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Letters

Acridine-Based Agents with Topoisomerase II Activity Inhibit Pancreatic Cancer Cell Proliferation and Induce Apoptosis

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Abstract: A series of substituted 9-aminoacridines is evaluated for antiproliferative activity toward pancreatic cancer cells. The results indicate that the compounds inhibit cell proliferation by inducing a G1-S phase arrest. A model is also developed that explains the molecular basis to inhibition through a DNA "threading" mechanism. We conclude that the drug–DNA complex formed blocks topoisomerase II binding and activity leading to catalytic inhibition of the enzyme and the induction of apoptosis and programmed cell death.

Acridine-based compounds have had a long and successful history as antibacterial, antimalarial, and more recently, antitumor agents. Most of these antitumor agents act by inhibiting the essential enzyme, topoisomerase II (topo II).[1,2] Topo II plays a critical role in actively replicating cells by affecting topological changes in DNA, allowing for replication, transcription, and decatenation.[3] Therefore, aggressive and rapidly replicating cancers, such as small-cell lung cancer, lymphomas, and a variety of leukemias, appear to be the most responsive to compounds that inhibit topo II.[4,5] Current topo II inhibitors are classified as topo II catalytic inhibitors or topo II poisons, with most of the current inhibitors being poisons. Topo II poisons have been well studied and act by stabilizing the "cleavable complex", resulting in the covalent linkage between the enzyme and DNA, ultimately leading to irreversible double strand breaks.[3–5] Catalytic inhibitors, which have received far less attention, act at any other stage in the seven step catalytic cycle, such as enzyme/DNA association occurring in the first step.[6]

Recent work in our laboratory investigating compounds with antiherpes activity resulted in the discovery of a small library of substituted 9-aminoacridine derivatives that appear to have novel topo II catalytic inhibitory activity.[7] These compounds demonstrated activity comparable to that of amsacrine in topo II relaxation assays but did not show activity in a topo II cleavage assay.[7] On the basis of their potency in preliminary cytotoxicity screens and effectiveness in inhibiting human topo II catalyzed relaxation reactions, four representative compounds, shown in Figure 1, were assayed for antiproliferative activity against three pancreatic cell lines. To further our understanding of the mechanism of these compounds at a cellular and molecular level, the compounds were investigated for their effect on cell proliferation, cell cycle progression, induction of apoptosis and modeled to investigate their interaction with DNA.

Three pancreatic cancer cell lines consisting of MiaPaCa-2, SU86.86, and BXPC-3 were used to allow for the investigation of growth inhibition based on their different rates of proliferation. MiaPaCa-2 has the fastest rate of proliferation, whereas BXPC-3 has the slowest. Percent growth inhibition experiments using a range of concentrations of **1**–**4** were conducted over a 72 h period using an MTT assay for cell viability quantification. The IC_{50} values for the three cell lines are shown in Table 1, with all of the compounds showing low micromolar activity in the three cell lines examined.

Previous studies have indicated that topo II poisons primarily cause irreversible damage during the S phase (when topo IIα levels are high), ultimately leading to an accumulation of cells in the G2 phase.[6,8] Similarly, the topo II catalytic inhibitor, aclarubicin, also induces DNA damage during the S phase by preventing relaxation of supercoiled DNA ahead of the replication fork.[6] However, a different morphologic appearance is observed with an enlarged nucleus (containing elongated and entangled DNA) compared to the small apoptotic nuclei (containing fragmented DNA) induced by topo II poisons.[6] Catalytic inhibitors therefore result in

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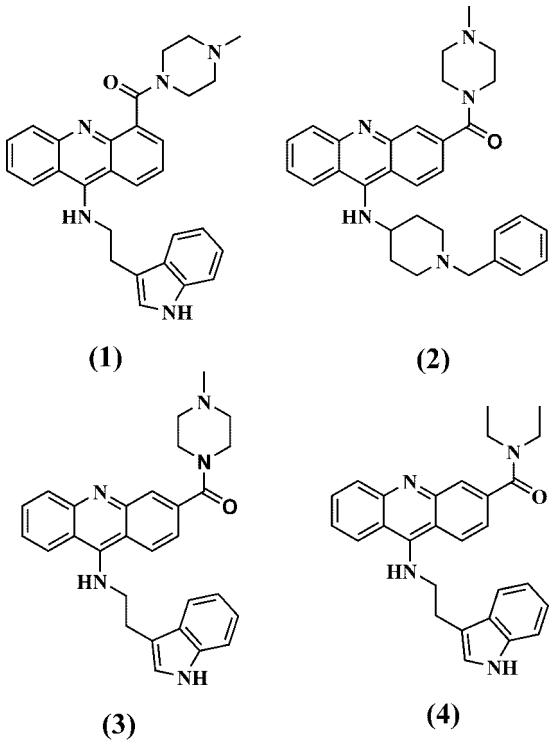

Figure 1. Structures of the compounds investigated in the current study. Syntheses of the compounds were previously reported.[7]

Table 1. Inhibition of Pancreatic Cell Lines by Acridine Compounds **1–4**[a]

	IC_{50} (μM)		
compd	MiaPaCa-2	SU86.86	BXPC-3
1	4 ± 0.7	10 ± 0.8	18 ± 2.8
2	1 ± 0.25	6 ± 0.4	7 ± 0.35
3	6 ± 1.3	8 ± 1.2	12 ± 1.5
4	11 ± 1.6	10 ± 1.5	14 ± 1.8

[a] IC_{50} estimated from MTT assay. The pancreatic cancer cell lines MiaPaCa-2, SU86.86, and BXPC-3 were obtained from ATCC (Rockville, MD) and were grown in DMEM medium containing 10% fetal calf serum and L-glutamine.

cellular arrest at the G2 phase by stopping the cell cycle at the catenation checkpoint. However, it is unclear at which stage in the cell cycle this novel set of acridine-based compounds is affecting tumor cell proliferation. To examine this, we treated the indicated pancreatic tumor cell lines with diluent or **1–4** and determined cell cycle accumulation at 36 h using propidium iodide staining and flow cytometry quantification. Two of the cell lines, SU86.86 and BXPC-3, demonstrate a loss of cells from the S and G2/M phases of the cell cycle with a corresponding accumulation of cells in G1 (Table 2). The MiaPaCa-2 cell line, on the other hand, shows an accumulation of cells in the S phase following treatment with **1–4** (Table 2). These data are consistent with the notion that these novel compounds are topo II inhibitors, as they would be predicted to impede the G1 → S transition, since topo II is involved in unwinding DNA during the initiation of replication. The reason that the MiaPaCa-2 cells demonstrate more of an S-phase arrest in response to treatment with the compounds is unclear but may be due to its relatively fast proliferation rate compared to the other two cell lines.

Since the compounds result in a replication block, we next investigated whether the novel compounds lead to programmed cell death, since stalling the proliferation of cancer cells can result in crisis and initiation of apoptosis. To examine this, we treated BXPC-3 cells with diluent or the indicated concentrations of **1–4**, measured apoptosis using Hoechst staining of nuclei from the treated cells, and scored them for the presence of apoptosis (fragmentation of the nucleus into multiple discrete fragments). The results, shown in Figure 2, demonstrate that the BXPC-3 cell line undergoes apoptosis in a concentration- and time-dependent manner. Cell lysates were also prepared and analyzed for PARP cleavage, a hallmark of cells undergoing apoptosis. Consistent with the Hoechst staining results, the BXPC-3 cells treated with the compound show PARP cleavage. Similar induction of apoptosis was seen in the SU86.86 and MiaPaCa-2 cell lines (see Table 3). Together, these results indicate that the acridine-based compounds not only inhibit pancreatic cancer cell proliferation but also induce apoptosis, which is a characteristic of catalytic topo II inhibitors. This is in contrast to topo II poisons, which can also induce cellular necrosis.[9]

Table 2. Cell Cycle Arrest by Acridine Compounds **1–4**[a]

	G1/S/G2M		
compd	MiaPaCa-2	SU86.86	BXPC-3
DMSO	46/36/17	39/42/19	50/37/13
1	32/47/21	56/27/17	83/10/7
2	28/52/20	53/29/18	77/16/7
3	29/51/20	50/26/24	81/12/7
4	32/50/18	60/24/16	76/20/4

[a] Results were tabulated after 36 h of treatment. The pancreatic cancer cells were incubated for 36 h with diluent or the indicated compounds **1**−**4** (15 μM final concentration). Samples were then fixed in 50% ethanol, rehydrated, treated with RNase, stained with 50 μg/mL propidium iodide in 0.1% sodium citrate, and analyzed (at least 20 000 events per sample) using a Becton Dickinson FACScan flow cytometer. Very small debris (forward scatter less than 10% of intact cells) was excluded from the analysis by gating. Cell-cycle profiles were determined using Modfit software (Verity, Topsham, ME).

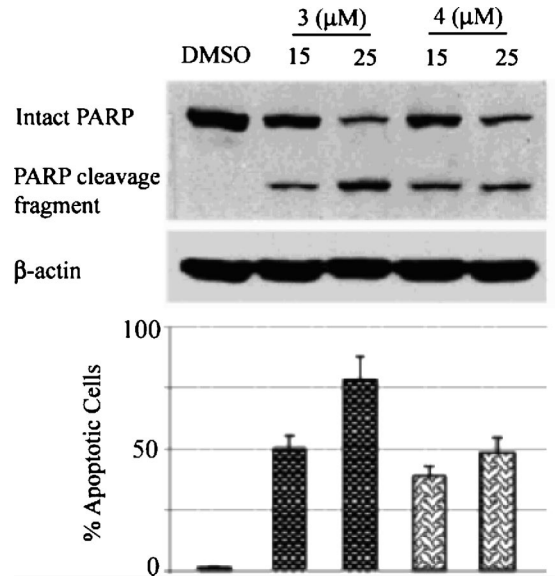

Figure 2. Induction of apoptosis following treatment with 9-aminoacridine derivatives. BXPC-3 cells were treated with diluent or the indicated concentrations of **3** and **4**. (Results for **1** and **2** are similar.) Forty-eight hours after exposure, cells were collected and stained with Hoechst. Three-hundred nuclei were counted per field and scored for apoptotic changes (fragmentation of the nucleus into multiple discrete fragments) and are graphically represented (lower panel). Cell lysates from the same treated cells were probed for PARP cleavage (upper panel), a hallmark of apoptosis. β-Actin was used as a loading control.

Table 3. Percentages of Cells in Apoptosis after 48 h of Exposure to Compounds **1**–**4**

compd	cells in apoptosis (%)		
	MiaPaCa-2	SU86.86	BXPC-3
1 (50 μM)	29 ± 4	23 ± 6	32 ± 8
2 (25 μM)	36 ± 9	30 ± 5	52 ± 11
3 (15 μM)	20 ± 5	34 ± 7	44 ± 6
4 (15 μM)	42 ± 7	17 ± 3	40 ± 5

The results are consistent with our previous work that linked the antiherpes virus activity of these compounds with catalytic inhibition of topo II.[7] As anticipated, the acridine-based compounds in the current investigation exhibited notable activity against all three pancreatic cell lines. Here, we have further shown that these compounds not only inhibit cancer cell proliferation but also induce a buildup of cells at the G1 → S transition of the cell cycle. Since topo II plays a critical role in the unwinding of DNA in front of the replication machinery, such a buildup is easily explained and is a well-known hallmark of topo II inhibition. Disruption or prevention of topo II activity results in a replication block that in turn leads to the initiation of programmed cell death, as indicated by the Hoechst staining and PARP cleavage results. This is also expected, since the stalling of cancer cell proliferation results in a cellular crisis and subsequent induction of apoptotic pathways.

While the results presented here support our proposed mechanism of action at a cellular level, there is still little known about how substituted tricycles of this type function at the molecular level. It has long been postulated that acridine- and anthracycline-based topo II inhibitors (e.g., doxorubicin) function via intercalation with DNA, resulting in the disruption of topo II activity. Most of these intercalating inhibitors, however, are classified as poisons because of their ability to produce irreversible double strand breaks. The important exception is aclarubicin, a catalytic inhibitor and intercalator that functions by preventing the association of DNA and topo II by blocking the minor groove.[6,10] This is, of course, the major difference in catalytic inhibitors and poisons; the former prevents topo II from binding, while the latter traps the enzyme−DNA complex in the cleavable form.

To begin elucidation of the structure-based mechanism to activity of the 9-aminoacridines, preliminary molecular modeling studies were conducted. Ethidium bromide displacement studies performed in our laboratory have shown that these compounds intercalate DNA with high affinity.[7] By utilization of a reported X-ray crystal structure of a related acridine derivative complexed with DNA as a template (9-amino-DACA with the hexanucleoside d(CGTACG)$_2$),[11,12] the compounds were modeled into the DNA followed by side chain relaxation/optimization using the Schrödinger suite of software.[13] The resulting complexes, shown in Figure 3, indicate that the acridine core intercalates DNA base pairs through π-stacking interactions, while the side chain substitutions extend into the major and minor grooves. This type of interaction is referred to as a "threaded" binding mode and has been proposed for other disubstituted acridines but never subjected to X-ray crystallography or molecular modeling.[14–16]

Given the prior work on anthracyclines, it is fair to conclude that the substituted acridines function by sterically blocking topo II recognition of the minor and/or major groove of DNA. A comparison of the structures of doxorubicin with aclarubicin shows, the latter contains two additional sugar moieties (see Figure 4). These sugars block topo association with the minor groove of DNA. A similar analogy can be drawn between

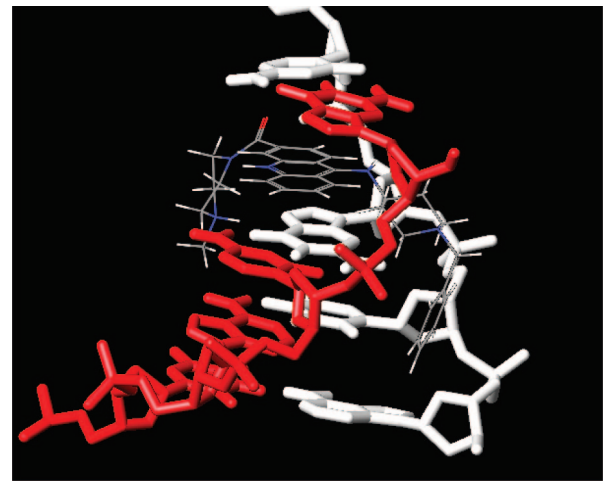

Figure 3. Drug–DNA complex of **2** threaded into a representative DNA fragment. The 9-amino substitution is in the minor grove, and the 3-amidesubstitution is in the major groove with maximal $\pi-\pi$ overlap between the acridine core tricycle and adjacent DNA base pairs.

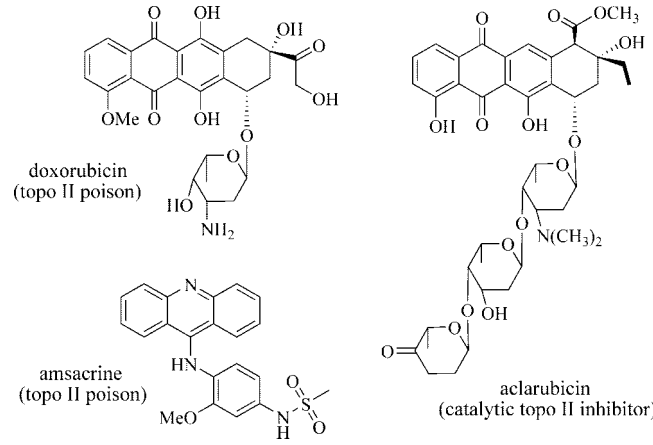

Figure 4. Structures of known topo II inhibitors.

amsacrine (a related acridine-based topo poison) and **1**–**4**. While both acridine cores are capable of strong intercalation, amsacrine lacks the steric bulk required to block minor or major groove binding of topo II to DNA. This not only explains the differences in the mechanism of action of these related compounds but also offers new insight to the structure-based design of catalytic inhibitors and poisons of topo II activity. A complete mechanistic evaluation of **1**–**4** and comparisons with related topo drugs are forthcoming.[17]

Since catalytic inhibitors may hold some inherent advantages over poisons in blocking topo II activity (stemming from the inability to induce DNA cleavage), the ability to rationally design catalytic inhibitors may prove to be extremely useful in the development of improved anticancer agents. The observation that **1**–**4** are active in all three cells lines, including the slowest growing cells (BXPC-3) is encouraging because a number of problematic solid tumors, prostate cancer in particular, have relatively slower rates of proliferation compared to small cell carcinomas and leukemias. Although not tested here, we fully expect the catalytic inhibitors reported here will in fact be active against a wide range of cancers, offering new strategies for the design of anticancer agents with lower toxicities.

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Supporting Information Available: Experimental procedures and descriptions of biology experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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