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Inhibition of DNA Topoisomerase II by Imidazoacridinones, New Antineoplastic Agents with Strong Activity Against Solid Tumors

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SUMMARY

Imidazoacridinones are new antitumor compounds that exhibit strong antitumor effect against solid tumors such as human colon and breast carcinomas. The mechanism of action of imidazoacridinones is unknown, although a similarity in the chemical structure between active imidazoacridinones and mitoxantrone suggests common cellular targets. We show that imidazoacridinones inhibit the catalytic activity of purified topoisomerase II as determined by both relaxation and decatenation assays. All biologically active compounds stimulated the formation of cleavable complexes *in vitro*, whereas inactive compounds did not. The pattern of DNA cleavage in SV40 DNA was similar to that obtained for 4′-(9-acridinylamino)methanesulfon-*m*-aniside, particularly within the matrix-associated region. Significant levels of DNA complexes were observed when DC-3F fibrosarcoma cells were treated with active compounds, whereas negligible amounts of these complexes were induced by inactive analogues. DC-3F/9-OHE cells, which are resistant to other topoisomerase II inhibitors, are 30–125-fold cross-resistant to active imidazoacridinones. The resistance is associated with a reduction in the formation of DNA/protein complexes and is highest for compounds that are potent topoisomerase II inhibitors *in vitro*. Interestingly, the two most active derivatives, C-1310 and C-1311, were equally cytotoxic toward fast-growing monolayer cultures and cells growing in three dimensions as multicellular spheroids, which have a slower growth fraction. In contrast, 4′-(9-acridinylamino)methanesulfon-*m*-aniside, mitoxantrone, and doxorubicin were more cytotoxic toward monolayer cultures. Taken together, the results suggest that DNA topoisomerase II is a major cellular target of biologically active imidazoacridinones and that these drugs show both similarities and dissimilarities compared with classic topoisomerase II inhibitors.

Despite great effort, the development of curative antitumor drugs has been only partially successful, especially for the frequent, slowly growing tumors of lungs, breast, and gastrointestinal tract. In a search for new drugs derived from acridines that was based on drug design derived from previous results of studies with mitoxantrone and its analogues (1), a series of imidazoacridinones with potent antitumor properties were developed at the Technical University of Gdansk, Poland (2, 3).

Imidazoacridinones display considerable cytotoxic activity toward various murine and human cell lines *in vitro* (2, 3) as well as a potent antitumor effect toward breast and colon adenocarcinomas *in vivo* (4). In addition, it has been demonstrated that active imidazoacridinone derivatives do not induce free radical formation; therefore, they should not be cardiotoxic, unlike doxorubicin and other anthracyclines. The most active imidazoacridinone derivative, C-1311, has been accepted for clinical trials in Great Britain.

The development of a new group of very active anticancer drugs raises important questions concerning their mechanism of action. In addition to the fundamental aspect, such studies are of practical value because they may provide information concerning optimal drug treatment and suitable drug combinations and result in the development of even more active derivatives. Given the structural similarities between imidazoacridinones and mitoxantrone, it was expected that DNA topoisomerase II would be a target for these compounds.

DNA topoisomerase II is an essential nuclear enzyme that regulates DNA topology and organization (5). The enzyme modulates the three-dimensional structure of chromatin and

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ABBREVIATIONS: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-aniside; VP-16, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DPC, DNA/protein complex; TBE, Tris/borate/EDTA; MAR, matrix-associated region.

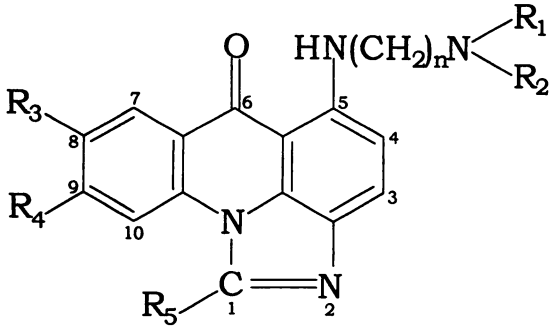
catalyzes interconversions between DNA topoisomers, such as relaxation of supercoiled DNA and decatenation of intertwined DNA molecules. Topoisomerase II may also play a structural role in the organization of chromatin in both interphase cells and mitotic chromosomes (6). Topoisomerase II is believed to be the primary target for a number of clinically useful antitumor drugs (5, 7). Most of these drugs reversibly stabilize covalent reaction intermediates (cleavable complexes) between topoisomerase II and nuclear DNA. Recently, two different isoforms of topoisomerase II have been found, and for at least some drugs, a different interaction with either isoform has been proposed (8, 9). Although the drug-induced stabilization of cleavable complexes is recognized to be important for cell killing, the series of events resulting from cleavable complex formation and culminating in cell death are still poorly understood.

We describe the inhibition of topoisomerase II by selected imidazoacridinones both *in vitro* and in DC-3F fibrosarcoma cells as well as in DC-3F/9-OHE cells, which are resistant to topoisomerase II inhibitors. The compounds studied differed both in their chemical structure and in their biological activities (2, 3), which allowed us to delineate structural components of imidazoacridinones that are important for their interactions with DNA topoisomerase II.

Materials and Methods

Chemicals. Imidazoacridinone derivatives (see Fig. 1 for chemical structures), mitoxantrone, ametantrone, and *m*-AMSA were synthesized at the Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdansk, Poland. [^{3}H]Thymidine, [^{14}C]leucine, and [α-^{32}P]dCTP were obtained from Amersham (Buckinghamshire, UK). All other chemicals were of reagent grade.

DNA substrates and enzymes. Supercoiled plasmid pBR322 DNA (>95% form I), pUC18, *Bst*XI, *Msp*I, and *Taq*I were purchased from Boehringer Mannheim (Mannheim, Germany). SV40 DNA and 123-base pair DNA ladder were obtained from GIBCO (Cergy Pontoise, France), whereas Klenow fragment polymerase was obtained from United States Biochemical (Cleveland, OH). Highly catenated kinetoplast DNA was isolated from *Crithidia fasciculata* kindly provided by Dr. Guy Riou (Institut Gustave Roussy, Villejuif, France) and purified through cesium chloride/ethidium bromide density centrifugation (10). DNA topoisomerase II was obtained from *Saccharomyces cerevisiae* overexpressing a multicopy plasmid (a generous gift from Dr. James Wang, Harvard University, Cambridge, MA) and purified according to the method of Worland and Wang (11).



compound	n	R_1, R_2	R_3	R_4	R_5
C-1263	2	CH_3	OH	H	H
C-1310	2	CH_2CH_3	OH	H	CH_3
C-1311	2	CH_2CH_3	OH	H	H
C-1371	3	CH_3	OH	H	H
C-1419	2	CH_2CH_3	H	OH	H
C-1492	5	CH_3	OH	H	H
C-1554	2	CH_2CH_3	CH_3	H	H
C-1558	2	CH_2CH_3	*t*-butyl	H	H

Fig. 1. Chemical structures of imidazoacridinones.

Relaxation assay. The reaction mixture contained 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 165 mM KCl, 1 mM ATP, and 150 ng of pBR322 DNA. The reaction was initiated by the addition of DNA topoisomerase II and allowed to proceed at 30° for 10 min. Reactions were terminated by the addition of loading buffer (1% SDS, 0.05% bromophenol blue, 50 mM EDTA, 10% sucrose, final concentrations). The samples were electrophoresed in 1% agarose gels at 2 V/cm for 18 hr in TBE buffer (90 mM Tris-borate, and 1 mM EDTA, pH 8). Gels were stained with 0.5 μg/ml ethidium bromide to visualize DNA and photographed under UV illumination. The peak areas of supercoiled DNA were determined by scanning photographic negatives with a Joyce-Loebl Chromoscan 3 densitometer.

Decatenation assay. The reaction conditions were as described above except that 200 ng of kinetoplast DNA was used as substrate and that the incubation time was 15 min. Reactions were stopped by the addition of loading buffer (1% SDS, 0.05% bromophenol blue, 50 mM EDTA, 30% glycerol, final concentrations). The samples were electrophoresed in 1.2% agarose gels at 1 V/cm for 16 hr, and liberated minicircles were quantified with the use of densitometry.

Formation of cleavable complexes *in vitro*. The experimental conditions were the same as for the relaxation assay except that ~50-fold more DNA topoisomerase II was used. The reaction was initiated with the addition of the enzyme. After 10 min at 30°, the reactions were terminated by the addition of 1% SDS and 0.05 mg proteinase K, followed by incubation at 50° for 30 min. Electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/ml) was carried out at 2 V/cm for 18 hr in TBE buffer containing 0.5 μg/ml ethidium bromide. All of the topoisomerase assays were done at least twice with two different enzyme preparations.

Topoisomerase II-induced cleavage of SV40 DNA. SV40 DNA was linearized with *Taq*I and end-labeled with Klenow fragment and [α-^{32}P]dCTP. The labeled DNA was then subjected to a second digestion with *Bst*XI, the fragments obtained were separated with agarose gel electrophoresis, and the larger fragment was used for DNA cleavage assays. Reactions mixtures contained 20 mM Tris·HCl, pH 7.4, 165 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, ~1×10^5 cpm (~50 ng) 3′-end-labeled SV40 DNA, and the indicated concentrations of drugs. The reactions were initiated by the addition of DNA topoisomerase II (15 ng) and were allowed to proceed for 10 min at 37°. Reactions were terminated by the addition of SDS, proteinase K, and EDTA (0.35%, 0.3 mg/ml, and 15 mM final concentrations, respectively) and incubated for 1 hr at 50°. Loading buffer was added, and samples were electrophoresed in 4% polyacrylamide gels with TBE as a running buffer at 20 V/cm for 2–3 hr. Gels were dried and autoradiographed with Hyperfilm MP (Amersham) for 1–2 days. We used 123-base pair DNA ladder and *Msp*I digest of pUC18 DNA plasmid to estimate the size of the DNA fragments.

Cell culture and growth inhibition. The DC-3F fibrosarcoma cell line and its 9-hydroxy-ellipticine-resistant subline DC-3F/9-OHE have been described previously (12, 13). The cells were maintained in minimal essential medium supplemented with 8% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37° in a 5% CO_2/95% air atmosphere.

Growth inhibition assays were carried out with exponentially growing cells and 72-hr drug exposure as described previously (14). The liquid overlay system was used to generate spheroids from DC-3F cells as described (15). Under these conditions, cells are

unable to attach to the tissue plates, which are covered with 1% agarose, and grow as multicellular spheroids in three dimensions.

Cell cycle analysis and confocal fluorescence microscopy. Flow cytometric analysis was carried out as described (16). Briefly, the cells were trypsinized, washed twice with cold PBS, fixed in 70% ethanol overnight at 4°, and stained for 30 min at room temperature with propidium iodide in the presence of RNase A (20 and 100 μg/ml final concentrations, respectively). The data from 10^4 cells were analyzed with the use of an Epics Profile II flow cytometer (Coulter Counter, Hieilah, FL), and the relative fractions of cells in different phases of the cell cycle were determined with the Multicycle program (Phoenix Flow Systems, San Diego, CA). Values are given as mean from three independent experiments.

For analyses of intracellular accumulation and distribution of imidazoacridinones, DC-3F and DC-3F/9-OHE cells were grown onto glass slides and incubated with 1 μg/ml imidazoacridinones for 30 min at 37° immediately before microscopic examination with an ACAS 570 confocal microscopy system (Meridian Instruments, Okemos, OH). To confirm nuclear accumulation of the drugs, nuclei were counterstained with Hoechst 33342 (5 μg/ml final concentration in the medium), and dual-parameter analysis was performed.

Topoisomerase II-associated DNA damage in intact cells. DPCs were quantified with use of the KCl/SDS coprecipitation assay (17). DNA and proteins of DC-3F cells (~5 × 10^5) were radiolabeled with 0.3 μCi/ml [^{3}H]thymidine and 0.1 μCi/ml [^{14}C]leucine, respectively, for 18 hr. The cells were exposed to various concentrations of drugs for 1 hr at 37° or otherwise, as specified. After incubation, cells were lysed in a solution of 1.25% SDS, 5 mM EDTA, pH 8, and 0.4 mg/ml salmon testes DNA. The lysates were passed through a 22-gauge needle five times and incubated at 65° for 15 min. The lysates were then adjusted to 65 mM KCl, vortexed for 10 sec, and placed on ice for 15 min, and the precipitates were collected with a microcentrifuge. The pellets were washed three times in 10 mM Tris·HCl, pH 8, 100 mM KCl, 1 mM EDTA, and 0.1 mg/ml salmon testes DNA at 65° before being dissolved in 0.5 ml water and mixed with 5 ml scintillation fluid for determination of radioactivity. Data are expressed as the ratio of [^{3}H]DNA to [^{14}C]protein, with the protein being an internal standard for the exact number of cells used.

Results

Inhibition of the catalytic activity of topoisomerase II *in vitro*. Imidazoacridinones inhibited the catalytic activity of purified topoisomerase II in both relaxation and decatenation assays. The most potent imidazoacridinones inhibited the topoisomerase II-mediated relaxation of supercoiled pBR322 plasmid DNA at a concentration of ~2.5 μg/ml, resulting in a 50% decrease in DNA relaxation activity (Table 1). Biologically inactive analogues such as C-1492, C-1554, and C-1558 inhibited the relaxation of supercoiled pBR322 DNA at considerably higher concentrations, with IC50 values as high as 50 μg/ml for the C-1558 derivative. At lower concentrations, the binding of imidazoacridinones to DNA produced a typical biphasic pattern (14), with increased relaxation at intermediate concentrations (Fig. 2). This effect was not observed for C-1558, a nonintercalating imidazoacridinone derivative, and confirms indirectly an intercalative binding mode of imidazoacridinone derivatives.[1]

We also examined the abilities of imidazoacridinones to interfere with decatenation of kinetoplast DNA by purified topoisomerase II. The compounds studied inhibited the decatenation activity of purified topoisomerase II at concentrations higher than those required to inhibit DNA relaxation, with IC_{50} values of 30–100 μg/ml (Fig. 3, A and B). Interestingly, C-1419, which is a close structural analogue of the lead compound C-1311 but has an inversion of the functional groups between positions 8 and 9 (see Fig. 1), was as effective as the parent compound in inhibiting both relaxation and decatenation mediated by purified topoisomerase II (Table 1).

TABLE 1

Inhibition of topoisomerase II activity *in vitro* by imidazoacridinones

Compound	Antitumor activity[a]	Inhibition of	
		DNA relaxation IC_{50}[b]	DNA decatenation IC_{50}[c]
		μg/ml	
C-1263	++	5	45
C-1310	+++	5	27
C-1311	++	2.5	48
C-1371	++	5	38
C-1419	–	10	38
C-1492	+	25	66
C-1554	–	25	80
C-1558	–	50	106

[a] Antitumor activity against P388 leukemia *in vivo* according to Cholody *et al.* (2): +++, very active; ++, active; +, marginally active; and –, not active.
[b] Concentration inhibiting 50% of the topoisomerase II-mediated relaxation of supercoiled pBR322 plasmid DNA.
[c] Concentration inhibiting 50% of the topoisomerase II-mediated decatenation of kinetoplast DNA.

Induction of cleavable complexes *in vitro*. Biologically active imidazoacridinones stabilized the formation of cleavable complexes between topoisomerase II and pBR322 DNA *in vitro*. This inhibition was dose dependent, with biphasic curves obtained for biologically active compounds (Fig. 4). Inactive compounds were unable to stabilize topoisomerase II/DNA complexes, except for C-1419, for which the dose-response curve was very similar to that obtained for biologically active compounds.

Topoisomerase II-induced cleavage of SV40 DNA. The pattern of DNA cleavage induced by imidazoacridinones in SV40 DNA was compared with the pattern induced by other classic topoisomerase II inhibitors: the nonintercalating compound VP-16 and DNA intercalators such as mitoxantrone and *m*-AMSA. The results show that the level of topoisomerase II-mediated DNA breaks induced in the presence of imidazoacridinones is significantly lower than that for other inhibitors tested at equimolar concentrations (Fig. 5). The pattern of DNA breaks differed considerably from those of both VP-16 and mitoxantrone and was rather similar to that obtained for *m*-AMSA, particularly within the MAR.

Cytotoxicity against DC-3F and DC-3F/9-OHE cells. We determined the cytotoxicity of imidazoacridinones toward sensitive DC-3F and DC-3F/9-OHE cells, which are resistant to classic topoisomerase II inhibitors. We found that DC-3F/9-OHE cells are 30–125-fold cross-resistant to imidazoacridinones with antitumor activity (Table 2). The level of cross-resistance was the highest for compounds that were more potent against topoisomerase II *in vitro* and comparable to resistance factors obtained for reference drugs such as *m*-AMSA and the anthracenediones mitoxantrone and ametantrone. For biologically inactive imidazoacridinones, which were also poor inhibitors of purified topoisomerase II, the resistance factor was ~1–5.

In addition, the growth inhibitory activity of imidazoacridi-

[1] J. Dziegielewski, B. Slusarski, A. Skladanowski, and J. Konopa, manuscript in preparation.

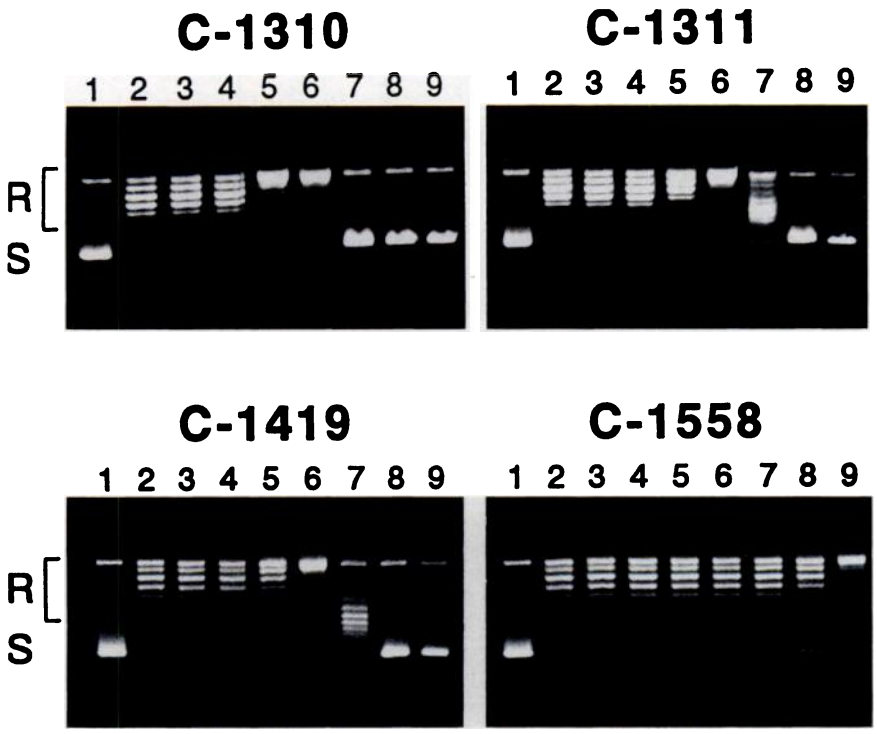


Fig. 2. Inhibition of the catalytic activity of purified yeast DNA topoisomerase II by imidazoacridinones as measured by relaxation. Supercoiled pBR322 DNA (*lane 1*) was relaxed by purified topoisomerase II in the absence (*lane 2*) or presence of imidazoacridinones at 0.1, 0.5, 1, 2.5, 5, 10, and 25 μg/ml (*lanes 3–9*). *S*, Native supercoiled DNA; *R*, relaxed topoisomers. Data shown are typical of three independent experiments.

nones toward DC-3F cells grown as monolayer cultures and as multicellular spheroids was determined. Cells grown in spheroids have a substantially different cell cycle distribution (G_1 = 76 ± 5%, S = 21 ± 4%, and G_2/M = 7 ± 2%) compared with monolayer cultures (G_1 = 38 ± 6%, S = 53 ± 5%, and G_2/M = 9 ± 1%). The results show that C-1310 and C-1311 were equally cytotoxic toward fast-growing monolayer cultures and slower growing three-dimensional spheroids (Table 3). This is in contrast to classic topoisomerase II inhibitors, which show a clearly reduced cytotoxicity against cells growing in spheroids.

Accumulation and distribution of imidazoacridinones within the cell. One of the most frequently observed changes in resistant cells is reduced drug accumulation due to expression of P-glycoprotein or multidrug-resistant proteins (18, 19). Therefore, the decreased cytotoxicity of imidazoacridinones toward DC-3F/9-OHE cells could be due to a diminished accumulation of the drugs in these cells. We exploited the facts that imidazoacridinones fluoresce both as free compounds and when complexed with DNA and that their accumulation and distribution within the cell can be observed with fluorescence microscopy. The compounds were preferentially accumulated within the nucleus, but no difference in the distribution of imidazoacridinones was observed between the parental DC-3F and resistant DC-3F/9-OHE cells (Fig. 6). Image analysis of the fluorescence intensities of the nuclear regions of 100 randomly chosen cells as observed with confocal microscopy showed no significant differences, as determined with the use of Student's *t* test, in imidazoacridinone-treated DC-3F and DC-3F/9-OHE cells (289 ± 79 and 265 ± 67 integrated pixel intensity/μm^2, respectively).

Formation of DNA/topoisomerase II complexes in intact cells. The ability of imidazoacridinones to inhibit topoisomerase II activity in DC-3F cells and their DC-3F/9-OHE resistant subline was determined. The induction of DPCs by imidazoacridinones was measured with the KCl/SDS coprecipitation assay after a 1-hr drug exposure. The results show that two biologically active compounds C-1310 and C-1311 produce significant levels of DPCs (Fig. 7), whereas the inactive analogues C-1419 and C-1558 induced negligible amounts of these complexes. Similar results were observed for four other derivatives. The levels of DPC was significantly higher in DC-3F-sensitive cells than in resistant cells. The relationship between the concentration and the level of DPC induced by imidazoacridinones is biphasic with a complete absence of DPC at concentrations of >50 μg/ml. This bell-shaped concentration profile has been observed for other DNA intercalators (20) and interpreted as self-inhibition of drug-induced DNA lesions (21).

In addition, the results show that imidazoacridinones are very cytotoxic per cleavable complex as, for example, at isotoxic doses C-1311 produces ~4-fold fewer cleavable complexes in DC-3F parental cells than *m*-AMSA, a classic topoisomerase II inhibitor (see Table 2).

We examined the kinetics of formation and reversibility of DPC induced by imidazoacridinones. Figs. 8 and 9 show the results of these experiments for the C-1311 derivative, the most potent compound with respect to DPC formation. The level of DPCs formed in the presence of C-1311 reaches a plateau after 15 min of drug incubation (Fig. 8). Topoisomerase II-mediated lesions were readily reversed when treated cells were incubated in drug-free medium with a substantial decrease of ~50% of the initial value after 30 min and only ~15% of the initial DNA damage after 2 hr after incubation (Fig. 9). This time course is similar to that obtained for *m*-AMSA regarding both the formation and reversal of DPC (data not shown).

Discussion

This study was undertaken to elucidate the mechanism of action of imidazoacridinones, new antitumor agents with

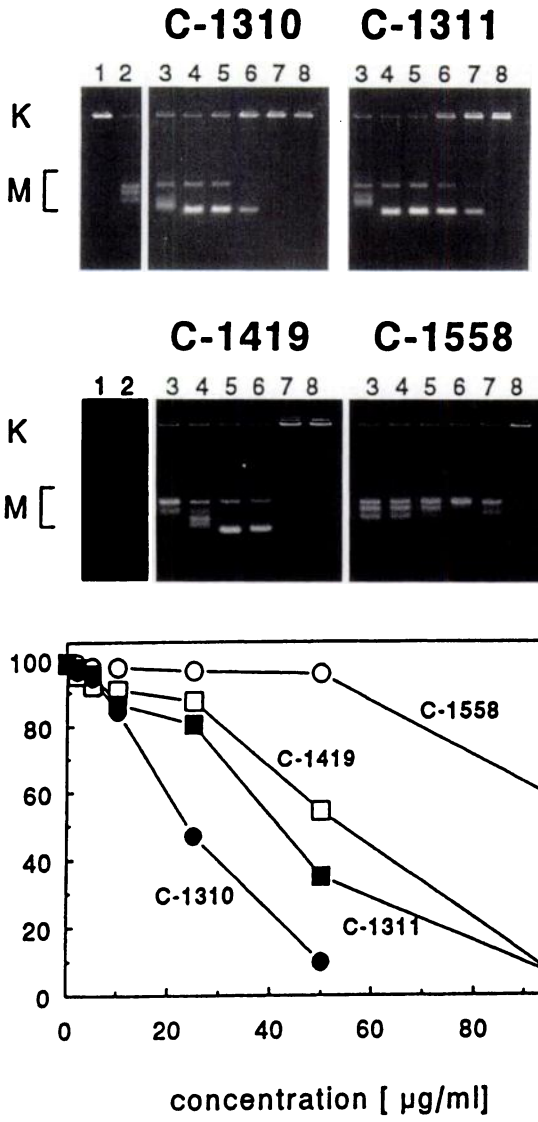


Fig. 3. Inhibition of the catalytic activity of purified yeast DNA topoisomerase II by imidazoacridinones as measured by decatenation. Kinetoplast DNA (*lane 1*) was decatenated by purified topoisomerase II in the absence (*lane 2*) or presence of imidazoacridinones at 2, 5, 10, 25, 50, and 100 µg/ml (*lanes 3–8*). *K*, catenated DNA; *M*, free minicircles. Data shown are typical of two independent experiments.

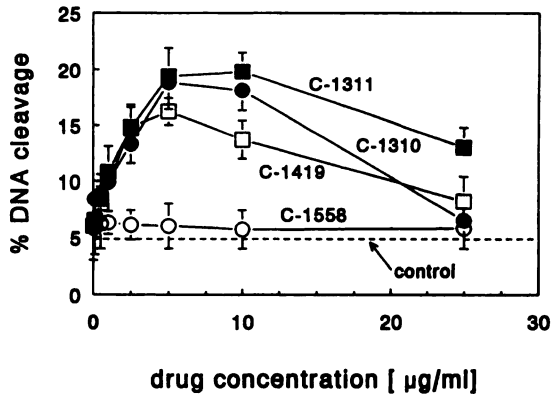


Fig. 4. Effect of imidazoacridinones on DNA cleavage mediated by purified yeast DNA topoisomerase II. Supercoiled pBR322 DNA was incubated with purified topoisomerase II in the presence of imidazoacridinones. The resulting topoisomerase II/DNA complexes were digested by proteinase K, and the different topological forms of DNA were separated by agarose gel electrophoresis. Data are presented as percentage of DNA cleavage calculated from densitometric scans and are the averages of two independent experiments. *Bars*, standard deviation.

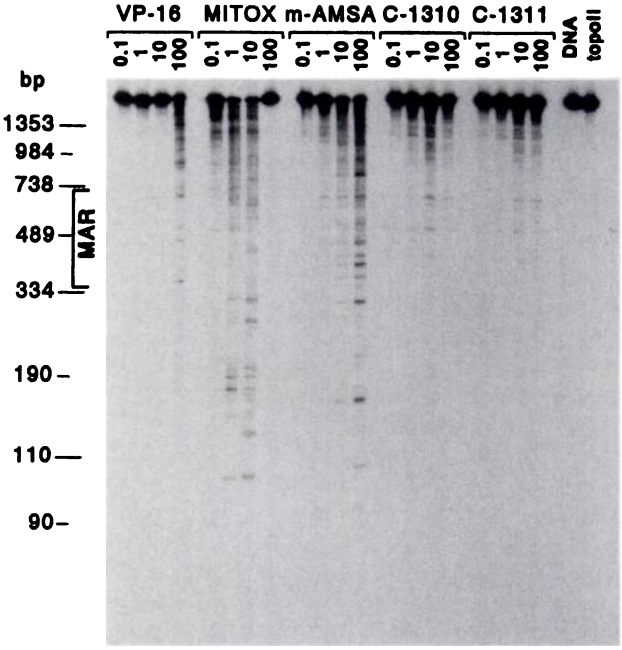


Fig. 5. DNA cleavage pattern induced by topoisomerase II inhibitors. The SV40 DNA was linearized, uniquely 3′-labeled, and incubated in the presence of purified topoisomerase II and VP-16, mitoxantrone, *m*-AMSA, and imidazoacridinones C-1310 and C-1311 at different concentrations (in µM). *DNA*, native SV40 DNA; *topoII*, SV40 DNA plus topoisomerase II (no drug). A molecular mass scale indicates base pair (*bp*) position and MAR in SV40 DNA.

strong activity against solid tumors. Their structural similarity to mitoxantrone suggested that their cytotoxic and antitumor effects might be due to inhibition of topoisomerase II. For these experiments, we selected a group of imidazoacridinone derivatives that differ in both their chemical structure and antitumor activities (2–4). We found that imidazoacridinones inhibit the catalytic activity of purified topoisomerase II *in vitro*. Like most topoisomerase II inhibitors, biologically active imidazoacridinones also stabilized cleavable complex formation between DNA and purified enzyme, whereas inactive derivatives did not.

We then wanted to determine whether the interaction with topoisomerase II plays a role in the cytotoxic action of imidazoacridinones. The cytotoxic effects were compared of imidazoacridinones toward sensitive DC-3F and 9-hydroxyellipticine-resistant DC-3F/9-OHE cells. The DC-3F/9-OHE cells are cross-resistant to classic topoisomerase II inhibitors and have been shown to have decreased amounts and activity of topoisomerase II (22–24). The results show that DC-3F/9-OHE cells are cross-resistant to imidazoacridinones with antitumor activities. The resistant cells used throughout this study express the multidrug-resistance-associated P-glycoprotein (13); however, this could not explain the differences in cytotoxicity between DC-3F and DC-3F/9-OHE as no differences in drug accumulation and distribution between sensitive and resistant cells were observed. The resistance levels are comparable to those observed for classic topoisomerase II

TABLE 2

Growth inhibition and DNA cleavage induced by imidazoacridinones in DC-3F fibrosarcoma cells and in DC-3F/9-OHE cells, which are resistant to topoisomerase II inhibitors

Compound	EC_{50}[a]		Fold resistance[b]	DPCs[c]
	DC-3F	DC-3F/9-OHE		
	ng/ml			L_{max}
C-1263	3.4	317.4	93	1.75
C-1310	12.2	384.8	31	2.16
C-1311	3.1	261.4	124	2.38
C-1371	1.8	213.6	119	1.57
C-1419	216.7	1088.7	5	1.2
C-1492	144.7	285.3	2	1.1
C-1554	224.8	446.1	2	1.1
C-1558	523.5	532.1	1	1.1
Mitoxantrone	0.4	38.5	96	[d]
Ametantrone	52.2	3479.5	67	[d]
m-AMSA	3.7	573.5	155	9.71

[a] Concentration inhibiting the growth of DC-3F cells by 50% compared with untreated control cells.
[b] Ratio between EC_{50} values obtained for DC-3F/9-OHE-resistant and DC-3F-sensitive cells, respectively.
[c] L_{max} is the maximal level of DPCs measured with the KCl/SDS coprecipitation assay, expressed as the ratio of [^{3}H]DNA to [^{14}C]protein values determined for treated and untreated control cells.
[d] Not determined.

TABLE 3

Growth inhibitory effect of imidazoacridinones and classic DNA topoisomerase II inhibitors toward DC-3F cells grown in monolayer culture and as multicellular spheroids

Compound	Growth inhibition EC_{50}[a]		Ratio
	Monolayer	Spheroids	
	ng/ml		
C-1310	12.0 ± 0.3	18.9 ± 0.6	1.6
C-1311	3.1 ± 0.3	3.7 ± 0.1	1.2
VP-16	41 ± 15	175 ± 20	4.3
m-AMSA	2.5 ± 0.3	7.9 ± 0.9	3.2
Mitoxantrone	0.36 ± 0.05	0.9 ± 0.4	2.8
Doxorubicin	2.9 ± 1	7.9 ± 0.4	2.7

[a] Concentration inhibiting the growth of DC-3F cells by 50% compared with untreated control cells.

inhibitors such as VP-16 and *m*-AMSA, which strongly suggests that topoisomerase II is an important target for imidazoacridinone action. However, we cannot exclude that part of the imidazoacridinone-induced cytotoxicity is derived from a topoisomerase II-independent mechanism.

Imidazoacridinones were highly cytotoxic toward DC-3F fibrosarcoma cells but induced relatively little cleavable complexes. For example, derivative C-1311 has a EC_{50} value comparable to that of *m*-AMSA but induces ~4-fold fewer cleavable complexes. Protein-linked DNA strand breaks induced by imidazoacridinones are rapidly reversed, which is a typical property of topoisomerase II-mediated DNA damage. In addition, the pattern of DNA damage induced by purified topoisomerase II in the presence of imidazoacridinones was very similar to that obtained for *m*-AMSA (Fig. 5). Therefore, the high toxicity of the cleavable complexes induced by these compounds can be related to neither an unusual persistence of DNA damage, as has been proposed for mitoxantrone (25), nor an inherently different selectivity toward DNA sequences.

Imidazoacridinones share some structural features with both mitoxantrone (side chain) and *m*-AMSA (acridine core). The structural similarities are reflected in the induction of DNA cleavage *in vitro*. Autoinhibition of topoisomerase II-associated DNA damage was observed at high concentrations (100 μM) for both imidazoacridinones and mitoxantrone but not for *m*-AMSA. On the other hand, the pattern of DNA breaks induced by imidazoacridinones is similar to that observed for *m*-AMSA, which suggests that it is probably the planar chromophore moiety that directs the sequence specificity of DNA cleavage by topoisomerase II in the presence of DNA intercalators. However, a major difference between imidazoacridinones and *m*-AMSA is that the latter drug has little activity toward solid tumors, which is in direct contrast to imidazoacridinones. It is interesting to note that topoisomerase-mediated damage observed for imidazoacridinones was preferentially induced in the MAR of SV40 DNA. It is therefore possible that these compounds specifically target DNA MARs, as has been suggested for certain other cleavable complex-inducing topoisomerase II inhibitors (26).

The cytotoxic effects of anticancer drugs are usually determined toward cells growing in suspension culture or in two dimensions on tissue culture plates. However, such results may not necessarily be representative of the *in vivo* situation, where most solid tumors grow as multicellular spheroids in three dimensions (27). Compared with two-dimensional cultures, cells growing in multicellular spheroids have a slower cell generation time and a different cell cycle distribution. In addition, three-dimensional cultures have a different cytoarchitecture and cell/cell interactions. We therefore compared the effects of C-1310 and C-1311 on cells growing in either two or three dimensions. Remarkably, the results show that these compounds are almost as potent toward cells growing in spheroids as toward cells growing as monolayer cultures. In contrast, other topoisomerase II inhibitors were 3–4-fold less active toward cells growing in spheroids. These results may, at least in part, explain the activity of C-1310 and C-1311 toward solid tumors *in vivo*. Taken together with the high cytotoxicity of C-1310- and C-1311-stabilized cleavable complexes, our results may also suggest that these imidazoacridinones affect topoisomerase II in a manner different from that of other cleavable complex inducers or, alternatively, that these drugs act preferentially on only one of the two topoisomerase II isoforms.

The comparative structure-activity data suggest that a hydroxyl group at position 8 of the chromophore ring, which is essential for biological activity of imidazoacridinones, is usually, but not always, associated with inhibitory activity against topoisomerase II *in vitro*. However, the position of this group is important for the inhibitory effect of imidazoacridinones *in vivo* cells because a compound with the hydroxyl group at position 9 (C-1419) was unable to stimulate DPCs, as measured with the KCl/SDS coprecipitation assay. On the other hand, the chemical structure of the side chain of these compounds, specifically a number of methylene groups in the aminoalkyl moiety, also plays a role in the interaction between topoisomerase II and imidazoacridinones because C-1492, a close analogue of C-1311 with a long aminoalkyl side chain, was inactive toward topoisomerase II both *in vitro* and *in vivo*. It is not clear why C-1419 is not effective against topoisomerase II in whole cells while it is as potent as biologically active imidazoacridinones when tested *in vitro*. We propose at least two explanations for this phenomenon. First, in our *in vitro* tests, we used yeast enzyme, which may have

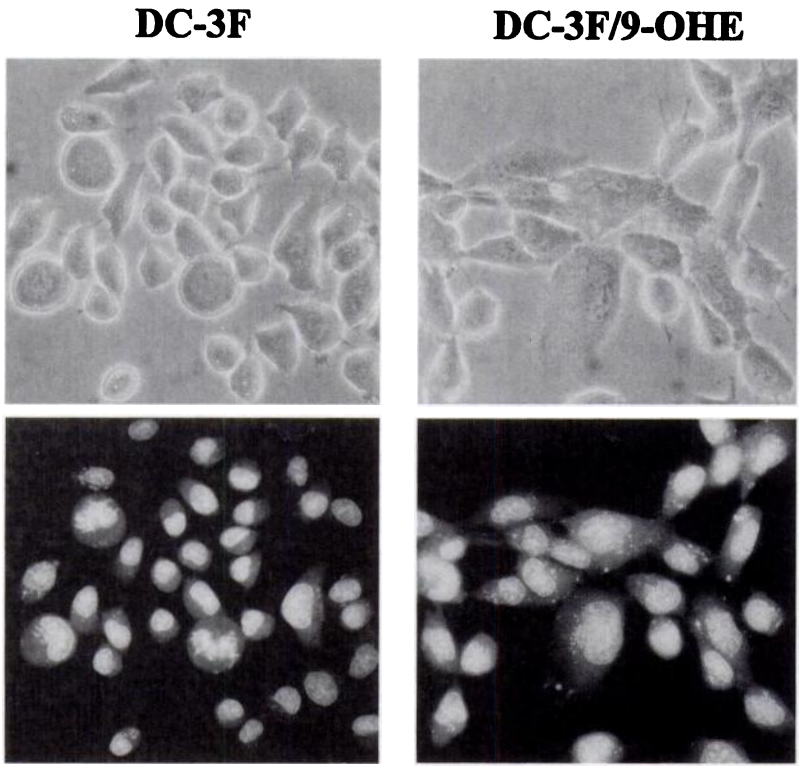


Fig. 6. Subcellular distribution of C-1311 in sensitive DC-3F and DC-3F/9-OHE cells resistant to topoisomerase II inhibitors. The cells were incubated with 1 μg/ml for 30 min at 37° and viewed with the use of phase contrast and fluorescence microscopy. Original magnification, 400×.

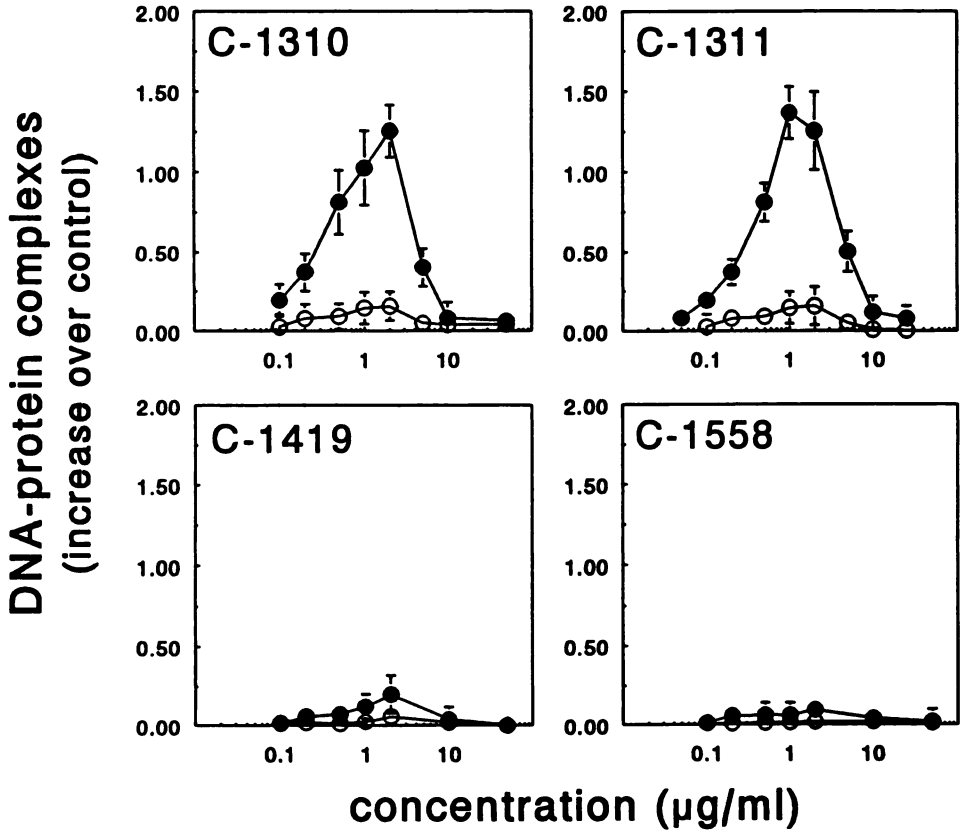


Fig. 7. DPC formation induced by selected imidazoacridinones in sensitive DC-3F (●) and DC-3F/9-OHE (○) cells resistant to topoisomerase II inhibitors. The DNA and protein were radiolabeled, and the cells were treated with the indicated concentrations of the drugs for 1 hr at 37°. Data are expressed as the amount of radiolabeled DNA precipitated with the cellular protein. Each point is the average of three individual experiments, each done in duplicate. *Bars*, standard deviation.

substrate specificity that is different than that of hamster. There is a high degree of heterogeneity between the carboxyl-terminal regulatory region of topoisomerase II from different eukaryotic cells (28), and this might explain why a compound that is inhibitory toward yeast topoisomerase II *in vitro* could be inactive against the hamster enzyme. It is also possible that imidazoacridinones interact with cellular topoisomerase II after metabolic activation by cellular enzymes. Imidazoacridinones have been shown to undergo enzymatic oxidation *in vitro* to a putative imino-quinoid structure (29). In contrast

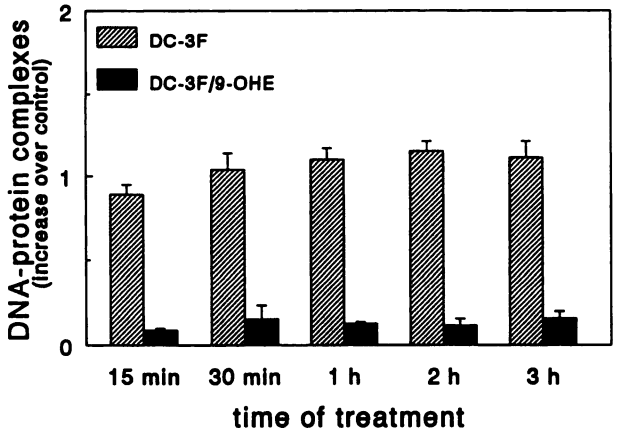


Fig. 8. Kinetics of DPC formation induced by C-1311 in sensitive DC-3F and DC-3F/9-OHE cells resistant to topoisomerase II inhibitors. The cells were exposed to the drug at 1 μg/ml for different times, and the number of DPCs were determined with the KCl/SDS coprecipitation assay. Each point is the average of three individual experiments, each done in duplicate. *Bars*, standard deviation.

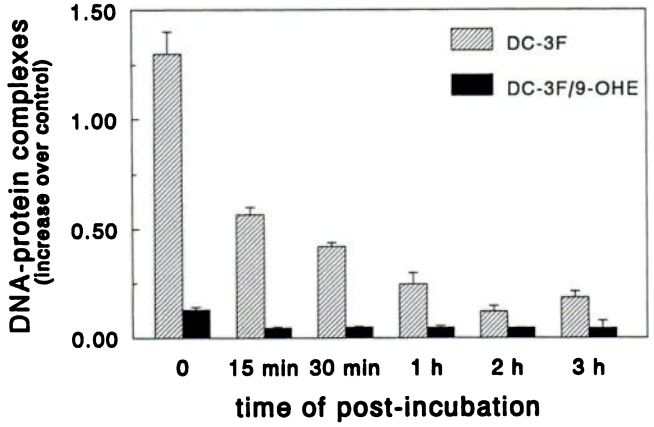


Fig. 9. Reversibility of DPC formation induced by C-1311 in sensitive DC-3F and DC-3F/9-OHE cells resistant to topoisomerase II inhibitors. The cells were exposed to C-1311 at 1 μg/ml for 1 hr and the number of DPCs were determined with the KCl/SDS coprecipitation assay after drug removal and incubation in drug-free medium for the times indicated. *Points*, average of three individual experiments, each done in duplicate. *Bars*, standard deviation.

to all biologically active imidazoacridinones, this imino-quinoid derivative cannot be formed in C-1419 because there is no hydroxyl group in a *para* position to a nitrogen atom in the acridinone moiety of this compound.

In conclusion, we have shown that biologically active imidazoacridinones inhibit the catalytic activity of topoisomerase II *in vitro* and in living fibrosarcoma cells. Like other classic topoisomerase II inhibitors, these compounds stabilize cleavable complex formation between topoisomerase II and DNA both with purified enzyme *in vitro* and in whole cells. The more cytotoxic compounds were also the most potent topoisomerase inhibitors. Imidazoacridinones show reduced activity against cells resistant to classic topoisomerase II inhibitors. A unique feature of the imidazoacridinones is their activity toward cells growing in three dimensions as multicellular spheroids. The latter effect may be responsible for the activity of these drugs toward solid tumors. Taken together, the presented data suggest that DNA topoisomerase II is a major cellular target of biologically active imidazoacridinones.

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