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The antitumor triazoloacridone C-1305 is a topoisomerase II poison with unusual properties

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Running title: Topoisomerase II inhibition by triazoloacridone C-1305

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ABSTRACT

C-1305 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. We here 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. Since 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 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 toward 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.

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INTRODUCTION

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, that 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 catalyses 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., 2003a). Anticancer drugs targeting topoisomerase II are among the most effective cytotoxic agents currently available for cancer therapy (Hande,

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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, that has no antitumor activity. The results show that C-1305 is a topoisomerase II poison with many original features compared to other agents of this class.

MATERIALS and METHODS

Drugs and Chemicals. Amsacrine (*m*-AMSA) 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) while 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 while amsacrine was prepared as a 10 mM stock solution in DMSO. 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

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(3000 Ci/mmol), [^{3}H]-thymidine (90 Ci/mmol) and [^{14}C]-leucine (300 mCi/mmol) Krzysztof, please verify 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, *Eco*RI and *Hind*III 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 (Worland and Wang, 1989; Escargueil et al., 2000).

Relaxation assay. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM DTT, 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 addition of loading buffer (0.1% SDS, 0.05% bromophenol blue, 2.5 mM EDTA, 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, 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 about 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

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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 *Eco*RI and end-labeled with Klenow fragment and α-[^{32}P]-dATP. The labeled DNA was then subjected to a second digestion with *Hind*III, the fragments obtained were separated by agarose gel electrophoresis and the larger fragment was used for DNA cleavage assays. Reactions mixtures contained 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, ~$2.5x10^4$ 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 allowed to proceed for 10 min at 30°C. Reactions were terminated by addition of SDS, proteinase K and EDTA (0.35%, 0.3 mg/ml and 15 mM final concentrations, 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 Pharmacia Biotech) for 1-2 days.

Cell lines. HL-60 leukemia and DC-3F fibrosarcoma cell lines have been characterized in detail previously (Côme et al., 1999; Larsen and Jacquemin-Sablon, 1989). A549 lung and DLD-1 colon carcinoma cells were purchased from American Type Culture Collection (Rockville, MD) while HT-29 colon carcinoma cells were

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provided by Richard Camalier, Division of Cancer Treatment and Diagnosis tumor repository, NCI. 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 Institute 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 MEM (DC-3F), McCoy's A (HCT-116, HCT-116/p21 -/-, HCT-116/p53 -/-), RPMI 1640 (HL-60, A549, DLD-1) or DMEM (HT-29, NIH3T3, NIH3T3-MDR-G185, NIH/7M, NIH/32) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2/air atmosphere (RPMI, MEM, McCoy's A) or 10% CO_2/air atmosphere (DMEM). All cell lines were screened routinely for Mycoplasma by the PCR 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 G-418. All resistant cell lines were kept in drug-free medium for at least one week before each experiment.

Cytotoxicity assays. The cytotoxicity was determined by the MTT assay as described previously (Poindessous et al., 2003). Briefly, 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

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microplate reader. The concentrations required to inhibit cell growth by 50% compared to untreated controls were determined from the curves plotting survival as a function of dose by use of the Slide Write program. 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 (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 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 post-incubation in drug-free media for 5-7 days. Alternatively, 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 co-precipitation assay as described (Zwelling et al, 1991). DNA and proteins of HT-29 cells (about 300,000 cells) were

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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 sec, 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 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 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 about 4 x 10^{6} cells per sample were embedded in low-melting agarose (0.75% final concentration). Plugs were treated with lysing buffer A (25 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8.0, 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, 1% SDS) was added and lysis continued overnight at 56°C. After lysis, plugs were washed 3 times for 30 min in final wash buffer (10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0) and embedded in 1% PFGE agarose (Seakem Gold agarose, BioWhittaker, Rockland, ME). DNA was separated in a CHEF-DRII apparatus (BioRad, Hercules, CA) in 0.5 x TBE (45 mM Tris-borate, 0.5 mM EDTA, pH 8.0) for 24 h at 14°C (180 V, 30 and 60 s initial, and final pulses,

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respectively, with linear ramp). After electrophoresis, gels were stained with 0.5 μg/ml ethidium bromide to visualize the DNA and photographed under UV illumination. Molecular weight standards (50 kbp lambda ladder) were from BioWhittaker (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. About 5 μM C-1533 was required to inhibit the relaxation reaction by 50% while 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).

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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 as 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 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 (for recent review, see Larsen et al., 2003b). 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 μM) 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 effect on the cytotoxicity of C-1533.

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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 while 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 can not be attributed to differential drug accumulation.

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 one hour drug exposure in the case of amsacrine and after 2 hours 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 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

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fragments migrating at the same position as the compression band (Fig. 6). The size of these fragments is estimated to be at least one million bp. Amsacrine exposure was also associated with the formation of very high molecular weight DNA fragments. In addition, concentrations above 2.5 μM amsacrine were accompanied by the formation of 50 to 350 kbp DNA fragments corresponding to the size of chromatin loops (Razin et al., 1991).

Time-dependence of the cytotoxic effect. Since 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 nM vs 38 nM, 85 nM vs 85 nM and 180 nM vs 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 drug exposure and up to 6 times more toxic after 24 h 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 incubation at 37°C.

Activity towards multicellular spheroids. An important difference between tissue culture conditions and tumor growth is that tissue culture usually is carried out

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with cells growing in 2-dimensions whereas tumor growth occurs in 3-dimensions (Sutherland, 1988). We therefore compared the cytotoxic effects of C-1305 and other topoisomerase II inhibitors on DC-3F cells growing in 3-dimensions as multicellular spheroids or in 2-dimensions as monolayer cultures. The cell cycle distribution of cells growing in spheroids was 65%, 22% and 13% for cells in G1, S or G2/M, respectively, whereas the cell cycle distribution of monolayer cultures was 35%, 55% and 10% for cells in G1, S and G2/M, respectively (data not shown). The results show that cells present in the slower growing, three-dimensional spheroids are about 3-fold more resistant to C-1305 and doxorubicin, and up to 5 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 NIH-3T3 cells and NIH-3T3 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 about 10-fold resistance to daunorubicin. Overexprexpression of MRP1 was associated with 2 to 3-fold resistance to C-1305 and amsacrine, compared with about 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.

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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, where the p53 or p21 genes have been deleted by homologous recombination. Unexpectedly, 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, while loss of p53 function was accompanied by about 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, while loss of p21 was accompanied by almost 6-fold increased sensitivity.

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 due to 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 classical topoisomerase II poison and the C-1533 derivative, that 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; Zwelling et al., 1991; Monnot et al., 1991; Skladanowski et al., 1996). In clear contrast, we observe no autoinhibition for amsacrine, that 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 because the C-1305 induced cleavable complexes are unusually toxic or alternatively, because the drug could have additional non-topoisomerase II mediated activities. A comparison between the closely related triazoacridones C-1305 and C-1533 indicates that both compounds and strong DNA binders and thus likely to influence a variety of DNA-binding enzymes and proteins. However, these activities do not appear 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). Interestingly, 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). Since 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 subject of further research.

Recent results suggest that 5-fluorouracil-refractive colorectal tumors have high levels of topoisomerase II (Shibao et al., 1999; Lazaris et al., 2002). Similarly, non-small cell lung cancers have consistently high topoisomerase II levels compared to the corresponding normal tissues (Mirski et al., 2000; Dingemans et al., 2001). Although we would expect the tumor-associated upregulation 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 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 to 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 resistance to daunorubicin. The activity of C-1305 toward cells growing in two dimensions as monolayer cultures or in

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three dimensions as multicellular spheroids was also determined. The results show that cells growing in multicellular spheroids are about 3-fold more resistant to both C-1305 and doxorubicin compared to the same cells growing in monolayer culture. In comparison, multicellular spheroids were 4 to 5 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). Unexpectedly, 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 due to a direct effect on the drug target, topoisomerase II. It has been shown previously, that both p53 and p21 inhibit the human topoisomerase II alpha promoter resulting in decreased expression of the topoisomerase II alpha 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 extend, 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 a relatively slow one. For example, we have previously shown 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 II alpha mRNA to have an immediate impact on the protein levels of topoisomerase II alpha. The p53-mediated downregulation of topoisomerase II alpha 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, where topoisomerase II would become downregulated, are more resistant to C-1305 compared to the p53- or p21-deficient variants, where 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 due to 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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FOOTNOTES

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FIGURE LEGENDS.

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

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.

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 to 10). The resulting DNA/topoisomerase II complexes were digested by proteinase K, and the different topological forms of DNA 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.

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.

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 (dark gray bars) and C-1533 (black bars) 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 duplicates. Bars, standard deviation.

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, 50 μM C-1305 (lanes 2-7) and 0.1, 0.25, 0.5, 1,

2.5, 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 grey bars), 24 h (dark grey bars) or continuous drug exposure (black bars). Cells were exposed to C-1305 or amsacrine for 3 or 24 h followed by post-incubation in drug-free media for 5-7 days. Alternatively, cells were grown in the continuous presence of drugs for the entire incubation period.

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 (circles), and the HCT-116 p53 -/- (full triangles) and HCT-116 p21 -/- sublines (open triangles) where 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 duplicates. Bars represent standard errors, and are indicated when they exceed the symbol size.

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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 (100μM) followed by post-incubation 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 duplicates.

	IC_{50} (μM)[a]		
compound	- ICRF	+ ICRF (100μM)	Δ[b]
C-1305	9.9	23.7	2.4
C-1533	46.5	44.4	0.9
m-AMSA	3.9	27.2	6.9
doxorubicin	0.7	2.0	2.8

[a] drug concentration causing 50% loss of cell viability compared to untreated controls
[b] ratio between IC_{50} concentrations for cells preincubated with or without ICRF-187

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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 determined by cell counting. All values are the average of at least two independent experiments, each done in duplicates.

	IC_{50} (nM)[a]		
compound	monolayer	spheroids	Δ[b]
C-1305	27	91	3.4
C-1533	3217	4090	1.3
m-AMSA	6.2	31	5.0
VP-16	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 inmonolayer cultures

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 post-incubation 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 duplicates.

	IC_{50} (μM)[a]			IC_{50} (μM)[a]		
compound	NIH 3T3-1	NIH/MDR1	Δ[b]	NIH 3T3-2	NIH/MRP1	Δ[b]
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
m-AMSA	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

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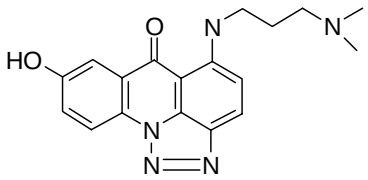
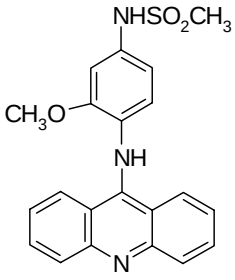
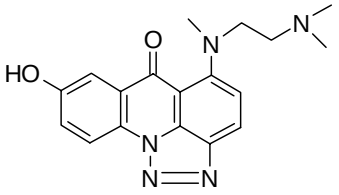


C-1305



***m*-AMSA**



C-1533

Fig. 1

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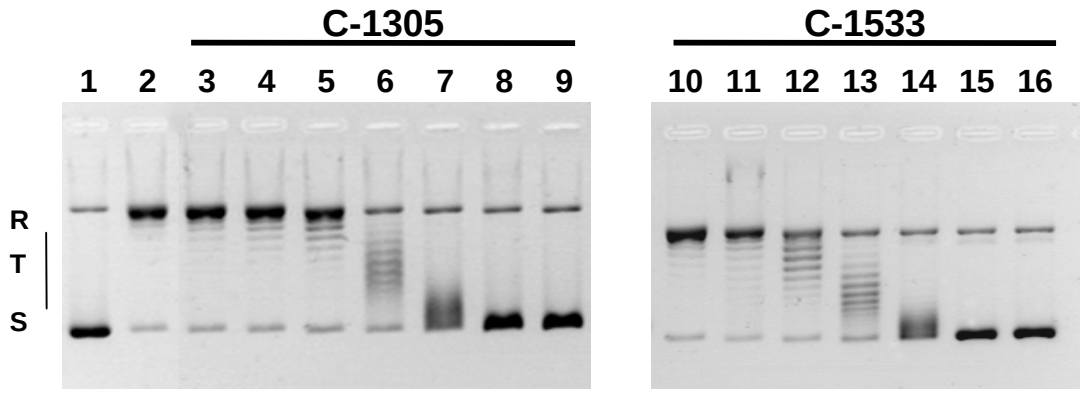

Fig. 2

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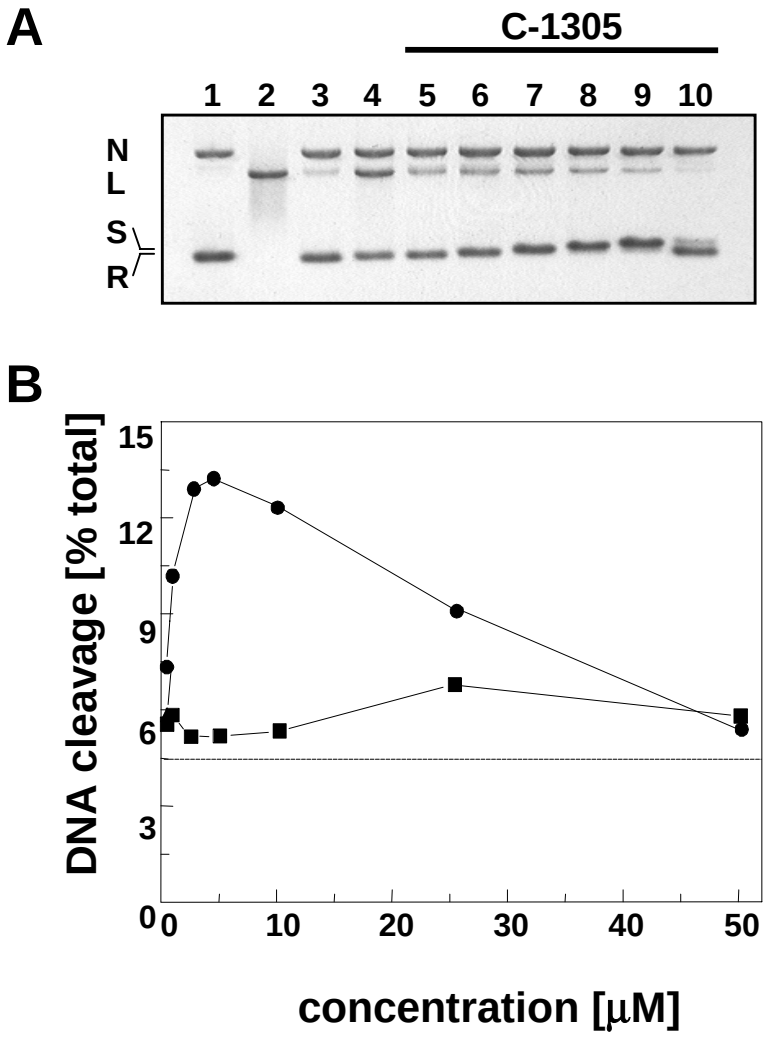

Fig. 3

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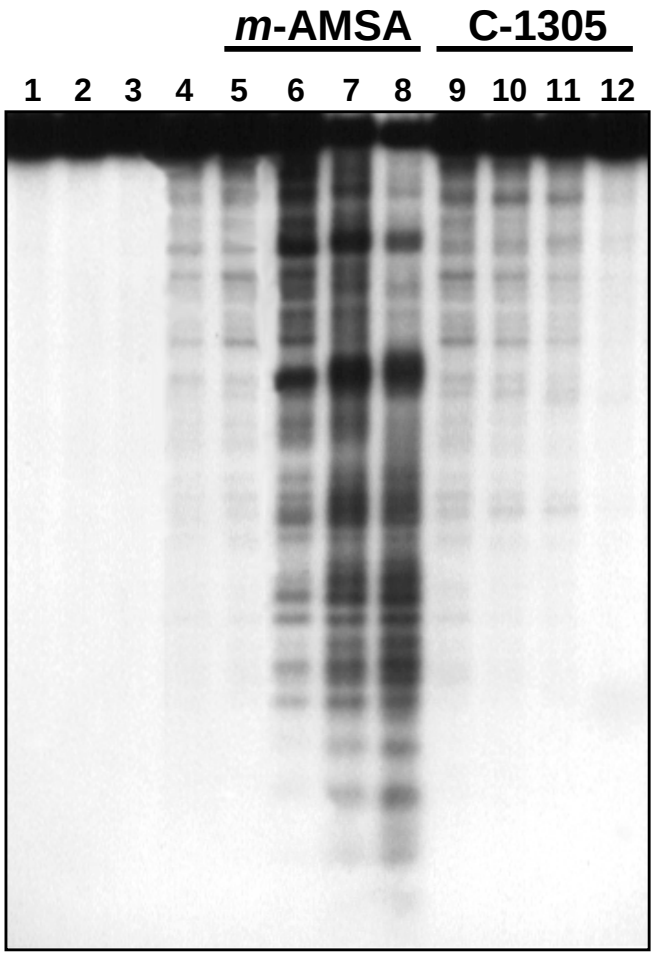

Fig. 4

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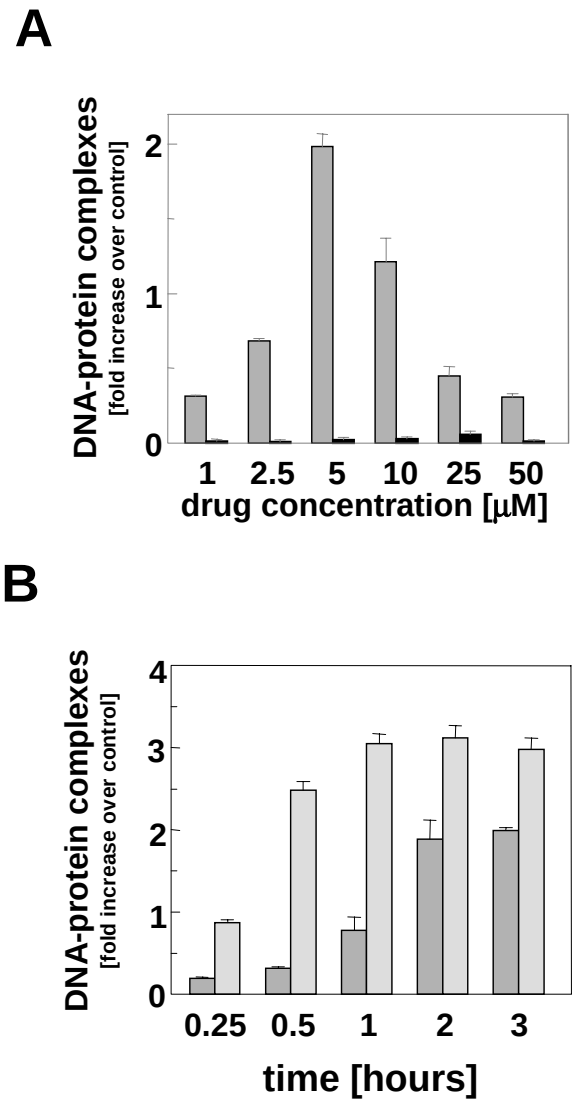

Fig. 5

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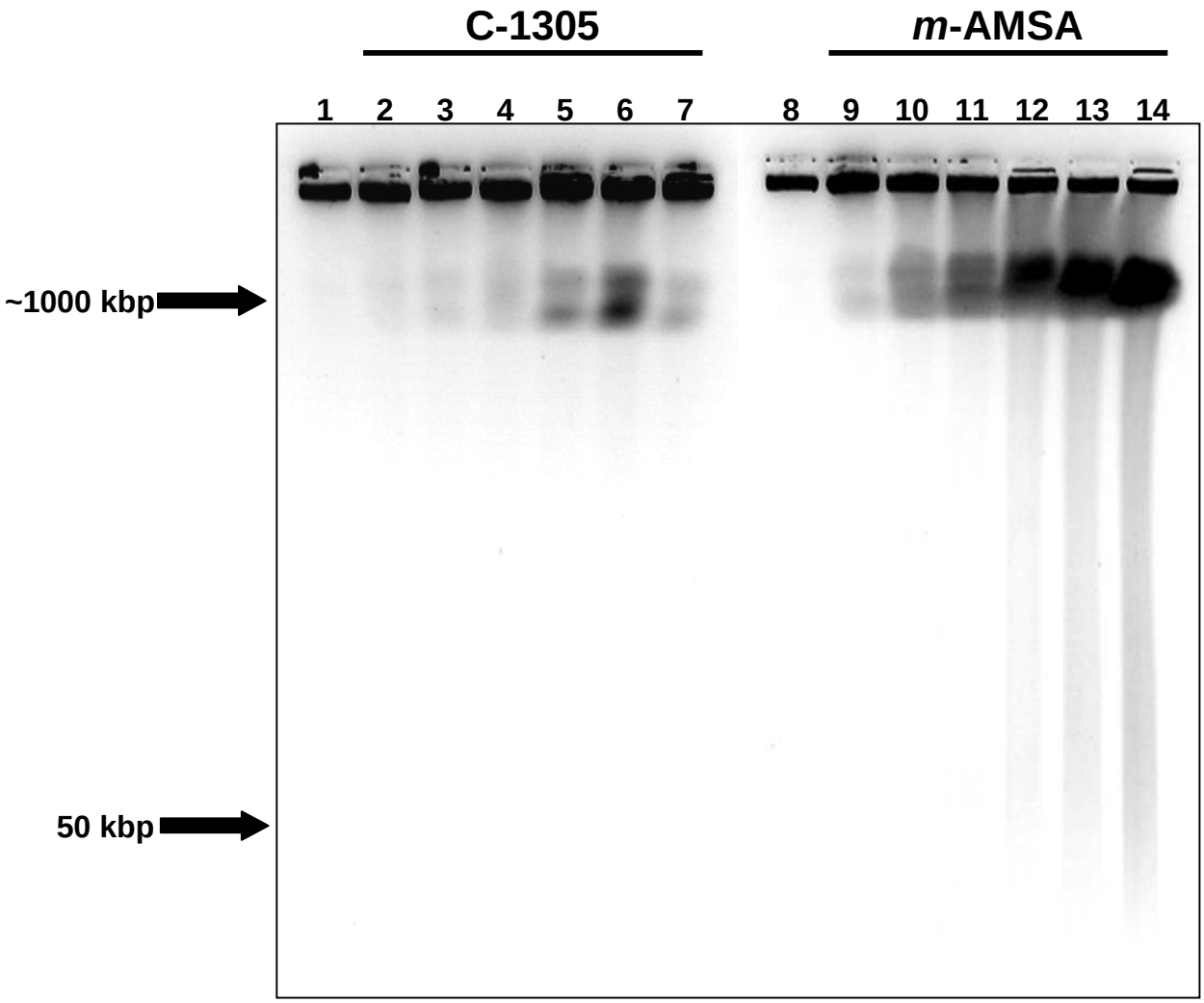

Fig. 6

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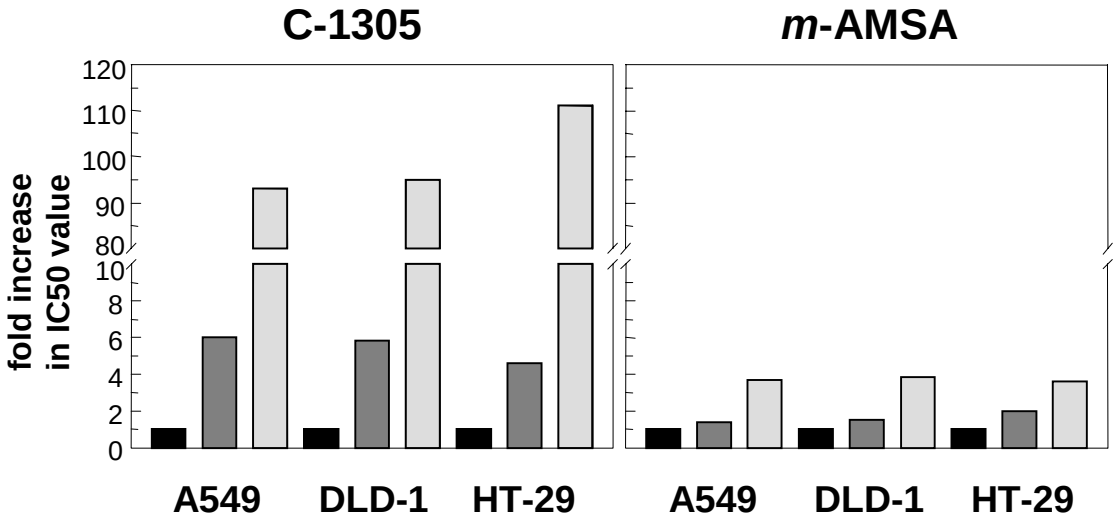

Fig. 7

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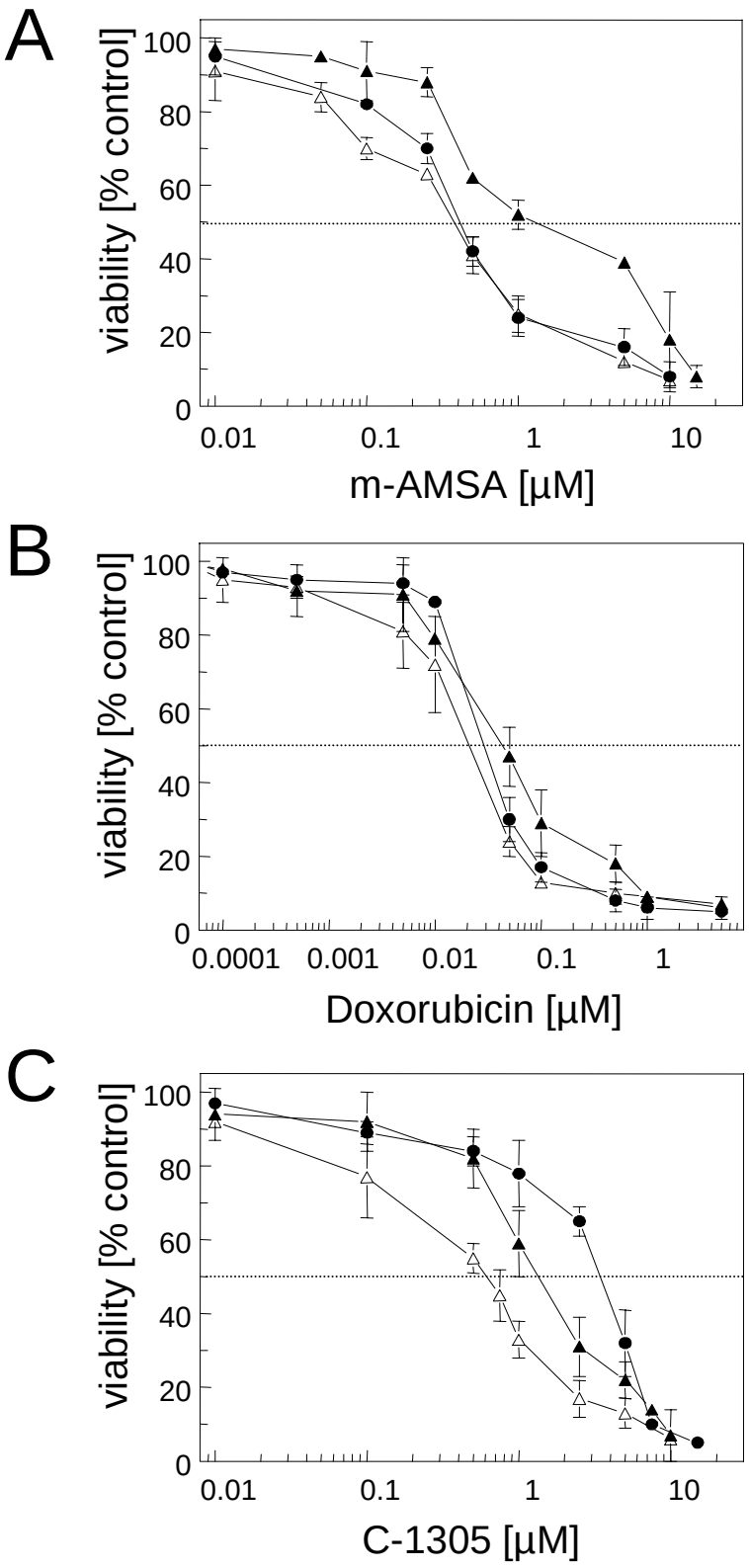

Fig. 8