A Green Synthesis of Carbene-Metal-Amides (CMAs) and Carboline-Derived CMAs with Potent *in vitro* and *ex vivo* Anticancer Activity

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The modularity and ease of synthesis of carbene-metal-amide (CMA) complexes based on the coinage metals (Au, Ag, Cu) and N-heterocyclic carbenes (NHCs) as ancillary ligands pave the way for the expansion of their applications beyond photochemistry and catalysis. Herein, we further improve the synthesis of such compounds by circumventing the use of toxic organic solvents which were previously required for their purification, and we expand their scope to include complexes incorporating carbolines as the amido fragments. The novel complexes are screened both *in vitro* and *ex vivo*, against several cancer cell lines and high-grade serous ovarian cancer (HGSOC) tumoroids, respectively. Excellent cytotoxicity values are obtained for most complexes, while the structural variety of the CMA library screened thus far, provides promising leads for future developments. Variations of all three components (NHC, metal, amido ligand), enable the establishment of trends regarding cytotoxicity and selectivity towards cancerous over normal cells.

Introduction

The recent surge of research on carbene-metal-amide (CMA) complexes of the coinage metals (Cu, Ag, Au) is a testament to their importance among organometallic materials with relevance to photonic applications.[1–3] These tunable emitters are comprised of three, simple components; an amido fragment acting as a donor which is usually based on the carbazole template, a linear d^{10} Cu, Ag or Au center and a carbene ligand occupying the second coordination position of the linear complex and acting as the acceptor. The carbene ligand is usually a cyclic (alkyl)(amino) carbene or monoamido- (MAC) or diamido-carbene DAC),[4–15] and less often a (benz)imidazolylidene or mesoionic carbene.[16–23] The ligand combination in the former case renders these materials ideal for Thermally Activated Delayed Fluorescence (TADF) applications. The latter cases have received comparatively much less attention because of limited potential for OLED materials applications, even though recent advances show that modifications of the acceptor imidazol(in)ylidene ligand enable through-space TADF.[24] Importantly, applications beyond TADF are possible when using imidazol(in)ylidene-based CMAs, with recent examples being long-lived room temperature phosphorescence (RTP),[18] and photocatalysis by triplet energy transfer.[22] For the latter application, the choice of the carbene ligand and the metal, Au in particular, is paramount as weaker π-acceptors enable longer-lived triplet states and the strong spin-orbit coupling (SOC) of the heavy metal atom enables efficient intersystem crossing (ISC), thus these compounds emit solely *via* phosphorescence, in addition to being more photolytically stable than their Ag congeners. An "outside the box" application, is the use of amido complexes (including CMAs) as simple Brønsted-basic complexes which act as pre-catalysts in certain catalytic reactions.[25–29] An early example was disclosed by Bergmann and co-workers involving more traditional and far more reactive/unstable amido complexes.[26] We recently applied this concept for the first time using a carbazole-based CMA in the gold-catalyzed hydrofluorination of alkynes.[29]

It must be stressed at this point, that the reasons which inspired the latter application are the ease of access to such CMAs, their indefinite stability as solids and the wide variety of structures which can be accessed. These features make gold-based CMAs very competitive Brønsted-basic complex alternatives to the gold-hydroxide and gold-hydrocarbyl

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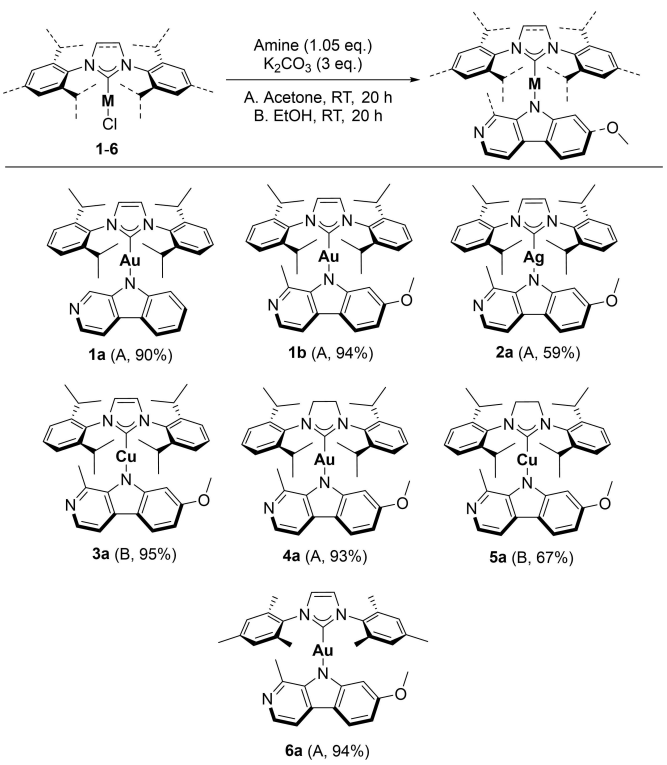
counterparts.[30–32] Until very recently however, the synthesis of CMAs and of the M–N bond in particular required the use of cumbersome organometallic techniques involving moisture-sensitive strong bases under inert atmosphere and in toxic organic solvents.[23] Alternative routes involve the use of pre-synthesized organometallic synthons such as the Au–OH and Au-Aryl complexes among others, decreasing atom- and step-economy.[32,33] In light of the interest surrounding CMAs, we disclosed a more sustainable, mild procedure for their synthesis which is based on the use of a cost-efficient, weak base, in air, in ethanol or acetone as desirable solvents or without a solvent, using mechanochemistry. The procedure is scalable and has a wide scope with regards to the carbene type, metal and amine, thus providing the tools to create various CMA libraries in a facile manner.[23] Based on this, we also developed a flow procedure, reducing the reaction time to a few minutes.[34] Despite these advances, the synthesis of CMAs still requires the use of toxic solvents, primarily tetrahydrofuran, diethyl ether and pentane for purification reasons. Herein, we circumvent this problem by focusing instead on the removal of reaction waste material from the desired CMA, which is a much more general approach (see below).

To expand the applications of CMAs, we shift our focus on other areas in which coinage metal-NHCs are of interest. Biological applications of such complexes are well known, since a wide range has been studied for antibacterial, antirheumatic and anticancer properties among others.[35] The stability of the M-NHC bond and the modularity of NHCs render such complexes ideal candidates as metallodrugs.[35f] Examples, with or without NHC, are found in the case of Au, such as auranofin, its analogues and other complexes which exhibit Thioredoxin Reductase (TrxR) inhibition, without however being limited to this function.[36] Their mechanisms of action can vary widely, however it has been determined that the ligands in such complexes, aside from the NHC, also have important effects.[35–37] Of relevance to our interests, complexes containing amido ligands have been evaluated as antiproliferative agents, although to the best of our knowledge no CMAs had been studied until our recent report.[35,36,38] In order to take full advantage of the CMA structural components, the amido ligand can be designed as the deprotonated form of a bioactive amine. Because of their structure and known antimicrobial and neuroprotective activity, carbolines, which are naturally occurring alkaloids are excellent candidates.[39] These compounds have also been shown to have antiproliferative activity, with harmine (7-methoxy-1-methyl-9H-pyrido[3,4-b]indole) displaying enhanced DNA intercalation.[40] Their complexes with Transition Metals have recently attracted ample attention for their applications in medicinal chemistry.[41] Although complexes with coinage metals have been reported,[41] CMAs of this type are not known, aside from one compound synthesized in our report as a proof of concept.[34] Herein, we report the sustainable synthesis of a range of carboline-derived CMAs and we evaluate their antiproliferative properties on cell lines and organoids. Importantly, we address for the first time the improvement of CMA purification, to render the entire process sustainable.

Results and Discussion

By employing the conditions which were disclosed in our previous report in combination with [Au(IPr)Cl] (**1**, IPr = *N,N'*-bis(2,6-diisopropylphenyl)imidazol-2-ylidene), it was quickly established that beta-carboline and harmine were amenable substrates and CMAs **1a** and **1b** were obtained in excellent yields. (Scheme 1) We recently reported the synthesis of compound **1b** using a flow reaction setup in just two minutes of residence time as a proof of concept dealing with carboline-derived CMAs.[34] We showed that the limitations of the original batch protocol, regarding prolonged reaction times when sterically demanding ligands were used,[23a] could be overcome by using flow technology. Therefore, the fact that a CMA derived from the sterically demanding harmine can be accessed under the standard conditions without requiring an exceedingly long reaction time is notable. Despite the advantages of a flow microreactor setup, such equipment is not widely available, and the scope of CMAs should not be limited by access to instruments such as flow or inert atmosphere setups (glovebox, drybox, Schlenk lines), which is the reason this mild methodology was developed. This is even more significant in the case of the Ag analogue, **2b**, since this stereochemical limitation of the system was more profound in the case of Ag in our previous report, yet it is shown herein that harmine reacts in a facile manner despite its increased steric bulk near the metal center. Lower isolated yield in this case is attributed to lower solubility of the compound in THF, which is the standard solvent used in the purification procedure. Of note, even though the stability



Scheme 1. Scope of the synthesis of carboline-derived CMAs.

test of this CMA in DMSO-d_6 showed no noticeable decomposition, the compound was unstable in chlorinated solvent solutions after just 20 hours. This is because of both the sensitivity of these complexes towards acids (i.e. HCl traces generated over time in chlorinated solvents) and photochemical instability and is in agreement with observations made for Ag-based CMAs in our previous report.[23] The Cu-based analogue **3a** was also obtained in excellent yield, under mild conditions. Changing to a Au–NHC complex with a saturated backbone, a structural feature that in our previous study proved to be of utmost importance for photochemical applications because of its higher π-accepting ability, led to CMA **4a** in 93% yield without requiring any modification of the conditions. Similarly, the Cu-based analog **5a** bearing the unsaturated NHC SIPr (SIPr = *N,N′*-bis(2,6-diisopropylphenyl)imidazolidin-2-ylidene) was successfully synthesized. Finally, the Au-based complex **6a** bearing another widely used NHC, IMes (IMes = *N,N′*-bis-(2,4,6-trimethylphenyl)imidazol-2-ylidene), was also obtained in high yield. To the best of our knowledge, a heterocyclic amine-based CMA with the metal/NHC combination of **5a** was only very recently reported for the first time,[24] while a heterocyclic amine-based CMA with the metal/NHC combination of **6a** is reported here for the first time.

To unambiguously establish atom connectivity and to obtain structural information about these novel complexes, single crystal X-ray diffraction analysis was performed (Figure 1). Suitable crystals of the harmine derivatives **1b** and **4a** were successfully grown *via* vapor diffusion and their solid-state structures were determined, showing that the metal-carbene/metal-nitrogen bonds and carbene-metal-nitrogen linearity values were similar to those found for the classical, carbazole-based CMAs despite the more sterically demanding structure of the amido fragment in the cases examined here. Interestingly, the coplanarity of the imidazolylidene and amido planes in the cases of **1b** and **4a** contrast with our previous findings regarding the perpendicular orientation of those planes for the majority of CMAs based on carbazole and N-Aryl substituted NHC ligands. This feature possibly results from the steric constraints imparted by the harminyl fragment and is in agreement with the case of one CMA reported in our previous study which contained an extremely sterically demanding NHC ligand. Of note, this structural feature is important for the photochemical properties of CMAs.[18,23a]

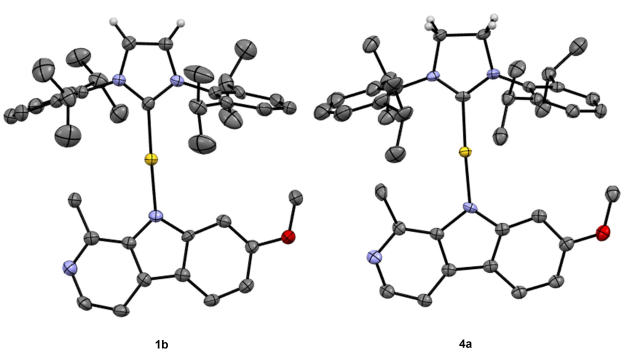
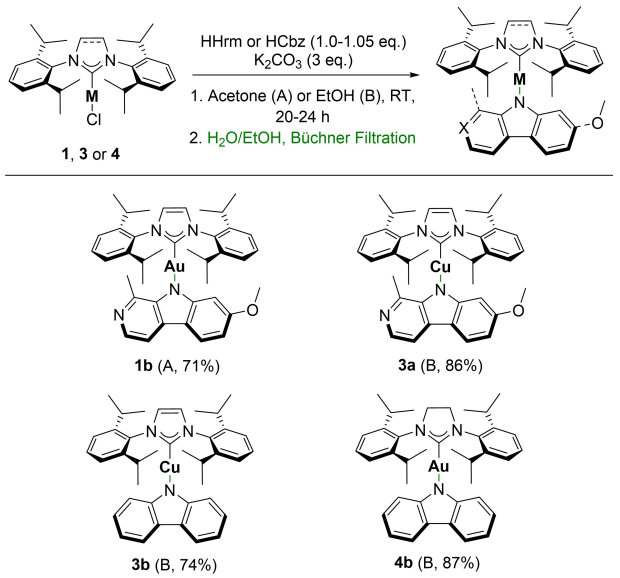


Figure 1. The X-ray molecular structure complexes **1b**, and **4a** are presented, showing thermal displacement ellipsoids at the 50% probability level and most hydrogen atoms omitted for clarity.[42] Selected bond lengths (Å) and angles (°): **1b** (Au–C_{NHC} = 1.989(4), Au–N = 2.026(4), C_{NHC}–Au–N = 177.9(1)), **4a** (Au–C_{NHC} = 1.988(7), Au–N = 2.05(2), C_{NHC}–Au–N = 173.6(8)).

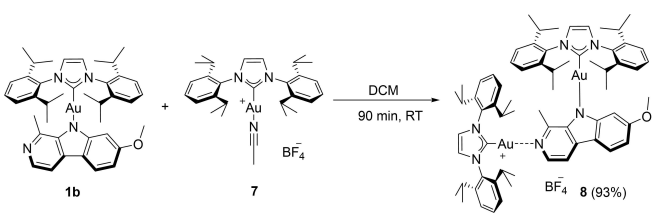
After establishing that our methods are sufficient in providing access to a variety of compounds, notably with exceptional ease, we aimed towards developing a completely sustainable methodology by targeting the purification aspect, which was the only part of the methodology requiring the use of toxic organic solvents such as tetrahydrofuran, diethyl ether and pentane. We reasoned that since the reactions are suspensions of acetone or ethanol and proceed to completion with equimolar or nearly equimolar amounts of the [M(NHC)Cl] and amine reactants, the only impurities that remain after the reaction is complete are inorganic in nature and specifically potassium salts and excess base. Combined with the hydrophobic nature of the products and their limited solubility in the reaction solvents, this prompted us to use water to dissolve the inorganic components of the mixture and obtain a suspension of the product which was then simply filtered using a Büchner filtration apparatus and collected as a microanalytically pure material. Any excess of the amine reactant, if required, can be removed by washing with acetone or an acetone/water mixture, although this will diminish the yield. This would constitute an improvement to this method and potentially render the synthesis of the valuable CMA complexes more sustainable both on a laboratory and on an industrial scale. Given that one of the applications these carboline-derived compounds are destined to have, is biological or pharmacological, complete elimination of toxic solvents which are not only harmful to the operator but may lead to traces of undesirable solvents in the final material, is an even more profound goal. To showcase this potential advancement, two representative complexes from the present work were synthesized and purified as shown in Scheme 2. The harmine-derived complexes **1b** and **3a** were obtained as pure materials in 71% yield and 86% yield respectively. Of note, the slightly lower product yield obtained when this purification method is employed is attributed solely to mechanical error, which is limited when the reactions are carried out on larger scale, as we exemplified in our previous report. These errors are more significant in this case because of the equipment that is required for the water-based, sustainable purification. The methodology in question was also applied to the synthesis of known CMAs (**3b** and **4b**), which are proven to be valuable as emissive materials and one of them as a next generation gold photocatalyst (**4b**). Thus, carbazole-based CMAs **3b** and **4b** were obtained in 74% yield and 87% yield respectively. This represents the first, truly green synthesis of CMAs as only mild reagents and desirable solvents are utilized throughout the entire procedure.

Finally, the potential for post-synthetic diversification of the carboline-derived CMAs is showcased via the assembly of a dinuclear gold complex. By taking advantage of the coordinating function of the pyridine moiety of **1b**, and the ease of access to well-defined cationic complexes of gold such as **7**, the synthesis of complex **8** was achieved by simply mixing the two



Scheme 2. A green synthesis of classical CMAs and carboline-derived CMAs.

components (Scheme 3). Note that silver is not required to obtain compound **7**, which is easily accessed through a commercially available synthon.[33a,b] The use of the non-coordinating dichloromethane solvent is however necessary as it ensures starting material solubility and product stability. The structural assignment of **8** is supported by the nuclear magnetic resonance (NMR) spectra, specifically the ^{13}C spectra which show two distinct, low field signals at 175.1 and 170.9 ppm, each corresponding to the C2 carbon of each imidazolylidene ligand. Despite numerous attempts, we were unable to obtain crystals of suitable quality for single crystal X-ray diffraction analysis, however the structure of this microanalytically pure compound in solution is clearly non-symmetrical, as it would be if both gold centers were coordinated by the negatively charged nitrogen atom of the amido fragment. With the vast libraries of cationic gold complexes available and CMAs such as **1 b**, a plethora of dinuclear compounds may be envisaged.[43]



Scheme 3. Synthesis of a dinuclear gold complex from a CMA and a cationic gold component.

Anticancer activity on ovarian and colon cancer cell lines and normal cells

The well-known biological properties of natural β-carboline derivatives and the ease of access to the NHC–M-amido complexes reported here prompted us to evaluate their potential anticancer activity. Specifically, three different human cancer cell lines (colon LOVO, ovarian A2780 and OVCAR5) and MRC-5 normal cells (human lung fibroblasts) were treated for 96 hours with the synthesized complexes and compared with cisplatin (positive control) and [Au(IPr)Cbz] (Cbz = carbazolyl). The latter was used as a reference to verify whether the use of natural biologically active heterocycles could be advantageous compared to a synthetic amine with a not intrinsic biological activity such as carbazole. Uncoordinated carboline and harmine derivatives, despite having a known biological activity, have a moderate/low antiproliferative activity against ovarian cancer cells, as demonstrated in some recent works.[44]

Preliminarily, the stability of all compounds examined was checked in DMSO-d^6 by NMR spectroscopy: after 24 hours at room temperature, no noticeable degradation was observed. The half inhibitory concentrations (IC_{50}) values summarized in Table 1 testify to the poor antiproliferative activity of [Au(IPr)Cbz], especially in the A2780 and OVCAR-5 ovarian cancer lines ($IC_{50} = 19 \pm 1$ and > 100 μM, respectively). A greater cytotoxicity against A2780 ovarian cancer cell line was instead observed for **1 a** and **1 b**. In particular, the former showed IC_{50} values in the micro- and sub-micromolar range on all tumor cell lines investigated.

From the data obtained for the three [Au(NHC)Hrm] derivatives (**1 b**, **4 a**, **6 a**, NHC=IMes, IPr and SIPr, Hrm = harminyl), it is possible to establish the following cytotoxicity trend: IMes > SIPr > IPr. Maintaining the carbene ligand (IPr) and the harmine residue constant, it is interesting to note the considerable influence of the metal center. In particular, the copper complex is more active than that of silver which in turn is more cytotoxic than the gold example.

On the other hand, the dinuclear cationic complex seems decidedly more active than its mononuclear congener (**1 b**). These interesting structure-activity relationships also provide us

Table 1. IC_{50} values (μM) of carbene-metal-amido (CMA) complexes and cisplatin on cancer and normal cell lines.[a]

Complex	A2780	OVCAR5	LOVO	MRC-5
Cisplatin	0.9 ± 0.1	1.2 ± 0.4	0.80 ± 0.05	2.2 ± 0.2
[Au(IPr)Cbz]	19 ± 1	> 100	2.2 ± 0.1	> 100
1 a	3.1 ± 0.4	3.6 ± 0.5	0.26 ± 0.02	> 100
1 b	4.3 ± 0.8	> 100	2.3 ± 0.2	21 ± 3
2 a	0.16 ± 0.04	0.13 ± 0.03	0.24 ± 0.04	1.5 ± 0.1
3 a	0.021 ± 0.002	0.20 ± 0.05	0.028 ± 0.009	0.3 ± 0.1
4 a	3.2 ± 0.1	3.9 ± 0.3	0.65 ± 0.05	10.1 ± 0.4
5 a	0.087 ± 0.009	0.13 ± 0.02	0.058 ± 0.003	0.09 ± 0.01
6 a	0.55 ± 0.03	3.1 ± 0.4	1.1 ± 0.2	2.5 ± 0.6
8	0.28 ± 0.02	2.7 ± 0.2	0.24 ± 0.01	1.8 ± 0.1

[a] Data after 96 h of incubation. Stock solutions in DMSO for all complexes; stock solutions in H_2O for cisplatin. A2780 and OVCAR-5 (ovarian cancer cell lines), LOVO (colon cancer cell line), MRC-5 (normal lung fibroblasts).

with an indication of the most promising compounds for each type of tumor. In the A2780 cell line (ovarian carcinoma) complexes **6a**, **2a**, **3a**, **5a** and **8** showed cytotoxicity comparable or even higher than cisplatin. On the other hand, in the OVCAR-5 cell line (high-grade serous ovarian cancer), compounds **2a**, **3a** and **5a** are the only ones with activity comparable or superior to cisplatin. Interestingly, the **1b** derivative is inactive against these tumor cells. All the examined compounds showed similar or lower IC_{50} values than cisplatin in the LOVO cell line (colon cancer), regardless of the metal, NHC or amido fragment used.

An important parameter in the development of new metallodrugs is the cytotoxicity towards normal cells. Although the approach to this problem is evaluated in a more realistic way on animal models and clinical trials, a preliminary evaluation can be carried out *in vitro* by treating cells derived from healthy tissues with the complexes of interest. To this end, the antiproliferative activity exerted by the synthesized complexes against human lung fibroblasts MRC-5 (normal cells) was evaluated. The IC_{50} values obtained show a certain selectivity towards cancer cells over normal ones (ca. one order of magnitude) for complexes **2a**, **3a** and **8**. The **1a** complex, although slightly less cytotoxic on cancer cells with respect to the silver and copper derivatives, is also very interesting as it is substantially inactive towards normal cells ($IC_{50} > 100$ μM). Overall, most of the investigated compounds exhibit higher antiproliferative activity than gold-NHC complexes recently tested by our group on the same tumor cell lines.[38c]

Antiproliferative activity on a high-grade serous ovarian cancer tumoroid

Encouraged by the results obtained with ovarian cancer cell lines, we wondered if further experiments on more complex biological models could provide us with important information on the real efficacy of the compounds synthesized in this work. In this context, organoids are lab-built mini-organs that can act as models to summarise cancer development.[45] The availability of innovative biobanks of organoids represents the last frontier of *ex vivo* testing of drugs.[46] As a matter of fact, the innate or acquired chemoresistance together with tumor heterogeneity are the causes of therapeutic failure in ovarian cancer[47] and innovative preclinical models are required to solve this complexity. A few leading groups in this field are developing animal and *ex vivo* organoid models of ovarian cancer to better replicate the response of clinical patients.[48]

It should be remembered that, among ovarian cancers, the high-grade serous sub-type (HGSOC) is the most common and has the worst 5-year survival rate.[46] Moreover, about 30% of HGSOC patients develop ascites, which are free-floating cells that are responsible for intraperitoneal metastasis.[49] Such patients are difficult to treat with classic chemotherapy and paracentesis is used to alleviate the symptoms.[50] For all these reasons, current lines of therapies are not effective, demanding novel drugs to overcome innate or acquired resistance that limit treatment efficacy and in turn, increasing toxicity. Thus, taking advantages from organoid biotechnology, one HGSOC tumoroid derived from ascites (Patient A), recently extracted and characterized by our group (Figure 2),[51] was selected to test the efficacy of some of the most promising Carbene-Metal-Amido (CMA) complexes (see Table 2).

The IC_{50} values obtained on this HGSOC patient-derived tumoroid show the poor cytotoxicity of [Au(IPr)Cbz] and carboplatin. The result obtained with carboplatin, which is the reference compound for clinical standard therapy, testifies to the high resistance of ascites to classical platinum-based anticancer drugs. Surprisingly, all complexes reported in this work exhibit excellent antiproliferative activity, with IC_{50} values in the micro- and sub-micromolar range. These results suggest the possible application of this class of complexes in chemotherapy and heavily treated patients who are resistant to most existing drugs. Based on such promising data, further *in vitro* and *in vivo* experiments and in-depth mechanistic studies are ongoing in our laboratories.

Conclusion

The synthesis of CMAs utilizing a mild base, sustainable solvents under ambient conditions has been shown as a versatile and viable alternative to the more frequently used, traditional routes, based on metal-alkoxides or metal-hydrides in THF, under inert atmosphere. In this work, the scope of this mild method has been further expanded, notably by using biorelevant amines. Inspired by the potential pharmacological applications of such CMAs, we showcased that the methodology may be improved further by modifying the work-up, avoiding the filtration step which required the use of THF and the recrystallization step which required diethyl ether or pentane. By using water to remove inorganic components of the reaction

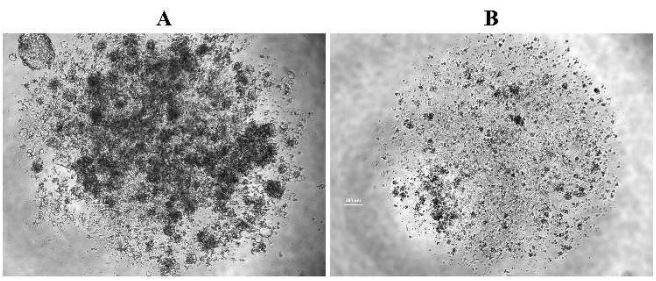


Figure 2. Patient derived organoids treated with **3a** (A) 0.001 μM and (B) 10 μM for 96 hours.

Table 2. IC_{50} values (μM) of carbene-metal-amido complexes and carboplatin on an ovarian cancer tumoroid after 96 h of incubation.

Complex	Patient A
Carboplatin	>200
[Au(IPr)Cbz]	>100
1a	2.2 ± 0.8
2a	2 ± 1
3a	1.0 ± 0.5
5a	0.3 ± 0.1

mixture, the operation sequence is rendered completely green. Furthermore, the synthesis of a dinuclear gold complex exemplifies the variety of structures that may be generated by using an amido fragment bearing additional functionalities. Beta-carbolines, with harmine being a representative case, are expected to provide additional stability to the CMAs since they impart steric protection and enhance the resistance to acids because of their basic/nucleophilic pyridine moiety. Their inherent biological activity, combined with that of coinage metal-NHC complexes prompted us to evaluate the novel CMAs as anticancer agents. This screening against multiple cell lines established the exceptional cytotoxicity of the Cu- and Ag-based, carboline-derived CMAs. It also enabled the identification of the smaller yet still significant effect of the NHC component, while it showcased the effect that the amido fragment has on selectivity towards cancer cell lines. Overall, these advances constitute a robust framework, based on which further screening of a wider library of such CMAs may lead to the identification of lead compounds for platinum resistant cancer treatment, and also enable the understanding of mechanistic parameters behind these cytotoxic effects.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Carbene-metal-amides · Coinage metal-N-heterocyclic carbene complexes · Cytotoxicity · Green chemistry · Synthetic methods

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