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COMMUNICATION

Toward multifunctional anticancer therapeutics: Post-synthetic carbonate functionalization of asymmetric Au(I) bis-*N*-heterocyclic carbenes

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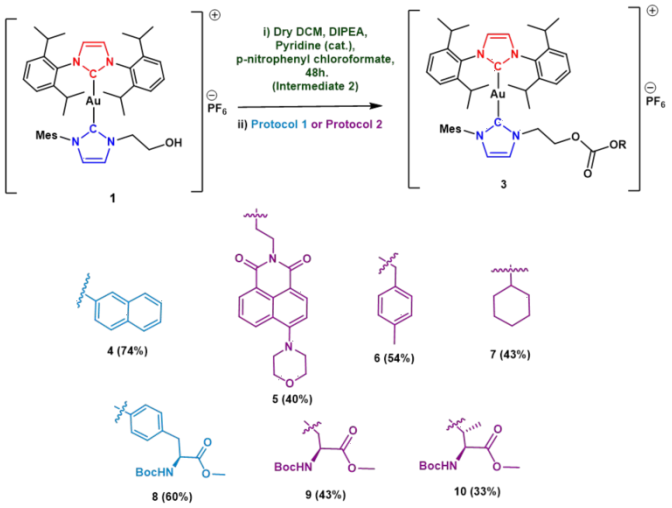
A post-synthetic strategy is reported that allows for functionalization of Au(I)-Bis NHCs via carbonate formation. The scope of this methodology was explored using both aromatic and aliphatic alcohols. As a demonstration of potential utility, the fluorescent Au(I)-bis NHC conjugate 5 was prepared; it was found to have enhanced stability when formulated with bovine serum albumin, localise within the mitochondria of A549 cells and do so without compromising the high cytotoxicity seen for the parent Au(I)-Bis NHC system.

Gold(I) bis-*N*-heterocyclic carbenes (Au(I) bis-NHCs) are emerging as a promising new class of potential metal-based chemotherapeutics.[1–5] Their inherent mitochondrial selectivity[1,6] combined with their ability to inhibit thioredoxin reductase (TrxR) has provided a platform for the development of potential tumour cell selective chemotherapeutics.[1–3,7–10] However, based on explorations of various Au(I)-NHCs[2–4,11–13], it is becoming increasingly appreciated that TrxR inhibition may not be the sole mechanism of action. Studies of structure-activity relationships (SAR) could help our understanding of this class of complexes. The ability to derivatise Au(I) bis-NHCs is viewed as essential to this latter effort.[14,15] The potential benefits of such SAR studies are underscored by the effectiveness achieved via the post-synthetic modification of Pt (II/IV)-based drugs.[16–20]

The asymmetric nature of heteroleptic Au(I) bis-NHCs provides significant advantages over symmetric homoleptic Au(I) bis-NHCs, as they can be used for further derivatisation.[14] However, the harsh conditions required to synthesize heteroleptic Au(I) bis NHCs often prevents the incorporation of linkers of varying anticipated stabilities, i.e., esters < carbonates < carbamates < amides.[21,22] Recently, we reported the asymmetric Au(I) bis-NHC **1**, which contains a single alkyl hydroxyethyl "arm". We found that complex **1** could be subject to post-synthetic carbamate functionalization with a series of different amines.[14] In an effort to expand the "toolbox" of methods amenable to Au(I)-bis NHC post-synthetic modification, we now detail functionalization of **1** via a carbonate linker using various aliphatic and aromatic alcohols as the reaction partners (Scheme 1). Carbonates are a common prodrug motif in therapeutics[23–26] and we considered it likely that their use might allow access to potentially promising new leads. As a proof-of-concept demonstration, the naphthalimide-based conjugate **5** was prepared. As detailed below, this system was found to bind well to bovine serum albumin (BSA), localize to mitochondria, as determined by fluorescence microscopy, and yield a strong cytotoxic response in the A549 human lung cancer cell line.



Scheme 1 - Synthesis of various carbonate-based Au(I) bis-NHCs using **1** as a synthetic platform. **Protocol 1**: Aromatic alcohol, dry dichloromethane (DCM), triethylamine (TEA), 24 h. **Protocol 2**: Aliphatic or benzylic alcohol, dimethylaminopyridine (DMAP), dry

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DCM, 24 h. Note – yields (shown in brackets) were calculated based on isolated product.

Scheme 1 summarizes the reaction chemistry used to produce carbonate functionalized Au(I) bis-NHCs of generalized structure **3** from the starting hydroxyethyl Au(I) bis-NHC **1**. In brief, complex **1** was reacted with 4-nitrophenyl chloroformate in a solution of DCM containing diisopropylethylamine (DIPEA) as a base and a catalytic quantity of pyridine to afford **2** in good yield (87%). With reactive intermediate **2** in hand two protocols were employed to effect conversion to various carbonate functionalized Au(I) bis-NHCs. Owing to the relatively high acidity of phenolic hydrogens, aromatic alcohols such as β-naphthol, were easily conjugated with **2** using dry dichloromethane (DCM) and NEt_3, conditions referred to as Protocol 1 (yields: 60 - 74 %). Unfortunately, Protocol 1 proved unsuccessful for the conjugation of aliphatic and benzylic alcohols. Success was encountered by mixing **2** in dry DCM along with dimethylaminopyridine (DMAP) in the presence of an aliphatic or benzylic alcohol. This procedure, Protocol 2, provided access to the desired carbonate aliphatic/benzylic derivatives (yields: 33 – 54 %). Using this latter protocol, Au(I)-bis-NHCs conjugates **5-7** were successfully synthesized and fully characterized (Scheme 1 and see Table S2). Single crystals of **4**-**6** suitable for X-ray diffraction analysis were grown via slow diffusion of diethyl ether into DCM solutions of the complex (for details see the SI). Two representative structures are shown in in Figure 1.

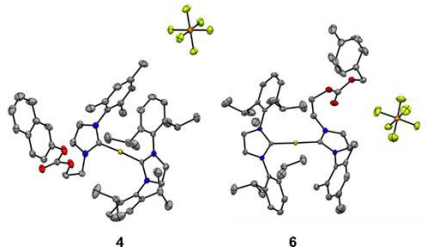

Figure 1 - ORTEP representations of **4** and **6**. Hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 50% probability level. Further details of these structures and that of 5 may be obtained from the Cambridge Crystallographic Centre by making reference to CCDC nos. 1999228-1999230.

Both Protocols 1 and 2 proved successful for the conjugation of alcohols containing amino acids yielding modified tyrosine, serine, and threonine linked Au(I)-bis NHCs **8-10**. Peptides and amino acid analogues have been widely used in anticancer therapy,[27,28] with amino-acids linked Pt(II) complexes displaying promise as anticancer agents.[29,30] Unfortunately, in spite of several attempts, the conjugation of tertiary alcohols with **2** proved unsuccessful. The naphthalimide-based conjugate **5** was of particular interest owing to the fact that it contains a fluorescent moiety tethered to the putative Au(I)-bis NHC therapeutic core. In addition, naphthalimide derivatives are recognised for their ability to stabilise serum albumin-drug interactions.[31,32] This was viewed as attractive in the context of developing Au(I) complexes as potential drug leads. In spite of a number of successful *in vitro* studies being reported, the highly lipophilic nature of most Au(I) bis-NHCs have limited their full biological evaluation.[33] It was thus envisioned that the naphthalimide-based Au(I) bis-NHC **5** may enhance binding to serum albumins, thus boosting its effective solubility and stability. As shown in Figs. 2a and S9, in their native form both **1** and **5** precipitate from aqueous solution after a 15-minute incubation period at a concentration relevant for *in vivo* studies (100 µM, 2% DMSO in phosphate buffered solution). However, in the presence of 0.6 mM BSA (physiological concentration, 6 equiv.), solutions of **5** remained transparent even after incubation for 6 days at 37° C (Fig. 2a).

To confirm this apparent binding and solubilisation by BSA, fluorescence titrations were performed with BSA (5 µM) against increasing equivalents of either **1** or **5** (cf. Figs. 2b, S3, and S5). The addition of **1** or **5**, led to a decrease in the inherent fluorescence intensity of BSA at 344 nm, which was taken as evidence of BSA binding. As expected for our design strategy, the extent of quenching was much higher in the case of **5** than for **1** (60% vs 26%). This finding is interpreted in terms of a higher level of binding for **5**. Quantitative analysis of the fluorescence data using the Stern-Volmer equation provided support for this conclusion**.** The bimolecular quenching constants, K_q, calculated from the associated Stern-Volmer plots were found to be (3.8 ± 0.3) x 10^{12} and (1.4 ± 0.2) x 10^{13} $M^{-1}s^{-1}$ for complexes **1** and **5**, respectively. These values are consistent with binding and thus static quenching between the BSA and the Au(I) complexes in question (See the SI for further analyses - Table S2).[34] The binding of **5** with BSA was further revealed through qualitative analyses, in which both BSA and, separately, **5** with BSA were precipitated from solution using cold EtOH. The resulting pellets were washed with PBS and checked for fluorescence with a handheld UV lamp (excitation: 365 nm). A clear distinction in the fluorescence intensity between the BSA only pellet and that produced from **5** and BSA was observed (Fig. 2c). Importantly, an enhanced fluorescence intensity was observed in the presence of BSA (Fig. S8). This latter finding was believed to be the result of BSA preventing aggregation and attendant quenching of a poorly soluble fluorophore.[35]

Carbonates are often seen as labile and prone to hydrolysis, which prevents their full therapeutic evaluation.[36] We believed that interaction with BSA could provide a relatively stable formulation that would also benefit from enhanced aqueous solubility. To test this hypothesis, complex **5** (100 µM) was dissolved in a 1:1 methanol/water mixture and subject to time dependent HPLC analysis. Over time, significant degradation of **5** was observed, which was ascribed to hydrolysis of the carbonate unit (Figs. 2d, and S2A). When a similar study was carried out in the presence of BSA, complex **5** remained largely intact even after incubation for 6 days at 37° C (Figs. 2d and S2B).

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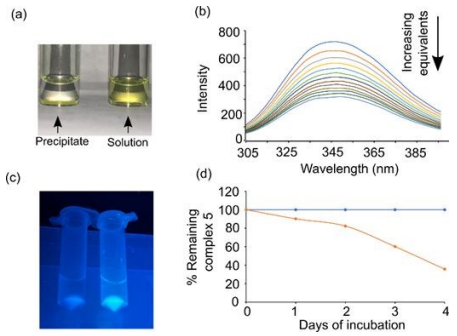

Figure 2 – (a) **5** (100 µM, 2% DMSO in PBS) photographed in the absence (left) and presence (right) of BSA after allowing to mix for 15 min. (b) Fluorescence quenching of BSA (5 µM) observed in the presence of increasing equivalents (0-3.96 equiv.) of **5**. (c) BSA pellets (left) without complex **5** (right) and with complex **5** illuminated with a hand-held UV lamp (excitation: 365 nm), and (d) stability comparison of **5** in the presence (blue) and absence (orange) of BSA.

As documented in previous literature reports, BSA binding can be seen with Au(I) bis-NHC complexes; however, this has led to a significant reduction to their cytotoxicity.[13] Therefore, an effort was made to test the antiproliferative activity of **1** and **5** (including each complex in the presence of BSA) against the A549 human lung cancer cell line (obtained from the ATCC, Manassas, Virginia, USA). The non-conjugated alcohol **1** and Auranofin were used as benchmark comparisons. Incubation was carried out for 72 h in all cases. The resulting IC_{50} values revealed an enhanced cytotoxicity for **5** as compared to **1** with no loss in cytotoxic potency being seen after **1** or **5** was treated with BSA for 1 h (Table 1).

Table 1: Cell proliferative data in A549 lung cancer cell line.

Compound	IC_{50} (µM)	Std. Error (+/-)
Auranofin	**2.23**	**0.12**
1	**0.149**	**0.018**
5	**0.072**	**0.004**
1 with BSA	**0.134**	**0.025**
5 with BSA	**0.059**	**0.003**

As noted above, complex **5** contains a naphthalimide subunit, which was expected to allow its cellular uptake to be followed via confocal microscopy. Moreover, the large stokes shift (~140 nm) seen for **5** could make it attractive for fluorescence imaging.[37] Most Au(I) bis-NHCs are cationic and, as a result, typically localize in the mitochondria.[1,14] This general expectation notwithstanding, certain naphthalimide functionalised Au(I) mono carbenes[38] and morpholine substituted naphthalimide-based probes[37,39] have been reported to localize in lysosomes. Which localization effect, if any, would dominate in the case of **5** was thus not clear. In addition, the presence of BSA may affect the cellular localisation relative to that seen for **5** alone. Cellular localisation studies were thus carried out using the A549 cell line as shown in Figure 3. It was found via confocal microscopy that both **5** and **5** + BSA localise within the mitochondria, as inferred from inspection of merged images recorded using MitoTracker® Red in the presence of either **5** or **5** + BSA.

Owing to the high cytotoxicity of **5**, A549 lung cancer cells were treated with low concentrations of **5** (1 µM). At these low concentrations, **5** remained soluble during the course of the experiment. Moreover, easy-to-discern images were obtained in the case of both **5** and **5** + BSA. In contrast, the control naphthalimide fluorophore (**Morpho-Np-OH**) produced little in the way of an observable emission signal. This lack of intracellular fluorescence is ascribed to the inability of **Morpho-Np-OH** to cross the cell membrane. In contrast, we suggest that **5** and **5** + BSA are able do so.

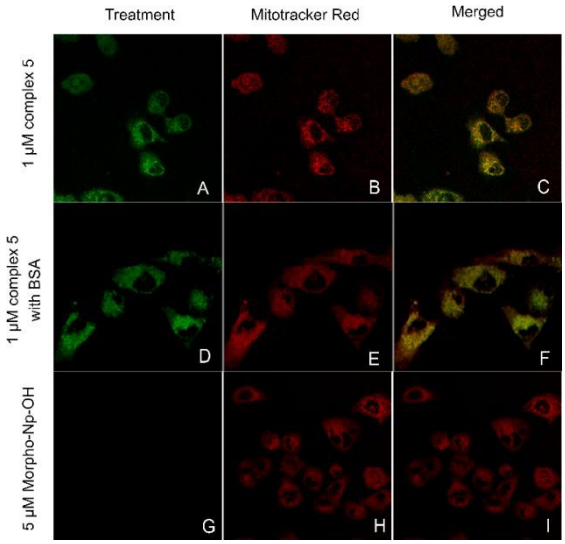

Figure 3 – Confocal microscopic images of A549 lung cancer cells after treatment with (A-C) 1 µM **5**, (D-F) 1 µM **5**, and BSA (G-I) 5 µM **Morpho-Np-OH**.

In conclusion, we report a general synthetic method for attaching various functional alcohols to the heteroleptic Au(I) bis-carbene **1** via carbonate conjugation. Two specific protocols were developed and used to conjugate different alcohol-containing subunits to **1**. Amongst the conjugates reported, the rationally designed naphthalimide functionalised **5** proved to be of special interest since it was found to interact with BSA to provide a soluble formulation that appears promising as a multifunctional Au(I) bis-NHC complex. In particular the combination of **5** and BSA proved capable of being imaged via confocal microscopy while also providing a high level of anti-proliferative activity in the A549 lung cancer cell line. We believe this work demonstrates the further promise of **1** to be used as a synthetic platform for SARs and the use of serum albumin to overcome the solubility issues that plague many Au(I) bis-NHC complexes. This is expected to facilitate future *in vivo* studies. The present approach may also allow for the attachment of tumour-localizing peptides to Au(I)-containing cytotoxic cores. Studies along these lines are in progress.

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Conflicts of interest

There are no conflicts to declare.

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