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A non-crosslinking platinum–acridine hybrid agent shows enhanced cytotoxicity compared to clinical BCNU and cisplatin in glioblastoma cells

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Abstract—Using clonogenic survival assays, we demonstrated that a new platinum–acridine hybrid agent, PT-ACRAMTU, is cytotoxic in SNB19 and U87MG glioblastoma cells at low-micromolar concentrations. PT-ACRAMTU is more cytotoxic than ACRAMTU (the platinum-free acridine), acts in a time and dose dependent manner, and appears to generate an apoptotic response in both cell lines on the basis of increased caspase-3 activity.

Each year approximately 190,000 people in the United States are diagnosed with a primary or metastatic brain tumor. Glioblastoma multiforme (GBM), the most aggressive form, will account for the majority of deaths (see, for instance, http://www.braintumor.org). Current treatment strategies for glioblastomas include surgical resection or debulking followed by radiation therapy alone or in combination with chemotherapy.[1] Despite this multi-modality therapy, the median survival of all patients with glioblastomas is only 9–12 months with negligible survival rates at three years.[1] Because current therapeutic strategies are largely ineffective, novel strategies, and therapies must be developed. Here, we report the biological activity of a novel platinum–acridine agent in two glioblastoma cell lines. Unlike classical platinum drugs, which cross-link purine bases in DNA,[2] our prototypical drug, PT-ACRAMTU (Fig. 1; ACRAMTU = 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea), acts through a dual 'monocoordinative'—intercalative binding mode.[3] (The Pt–S linkage in PT-ACRAMTU is resistant to nucleophilic attack by nucleobase nitrogen, rendering ACRAMTU a typical nonleaving group.[3,4]) Furthermore, the conjugate exhibits its unprecedented binding to adenine at 5′-TA and 5′-GA sites in ~20 percent of the adducts formed in native DNA.[4,5] PT-ACRAMTU has shown activity in a variety of cancers including cisplatin sensitive and resistant ovarian, nonsmall cell lung, colon, leukemic, and pancreatic cell lines.[6–8] Because of the unprecedented DNA damage profile and the previously demonstrated ability of PT-ACRAMTU to induce cytotoxicity in various cell lines, we studied the effect of PT-ACRAMTU on glioblastoma cells.

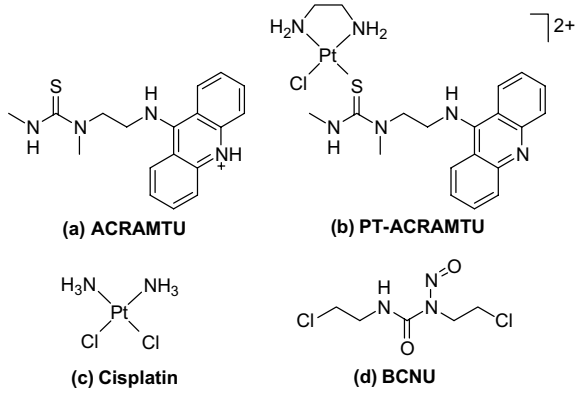

Figure 1. Structures of the drugs studied.

In order to assess the cytotoxicity of ACRAMTU and PT-ACRAMTU in SNB19 and U87MG glioblastoma

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Table 1. IC_{50} values (μM) for glioblastoma cell lines[a]

Compounds	SNB19	U87MG
ACRAMTU	2.46 ± 0.01	1.83 ± 0.04
PT-ACRAMTU	0.37 ± 0.06	0.75 ± 0.04
BCNU	2.37 ± 0.74	1.42 ± 0.11
Cisplatin	0.81 ± 0.03	0.49 ± 0.09

[a] Determined in clonogenic survival experiments. Cells were treated for 24 h. IC_{50} (μM) values are concentrations of drug required to inhibit cell growth by 50%. IC_{50} values were determined using CalcuSyn. Each IC_{50} is the average of three individual experiments ±SEM.

cell lines, clonogenic survival assays were conducted.[9] As in previous in vitro assays,[6–8] 24 h drug incubations were performed. IC_{50} values were determined from surviving fractions and are reported in Table 1. PT-ACRAMTU showed a cytotoxic effect in both cell lines with IC_{50} values of 0.37 ± 0.06 and 0.75 ± 0.04 for SNB19 and U87MG cells, respectively. These values are 6.7-fold and 2.5-fold lower, respectively, than the IC_{50} values for ACRAMTU, the platinum-free intercalator. As had been observed previously,[7,8] the linking of the monofunctional platinum moiety to ACRAMTU results in a significant enhancement of the cell-killing effect.

To determine if the length of PT-ACRAMTU exposure affected cell survival, additional clonogenic survival assays were conducted with both SNB19 and U87MG cell lines (Fig. 2). Cells were treated for various incubation times (1, 8, and 24 h) with PT-ACRAMTU at drug concentrations ranging from 0–10 μM. A 1 h PT-ACRAMTU drug incubation did not have a significant effect on cell survival in either SNB19 or U87MG cells. A dose and time dependent effect was observed however in SNB19 cells with longer incubation times. While the IC_{50} value for the 1 h incubation was 13.3 μM, the IC_{50} value for the 8 h incubation was 1.58 μM. The lowest IC_{50} value observed (0.37 μM) in SNB19 cells was following the 24 h PT-ACRAMTU incubation. U87MG cells also showed low IC_{50} values following a 24 h incubation (0.75 μM). Curiously, in this cell line the surviving fraction of cells at the 8 h time point showed values similar to the 1 h data.

Using clonogenic survival experiments, the cytotoxicity of PT-ACRAMTU was also compared with that of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a chemotherapeutic agent typically used in the clinic to treat glioblastoma patients, and cisplatin, the first generation platinum drug. SNB19 and U87MG cells were incubated with PT-ACRAMTU, BCNU, and cisplatin for 24 h. IC_{50} values are reported in Table 1. PT-ACRAMTU appears to be a more cytotoxic drug in both cell lines as evidenced by the lower IC_{50} values; however, there appears to be a differential effect of BCNU and cisplatin in the glioblastoma cell lines. BCNU is a more effective drug in U87MG cells compared to SNB19 cells. While there is a 6-fold higher IC_{50} value for BCNU in the SNB19 cell line, the difference in the U87MG cell line is only 2-fold. Cisplatin appears to be a more effective drug in SNB19 cells compared to U87MG cells, with an almost 2-fold difference between the two cell lines. Shorter incubation times with both drugs produced much higher IC_{50} values in both cell lines (data not shown).

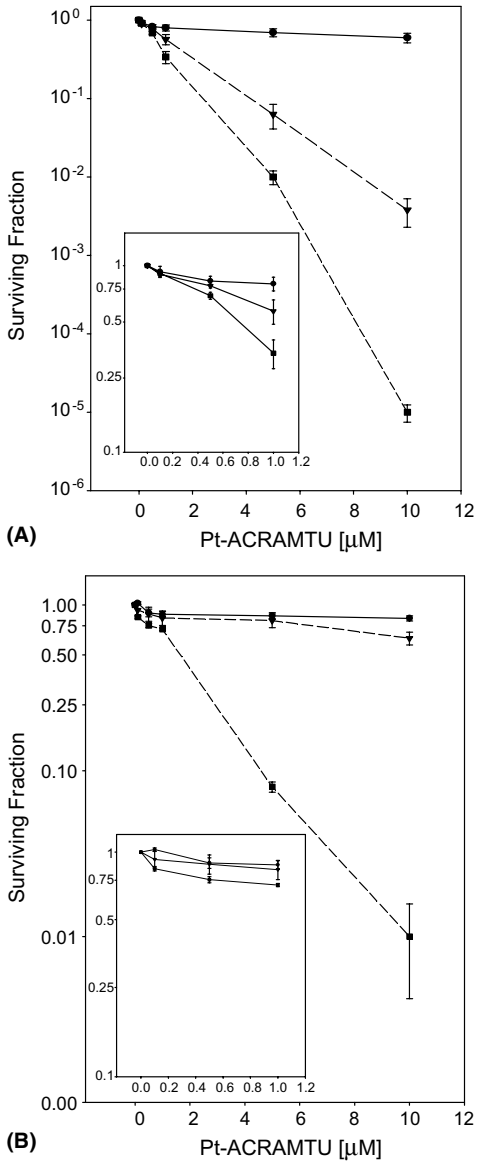

Figure 2. Clonogenic survival assay. (A) SNB19 and (B) U87MG glioblastoma cells were plated at various densities and exposed to PT-ACRAMTU for various incubation times [1 h (circles), 8 h (triangles), and 24 h (squares)]. No treatment controls were also plated. Drug was then removed and following a 14 day incubation, cells were fixed and stained. IC_{50} values were determined using CalcuSyn. Each IC_{50} is the average of three individual experiments ±SEM.

A cell viability assay was used to study the effect of ACRAMTU and PT-ACRAMTU on normal cells:[10] SNB19 cells and rat astrocytes were incubated with ACRAMTU, PT-ACRAMTU, and cisplatin. Cell viability was determined by trypan blue exclusion and is reported in Figure 3. Neither ACRAMTU nor PT-ACRAMTU affected the viability of normal astrocytes even at the highest concentration of 16 μM. In contrast, both drugs reduced viability in the SNB19 cells in a dose-dependent fashion. In contrast, cisplatin had very little effect on

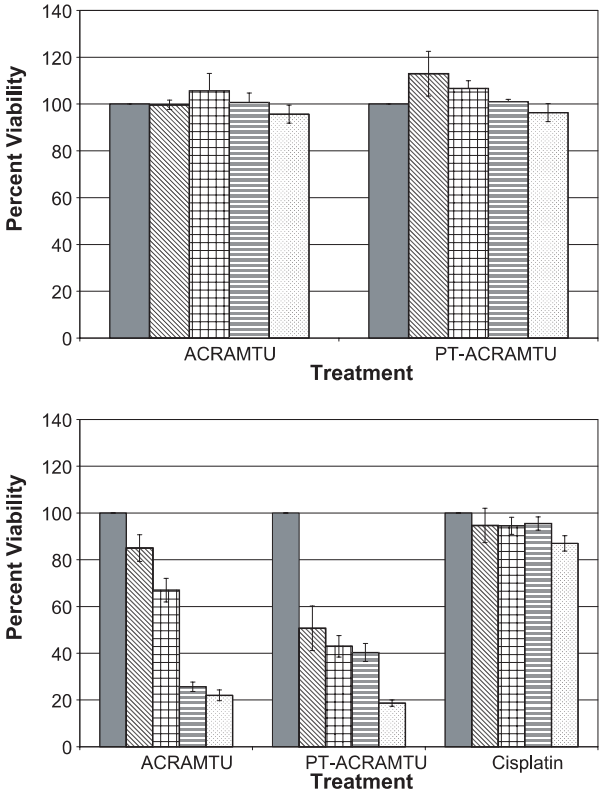

Figure 3. Trypan blue exclusion assays of primary rat astrocytes (top panel) and SNB19 glioblastoma cells (bottom panel). Preliminary data were generated with rat astrocytes due to difficulties with identifying growth media for normal human astrocytes (NHA). Cells were plated and treated with ACRAMTU, PT-ACRAMTU, or cisplatin for 24 h at various concentrations (0 μM, solid columns; 2 μM, diagonal columns; 4 μM, cross-hatched columns; 8 μM, horizontal columns; 16 μM, dotted columns). Cell viability was determined by trypan blue exclusion. The average of three individual experiments ±SEM is shown.

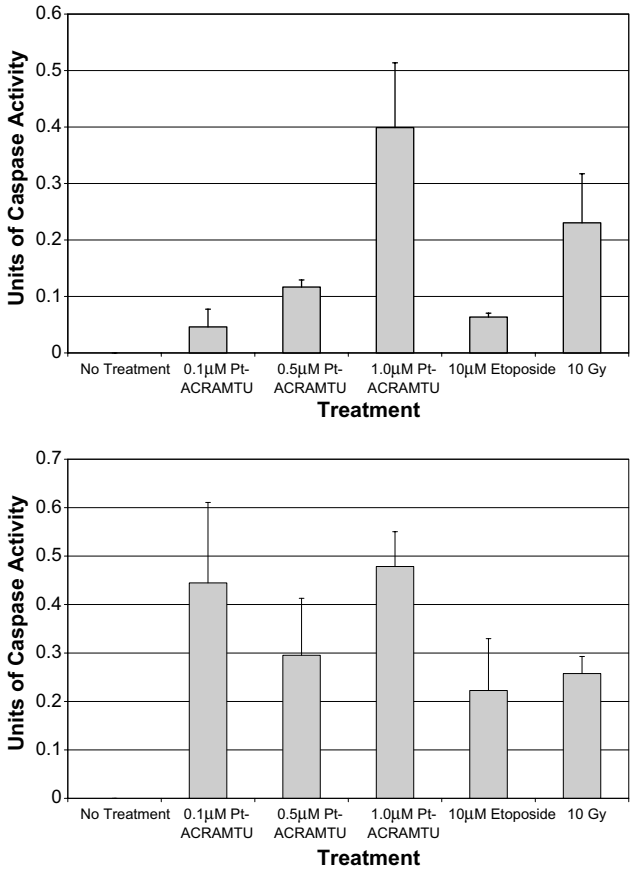

Figure 4. Caspase-3 Activity Assay. SNB19 (top panel) and U87MG (bottom panel) glioblastoma cells were plated at a density of 2×10^6 cells and then treated with various concentrations (0.1, 0.5, and 1.0 μM) of PT-ACRAMTU for 24 h. A 'no treatment', a 10 μM etoposide, and a 10 Gy radiation control were also plated. At 24 h, all cells were harvested and the caspase-3 activity was measured using the Clontech ApoAlert Caspase-3 Colorimetric Assay kit. The average of three individual experiments ±SEM is shown.

SNB19 cells. Similar results have been reported previously for the structurally related platinum-bisintercalating agent, PT-$(ACRAMTU)_2$.[11]

In order to determine if an apoptotic response was generated following PT-ACRAMTU treatment, activation of caspase-3, an enzyme involved in the apoptotic pathway, was monitored.[12] In SNB19 cells, a dose dependent response of caspase-3 was observed, while U87MG cells also had increased levels of caspase-3. Similar levels of caspase-3 were observed at 1 μM PT-ACRAMTU in both cell lines (Fig. 4).

Glioblastoma multiforme is difficult to control clinically with median survival of only 9–12 months. While several chemotherapy drugs are currently used in the treatment of GBM including platinum based drugs (cisplatin, carboplatin), nitrosoureas (BCNU, CCNU), temozolomide, procarbazine, and taxol, not all tumors respond to these therapies and efficacy has been limited due to issues of drug availability (blood brain barrier), associated toxicities, and drug resistance mechanisms.[1] While new methods are currently being developed in regard to drug delivery, additional chemotherapy agents with unique mechanisms of action need to be tested. PT-ACRAMTU, breaks the existing paradigm for active complexes that cross-link formation occurs with the N7 positions of adjacent purine bases in the major groove.[2] In addition to its distinct dual mode of binding (metalation plus intercalation), it appears to act by multiple mechanisms including possible topoisomerase inhibition and inhibition of transcription.[13,14]

In this study, we have demonstrated that ACRAMTU and PT-ACRAMTU show cytotoxic activity in two glioblastoma cell lines that are typically difficult to kill. As in previous studies, it appears addition of the Pt moiety produces a drug that is more active than the unmodified reversible intercalator, ACRAMTU. IC_{50} values of 0.37 μM and 0.75 μM in SNB19 and U87MG cells, respectively, were obtained. Previous IC_{50} values for PT-ACRAMTU ranged from 80 nM in pancreatic PANC-1 cells to 9.6 μM in C13* cisplatin resistant ovarian cells.[6–8,15] PT-ACRAMTU also appeared to be more cytotoxic than BCNU in the cell lines tested, however, when PT-ACRAMTU was compared to cisplatin, the

IC_{50} for PT-ACRAMTU was 2-fold lower and 1.6-fold higher than cisplatin in SNB19 cells and U87MG cells, respectively (Table 1).

We have shown that there is a time and dose dependent cytotoxic effect with PT-ACRAMTU; a 24 h incubation with PT-ACRAMTU generates the best cytotoxic response. While both ACRAMTU and PT-ACRAMTU intercalate quickly into the DNA base stack, irreversible covalent binding of the platinum in PT-ACRAMTU, a relatively slow binding step,[7] appears to be responsible for the greatly enhanced cytotoxicity. This data correlates well with data acquired in cell-free systems, which indicate PT-ACRAMTU binds to and inhibits transcription and endonucleolytic cleavage of plasmid DNA in a time dependent manner.[14]

To determine if an apoptotic response was triggered following PT-ACRAMTU treatment, a marker of apoptosis was monitored. Caspase-3 is activated in both cell lines following exposure to PT-ACRAMTU. In SNB19 cells, the caspase-3 levels are dose dependent and mirror the cell kill observed in the clonogenic survival experiments at drug concentrations <1 μM (see inset of Fig. 2A). This contrasts the situation for U87MG (Figs. 2B and 4, bottom), where high caspase-3 levels do not seem to translate into enhanced cell growth inhibition. In addition, the cytotoxic response in U87MG appears to be delayed compared to SNB19, possibly indicating differences in the apoptotic pathways[16] in the two cell lines.

In conclusion, we have shown that PT-ACRAMTU and ACRAMTU can generate a cytotoxic response in GBM cell lines that is similar to other solid tumor cell lines, but may not affect normal cells. Thus, PT-ACRAMTU and its derivatives[7] may be useful chemotherapy agents in the treatment of GBM, as well as other solid tumors. Further studies will be performed using a nude mouse model to determine the in vivo effects of this hybrid drug.

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9. Clonogenic Survival Assays (see also Refs. 6 and 8): glioblastoma cells were harvested and plated at various densities in 60 mm dishes. Cells were then either not treated or treated with various concentrations of ACRAMTU, PT-ACRAMTU, cisplatin, and BCNU. After 1, 8, or 24 h incubations, drug was removed, plates were rinsed twice with 1 × phosphate buffered saline (PBS), and fresh media added. Cells were incubated for 14 days to allow for colony formation. After 14 days, colonies were rinsed with PBS, fixed with methanol:acetic acid (3:1), and stained using 0.4% crystal violet. Colonies having greater than 50 cells were counted. Surviving fractions were determined. CalcuSyn. (BIOSOFT) was used to determine IC_{50} values.
10. Trypan Blue Exclusion Assay (see also Ref. 11): SNB19 cells or rat astrocytes were plated at a density of 10^5 cells in 60 mm dishes and allowed to grow to 70–80% confluency. Cells were either not treated or treated with various concentrations of ACRAMTU, PT-ACRAMTU or cisplatin. After 24 h incubation, cells were rinsed twice with 1 × PBS and fresh media was added. Cells were incubated an additional 48 h. After 48 h, both media and cells were harvested. Cell viability was determined using trypan blue exclusion.
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12. The ApoAlert Caspase-3 Assay Kit was obtained from Clontech. Glioblastoma cells were plated at a density of 2×10^6 cells in 100 mm dishes for each treatment group in duplicate. Cells were treated with drug at appropriate dose and incubation time. Cells were harvested and centrifuged at 1000 rpm for 5 min. Cells were resuspended in 50 μL chilled lysis buffer and placed on ice for 10 min. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatants (50 μL) were mixed with 50 μL 2 × reaction buffer/DTT mix and 3 μL caspase-3 substrate and incubated for 1 h in a water bath at 37 °C. Samples were transferred to a 96-well plate and optical density (OD) read along with standards (0, 2.5, 5, 10, and 20 nmol pNA) at 405 nm using a microplate reader. A calibration curve was prepared with x = nmol pNA and y = OD units (ODU). The slope (ΔODU/Δnmol of pNA) of this curve was used to calculate units of caspase activity with the following formula: Units caspase activity=ΔODU × 1/curve slope; where ΔODU = difference in ODU between uninduced control and induced sample.
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