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The impact of albumin conjugation on the cytotoxic properties of cisplatin, oxaliplatin and auranofin in cancer cells

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The role of human serum albumin (HSA) in the delivery of anticancer metallodrugs remains unclear and requires further investigation. To this end, bioconjugates of HSA with the metallodrugs cisplatin (CIS), oxaliplatin (OXA) and auranofin (AF) were prepared, characterised by ESI-MS and ICP, and tested for their cytotoxic properties in A2780 and HCT116 cancer cells. Significant differences in the biological activities of the two Pt bioconjugates compared to that of the Au bioconjugate emerged, and they are interpreted and discussed in the context of the available literature.

Protein–metallodrug bioconjugates have emerged as promising candidates for the development of novel cancer therapeutics, exploiting the unique properties of their constituent parts. Several studies have already demonstrated the great potential of such systems, including gold-based ferritin bioconjugates.[1,2]

The strength of these systems lies in the synergistic combination of the metal center – the "pharmacophore" – which confers potent cytotoxic properties, and the protein scaffold, which can enhance its selectivity and bioavailability while mitigating off-target effects. Although metal-based drugs are highly effective at inducing cancer cell death, their therapeutic application is often hindered by poor selectivity and severe systemic toxicity. Therefore, conjugating these compounds with biomolecules, particularly proteins acting as carriers, seems to be an effective strategy to improve their pharmacokinetic and pharmacodynamic profiles.

Of the various protein carriers, human serum albumin (HSA) – by far the most abundant plasma protein – has attracted considerable interest in the field of drug delivery. This monomeric globular protein, which has a molecular weight of ~66.5 kDa, plays a fundamental role in the transport of many endogenous and exogenous compounds through the bloodstream.[3] Several factors contribute to the suitability of HSA for metallodrug conjugation. Firstly, due to the high plasma concentration and the usual intravenous administration route of many chemotherapeutic agents, metal-based drugs are expected to interact significantly with HSA soon after injection, potentially forming stable adducts. Moreover, HSA has several metal-binding sites, including key residues such as Cys34, Trp214 and Tyr411, as well as a few methionines and histidines, which facilitate coordination to metal centers.[4]

In addition, HSA naturally accumulates in tumour tissue due to the Enhanced Permeability and Retention (EPR) effect.[5] This phenomenon arises from structural differences between normal and tumour-associated vasculature. Leaky blood vessels with larger pore sizes and impaired lymphatic drainage allow macromolecules, such as albumin, to accumulate at tumour sites preferentially. This property can be exploited to achieve targeted drug delivery, thereby increasing the concentration of the local therapeutic agent while minimizing the risk of systemic toxicity.

Platinum(II) compounds, such as cisplatin (CIS) and oxaliplatin (OXA), are widely used in clinical oncology to treat various types of cancer, including ovarian, testicular, colorectal, and lung cancer.[6] CIS (see Fig. 1), the first platinum drug to be approved by the FDA, exerts its cytotoxic effects primarily by forming DNA adducts. This interferes with DNA replication and transcription, eventually inducing apoptotic cell death.[7] Despite its efficacy, CIS has significant drawbacks, including severe systemic toxicity, the rapid development of drug resistance, and dose-limiting side effects. OXA (see Fig. 1), a third-generation platinum drug, circumvents some of the resistance mechanisms encountered with CIS to a certain extent. However, its long-term use is still limited due to severe neurotoxicity and other adverse effects.[8]

Unlike platinum-based drugs, gold(I) compounds such as auranofin (AF) (see Fig. 1) have emerged as a promising new

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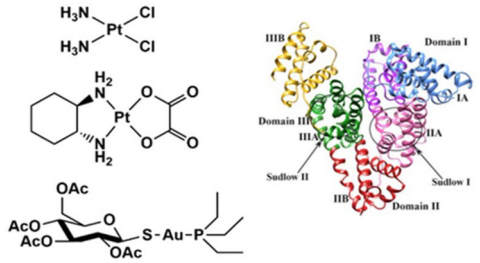

Fig. 1 Chemical structures of CIS (top), OXA (middle), and AF (bottom). On the right side, the 3D structure of the HSA with its domains, subdomains and Sudlow's binding sites I and II are shown. This latter, reproduced from ref. 9 with permission from Elsevier, copyright (2019).

class of anti-cancer agents with distinct mechanisms of action. Some of these compounds have entered clinical trials for cancer treatment.[10] Originally developed to treat rheumatoid arthritis, AF was later repurposed as an anti-cancer agent due to its potent inhibition of the selenoenzyme thioredoxin reductase (TrxR), which plays a key role in cellular redox homeostasis. The strong affinity of gold(I) for the selenocysteine residue in TrxR leads to the enzyme being profoundly inhibited, thereby promoting severe oxidative stress and subsequent apoptosis in cancer cells.[11,12] These unique properties make AF and related gold(I) compounds highly attractive candidates for targeted delivery *via* protein conjugation, which could enhance the therapeutic efficacy while reducing the systemic toxicity.

Although conjugating therapeutic metal complexes to HSA may offer several advantages, such as improved stability, solubility and circulation time, which may favor selective tumour accumulation, the strong binding of the metal center to albumin could result in the drug becoming sequestered in an inactive form. This would reduce its free concentration and impair its intrinsic cytotoxic efficacy. Therefore, the efficacy of the bioconjugate ultimately depends on a combination of many favorable and unfavorable factors, not easily predictable. Previous studies have investigated the formation of adducts between metallodrugs and serum albumin, as well as their potential pharmacological role.[13,14] However, the role of these adducts remains controversial and requires further investigation.[9]

A few comprehensive reviews have examined the interaction between platinum drugs and HSA in detail, highlighting that this issue has remained unresolved for years.[9] While some researchers have argued that binding to HSA mainly deactivates the cytotoxic activity of platinum drugs by preventing its interaction with the true cellular targets, such as DNA,[15] others have proposed that this interaction may result in the formation of a drug reservoir of some sort.[16,17] This would prolong systemic circulation and enhance therapeutic efficacy over time.

In the present study, our aim was to shed light on this long-standing controversy by systematically investigating the formation of HSA bioconjugates with CIS, OXA and AF, and their cellular effects, through a series of focused experiments. First, we characterised the binding profiles of these drugs to HSA using electrospray ionisation mass spectrometry (ESI-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES). This enabled us to evaluate the metal/protein binding stoichiometry, the nature of the protein-bound metal fragments, and the potential binding sites. Next, we measured the antiproliferative activity of the bioconjugates against A2780 (ovarian cancer) and HCT116 (colorectal cancer) cells, comparing their cytotoxicity with that of the free drugs. Metal uptake determinations helped us to elucidate the underlying mechanisms. In summary, our study provides valuable insight into the impact of HSA binding on the therapeutic efficacy of metal-based anticancer agents and could inform the future development of optimised albumin-based drug delivery systems.

The HSA conjugates with the three metallodrugs were prepared according to a standardised protocol involving the direct reaction of each metallodrug with the protein under tightly controlled solution conditions. Further details are provided in the SI. Briefly, freshly prepared solutions of CIS, OXA and AF were added to an aqueous solution of HSA at a metal-to-protein molar ratio of 1 : 1. The kinetics of adduct formation were evaluated by recording ESI-MS spectra at different incubation times (1, 3, and 24 hours) at 37 °C. Under these conditions, AF was found to bind rapidly to HSA, with spectra at 3 h already comparable to those at 24 h (Fig. S1). In contrast, the Pt-based drugs (CIS and OXA) exhibited markedly slower kinetics, with only weakly detectable adduct signals at 1 h and 3 h and well defined adducts forming only after 24 h (Fig. S2 and S3). To ensure consistent and comparable conditions across all metallodrugs, a 24 h incubation time was therefore adopted for the preparation of all bioconjugates (see SI, Section S1.4). Additional reactions were also carried out using higher protein to metal ratios (1 : 2 and 1 : 3); the corresponding ESI-MS spectra are reported in the SI (Fig. S4–S6). However, the best defined adducts and most reproducible results were obtained at a 1 : 1 ratio, which was therefore selected for all subsequent experiments (see SI, Section S1.5). After incubation, the samples were extensively dialyzed against water to remove excess, unreacted metal complexes. The purified conjugates were then collected for further analytical determinations.

The formation of HSA-metallodrug adducts was clearly demonstrated by ESI-MS analysis. The ESI-MS spectra of the conjugates are shown in Fig. 2, alongside the spectrum of native HSA.

The control spectrum of free HSA shows three main recognizable peaks, which is consistent with previous reports from our laboratory.[18,19] The first peak, at 66 439 Da, corresponds perfectly to the HSA sequence (blue dot). The two higher-mass peaks, on the other hand, can be associated with HSA species that have undergone post-translational modifications. Specifically, the peak at 66 557 Da is associated with cysteinylated HSA (red dot), while the peak at 66 719 Da is associated with glycosylated HSA (orange dot). The peak at 66 471 Da (highlighted by a blue arrow) could not be clearly assigned. We would like to emphasize that the protein was purchased com-

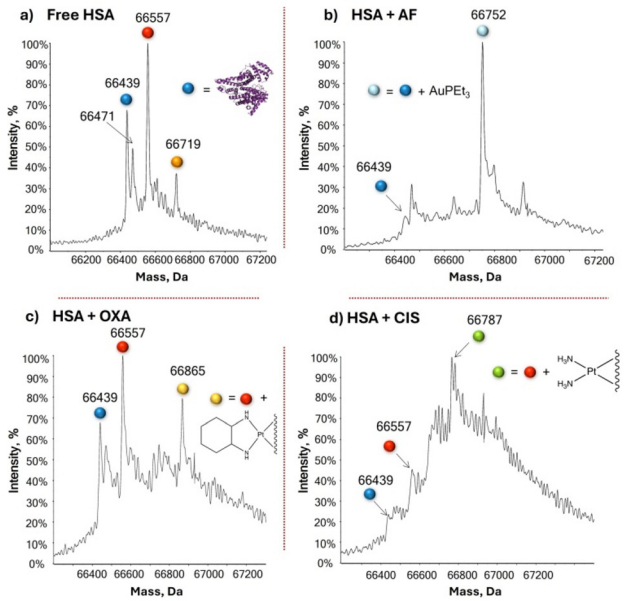

Fig. 2 (a) ESI-MS spectra of HSA (blue dot) with a peak corresponding to 66 439 Da, cysteinylated HSA (red dot) with a peak at 66 557 Da and the glycosylated HSA with a peak at 66 719 (orange dot); (b) ESI-MS spectrum of HSA after 24 h incubation with AF. The AF fragment attached to the protein corresponds to $AuPEt_3$ (light blue dot) with a peak at 66 752 Da; (c) ESI-MS spectrum of HSA after 24 h incubation with OXA. The yellow dot (66 865 Da) represents the cysteinylated HSA plus an oxalate moiety deriving from OXA fragmentation; (d) ESI-MS spectrum of HSA after 24 h incubation with CIS. The green dot represents the cysteinylated HSA plus a CIS fragment, corresponding to a peak of 66 787 Da.

mercially and that these post-translational modifications were intrinsic to the HSA stock.

Notably, adding the three metal drugs individually produced significant alterations to the mass spectrum of the native protein, as detailed below. When free HSA was reacted with AF, the intensity of several of the aforementioned peaks was greatly reduced and a new peak appeared at 66 752 Da (+314 Da), which belongs to HSA conjugated with the triethylphosphine gold $[AuPEt_3]^+$ moiety of AF (light blue dot). The spectrum obtained from the reaction with OXA was slightly different; all the peaks of free HSA were still present, along with a new peak at a higher mass (66 865 Da; +307 Da) (yellow dot). This corresponds to the binding of a specific OXA fragment, *i.e.* (*R*,*R*)-*trans*-1,2-diaminocyclohexane platinum(II), referred to as DACH-Pt ($C_6H_{12}N_2Pt$). Lastly, interpreting the mass spectrum of the HSA sample reacted with CIS proved to be the most challenging task. The X-ray crystal structure of HSA in the complex with CIS previously reported revealed that the protein displays up to five distinct platinum-binding sites, primarily involving methionine and histidine residues.[20] This high degree of metal binding heterogeneity and the consequent multiplicity of species contribute to the complexity of the resulting mass spectrum, leading to signal broadening, spectral distortion and loss of resolution. Indeed, in our measurements, we observed a general reduction in peak intensity; however, these spectral alterations are indicative of significant interactions between HSA and CIS. The signal detected at 66 787 Da (green dot) is associated with the interaction between the cysteinylated HSA and $[Pt(NH_3)_2]^{2+}$.

Overall, a careful examination of these ESI-MS spectra reveals adduct formation with each of the three metallodrugs. To provide additional support for this finding, the samples were analysed using ICP-OES to determine the exact amount of gold or platinum present in each sample. The results are shown in the SI (Table 1), with values expressed in relation to protein concentration. The results show that the percentage of protein metalation is around 70–80% for platinum drugs and 30% for AF. These results are consistent with previous reports from Shaw and Gailer. Indeed, Frank Shaw demonstrated that the degree of protein metalation exerted by AF towards HSA depends on the experimental conditions.[19] Gailer and colleagues also showed that CIS binds to HSA to a large extent after three hours of incubation in plasma, with an unbound drug amount comparable to those found in our experiments.[9]

These ICP data revealed significant differences among the binding of the distinct compounds. AF exhibited a binding ratio of approximately 0.328 gold atoms per HSA molecule, while CIS and OXAHSA adducts showed higher values, with 0.952 and 0.825 platinum atoms per protein, respectively. These differences may reflect the distinct coordination behaviour of the two metals toward albumin. Gold(I), as delivered by AF, is known to form a highly selective and covalent interaction with the free thiol group of Cys34, resulting in a 1 : 1 stoichiometry that is typically well-defined and limited to a single high-affinity site. In contrast, platinum(II) complexes such as CIS and OXA tend to exhibit a broader and less selective binding profile, involving multiple nucleophilic residues including methionines and histidines. This multisite coordination likely contributes to the higher metal/protein ratios observed for the platinum adducts, which may reflect partial occupancy of several lower-affinity binding sites on the albumin surface.

The cytotoxic effects of the three bioconjugates described above were then measured in two representative cancer cell lines, *i.e.* the ovarian cancer line A2780 and the colorectal cancer line HCT116, in comparison with those of the free drugs. The resulting IC_{50} values are given in Table 2 (see also Table S1, Fig. S13 and 14).

Examination of these data shows that, upon HSA conjugation, the two platinum drugs essentially lose their antiproliferative activity in the two cancer cell lines. The IC_{50} values for

Table 1 Average number of metal atoms bound per protein molecule, with standard deviations

Adduct	Metal[a] atoms for HSA molecules
AF	0.462 ± 0.03
CIS	0.972 ± 0.03
OXA	0.693 ± 0.02

[a] Au for AF and Pt for CIS and OXA.

Table 2 Comparative IC_{50} data in A2780 and HCT116 treated with the adduct for 24 or 72 hours. Results are reported as the mean ± SD of four independent experiments

	A2780		HCT116	
Drug	IC_{50} (μM)	±SD	IC_{50} (μM)	±SD
HSA–AF 24 h	4.320	0.237	2.802	0.080
AF 24 h	1.295	0.201	1.218	0.205
HSA–AF 72 h	1.563	0.275	2.170	0.310
AF 72 h	0.327	0.047	1.249	0.087
HSA–OXA 24 h	>100	—	>100	—
OXA 24 h	69.60	5.858	74.58	10.98
HSA–OXA 72 h	>100	—	>100	—
OXA 72 h	1.435	0.276	2.274	0.458
HSA–CIS 24 h	>100	—	>100	—
CIS 24 h	15.08	2.983	33.51	5.019
HSA–CIS 72 h	>100	—	>100	—
CIS 72 h	2.074	0.071	9.797	0.945

the bioconjugated drugs are indeterminable and, in any case, greater than 100 μM. In contrast, AF retains a considerable degree of biological activity when bound to HSA. We wondered whether this significant difference could be attributed to differences in metal uptake. Therefore, we conducted additional ICP-OES experiments on the A2780 cell line to measure metal uptake. For these experiments, we selected CIS and AF only. Specifically, we measured the cellular uptake of the metal for the free drug and its respective HSA conjugate. The experimental details are provided in Table S2, and the results are summarised in Fig. 3.

Interestingly, there is a moderate (approximately 20%) reduction in cellular uptake of gold when switching from the free drug to the HSA adduct. A far more pronounced decrease in platinum uptake is detected when comparing free CIS with its HSA conjugate. In the latter case, platinum uptake is reduced to approximately one third compared to that of the free drug. These results are broadly consistent with previous literature reports, indicating that binding metals to HSA leads to reduced cellular internalisation compared to that of the free drug.[15] It is tempting to establish possible correlations between the results of the cytotoxicity tests and the metal uptake results and try to provide a mechanistic explanation. In

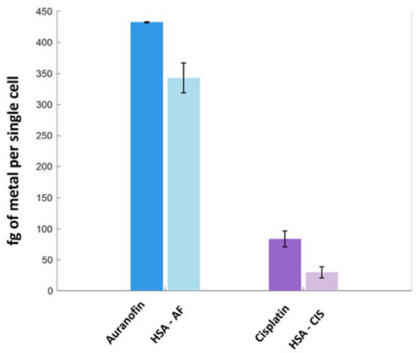

Fig. 3 Comparative assessment of intracellular metal content by ICP-OES analysis. AF and CIS *vs.* their HSA-adducts in the A2780 line.

the case of AF, gold uptake decreases by around 20% upon HSA conjugation, while cytotoxicity decreases by a factor of five. In contrast, platinum uptake is reduced to approximately one-third that of the free drug when CIS is conjugated to HSA, but the cytotoxicity decreases by more than a factor of 50.

These observations enable us to formulate specific mechanistic hypotheses. The reduction in AF cytotoxicity cannot be attributed solely to reduced gold uptake. It is reasonable to assume that, once inside the cells, the AF–HSA bioconjugate remains sufficiently stable and exhibits an intrinsically lower level of cytotoxicity than free AF. This reduced activity may be due to the gold pharmacophore being slowly released from the HSA conjugate. Interestingly, in the case of CIS, we observed that the bioconjugate can enter cells, albeit in significantly smaller amounts than the free drug. However, it is now virtually devoid of biological activity.[13]

We can assume that the inability of the intracellular CIS–HSA bioconjugate to cause cancer cell death is due to either the inability of the platinum center to be released from the protein or its inability to reach nuclear DNA, where platinum is believed to exert its pharmacological action.[15] Notably, a similar difference between Pt and Au bioconjugates was recently found in the case of human ferritin,[1,2,21] the gold derivatives still manifesting a high cytotoxic activity in contrast to the inactive Pt derivatives. This observation likely implies that the binding to these proteins (specifically HSA and human ferritin) severely interferes with the usual intracellular movement of anticancer Pt compounds, thereby preventing their interaction with the final targets and abolishing their cytotoxic action. To further explore this hypothesis, we evaluated the stability of the HSA–metal bioconjugate adducts under conditions mimicking the intracellular environment, namely at acidic pH (to mimic the endosomal–lysosomal pathway) and in the presence of glutathione (GSH). The experiments were conducted kinetically by monitoring the evolution of the ESI-MS spectra at different time points after incubation with GSH or under acidic conditions (further details are provided in the SI, Section S1.6). For the HSA–AF adduct, no significant spectral changes were observed under acidic conditions, indicating that the complex remains stable at low pH (Fig. S10). In contrast, upon incubation with GSH, a marked decrease in the signal corresponding to the conjugate and a concomitant increase in the peak of free HSA were detected over time, consistent with a progressive, partial dissociation of the gold compound from the protein (see Fig. S7). No significant spectral changes were detected for the HSA–Pt adducts throughout the monitored time window, under either reducing or acidic conditions, suggesting that the platinum moieties remain firmly bound to the protein (see Fig. S8 and S9; Fig.S11 and S12). This behaviour provides additional support for the hypothesis that the HSA–AF bioconjugate can release the gold pharmacophore within the cell, thus retaining its anticancer activity. Conversely, the strong and irreversible binding of platinum to HSA likely prevents drug release and accounts for the complete loss of cytotoxic activity observed for the platinum bioconjugates.

Conclusions

The extent to which the binding of anticancer metallodrugs to plasma proteins affects their activity is a subject of intense debate, with conflicting reports in the literature. This prompted us to clarify this controversial issue. To this end, conjugates were prepared between three well-established metallodrugs and HSA, which were then characterised in detail using MS and ICP measurements. The antiproliferative effects of these bioconjugates were then evaluated in two distinct cancer cell lines, and the results were compared with those of the respective free drugs. Interestingly, relevant differences in cellular effects were observed between the two platinum (Pt) drugs and the gold (Au) drug AF. Indeed, we found that conjugating Pt drugs to HSA resulted in a total loss of biological activity, which cannot be explained by the reduced cellular uptake of Pt alone. We therefore conclude that Pt–HSA conjugates are intrinsically inactive, most likely because the Pt centre strongly binds to the protein, preventing subsequent intracellular release. In contrast, the AF–HSA conjugate retained significant antiproliferative activity following albumin conjugation, suggesting that gold release is still possible in the latter case. Despite other experiments needed to better characterize the molecular mechanisms, significant support for our interpretation comes from the recent work by Zou *et al.*,[22] which demonstrated that the cytotoxicity of the AF–HSA adduct is restored upon thiol addition. The TGTA ligand and other small-molecule thiols enhance AF's antitumour activity by releasing the protein-bound metal fragment, indicating that the HSA–AF interaction is reversible in the presence of thiols. In contrast, Ivanov, Sadler *et al.*[23] showed that thiols do not demetalate preformed CIS–HSA adducts. These findings support our view that the gold–albumin adduct is less stable than the platinum ones. Consistently, our metalation experiments (1 : 1 drug : HSA) showed ~30% metallation for AF *vs.* 80–100% for CIS and OXA, confirming the higher affinity of albumin for platinum drugs. Also, the role of albumin binding in platinum drug inactivation is underscored by the enhanced kidney toxicity observed under hypoalbuminaemic conditions.[24] Although reliable pharmacokinetic and pharmacodynamic data require animal models, this lies beyond the scope of the present study, which focuses on generating cellular data with predictive value for future *in vivo* validation. The pharmacokinetic aspects of platinum and gold drug–protein interactions have been previously described.[25] Shaw *et al.* showed that gold efflux from red blood cells in plasma follows first-order kinetics (k = 0.81 ± 0.18 h^{-1}), driven by serum albumin and glutathione, providing the first evidence that the AlbSAuSG complex can circulate as an AF metabolite, corroborating our findings.[26] Finally, this study has important implications for the design of new metallodrug albumin conjugates and drug delivery systems/formulations that can shield selected drugs from interacting with plasma proteins, thereby preserving their pharmacological activity. Our findings suggest that the nature of the metal center and its interactions with the carrier protein play a crucial role.

Author contributions

TM and LM conceived the original idea for this paper and prepared its original version. LMa and VV performed the ESI-MS experiments and carried out metallodrug–HSA adduct preparation. LC, MLT and CG with the support of TM carried out cellular experiments. MS performed the ICP experiments. All the authors contributed to writing and revision approving the submitted version.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: adduct preparation and characterization; ESI MS experiments; ICP-OES experiments and Au–Pt cellular uptake; cell culture; and IC_{50} determination. See DOI: **https://doi.org/10.1039/d5qi01487e**.

Further data underlying this article will be shared on reasonable request to the corresponding authors.

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