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Synthesis and anticancer activity of novel NHC-gold(I)-sugar complexes

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ABSTRACT

Gold(I) complexes containing stabilising ligands such as phosphines or *N*-heterocyclic carbenes (NHCs) are known to be inhibitors of the enzyme thioredoxin reductase (TrxR) and therefore act as potential apoptosis-inducing anticancer drug candidates. The conjugation of biomolecules overexpressed in cancer cells to the gold complexes makes them semi-targeted metabolites. Auranofin, an anti-arthritis agent, encompasses this property and exhibits anti-tumour activities. The synthesis, characterization and biological evaluation of four novel *N*-heterocyclic carbene-gold(I)-thiosugar complexes derived from glucose, lactose and galactose is reported. The reactions of 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene gold(I) chloride (NHC∗-Au-Cl) with pre-synthesized glycosyl thiols under mildly basic conditions gave the desired NHC-Au-thiosugar complexes in high to excellent yields (79–91%). The complexes retain the strong and redox-active Au-S bond contained in Auranofin. All complexes showed good solubility in biological media and were tested against the NCI 60 cancer cell panel for cytotoxicity. The synthesized NHC∗-Au derivatives showed good activity in the medium to low micromolar region, while complex **2** showed activity in the low micromolar to nanomolar region against the tested cell lines. To provide a theoretical structure of **4**, computational calculations were carried out based on the crystal structures of NHC-Au-SCN and NHC-Au-S-C_6H_4OMe.

Significant attention has been focused in the past decades on the synthesis and biological study of NHC-metal complexes since the successful synthesis of the first NHC derivative by Arduengo and co-workers in 1991 [1]. NHC-metal complexes have emerged as a research frontier for the development of novel metallodrugs due to their high stability and ease of modification [2]. Metallodrugs such as auranofin which is used in the treatment of rheumatoid arthritis could exhibit a wide range of biological activities and their applications in medicinal chemistry have attracted great interest [3]. Auranofin consists of a gold(I) center linearly coordinated to triethylphosphine and to the thiosugar 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-1-thiolate. Recently, auranofin was also found to be a potent inhibitor of thioredoxin reductase (TrxR) with an IC_{50} value in the low nanomolar region, which is often overexpressed in many cancer cells and has been identified as a potential target for anticancer drugs [4,5]. TrxR is essential in normal cells for redox homeostasis and protection against oxidative damage and mutation. Once transformed into malignant cells, TrxR supports tumour growth and progression. Auranofin is thus a promising anticancer agent, and it could induce cancer cell apoptosis *via* increased levels of reactive oxygen species (ROS) [6].

Compared to normal cells, the rapid growth and proliferation of cancer cells demand a drastic increase in glucose uptake and metabolite flux through glycolysis, which is termed the Warburg effect [7], and as such, altered glucose intake and glucose transporter (GLUT) overexpression are common in cancers and provide clinically validated targets for cancer treatment. Therefore, the bioconjugation of chemotherapeutics including metal-containing anticancer agents to glucose-like or glycomimetic substrates serves as a new method of semi-targeted chemotherapy. The rationale of designing such a strategy relies on the assumption that the glucose-like scaffold attached to a bioactive molecule could be recognized by the glucose transporter and the glycoconjugate internalized as a whole. Once taken up the cytotoxic metallodrug could exert its anticancer activity directly inside the tumour cells, which would greatly increase the drug effectiveness as well as selectivity [8]. Moreover, such bioconjugation could also potentially improve bioavailability, water solubility, enantiomeric purity and biocompatibility of the metallodrugs. For example, a series of glycoconjugates of cisplatin with monsaccharides were synthesized and the one with a β-D-glucose derivative showed promising activity against human A2780 ovarian carcinoma and MeWo

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melanoma cell lines [9]. Also, structure-activity relationship studies revealed that complexes containing α-anomers were overall more active than the corresponding β-anomers, whereas no difference was observed between the L- and G-isomers [9b]. A gold(I) complex with thioglucose also exhibited increased stability under physiological conditions, water solubility, bioavailability and antiproliferative activity compared with the non-glycosylated counterpart. Moreover, it proved cytotoxic towards human MCF7 breast adenocarcinoma, NCH89 and NCH82 glioblastoma, and murine C6 glioma cell lines with IC_{50} values in the low micromolar range [10]. A series of ruthenium complexes with sugar-phosphite ligands were synthesized and the influence of the different sugar ligands on their *in vitro* anticancer activity was evaluated. Such complexes could also have improved water solubility and showed enhanced selectivity towards cancer cells with moderate growth inhibition capability against several human tumour cell lines [11].

Recently, we reported the synthesis and bioactivity of NHC analogues of auranofin in which the alkyl substituents on the carbene were specifically modified. Bioactivity studies were very encouraging especially in human MCF7 breast adenocarcinoma and Caki-1 skin carcinoma cells, in particular in comparison to analogues where either the carbene or the thioglucose ligands are replaced by chlorides [12]. As an extension of this project, we report herein the synthesis and cytotoxicity studies of four new NHC-Au(I)-sugar complexes.

The success of auranofin as a treatment for arthritis as well as in a currently ongoing preclinical trial for the treatment of chronic lymphocytic leukaemia makes auranofin-like molecules potential anticancer agents [13]. The incorporation of tumour-targeting ligands such as sugars into metal complexes would thus be a promising approach for targeted anticancer chemotherapy. This could boost the impact on cancer cells and minimize the occurrence of adverse side effects [14]. Our previous work focused on the *in vitro* and *in vivo* activity of NHC-gold(I)-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-1-thiolate (**1** Fig. 1) [15]. This complex exhibited very good activity against a wide range of human cancer cell lines carried out on NCI 60 as well as promising growth inhibitory activity against the xenografted renal cancer cell line Caki-1 in mice. Moreover, inhibitory activity against the mammalian thioredoxin reductase enzyme was also observed with IC_{50} values in the low micromolar range [15], indicating that apoptosis induction through elevated oxidative stress could be the main mechanism for the complex. Encouraged by the promising *in vitro* and *in vivo* anticancer activity of **1**, another four complexes with the general structural formula NHC-Au(I)-SR, as shown in Fig. 1, were designed and synthesized in this work, in which NHC is the 1,3-dibenzyl-4,5-diphenyl-imidazole ligand and SR represents the thiosugars. Structure **2** containing α-thioglucose was designed because the anomeric configuration could have significant impact on the cellular uptake and activity profiles of metallodrugs as revealed in a recent report [16]. Also, it is known that other sugars besides D-glucose can be substrates for GLUT transporters, and can thus be considered as candidates for GLUT-targeting approach as well. Different sugars have been conjugated to anticancer agents to take advantage of GLUT-mediated cellular entry [17], for instance, D-galactose, the C4 epimer of glucose, has been found to possess an equivalent affinity and uptake rate by GLUT-1 compared to glucose [18]. Hence, galactose was also chosen as a sugar ligand leading to structure **3**.

Our recent work on glycolipids disclosed structural extension with an extra sugar could sometimes be beneficial to bioactive molecules [19]. Lactose-containing complex **4** was thus designed as another target structure. Finally, in order to explore the impact of a spacer on the bioactivity profile, a three-carbon spacer was inserted between the sugar and the metal core to give complex **5**.

Based on our previous work [12], the synthesis of the above four complexes **2**–**5** was relatively straightforward. Commercially

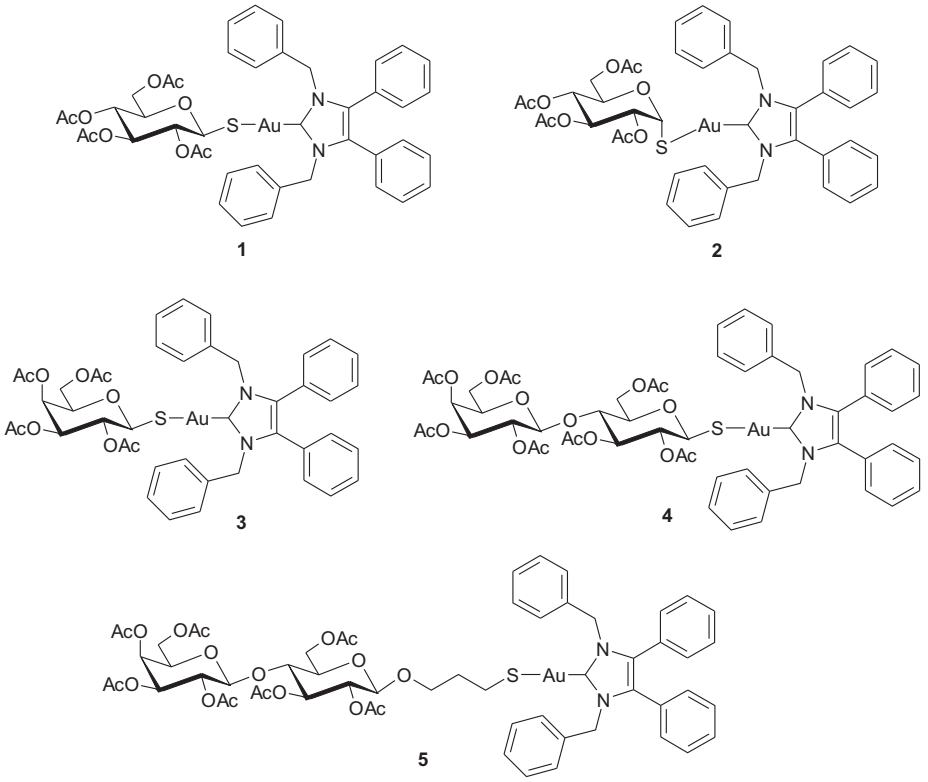

Fig. 1. Structures of NHC-Au(I)-SR complexes **1**–**5**.

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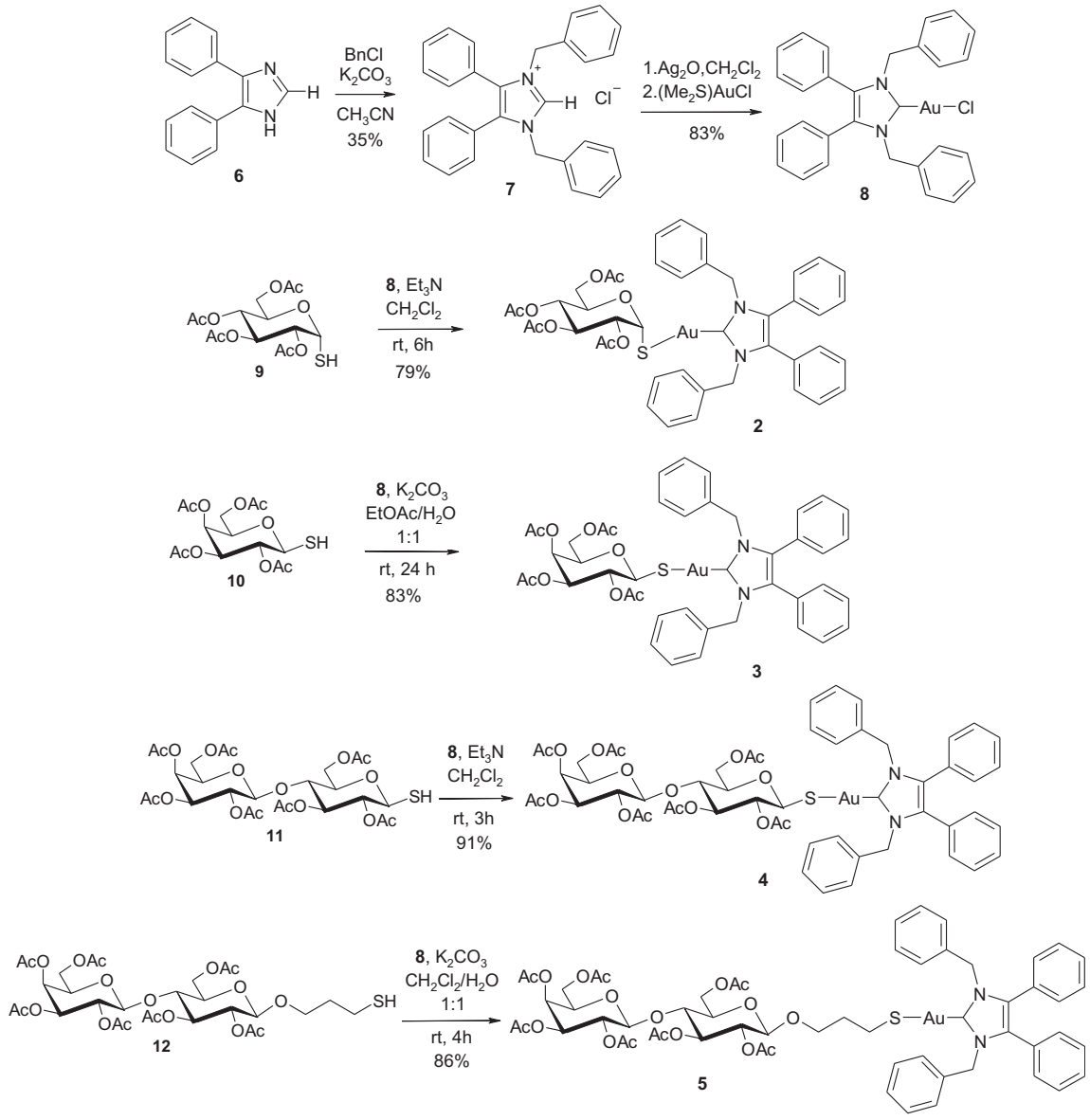
available 4,5-diphenylimidazole **6** (Scheme 1) was first di-benzylated with BnCl in the presence of K_2CO_3 to give imidazolium chloride **7** in 35% yield following the literature procedure [20]. Chloride **7** was then treated with Ag_2O in CH_2Cl_2 under the exclusion of light at room temperature to generate the NHC-Ag(I) chloride complex, which was *in situ* subjected to metal exchange with chloro (dimethylsulfide)gold(I) to give NHC-Au(I) chloride **8** [12] in 83% yield. Chloride **8** then served as the precursor to all four target molecules **2**–**5**. Meanwhile, as needed, four different thio-containing sugars **9**–**12** were prepared following different procedures [21–24]. α-Thioglucose **9** [21b] was prepared from tribenzyl levoglucosan in four steps in very good yield involving a highly stereoselective ring-opening procedure developed in our laboratory [21a]. It is worth mentioning that the procedure was a significant advance in glycosyl thiol chemistry because there had not been any reports on the direct stereoselective preparation of α-glycosyl thiols prior to our work. β-Glycosyl thiols **10** [22] and **11** [23] were prepared from the corresponding α-bromides following a standard two-step procedure [24], i.e. treatment with thiourea followed by hydrolysis with an alkali metal disulfite. The α-anomeric configuration of thioglucose **9** was readily determined by the coupling constant $^3J_{H1\text{-}H2}$ value, which is about 5.7 Hz, whereas analogous β-thiosugars, such as **10** and **11**, have $^3J_{H1\text{-}H2}$ value of 7–10 Hz. Thiol **12** [25] was synthesized from β-allyl per-acetyllactoside in two steps: thiol-ene addition of thioacetic acid to the double bond followed by selective *S*-deacetylation.

All the above thiols were then coupled with NHC-Au(I) chloride **8** to form the desired complexes (Scheme 1). The acetyl protecting groups were retained in the target molecules **2**–**5** as they could be labile under physiological conditions and easily hydrolysed to give the true active form of the NHC-Au(I) complexes. α-Thioglucose **9** was first coupled with chloride **8** in the presence of Et_3N in CH_2Cl_2 to produce compound **2** in very good yield. Notably, the α-anomeric thiol, which is known to be less nucleophilic than its corresponding β-counterpart [26], exhibited fairly good reactivity towards the NHC-Au(I) chloride. The coupling between β-thiol **10** and chloride **8** were conducted under the same conditions, but surprisingly, the reaction failed to give the desired product even after prolonged reaction times. Hence, we turned to the biphasic conditions, which has been successfully applied to the synthesis



Scheme 1. Synthesis of NHC-Au(I)-SR complexes **2**–**5**.

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of *S*-glycopeptides and lanthionine derivatives [27]. A mixture of **10** and **8** in EtOAc was treated with an aqueous solution of K_2CO_3, and as expected, the desired complex **3** was produced in very high yield (Scheme 1). Both conditions were thus applied to the formation of compounds **4** and **5**, and fortunately, all reactions worked well with the highest-yielding shown in Scheme 1. Thioglycosylation of **8** with thiol **11** under the action of Et_3N gave compound **4** in 91% yield, while with thiol **12** the reaction provided compound **5** in 86% yield under the biphasic conditions. All of the NHC-Au(I) complexes **2**–**5** were isolated as off-white solids and fully characterized by spectroscopic techniques and microanalysis. The formation of complexes **2**–**4** could be readily determined by comparing the ^{1}H NMR spectra of the complexes and their corresponding glycosyl thiols **9**–**11**. A triplet peak corresponding to the anomeric protons of the thiols disappeared and was replaced with a doublet peak in the complexes. The chemical shifts of these protons were also clearly shifted. Also, the benzylic protons observed as a singlet peak in the precursor chloride **8**, became diastereotopic and showed doublet of doublet peaks in complexes **3** and **4**, which was in accordance with the previous report [12]. Interestingly, the benzylic protons remain a singlet peak for complexes **2** and **5** which is likely due to the α-anomeric configuration of the sugar in **2** and the extra three-carbon linker in **5**. The most conspicuous change in the ^{13}C NMR spectra was the migration of the chemical shift of the carbene carbon (N***C***N). The peak, observed at around 170 ppm for chloride **8**, was shifted downfield to around 180 ppm for the NHC-Au(I) complexes **2**–**5**, indicating the different chemical environment of the carbon before and after the reaction.

The *in vitro* cytotoxicity of the four synthesized complexes **2**–**5** were subsequently carried out at the National Cancer Institute (NCI), and the results are given in the ESI. In all tested cell lines, complex **2** had better activity in comparison with the other tested compounds and is therefore a potential new anticancer drug candidate.

Computational results

Computational calculations were carried out in order to provide a theoretical structure of **4**.

Structures were optimized by DFT using different functionals (B3LYP, M06-2X, WB97XD and MN15) with the 6–31 + G(d) basis set for all the atoms and pseudopotentials LANL2DZ on the heavy Au atom as described in the computational methods. Firstly, the performance of each functional was benchmarked on the crystal structure of NHC-Au-SCN [28]. For such purpose, the crystal structure was used as an input (Isomer 1) and optimised without any constraints. Additionally, a second isomer was considered to explore the possible influence of the different arrangements on the structural parameters, i.e., Au-S distances, angles, etc, within both isomers (Fig. 2).

It is worth noting that the aim of this study was not to perform a thorough investigation on the relative stability of all the possible conformations but to evaluate the C-Au and Au-S distances and benchmark the different functionals against the crystal structure in order to select the best computational level to be used in larger systems. All the functionals utilised provided reliable parameters when compared with the crystal structure (see ESI for details). The calculated C-Au distances range between 2.012 and 2.030 Å. The worst case corresponds to B3LYP while MN15 and WB97XD presented a good agreement with respect to the actual crystal structure. Similar results are found for the Au-S distances in which MN15 and WB97XD provided the closest values with respect to the crystal structure in both conformers 1 and 2. Values of the Au-S-C angle are very close to the crystal structure with deviations of ±1°. Fig. 4 shows the overlap between the calculated models and the crystal structure (blue). As observed, all the functionals present a good overlap with no significant deviations. As previously observed, B3LYP presented the largest variations (Fig. 3, red) with a total overall RMSD of the position of each atom with respect to the crystal structure of 0.674 Å. The total overall RMSD for the rest of the functionals were 0.428 (M06-2X, orange), 0.501 (MN15, green) and 0.381 Å (WB97XD, cyan). Considering these results, MN15 and WB97XD provided the most reliable results (less total RMSD and distance deviation), and reproduce the crystal structure more accurately. However, M06-2X could also describe the structure within a reasonably good accuracy.

A similar benchmark study was also carried out using the crystal structure for *p*-methoxythiophenolate conjugated to the NHC*-Au complex [28]. As observed in the case of NHC-Au-SCN, WB97XD and MN15 functional provide the most accurate results, i.e. structural parameters such as bonding distances and angles from those functionals are the closest to the crystal structure (see ESI). Once the functionals are benchmarked and the computational level selected, we obtained a model structure for compound **4**. Fig. 4 shows the optimised structure and all the selected structural parameters were gathered in Table 3 (see ESI). It is shown that the Au-S bond distance ranges from 2.404 to 2.339 Å with an average Au-S bond distance of 2.372 ± 0.027 Å. The C-Au distance is less sensitive to the method with an average distance across the method of 2.027 ± 0.007 Å. Finally, the Au-S-C angle is very stable showing an average value of 94.5 ± 0.4°. Of course, there is a wide range of different conformers to be considered since the OH groups can have free rotation. But based on the previous crystal structures and the benchmarks, we believe that this can be a very reliable and possible structure.

In summary, as an extension of our previous project, four new monomeric NHC-Au(I)-sugar complexes **2**–**5** were synthesized in very high to excellent yields by reacting the appropriate pre-synthesized acetylated thiosugars with NHC-Au(I) chloride. Two different conditions were attempted for the reaction and the

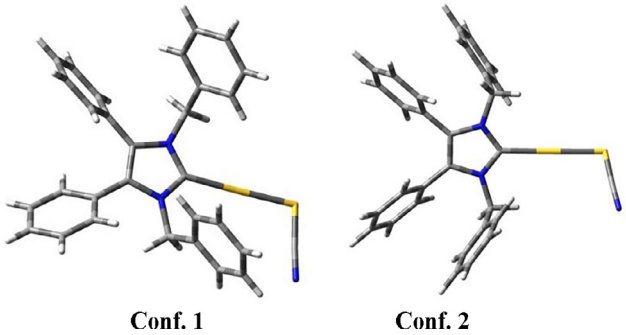

Fig. 2. Two different conformers evaluated using DFT. Conformer 1 (Conf.1) corresponds to the crystal structure.

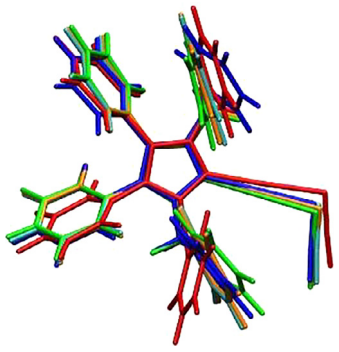

Fig. 3. Overlap between the crystal structure (blue), and those calculated using B3LYP (red), M06-2X (orange), MN15 (green) and WB97XD (cyan) functionals.

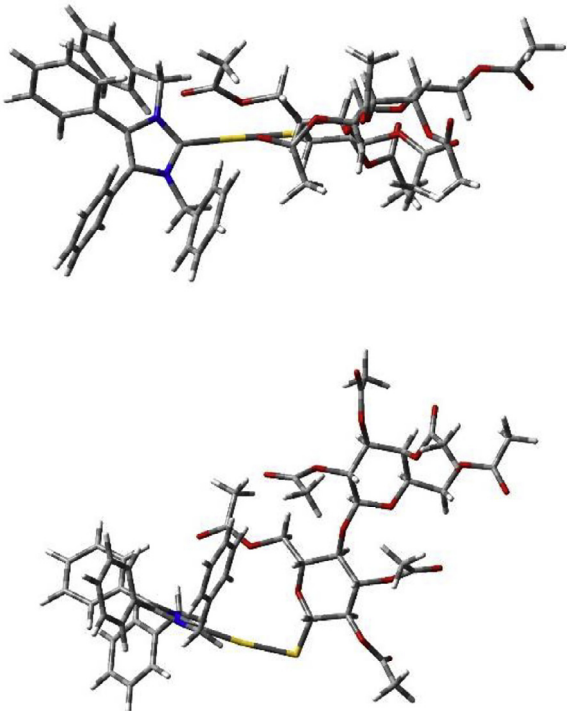

Fig. 4. Structure of compound **4** at the WB97XD/6–31 + G(d)/Lanl2dz.

biphasic conditions were found to be more reliable and gave the complexes in invariably high yields. The preliminary cytotoxic activity of the complexes was tested using the NCI60 cancer cell line panel. All compounds showed good activity against a wide range of the tested human cancer cell lines. Among them, complex **2** showed the best overall activity in the series and exhibited better activity than its β-counterpart **1** on all cell lines. Complex **4** exhibited better activity than **3** by a factor of four and better activity than **5** by a factor of six or seven on specific cell lines. Complex **4** also showed better activity than previously reported complex **1**. This finding indicates that appropriate substitution of the sugar moiety on the NHC-gold(I) complex can improve the activity against certain cancer cells. Therefore, there is potential for more gold(I) complexes in therapy in the future from the rational design of NHC-gold(I) complexes that are slightly more targeted. With the use of computational calculations, we were also able to predict the potential crystal structure of compound **4**.

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A. Supplementary data

Supplementary data (experimental procedure, characterization data, as well as copies of the 1H and 13C NMR of new compounds) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tetlet.2018.06.040.

References

[1] A.J. Arduengo, R.L. Harlow, M. Kline, J. Am. Chem. Soc. 113 (1991) 361.
[2] (a) W.A. Herrmann, Angew. Chem., Int. Ed. 41 (2002) 1290;
(b) D. Bourissou, O. Guerret, F.P. Gabbaï, G. Bertrand, Chem. Rev. 100 (2000) 39;
(c) C.M. Crudden, D.P. Allen, Coord. Chem. Rev. 248 (2004) 2247.
[3] (a) C.F. Shaw, Chem. Rev. 99 (1999) 2589;
(b) I.E. Leon, J.F. Cadavid-Vargas, A.L.D. Virgilio, S.B. Etcheverry, Curr. Med. Chem. 24 (2017) 112.
[4] C. Fan, W. Zheng, X. Fu, X. Li, Y.-S. Wong, T. Chen, Cell Death Dis. 5 (2014) e1191.
[5] T.C. Karlenius, K.F. Tonissen, Cancers (Basel) 2 (2010) 209.
[6] (a) C. Marzano, V. Gandin, A. Folda, G. Scutari, A. Bindoli, M.P. Rigobello, Free Radic. Biol. Med. 42 (2007) 872;
(b) R. Rubbiani, I. Kitanovic, H. Alborzinia, S. Can, A. Kitanovic, L.A. Onambele, M. Stefanopoulou, Y. Geldmacher, W.S. Sheldrick, G. Wolber, A. Prokop, S. Wölfl, I. Ott, J. Med. Chem. 53 (2010) 8608;
(c) C. Liu, Z. Liu, M. Li, X. Li, Y.-S. Wong, S.-M. Ngai, W. Zheng, Y. Zhang, T. Chen, PLoS One 8 (2013), e53945.
[7] (a) O. Warburg, Science 123 (1956) 309;
(b) M.V. Liberti, J.W. Locasale, Trends Biochem. Sci. 41 (2016) 211.
[8] A. Pettenuzzo, R. Pigot, L. Ronconi, Metallodrugs 1 (2015) 36.
[9] (a) Y. Chen, M.J. Heeg, P.G. Braunschweiger, W. Xie, P.G. Wang, Angew. Chem. Int. Ed. 38 (1999) 1768;
(b) Y. Mikata, Y. Shinohara, K. Yoneda, Y. Nakamura, I. Brudzinska, T. Tanase, T. Kitayama, R. Takagi, T. Okamoto, I. Kinoshita, M. Doe, C. Orvig, S. Yano, Bioorg. Med. Chem. Lett. 11 (2001) 3045.
[10] E. Viry, E. Battaglia, V. Deborde, T. Müller, R. Réau, E. Davioud-Charvet, D. Bagrel, ChemMedChem 3 (2008) 1667.
[11] I. Berger, M. Hanif, A.A. Nazarov, C.G. Hartinger, R.O. John, M.L. Kuznetsov, M. Groessl, F. Schmitt, O. Zava, F. Biba, V.B. Arion, M. Galanski, M.A. Jakupec, L. Juillerat-Jeanneret, P.J. Dyson, B.K. Keppler, Chem. Eur. J. (2008) 9046.
[12] F. Hackenberg, H. Müller-Bunz, R. Smith, W. Streciwilk, X. Zhu, M. Tacke, Organometallics 32 (2013) 5551.
[13] W. Fiskus, N. Saba, M. Shen, M. Ghias, J. Liu, S. Das Gupta, L. Chauhan, R. Rao, S. Gunewardena, K. Schorno, C.P. Austin, K. Maddocks, J. Byrd, A. Melnick, P. Huang, A. Wiestner, K.N. Bhalla, Cancer Res. 74 (2014) 2520.
[14] C. Sanchez-Cano, M.J. Hannon, Dalton Trans. (2009) 10702.
[15] W. Walther, O. Dada, C. O'Beirne, I. Ott, G. Sánchez-Sanz, C. Schmidt, C. Werner, X. Zhu, M. Tacke, Lett. Drug Des. Discov. 14 (2017) 125.
[16] M. Patra, S.G. Awuah, S.J. Lippard, J. Am. Chem. Soc. 138 (2016) 12541.
[17] P.G. LeFevre, Pharmacol. Rev. 13 (1961) 39.
[18] D. Melisi, A. Curcio, E. Luongo, E. Morelli, M.G. Rimoli, Curr. Top. Med. Chem. 11 (2011) 2288.
[19] (a) L. Zhang, C. Mc Carthy, X. Zhu, Carbohydr. Res. 448 (2017) 43;
(b) A.M. Melo, L. Zhang, E.F. Dockry, A. Petrasca, Y.G. Ghnewa, E.P. Breen, M.E. Morrissey, C. O'Reilly, R. Bruen, A. O'Meara, J. Lysaght, X. Zhu, D.G. Doherty, Glycobiology 28 (2018) 512.
[20] (a) F. Hackenberg, G. Lally, H. Müller-Bunz, F. Paradisi, D. Quaglia, W. Streciwilk, M. Tacke, J. Organomet. Chem. 717 (2012) 123;
(b) F. Hackenberg, G. Lally, H. Müller-Bunz, F. Paradisi, D. Quaglia, W. Streciwilk, M. Tacke, Inorg. Chim. Acta 395 (2013) 135.
[21] (a) X. Zhu, R.T. Dere, J. Jiang, L. Zhang, X. Wang, J. Org. Chem. 76 (2011) 10187;
(b) H. Wang, X. Zhu, Org. Biomol. Chem. 12 (2014) 7119.
[22] M.B. Haque, B.P. Roberts, D.A. Tocher, J. Chem. Soc., Perkin Trans. 1 (1998) 2881.
[23] D.A. Fulton, J.F. Stoddart, J. Org. Chem. 66 (2001) 8309.
[24] M. Černý, J. Vrkoč, J. Staněk, Collect. Czech. Chem. Commun. 24 (1959) 64.
[25] T.M. Altamore, C. Fernández-García, A.H. Gordon, T. Hübscher, N. Promsawan, M.G. Ryadnov, A.J. Doig, D.N. Woolfson, T. Gallagher, Angew. Chem. Int. Ed. 50 (2011) 11167.
[26] R. Smith, H. Müller-Bunz, X. Zhu, Org. Lett. 18 (2016) 3578.
[27] (a) X. Zhu, K. Pachamuthu, R.R. Schmidt, J. Org. Chem. 68 (2003) 5641;
(b) X. Zhu, R.R. Schmidt, Eur. J. Org. Chem. (2003) 4069.
[28] O. Dada, D. Curran, C. O'Beirne, H. Müller-Bunz, X. Zhu, M. Tacke, J. Organomet. Chem. 840 (2017) 30.