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The Antitumor Triazoloacridone C-1305 Is a Topoisomerase II Poison with Unusual Properties

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ABSTRACT

C-1305 [S-[[3-(dimethylamino)propyl]amino]-8-hydroxy-6H-v-triazolo[4,5,1-*de*]acridin-6-one] is a triazoloacridone with excellent activity in colon cancer models. The mechanism of C-1305 is unknown, although similarities in the chemical structure between C-1305 and amsacrine suggest common cellular targets. Here, we report that C-1305 is a topoisomerase II poison that is able to induce cleavable complexes with topoisomerase II in vitro as well as in living cells. Even at optimal concentrations, C-1305 is a much weaker inducer of cleavable complexes than amsacrine. Because the cytotoxic activities of the two compounds after continuous drug exposure are comparable, these findings suggest that the low levels of cleavable complexes induced by C-1305 may be unusually toxic. In contrast to amsacrine, the cytotoxicity of C-1305 is strongly time-dependent, with at least 24 h of drug exposure required for optimal cytotoxicity. The p53 tumor suppressor is inactivated in the majority of human tumors, including colorectal cancers. We therefore compared the long-term cytotoxic effects of C-1305, amsacrine, and doxorubicin on human cell lines in which the p53 or p21 pathways have been specifically disrupted by targeted homologous recombination. Disruption of p53 and p21 had minor influence on the cytotoxicity of doxorubicin, whereas p53 but not p21 disruption was associated with increased resistance to amsacrine. In marked contrast, disruption of p53 and p21 was associated with increased sensitivity to C-1305. Taken together, our results show that exposure to C-1305 is accompanied by the formation of low levels of potent cleavable complexes that are selectively toxic toward tumor cells with defective p53 function.

Despite great efforts, the development of curative anticancer agents has been only partially successful, especially for the treatment of the frequent carcinomas of the lung, breast, and colon. In a search for new acridine-based antineoplastic agents, a series of triazoloacridones with potent antitumor activities were developed at the Gdansk University of Technology in Poland (Cholody et al., 1990). The most active triazoloacridone derivative, C-1305, showed strong activity toward colon carcinomas in animal models, which in most cases was associated with complete tumor regression (Kusnierczyk et al., 1994).

The development of a new, very active anticancer agent raises important questions about its mechanism of action. In addition to the fundamental aspect, such studies are of practical value because they may provide information concerning optimal drug exposure and suitable drug combinations and could result in the development of even more active derivatives. The structural similarity of C-1305 to known topoisomerase II inhibitors such as amsacrine, mitoxantrone, and the C-1311 imidazoacridinone characterizes it as a potential topoisomerase II inhibitor (for chemical structures, see Fig. 1).

DNA topoisomerase II is an essential nuclear enzyme that regulates DNA topology and organization (Wang, 2002). The enzyme modulates nuclear architecture and 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 both during interphase and in mitotic chromosomes (Razin et al., 1991; Escargueil et al., 2001; Larsen et al., 2003b). Anticancer drugs targeting topo-

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ABBREVIATIONS: C-1305, 5-[[3-(dimethylamino)propyl]amino]-8-hydroxy-6H-v-triazolo[4,5,1-*de*]acridin-6-one; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (amsacrine); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ABC, ATP-binding cassette; MDR, multidrug resistance.

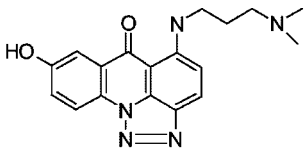
isomerase II are among the most effective cytotoxic agents currently available for cancer therapy (Hande, 1998). Most clinically active topoisomerase II inhibitors are so-called topoisomerase II poisons that reversibly stabilize covalent complexes (cleavable complexes) between DNA and topoisomerase II (Wilstermann and Osheroff, 2003). Although it is well established that cleavable complex formation is a crucial step in the cytotoxic activity of these agents, the additional series of events resulting from cleavable complex formation and culminating in cell death are still poorly understood (Larsen and Skladanowski, 1998).

We have determined the influence of C-1305 on topoisomerase II in vitro and in human tumor cells in comparison with amsacrine and the closely related C-1533 triazoloacridone derivative, which has no antitumor activity. The results show that C-1305 is a topoisomerase II poison with many original features compared with other agents of this class.

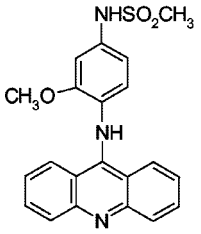
Materials and Methods

Drugs and Chemicals. Amsacrine (mAMSA) and the C-1305 and C-1533 triazoloacridone derivatives were synthesized by Barbara Horowska in the Department of Pharmacological Technology and Biochemistry at Gdansk University of Technology (Poland). Daunorubicin and doxorubicin were purchased from Farmitalia (Milan, Italy), and ICRF-187 (Cardioxane) was obtained from Chiron BV (Amsterdam, the Netherlands). C-1305 and C-1533 (free bases) were prepared as 10 mM stock solutions in 0.2% lactic acid (v/v in water), doxorubicin and daunorubicin (HCl salts) were prepared as 10 mM stock solutions in water, and amsacrine was prepared as a 10 mM stock solution in dimethyl sulfoxide. Stock solutions were kept at −20°C until use. ICRF-187 was prepared as a 10 mM stock solution in water and used immediately. Proteinase K was from Merck (Darmstadt, Germany). [α-^{32}P]dATP (3000 Ci/mmol), [^{3}H]thymidine (90 Ci/mmol), and [^{14}C]leucine (300 mCi/mmol) were purchased from Amersham Biosciences AB (Uppsala, Sweden). All other reagents were of analytical grade.

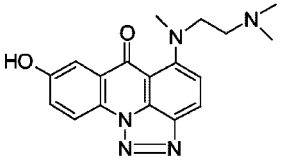
DNA Substrates and Enzymes. Supercoiled plasmid pBR322 DNA (>95% form I), Klenow fragment DNA polymerase, EcoRI, and HindIII were purchased from Promega (Madison, WI). Yeast DNA topoisomerase II was isolated from *Saccharomyces cerevisiae* overexpressing a multicopy plasmid (a generous gift from James Wang, Cambridge, MA) and purified as described previously (Worland and Wang, 1989; Escargueil et al., 2000).



C-1305



m-AMSA



C-1533

Fig. 1. Chemical structures of amsacrine and the triazoloacridones C-1305 and C-1533.

Relaxation Assay. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 mM KCl, 1 mM ATP, and 200 ng of pBR322 DNA. The reaction was initiated by the addition of DNA topoisomerase II and allowed to proceed at 30°C for 15 min. Reactions were terminated by the addition of loading buffer (0.1% SDS, 0.05% bromphenol blue, 2.5 mM EDTA, and 10% sucrose, final concentrations). The samples were electrophoresed in 1.2% agarose gels at 0.5 V/cm for 18 h 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 were photographed under UV illumination as described previously (Bojanowski et al., 1992).

Formation of Cleavable Complexes In Vitro. The experimental conditions were the same as for the relaxation assay except that approximately 50-fold more DNA topoisomerase II was used. The reaction was initiated with the addition of the enzyme. After 10 min at 30°C, the reactions were terminated by adding 0.35% SDS and 0.3 mg/ml proteinase K (final concentrations) followed by incubation at 56°C for 1 h. The DNA cleavage products were separated by electrophoresis in 1.2% agarose gels containing ethidium bromide (0.5 μg/ml) at 1 V/cm for 18 h in TBE buffer and were visualized and photographed under UV illumination as described previously (Larsen et al., 1993).

Topoisomerase II-Induced Cleavage of Linear pBR322. pBR322 plasmid DNA was linearized with EcoRI and end-labeled with Klenow fragment and [α-^{32}P]dATP. The labeled DNA was then subjected to a second digestion with HindIII, the fragments obtained were separated by agarose gel electrophoresis, and the larger fragment was used for DNA cleavage assays. Reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, ~2.5×10^4 cpm (~50 ng) 3′-end–labeled pBR322 DNA, and the indicated drug concentrations. The reactions were initiated by adding DNA topoisomerase II (75 ng) and were allowed to proceed for 10 min at 30°C. Reactions were terminated by the addition of SDS, proteinase K, and EDTA (final concentrations of 0.35%, 0.3 mg/ml, and 15 mM, respectively) and incubated for 1 h at 56°C. Loading buffer was added, and samples were electrophoresed in 1.2% agarose gels containing 0.1% SDS with TBE as a running buffer at 2 V/cm for 18 h. Gels were dried and autoradiographed with Hyperfilm MP (Amersham Biosciences) for 1 to 2 days.

Cell Lines. HL-60 leukemia and DC-3F fibrosarcoma cell lines have been characterized in detail previously (Larsen and Jacquemin-Sablon, 1989; Côme et al., 1999). A549 lung and DLD-1 colon carcinoma cells were purchased from American Type Culture Collection (Manassas, VA), and HT-29 colon carcinoma cells were provided by Richard Camalier (Division of Cancer Treatment and Diagnosis tumor repository, National Cancer Institute, Bethesda, MD). HCT-116 colon carcinoma cells and its p53−/− and p21−/− variants (Bunz et al., 1998) were a generous gift from Bert Vogelstein (Baltimore, MD). NIH3T3 parental cells and NIH3T3-MDR-G185 transfectants overexpressing the human MDR1 gene (Cardarelli et al., 1995) were kindly provided by Carol Cardarelli (National Institutes of Health, Bethesda, MD). NIH/7M wild-type cells transfected with empty vector and NIH/32 transfectants overexpressing the human MRP1 gene (Breuninger et al., 1995) were a kind gift from Gary Kruh (Philadelphia, PA).

The cells were maintained in minimal essential medium (DC-3F), McCoy's A (HCT-116, HCT-116/p21−/−, and HCT-116/p53−/−), RPMI 1640 medium (HL-60, A549, and DLD-1), or Dulbecco's modified Eagle's medium (HT-29, NIH3T3, NIH3T3-MDR-G185, NIH/7M, and NIH/32) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2/air atmosphere (RPMI 1640, minimal essential medium, and McCoy's A) or 10% CO_2/air atmosphere (Dulbecco's modified Eagle's medium). All cell lines were screened routinely for *Mycoplasma* species by the polymerase chain reaction

method with Mycoplasma Plus PCR Primer Set (Stratagene, La Jolla, CA). NIH3T3-MDR-G185 cells were maintained in the presence of 60 ng/ml colchicine, whereas NIH/32 cells were grown in the presence of 750 μg/ml G418. All resistant cell lines were kept in drug-free medium for at least 1 week before each experiment.

Cytotoxicity Assays. The cytotoxicity was determined by the MTT assay as described previously (Poindessous et al., 2003). In brief, exponentially growing cells were continuously exposed to different drug concentrations, and the cellular viability was determined after three to four generation times. Cells were exposed to the MTT tetrazolium salt for 4 h at 37°C, and the formation of formazan was measured by a microplate reader. The concentrations required to inhibit cell growth by 50% compared with untreated controls were determined from the curves plotting survival as a function of dose by use of SlideWrite software (Advanced Graphics Software, Inc., Encinitas, CA). All values are averages of at least two independent experiments, each done in duplicate.

The liquid overlay system was used to generate spheroids from DC-3F cells as described previously (Kobayashi et al., 1993). Under these conditions, cells are unable to attach to the tissue plates that are covered by 1% agarose and grow as multicellular spheroids in three dimensions. The comparison between cells growing in monolayer culture and in multicellular spheroids was determined after 72 h of continuous drug exposure followed by counting of viable cells as described earlier (Skladanowski et al., 1996). All values are averages of at least two independent experiments, each done in duplicate.

The colony formation assay was used to compare cellular survival after 3 h, 24 h, or continuous drug exposure. Exponentially growing cells (250 to 500) were plated in 60-mm Petri dishes and incubated at 37°C for 24 h before drug treatment. Cells were treated with C-1305 or amsacrine for 3 or 24 h followed by postincubation in drug-free media for 5 to 7 days. Otherwise, cells were grown in the continuous presence of drugs for the entire incubation period. Colonies were washed with 0.9% NaCl, fixed with 100% ethanol, and stained with crystal violet, and colonies with more than 50 cells were counted. All values are averages of at least two independent experiments, each done in triplicate.

Topoisomerase II-Associated DNA Damage in Intact Cells. DNA/protein complexes were quantified by the KCl/SDS coprecipitation assay as described previously (Zwelling et al., 1991). DNA and proteins of HT-29 cells (approximately 300,000 cells) were radiolabeled with 0.6 μCi/ml [^{3}H]thymidine and 0.2 μCi/ml [^{14}C]leucine, respectively, for 24 h. Radiolabeled cells were exposed to various drug concentrations at 37°C for the indicated times. 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°C for 15 min. The lysates were then adjusted to 65 mM KCl, vortexed for 10 s, 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°C before being dissolved in 0.5 ml of water and mixed with 5 ml of scintillation fluid for determination of radioactivity. Data are expressed as the ratio of [^{3}H]DNA to [^{14}C]protein, with protein being an internal standard for the exact number of cells used.

Pulse-Field Gel Electrophoresis. For DNA fragmentation studies, exponentially growing HT-29 cells were exposed to the indicated drug concentrations for 3 h. After incubation, cells were harvested, and approximately 4×10^6 cells/sample were embedded in low-melting agarose (final concentration, 0.75%). Plugs were treated with lysing buffer A (25 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8.0, and 1% SDS) containing 1 mg/ml proteinase K for 1 h at 37°C. An equal volume of lysing buffer B (25 mM Tris-HCl, 0.4 M EDTA, pH 8.0, and 1% SDS) was added, and lysis continued overnight at 56°C. After lysis, plugs were washed three times for 30 min in final wash buffer (10 mM Tris-HCl, pH 8.0, and 50 mM EDTA, pH 8.0) and embedded in 1% pulse-field gel electrophoresis agarose (Seakem Gold agarose; Cambrex Bio Science Walkersville, Inc., Walkersville, MD). DNA was separated in a CHEF-DRII apparatus (Bio-Rad, Hercules, CA) in 0.5× TBE (45 mM Tris-borate and 0.5 mM EDTA, pH 8.0) for 24 h at 14°C (180 V, 30- and 60-s initial and final pulses, respectively, with linear ramp). After electrophoresis, gels were stained with 0.5 μg/ml ethidium bromide to visualize the DNA and were photographed under UV illumination. Molecular weight standards (50 kilobase pair λ ladder) were from Cambrex Bio Science Rockland, Inc. (Rockland, ME).

Results

Inhibition of the Catalytic Activity of Topoisomerase II In Vitro. The relaxation of supercoiled plasmid DNA by topoisomerase II was studied in the presence of different concentrations of C-1305 or C-1533. C-1305 diminished the relaxation reaction by 50% at a concentration of 2.5 μM and totally inhibited the topoisomerase II-mediated relaxation at 10 μM (Fig. 2, left). The closely related C-1533 compound also inhibited the catalytic activity of topoisomerase II, but to a lesser extent. Approximately 5 μM C-1533 was required to inhibit the relaxation reaction by 50%, whereas 25 μM was needed to completely inhibit the catalytic activity (Fig. 2, right).

Induction of Cleavable Complexes In Vitro. The biologically active C-1305 triazoloacridone is able to stabilize the formation of cleavable complexes between topoisomerase II and pBR322 plasmid DNA (Fig. 3A). Whatever the concentration, C-1305 induced less cleavable complexes than 10 μM amsacrine. The cleavable complex formation was dose-dependent, reaching a maximum in the presence of 2.5 to 10 μM C-1305. A similar biphasic dose response has previously been reported for the structurally related anthracenediones and imidazoacridones and is characteristic of compounds that are strong DNA binders (Zwelling et al., 1991; Skladanowski et al., 1996).

The capacity of the biologically inactive C-1533 triazoloacridone to stabilize cleavable complexes was also determined (Fig. 3B). In contrast to C-1305, the structurally similar C-1533 compound is not able to stabilize cleavable complexes between topoisomerase II and DNA. Therefore, C-1305 can be considered to be a topoisomerase II poison, whereas C-1533 is a catalytic topoisomerase II inhibitor.

Topoisomerase II-Induced Cleavage of pBR322 DNA. The pattern of DNA cleavage induced by C-1305 in pBR322 plasmid DNA was compared with the pattern induced by amsacrine (Fig. 4). The results show that the degree of topoisomerase II-mediated DNA cleavage was much lower for C-1305 than for amsacrine. However, at comparable levels of

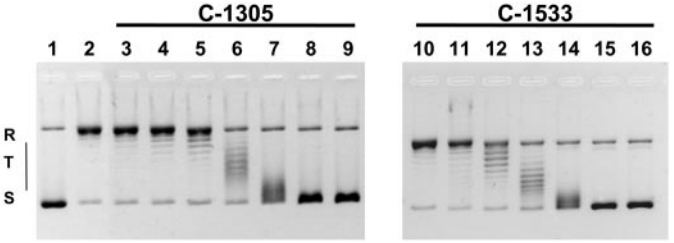


Fig. 2. Inhibition of the catalytic activity of purified DNA topoisomerase II by triazoloacridones C-1305 and C-1533 as measured by relaxation. Supercoiled pBR322 plasmid DNA (lane 1) was relaxed by purified topoisomerase II in the absence (lane 2) or presence of C-1305 at 0.25, 0.5, 1, 2.5, 5, 10, or 25 μM (lanes 3–9) or with C-1533 at 0.5, 1, 2.5, 5, 10, 25, or 50 μM (lanes 10–16). The resulting topological forms of DNA were separated by gel electrophoreses. S, supercoiled DNA; R, relaxed DNA; T, DNA topoisomers. Data shown are typical of three independent experiments.

DNA cleavage, no obvious differences in cleavage site preference were observed between the two compounds.

The Influence of ICRF-187 on the Cytotoxicity of C-1305. Coincubation of cells with a topoisomerase II poison and a catalytic topoisomerase II inhibitor is associated with decreased cleavable complex formation and reduced cytotoxicity (Larsen et al., 2003a). To determine the importance of topoisomerase II as a target for C-1305 in living cells, HT-29 cells were incubated with C-1305, C-1533, and, as controls, amsacrine and doxorubicin in the absence or presence of the catalytic topoisomerase II inhibitor ICRF-187 (Table 1). The presence of ICRF-187 (100 mM) reduced the cytotoxicity of amsacrine almost 7-fold, whereas the cytotoxic effects of C-1305 and doxorubicin were both reduced 2- to 3-fold. In contrast, ICRF-187 had no influence on the cytotoxicity of C-1533.

Formation of DNA/Topoisomerase II Complexes in Living Cells. The ability of C-1305 to induce cleavable complexes in living cells was determined by the KCl/SDS coprecipitation assay (Fig. 5A). The results show a biphasic relationship between drug concentration and the levels of DNA/protein complexes, similar to the in vitro findings with purified topoisomerase II (Fig. 3). The highest levels of DNA/protein complexes were observed at concentrations of C-1305 ranging from 2.5 to 10 μM, whereas concentrations higher than 10 μM were associated with increasing autoinhibition of cleavable complex formation. In comparison, only background levels of DNA/protein complexes were observed for the structurally similar C-1533 compound at all concentrations studied (Fig. 5A). The striking similarity between the in vitro results (Fig. 3) and the findings observed for living cells (Fig. 5A) indicates that the in vivo differences in the levels of cleavable complexes induced by amsacrine or by C-1305 cannot be attributed to differential drug accumulation.

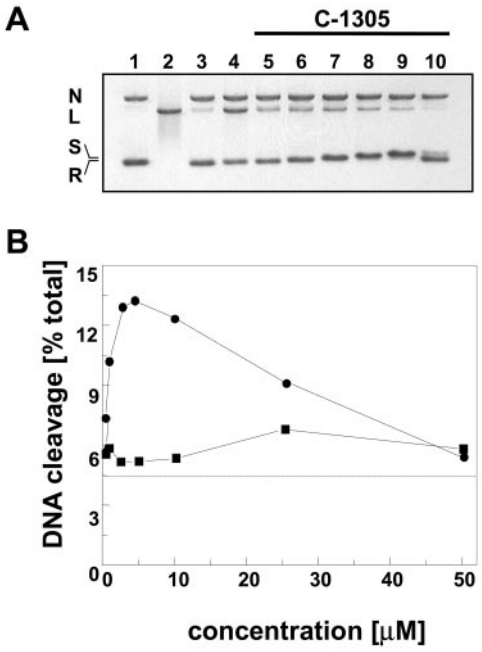


Fig. 3. The influence of C-1305 and C-1533 on the formation of covalent DNA-topoisomerase II complexes. A, supercoiled pBR322 plasmid DNA (lane 1) was incubated with purified topoisomerase II in the absence (lane 3) or in the presence of 10 μM amsacrine (lane 4) or with 1, 2.5, 5, 10, 25, and 50 μM C-1305 (lanes 5–10). The resulting DNA/topoisomerase II complexes were digested by proteinase K, and the different topological forms of DNA were separated by agarose gel electrophoresis in the presence of ethidium bromide. Lane 2, linearized pBR322 DNA. S, supercoiled DNA; R, relaxed DNA; L, linear DNA; N, nicked circular DNA. Data shown are typical of two independent experiments. B, topoisomerase II-mediated DNA cleavage in the presence of C-1305 and C-1533. Plasmid DNA and purified topoisomerase II were incubated with different concentrations of C-1305 or C-1533, and the formation of linear DNA determined by gel densitometry. Data shown are the averages of two independent experiments. ●, cleavage induced by C-1305; ■, cleavage induced by C-1533. The dotted line indicates the background levels of cleavable complexes formed in the absence of drug.

Next, the formation of DNA/protein complexes was determined as a function of exposure time (Fig. 5B). The results show that the maximal formation of DNA/protein complexes was obtained after 1 h of drug exposure in the case of amsacrine and after 2 h of drug exposure for C-1305. Further drug exposure did not alter the levels of DNA/protein complexes for either amsacrine or C-1305.

Size Distribution of DNA/Topoisomerase II Complexes in Living Cells. To compare the topoisomerase II interaction of

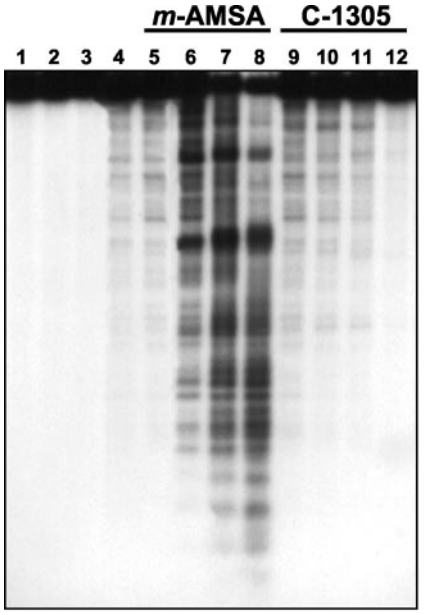


Fig. 4. DNA cleavage patterns induced by topoisomerase II in the presence of amsacrine and C-1305. pBR322 DNA was linearized, uniquely 3′-labeled (lane 1), and incubated in the presence of 100 μM amsacrine (lane 2), 100 μM C-1305 (lane 3), or with topoisomerase II in the absence (lane 4) or presence of 0.1, 1, 10, and 100 μM amsacrine (lanes 5 to 8) or 0.1, 1, 10, and 100 μM C-1305 (lanes 9–12). Data shown are typical of two independent experiments.

TABLE 1

Cytotoxicity of C-1305 and other topoisomerase II inhibitors toward HT-29 carcinoma cells in the presence or absence of the catalytical topoisomerase II inhibitor ICRF-187

Cells were incubated with the indicated compounds for 3 h in the absence or presence of ICRF-187 (100 μM) followed by postincubation in drug-free medium for three to four generation times, and the cytotoxicity was determined by MTT. All values are the average of at least two independent experiments, each done in duplicate.

Compound	IC_{50}[a]		Δ[b]
	−ICRF	+ICRF (100 μM)	
	μM		
C-1305	9.9	23.7	2.4
C-1533	46.5	44.4	0.9
mAMSA	3.9	27.2	6.9
Doxorubicin	0.7	2.0	2.8

[a] Drug concentration causing 50% loss of cell viability compared with untreated controls.

[b] Ratio between IC_{50} concentrations for cells preincubated with or without ICRF-187.

C-1305 and amsacrine in living cells, HT-29 colon cancer cells were incubated with different concentrations of the two compounds for 3 h followed by cell lysis and treatment with proteinase K to reveal the protein-associated DNA strand breaks. Pulse-field electrophoresis revealed that C-1305 exposure was associated with the formation of very-high-molecular-weight fragments migrating at the same position as the compression band (Fig. 6). The size of these fragments is estimated to be at least 1 million base pairs. Amsacrine exposure was also associated with the formation of very-high-molecular-weight DNA fragments. In addition, concentrations greater than 2.5 μM amsacrine were accompanied by the formation of 50- to 350-kilobase pair DNA fragments corresponding to the size of chromatin loops (Razin et al., 1991).

Time Dependence of the Cytotoxic Effect. Because the maximal formation of both amsacrine and C-1305–induced cleavable complexes in living cells was much slower than what has been described earlier for other topoisomerase II inhibitors such as the structurally related C-1311 imidazoacridinone compound (Skladanowski et al., 1996), we wished to establish the influence of incubation time on the cytotoxic activities of the two compounds. Unexpectedly, the time dependence was entirely different for C-1305 and amsacrine (Fig. 7). Continuous drug exposure was associated with comparable activity for C-1305 and amsacrine, with IC_{50} values of 47 versus 38 nM, 85 versus 85 nM, and 180 versus 150 nM toward A549, DLD-1, and HT-29 cells, respectively. In marked contrast, comparison of IC_{50} values for amsacrine and C-1305 showed that amsacrine was up to 37 times more toxic than C-1305 after 3 h of drug exposure and up to 6 times more toxic after 24 h of drug exposure. These results show that the optimal conversion of the C-1305–induced cleavable complexes into lethal lesions requires prolonged drug exposure. The results also indicate that C-1305 is still cytotoxic to tumor cells even after 24 h of incubation at 37°C.

Activity toward Multicellular Spheroids. An important difference between tissue culture conditions and tumor growth is that tissue culture usually is carried out with cells growing in two dimensions, whereas tumor growth occurs in three dimensions (Sutherland, 1988). We therefore compared the cytotoxic effects of C-1305 and other topoisomerase II inhibitors on DC-3F cells growing in three dimensions as multicellular spheroids or in two dimensions as monolayer cultures. The cell-cycle distribution of cells growing in spheroids was 65, 22, and 13% for cells in G_1, S, or G_2/M, respectively, whereas the cell-cycle distribution of monolayer cultures was 35, 55, and 10% for cells in G_1, S, and G_2/M, respectively (data not shown). The results show that cells

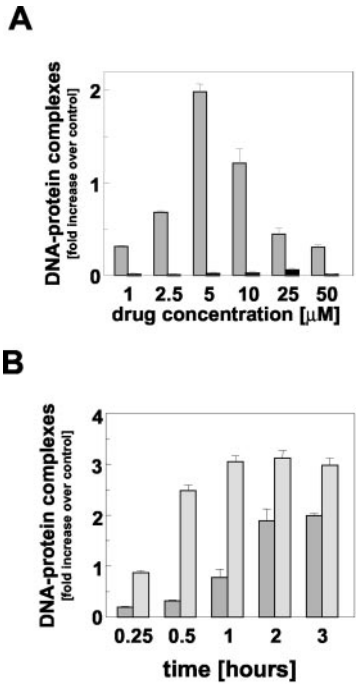


Fig. 5. Induction of DNA/protein complexes (DPC) in HT-29 cells by C-1305, C-1533, and amsacrine. A, the DNA and protein were radiolabeled, and the cells were treated with the indicated concentrations of C-1305 (▨) and C-1533 (■) for 3 h at 37°C. B, kinetics of DPC formation in HT-29 cells after exposure to 5 μM C-1305 (dark gray bars) or 2.5 μM amsacrine (light gray bars) for the indicated times. Data are expressed as the amount of radiolabeled DNA precipitated with the cellular protein in drug-treated cells compared with untreated control cells. Each point is the average of three independent experiments, each done in duplicate. Bars, S.D.

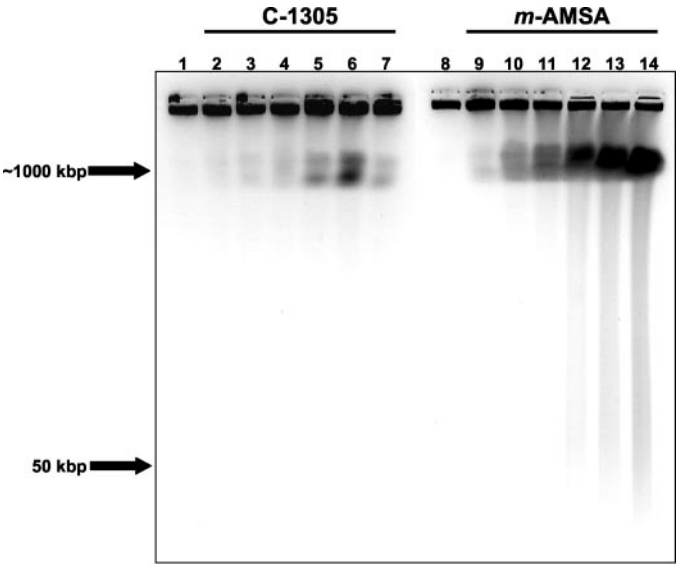


Fig. 6. Induction of DNA cleavage in HT-29 cells by C-1305 and amsacrine as determined by pulse-field electrophoresis. Cells (untreated controls, lanes 1 and 8) were exposed to 1, 2.5, 5, 10, 25, and 50 μM C-1305 (lanes 2–7) and 0.1, 0.25, 0.5, 1, 2.5, and 5 μM amsacrine (lanes 9–14) for 3 h followed by cell lysis and pulse-field gel electrophoresis. Data shown are typical of two independent experiments.

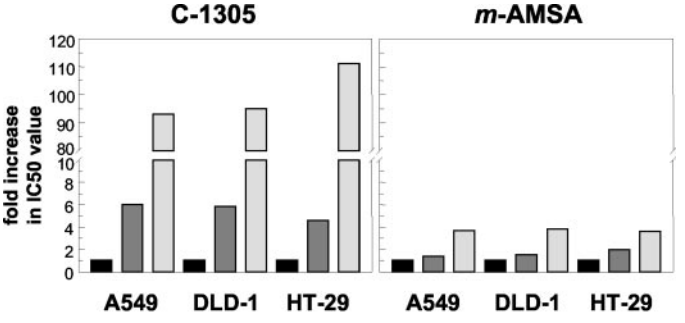


Fig. 7. Time dependence of the cytotoxicity of C-1305 and amsacrine toward A549, DLD-1, and HT-29 carcinoma cells as determined by colony formation assays after 3 h (light gray bars), 24 h (dark gray bars), or continuous drug exposure (black bars). Cells were exposed to C-1305 or amsacrine for 3 or 24 h followed by postincubation in drug-free media for 5 to 7 days. Otherwise, cells were grown in the continuous presence of drugs for the entire incubation period.

present in the slower growing, three-dimensional spheroids are approximately 3-fold more resistant to C-1305 and doxorubicin and up to five times more resistant to etoposide and amsacrine (Table 2). These results suggest that the cytotoxicity of C-1305 is not limited to rapidly dividing cells and is not strictly dependent on the cell-cycle distribution.

Activity toward Cells Overexpressing the MDR1 and MRP1 ABC Drug Transporters. Overexpression of the MDR1 or MRP1 ABC drug transporters are associated with resistance to most topoisomerase II inhibitors. We compared the cytotoxic effects of C-1305, C-1533, amsacrine, and daunorubicin toward parental NIH3T3 cells and NIH3T3 transfectants overexpressing either the MDR1 or MRP1 drug transporters (Table 3). Overexpression of MDR1 was associated with 2- to 3-fold resistance to C-1305 and amsacrine compared with approximately 10-fold resistance to daunorubicin. Overexpression of MRP1 was associated with 2- to 3-fold resistance to C-1305 and amsacrine compared with approximately 20 times resistance to daunorubicin. Thus, C-1305 is a poor substrate for both drug transporters. In comparison, C-1533 showed modest toxicity toward all the cell lines with no clear differences in the activities toward transfected and parental cells.

Influence of p53 and p21 Status. It has been reported that loss of p53 function is associated with increased resistance to topoisomerase II inhibitors (Bunz et al., 1998). We have compared the influence of C-1305, amsacrine, and doxorubicin toward parental HCT-116 human colon adenocarcinoma cells and the HCT-116 p53−/− and HCT-116 p21−/− sublines, in which the p53 or p21 genes have been deleted by homologous recombination. It was unexpected that the three topoisomerase II inhibitors showed a different activity spectrum toward the different HCT-116 sublines (Fig. 8). For amsacrine, essentially no differences were observed between parental and p21−/− cells, whereas loss of p53 function was accompanied by approximately 4-fold resistance. In comparison, loss of p53 function was only associated with slightly increased resistance to doxorubicin, whereas loss of p21 had no obvious effect on the sensitivity. In marked contrast, loss of p53 sensitized the cells 2- to 3-fold to C-1305, whereas loss of p21 was accompanied by almost 6-fold increased sensitivity.

TABLE 2

Cytotoxicity of C-1305 and other topoisomerase II inhibitors toward DC-3F cells growing in two dimensions as monolayer cultures or in three dimensions as multicellular spheroids

Cells were incubated with the indicated compounds for three to four generation times, and the cytotoxicity was determined by cell counting. All values are the average of at least two independent experiments, each done in duplicate.

Compound	IC_{50}[a]		Δ[b]
	Monolayer	Spheroids	
	nM		
C-1305	27	91	3.4
C-1533	3217	4090	1.3
mAMSA	6.2	31	5.0
Etoposide	70	297	4.3
Doxorubicin	5.0	14	2.7

[a] Concentration causing 50% cell growth inhibition.
[b] Ratio between IC_{50} values of cells growing in multicellular spheroids and cells growing in monolayer cultures.

Discussion

This study was undertaken to elucidate the mechanism of action of C-1305, a triazoloacridone derivative with potent activity in colon cancer models. The structural similarity of C-1305 to amsacrine, mitoxantrone, and the C-1311 imidazoacridone suggested that the cytotoxic and antitumor effects of C-1305 might be caused by interaction with DNA topoisomerase II. For these experiments, we selected three compounds that are structurally related but have different biological activities. Besides C-1305, we included amsacrine, a classic topoisomerase II poison, and the C-1533 derivative, which is very similar structurally to C-1305 but has no antitumor activity. We found, that both C-1305 and C-1533 inhibited the catalytic activity of topoisomerase II in vitro. However, only C-1305 was able to stabilize cleavable complex formation between DNA and topoisomerase II in vitro and in living cells. Thus, for the closely related C-1305 and C-1533 compounds, antitumor activity was not associated with the inhibition of topoisomerase II as such but rather with the ability to induce topoisomerase II-associated cleavable complexes.

The induction of cleavable complexes by C-1305 follows a biphasic curve that is similar in vitro and in living cells. Maximal cleavable complex formation is obtained with concentrations of C-1305 between 2.5 and 10 μM. Higher concentrations of C-1305 lead to increasing autoinhibition, and at 50 μM, only low levels of cleavable complexes are detected. A similar dose-response has been described previously for several topoisomerase II inhibitors that are strong DNA intercalators, including doxorubicin, mitoxantrone, imidazoacridones, and ellipticines (Tewey et al., 1984; Monnot et al., 1991; Zwelling et al., 1991; Skladanowski et al., 1996). In clear contrast, we observed no autoinhibition for amsacrine, which is a weak DNA intercalator (Denny and Wakelin, 1986).

Although C-1305 is a much weaker topoisomerase II poison than amsacrine, the two compounds show comparable cytotoxic effects after continuous drug exposure. This might be

TABLE 3

Cytotoxicity of C-1305 and other topoisomerase II inhibitors toward parental NIH3T3 cells and NIH3T3 transfectants overexpressing the MDR1 (NIH/MDR1) or MRP1 (NIH/MRP1) ABC transporters

Cells were exposed to the indicated drugs for 3 h followed by postincubation in drug-free media for three to four generation times, and the cytotoxicity was determined by counting of viable cells. All values are the average of at least two independent experiments, each done in duplicate.

Compound	IC_{50}[a]		Δ[b]	IC_{50}[a]		Δ[b]
	NIH3T3–1	NIH/MDR1		NIH3T3–2	NIH/MRP1	
	μM			*μM*		
C-1305	0.35	0.82	2.3	0.38	1.0	2.6
C-1533	32.0	41.9	1.3	20.4	31.1	1.5
mAMSA	0.078	0.21	2.7	0.058	0.14	2.4
Daunorubicin	0.015	0.14	9.6	0.017	0.35	21

[a] Drug concentration causing 50% growth inhibition.
[b] Resistance index, ratio between IC_{50} values obtained for MDR1 or MRP1-transfected cells and parental cells.

because the C-1305–induced cleavable complexes are unusually toxic or, alternatively, because the drug could have additional nontopoisomerase II-mediated activities. A comparison between the closely related triazoacridones C-1305 and C-1533 indicates that both compounds are strong DNA binders and thus are likely to influence a variety of DNA-binding enzymes and proteins. However, these activities do not seem to be very cytotoxic, given the modest cytotoxicity of C-1533 after continuous drug exposure (Tables 2 and 3). An alternative explanation is that the C-1305–induced cleavable complexes could be more stable than the complexes induced by amsacrine, a possibility supported by initial experiments in our laboratory (K. Lemke, unpublished results). It is interesting to note that recent experiments show that C-1305 binds strongly to DNA at guanine-rich regions, resulting in unique conformational changes (Lemke et al., 2004). Previous results indicate that unusual structures formed in guanine-rich DNA are recognized by topoisomerase II (Chung et al., 1992). Because promoter regions often contain guanine-rich elements, we speculate that C-1305 might favor the induction of topoisomerase II-induced cleavable complexes in such regions, resulting in local transcriptional perturbations. These possibilities are the subject of further research.

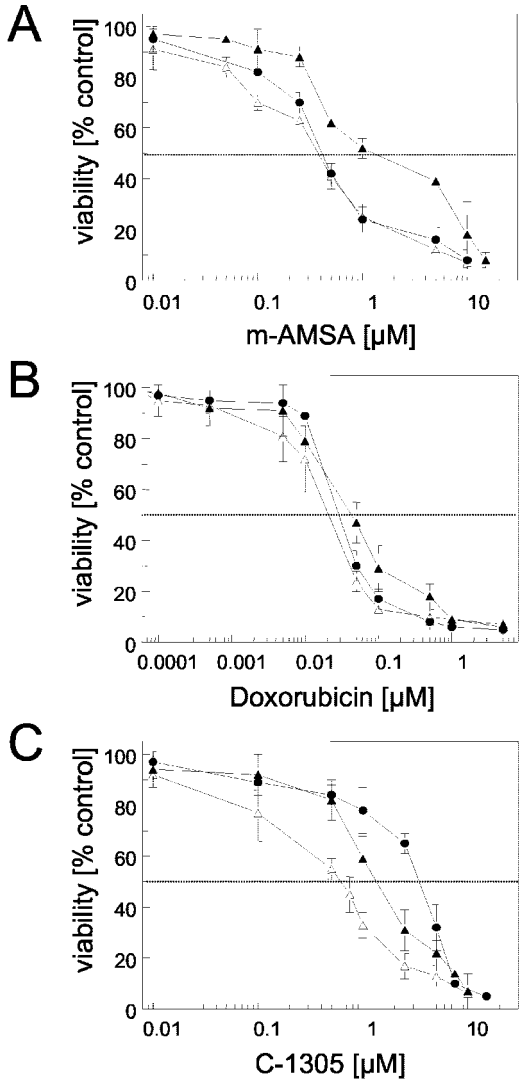


Fig. 8. The influence of p53 and p21 function on the cytotoxicity of amsacrine, doxorubicin, and C-1305 on the viability of parental HCT-116 cells (●) and the HCT-116 p53 −/− (▲) and HCT-116 p21 −/− sublines (△) in which the p53 or p21 genes, respectively, have been deleted by homologous recombination. The cytotoxicity was determined by the MTT assay after continuous drug exposure. A, amsacrine; B, doxorubicin; C, C-1305. Each point is the average of at least two independent experiments, each done in duplicate. Bars represent standard errors and are indicated when they exceed the symbol size.

Recent results suggest that 5-fluorouracil–refractive colorectal tumors have high levels of topoisomerase II (Shibao et al., 1999; Lazaris et al., 2002). Likewise, non–small-cell lung cancers have consistently high topoisomerase II levels compared with the corresponding normal tissues (Mirski et al., 2000; Dingemans et al., 2001). Although we would expect the tumor-associated up-regulation of topoisomerase II levels in these tumor types to be accompanied by increased sensitivity to topoisomerase II inhibitors, this is not what has been observed clinically. Several explanations have been provided to explain this observation, including the recognition of topoisomerase II inhibitors by ABC transporters such as MDR1 (Spoelstra et al., 1991) as well as the reduced activity of topoisomerase II inhibitors toward tumor cells growing in three dimensions (Shain and Dalton, 2001). The activity of C-1305 toward parental cells and transfectants overexpressing the MDR1 or MRP1 ABC transporters was determined and compared with other topoisomerase II inhibitors. The results show that the transfected cell lines are at the most 3-fold–resistant to both C-1305 and amsacrine compared with 10 to 20 times resistant to daunorubicin. The activity of C-1305 toward cells growing in two dimensions as monolayer cultures or in three dimensions as multicellular spheroids was also determined. The results show that cells growing in multicellular spheroids are approximately 3-fold more resistant to both C-1305 and doxorubicin compared with the same cells growing in monolayer culture. In comparison, multicellular spheroids were four to five times resistant to amsacrine and etoposide. These results suggest that the cytotoxicity of C-1305 is not limited to rapidly dividing cells and is not strictly dependent on the cell-cycle distribution.

The p53 tumor suppressor gene is inactivated in the majority of human cancers, including colorectal tumors. We therefore compared the long-term (120 h) cytotoxic effects of C-1305, amsacrine, and doxorubicin on human colon cancer cell lines in which the p53 or p21 pathways have been specifically disrupted by targeted homologous recombination (Bunz et al., 1998). It was unexpected that the three topoisomerase II inhibitors gave different results. Disruption of p53 and p21 function had minor influence on the cytotoxicity of doxorubicin, whereas p53 but not p21 disruption was associated with increased resistance to amsacrine. In marked contrast, disruption of p53 or p21 was associated with increased sensitivity to C-1305.

An intriguing possibility is that the influence of p53 and p21 on the cytotoxicity of C-1305 might be caused by a direct effect on the drug target, topoisomerase II. It has been shown previously that both p53 and p21 inhibit the human topoisomerase IIα promoter, resulting in decreased expression of the topoisomerase IIα gene (de Toledo et al., 1998; Zhu et al., 2002; Joshi et al., 2003). It is important to note that p53 and, to a lesser extent, p21 are only activated and functional under conditions of cellular stress. Furthermore, the transcriptional effects of p53 are not an immediate response to stress but rather a relatively slow one. For example, we have shown previously that nuclear translocation and build up of transcriptionally active p53 protein

after exposure to cytotoxic anticancer agents requires at least 4 h (Gobert et al., 1999; Poindessous et al., 2003). In addition, we would not expect the p53- or p21-mediated decrease in the cellular levels of topoisomerase IIα mRNA to have an immediate impact on the protein levels of topoisomerase IIα. The p53-mediated down-regulation of topoisomerase IIα is therefore expected to have relatively little effect on fast-acting drugs like amsacrine. In contrast, a slow drug like C-1305 would be much more sensitive to long-term variations of topoisomerase II levels. This model could explain why the parental cells, in which topoisomerase II would become down-regulated, are more resistant to C-1305 compared with the p53- or p21-deficient variants, in which the topoisomerase II levels are expected to remain constant. In any case, given the high frequency of p53 inactivation in most human tumors, selective cytotoxicity of an anticancer agent toward p53-defective cells would be a desirable feature and might, at least in part, explain the unusual antitumor activity of C-1305 in animal models.

In conclusion, we here report that the anticancer C-1305 triazoloacridone compound inhibits the catalytic activity of topoisomerase II. Like most clinically active topoisomerase II inhibitors, C-1305 is able to stabilize the formation of cleavable complexes between topoisomerase II and DNA, both with purified enzyme in vitro and in living cells. A unique feature of C-1305 is the induction of low levels of unusually toxic cleavable complexes. The toxicity of these complexes might be a result of their enhanced stability and/or caused by an original sequence-specificity of the drug-induced topoisomerase II cleavage sites. Another interesting property of C-1305 is its preferential cytotoxic activity toward cells with defective p53 function. Future studies aim to identify the C-1305–induced cleavage sites and to characterize the cellular pathways induced by this unusual new compound.

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