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Gold(I)–indomethacin anticancer candidates with anti-breast cancer stem cell properties

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The rational design, sustainable synthesis, and comprehensive biological evaluation of a new family of gold(I)–indomethacin complexes bearing N-heterocyclic carbene (NHC) ligands are reported. The mild, scalable synthetic protocol affords the first reported examples of well-defined [Au(NHC)(indomethacin)] complexes that display robust chemical and thermal stability and exhibit potent antiproliferative activity across a panel of human cancer cell lines, frequently outperforming cisplatin, including in cisplatin-resistant models. Notably, these gold(I)–indomethacin derivatives retain strong cytotoxicity against breast cancer stem cells (CSCs)-like populations and demonstrate superior efficacy in physiologically relevant 3D spheroid models. Mechanistic investigations reveal that their anticancer activity correlates with efficient inhibition of thioredoxin reductase, disruption of intracellular thiol homeostasis, and induction of oxidative stress through reactive oxygen species generation.

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Introduction

Cancer remains one of the most formidable health challenges of the 21st century, accounting for millions of deaths worldwide each year. Traditional treatments, including surgery, radiotherapy, and chemotherapy, have significantly improved survival rates.[1] However, these approaches often come with severe side-effects and limitations. Chemotherapy, in particular, while effective at killing rapidly dividing cells, lacks specificity, leading to collateral damage to healthy tissues and significant adverse effects.[2] Moreover, the development of resistance to chemotherapeutic drugs is a common and serious issue, necessitating the search for new and more effective treatments.[3]

Metal-based compounds have a rich history in cancer therapy, with the platinum-based drug *cisplatin* being one of the most successful examples.[4] The latter and its derivatives have been widely used to treat various cancers and are highly effective against several malignancies, including testicular, ovarian, bladder, lung, head and neck cancers. However, they exhibit several drawbacks, including nephrotoxicity, ototoxicity,[5] as well as limited efficacy or unsuitability in certain cancer types and/or the occurrence of resistance phenomena.[6] This has spurred interest in other metal complexes as potential therapeutic agents. Among these, gold complexes have garnered significant attention due to their unique chemical properties and biological activities. In particular, gold has been used in medicine for some time, primarily in the treatment of rheumatoid arthritis through gold salts like aurothiomalate.[7] The therapeutic potential of gold compounds extends beyond anti-inflammatory properties to include antiviral, antibacterial, and anticancer activities.[8–10] In fact, the ability of gold to form stable complexes with a variety of ligands makes it a versatile candidate for drug development. Among the various types of gold complexes, those featuring N-heterocyclic carbene (NHC) ligands have shown particular promise in the realm of anticancer therapy.[11–16] The strong covalent bond between gold and the carbene carbon imparts significant stability to these complexes, making them less prone to decomposition in biological environments compared to their phosphine congeners. This stability is crucial for maintaining therapeutic efficacy and minimizing off-target effects. Furthermore, the cellular uptake of gold–NHC complexes can be influenced by the nature of the *N*-substituents on the carbene ligand. In particular, the introduction of lipophilic groups at the nitrogen atoms may enhance membrane permeability and promote intracellular accumulation.[17] The ability to fine-tune the electronic and steric properties of NHC ligands through synthetic modifications enables researchers to optimize the pharmacokinetic

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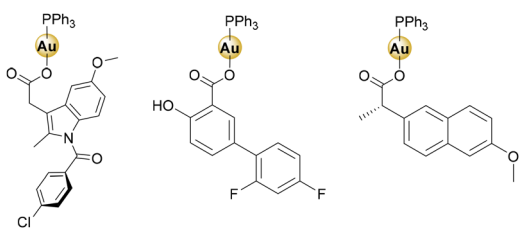
and pharmacodynamic profiles of gold–NHC complexes, enhancing their anticancer activity and selectivity.[14,15,17]

The anticancer activity of gold–NHC complexes is mediated through multiple and potentially complementary mechanisms. Among these, inhibition of thioredoxin reductase (TrxR), an enzyme frequently overexpressed in cancer cells and involved in the maintenance of cellular redox homeostasis, has been widely reported.[18,19] In many cases, TrxR inhibition leads to increased oxidative stress and can promote apoptotic cell death. However, this mechanism is not universal, as some Au–NHC complexes exhibit significant cytotoxicity without strong TrxR inhibition, suggesting the involvement of additional molecular targets and pathways.

In addition to protein targeting, gold–NHC complexes have also been reported to interact with DNA;[20] however, for Au(I)–NHC species this interaction is generally weaker and not considered a dominant mechanism of action. These complexes typically display a higher affinity for protein thiols and selenols, and any effects on DNA are often indirect, arising from oxidative stress or disruption of redox homeostasis. Nevertheless, in certain cases, DNA perturbation may contribute to cell cycle arrest and apoptosis, thereby supporting their overall anticancer activity. Moreover, gold–NHC complexes can disrupt mitochondrial function, leading to the release of cytochrome c and the activation of caspases, which are central to the intrinsic pathway of apoptosis.[21]

This multifaceted approach to inducing cell death makes gold–NHC complexes highly effective against a range of cancer types, including those resistant to conventional therapies. With the aim of contributing to this fascinating research topic, we recently became interested in the work of Johnson, Awuah, and Suntharalingam, who reported a notable example of gold(I) complexes specifically investigated for anti-breast cancer stem cell (CSC) activity (Scheme 1).[22]

It should be noted that breast cancer recurrence is strongly associated with the presence of CSCs, a subset of breast cancer cells capable of self-renewal, differentiation, and forming secondary tumors.[23,24] As a confirmation of this, basal-like and claudin-low breast tumors—subtypes frequently enriched in cancer stem cells (CSCs)—are generally associated with lower survival rates.[25] In contrast, HER2-positive tumors are not consistently CSC-enriched, and their prognosis has improved significantly with targeted therapies such as trastuzumab.



Scheme 1 Au(I) complexes with anti-breast cancer stem cell properties.

CSCs are also implicated in metastasis, with clinical studies showing higher proportions of CSC-like cells in metastatic tumors compared to primary tumors.[26–28] CSCs divide more slowly than other breast cancer cells, allowing them to evade conventional chemotherapy and radiotherapy, which target rapidly dividing cells.[29–32] Additionally, the small number of CSCs within a tumor and their tendency to reside in difficult-to-access areas mean they can be missed during surgery. After treatment, CSCs can regenerate tumors at the original site or produce invasive cells that spread to other organs.[33] Therefore, effective treatments must eliminate all cancer cell populations, including CSCs, to prevent relapse. While potential targets for CSC therapy, such as cell surface markers, signaling pathways, and microenvironment components, have been identified,[26] no clinically approved drug currently exists that can fully eradicate CSCs at administered doses.

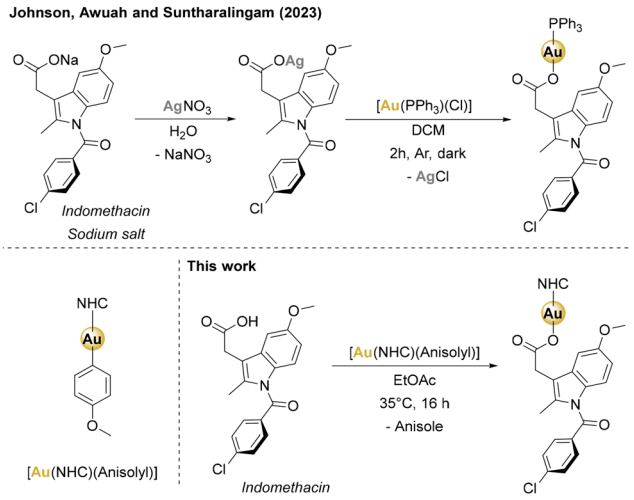
In their seminal work, Johnson, Awuah, and Suntharalingam demonstrated that gold(I) complexes containing one triphenylphosphine and one non-steroidal anti-inflammatory drug—specifically indomethacin, diflunisal, or naproxen, as other NSAIDs proved unstable under physiological conditions due to oxidation to triphenylphosphine oxide—exhibit promising anti-breast cancer stem cell (CSC) properties (Scheme 1).[22] Notably, the most encouraging results were obtained with the complex containing indomethacin. This gold(I) complex demonstrates significantly higher potency against breast cancer stem cells (CSCs) compared to bulk breast cancer cells, with up to 80 times greater effectiveness. Mechanistic studies indicate that this gold–indomethacin derivative causes breast CSC death through localization in the cytoplasm, production of intracellular reactive oxygen species levels, downregulation and inhibition of cyclooxygenase-2, and induction of apoptosis.

Inspired by these promising results, we present in this work the synthesis and antitumor properties of gold(I)–indomethacin complexes in which the triphenylphosphine ligand has been replaced by an NHC ligand. The possibility to fine tune the electronic and steric characteristics of the target complexes by varying the type of NHC ligand used, along with the greater stability of gold–NHC complexes compared to their triphenylphosphine congeners, motivated our efforts and results are presented here.

Additionally, with the aim of making these gold(I)–indomethacin derivatives more accessible, we have developed an original synthetic approach to these gold–carboxylate complexes that avoids the use of silver salts or external bases.

Results and discussion

Before embarking on our diversification of the [Au(NHC)(indomethacin)] architecture, we initially investigated alternative synthetic routes distinct from the reported method involving silver as the transmetalation agent and dichloromethane as solvent (Scheme 2).[22] Our objective was to identify a simpler pathway devoid of the drawbacks associated with metal usage,



Scheme 2 Synthetic procedure to target Au(I)–NHC indomethacin complexes.

Table 1 Research on optimal conditions

Entry	[Au]	Base	Solvent	Time [h]	*T* [°C]	Conv. (yield) [%]
1	[Au(IPr)Cl]	K_2CO_3	EtOH	16	RT	NR
2	[Au(IPr)Cl]	K_2CO_3	EtOH	16	60	NR
3	[Au(IPr)Cl]	K_2CO_3	Toluene	16	60	NR
4	[Au(IPr)Cl]	K_2CO_3	Acetone	16	60	NR
5	[Au(IPr)Cl]	K_2CO_3	EtOAc	16	RT	NR
6	[Au(IPr)(anisolyl)]	—	Toluene	16	RT	NR
7	[Au(IPr)(anisolyl)]	—	THF	16	RT	NR
8	[Au(IPr)(anisolyl)]	—	DCM	16	RT	75
9	[Au(IPr)(anisolyl)]	—	DCM	30	RT	100
10	[Au(IPr)(anisolyl)]	—	EtOAc	30	RT	100
11	[Au(IPr)(anisolyl)]	—	EtOAc	16	RT	67
12	[Au(IPr)(anisolyl)]	—	EtOAc	16	35	100 (78)

Conditions: reactions conducted in air. 10 mg of the Au source and 1 eq. of indomethacin. 3 eq. of base in the case of [Au(IPr)Cl]. Solvents were used as received except for DCM which was previously passed through a plug of basic alumina.

given its relative expense and limited scalability. The high cost of silver, susceptible to market fluctuations and potential supply constraints, can significantly impact the feasibility and reliability of any synthetic process. Additionally, the use of silver salts and dichloromethane requires careful handling and appropriate waste disposal to minimize potential health and environmental impacts, although toxicity is context-dependent and generally manageable at laboratory scale.[34–36] Considering these challenges, we explored alternative routes that could offer more cost-effective, environmentally sustainable, and practical solutions for synthesizing the targeted Au–carboxylate complexes.

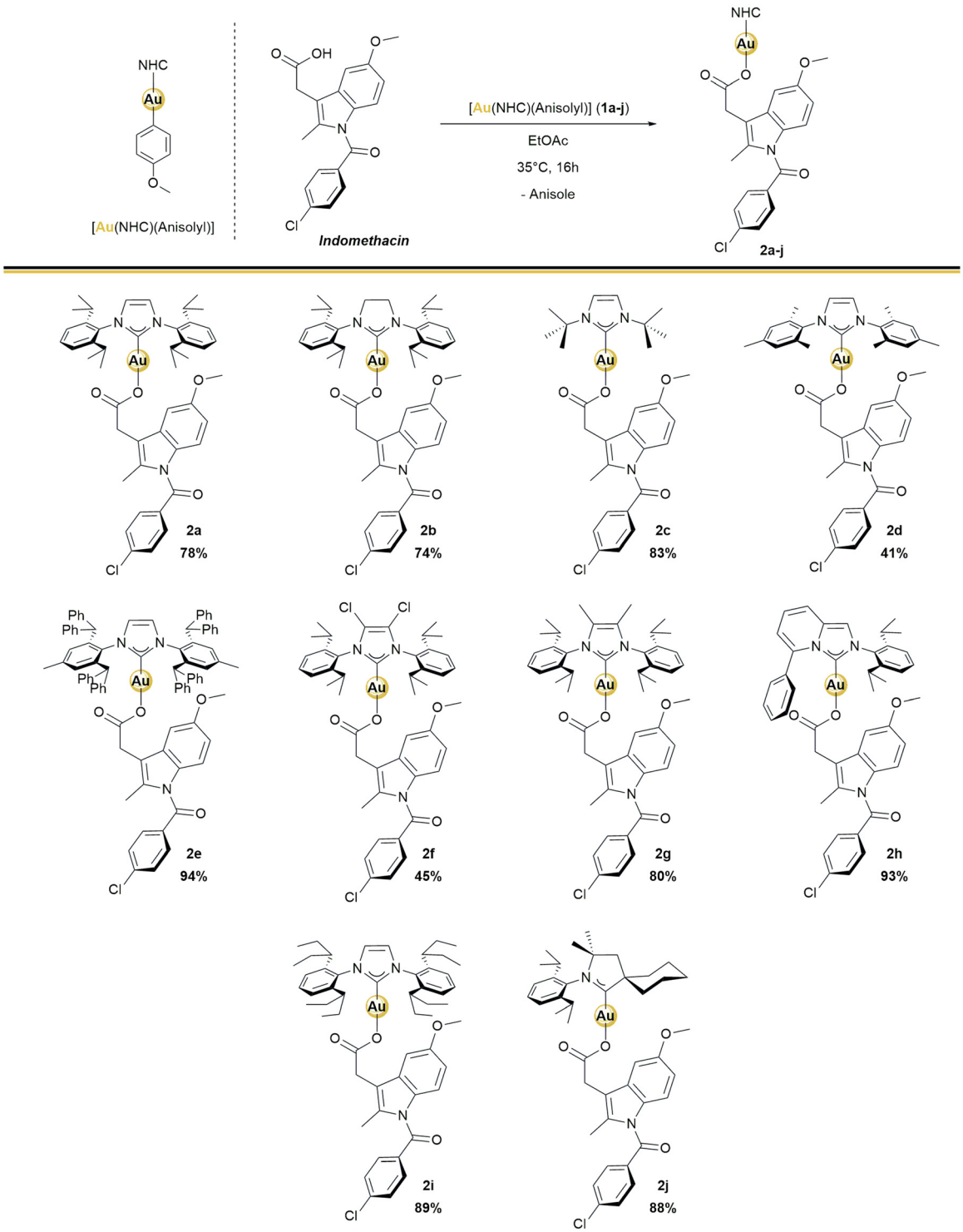
The synthesis of Au–carboxylate complexes bearing NHC ligands has previously been approached through silver transmetalation strategies,[37] as well as through the use of pre-functionalized gold synthons.[38,39] However, the reported examples are largely confined to gold–acetate complexes and closely related derivatives, rather than representing a general and systematic access to the broader class of [Au(NHC)(carboxylate)] species. This narrow scope also reflects the very limited range of NHC ligands that can be employed, which is mainly dictated by the poor availability and restricted scope of gold synthons such as NHC–gold–acetonyls and NHC–gold–hydroxides. Because of these limitations, the chemical space explored thus far remains extremely narrow, despite the apparent potential of this structural motif. In this context, [Au(NHC)(carboxylate)] complexes constitute an intriguing yet still largely underdeveloped family of gold derivatives. Notably, although a number of Au–NHC–carboxylate complexes have been reported, a comprehensive study offering a general, elegant, and silver-free protocol for their synthesis, along with systematic evaluation in biological applications, has not yet been performed.

Initial attempts to synthesize the desired [Au(NHC)(carboxylate)] complexes from [Au(NHC)Cl] precursors using the *weak base route*[14] were met with failure, as the starting materials decomposed rather than forming the target products. We conducted extensive experiments, varying the temperature and solvent in hopes of finding the right conditions, but these efforts proved unsuccessful (Table 1, entries 1–5). It became evident that the reaction between [Au(NHC)Cl] and indomethacin in the presence of potassium carbonate was not a viable pathway for our target synthesis.

The use of gold-aryl synthons with an integrated base (anisolyl) emerged as an appealing strategy. This method drives the reaction irreversibly toward the desired product through protonolysis of the Au–aryl bond, generating anisole as a byproduct while installing the target carboxylate. Such an innovative approach not only simplifies the synthesis process but also enhances efficiency and selectivity in achieving the desired outcome. We selected the [Au(NHC)(Anisolyl)] synthons as starting reagents (Scheme 2) for their remarkable substrate availability.[15,40–42] These valuable gold synthons can be easily obtained on large scale from [Au(NHC)Cl] complexes and 4-methoxy-phenylboronic acid in the presence of potassium carbonate.[15,40]

Expanding upon previous studies, we successfully synthesized 10 distinct carboxylate complexes, highlighting the versatility of the methodology. This simple protocol allowed us to achieve our synthetic goals under mild conditions (35 °C, 16 h), bypassing the need for a base and utilizing technical grade ethyl acetate as the solvent (Table 1). Moreover, all reactions proceed under aerobic conditions. Notably, this process is strongly dependent on the solvent used, since the experiments performed in toluene or tetrahydrofuran (THF) resulted in no conversion of the starting materials (Table 1, entries 6 and 7). Conversely, despite its toxicity, dichloromethane can be used as an alternative to ethyl acetate (Table 1, entries 8 and 9) but remains a distant alternative.

Focusing on the scope of NHC ligands (Scheme 3), the gold-anisolyl precursors as well as their corresponding gold–indomethacin derivatives were successfully obtained using: (i) classical imidazol-2-ylidenes (IPr, **1–2a**; I^{t}Bu, **1–2c**; IMes, **1–2d**;



Scheme 3 [Au(NHC)indomethacin] complexes (**2a–2j**) synthesized in this work.

IPr*, **1–2e**; IPent, **1–2i**), (ii) backbone functionalized imidazol-2-ylidenes (IPr^{Cl}, **1–2f**; IPr^{Me}, **1–2g**), (iii) imidazolin-2-ylidenes (SIPr, **1–2b**); (iv) imidazo[1,5-*a*]pyridine-3-ylidenes (ImPy, **1–2h**); and (v) cyclic(alkyl)(amino)carbenes ($CAAC^{Cy}$, **1–2j**).

All compounds were thoroughly characterized by NMR, HRMS or elemental analyses. Specifically, the formation of the target [Au(NHC)(indomethacin)] complexes was supported by ^{1}H and ^{13}C NMR spectroscopy, which confirm ligand substitution and the presence of the expected organic moieties, although these techniques do not unambiguously establish metal–ligand connectivity or the Au–O bond (see Fig. S9–S78 in SI).

In these spectra, a shift in signals compared to the starting reagents was observed, along with the disappearance of the aromatic signals of the aryl fragment (anisole) and the disappearance of the carboxylic proton signal of indomethacin (in the ^{1}H NMR spectra). The structures of complexes **2b**, **2c**, **2g** and **2h** have been unambiguously confirmed through X-ray crystallographic analysis on single crystals. The effective formation of the NHC–Au–carboxylate linkage and the characteristic linear geometry of gold is clear from structures shown in Fig. 1 and Fig. S3–S8 in SI.

To assess thermal stability, compounds **2a** and **2b** were heated in toluene at 110 °C. After 40 hours, **2a** (IPr) showed less than 10% decomposition, while **2b** (SIPr) remained largely intact. These results indicate that the complexes display reasonable thermal stability under the tested conditions, although further studies and comparison with benchmark systems would be needed to draw more general conclusions about the thermal robustness of this family (see Fig. S1 and S2 in SI). The physical appearance of the complexes remained unchanged over time when stored in air at room temperature, and their purity, estimated by ^{1}H NMR spectroscopy, remained above 95% after 2 years for compounds **2d**, **2e**, **2f**, and **2h**.

Having established a scope, we conducted solubility and stability tests in a 2 : 1 mixture of DMSO-d_6/D_2O, finding that all complexes remained soluble and stable for at least 48 hours (Fig. S79–S88 in SI). No release of indomethacin was observed, as its characteristic signals in the free form were clearly distinguishable even in this solvent mixture. However, when utilizing the CAAC^{Cy} ligand instead of the standard NHCs, a solvent switch from DMSO to DMF was necessary, to address solubility issues.

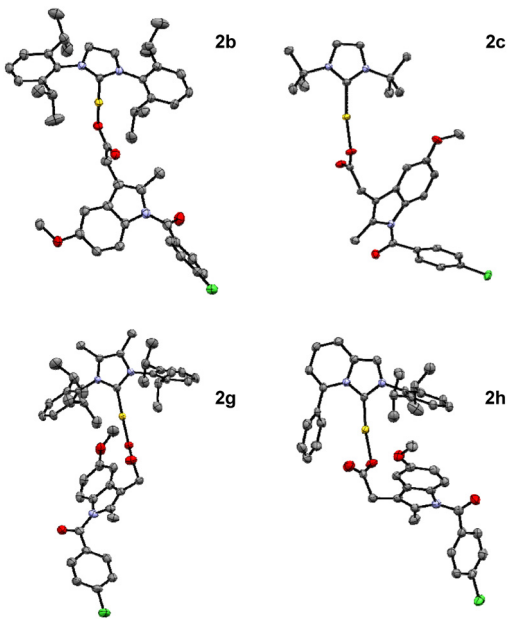


Fig. 1 Molecular structure of **2b** (CCDC: 2367333), **2c** (CCDC: 2367331), **2g** (CCDC: 2521381) and **2h** (CCDC: 2367330) determined by single-crystal X-ray diffraction analysis. Hydrogen atoms and solvent molecules are omitted for clarity. Thermal displacement ellipsoids are shown at the 50% probability level.

We strategically targeted a range of NHC gold complexes, each tailored with distinct stereoelectronic parameters. This deliberate variation not only enriched the diversity of our compounds but also permits future modelling of their well-established electronic and steric properties,[43,44] with potential significance in various biological applications.

The novel gold(I) complexes **2a–2j** were evaluated for their cytotoxic activity towards a series of human cancer cell lines in bidimensional (2D) assays. In particular, the in-house cancer cell line panel contains examples of human lung (A549), breast (MDA-MB-231), pancreatic (BxPC-3), germinal (NTERA-2) and ovarian (2008) cancers. As reported in the Experimental section, cisplatin was selected as the reference metallodrug and was tested under the same experimental conditions. The cytotoxicity parameters obtained by means of the MTT assay after 72 h of drug exposure, and expressed in terms of IC_{50}, are listed in Table 2.

Overall, the 2D cytotoxicity studies highlighted that all gold(I)–indomethacin complexes elicited IC_{50} values in the micromolar range. Within the series, complexes **2c** and **2f** proved to be the most effective, with an average IC_{50} value over the five cancer cell lines of some 10 μM. Similarly, derivatives **2d** and **2g** showed very promising cytotoxic profiles, with average IC_{50} lower compared with that of the reference metal-based chemotherapeutic drug *cisplatin*. On the contrary, derivatives **2h** and **2i** displayed the weakest cytotoxic activity, with average IC_{50} value of about 30 μM. It is interesting to note that many compounds (**2a–2d** as well as **2f–g**) were significantly more effective than *cisplatin* against human triple negative breast cancer cells MDA-MB-231.

The antiproliferative cytotoxicity of the novel gold(I)–indomethacin compounds was also investigated in a *cisplatin*-resistant cancer cell subline (C13* cells). All tested complexes exhibited a remarkably similar activity level in both the *cisplatin*-sensitive (2008) and *cisplatin*-resistant (C13*) cell lines. The resulting resistance factor (RF) values, close to 1, indicate a lack of cross-resistance with cisplatin, although they do not provide mechanistic insight into overcoming resistance. For comparison purposes, literature data for the clinically used gold(I) compound auranofin report IC_{50} values of 1.4 μM and 1.0 μM in the 2008 and C13* ovarian cancer cell lines, respectively (RF = 0.73),[45] as well as IC_{50} values of 0.75 μM in A549 lung cancer cells and 9.2 μM in MDA-MB-231 breast cancer cells.[46,47] On this basis, the most active derivatives identified in this study (**2c**, **2f** and **2g**) display an antiproliferative activity that is of the same order of magnitude as auranofin in the MDA-MB-231 breast cancer model, whereas auranofin appears somewhat more cytotoxic in the ovarian (2008/C13*) and lung (A549) cancer cell lines. It should be noted, however, that these values derive from literature reports obtained under potentially different experimental conditions; therefore, the comparison should be regarded as indicative and limited to the general order of magnitude rather than as a strict quantitative benchmark.

Table 2 Cytotoxic activity evaluated by the MTT test at 72 h. IC_{50} values were calculated with a four-parameter logistic model ($p < 0.05$). R.F. = IC_{50} (resistant subline)/IC_{50} (wild-type cells). S.D: standard deviation

IC_{50} (μM) ± S.D.							
	NTERA-2	MDA-MB-231	A549	BxPC-3	2008	C13* (R.F.)	CSC MDA-MB-231
[Au(IPr)(indomethacin)] **2a**	15 ± 2	21 ± 2	31.2 ± 0.8	12.6 ± 0.5	14 ± 2	21 ± 2 (1.4)	43 ± 2
[Au(SIPr)(indomethacin)] **2b**	16 ± 3	19 ± 2	20 ± 1	15.1 ± 0.8	14 ± 3	16 ± 4 (1.1)	31 ± 2
[Au(I^{t}Bu)(indomethacin)] **2c**	1.2 ± 0.5	7.8 ± 0.9	19 ± 2	9.1 ± 0.8	11 ± 1	11.8 ± 0.6 (1.0)	12 ± 3
[Au(IMes)(indomethacin)] **2d**	10 ± 2	14 ± 2	18.1 ± 0.5	14 ± 4	11.9 ± 0.5	10.0 ± 0.9 (0.8)	23 ± 2
[Au(IPr*)(indomethacin)] **2e**	>50	>50	>50	>50	>50	>50	>50
[Au(IPr^{Cl})(indomethacin)] **2f**	2.9 ± 0.7	12 ± 2	13 ± 1	7.0 ± 0.8	3.8 ± 0.5	6.9 ± 0.6 (1.8)	19 ± 3
[Au(IPr^{Me})(indomethacin)] **2g**	7 ± 2	12 ± 2	9.7 ± 0.2	17 ± 2	17 ± 2	15.2 ± 0.3 (0.9)	17 ± 2
[Au(ImPy)(indomethacin)] **2h**	26 ± 1	>50	>50	23.0 ± 0.1	26 ± 1	26.7 ± 0.9 (1.0)	>50
[Au(IPent)(indomethacin)] **2i**	35 ± 2	>50	>50	35 ± 2	41 ± 4	15 ± 2 (0.4)	>50
[Au(CAAC^{Cy})(indomethacin)] **2j**	24 ± 2	45 ± 4	41 ± 1	26 ± 4	22 ± 3	26 ± 3 (1.2)	>50
Cisplatin	16 ± 4	28 ± 3	8.4 ± 0.8	7 ± 1	2 ± 1	29 ± 3 (13.6)	41 ± 2

A CSC-enriched population was derived from MDA-MB-231 cells, one of the most widely used models for CSC studies,[48] and employed to evaluate the cytotoxicity of the newly developed indomethacin-based derivative toward cells with stem-like properties. Remarkably, the response pattern observed for the tested compounds was comparable to that displayed in the parental cell line, whereas *cisplatin* proved to be less effective against CSCs; collectively, these findings emphasize that the new compounds retain potent cell-killing activity toward CSC populations.

The cytotoxic activity of the newly developed gold(I) complexes observed in 2D monolayer cultures was also assessed in established 3D cell culture models. Actually, 3D spheroid cell culture systems are widely recognized to better reproduce the cancer *in vivo* microenvironment, both in terms of physiopathological characteristics (*e.g.*, gene expression, cell–cell interactions and metabolism) and drug-related interactions (drug permeation, retention and intracellular trafficking), making them more predictive models for anticancer drug screening.[49] The IC_{50} values acquired after treatment of 3D cell spheroids derived from human breast and lung cancer cells are reported in Table 3.

Table 3 Spheroids from MDA-MB-231 or A549 cells were treated for 72 h with increasing concentrations of **2a–2j** or cisplatin. Cytotoxicity was assessed by means of a modified APH assay. IC_{50} values were calculated from the dose–survival curves by the four-parameter logistic model ($p < 0.05$). S.D. = standard deviation

IC_{50} (μM) ± S.D.		
	A549	MDA-MB-231
[Au(IPr)(indomethacin)] **2a**	>50	>50
[Au(SIPr)(indomethacin)] **2b**	35 ± 6	19 ± 4
[Au(I^{t}Bu)(indomethacin)] **2c**	>50	47 ± 10
[Au(IMes)(indomethacin)] **2d**	35 ± 8	22 ± 1
[Au(IPr*)(indomethacin)] **2e**	>50	>50
[Au(IPr^{Cl})(indomethacin)] **2f**	>50	44 ± 17
[Au(IPr^{Me})(indomethacin)] **2g**	35 ± 5	31 ± 6
[Au(ImPy)(indomethacin)] **2h**	>50	>50
[Au(IPent)(indomethacin)] **2i**	>50	26 ± 3
[Au(CAAC^{Cy})(indomethacin)] **2j**	>50	>50
Cisplatin	60 ± 1	107 ± 3

Surprisingly, in both 3D cancer models the pattern of response was quite different for the newly developed compounds, with compounds **2b**, **2d** and **2g** displaying the highest antitumor potential, with IC_{50} values approximately 2-fold lower than those of *cisplatin*.

Gold(I) complexes are well-known inhibitors of redox-active selenoenzyme Thioredoxin Reductase (TrxR).[50] On these bases, we estimated the ability of the newly synthesized gold(I)–indomethacin derivatives to inhibit TrxR activity, both in cell-free experiments and in human breast cancer cells. *Auranofin*, a well-characterized metal-based TrxR inhibitor, was employed as a positive control. The inhibitory effects on TrxR activity were measured using standard procedures detailed in the Experimental section, and results (reported as the percentage of residual enzyme activity relative to the untreated control) are shown in Fig. 2(A and B).

In cell-free assays, all gold(I) complexes were highly efficient in inhibiting cytosolic mammalian TrxR, although with varying degrees of potency. Among them **2c** and **2i** exhibited the strongest TrxR inhibition, albeit their efficacy remained lower than that of the reference inhibitor *auranofin* (Fig. 2A). In MDA-MB-231 cells, complexes **2c**, **2f** and **2g** showed the strongest inhibitory effects on the selenocysteine-containing redox enzyme TrxR, reducing its activity by approximately 70% (Fig. 2B). Similarly, compounds **2b** and **2d** were able to decrease cellular TrxR activity by about 40%. Taken together, these results are consistent with the hypothesis that the complexes may exert part of their activity through TrxR inhibition in intact cancer cells, although direct causation and selectivity *versus* other redox proteins have not been established. Notably, TrxR inhibition was directly correlated with the cytotoxic profile of the compounds (see the insert in Fig. 2B), thus underlying that TrxR can represent a key molecular target of these complexes.

It is well established that the thioredoxin reductase (TrxR) system is a key regulator of cellular redox reactions and plays a central role in maintaining intracellular redox homeostasis. Inhibition of this redox-regulating pathway disrupts the thiol–disulfide balance, promotes the accumulation of reactive

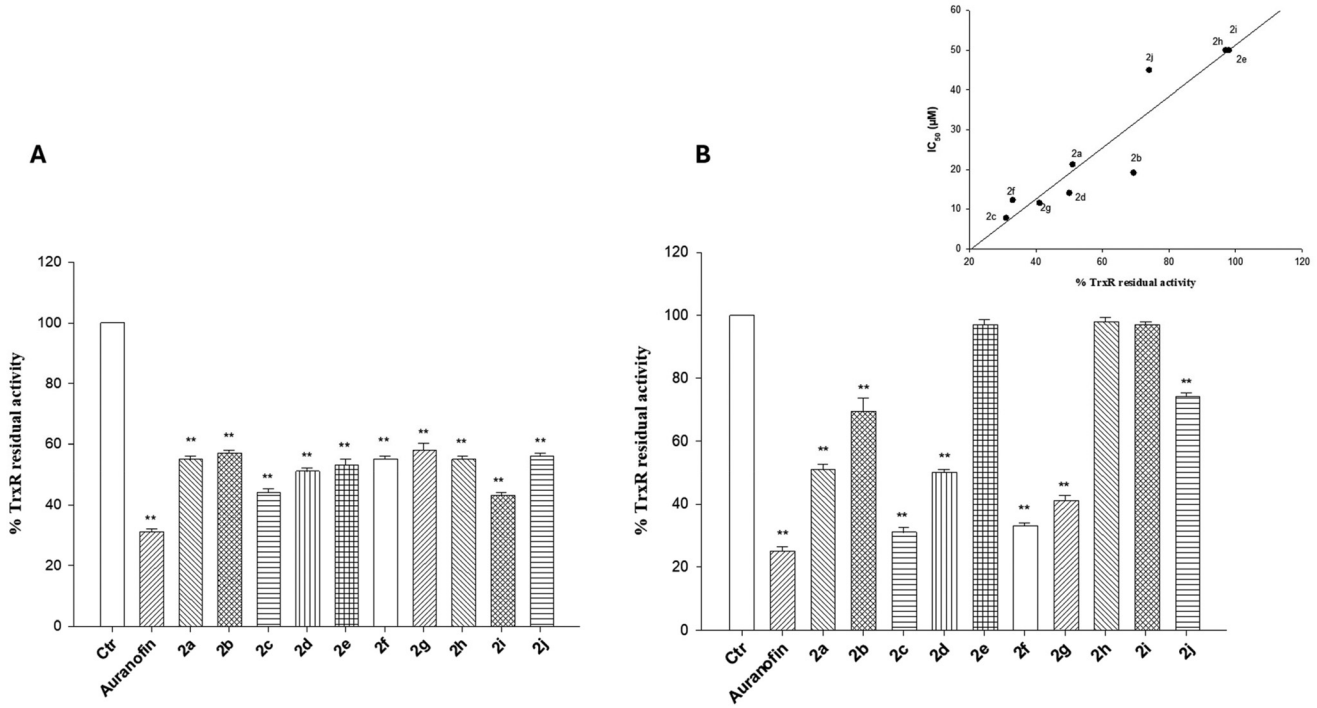


Fig. 2 TrxR inhibition. (A) TrxR1 activity was assayed by measuring NADPH-dependent reduction of DTNB at 412 nm as described in the Experimental section. Error bars indicate S.D. (B) MDA-MB-231 cells were incubated for 24 h with tested complexes (5 μM) or auranofin (1 μM). Subsequently, TrxR activity was assessed in cell lysates by measuring NADPH-dependent reduction of DTNB at 412 nm. The insert shows the correlation between the IC_{50} values calculated for MDA-MB-231 cells and the percentage of residual TrxR activity. Error bars indicate SD. ** $p < 0.01$.

oxygen species (ROS), and ultimately impairs cell viability.[51] Indomethacin has been shown to induce the generation of reactive oxygen species (ROS), a feature that underpins its rationale for inclusion in multi-target anticancer therapeutic approaches.[52] The effects of the newly synthesized complexes on cellular thiol contents and ROS generation were investigated (Fig. 3).

Total free sulfhydryl groups, including glutathione, cysteine residues, and protein thiols, were quantified using the DTNB assay after treatment of MDA-MB-231 cells with the newly developed gold(I) complexes (10 μM), with *auranofin* (3 μM) used as a positive control.

As shown in Fig. 3A, compound that elicited the highest TrxR inhibitory effect were those inducing a more pronounced

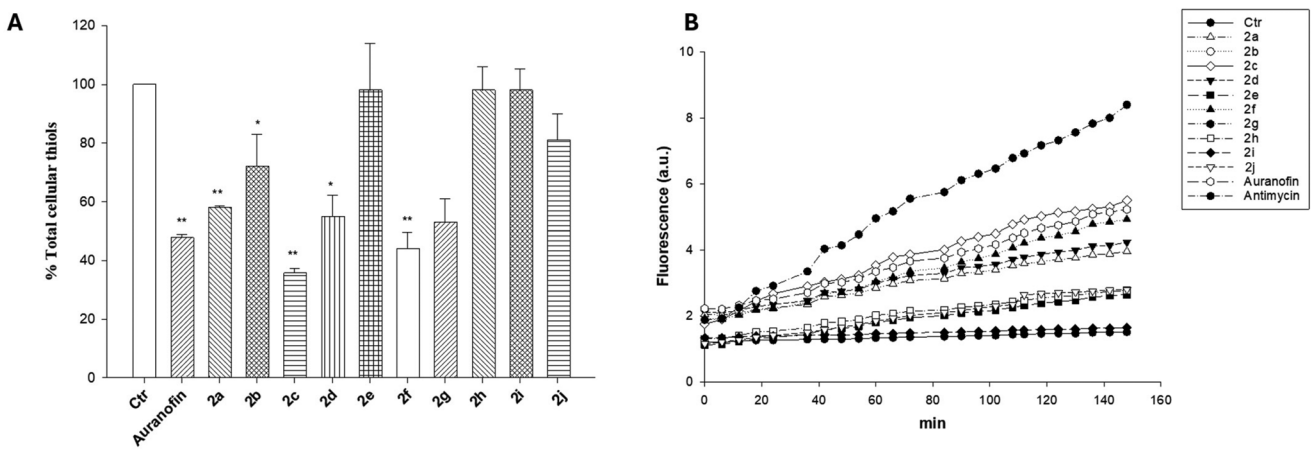


Fig. 3 Effects on sulfhydryl content and ROS production. (A) Sulfhydryl content in MDA-MB-231 cancer cells incubated for 24 h with Au(I) complexes (5 μM) and auranofin (1 μM). The sulfhydryl group amount was determined by the DTNB assay. Error bars indicate S.D. * $p < 0.05$, ** $p < 0.01$. (B) Effect of gold(I) compounds on hydrogen peroxide formation in MDA-MB-231 cells. Cells were pre-incubated in PBS/10 mM glucose medium for 20 min at 37 °C in the presence of 10 μM CM–DCFDA and then treated with tested complexes (25 μM) or antimycin (3 μM).

depletion of intracellular thiols. In addition, they were also the most effective in promoting ROS accumulation. Specifically, treatment of MDA-MB-231 cells with **2c** and **2f** complexes resulted in a marked, time-dependent increase in hydrogen peroxide levels (Fig. 3B), like that induced by *auranofin*. However, all compounds induced a ROS increase which was lower than that induced by antimycin A, a well-known complex III inhibitor of the mitochondrial respiratory chain.

Conclusions

In this study, we report the development of a novel and chemically distinct class of gold(I)–indomethacin complexes supported by N-heterocyclic carbene ligands, conceived to overcome both synthetic and biological limitations of existing gold-based anticancer agents. Central to this work is the implementation of an original, silver-free and base-free synthetic strategy exploiting gold–anisolyl synthons, which enables direct and efficient access to the first structurally authenticated [Au(NHC)(indomethacin)] complexes. This mild and scalable methodology, operating under aerobic conditions and using environmentally more benign solvents, substantially broadens the scope of gold carboxylate chemistry and facilitates systematic structure–activity investigations.

By varying the nature of the NHC ligands, we have generated a structurally diverse library of complexes characterized by outstanding thermal stability. Among them, derivatives bearing I^{t}Bu (**2c**), IPr^{Cl} (**2f**), IMes (**2d**), and IPr^{Me} (**2g**) ligands emerged as the most biologically performant compounds, consistently displaying IC_{50} values in the low- to mid-micromolar range across multiple cancer cell lines, and surpassing *cisplatin* in several models, including triple-negative breast cancer cells.

Importantly, all selected gold(I)–indomethacin complexes retained full cytotoxic potency in *cisplatin*-resistant ovarian cancer cells, demonstrating their ability to overcome platinum-based drug resistance. Even more strikingly, the most active derivatives exhibited robust activity against breast cancer stem cell–like populations, a cellular subset largely refractory to conventional chemotherapeutics and strongly implicated in tumor relapse and metastasis. These findings were further corroborated in 3D spheroid models, where some compounds displayed superior antitumor efficacy, with IC_{50} values approximately two-fold lower than *cisplatin*, highlighting their effectiveness under conditions that closely resemble the *in vivo* tumor microenvironment.

Mechanistic studies consistently point to thioredoxin reductase as a key intracellular target. The most active complexes, notably **2c**, **2f**, and **2g**, induced pronounced inhibition of cellular TrxR activity, accompanied by intracellular thiol depletion and a time-dependent accumulation of reactive oxygen species. The strong correlation between TrxR inhibition and cytotoxicity underscores a redox-mediated mode of action, synergistically reinforced by the intrinsic ROS-inducing properties of the indomethacin moiety.

Taken together, this work establishes gold(I)–NHC–indomethacin complexes as a robust, tunable, and mechanistically coherent anticancer platform. The identification of highly performant derivatives capable of targeting bulk tumor cells, drug-resistant phenotypes, and cancer stem cell populations provides a compelling foundation for further preclinical development and positions this class of compounds at the forefront of next-generation gold-based anticancer therapeutics.

Conflicts of interest

There are no conflicts to declare.

Data availability

All data included and leading to conclusions presented in this manuscript are included in the manuscript.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6dt00194g.

CCDC 2367330, 2367331, 2367333 and 2521381 contain the supplementary crystallographic data for this paper.[53a–d]

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