Titanocene–Gold Complexes Containing N-Heterocyclic Carbene Ligands Inhibit Growth of Prostate, Renal, and Colon Cancers in Vitro

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ABSTRACT: We report on the synthesis, characterization, and stability studies of new titanocene complexes containing a methyl group and a carboxylate ligand (mba = −OC(O)−p-C6H4−S−) bound to gold(I)−N-heterocyclic carbene fragments through the thiolate group: [(η5-C5H5)2TiMe(μ-mba)Au(NHC)]. The cytotoxicities of the heterometallic compounds along with those of novel monometallic gold−N-heterocyclic carbene precursors [(NHC)Au(mbaH)] have been evaluated against renal, prostate, colon, and breast cancer cell lines. The highest activity and selectivity and a synergistic effect of the resulting heterometallic species was found for the prostate and colon cancer cell lines. The colocalization of both titanium and gold metals (1:1 ratio) in PC3 prostate cancer cells was demonstrated for the selected compound 5a, indicating the robustness of the heterometallic compound in vitro. We describe here preliminary mechanistic data involving studies on the interaction of selected mono- and bimetallic compounds with plasmid (pBR322) used as a model nucleic acid and the inhibition of thioredoxin reductase in PC3 prostate cancer cells. The heterometallic compounds, which are highly apoptotic, exhibit strong antimigratory effects on the prostate cancer cell line PC3.

INTRODUCTION

The potential of heterometallic complexes as cancer chemotherapy has been recently highlighted.1 The improved activity of heteronuclear complexes as antitumor agents by incorporation of two different cytotoxic metals within the same molecule has been demonstrated. The beneficial influence may be due to a synergistic or cooperative effect. Bimetallic and trimetallic compounds with anticancer properties have been described. There have been reports on titanocenes incorporating Ru(II), Pt(II), and Pd(II) centers2,3 and a number of complexes containing ferrocene moieties and other metals.4 Bimetallic systems based on Ru(II)−Pt(II)5 or Ru(II)−Ir(III)6 have also been described. Ferrocenyl phosphanes were incorporated in the iminophosphorane skeleton of gold(III) and palladium(II) coordination complexes.4 Heterometallic compounds based on gold(I) fragments have been reported for titanocene,1,3,8 ruthenium(II),9−12 platinum(II),13 rhenium−(I),14 and copper(II)15 derivatives.

We have reported on a number of titanocene−gold derivatives with potential as anticancer agents (zero-, first-, and second-generation derivatives 1−3 in Chart 1).1,3,8 We described cytotoxic species in which gold fragments coordinate to cyclopentadienyl−phosphane ligands that displayed a synergistic effect (such as 1 in Chart 1).3 In order to improve...
stability in physiological media and prevent the loss of cyclopentadienyl–gold fragments, first-generation derivatives (such as 2) were developed. While Ti–Cp hydrolysis still occurs at pH 7, the gold fragment remains linked to titanium by the carboxylate–phosphane ligand. These first-generation compounds showed excellent activity against renal cancer cell lines. In addition, the compounds were more selective toward first-generation such as C5H5)2TiMe(OC(O)C5H5)2 (such as 23) were developed. While Ti–Cp hydrolysis still occurs at pH 7, the gold fragment remains linked to titanium by the carboxylate–phosphane ligand. These first-generation complexes displayed strong antimitotic effects,15,16 and have demonstrated excellent inhibitory properties of certain enzymes such as thioredoxin reductase. They have also relevant anticancer effects in vitro15-25 (including some recent examples on heterometallic complexes9,10), and in vivo effects on melanoma have recently been described.26 We report here on the synthesis, characterization, and stability studies of the novel monometallic gold–N-heterocyclic carbene precursors [NHC]Au(mbaH)] and heterometallic titanocene complexes of the type [(η5-C5H5)2TiMe(μ-mba)Au(PR3)] (sec-triangular faces of two neighboring units (Figure 2)) with Z = 4 formula units in the unit cell. The thiol group on the bifunctional ligand H2mba (1 equiv) was deprotonated by reaction with 1 equiv of KOH for 20 min at room temperature. Subsequent addition of 1 equiv of the gold(I) N-heterocyclic carbene complexes a–d in situ, in a mixture of ethanol and water (4/1), led to the formation of the corresponding monometallic gold complexes [Au(NHC)-Hmba] (NHC = SPr (4a), IPPr (4b), IMes (4c), ICy (4d)). Compounds 4a–d were isolated as pale orange solids in high yield and characterized by NMR and UV–vis spectroscopy, mass spectrometry, and elemental analysis (see the Experimental Section). The chemical shifts of the carbene carbons in the new derivatives (13C{1H} NMR spectra) appear at fields lower (~10 ppm) than those reported for the parent.

## RESULTS AND DISCUSSION

### Synthesis and Characterization

The synthesis of the new monometallic gold(I)–NHC compounds bearing the bifunctional ligand Hmba (Scheme 1) was carried out following the same strategy reported for the synthesis of species [Au(Hmba)(phosphane)].

The thiol group on the bifunctional ligand H2mba (1 equiv) was deprotonated by reaction with 1 equiv of KOH for 20 min at room temperature. Subsequent addition of 1 equiv of the gold(I) N-heterocyclic carbene complexes a–d in situ, in a mixture of ethanol and water (4/1), led to the formation of the corresponding monometallic gold complexes [Au(NHC)-Hmba] (NHC = SPr (4a), IPPr (4b), IMes (4c), ICy (4d)). Compounds 4a–d were isolated as pale orange solids in high yield and characterized by NMR and UV–vis spectroscopy, mass spectrometry, and elemental analysis (see the Experimental Section). The chemical shifts of the carbene carbons in the new derivatives (13C{1H} NMR spectra) appear at fields lower (~10 ppm) than those reported for the parent chloro derivatives a–d,27,28 indicating a smaller electron-donating character of the Hmba ligand (σ donor) with respect to the chloride ligand (σ and π donor).

In the case of compound 4c, crystals suitable for X-ray diffraction (Figure 1) were obtained by layering n-pentane over a solution of compound 4c in tetrahydrofuran.

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**Figure 1.** ORTEP view of the molecular structure of 4c showing the labeling scheme. The labels for hydrogen and some carbon atoms are omitted for clarity. A drawing of the molecular structure containing all labeled carbon atoms is provided in the Supporting Information.

The crystals of compound 4c were determined to be triclinic (space group P1) with Z = 4 formula units in the unit cell. The environment of the gold atoms is close to linear (C–Au–S 177.76(18)°) (Figure 1). A selection of structural parameters is given in Table 1. The individual monomeric units (Figure 1) show hydrogen bonds (~1.83 Å) between the carboxyl groups of two neighboring units (Figure 2).

### Table 1. Selected Structural Parameters of Complex 4c Obtained from X-ray Single-Crystal Diffraction Studies

<table>
<thead>
<tr>
<th>Bond</th>
<th>Distance (Å)</th>
</tr>
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<tbody>
<tr>
<td>Au–C(1)</td>
<td>1.995(6)</td>
</tr>
<tr>
<td>Au–S(1)</td>
<td>2.2790(17)</td>
</tr>
<tr>
<td>S(1)–C(1)</td>
<td>1.746(6)</td>
</tr>
<tr>
<td>C(1)–N(1)</td>
<td>1.353(7)</td>
</tr>
<tr>
<td>C(1)–N(2)</td>
<td>1.341(7)</td>
</tr>
<tr>
<td>C(1)–Au(1)</td>
<td>1.335(9)</td>
</tr>
<tr>
<td>C(1)–Au(1)–S(1)</td>
<td>177.76(18)</td>
</tr>
<tr>
<td>C(1)–Al(1)–Au(1)</td>
<td>105.92(19)</td>
</tr>
<tr>
<td>C(1)–N(1)–C(2)</td>
<td>111.4(5)</td>
</tr>
<tr>
<td>C(1)–N(1)–C(3)</td>
<td>111.1(5)</td>
</tr>
<tr>
<td>C(1)–N(2)–C(3)</td>
<td>123.4(5)</td>
</tr>
<tr>
<td>C(2)–N(2)–C(3)</td>
<td>124.8(5)</td>
</tr>
<tr>
<td>N(2)–C(1)–N(1)</td>
<td>104.2(5)</td>
</tr>
</tbody>
</table>

*Bond lengths are given in Å and angles in deg.*
mononuclear gold(I) complexes $4a$–$d$ with 1 equiv of ($\eta^5$-C$_5$H$_5$)$_2$TiMe$_2$ afforded the corresponding heterometallic complexes [(($\eta^5$-C$_5$H$_5$)$_2$TiMe($\mu$-mba)Au(NHC))] (NHC = SIPr ($5a$), IPr ($5b$), IMes ($5c$), ICy ($5d$)), with concomitant elimination of 1 equiv of methane. Compounds $5a$–$d$ were obtained in moderate to high yields as air- and moisture-stable yellow solids. These compounds are less acidic than tincocene dichloride and soluble in DMSO/H$_2$O, DMSO/PBS, or DMSO/media (1/99) mixtures at micromolar concentrations, which is relevant for subsequent biological testing. Moreover, they are more soluble at higher concentrations in DMSO/PBS mixtures than the previously described [(($\eta^5$-C$_5$H$_5$)$_2$TiMe($\mu$-mba)Au(PR$_3$))] counterparts. Compounds $5a$–$d$ are stable as solids in air and at 5 °C for months and in CDCl$_3$ solution for at least 3 days. They are stable in DMSO-$d_6$ solution for weeks.

The structures of complexes $5a$–$d$ depicted in Scheme 1 have been proposed on the basis of NMR and UV–vis spectroscopy, mass spectrometry, and elemental analysis (see the Experimental Section). Moreover, IR experiments and DFT calculations were carried out in order to shed light on the coordination mode of the carboxylate groups. The differences found between the symmetric and antisymmetric stretching bands for the carboxylate groups in the solid state IR spectra (ranging from 210 to 351 cm$^{-1}$) are greater than 200 cm$^{-1}$, indicating a monodentate coordination mode. DFT calculations (e.g., Figure 3) also confirmed the monodentate nature of the carboxylate functionality. In the Supporting Information, data on different optimizations are provided. All calculations performed led to the species containing a monodentate carboxylate. Similar difference values (ranging from 200 to 328 cm$^{-1}$) were found between the symmetric and antisymmetric stretching bands for the carboxylate groups in the IR calculated on the basis of the DFT studies.

The stability of compounds $5a$–$d$ was evaluated by $^1$H NMR spectroscopy in DMSO-$d_6$ and DMSO-$d_6$/PBS-D$_2$O (3/2) and by mass spectrometry over time (see the Supporting Information). NMR experiments were performed in DMSO-$d_6$ and in DMSO-$d_6$/PBS-D$_2$O mixtures. The stability study of compounds $5a$–$d$ by $^1$H NMR in DMSO-$d_6$ showed half-life values of 1, 3, 2, and 2 h, respectively; i.e., shorter than those for the corresponding phosphane derivatives$^3$ (3, Chart 1). However, as shown in Table 2, compounds $5a$–$d$ exhibited longer half-lives in 3/2 of DMSO-$d_6$/PBS-D$_2$O mixtures. Tincocene dichloride is also known to hydrolyze with a higher rate in DMSO than in water.$^2$ Mass spectrometry further supports the presence of species containing both titanium and gold in 1% DMSO/PBS solution after 24 h (see the Supporting Information).

**Biological Activity. Assays of Cytotoxicity and Cell Death.** The cytotoxicity of the heterometallic complexes [(($\eta^5$-C$_5$H$_5$)$_2$TiMe($\mu$-mba)Au(NHC))] (NHC = SIPr ($5a$), IPr ($5b$), IMes ($5c$), ICy ($5d$)), monometallic gold(I) complexes [Au(NHC)(Hmba)] (NHC = SIPr ($4a$), IPr ($4b$), IMes ($4c$), ICy ($4d$) in Scheme 1), and tincocene Y$^{33}$ used as control was assayed by monitoring their ability to inhibit cell growth using the PrestoBlue Cell Viability assay (see the Experimental Section). The cytotoxic activity of the compounds was determined as described in the Experimental Section. In this assay, human cancer cell lines such as prostate PC3 and DU145, renal Caki-1, colon DLD1, triple negative breast MDA-MB-231, and nontumorigenic human embryonic kidney cell lines HEK-293T were incubated with the indicated compound for 72 h. The results are summarized in Table 3.

The heterometallic compounds are considerably more toxic to the prostate cancer cell lines (PC3 and DU145) and the colon cancer cell line (DLD1) than tincocene Y. In addition, the heterometallic compounds $5a$–$d$ are more toxic in all the cell lines (excluding the triple negative breast cancer cell lines)
than the monometallic gold compounds 4a–d on these cells. None of the heterometallic or monometallic gold compounds are toxic in the triple negative breast MDA-MB-231 cancer cell lines in concentrations lower than 100 μM, as opposed to titanocene Y. While the heterometallic compounds are toxic on renal Caki-1 cancer cell lines, the IC50 values are only comparable to those for titanocene Y for compounds 5a,c. These IC50 values are larger than those found for the first-generation titanocene—gold compounds previously described by us3 and larger than the IC50 value of the second-generation compounds of the type [(η5-C5H5)2TiMe(μ-mba)Au(PR3)] (especially 3). In terms of selectivity, the heterometallic compounds exhibit selectivity for the cancer cell lines (excluding the triple negative breast cancer cell line, MDA-MB-231), with compound 5b having a better selectivity in comparison to nontumorigenic human embryonic kidney cell lines HEK-293T. The new compounds display a better selectivity toward the HEK-293T cell line than the phosphane [(η5-C5H5)2TiMe(μ-mba)Au(PR3)] derivatives described before.

We did not find a strong correlation between the type of NHC ligand employed and the biological activity.

Lysates of PC3 cells treated with 5a were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) to determine the colocalization and amounts of Au and Ti metals in these prostate cancer cell lines (see the Experimental Section for details). Lysate of untreated cells was employed as a control. It was observed that these cells have some basal levels of Au present (0.1 μg Au per mg cell protein). The cellular uptake of this compound increases with the increase in drug concentration in the media, indicating a dose-dependent uptake of compound 5a by PC3 cells (Figure 4).

Increasing the drug concentration from 10 to 20 μM resulted in a 2.6-fold increase in the cellular levels of compound 5a. More importantly, on correction for background levels of Au and Ti in cell lysates, the stoichiometric ratios of these elements were close to unity, suggesting that the compound remains stable in the intracellular environment after 72 h (or that at least uptake of both metals occurs concurrently).

We had found that the compound [(η5-C5H5)2TiMe(μ-mba)Au(PhP)]3 exerted cell death by inducing apoptosis.1 Titanocenes C, X, and Y are also known to induce apoptosis in different cancer cell lines.34 In order to gain some insight into the nature of the cytotoxicity of the new heterometallic compounds containing N-heterocyclic carbenes, we performed some experiments. The effect of titanocene Y, selected monometallic compounds 4a,b, and bimetallic 5a,b on necrosis and apoptosis on Caki-1 and PC3 was assessed by measuring protease activity using non-cell-permeable substrates and cell-permeable substrates and by measuring the total caspase-3 and -7 activities with the ApoTox-Glo triplex assay (see the Experimental Section). The effect of each treatment was determined by comparing treated and untreated cells after 72 h incubation. The results for each treatment were expressed as fold changes between nontreated (0.1% DMSO) and treated samples. ApoTox-Glo triplex assays were repeated twice (n = 2), and each repetition was run in quadruplicate.

The average of the four values was used for statistical calculations. The data (Figure 5) are presented as the mean ± the standard error of the mean.

### Table 3. IC50 Values (μM) in Human Cell Lines Determined with Heterometallic Ti–Au Compounds 5a–d, Monometallic Au Compounds 4a–d, and Titanocene Y as Control

<table>
<thead>
<tr>
<th>compound</th>
<th>PC3</th>
<th>DU-145</th>
<th>Caki-1</th>
<th>DLD1</th>
<th>MDA-MB-231</th>
<th>HEK-293T</th>
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<tr>
<td>titanocene Y</td>
<td>58.1</td>
<td>55.2</td>
<td>29.4</td>
<td>56.2</td>
<td>18.0</td>
<td>&gt;200</td>
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<tr>
<td>5a</td>
<td>9.8</td>
<td>11.8</td>
<td>21.0</td>
<td>13.9</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5b</td>
<td>10.3</td>
<td>18.9</td>
<td>51.5</td>
<td>30.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5c</td>
<td>17.1</td>
<td>13.76</td>
<td>29.11</td>
<td>19.9</td>
<td>&gt;100</td>
<td>69.7</td>
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<tr>
<td>5d</td>
<td>11.8</td>
<td>16.7</td>
<td>42.9</td>
<td>21.5</td>
<td>&gt;100</td>
<td>77.1</td>
</tr>
<tr>
<td>4a</td>
<td>66.3</td>
<td>74.8</td>
<td>81.4</td>
<td>78.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4b</td>
<td>70.4</td>
<td>60.9</td>
<td>79.2</td>
<td>82.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>4c</td>
<td>57.1</td>
<td>67.6</td>
<td>97.2</td>
<td>73.1</td>
<td>&gt;100</td>
<td>87.9</td>
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<tr>
<td>4d</td>
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<td>59.9</td>
<td>88.9</td>
<td>77.5</td>
<td>&gt;100</td>
<td>97.2</td>
</tr>
</tbody>
</table>

All compounds were dissolved in 1% of DMSO and diluted with water before addition to cell culture medium for a 72 h incubation period.

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**Figure 4.** Cellular uptake of compound 5a in PC3 cells. The concentrations of compound 5a calculated on the basis of Ti and Au content in the cell lysates are similar, suggesting that the compound is robust and that both elements are colocalized in the cells.
The effect of titanocenes on the migratory capability of cancer cells has been scarcely studied. We previously found that first-generation \( \left( \eta^5\text{C}_5\text{H}_5 \right)_2\text{Ti}\{\text{OC(O)}-p\text{-C}_6\text{H}_4-\text{PPh}_2\text{AuCl}\} \) and second-generation \( \left( \eta^5\text{C}_5\text{H}_5 \right)_2\text{TiMe}(\mu\text{-mba})\text{Au}(\text{PPh}_3) \) titanocene–gold complexes displayed relevant antimigratory properties. We evaluated the anti-invasive properties of the most active heterometallic complexes \( \left( \eta^5\text{C}_5\text{H}_5 \right)_2\text{TiMe}(\mu\text{-mba})\text{Au(NHC)} \) (NHC = SIPr (5a), IPr (5b)) and titanocene Y, by using the same wound-healing scratch assay (Experimental Section) on prostate cancer PC3 cell lines. Twenty-four hours following a scratch through an in vitro confluent monolayer of prostate carcinoma PC3, cells treated with 15 μM titanocene Y invaded 69% of the scratch and cells treated with 5 μM of 5a or 5b invaded 42% or 33% of the scratch, respectively, while cells treated with 0.1% DMSO control invaded 88% of the scratch (Figure 6A). Figure 6B shows a comparison in terms of total reduction of migration among compounds 5a–d and titanocene Y. A similar assay was performed with gold monometallic compounds 4a, b (see Figure S60 in the Supporting Information), and it was found that under the same conditions (in vitro confluent monolayer of prostate carcinoma PC3 cells treated with 15 μM of the gold monometallic compounds) 4a, b invaded 73% and 80% of the scratch, respectively.

We can conclude from this experiment that heterometallic compounds 5a–d possess antiinvasive properties in PC3 cells in comparison to the control and are twice as powerful in inhibiting migration as titanocene Y. Recently enantiopure cyclopentadienyl Ti(IV) oximate compounds have been shown to affect the cell adhesion and migration of PC3 cancer cell lines. In this case the compounds (50 μM) showed a migration capability (62–72% of wound healing) significantly lower than that of control cells. The heterometallic complexes described here (5a, b) thus have strong antiinvasive properties on PC3 cancer cell lines.

Interaction with Plasmid pBR322 DNA. We and others have previously found that titanocene–gold compounds interact weakly with calf thymus DNA or do not interact with plasmid pBR322 DNA, as is the case for many other gold compounds. Recent reports on titanocene dichloride and titanocene Y also indicate a weak interaction with DNA and the lack of suppression for DNA-processing enzymes. DNA interactions were tested with heterometallic compounds 5a–d or cisplatin by using plasmid (pBR322) DNA (Figure 7). This plasmid has two main forms: OC (open circular or relaxed form, form II) and CCC (covalently closed or supercoiled form, form I). Agarose gel electrophoresis assays were performed whereby decreased electrophoretic mobilities of both forms were taken as evidence of metal–DNA binding.

Figure 5. (A) Impairment of the viability of Caki-1 cells by compounds 4a (80 μM), 5a (20 μM), 4b (80 μM) 5b (50 μM), and titanocene Y (30 μM) by inducing apoptosis. (B) Impairment of the viability of PC3 cells by compounds 4a (60 μM), 5a (10 μM), 4b (70 μM), 5b (10 μM), and titanocene Y (30 μM) by inducing apoptosis.

Figure 6. Cell migration in 5a or 5b treated PC3 cells. Migration of PC3 cells was assessed using a wound-healing assay following treatment with 15 μM titanocene Y or 5 μM of 5a or 5b incubated for 24 h (values normalized against 0.1% DMSO control): (A) absolute migration (%); (B) reduction of migration (%).

Figure 7. Electrophoresis mobility shift assays for cisplatin and heterometallic Ti–Au compounds 5a–d (see the Experimental Section for details). DNA refers to untreated plasmid pBR322. a, b, c, and d correspond to metal/DNA bp ratios of 0.25, 0.5, 1.0, and 2.0, respectively.
Generally, the slower the mobility of supercoiled DNA (CCC, form I), the greater the DNA unwinding produced by the drug. For example, binding of cisplatin to plasmid DNA results in decreased mobility of the CCC form and increased mobility of the OC form. Treatment of plasmid DNA with increasing amounts of the new heterometallic compounds 5a–d did not affect the mobility of the faster-running supercoiled form (form I) even at the highest molar ratios (d). This result is in accordance with the lack of interaction shown by titanocene–gold compounds (zero, first, and second generation) and the lack of interaction displayed by monometallic gold compounds 4a–d (see Figure S59 in the Supporting Information).

**Inhibition of Thioredoxin Reductase in PC3 Cancer Cells.**

Many chemoresistant cancers produce changes in the cell antioxidant capacity. The overexpression of thioredoxin reductase (TrRx) is among the key defense and survival mechanisms of cisplatin-resistant cells. Thioredoxin reductase has become a potential target in cancer chemotherapy.43,44 We have reported on the inhibition of TrRx in Caki-1 cells by aurano and the heterometallic titanocene–gold complex [(η⁵-C₅H₅)₂TiMe(μ-mba)Au(PR₃)] (3). Since Au–NHC compounds are known to inhibit TrRx, we measured the activity of thioredoxin reductase in PC3 prostate cancer cells, following incubation with monometallic compounds [Au(NHC)(Hmba)] (NHC = SIPr (4a), IPr (4b)) and bimetallic compounds [(η⁵-C₅H₅)₂TiMe(μ-mba)Au(NHC)] (NHC = SIPr (5a), IPr (5b)). We found thioredoxin reductase activity to be lower in cells treated with 5 μM of 5a and 5b with observed inhibitions of 31% and 30%, respectively, after a 5 h incubation period (Figure 8). The inhibition was 61% (5a) and 76% (5b) after 24 h incubation. In the case of PC3 cells treated with 30 μM of monometallic gold compounds (4a,b) there was inhibition of thioredoxin reductase but to a lower extent (30% and 36% for 4a and 4b, respectively, after 24 h incubation). Surprisingly, we found that titanocene Y (15 μM) was also a strong TrRx inhibitor in PC3 cells (with a 57% or 80% reduction after 5 or 24 h of treatment, respectively, see Figure S61 in the Supporting Information). This experiment showed that the inhibition of TrRx is involved in the cell death mechanism of the new compounds and that the titanocene component has an influence on this target, although other cellular targets may not be excluded. In the past years, a number of other targets (such as glutathione reductase, cystine proteases such as cathepsins K and S, protein tyrosine phosphatases, glutathione peroxidase (GPx),44 iodothyronine deiodinase (ID),45 and IκB kinase) have been identified for gold(I) complexes. Helicases/topoisomerases and HIST1H4 core histones have been pointed out as targets of titanocene C, and we reported on the strong inhibitory effect of titanocene dichloride against PI3 protein kinases from a panel of 35 kinases of oncological interest. For titanocene–gold(I) heterometallic complexes, we have shown that the compound [(η⁵-C₅H₅)₂TiMe(μ-mba)Au(PR₃)] (3) not only inhibited TrRx in Caki-1 renal cancer cells with an IC₅₀ value very similar to that of Aurano but also was considerably more cytotoxic than aurano in this cell line due to a more potent inhibition of the specific protein kinases AKT, p90-RSK, and MAPKAPK3 in vitro. All of these results warrant further studies on the mode of action of the new heterometallic compounds.

![Figure 8. Thioredoxin reductase activity in 4a, 4b, 5a, or 5b treated PC3 cells: (A) activity of endogenous PC3 thioredoxin reductase from soluble whole cell lysates following incubation with 30 μM of 4a or 4b for 5 and 24 h (values normalized against DMSO control); (B) activity of endogenous PC3 thioredoxin reductase from soluble whole cell lysates following incubation with 5 μM of 5a or 5b for 5 h and 24 h (values normalized against DMSO control).](image)

**CONCLUSIONS**

In conclusion, we have described the preparation of novel heterometallic titanocene–gold compounds incorporating gold(I)–N-heterocyclic carbene fragments. The exchange of the phosphate ligands by NHC ligands (L) in complexes of the type [(η⁵-C₅H₅)₂TiMe(μ-mba)Au(L)] did result in lower IC₅₀ values in renal Caki-1 cancer cell lines, although a significant activity and a considerably higher selectivity with respect to noncancerous cell lines was obtained in prostate and colon cancer cell lines for the new Ti–Au–NHC complexes. As for the analogous titanocene–gold compounds containing phosphines, the new heterometallic carbene derivatives did not display a significant interaction with plasmid (pBR322) used as a model nucleic acid. Two selected compounds (5a,b) were found to be highly apoptotic and to inhibit TrRx in prostate PC3 cancer cell lines. These complexes also display strong antimigratory properties. The work presented here is the proof of concept that the substitution of PR₃−gold(I) by NHC–gold(I) fragments in titanocene–gold complexes may afford derivatives with potential as cancer chemotherapeutics which will allow for further modification. With the NHC ligands described in this work we did not find a strong SAR correlation. Further optimization of the NHC ligands employed and more detailed mechanistic studies are needed in order to find candidates with improved pharmacological properties. These studies are currently underway in our laboratories.
**EXPERIMENTAL SECTION**


Imidazolium salts ([IPrHCl][HCl])<sup>4d</sup> and [IPrCl][HCl]<sup>4d</sup> were prepared as previously reported. C<sub>24</sub>H<sub>44</sub>TiCl<sub>2</sub> and H[AuCl] were purchased from Strem Chemicals. Tetrahydrothiophene was purchased from Sigma-Aldrich.

**Reaction Solvents:**

Reaction solvents were purchased anhydrous from Fisher Scientific (ACS grade) and purified over molecular sieves (3 Å, 0.5 mm M4). Aliquots of the solvents were kept in a desiccator over silica gel, degassed by the freeze—pump—thaw method.

**H NMR spectra were recorded using Bruker AV400 (1H NMR at 400 MHz, 13C NMR at 125 MHz) at 0 ppm using tetramethylsilane as the solvent, unless otherwise stated.**1H and 13C NMR spectra (4000−1 MHz) were recorded using a Bruker AV400. Reaction solvents were kept over molecular sieves (3 Å, 0.5 mm M4) and purified by use of a PureSolv purification unit from Innovative Technology, Inc. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc., kept over molecular sieves (3 Å, 0.5 mm M4), and degassed by the freeze—pump—thaw method.

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**EXPERIMENTAL SECTION**


Imidazolium salts ([IPrHCl][HCl])<sup>4d</sup> and [IPrCl][HCl]<sup>4d</sup> were prepared as previously reported. C<sub>24</sub>H<sub>44</sub>TiCl<sub>2</sub> and H[AuCl] were purchased from Strem Chemicals. Tetrahydrothiophene was purchased from Sigma-Aldrich.

**Reaction Solvents:**

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Organometallics

Sd (NHC = ICy): 79% yield (0.25 g). Anal. Calcld for C5,H12,N2: C, 50.01; H, 6.19; N, 20.00. The NMR (CDCl3; δ 7.96 (s, 4H, CH2), 7.12-7.16 (2H, 1-CH2), 7.80 (d, 2H, ArH), 8.00 (d, 2H, ArH)) was used to calculate the solvent disorder area and remove its contribution to the residual peak. The program SQUEEZE, a modified version of the SHELXTL suite, was used to refine the structure. The calculations have been performed using the SHELXTL suite of programs.

X-ray Crystallography. Suitable single crystals of compound 4c were obtained by slow evaporation of a solution of the compound in a mixture of chloroform and methanol. The crystals were analyzed using an X-ray diffractometer equipped with a graphite monochromator. The data were collected at 100 K using Mo Kα radiation (λ = 0.71073 Å). The structure was solved using direct methods and refined by least-squares techniques on weighted F² values for all reflections (SHELXTL, 6.14). All non-hydrogen atoms were assigned anisotropic displacement parameters and refined without positional constraints. All hydrogen atoms were calculated using a riding model. Complex neutral-atom scattering factors were used.

Biological Effects. The biological effects of the compounds were studied in vitro using a cell line derived from human prostate cancer. The cell line was cultured in a humidified incubator at 37 °C in 5% CO₂. The cells were treated with increasing concentrations of the compounds and the viability was determined using an MTS assay. The IC₅₀ (μM) value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean ± SEM of at least two independent experiments each with triplicate measurements.

Cell Death for Titanocene Y, 4a, and 5a. For apoptosis, viability, and necrosis assays, the Caki-1 and PC3 cells were seeded in 96-well opaque-walled tissue culture plates with clear bottoms (Thermo Scientific Nunc; Somerset, NJ) at an initial density of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. For evaluation of cell viability, cells were seeded at a concentration of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. For evaluation of cell viability, cells were seeded at a concentration of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. For evaluation of cell viability, cells were seeded at a concentration of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. For evaluation of cell viability, cells were seeded at a concentration of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator. For evaluation of cell viability, cells were seeded at a concentration of 5 × 10⁴ cells/well in 90 μL of DMEM or RPMI without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLite MicroWell Plate, Fisher Scientific, Waltham, MA) and grown for 24 h at 37 °C under 5% CO₂ and 95% air in a humidified incubator.
for 1 h. Luminescence was measured for 1 s and is proportional to the amount of caspase activity present (BioTek U.S., Winooski, VT). The results for each treatment were expressed as fold change between nontreated (0.1% DMSO) and treated samples. ApoTox-Glo triplex assays were repeated twice (n = 2), and each repetition was run in quadruplicate. The average of the four values was used for statistical calculations. The data are presented as the mean values.

In Vitro Migration Assay (Wound Healing Assay). For the assessment of cell migration, confluent PC3 cells maintained in standard medium were wounded with a plastic micropipette tip (tip 20–200 μL). After washing, the medium was replaced by fresh medium containing 5 μM of either 5a or 5b, 15 μM of titancene Y, and 15 μM of 4a or 4b or 0.1% DMSO solution (control). Photographs of the wounded area were taken after 0 and 24 h using phase-contrast microscopy. For evaluation of wound closure, four randomly selected points along each wounded area were marked and the horizontal distance of migrating cells from the initial wound was measured (Labomed TCM400 Inverted Phase Microscope Series, equipped with a digital camera (Fisher Scientific Moticam 10). The assays were done twice, and for each trial two images were analyzed per time point.

Method for Thioredoxin Reductase Activity Assay. Whole cell lysates was assayed using PC3 cells treated in vitro with 5 μM of 5a or 5b, 15 μM of titancene Y, 30 μM of 4a or 4b, or 0.1% DMSO solution (control). After 5 or 24 h of treatment, cells were washed three times in PBS and lysed by douncing using scrapers and sheer force through a syringe with a 34 gauge in assay buffer (Abcam Thioredoxin Reductase Assay Kit, ab83463) with 1 mM Protease Inhibitor Cocktail (Abcam, ab65621). The lysates were centrifuged at 10000 rcf for 15 min at 4 °C to isolate insoluble material. The total protein concentrations of soluble lysates were measured using the Bradford assay. The soluble lysates were incubated for 20 min in assay buffer or assay buffer with a proprietary thioredoxin reductase specific inhibitor before adding a specific substrate, DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), and measuring the activity at 1 min intervals for 30 min using a BioTek Fluorescence Microplate Reader (BioTek U.S., Winooski, VT) at λ 412 nm. Lysates were tested in duplicate. TrxR activity was calculated on the basis of the linear amount of TNB (2-nitro-S-thio benzoic acid) produced per minute per milligram of total protein and adjusted for background activity from enzymes other than TrxR in the lysates.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.organomet.6b00051.

NMR, IR and UV–vis spectra of all new compounds, MS ESI+ spectra of all compounds and theoretical isotopic distributions of relevant peaks, DFT calculations for all new compounds, crystallographic data for 4c, interaction of monometallic gold compounds 4a–d with plasmid pBR322 DNA, migration assays with compounds 4a,c, and inhibition of thioredoxin reductase (TrxR) studies of titancene Y at S and 24 h (PDF)

Crystallographic data for 4c (CIF)

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Notes
The authors declare no competing financial interest.

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REFERENCES


