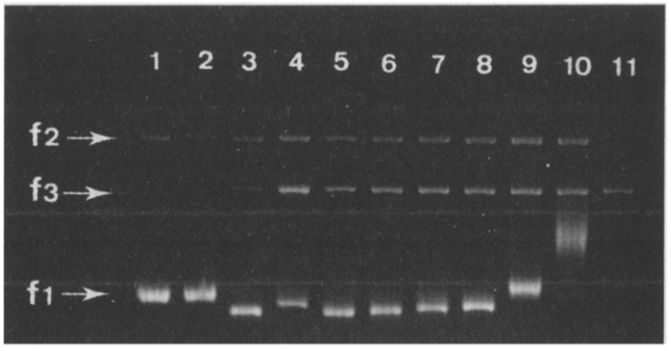

Fig. 5. Cleavable complex stabilization by DiMIQ. Cleavage reaction and gel electrophoresis were performed as described in Materials and Methods. Lane 1 contains unreacted pSP65 DNA, lane 2 pSP65 DNA + 50 μM DiMIQ, lane 3 pSP65 DNA reacted with topoisomerase II, lane 4 identical to lane 3 with 50 μM *m*-AMSA, lanes 5–10 identical to lane 3 with DiMIQ at a concentration of 0.1, 1, 5, 10, 50 and 100 μM, respectively. Lane 11 linear pSP65. Per cent of form III (f3) is 2.6 in the absence of drug, 36.1 in the presence of *m*-AMSA and 12.9, 18.1, 22.0, 16.7, 16.1 and 11.8 in the presence of DiMIQ in the order of increasing concentration. Forms I and II are marked as f1 and f2, respectively.

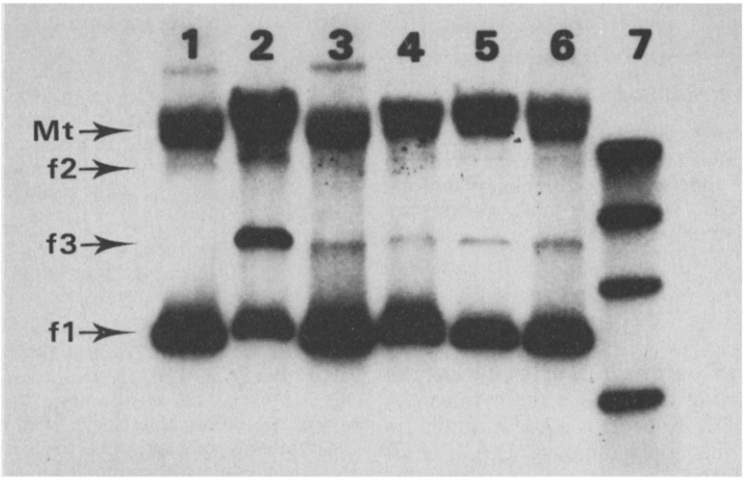

Fig. 6. Intracellular cleavage of BPV I DNA by DiMIQ. DNA extraction, gel electrophoresis and Southern blot analysis were performed as described in Materials and Methods. DNA from untreated cells (lane 1); cells treated with 20 μM *m*-AMSA (lane 2) or with DiMIQ at a concentration of 0.1, 1, 10 and 50 μM, respectively (lanes 3–6). Molecular mass markers were run in lane 7. The forms I, II and III are marked as f1, f2 and f3, respectively. The BPV I DNA multimers are marked as Mt.

commonly observed for the stimulation by strong DNA intercalators of topoisomerase II-mediated DNA cleavage [14, 15]. The electrophoretic mobility shift of form I observed with *m*-AMSA and DiMIQ at the highest concentrations results from drug intercalation into DNA.

Cleavable complex induction by DiMIQ in vivo

BPV I DNA which replicates autonomously in rat fibroblasts and is stably maintained at high copy number per cell [16] is a convenient target for analysing the effect of DNA topoisomerase II poisons *in vivo*. The extent of conversion of circular BPV I DNA to linear form can be determined after separation by agarose gel electrophoresis followed by Southern blot hybridization with an appropriate probe. As shown in Fig. 6, only 1.2% of BPV I was cleaved in the absence of drug, whereas in the presence of 20 μM *m*-AMSA, 40% of DNA was cleaved. When the effect of DiMIQ at concentrations ranging from 0.1 to 50 μM was studied, 6.5% of cleavage was observed on average for all the concentrations tested, with no significant variation as a function of the concentration. Because of the pH of the cell culture medium (between 7.5 and 8.0), the uncharged form of DiMIQ is predominant which may be responsible of the low cleavage observed under these conditions.

These observations indicate that DiMIQ, as well as *m*-AMSA, is able to cross the cellular membrane to reach the nucleus where it stabilizes the topoisomerase II–DNA cleavable complex.

DiMIQ cytotoxicity

To confirm the hypothesis that cleavable complex

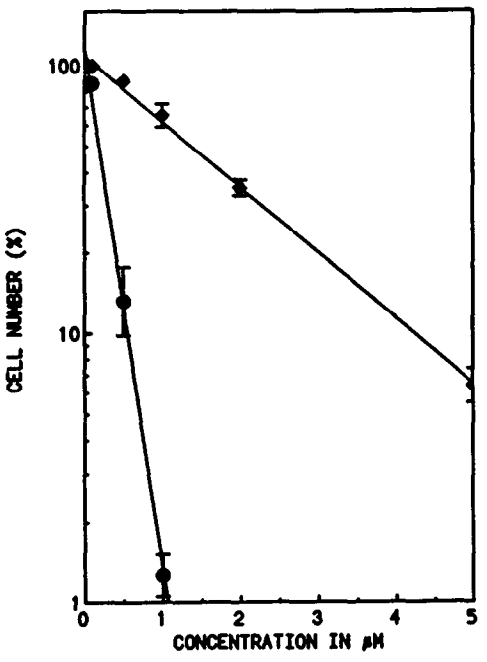

Fig. 7. Cytotoxicity of DiMIQ to sensitive and resistant cells. Survival of hamster lung cell line DC3F treated with DiMIQ (●) and the topoisomerase II-inhibitor resistant subline (DC3F/9-OH-E) (◆) was determined as described in Materials and Methods.

stabilization plays an essential role in the mechanism of the cytotoxic action of DiMIQ, the cytotoxicity of the drug on DC3F cells was determined in comparison with the topoisomerase II inhibitor-resistant cell line DC3F/9-OH-E. As shown in Fig. 7, the ID_{50} was found to be 0.2 and 1.4 μM for the drug-sensitive and the drug-resistant cells, respectively, supporting an involvement of topoisomerase II in the mechanism of cytotoxicity.

DISCUSSION

We have studied some physical–chemical an biological properties of a new compound DiMIQ which, due to the analogy of its structure with ellipticine derivatives, is a potential antitumor drug. The interaction with DNA was studied at pH < 7 to avoid complications due to the presence of two different forms of the drug in equilibrium. The interaction with topoisomerase II and the cytotoxicity studies were carried out at physiological pH where the quinolinium form is present at a level sufficient to produce detectable effects.

Like ellipticine, DiMIQ binds to DNA with a high affinity. DNA length increase measurements indicate that the drug binds by intercalation between DNA base pairs. The unwinding angle of 7.5° associated with this process is small when compared to other intercalating compounds, but is not very different from the Adriamycin® unwinding angle [17]. *In vitro*, DiMIQ stabilizes efficiently the topoisomerase II–DNA cleavable complex with a pH dependence indicating that the protonated form of the drug is the active form for inducing this effect.

Cleavage of circular BPV I DNA maintained autonomously in rat fibroblasts is observed after treatment of the cells with DiMIQ at a concentration as low as 0.1 μM, at which *m*-AMSA is not efficient. Furthermore, the cross-resistance to DiMIQ of the ellipticine-resistant cell line DC3F/9-OH-E, as well as the *in vitro* observation, strongly suggests that DNA topoisomerase II is involved in the mechanism of cytotoxicity of the drug.

DiMIQ is 10 times more cytotoxic than ellipticine to KB cells [5] and exhibits a slightly greater antitumor activity than 9-OH-E against mouse leukemia L1210 [2, 5]. Furthermore, the DiMIQ lethal dose determined for mice is very high (unpublished data) indicating that this molecule is well tolerated by animals. Some DiMIQ derivatives display a very low optimum concentration for cleavable complex induction (less than 0.1 μM) [18] and are promising for antitumoral activity.

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