Probing Platinum−Adenine-N3 Adduct Formation with DNA Minor-Groove Binding Agents

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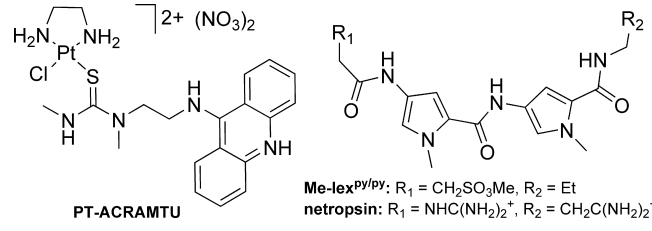
Me-lex$^{py/py}$, an adenine-N3-selective alkylating agent, and the reversible minor-groove binder netropsin were used to probe the formation of unusual minor-groove adducts by the cytotoxic hybrid agent PT-ACRAMTU ([PtCl(en)(ACRAMTU)]$(NO_3)_2$; en = ethane-1,2-diamine, ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea). PT-ACRAMTU was found by chemical footprinting to inhibit specific Me-lex-mediated DNA cleavage at several adenine sites but not at nonspecific guanine, which is consistent with the platination of adenine-N3. In a cell proliferation assay, a significant decrease in cytotoxicity was observed for PT-ACRAMTU, when cancer cells were pretreated with netropsin, suggesting that minor-groove adducts in cellular DNA contribute to the biological activity of the hybrid agent.

The development of nonclassical platinum-containing drugs in recent years that interact with DNA radically differently than *cis*-diamminedichloroplatinum(II) (cisplatin[1]) (*1*) requires novel biochemical tools to study the DNA damage caused by these agents and their biological effects. One such compound, PT-ACRAMTU ([PtCl(en)(ACRAMTU)]$(NO_3)_2$; en = ethane-1,2-diamine, ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea); Chart 1) and its derivatives have demonstrated promising activity in chemoresistant cancers (*2, 3*). Structure−activity relationship studies suggest that the cytotoxic effect of the platinum−acridines is mediated by DNA adducts (*2, 4*−*6*). Unlike cisplatin, PT-ACRAMTU and its derivatives do not induce nucleobase−nucleobase cross-links but form monofunctional-intercalative DNA adducts. The DNA damage profile of PT-ACRAMTU differs significantly from that of clinical platinum-based drugs. While the latter agents primarily target sequences of contiguous guanine (G) bases (*7*), the hybrid agent's adducts form with single G bases at pyrimidine−purine steps, and with adenine (A), mainly at 5′-TA sites (*5, 8*). The most striking DNA-binding feature of PT-ACRAMTU proved to be its ability to form adducts with A-N3, a binding mode previously unknown in platinum−DNA interactions (*3*).

We previously demonstrated that more than 30% of the adenine adducts are formed with the minor-groove site, A-N3, but the local sequence context of these adducts remained elusive (*3*). On the basis of the presence of the platinated deoxydinucleotide fragment d(TpA*) in mixtures of enzymatically digested drug-modified DNA, we proposed that PT-ACRAMTU forms adducts in the minor groove in this specific sequence (*9*). The reasoning behind this explanation was that platination of A-N3 might interfere with the DNA cleavage chemistry of DNase I, the minor-groove binding endonuclease (*10*) used in our assay, resulting in the undigested, platinated deoxydinucleotide.

Chart 1



To test our hypothesis of minor-groove platination in TA-rich sequences, we took advantage of two DNA minor-groove directed agents, the dipyrroledipeptide−methylsulfonate ester conjugate Me-lex$^{py/py}$, a minor-groove-specific alkylating agent (*11*−*14*), and netropsin, the classical dipyrroledipeptide natural product (*15*) (Chart 1). The data presented here suggest that in a synthetic 46-base-pair DNA fragment treated with PT-ACRAMTU, platinum efficiently protects A-N3 from methylation by Me-lex$^{py/py}$, providing evidence for platinum binding to this site. Additionally, a cell proliferation assay was performed, demonstrating that netropsin partially protects cancer cells from PT-ACRAMTU (but not from cisplatin), corroborating the notion that A-N3 adducts form intracellularly and contribute to the cytotoxic effect of the hybrid agent.

Alkylating agents have potential applications in probing the sequence and base selectivity of DNA interactive molecules (*16*−*18*). Me-lex$^{py/py}$ methylates A-N3 in double-stranded DNA with high selectivity (*14*). This produces 3-methyladenine (3-MeA) in TA-rich sequence contexts. 3-MeA is a labile adduct, which undergoes thermal depurination. Subsequent base treatment leads to single-strand breaks, which can be monitored in radioactively labeled DNA fragments by polyacrylamide gel electrophoresis (PAGE).

The footprinting assay reported here is based on the expectation that platination of A-N3 will protect this minor-groove site from alkylation by Me-lex$^{py/py}$, ultimately inhibiting the DNA-cleavage chemistry of the methylating agent. Thus, platination

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[1] Abbreviations: ACRAMTU, 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea; cisplatin, *cis*-diamminedichloroplatinum(II); DMS, dimethyl sulfate; IC_{50}, 50% inhibitory concentration; MAR, matrix attachment region; Me-lex$^{py/py}$, methyl-3-(1-methyl-5-(1-methyl-5-(propylcarbamoyl)-1*H*-pyrrol-3-ylcarbamoyl)-1*H*-pyrrol-3-ylamino)-3-oxopropane-1-sulfonate; PAGE, polyacrylamide gel electrophoresis.

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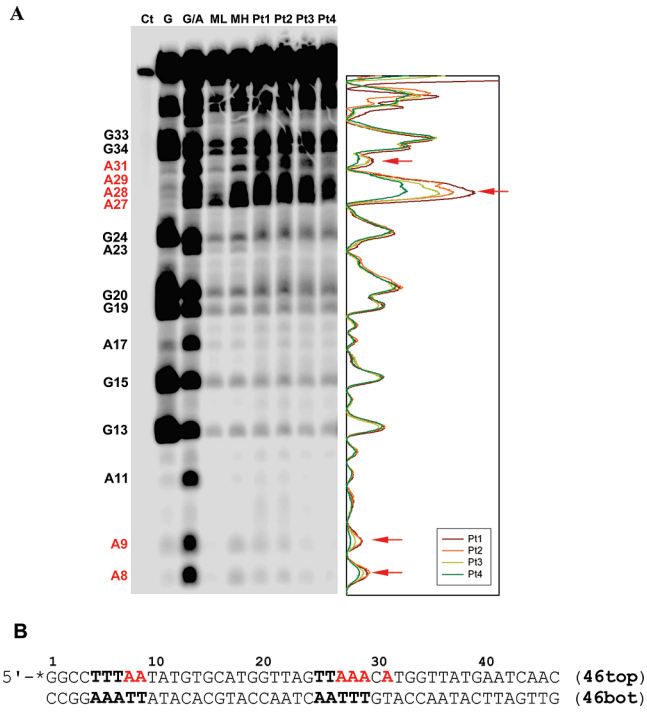

Figure 1. Chemical footprinting analysis of a 46-base-pair DNA fragment reacted with PT-ACRAMTU and subsequently subjected to Me-lex$^{py/py}$-mediated DNA cleavage chemistry. (A) Polyacrylamide gel giving integrated band intensities for lanes Pt1−Pt4. Lane assignments: Ct, control, full-length fragment; G, guanine sequencing reaction; G/A, guanine and adenine sequencing reaction; ML, 20 μM Me-lex$^{py/py}$; MH, 50 μM Me-lex$^{py/py}$; Pt1, 10 μM PT-ACRAMTU and 50 μM Me-lex$^{py/py}$; Pt2, 20 μM PT-ACRAMTU and 50 μM Me-lex$^{py/py}$; Pt3, 40 μM PT-ACRAMTU and 50 μM Me-lex$^{py/py}$; Pt4, 60 μM PT-ACRAMTU and 50 μM Me-lex$^{py/py}$. (B) Sequence of the 46-base-pair fragment with residues in the top strand numbered. Me-lex$^{py/py}$ target sites and the sites of cleavage inhibition are highlighted in bold and red, respectively. The asterisk denotes the ^{32}P label.

of Me-lex-specific A-N3 prior to treatment of the probe sequence with the minor-groove alkylator should result in a weakening or complete disappearance of the corresponding band on the gel. (Unlike 3-MeA, heat-induced depurination of platinated adenine is not observed under the conditions of this assay (*19*).) Varadarajan et al. demonstrated that Me-lex$^{py/py}$ preferentially alkylates A-N3 in 5′-(T)$_n$(A)$_m$ ($n = 2/m = 3$ or $n = 3/m = 2$) sequences in which the A base directly adjacent to T showed the highest propensity for 3-MeA formation (*14*). Since the 5′-TA step is also targeted by PT-ACRAMTU (*8, 9*), we designed a DNA fragment containing the above high-affinity sites to detect the formation of A-N3 adducts in this sequence context.

The 46-base-pair sequence, whose top strand (**46top**, Figure 1B) was 5′ end-labeled with ^{32}P, was incubated with four different concentrations of PT-ACRAMTU, treated with Me-lex$^{py/py}$, and subjected to piperidine cleavage after the removal of bound platinum. The resulting fragments were then identified and quantified by running the samples on a denaturing polyacrylamide gel along G and G + A sequencing lanes, as well as Me-lex-only footprinting lanes. The DNA fragment was modified with platinum at drug-to-nucleotide ratios of 0.022, 0.044, 0.087, and 0.13 (lanes Pt1−Pt4, Figure 1A), assuming that platinum binding is complete after 24 h (*8*), which corresponds to a total of 6 adducts per double-stranded fragment at the highest drug concentration.

To test the alkylation activity of Me-lex$^{py/py}$ in the absence of platinum, the 46-base-pair sequence was incubated with 20 μM and 50 μM alkylating agent, respectively (lanes ML and MH in Figure 1A). The strongest damage caused by Me-lex$^{py/py}$ in the portion of **46top** suitable for monitoring nucleobase alkylation (A8-G33) was observed at adenine residues A27−A29. When the fragment was incubated at a concentration of 50 μM, the minor-groove alkylator had a higher affinity for the adenine triplet than for the two adjacent A bases, A8 and A9. The agent also caused damage at A31 and at several G bases, the latter resulting from nonspecific alkylation, which is in agreement with the damage profile reported in the literature (*14*).

Pretreatment of the probe sequence with PT-ACRAMTU has a dramatic effect on the alkylation chemistry of Me-lex$^{py/py}$ at A8, A9, A27−A29, and A31. Cleavage is significantly reduced at these sites as evidenced by a gradual decrease of the corresponding band intensities on the gel with increasing platinum concentration (Pt1−Pt4, Figure 1A). This confirms that PT-ACRAMTU and Me-lex$^{py/py}$ target adenine bases with the same sequence specificity and comparable affinity. The pronounced inhibitory effect of PT-ACRAMTU on A-N3 alkylation is consistent with the formation of platinum adducts with the minor-groove donor. By contrast, the bands resulting from nonspecific, Me-lex-initiated cleavage at G residues show no decrease in intensity. Cleavage at G13 and G15, for instance, is completely unaffected by platination, which is surprising because PT-ACRAMTU has been shown to target 5′-TG sites (*5*). Since G is the major target for PT-ACRAMTU (∼80%) (*9*) but only a minor target for Me-lex$^{py/py}$ (∼10%) (*14*), platinum might be expected to interfere with the alkylation chemistry of the minor-groove binder, which is not the case. This suggests that depurination of G in this sequence context is mediated by minor-groove alkylation of G-N3, which does not seem to be affected by the platinum adducts formed exclusively with G-N7 in the major groove. (While methylation of G-N3 by Me-lex$^{py/py}$ is feasible (*14*), platination of this site is sterically prohibited (*3*).)

To test our hypothesis that PT-ACRAMTU forms minor-groove adducts in nuclear DNA, which might contribute to the cytotoxic effect of this agent, we designed a cell protection assay. The design is based on the supposition that minor-groove binders should compete with PT-ACRAMTU for minor-groove binding sites, thereby preventing the platination of A-N3. Since Me-lex$^{py/py}$ itself is very cytotoxic (*13*), a less toxic, noncovalent binder, netropsin (Chart 1), was chosen in this study. (Netropsin has previously been used to demonstrate that methylation of A-N3 by Me-lex$^{py/py}$ is the major cause of the alkylating agent's cell toxicity (*20*).) The cell line chosen for this experiment is NCI-H460, a nonsmall cell lung cancer, which has proven highly sensitive to PT-ACRAMTU-type agents. To determine if netropsin can be used as a modulator of cytotoxicity in this assay, NCI-H460 cells were incubated with the minor-groove binder for 72 h in the absence of the platinum drug. It was confirmed that netropsin itself does not affect H460 cell proliferation at concentrations below 50 μM. H460 cells were treated for 72 h with PT-ACRAMTU and, in a control experiment, with cisplatin, both in the absence of netropsin and at three concentrations (6.25, 12.5, and 25 μM) of netropsin that have no effect on cell survival. Selected drug−response curves are shown in Figure 2. In the absence of netropsin, an IC$_{50}$ value of 0.068 ± 0.003 μM was calculated for PT-ACRAMTU. In the presence of the minor-groove binder, PT-ACRAMTU's cell kill efficiency decreases, resulting in higher IC$_{50}$ values. At 25 μM netropsin, a statistically significant ($P < 0.05$) higher concentration of PT-ACRAMTU (0.163 ± 0.013 μM) is needed compared to that of netropsin-free H460 to inhibit cell proliferation by 50%. These results suggest that a relationship exists between PT-ACRAMTU's ability to interact with the minor groove in nuclear DNA and the drug's cell kill potential. By contrast, cisplatin in netropsin-treated

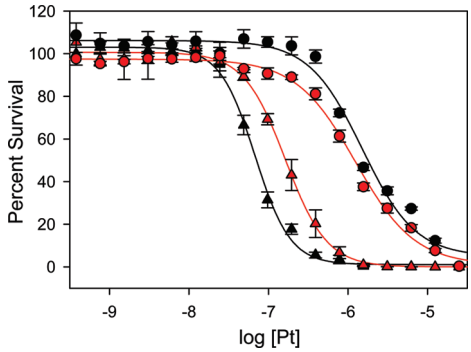

Figure 2. Drug−response curves for cell proliferation assays with NCI-H460 cells treated with PT-ACRAMTU (triangles) and cisplatin (spheres) in the absence of netropsin (black traces) and in the presence of 25 μM netropsin (red traces). The plotted data are averages (± standard errors) of three independent experiments for PT-ACRAMTU and two independent experiments for cisplatin.

cells shows the opposite trend: a slightly lower IC_{50} value of 1.245 ± 0.090 μM was determined for cisplatin in cells treated with 25 μM netropsin compared to that of untreated cells (IC_{50} = 1.506 ± 0.16 μM). These findings are in agreement with the fact that while PT-ACRAMTU and netropsin target common regions in nuclear DNA, cisplatin (G, major groove) and netropsin (A, minor groove) target DNA at mutually exclusive sites.

PT-ACRAMTU is the only platinum agent known to date that targets TA-rich sequences. However, several bioactive A-N3 alkylating natural and synthetic DNA binders exist, such as bizelesin, a hypercytotoxic bifunctional chloromethyl derivative of the family of cyclopropylpyrroloindole antitumor antibiotics (*21*). Bizelesin induces long-range A-N3/A-N3 interstrand cross-links in untranscribed, adenine-rich regions (AT islands) of the genome known as matrix attachment regions (MARs) (*22*−*24*). MARs play important roles in chromatin organization and gene expression, and disruption of MAR function has been shown to stall cell proliferation (*22*). The potent antiproliferative properties of PT-ACRAMTU may be, in part, due to the formation of adducts in these sequences. The decrease in cytotoxicity of PT-ACRAMTU in the presence of netropsin at noncytotoxic concentrations supports this notion. Future work will explore the possibility of using Me-lex$^{py/py}$ to detect platinum-modified A-N3 in nuclear DNA extracted from drug-treated cells.

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Supporting Information Available: Experimental details and summary of cytotoxicity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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