Full paper

Applied Organometallic Chemistry

Received: 2 October 2015 Revised: 29 October 2015 Accepted: 17 December 2015 Published online in Wiley Online Library: 4 February 2016

(wileyonlinelibrary.com) DOI 10.1002/aoc.3434

1,2,4-Triazole-based *N*-heterocyclic carbene complexes of gold(I): synthesis, characterization and biological activity

Jan Turek[a], Zdeňka Růžičková[a], Eva Tloušťová[b], Helena Mertlíková-Kaiserová[b], Jana Günterová[b], Lubomír Rulíšek[b] and Aleš Růžička[a]*

Two gold(I) complexes of the (NHC)AuX type bearing a triazole-based *N*-heterocyclic carbene (NHC) ligand (1-*tert*-butyl-4-(4-methylphenyl)-3-phenyl-1*H*-1,2,4-triazol-4-ium-5-ylidene) and various halide ligands (X = Br, I) were synthesized and characterized in solution using NMR spectroscopy as well as in the solid state using X-ray diffraction techniques. The cytotoxic properties of both compounds and the precursor, (NHC)AuCl, were screened against a panel of human tumour cell lines including liver cancer (HepG2), cervical cancer (HeLa S3) and leukaemia (CCRF-CEM, HL-60) and compared to cisplatin and auranofin. It was found that the activities of the chloro and bromo derivatives were generally superior to that of cisplatin and slightly less effective compared to auranofin, except for HepG2 cells where auranofin was not as effective. In addition, the ability to induce membrane phosphatidyl serine externalization as a hallmark of apoptosis in CCRF-CEM leukaemic cells was investigated. **Copyright © 2016 John Wiley & Sons, Ltd.**

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Keywords: gold(I) complexes; *N*-heterocyclic carbenes; cytotoxicity; apoptosis

Introduction

During the last decade, *N*-heterocyclic carbenes (NHCs) have had an impact on nearly all fields of chemistry,[1] becoming a serious replacement to the widely used tertiary phosphines.[1a,2] This is mainly because of their unique σ-donating properties and therefore their ability to form stable metal complexes with transition metals, in either low or high oxidation state,[3] as well as main group elements.[4] Free and stable NHCs were initially predicted and investigated by Wanzlick *et al.* in the 1960s;[5] nevertheless it took almost thirty years until the isolation of the first stable NHC was described in 1991 by Arduengo *et al.*[6]

Metal complexes with NHCs as ligands were first synthesized by the groups of Wanzlick[7] and Öfele[8] followed by Lappert and co-workers.[9] But the real breakthrough causing an ever-growing interest in the field of NHC chemistry came with the aforementioned work by Arduengo *et al.*[6] The first NHC–gold(I) complex was described in 1973,[10] but it is only recently that these compounds, mainly of general formula (NHC)AuCl with gold oxidation state of +1, have attracted attention, benefiting from recently developed straightforward synthetic routes.[11] Nolan and co-workers reported an efficient synthetic method for the preparation of (NHC)AuX (X = Cl, Br and I) from (NHC)HX.[12]

NHC–metal complexes are typically used in catalytic applications.[13] In addition, NHCs were recently found to be suitable carrier ligands for cytotoxic metal complexes because they perfectly fulfil the requirements for an efficient drug design and fast optimization.[13a,d,14] NHCs mainly benefit from convenient and efficient synthetic procedures allowing a wide variation of substituents and therefore fine-tuning of both physicochemical properties and reactivity in biological media of the final NHC–metal complexes. Furthermore, their relatively high stability and ease of derivatization make them promising candidates for drug development.[13a,d,14]

The utilization of NHC ligands was the next logical step after the successful application of gold phosphine complexes as anti-tumour agents. A number of NHC–gold(I) complexes have been synthesized and characterized for their biological properties[14b,15] since the pioneering work of Berners-Price and co-workers that established the application of a variety of cationic mononuclear NHC–gold(I) biscarbene complexes as potential chemotherapeutic agents.[16] As mentioned, NHC–gold(I) compounds hold the potential of various derivatizations associated with the possible presence of an ancillary ligand coordinated to gold, in addition to the Au-$C_{carbene}$ bond (e.g. phosphines, thiols, as well as a second NHC ligand).[17] Unlike for platinum derivatives, the induction of apoptosis via targeting mitochondria, but also through the interaction with various proteins/enzymes (e.g. thioredoxin reductase), has been shown for several NHC–gold(I) complexes.[18]

In this paper, we report the synthesis and structural characterization of three gold(I) complexes of the (NHC)AuX type bearing a

* *Correspondence to: Aleš Růžička, Department of General and Inorganic Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, CZ-532 10, Pardubice, Czech Republic. E-mail: ales.ruzicka@upce.cz*

a *Department of General and Inorganic Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, CZ-532 10, Pardubice, Czech Republic*

b *Institute of Organic Chemistry and Biochemistry, Gilead Sciences Research Center and IOCB, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10, Praha 6, Czech Republic*

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triazole-based NHC ligand (1-*tert*-butyl-4-(4-methylphenyl)-3-phenyl-1*H*-1,2,4-triazol-4-ium-5-ylidene) and various halido ligands (X = Cl, Br, I). In addition, the cytotoxic properties of all compounds were investigated employing the screening against a panel of human tumour cell lines including liver cancer (HepG2), cervical cancer (HeLa S3) and leukaemia (CCRF-CEM, HL-60).

Experimental

General Comments

All reactions were performed under an argon atmosphere using standard Schlenk techniques. The solvent purification system PureSolv MD 7 (Innovative Technology Inc.) was used to dry the solvents that were then degassed using the freeze–pump–thaw method and stored under an argon atmosphere.

Materials

1-(*tert*-Butyl)-4-(4-methylphenyl)-3-phenyl-1*H*-1,2,4-triazol-4-ium-5-ide gold(I) chloride (**1**),[19] [2-(*N*,*N*-dimethylaminomethyl)phenyl]di-*n*-butyltin(IV) bromide[20] and [2-(*N*,*N*-dimethylaminomethyl)phenyl]di-*n*-butyltin(IV) iodide[21] were prepared according to literature procedures.

NMR Spectroscopy

NMR spectra were recorded from solutions in $CDCl_3$ with a Bruker Ascend™ 500 MHz spectrometer (equipped with Z-gradient 5 mm probe) operating at frequencies of 500.13 MHz for ^1H, 125.76 MHz for $^{13}C\{^1H\}$ and 186.50 MHz for $^{119}Sn\{^1H\}$ at 295 K. $CDCl_3$ was distilled under reduced pressure, degassed using the freeze–pump–thaw method and finally stored over molecular sieves under an argon atmosphere. The solutions were obtained by dissolving approximately 20 mg of the analysed compound in *ca* 0.7 ml of deuterated solvent. The ^1H NMR chemical shift values are given relative to internal tetramethylsilane ($\delta(^1H) = 0.00$ ppm) or to residual solvent signal ($CDCl_3$, $\delta(^1H) = 7.26$ ppm). The ^{13}C NMR chemical shifts were calibrated similarly ($CDCl_3$, $\delta(^{13}C) = 77.16$ ppm). All ^{13}C NMR spectra were measured using the standard proton-decoupled experiment and CH and CH_3 versus C and CH_2 groups were distinguished by the APT method.[22] The ^{119}Sn NMR chemical shift values are referred to external neat tetramethylstanane ($\delta(^{119}Sn) = 0.0$ ppm). Positive chemical shift values denote shifts to higher frequencies relative to the standards.

Elemental Analysis

Elemental compositions were determined under an argon atmosphere using an automatic combustion analyser (EA 1108, Fisons Instruments).

X-ray Crystallography

The diffraction data (see supporting information, Table S1) were obtained at 150(2) K with an Oxford Cryostream low-temperature device using a Nonius KappaCCD diffractometer with Mo K_α radiation ($\lambda = 0.71073$ Å) using a graphite monochromator, and the φ and χ scan mode. Data reductions were performed with DENZO-SMN.[23] The data were corrected for absorption by integration methods.[24] The structures were solved by direct methods (SIR92)[25] and refined by full matrix least squares based on F_2 (SHELXL97).[26] Hydrogen atoms could be mostly localized on the difference Fourier maps. However, to ensure uniformity of the treatment of the crystal structures, all hydrogen atoms were recalculated into their idealized positions (riding model) and assigned temperature factors of $U_{iso}(H) = 1.2U_{eq}$ (pivot atom) or of $1.5U_{eq}$ for the methyl moiety with C&bond;H = 0.96, 0.97 and 0.93 Å for methyl, methylene and hydrogen atoms in aromatic rings or allyl moiety, respectively. CCDC 1428999 (**2**) and 1429000 (**3**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.

Synthesis

Complexes **2** and **3** were prepared according to a previously published procedure.[19] Complex **1** (0.78 mmol) and [2-(*N*,*N*-dimethylaminomethyl)phenyl]di-*n*-butyltin(IV) bromide[20] or [2-(*N*,*N*-dimethylaminomethyl)phenyl]di-*n*-butyltin(IV) iodide[21] (0.82 mmol) were dissolved in benzene (*ca* 30 ml) and the reaction mixture was stirred at 55°C for 24 h. After cooling to room temperature, the reaction mixture was evaporated to dryness and the resulting crude products were washed with hexane to give off-white powders of **2** and **3**. Single crystals suitable for X-ray diffraction were obtained by slow diffusion of hexane into a saturated dichloromethane solution of the respective complex at room temperature.

1-(*tert*-Butyl)-4-(4-methylphenyl)-3-phenyl-1*H*-1,2,4-triazol-4-ium-5-ide gold(I) bromide (**2**). Yield 87%; m.p. 262–263°C. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm): 7.42–7.41 (m, 1H, Ar*H*), 7.34–7.32 (m, 4H, Ar*H*), 7.27–7.22 (m, 4H, Ar*H*), 2.40 (s, 3H, CH_3), 1.95 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 177.8 ($NC_{carb}N$), 150.7 (NCN), 140.4 (ArC), 134.2 (ArC), 130.9 (ArC), 130.6 (ArC), 128.9 (ArC), 128.8 (ArC), 127.0 (ArC), 125.0 (ArC), 63.2 ($C(CH_3)_3$), 31.3 ($C(CH_3)_3$), 21.5 (CH_3). Anal. Calcd for $C_{19}H_{21}AuClN_3$ (%): C, 39.01; H, 3.46; N, 7.58. Found (%): C, 39.03; H, 3.48; N, 7.59.

1-(*tert*-Butyl)-4-(4-methylphenyl)-3-phenyl-1*H*-1,2,4-triazol-4-ium-5-ide gold(I) iodide (**3**). Yield 81%; m.p. 260–261°C. ^1H NMR (500 MHz, $CDCl_3$, δ, ppm): 7.42–7.41 (m, 1H, Ar*H*), 7.35–7.31 (m, 4H, Ar*H*), 7.28–7.22 (m, 4H, Ar*H*), 2.40 (s, 3H, CH_3), 1.95 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 184.4 ($NC_{carb}N$), 150.6 (NCN), 140.4 (ArC), 134.2 (ArC), 130.9 (ArC), 130.5 (ArC), 128.9 (ArC), 128.8 (ArC), 126.9 (ArC), 125.0 (ArC), 63.2 ($C(CH_3)_3$), 31.3 ($C(CH_3)_3$), 21.5 (CH_3). Anal. Calcd for $C_{19}H_{21}AuClN_3$ (%): C, 35.96; H, 3.19; N, 6.99. Found (%): C, 35.98; H, 3.20; N, 7.01.

Cytotoxicity Assay

All cell lines were obtained from ATCC (Manassas, VA). CCRF-CEM (T-lymphoblastic leukaemia), HL-60 (promyelocytic leukaemia), HepG2 (hepatocellular carcinoma) and HeLa S3 (cervical carcinoma) cells were maintained in RPMI-1640 or Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, antibiotics (200 μg ml^{-1} of streptomycin and 200 U ml^{-1} of penicillin G) and 4 mM glutamine at 37°C under a humidified atmosphere containing 5% CO_2. Subconfluent cells were seeded in a 96-well plate at a density of 13 500 cells per well 24 h prior to an experimental treatment. Compounds (15 mM stock solutions in dimethylsulfoxide (DMSO)) were added to the culture media and incubated for 72 h. The final concentration of DMSO in culture media was up to 0.1%. The cytotoxicity of the synthesized compounds was then assessed using the XTT cell proliferation kit II (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The absorbance at 495 nm was recorded following 1 h incubation with the dye. IC_{50} values were determined

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using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA).

Cell Cycle Analysis

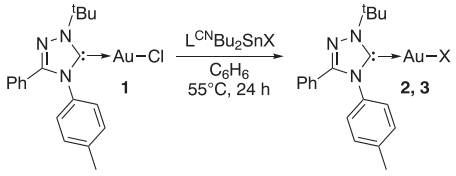
Subconfluent CCRF-CEM cells seeded at a density of 1×10^5 ml^{-1} in six-well plates were left to rest for 24 h and treated with GIC_{50} of tested compounds for 72 h. The cells were then collected by centrifugation, and resuspended with phosphate-buffered saline (PBS). Cells were centrifuged at 250*g* for 5 min, washed again with PBS and then fixed with 70% ice-cold ethanol for 30 min. The fixed cells were centrifuged, washed in PBS, treated with RNase A (500 μg ml^{-1}) at 37°C for 30 min and incubated with a staining solution containing 0.1% Triton® X-100 and propidium iodide (100 μg ml^{-1}) in PBS for 1 h at a final concentration of 10^6 cells ml^{-1}. The cellular DNA content was determined using a flow cytometer (FACSAria, BD Biosciences, San Jose, CA). At least 30 000 cells were used for each analysis, and the results were displayed as histograms. The cell cycle distribution was analysed using ModFit LT 3.0 (Verity Software House, Topsham, ME).

Apoptosis Evaluation

The phosphatidyl serine externalization in the cells was quantified using an ApoAlert® annexin V-FITC apoptosis kit (Clontech) according to the manufacturer's instructions. Briefly, 10^6 cells were harvested and washed with PBS (250*g* for 5 min). The pellet was resuspended in 0.2 ml of the Ca^{2+}-containing binding buffer supplemented with annexin V-FITC conjugate at a concentration of 1 μg ml^{-1} and incubated for 15 min in the dark. The cells were then centrifuged, washed twice with binding buffer and finally resuspended in 0.5 ml of the same buffer. Propidium iodide was added at a concentration of 5 μg ml^{-1}, vortexed and the cells were immediately subjected to flow cytometry. The subpopulations were gated into quadrants and interpreted as follows: double negative population was considered healthy, FITC-positive/propidium iodide-negative population was considered early apoptotic and double positive population was considered late apoptotic or necrotic.

Results and Discussion

Au(I) complexes **2** and **3** were successfully synthesized according to a published method[19] comprising the treatment of **1** with an appropriate triorganotin(IV) halide $L^{CN}Bu_2SnX$[20,21] (L^{CN} = 2-(*N*,*N*-dimethylaminomethyl)phenyl; X = Br for **2** or I for **3**) in benzene at 55°C for 24 h (1) while the standard Finkelstein procedures using KBr and NaI in coordinating solvents produced only mixtures of complexes. As mentioned previously, the use of the organotin(IV) agent is very efficient and enables a convenient monitoring of the halogen exchange completion using ^{119}Sn NMR spectroscopy (singlet signals in $CDCl_3$ at −42 ppm for $L^{CN}Bu_2SnBr$ and −33 ppm for $L^{CN}Bu_2SnI$), where only one signal corresponding to the $L^{CN}Bu_2SnCl$ (−48 ppm) is present when a full conversion is achieved. The resulting $L^{CN}Bu_2SnCl$ as well as starting tin(IV) bromide or iodide are extremely soluble in non-polar solvents, e.g. hexane, and therefore easily separable from the reaction mixture.



L^{CN} = *N*,*N*-dimethyl-1-phenylmethanamine
X = Br (**2**) or I (**3**)

Scheme 1. Synthesis of Au(I) complexes 2 and 3.

The formation of the desired NHC–Au complexes **2** and **3** was elucidated by means of ^{1}H NMR and ^{13}C NMR spectroscopy showing spectral patterns expected for these types of compounds. The chemical shift values of the metal-bound carbene carbon (177.8 ppm for **2**, 184.4 ppm for **3**) in the ^{13}C NMR spectra correlate well with those of analogous complexes published recently.[19] In both cases, single crystals suitable for X-ray structure determination were obtained.

The X-ray structures of **2** and **3** reveal the formation of monocarbene linear-type geometry (Figs. 1, 2). The carbine–metal bonds ($C_{carbene}$-Au = 1.992(5) Å for **2**; 1.993(7) Å for **3**) as well as metal–halide bonds (Au-Br = 2.4014(5) Å; Au-I = 2.5475(5) Å) are comparable with values in other reported gold(I) bromides and iodides stabilized by NHC ligands.[19,27] Besides, the packing diagrams of both complexes show the formation of sets of two head-to-tail oriented molecules aggregated via a weak Au···Au interaction (3.649 Å for **2**; 3.698 Å for **3**) along with an electrostatic (dipole–dipole) interaction between the negatively charged halide

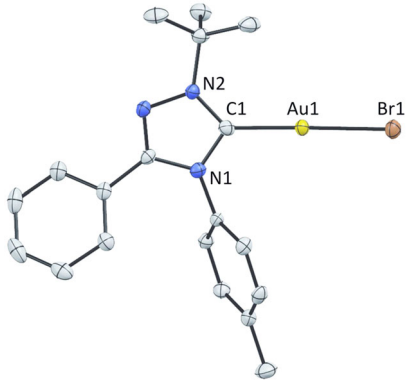


Figure 1. Molecular structure (ORTEP, 50% probability level) of **2**. Hydrogen atoms have been omitted for clarity. Selected interatomic distances (Å) and angles (°): Au1–C1 1.992(5), Au1–Br1 2.4014(5), N1–C1 1.364(6), C1–N2 1.344 (6), C1–Au1–Br1 177.66(13), N2–C1–N1 103.7(4).

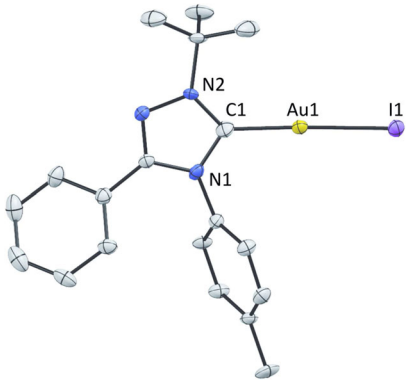


Figure 2. Molecular structure (ORTEP, 50% probability level) of **3**. Hydrogen atoms have been omitted for clarity. Selected interatomic distances (Å) and angles (°): Au1–C1 1.993(7), Au1–I1 2.5475(5), N1–C1 1.362(9), N2–C1 1.331 (8), C1–Au1–I1 176.4(2), N2–C1–N1 103.6(6).

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ion of one of the monomers and moderately positively charged carbene ring of the other monomer and vice versa (see supporting information, Fig. S1).[28]

Biological Activity

Cytotoxic activity

The compounds were examined for their cytotoxic properties against a panel of human tumour cell lines. Cisplatin and auranofin served as reference compounds, cisplatin as a representative of clinically used anticancer platinum(II)-based metallocomplexes and auranofin as phosphine gold(I) complex. Nolan and co-workers[29] performed screening on set of known gold(I) imidazole or benzimidazole NHC complexes against human tumour cell lines – prostate, breast, urothelial and bladder carcinoma. All these complexes revealed activity (IC_{50}) in the low micromolar range similarly to the studied complexes but on different cell lines.

Exposure of the tumour cells to all tested compounds leads to a concentration-dependent decrease in cell viability as determined by XTT assay with (NHC)AuI being the least effective of the series (Table 1). All activities are in the low micromolar range, generally superior to the activity of cisplatin and slightly less effective compared to auranofin (with the exception of HepG2 cells where auranofin is not as effective as (NHC)AuBr and (NHC)AuCl). Considering the stability and relative insolubility of the tested compounds in aqueous solutions we can hypothesize that an improved formulation of the compounds (liposomal encapsulation, chemical derivatization, etc.) may significantly increase the cytotoxic potential of the compounds under investigation compared to auranofin.

Apoptosis and cell cycle effects

Apoptotic effects of gold(I) complexes have been previously demonstrated in a variety of tumour cells indicating the anticancer potential of this class of compounds.[30] We have therefore investigated the ability of the tested compounds, at their respective GIC_{50}, to induce membrane phosphatidyl serine externalization as a hallmark of apoptosis in CCRF-CEM leukaemic cells. We have found that upon 72 h incubation all compounds tested increased the proportion of apoptotic cells compared to the untreated sample. Total number of annexin V-positive cells was 34% for (NHC)AuBr, 27% for (NHC)AuCl and 25% for (NHC)AuI versus 16% in control (Fig. 3).

The apoptotic properties of the compounds are also confirmed using cell cycle analysis where they induce a significant increase in sub-G1 population by 44–58% compared to the untreated control. In both assays, (NHC)AuBr is the most potent in apoptosis induction. All compounds also affect the cell cycle distribution by slightly increasing the percentage of G2/M phase (Table 2). A similar pattern has been previously observed for auranofin-like triethylphosphine complexes.[31] On the contrary, studies with auranofin in MCF-7 and myeloma cells demonstrated a cell cycle arrest in G1-phase.[32] This suggests that the mechanism of the anticancer activity of the tested compounds may differ from that of auranofin.

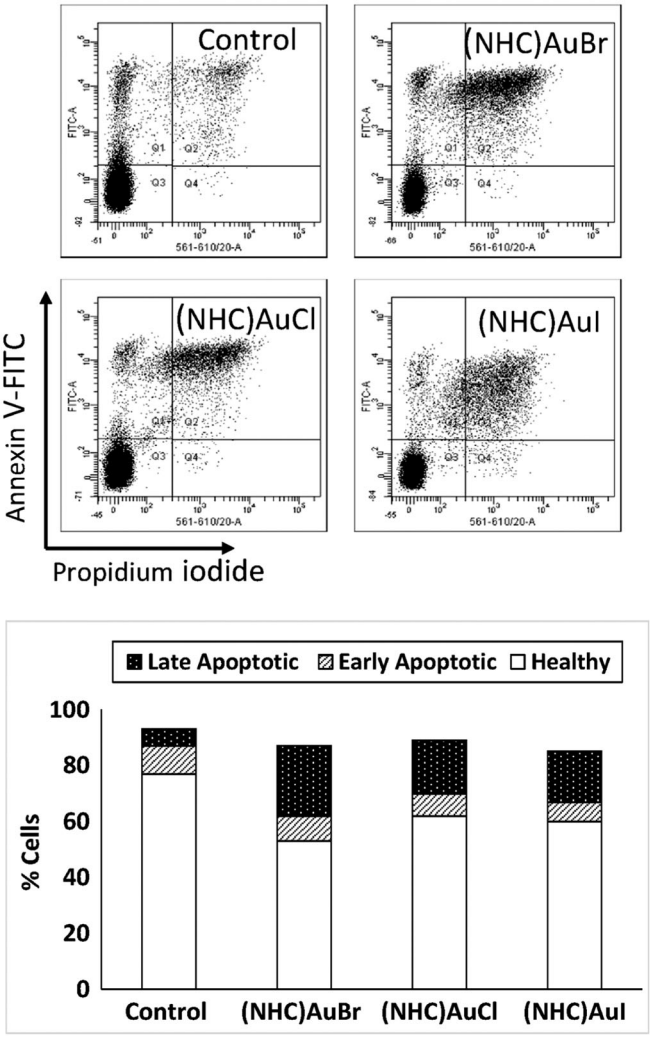

Figure 3. Flow cytometric analysis of apoptosis by annexin V-FITC/propidium iodide (PI) dual staining in CCRF-CEM cells following a 72 h incubation with (NHC)AuBr, (NHC)AuCl and (NHC)AuI. (a) Representative dot plots showing CCRF-CEM cell distribution. *Lower-left quadrant*: healthy (A−/PI−) cells; *upper-left quadrant*: early apoptotic (A+/PI−) cells; *upper-right quadrant*: late apoptotic/necrotic (A+/PI+) cells; *lower-right quadrant*: necrotic (A−/PI+) cells. (b) Relative frequency (percentage) of apoptotic cells in each sample.

Table 1. Cytotoxicity of the compounds in tumour cell lines

	IC_{50} (μM)[a]			
	HepG2	HL60	HeLa S3	CCRF-CEM
(NHC)AuBr	3.51 ± 0.17	1.54 ± 0.08	2.61 ± 0.12	1.54 ± 0.13
(NHC)AuCl	3.74 ± 0.18	1.40 ± 0.06	3.37 ± 0.15	1.25 ± 0.03
(NHC)AuI	7.73 ± 0.09	2.52 ± 0.10	4.85 ± 0.07	2.13 ± 0.18
Auranofin	7.08 ± 0.05	1.00 ± 0.14	1.82 ± 0.09	0.60 ± 0,02
Cisplatin	14.42 ± 1.93	5.33 ± 0.10	3.69 ± 0.03	5.02 ± 0.15

[a] Data expressed as means ± standard deviation from four experiments (XTT test).

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Table 2. Cell cycle effects of the compounds in CCRF-CEM cells following a 72-h incubation with (NHC)AuBr, (NHC)AuCl and (NHC)AuI - relative frequencies (percentage) of the cells in the respective cell cycle phases. SubG1 population refers to apoptotic cells or fragmented cells

% cells	G1/G0	S	G2/M	SubG1
(NHC)AuBr	45	44	11	7
(NHC)AuBr	38	38	24	65
(NHC)AuCl	39	43	18	51
(NHC)AuI	38	40	22	57

Conclusions

The complexes (NHC)AuX (X = Br for **2** or I for **3**) were obtained from a straightforward reaction of (NHC)AuCl (**1**) with an appropriate triorganotin(IV) halide and fully characterized using spectroscopic and structural methods. The cytotoxic properties of all three complexes were screened against a panel of human tumour cell lines including liver cancer (HepG2), cervical cancer (HeLa S3) and leukaemia (CCRF-CEM, HL-60) and compared with cisplatin and auranofin as reference compounds. Among the investigated complexes, the iodo derivative **3** was shown to be the least effective. The activities of chloro derivative **1** and bromo derivative **2** were generally superior to the activity of cisplatin and slightly less effective compared to auranofin, except for the HepG2 cells where auranofin was not as effective. Also, the apoptotic properties of **1**, **2** and **3** were assessed, revealing that the anticancer activity mechanism of the tested compounds may differ from that of auranofin. An improved formulation of the compounds (chemical derivatization, liposomal encapsulation, etc.), which may significantly increase the cytotoxic potential of the presented compounds, is currently being investigated.

Acknowledgments

The authors thank the Czech Science Foundation for financial support (project nos. P207/12/0223 and 14-31419S). The support of the Czech Ministry of Education, Youth and Sports (NPU project no. LO1302) is also acknowledged.

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