

# Ex vivo plasma protein binding and in vitro evaluations of AP5346, a novel platinum-bound biopolymer: Evidence showing that ≥72hr DACH-platinum release may play a major role in cytotoxicity.

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## ABSTRACT

**Background**  
AP5346 displays activity in a wide variety of solid tumors in preclinical models and clinical trials. AP5346 is designed to selectively release DACH platinum (Pt) into the acidic tumor environment. AP5346 has a cytotoxic profile similar to that of oxaliplatin in our human cancer cell line panel. We investigated AP5346 binding to plasma proteins and the kinetics of Pt release.

### Materials and Methods

Plasma protein binding and Pt release were evaluated ex-vivo in plasma at 300 and 30 μg/mL (concentrations representing the therapeutic range, Cmax and Cmin) at 37°C with adjusted pH (7.35-7.4) using Ultra-4 filters (Amicon) with 50 and 3 kDa cut-off. Reversibility of binding was investigated by protein precipitation with acetonitrile. Pt levels were measured by atomic absorption. Antiproliferative effects were evaluated in HT29 and HCT116 human cancer cell lines by MTT assay after 1-72h of exposure.

### Results

Both AP5346 and oxaliplatin bind plasma proteins. AP5346 induces non-covalent protein binding, addition of acetonitrile caused dissociation of all weakly bound ligands. AP5346 binding to proteins was sustained for up to 244h (6 days). In these experiments, AP5346 protein binding was about 94% immediately after AP5346 addition. Unbound Pt was 2.96% (6.3 μg/mL) and 5.73% (1.7 μg/mL) for Cmax and Cmin, respectively. Interestingly, Pt release from plasma-protein bound AP5346-polymer increased progressively over time reaching a steady-state at >72-96h. This slow Pt release was consistent with exposure cytotoxicity kinetics. In vitro, AP5346 also displayed time-dependent cytotoxicity in HT29 and HCT116 colon cancer cells. AP5346 exposure >72h showing higher antiproliferative effects than shorter exposures (<48h). At equimolar concentrations, oxaliplatin was slightly more active than AP5346 for short exposure durations (<48h). Conversely, for duration of exposure >72h, AP5346 displayed IC50 ranging from 0.34-0.5 μM in colon cancer cells while oxaliplatin IC50 ranged from 0.5-0.9 μM. Similarly, AP5346-induced Pt DNA incorporation was time-dependent, with a higher level of Pt bound to DNA observed for exposure >72h in human cancer cells.

### Conclusions

Together, our data strongly suggest that protein-bound AP5346 polymers progressively release free-Pt in plasma, reaching a sustained steady state after >72 h, resulting in sustained exposure to Pt. Considering that extended duration of exposure is essential for AP5346 cytotoxicity, our data may help optimize dosing schedules in the design of future combination clinical trials.

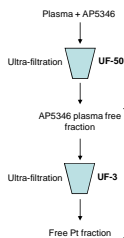
## AIMS OF THE STUDY

- To develop a new methodology to measure AP5346 binding and bioavailability through measurement of its release rate.
- To compare the extent to which AP5346 and oxaliplatin bind to plasma protein ex vivo.
- To assess the reversibility of such plasma protein binding protein.
- To determine the rate of release of platinum from plasma protein-bound AP5346.
- To determine the antiproliferative effects of AP5346 and oxaliplatin in HT29 and HCT116 human cancer cell lines over 1-72hr exposure.

## MATERIALS AND METHODS

### Plasma protein binding:

AP5346 protein binding was studied using plasma or HSA solutions by ultra-filtration. Plasma binding and Pt release were evaluated ex vivo in plasma at concentrations of 30 μg/mL and 300 μg/mL and Cmin and Cmax for the therapeutic concentrations range) at 37°C (pH 7.35-7.40) using Ultra-4 filters (Amicon) with 3 kDa (UF-3) and 50 kDa (UF-50) cut-off. Following ultra-filtration, the concentrations of unbound (free) AP5346 (UF-C) and platinum (UF-P) in the filtrate were measured and used to calculate the amount of bound ligand.



## INTRODUCTION

Platinum-based drugs are among the most active anticancer agents and have been widely used in the treatment of a variety of human tumors. They exert their antitumor activity primarily by creating DNA adducts, leading to cell death by apoptosis or necrosis<sup>1-4</sup>. The effectiveness of first- and second-generation platinum agents such as cisplatin or oxaliplatin is limited, as some tumors are either resistant to these agents or become resistant during treatment.

Oxaliplatin is a third generation platinum compound, which has demonstrated a broad spectrum of activity in a wide range of human tumors in vitro and in vivo and remains effective against cisplatin-resistant tumors. Like cisplatin, oxaliplatin has side effects such as acute and cumulative neurotoxicity, which can be so severe as to limit treatment. Furthermore, the antitumor potential of these drugs is significantly reduced by their readiness to bind to plasma proteins. In tests, following a 2-hour incubation of oxaliplatin in human plasma at 37°C, between 85 and 88% of the platinum was bound to plasma proteins such as albumin<sup>5</sup>. Cisplatin and oxaliplatin have also been found to accumulate in erythrocytes<sup>6</sup>. This leads to cumulative toxicity and an increased rate of hemolysis which may play a role in the incidence of anemia during treatment with these drugs.

AP5346 is a structural analog of oxaliplatin, having the DACH-Pt moiety in common. It is comprised of bioactive DACH-Pt linked via a pH-sensitive chelator and tri-glycine spacer group<sup>7</sup> to a hydrophilic biocompatible biopolymer, which acts as a macromolecular carrier. AP5346 was rationally designed to exploit the enhanced permeability retention effect (EPR) observed in tumors due to their "leaky" vasculature, and to release the DACH-Pt in low pH conditions such as in the extracellular space of hypoxic tumors and the endosome.

Previous pharmacokinetic (PK) studies of AP5346 to determine free bioactive platinum were done by measuring the platinum recovered following filtration through a protein separating filter. This method does not take into account the rate of platinum release from the polymer nor the amount of polymer bound to serum proteins. To measure more precisely the PK and pharmacodynamics of AP5346 we have developed a new approach.

Here we determine the extent of plasma protein binding of AP5346 ex vivo, and compare the rate of release of free-Pt into plasma to the cytotoxicity kinetics of AP5346 in two human cancer cell lines.

### Platinum Binding

The kinetics of platinum release from AP5346 has been well characterized in non-physiological conditions (Figure 1) but to date has never been tested in plasma.

Assessment of the amount of platinum in samples following filtration through a 50 kDa filter found that at either concentration tested (30 μg/mL or 300 μg/mL) approximately 92% of the total amount of platinum administered was bound to plasma proteins which do not pass through the 50 kDa filter. As can be seen (Figure 2) binding was sustained over the 96hr tested, in other experiments (data not shown) stable binding was maintained at time points up to 144 days.

It was expected that the 50 kDa filtrate would contain free platinum and AP5346 plasma-polymer complexes. Likewise, platinum associated with plasma proteins consists of protein-AP5346 and protein-platinum complexes.

Use of a second separation step using the smaller 3 kDa filter allowed estimation of the amount of free-Pt in the 50 kDa filtrate. As can be seen (Figure 3) at both concentrations tested, initially only a very small fraction (<1%) of total platinum administered is free, but the amount of free-Pt increases over time as it is released from the AP5346, with a maximum of between 3-5% after 96hr.

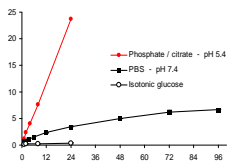


Figure 1: AP5346 – Platinum release is pH sensitive

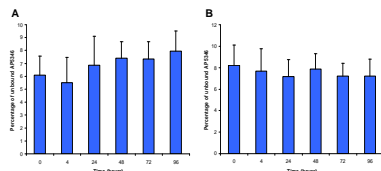


Figure 2: Time course of unbound AP5346  
Percentage of unbound AP5346 measured in samples taken at time points indicated following incubation of AP5346 at concentrations of (A) 30 μg/mL, with plasma (37°C, ex vivo).

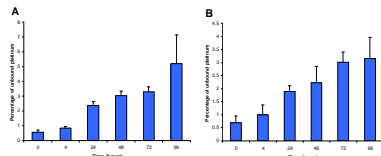


Figure 3: Time course of unbound platinum  
Percentage of unbound platinum measured in samples taken at time points indicated following incubation of AP5346 at concentrations of (A) 30 μg/mL, or (B) 300 μg/mL, with plasma (37°C, ex vivo).

## RESULTS

### Cytotoxicity

AP5346 displayed a time-dependent cytotoxicity in HT29 and HCT116 colon cancer cells, exposure of AP5346 for >72hr showing higher antiproliferative effects than at shorter exposures (<24hr). At equimolar concentrations, oxaliplatin was slightly more active than AP5346 for short duration of exposure (<48hr). Conversely, for duration of exposure >72hr, AP5346 displayed IC50 from 0.3-0.5 μM in colon cancer cells, while oxaliplatin IC50 was 0.5-0.9 μM (Figure 4).

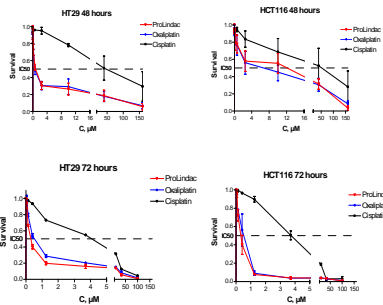
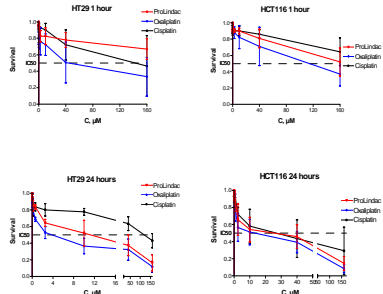


Figure 4: Time-dependent cytotoxicity of AP5346, oxaliplatin and cisplatin in HT29 and HCT116 colon cancer cell lines

### RBC Partitioning

AP5346 RBC partitioning experiments show that at either concentration tested, initially approximately 10% of the total platinum added to blood samples associates with RBCs. This increased slightly over the first 30 minutes of incubation to approximately 15% of platinum associated with RBCs but remained relatively stable at later time points (Figure 5). There is as yet no data available about the topologic nature of the platinum versus RBC association (internalized or surface bound) nor the nature or type of binding (membranes or globins, reversible or weak binding etc.). This work is ongoing.

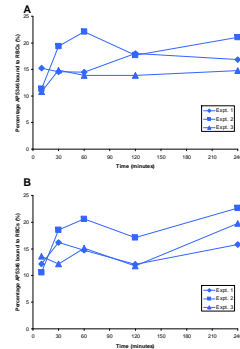


Figure 5. The percentage of total platinum administered associated with RBCs over time  
AP5346 at concentrations of (A) 30 μg/mL or (B) 300 μg/mL was incubated with samples of whole blood in 3 separate experiments.

## CONCLUSIONS

Together, data strongly suggest that protein-bound AP5346 polymers progressively release free-Pt in plasma. This release reaches a sustained steady state after >72 hours, resulting in sustained exposure to Pt.

AP5346 and small Pt species are highly bound, >90%, to plasma and HSA. This binding is time dependent, and this effect will be investigated further.

It is interesting to note that after 96 hours incubation with whole plasma, a similar percentage of bioavailable free-Pt was released, as previously reported in non-physiological pharmacokinetic studies (i.e. 5-10%).

Evaluations of red blood cell partitioning, and biotransformation of AP5346 are ongoing to determine which Pt species are released from polymer.

## REFERENCES

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