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In vivo investigations into the carbene gold anticancer drug candidates NHC*-Au-SCN and NHC*-Au-Scyclo

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ABSTRACT

The anticancer drug candidate 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene gold(I) thiocyanate (NHC*-Au-SCN) and its cyclohexane thiolate derivative (NHC*-Au-Scyclo) exhibited very good activity against human colon cancer with GI_{50} values against human HCT116 colon cancer cells of 0.40 and 1.65 μM, respectively. In addition, inhibition of the mammalian thioredoxin reductase (TrxR) was observed with IC_{50} values of 0.77 ± 0.34 μM for NHC*-Au-SCN and 13 ± 4 μM for NHC*-Au-Scyclo. This encouraged maximum tolerable dose (MTD) experiments in mice, where MTD values of 10 mg/kg for NHC*-Au-SCN and 30 mg/kg for NHC*-Au-Scyclo were determined with single injections to groups of 2 mice. In the subsequent tumor xenograft experiment NHC*-Au-SCN and NHC*-Au-Scyclo were applied three times at two doses in groups of 6 HCT116 tumor-bearing NMRI:nu/nu mice. The control group comprising 6 mice was treated with the solvent only. NHC*-Au-SCN at the dose of 5 and 10 mg/kg and NHC*-Au-Scyclo at the higher dose of 15 and 30 mg/kg showed tolerability towards the drugs, while no significant body weight loss was seen in both groups. NHC*-Au-SCN exerted only weak antitumoral activity reflected by T/C values of 0.81 and 0.65. The tumor volume growth reduction induced by NHC*-Au-Scyclo was better, with optimal T/C values of 0.58 and 0.31 being observed at doses of 15 mg/kg and 30 mg/kg, respectively. Alterations in dosing and/or application schedules might further improve the antitumoral activity, particularly for NHC*-Au-Scyclo.

KEYWORDS: carbene-gold anticancer drug, NCI 60 cancer cell panel, thioredoxin reductase, HCT116 colon cancer, xenograft mouse model.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide, with almost 1.4 million new cases diagnosed every year [1]. If not detected early and removed by surgery, these cancers have a reasonable number of chemotherapeutic treatment options like FOLFOX (leucovorin, 5-FU, and oxaliplatin) or FOLFIRI (leucovorin, 5-FU, and irinotecan). New targeted compounds like Bevacizumab, Ramucirumab, Cetuximab or Panitumumab and others give a certain amount of hope to patients with advanced colorectal cancer, since these compounds can block the vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) pathways. Nevertheless, one out of three colorectal cancer

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patients dies in countries with advanced treatment options within five years [2]. These clinical facts suggest that new therapeutic regimens must be explored in the quest to develop an effective therapy for these metastatic or advanced forms of colorectal cancer.

There is significant unexplored space for chemotherapeutic coinage metal-based drugs [3] targeting difficult-to-treat cancers. Already in 2008 a publication by Youngs suggested that carbene-silver acetato complexes derived from 4,5-dichloro-imidazole may have the stability and antitumoral activity to become anticancer drug candidates [4]. The idea was further pursued and led to the development of more lipophilic benzyl-substitute dimidazole- and benzimidazole-derived carbene-silver, -gold, and -ruthenium complexes showing activity against the human renal cancer line CAKI-1 [5-23]. So far, the most promising derivative 1-methyl-3-(*p*-cyanobenzyl)benzimidazole-2-ylidene silver(I) acetate (SBC1) showed activity against CAKI-1 cells with an IC_{50} value of 1.2 μM [20], which is superior when compared to cisplatin. Due to its lipophilicity and suitable shape, the anticancer drug candidate SBC1 binds well to albumin and interacts with DNA *in vitro*, but failed to show an antitumoral effect *in vivo* [24]. However, further synthesis led to 1,3-di(*p*-methoxybenzyl)-4,5-di (*p*-isopropylphenyl)-imidazol-2-ylidene copper(I) bromide (WBC4), which shows nanomolar activity with an IC_{50} value of 0.65 μM against CAKI-1 cells [25] and a significant antitumoral effect against renal-cell cancer *in vivo* [26]. Recently, we published the antitumoral effects of 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene gold(I) chloride(NHC*-Au-Cl) and its 2',3',4',6'-tetra-O-acetyl-beta-D-glucopyranosyl-1'-thiolate derivative (NHC*-Au-SR) [11]; both compounds showed good activity against the renal cell line CAKI-1 with GI_{50} values of 1.74 μM and 2.00 μM *in vitro* and significant activity in a xenograft CAKI-1 mouse model *in vivo* [27, 28].

The present study investigates the anti-proliferative effect of further two carbene-gold anticancer drug candidates; gold derivatives are known for showing an interesting apoptosis mechanism targeting mitochondria [29] *via* thioredoxin reductase (TrxR) inhibition [30]. Thus, 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene gold(I) thiocyanate (NHC*-Au-SCN) and its cyclohexane thiolate derivative (NHC*-Au-Scyclo) exhibited very good activity against human colon cancer with GI_{50} values against HCT116 cells of 0.40 and 1.65 μM, respectively [31]. NHC*-Au-SCN and NHC*-Au-Scyclo were tested as TrxR inhibitors *in vitro* as well as for their activity and toxicity in a HCT116 colon cancer xenograft mouse model *in vivo*. The structures of NHC*-Au-SCN, NHC*-Au-Scyclo, NHC*-Au-Cl and NHC*-Au-SR are shown in Figure 1.

MATERIALS AND METHODS

Synthesis of NHC*-Au-SCN and NHC*-Au-Scyclo

NHC*-Au-SCN and NHC*-Au-Scyclo were freshly prepared from the corresponding NHC*-Au-Cl precursor as reported earlier [31].

Inhibition of mammalian thioredoxin reductase

To determine the inhibition of mammalian TrxR an established microplate reader-based assay was performed. For this purpose, commercially available rat liver TrxR (from Sigma-Aldrich) was used and diluted with distilled water to achieve a concentration of 3.58 U/mL. The compounds, NHC*-Au-SCN and NHC*-Au-Scyclo, were freshly dissolved as stock solutions in dimethylformamide (DMF). 25 μL aliquots of the enzyme solution and 25 μL of either potassium phosphate buffer pH 7.0 containing the compounds in graded concentrations or 25 μL buffer without compounds but DMF (positive control) were added. 50 μL of a blank solution (DMF in buffer) was also prepared (final concentrations of DMF: 0.5% V/V). The resulting solutions were incubated with moderate shaking for 75 min at 37 °C in a 96-well plate. To each well, 225 μL warm reaction mixture (1 mL reaction mixture consists of 500 μL potassium phosphate buffer pH 7.0, 80 μL ethylenediamine tetraacetate (EDTA) solution (100 mM, pH 7.5), 20 μL bovine serum albumin (BSA) solution (0.2%), 100 μL of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) solution (20 mM) and 300 μL distilled water) were added and the reaction started immediately by addition of 25 μL of 20 mM ethanolic solution of 5,5'-dithio-bis-[2-nitrobenzoic acid] (DTNB). After proper mixing, the formation of 5-TNB

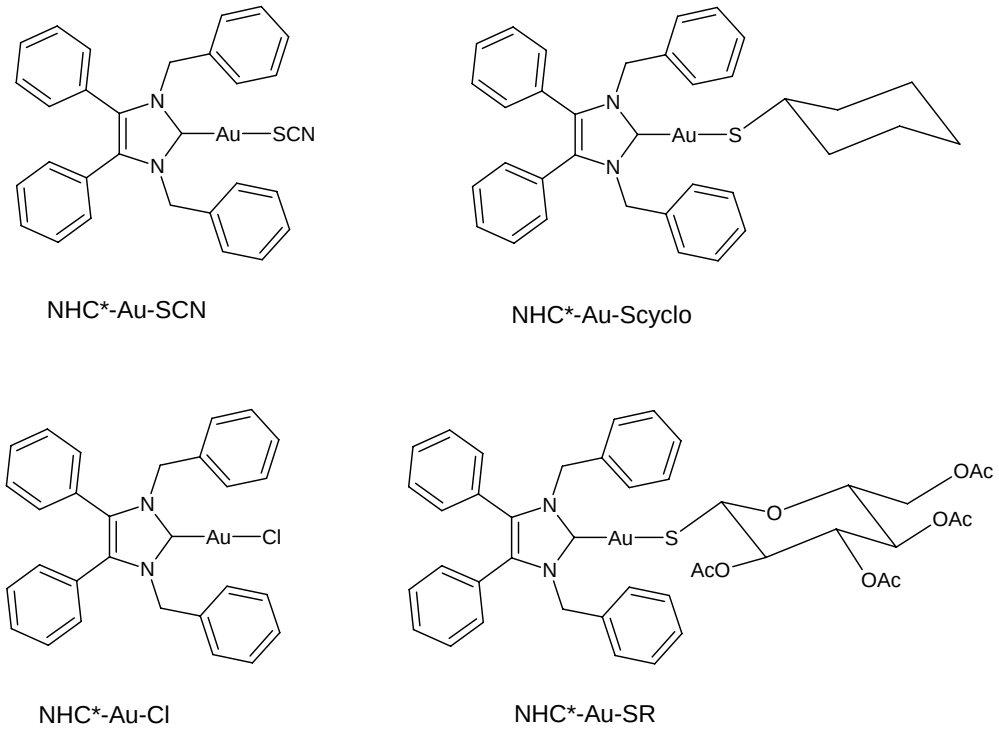

Figure 1. Molecular structure of the carbene-gold compounds NHC*-Au-SCN, NHC*-Au-Scyclo, NHC*-Au-Cl and NHC*-Au-SR.

was monitored with a microplate reader at 37 °C at 405 nm for about 6 min (10 times in 35 s intervals). The increase in 5-TNB concentration over time followed a linear trend ($r^2 \geq 0.990$), and the enzymatic activities were calculated as the slopes (increase in absorbance per second) thereof. For each tested compound, the noninterference with the assay components was confirmed by a negative control experiment using an enzyme-free test solution. The IC_{50} values were calculated as the concentration of compound decreasing the enzymatic activity of the untreated control by 50% and are given as the means and error of three repeated experiments.

HCT116 human colon carcinoma xenograft model

The *in vivo* experiments were performed at EPO GmbH Berlin in a single study. All animals were received from Janvier Labs; housing and animal care was in accordance to all legal and ethical regulations. In the first animal experiment the maximum tolerable dose (MTD) of NHC*-Au-SCN and NHC*-Au-Scyclo was determined in female NMRI:nu/nu mice. Both compounds were dissolved in dimethylsulfoxide (DMSO) (final concentration 10%) and further diluted with 5% Tween 80 in saline. Female NMRI:nu/nu mice (n = 2 mice per group) were administered with 2.5, 5, 10, 20, 30 and 40 mg/kg of NHC*-Au-SCN or NHC*-Au-Scyclo intraperitoneally (i.p.) in single injections of 0.1 ml in order to determine the approximate MTD. In this experiment maximum body weight loss and side effects were documented.

In the second *in vivo* experiment 1 x 10^7 HCT116 cells (expanded *in vitro* in RPMI medium + 10% fetal bovine serum) were injected subcutaneously (s.c.) in a volume of 0.1 ml to female NMRI:nu/nu mice (n = 6 mice per group) on day 0. When tumors were grown to a palpable size of around 0.1 cm^3 mice were randomised and treatment was initiated on day 9. For groups B and C the experimental anticancer drug NHC*-Au-SCN and for groups D and E NHC*-Au-Scyclo were injected into mice i.p. at doses of 5 or 10 mg/kg (group B/C) and 15 or 30 mg/kg (groups D/E) on days 9, 13, and 17, while the control group (group A) of mice was treated with the solvent

only. Tumor volumes (TV) were measured with a caliper instrument at indicated time points and calculated using the formula: TV = (width^2 x length)/2. Tumor volumes, relative tumor volumes (relation to the first treatment day) and treated to control (T/C) values were calculated. Body weight and health conditions of the mice were determined continuously during the experiments to estimate tolerability of the drug. Mice were sacrificed 7 days after their last treatment and necropsy was performed for evaluation of side effects.

The animal experiments were performed according to the German Animal Protection Law and with approval from the responsible local authorities (LaGeSo Berlin, Germany). The *in vivo* procedures were consistent and in compliance with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines.

Statistical analysis

Statistical evaluation of all experiments was performed using the one-way anova test and Bonferroni-correction. The level of statistical significance was defined with a p-value of $p \leq 0.05$.

RESULTS AND DISCUSSION

NHC*-Au-SCN and NHC*-Au-Scyclo effects on the inhibition of mammalian thioredoxin reductase

The inhibition of mammalian TrxR through NHC*-Au-SCN is significant and reaches an IC_{50} value of 0.77 ± 0.34 μM, while in NHC*-Au-Scyclo the inhibition effect was weaker and reaches an IC_{50} value of 13 ± 4 μM. This is significantly weaker than the inhibition of TrxR by Auranofin (Triethylphosphino gold(I) 2,3,4,6-tetra-O-acetyl-alpha-D-glucopyranosyl-1-thiolate) with an IC_{50} value of 90 nM [32], but compares well to other NHC-Au(I)-X complexes, which have typical values in the single digit micromolar region [33].

NHC*-Au-SCN and NHC*-Au-Scyclo mediated growth inhibition on HCT116 xenograft tumors

In the mouse experiment for the determination of MTD female NMRI:nu/nu mice (n = 2 mice per group) were treated with single doses of NHC*-Au-SCN or NHC*-Au-Scyclo ranging from 2.5-40 mg/kg. In general, both compounds were tolerated well by the animals. For NHC*-Au-SCN, doses of up to 15 mg/kg were tolerated without body weight loss, while for NHC*-Au-Scyclo the dosage could be extended to 40 mg/kg without generation of side effects for the animals. From these investigations doses of 5 and 10 (NHC*-Au-SCN) or 15 and 30 (NHC*-Au-Scyclo) mg/kg were derived as well-tolerated doses for further therapeutic experiment with an extended treatment period for the tumor-bearing mice.

In the HCT116 s.c. tumor xenograft experiment all tumors grew progressively and the tumors reached a palpable size of around 0.1 cm^3 on day 9. Therefore, five groups of 6 mice each were treated at days 9, 13 and 17 intraperitoneally with the solvent (group A), NHC*-Au-SCN (groups B/C) or NHC*-Au-Scyclo solution (group D/E). One of the mice in the high dosage group E died on day 14, while another from group B died after the treatment period on day 21. The treated mice from groups B to E showed no significant body weight loss when compared to the mice of the untreated group A. Figure 2A shows the body weight development of all groups during the experiment, while dosing and application schedules of the HCT116 xenograft experiment are shown in Table 1.

As shown in Figure 2B, all treatment groups B-E showed a tumor volume increase after the first treatment on day 9 and reached mean tumor volumes of 0.417 cm^3 (group B), 0.333 cm^3 (group C), 0.300 cm^3 (group D) and 0.163 cm^3 (group E) on day 13, while the control cohort (group A) showed continued tumor growth and reached a significantly higher tumor volume of 0.514 cm^3. After that, treatments were given on day 13 and the tumors grew to mean tumor volumes of 0.749 cm^3 (group B), 0.565 cm^3 (group C), 0.449 cm^3 (group D) and 0.370 cm^3 (group E) on day 16, while the tumors in the control animals (group A) reached a higher tumor volume of 0.702 cm^3. After another injection on day 17 the tumors in the treated groups showed further increased volumes of 1.024 cm^3 (group B), 0.953 cm^3 (group C), 0.741 cm^3 (group D) and 0.696 cm^3 (group E) on day 20. However, for the

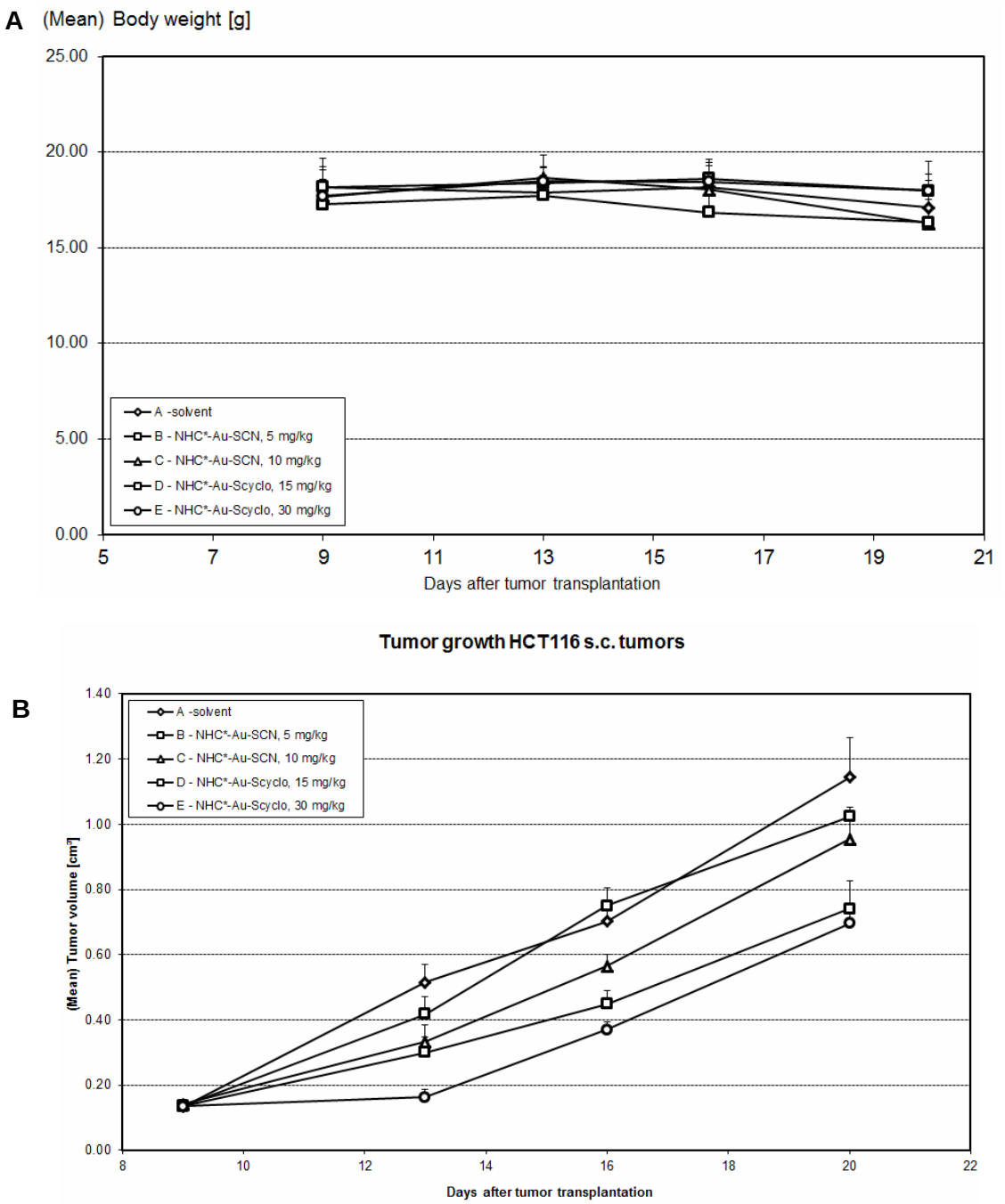

Figure 2. Influence of NHC*-Au-SCN and NHC*-Au-Scyclo treatments on body weight (A) and growth of HCT116 xenotransplant tumors (B) in NMRI nu/nu mice. The animals were treated with 5 mg/kg and 10 mg/kg of NHC*-Au-SCN and with 15 mg/kg and 30 mg/kg of NHC*-Au-Scyclo at days 9, 13 and 17. The therapeutic effects were determined by measuring the tumor volumes. The standard deviations are given as SEM.

control cohort (group A) a higher tumor volume of 1.145 cm^3 was measured. The treatment with NHC*-Au-SCN generated T/C values of 0.81 and 0.65 for groups B and C, both on day 13. The NHC*-Au-Scyclo treatment achieved optimal T/C values of 0.58 for the 15 mg/kg dose (group D) and 0.31 for the animals that received 30 mg/kg (group E), both on day 13.

Table 1. Overview of results obtained in the HCT116 xenograft experiment. Female NMRI nu/nu mice received subcutaneous tumor cell injections on day 0. Starting at palpable tumor size the mice were treated with NHC*-Au-SCN, NHC*-Au-Scyclo or the solvent at days 9, 13 and 17. Tumor size in the treated group in relation to the control group (T/C) was measured as a therapeutic marker, while the number of deaths and the body weight change were used as toxicity parameters.

Group	Number of mice	Substance	Treatment [on day]	Route	Dose (mg/kg)	Opt. T/C [on day]	Deaths [on day]
A	6	Solvent	9,13,17	i.p.			0/6
B	6	NHC*-Au-SCN	9,13,17	i.p.	5	0.81 [13]	1/6 [21]
C	6	NHC*-Au-SCN	9,13,17	i.p.	10	0.65 [13]	0/6
D	6	NHC*-Au-Scyclo	9,13,17	i.p.	15	0.58 [13]	0/6
E	6	NHC*-Au-Scyclo	9,13,17	i.p.	30	0.31 [13]	1/6 [14]

CONCLUSION

The experimental anticancer drug candidates NHC*-Au-SCN and NHC*-Au-Scyclo showed very good cytotoxic activity against the human colon cancer cell line HCT116 exhibiting GI_{50} values of 0.40 and 1.65 μM and significant inhibition of mammalian TrxR reaching IC_{50} values of 0.77 ± 0.34 μM and 13 ± 4 μM, respectively. This encouraged further preclinical development by testing the antitumoral activities in xenografted HCT116 tumors in mice. From the first *in vivo* experiments using non-tumor bearing mice and single injections, MTD values of 10 mg/kg and 30 mg/kg were determined for NHC*-Au-SCN and NHC*-Au-Scyclo. In the therapeutic xenograft experiment using HCT116 tumor-bearing mice these MTD values proved to be (moderately) effective and of tolerable toxicity. When exposed to 3 MTD dosages of 10 or 30 mg/kg of NHC*-Au-SCN or NHC*-Au-Scyclo, respectively, only one mouse in the NHC*-Au-Scyclo cohort died during the treatment on day 14, while a moderate T/C value of 0.65 was reached on day 13 for NHC*-Au-SCN and a good T/C value of 0.31 for NHC*-Au-Scyclo on day 13. Such activity compares well with other gold-based drugs tested *in vivo* recently [34-36]. This treatment demonstrated that NHC*-Au-Scyclo has a useable therapeutic index probably in an optimised formulation.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a financial interest in developing NHC*-Au-SCN and NHC*-Au-Scyclo into anticancer drugs.

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