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On the role of topoisomerase I in mediating the cytotoxicity of 9-aminoacridine-based anticancer agents

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ABSTRACT

The cytotoxicity and mechanism of action of a series of substituted 9-aminoacridines is evaluated using topoisomerase I and cancer cell growth inhibition assays. In previous work, compounds of this type were shown to catalytically inhibit topoisomerase II, leading to a G1-S phase arrest of the cell cycle and apoptosis in pancreatic cancer cells in vitro and in vivo. The present study expands the potential utility of these compounds in the development of cancer therapeutics by showing that these compounds inhibit proliferation of cell lines derived from the nine most common human cancers. Further results show that at least one of the compounds effectively stabilizes topoisomerase I–DNA adduct formation in intact cells. RNA interference experiments, however, indicate that this interaction does not contribute to the drug-induced killing of cancer cells indicating the compounds may be non-lethal poisons of topoisomerase I.

Topoisomerases I and II are well established targets in the development of anti-cancer agents.[1–4] The roles of these enzymes in processes that involve changes in DNA topology, including replication, transcription and chromosome segregation, are well documented, as are the mechanisms of action of most, if not all, topoisomerase-targeted drugs. In general, topoisomerase inhibitors are divided into two main categories: poisons and catalytic inhibitors. Poisons, which include the most widely used drugs clinically for treating cancers, function by binding to the covalent DNA–topoisomerase enzyme complex (cleavable complex) and preventing DNA religation after DNA strand cleavage occurs. In susceptible cells, events triggered by this cleavable complex stabilization lead to apoptosis and cell death. Well established examples of poisons include doxorubicin, etoposide, mitoxantrone, and amsacrine, which primarily target topoisomerase II, and the camptothecins topotecan (TPT) and 7-ethyl-10-hydroxycamptothecin, which target topoisomerase I. Catalytic inhibitors are not as well defined mechanistically and can function through inhibiting any other step of the topoisomerase enzymatic cycles. Examples of catalytic inhibitors include the anthracycline aclarubicin, which blocks formation of DNA–enzyme complexes, and the bisdioxopiperazine ICRF-193, which stabilizes the close-clamped form of topoisomerase II.[3,4]

We have recently described a new series of potential anticancer agents that share a common structural core with amsacrine but function through a mechanism similar to that of aclarubicin.[5–7] Detailed mechanistic studies have shown that the compounds in Figure 1 intercalate into DNA and inhibit the catalytic activity of topoisomerase II without poisoning it. The compounds have been also shown to induce a G1-S phase arrest followed by apoptotic cell death (a common consequence of topoisomerase II inhibition) in both SU86.86 and BXPC-3 pancreatic cancer cells in vitro.[6,7] In vivo, the compounds were found to significantly reduce the proliferation of SU86.86 cells grown as xenografts in mice. The relative activity of the compounds against other cancers and potential function at related enzymatic targets (such as topoisomerase I), however, has not yet been determined.

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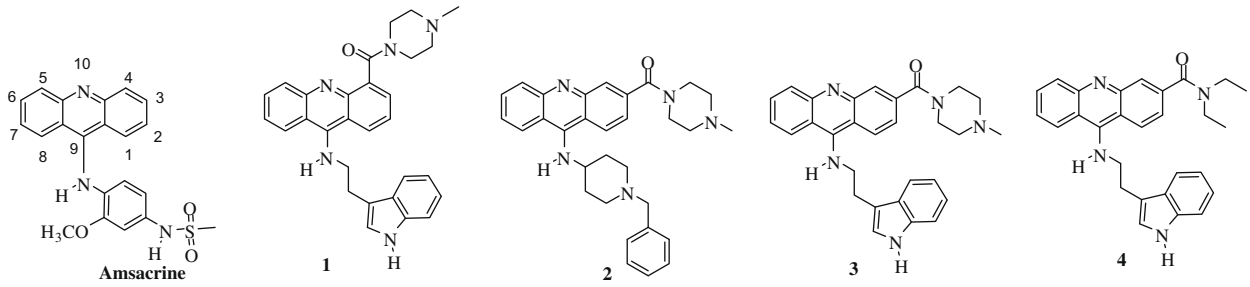

Figure 1. Substituted 9-aminoacridines.

Given the utility of other topoisomerase-targeting agents (e.g., etoposide, doxorubicin or the camptothecins) in treating a variety of cancers, compounds **1–4** have now been further evaluated for activity against cell lines derived from the nine most common human cancers. In standard cytotoxicity assays (Table 1), the compounds exhibit relatively similar potency across all cancer cell lines, with some notable exceptions. The diethylamido tryptamine derivative **4**, for example, shows a consistently higher potency across all nine cancer cell lines. It is interesting to note that this compound bears the weakest charge within the group, having only two protonatable nitrogens. The reduced charge or polarity of this compound may increase cellular uptake (through passive diffusion across the membrane) which may, in part, explain the general gain in potency noted. In terms of relative activity across the cell lines, the methylpiperizine **3** shows three- to fourfold greater potency against MEL melanoma and REH renal carcinoma cells, whereas compound **1** exhibits greater potency against SU86.86 pancreatic cancer cells. These cell line-specific differences are somewhat surprising because the only difference between **1** and **3** is the position of the amido substitution of the ring.

Although a clear connection between topoisomerase II inhibition and the anticancer activity of the compounds has been established,[6,7] it is unclear what role, if any, topoisomerase I inhibition plays in their cytotoxicity. It has previously been reported that acridine dyes and other intercalating agents can interfere with the catalytic activity of topoisomerase I. In particular, morpholinodoxorubicin and actinomycin D trap topoisomerase I–DNA covalent complexes;[8,9] and pyrazoloacridine as well as doxorubicin inhibit topoisomerase I catalytic activity without trapping cleavable complexes.[9–11] To assess the potential importance of topoisomerase I as a target of compounds **1–4**, we have analyzed the effect of these agents on topoisomerase I catalytic activity under cell-free conditions and on topoisomerase I–DNA complexes in intact cells. In addition, we have utilized RNA interference to manipulate topoisomerase I levels and assess the impact on the antiproliferative effects of these compounds.

Table 1
Growth inhibition of **1–4** against lines derived from the nine most common human cancers[a]

Cell line	EC_{50} ± SE (μM)			
	1	**2**	**3**	**4**
DU145	37.2 ± 12.1	32.4 ± 8.8	16.6 ± 5.0	11.9 ± 2.9
HCT-116	29.0 ± 8.7	29.2 ± 4.1	27.6 ± 5.3	11.2 ± 2.6
Hepa-1c1c7	55.9 ± 5.3	26.9 ± 6.6	24.8 ± 9.0	11.2 ± 3.0
H460	16.0 ± 6.9	38.5 ± 3.6	31.8 ± 3.2	18.8 ± 2.3
MCF-7	37.2 ± 6.2	25.7 ± 5.0	19.2 ± 3.1	13.5 ± 2.7
SU86.86	6.8 ± 2.2	21.2 ± 5.1	16.3 ± 3.5	16.2 ± 2.9
MEL	16.7 ± 4.1	14.6 ± 3.2	5.3 ± 2.5	16.0 ± 3.2
OCL-3	39.8 ± 8.6	20.3 ± 4.6	15.3 ± 3.8	18.3 ± 2.8
REH	19.7 ± 4.9	21.0 ± 4.5	4.1 ± 0.5	15.5 ± 2.8

[a] Values reported are EC_{50} (μM) ± SE, $n = 3$. Cancer cells are as follows: DU-145 (prostate), HCT-116 (colon), Hepa-1c1c7 (liver), H460 (non-small lung), MCF-7 (breast), SU86.86 (pancreatic), MEL (melanoma), OCL-3 (ovarian), and REH (kidney).

The effects of compounds **1–4** on the catalytic activity of topoisomerase I are shown in Figure 2A. In contrast to pyrazoloacridine, which completely suppressed the ability of topoisomerase I to relax supercoiled DNA at a concentration of 8 μM (Fig. 2A, lanes 1–3), compounds **1–4** failed to significantly inhibit relaxation of supercoiled DNA even at concentrations as high as 100 μM. Because topoisomerase I poisons such as camptothecin are poor inhibitors of topoisomerase I catalytic activity,[12] the compounds were also assayed for the ability to stabilize covalent topoisomerase I–DNA adducts under cell-free conditions. Like the camptothecin derivative topotecan (TPT, Fig. 2B, lane 3), compound **2** stabilized these adducts, as indicated by the appearance of nicked plasmid in the presence of topoisomerase I (Fig. 2B, lane 7). To further evaluate this result, the ability of these compounds to stabilize topoisomerase I cleavable complexes in intact cells was assessed using the more sensitive ICE assay. Examination of $CsCl_2$–purified DNA fractions for the presence of topoisomerase I (Fig. 2C) demonstrated that compound **2**, like topotecan, stabilized topoisomerase I–DNA adducts in intact cells. To a smaller and more variable extent, compounds **1** and **4** also stabilized small numbers of topoisomerase I–DNA adducts.

While the data indicate that compound **2** (and to a lesser extent **1** and **4**) can trap topoisomerase I–DNA adducts in vitro and in intact cells, these observations do not establish whether the stabilization of these complexes is critical for inhibition of cancer cell growth. To address this question, HeLa cells treated with control or topoisomerase I-directed siRNA were exposed to compound **2** for 24 h, washed, and assayed for the ability to form colonies. Consistent with previous results in gene-targeted yeast[13,14] and siRNA-treated mammalian cells,[15] topoisomerase I siRNA diminished the cytotoxicity of camptothecin (Fig. 3A). In contrast, the cytotoxicity of compound **2** was relatively unaffected (Fig. 3B), indicating that stabilization of topoisomerase I–DNA complexes is not the major mechanism by which compound **2** kills cells. These results are consistent with the previous conclusion that compounds **1–4** act predominantly as topoisomerase II inhibitors.[7]

Collectively, these results provide important new insight into the cytotoxic action of compounds **1–4**. Our previous work has suggested that these compounds function by intercalating DNA through a threading mechanism that sterically hinders interactions with both the major and minor grooves of DNA. Given that this mechanism is typically considered to be non-specific, the finding that only one of the compounds binds to and strongly stabilizes the topoisomerase I–DNA complex is surprising. Furthermore, all four compounds failed to show catalytic inhibition of topoisomerase I activity. This suggests that the binding and stabilization of topoisomerase I–DNA complexes by the 9-aminoacridines may,

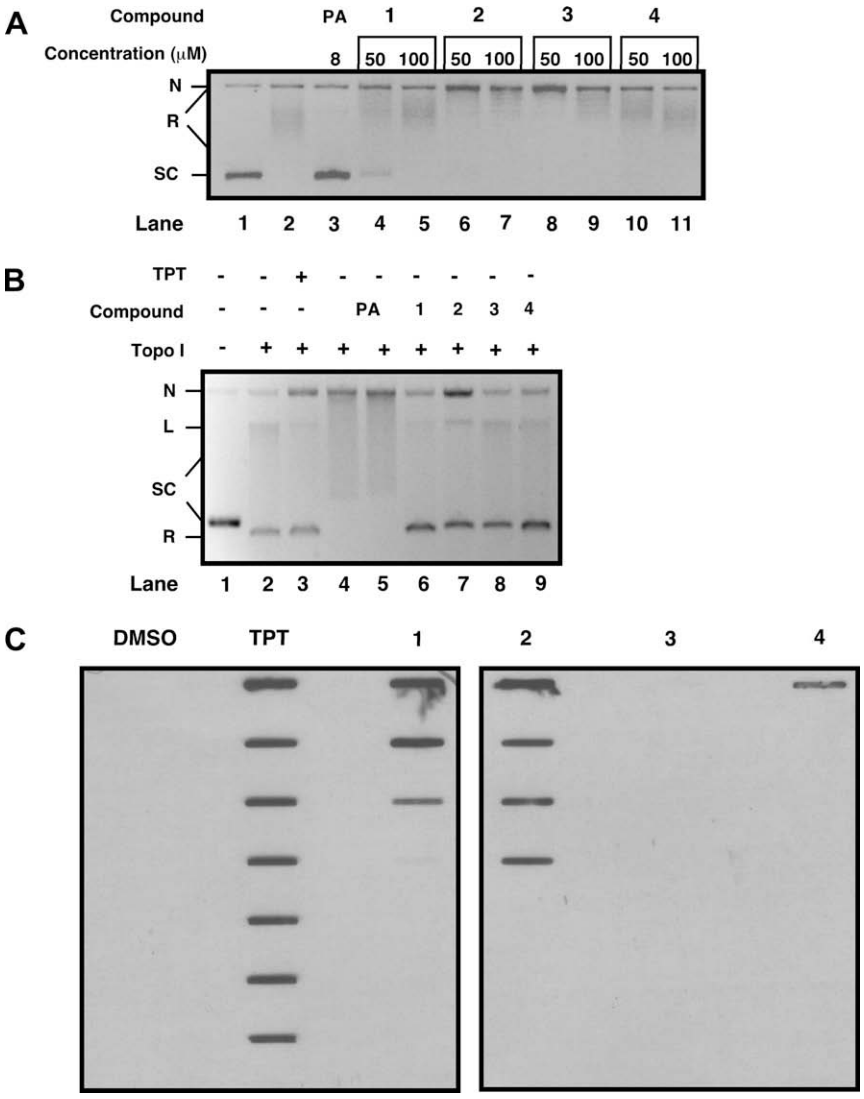

Figure 2. Effects of compounds **1**, **2**, **3**, and **4** on topoisomerase I catalytic activity (A), formation of covalent topoisomerase I–DNA complexes (B) and ICE assay (C). (A) Effect on topoisomerase I activity. Supercoiled plasmid was incubated with 2 units of topoisomerase I in the absence (lane 2) or presence of the positive control pyrazoloacridine (8 μM) or compounds **1**, **2**, **3**, and **4** (lanes 4–11) at a final concentration of 50 or 100 μM as indicated. Lane 1, DNA incubated without enzyme. SC, supercoiled DNA; R, relaxed DNA. Results are representative of four experiments. (B) Stabilization of topo I–DNA complexes. Aliquots of supercoiled plasmid were incubated for 30 min at 37 °C with nuclear extract containing 50 units of topoisomerase I in the presence of TPT (1.6 μM), PA (200 or 100 μM) or compounds **1**, **2**, **3**, and **4** (100 μM), as indicated. After treatment with SDS and proteinase K, samples were separated on 1% agarose gels in the presence of 0.5 μg/ml ethidium bromide to better separate nicked (*N*) from relaxed (*R*) DNA. Compare the increased amount of nicked DNA (lanes 3–9) with the nuclear extract only (lane 2). (*L*), linear DNA. Results were representative of three independent experiments. (C) Stabilization of DNA–topoisomerase I complexes in vivo. MiaPACA cells treated with 0.1% DMSO, 20 μM TPT or 100 μM Compounds **1**, **2**, **3**, and **4** for 1 h at 37 °C were lysed with sarkosyl and subjected to the ICE bioassay as described in the Supplementary data. Immunoblot of gradient fractions that were positive for DNA is shown (starting from the bottom of the tube). Because of the limited slots available, two membranes (blotted simultaneously) were used to test all the compounds at once. Results are representative of four independent experiments.

in fact, be structure-based which points to the existence of a specific binding site within the enzyme–DNA complex. A similar conclusion was reached in prior work evaluating the topoisomerase II activity of a larger library of substituted acridines.[6] It is important to point out, however, that this trapping of the complex by **2** does not appear to lead to cytotoxicity. Unlike camptothecin, which becomes less toxic when topoisomerase I is downregulated (Fig. 3A), compound **2** shows little sensitivity to the siRNA knockdown (Fig. 3B). One explanation for this apparent lack of sensitivity is that compound **2** effectively diminishes DNA synthesis. Prior work has shown that active DNA synthesis is required to convert topoisomerase I cleavage complexes into lethal damage.[16,17] Based on the effects of other intercalating agents, it is possible that decreased DNA synthesis in the presence of compound **2** reflects inhibition of helicases or polymerases that are critical for DNA replication, thereby antagonizing any effect of stabilizing topoisomerase I cleavable complexes. Alternatively, the failure of topoisomerase I downregulation to affect the cytotoxicity of compound **2** may simply reflect the fact that catalytic inhibition of topoisomerase II (as previously postulated) rather than trapping of topoisomerase I is the primary mechanism of action of cell killing at the concentrations used in the clonogenic assays shown in Figure 3. In either case, compound **2** appears to be a non-lethal poison of topoisomerase I. Non-productive binding of this type can have a significant impact on efficacy (as well as toxicity and the development of resistance), underscoring the importance of verifying the mechanism of action in the design and development of cytotoxic drugs.

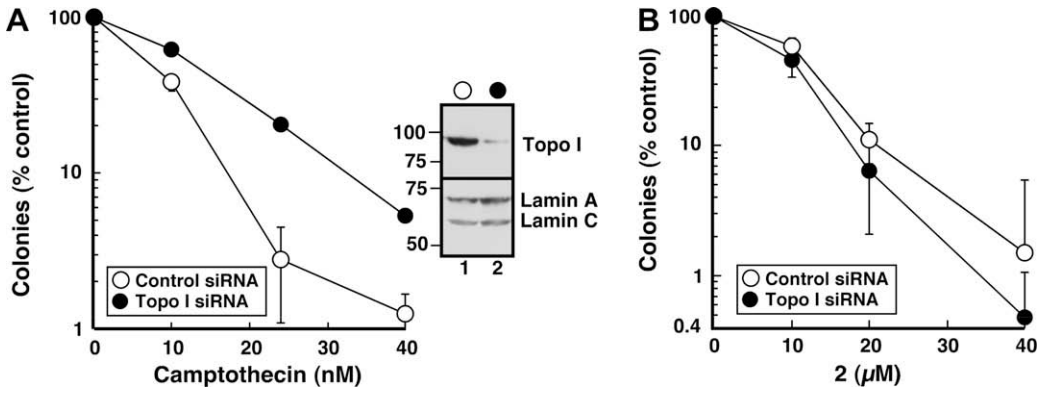

Figure 3. Effect of topoisomerase I siRNA on drug sensitivity. After transfection with luciferase siRNA (open circles) or topoisomerase I siRNA (filled circles), HeLa cells were treated for 24 h with diluent (0.1% DMSO) or the indicated concentration of camptothecin (A) or compound **2** (B) for 24 h, washed and incubated in drug-free medium for 8 d to allow colonies to form. The *Y* axis is graphed on a logarithmic scale as is common practice with colony forming assays to emphasize the log-linear effects of these agents. Error bars ± s.d. are from triplicate plates. Inset in A, whole cell lysates were prepared from additional transfected cells at the time of drug treatment, subjected to SDS–PAGE and probed for topoisomerase I. Lamins A and C served as loading controls.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.037.

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