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Cytotoxic Acridinylthiourea and Its Platinum Conjugate Produce Enzyme-Mediated DNA Strand Breaks

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Abstract—The reactions of plasmid DNA modified with the novel acridinylthiourea, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea (**1**), and the corresponding intercalator-tethered platinum complex (**2**) with human type I and type II topoisomerases have been studied. Assays were based on evaluating DNA cleavage products resulting from incubations of drug-modified DNA in cell-free systems. **2** produces double-strand breaks in the presence of topo II while **1** proved to be a dual topo I/topo II poison.

There is a continued interest in the design of anticancer drugs that produce their cytotoxic effect by attacking multiple cellular targets.[1] In an effort to generate novel DNA-targeted platinum-based agents that circumvent cross-resistance to current clinical treatments, we have developed a structurally unique platinum–acridine conjugate, **2** (Fig. 1).[2] Compound **2** contains the novel acridinylthiourea, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea (**1**) (Fig. 1), a high-affinity DNA intercalator.[3] Compounds **1** and **2** were active at micromolar concentrations in leukemia and ovarian cancer cell lines, and **2** showed only partial cross-resistance with cisplatin in clonogenic survival assays.[2] Biophysical studies indicated that **2**, unlike cisplatin derivatives, does not induce cross-links but damages target DNA through a unique dual binding mode involving intercalation of acridine and monofunctional platination of the duplex.[3] Conjugate **2** unwinds pUC19 plasmid by 21° per adduct,[3] consistent with (partial) intercalation of the planar chromophore. Although this type of binding has been suggested to mimic, to some extent, cisplatin-induced DNA crosslinking,[4] other types of damage may exist that dominate or contribute to the biological activity of this drug prototype. To examine the possibility of alternative mechanisms of action, we study the interactions of DNA-processing enzymes with drug-modified DNA in cell-free systems. Here we report the effects of drug–DNA adducts on the DNA cleavage and relaxation activity of human type I and type II topoisomerases, which are known to be critical cellular targets for numerous clinical and experimental cancer therapeutics, such as acridine derivatives.[5]

Incubations of drug-modified supercoiled plasmid with enzyme and electrophoretic separations of DNA cleavage products were used to study the effects of **1** and **2** on topoisomerase activity.[6] In assays that favor cleavage of DNA, **1** was efficient at inducing enzyme-associated DNA-strand breaks. In the presence of topoisomerase I (Fig. 2a) **1** produced single-strand breaks (nicked open-circular DNA, form II) at a concentration of 1 μM. At drug concentrations >1 μM, reduced cleavage activity was observed, which resulted in complete inhibition of relaxation of supercoiled plasmid (form I) at concentrations >25 μM. The dose-dependent inhibition of enzyme acitivity was confirmed with assays that favor relaxation of plasmid (not shown). In analogous experiments, conjugate **2** inhibited relaxation at concentrations >5 μM but no single-strand breaks were detected. In a second set of experiments, the ability of **1** and **2** to poison topoisomerase II was assessed (Fig. 2b). Acridine **1** proved to be an inducer of DNA double-strand breaks at concentrations of 0.1 and 0.5 μM as evidenced by the appearance of a band on the gel assigned to linear DNA (form III). Likewise, platinum–acridine (**2**) showed noticeable cleavage activity at concentrations of 0.05–0.35 μM (Fig. 2b), while little formation of form III was observed at 0.5 μM. Similar to the case of topoisomerase I, the poisoning of topoisomerase II proved to be highly dependent on drug

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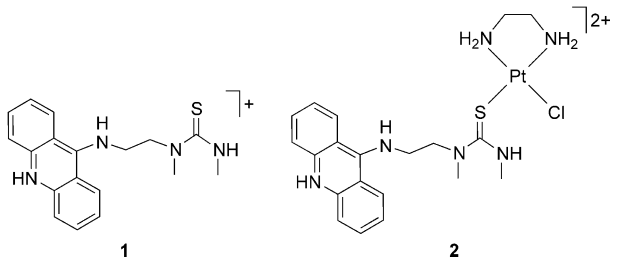

1 2

Figure 1. Structures of the novel acridine, **1**, and the platinum–acridine conjugate, **2**.

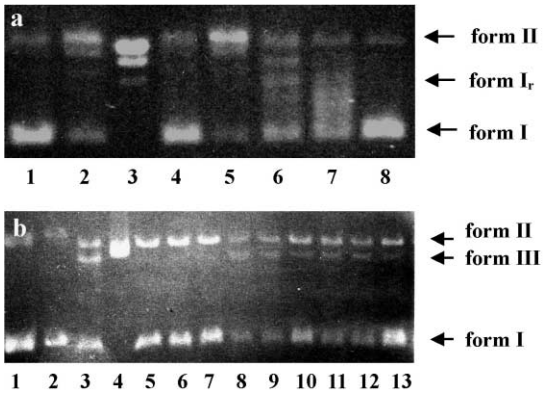

Figure 2. (a) Topoisomerase I cleavage assay of pHOT1 DNA modified with **1**. Lanes: plasmid reacted with enzyme in the absence of drug (1), 100 μM camptothecin + topoisomerase I (2), relaxed DNA marker (3), topo I + **1** at 0.5 μM (4), 1 μM (5), 5 μM (6), 10 μM (7), 25 μM (8). No ethidium bromide was added to this gel and running buffer to resolve relaxed topoisomers (form I_r). (b) Topoisomerase II cleavage assays of pRYG DNA modified with **1** and **2**. Lanes: free plasmid (1), plasmid reacted with enzyme in the absence of drugs (2), 100 μM etoposide + topoisomerase II (3), linear DNA marker (4), topo II + **1** at 0.005 μM (5), 0.01 μM (6), 0.05 μM (7), 0.1 μM (8), 0.5 μM (9); topo II + **2** at 0.05 μM (10), 0.2 μM (11), 0.35 μM (12), 0.5 μM (13). Gel and buffer contained 0.5 μg/mL ethidium bromide to enhance the resolution of linear (form III) and nicked open circular (form II) DNA. As observed previously, staining of the bands of plasmid treated with **2** proved to be difficult due to competition of irreversibly bound platinum with ethidium bromide for DNA binding sites.

concentration. Enzymatic activity (relaxation of pRYG plasmid) was inhibited for **1** and **2** at concentrations of 5 and 0.5 μM, respectively.

DNA-binding agents that increase the level of covalent topoisomerase cleavable complexes are termed topoisomerase poisons.[7] While these DNA–enzyme adducts are normal intermediates in topoisomerase-catalyzed conversions of DNA topoisomers, such complexes are turned into deadly cytotoxins in the presence of certain drugs that are able to trap the transiently cleaved DNA and the enzyme in a ternary complex. The induction of long-lived single-strand (topo I) and double-strand (topo II) breaks triggers events that ultimately lead to cell death.[5,7] For certain intercalating agents (9-aminoacridine, ethidium bromide), inhibition of DNA relaxation by topoisomerases has been observed, which indicates reduced binding affinity of the enzyme to the drug-modified DNA. This specific type of enzyme inhibition may lead to a reduced level of cleavable complex formation and to concentration-dependent self-inhibition of the topoisomerase poison.[8]

In our assays, acridine **1** was a potent poison of topoisomerase I and II activity at micromolar drug concentrations, suggesting that this agent may produce its cytotoxic activity by targeting both enzymes simultaneously. Compound **1** induced single-strand breaks at significantly lower concentration than the controls camptothecin and etoposide. Dual topoisomerase poisons are of interest for their use in the treatment of cells exhibiting multi-drug resistance (MDR).[9] Inhibition of enzyme activity by **1** was observed in both cases at higher concentrations, indicating that a threshold concentration exists above which **1** acts as an antagonist of topoisomerase I and II poisoning. Compound **2** poisoned topoisomerase II at nano-to-micromolar concentrations. The relatively low concentration necessary to effect topo II poisoning and inhibition compared to the case of **1** (0.05 μM vs 0.1 μM, and 0.5 μM vs 5 μM, respectively) may be a consequence of the covalent binding of platinum–acridine.[3] This binding mode is expected to reduce the dissociation ('off-rates') of the planar chromophore from DNA, which might enhance the efficiency of the drug. On the other hand, based on inhibitory concentrations, irreversible binding appears to occlude potential binding sites of the enzyme on DNA more efficiently than the simple intercalator **1**. Clonogenic survival data[2] for **1** and **2** in human leukemia (HL-60) and ovarian cancer cells (2008, C13*) did not show dose-dependent self-inhibitory effects. Characteristically, IC_{50} values were found to be significantly lower (0.01–10 μM^2) for both compounds than inhibitory concentrations established in this study.

Recently, several preclinical and phase I clinical studies have indicated that promising synergistic interactions exist between cisplatin[10] and its second-generation analogues[11] and topoisomerase poisons in combination chemotherapy. Our results suggest that **2** (platinum-tethered **1**) may act both as a pseudo-cisplatin 'alkylating agent' and as a topo II poison, whose activity is potentiated by covalent platinum–DNA interactions. In summary, the findings presented here corroborate the view that **1** and **2** may have clinical utility in the chemotherapy of tumors resistant to classical single-drug treatments and warrant further in vitro studies on the cell-cycle specificity and type of DNA damage of these compounds.

Acknowledgements

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6. Compounds **1** and **2** were synthesized in this laboratory according to published procedures (see ref 2). Topoisomerase drug screening kits and human topoisomerases were obtained from TopoGen Inc. (Columbus, OH). Drugs were prepared as 100 μM stock solutions and stored at −20 °C. Buffers used in topoisomerase II assays were supplemented with adenosine triphosphate (Sigma). Topoisomerase I assays were carried out in 30-μL assemblies in cleavage/assay buffer using 0.25 μg/μL pHOT1 supercoiled plasmid DNA and human type I topoisomerase (5 units for relaxation and 15 units for cleavage). Topoisomerase II assays were carried out analogously in 20-μL reactions containing relaxation or cleavage buffer, 0.25 μg/μL pRYG plasmid, and human type II topoisomerase (4 units for relaxation and 10 units for cleavage). Reactions were assembled on ice and incubated for 30 min at 37 °C. **2** was preincubated with plasmid at 37 °C in the dark for 24 h before reactions with topoisomerase. Camptothecin and etoposide were used as controls. Following the incubation, the DNA–enzyme complex was denatured with 1% sodium dodecyl sulfate (SDS) and the enzyme digested with 50 μg/mL proteinase K for 30 min at 37 °C. Assays were loaded onto 1% agarose gels in 1×TAE buffer (pH 8.3) (Fisher). Topoisomerase I assays were subjected to electrophoresis at 22 V for 16 h at 4 °C. Topoisomerase II assays were electrophoresed at 47 V for 8 h at 4 °C. To resolve linear from nicked open circular DNA in cleavable complex formation assays, 0.5 μg/mL ethidium bromide was added to the gel and running buffer. Gels were stained with ethidium bromide (0.5 μg/mL) and photographs were taken of the UV-illuminated bands.
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