European Journal of Medicinal Chemistry 46 (2011) 5927–5934

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and biological studies of silver *N*-heterocyclic carbene complexes derived from 4,5-diarylimidazole

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ARTICLE INFO

Article history:
Received 21 April 2011
Received in revised form
26 September 2011
Accepted 2 October 2011
Available online 7 October 2011

Keywords:
Silver *N*-heterocyclic carbene complexes
Tumor cell growth inhibition
Estrogen receptor binding
COX inhibition
Antibacterial properties

ABSTRACT

A novel class of silver *N*-heterocyclic carbene complexes (**5a**–**f**) were synthesized in high yield by reacting silver(I) oxide with 4,5-diarylimidazolium halides (**4a**–**f**). The complexes were characterized using NMR and IR spectroscopy. The structure was confirmed on the example of bromo[1,3-diethyl-4,5-bis(4-fluorophenyl)imidazol-2-ylidene]silver(I) (**5c**) by crystal structure analysis. The X-ray structure indicated a three-dimensional coordination polymer with a repeating unit consisting of a C_{carben}-Ag_2-Br_2-C_{carben} cluster. Pharmacological investigations revealed that all silver complexes possessed growth inhibitory effects against breast cancer (MCF-7 and MDA-MB-231) as well as colon carcinoma (HT-29) cells. The most active compound **5c** was slightly less active against MCF-7 cells, more active against MDA-MB-231 cells and comparable active as cisplatin against HT-29 cells. Further pharmacological investigations were performed with selected compounds on estrogen receptor (ER) binding, DNA intercalation, cyclooxygenase (COX) inhibition and antibacterial activity. The complexes were only marginally active at the DNA, ER and the COX enzymes, so these targets can be excluded to be involved in the mode of action. However, the growth of bacteria was significantly inhibited by **5c** and **5f** and opens a new application of this complex type.

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1. Introduction

Platinum-based drugs such as cisplatin or oxaliplatin are widely used in the treatment of cancer. A great disadvantage is the possible development of resistance during the therapy. Therefore, many groups focus their attention on the design of platinum complexes for the treatment of tumors with intrinsic (e.g the mammary carcinoma) or acquired resistance [1]. A very promising strategy to overcome resistance phenomena is the use of specific carriers and the change from platinum to other transition metals [2–4].

N-Heterocyclic carbenes (NHCs) came into the focus as carrier ligands for cytotoxic metal complexes because they perfectly fit prerequisites for an efficient drug design and fast optimization [2–4]. NHCs are readily accessible in few steps and their substituents can be widely varied allowing an easy fine-tuning of both the physicochemical properties and the reactivity in biological medium of the final NHC metal complexes. Despite extensive studies on NHC complexes in organometallic chemistry and catalysis, only a restricted array of biomedical applications have been reported so far for gold, palladium, copper, ruthenium, platinum, silver and rhodium derivatives [2–14].

Silver salts have enjoyed a long history as antimicrobial agents and have proved to exhibit low toxicity for humans [2–6,9–14]. Recently, an increasing number of reports deal with silver complexes showing properties relevant for the design of novel anticancer therapeutics [3,4,9–13]. For instance, Youngs et al. described silver NHC complexes with 4,5-dichloro-1*H*-imidazole ligands as selective cytostatics in ovarian (OVCAR-3) and breast (MB-157) cancer cell lines. Activity was even demonstrated *in vivo* for acetato [4,5-dichloro-1,3-dimethylimidazol-2-ylidene] silver(I) (Scheme 1) against an ovarian cancer xenograft model [10]. The groups of Gautier and Morel reported an *N,N′*-diaryl-substituted carbene of high lipophilicity as suitable ligand for metal complexes. The cytotoxicity of the resulting silver complex (Scheme 1) was 40-fold (MCF-7 and HL60) to 7-fold (MCF-7R) higher than that of cisplatin [4].

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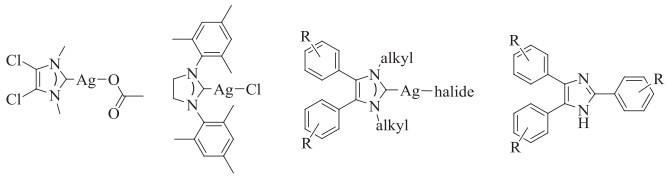
doi:10.1016/j.ejmech.2011.10.002



Scheme 1. Selected silver NHC complexes and 2,4,5-triarylimidazoles.

Encouraged of these and other previously reported results of metal NHC complexes with high antitumor activity [2–10], we started the examination of the antitumor activity of silver NHC complexes with 4,5-diarylimidazole ligands (Scheme 1). To achieve a high accumulation grade in tumor cells, the aromatic rings were 2-F, 3-F, 4-F, 4–OH, or 4–OCH_3 substituted. Such substitution patterns were already very successfully used in the case of [1,2-diarylethylenediamine]platinum(II) complexes [1,15]. The related NHC complexes can be considered as plain derivatives of these compounds.

2. Chemistry

In previous studies, we already synthesized substituted 4,5-diarylimidazoles by oxidation of respective substituted 4,5-diaryl-2-imidazolines, which were obtained from substituted benzaldehydes in a 6 step sequence, unfortunately, in low yields [16]. Therefore, a new synthesis (Scheme 2) based on a procedure reported by Sharpe *et al.* was chosen [17]. Compounds **1a**–**d** were synthesized from commercially available benzaldehydes via the catalysis of thiamine. For ring closure, yielding the respective imidazoles **2a**–**d**, the benzoines **1a**–**d** were heated in formamide to reflux for 3 h. Reaction of **2a**–**d** with NaH and ethyl bromide in absolute THF afforded the corresponding *N*-alkylation (**3a**–**d**). The hydroxyl-substituted imidazole **3e** was generated from **3d** by ether cleavage with BBr_3 [16]. Subsequent reaction of **3a**–**e** with ethyl bromide in CH_3CN yielded the imidazolium salts **4a**–**e**. The same reaction of **3c** with benzyl chloride resulted in 3-benzyl-1-ethyl-4,5-diarylimidazolium chloride **4f**. Finally, the imidazolium salts **4a**–**f** were treated with silver oxide in a mixture of CH_2Cl_2 and CH_3OH at room temperature to form the corresponding silver NHC complexes **5a**–**f** in analogy to the reported procedure by Wang and Lin [5,6,18].

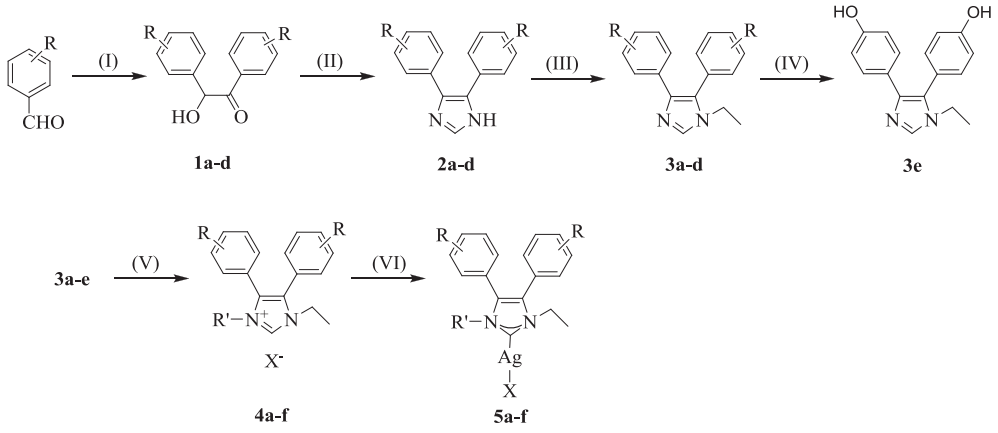
Precursors and the silver complex were characterized by IR, NMR and MS spectra. The IR spectra of ligands and complexes are very similar and not suitable to confirm the binding of NHC to silver. In silver NHC complexes, the methine proton at C2 is exchanged by the metal. Consequently, the NCHN signal at $\delta = 9$–11 disappeared in the ^1H NMR spectra. In accordance with the literature [5,6], the metal bound carbine (NCN–Ag) is not observable in the ^{13}C NMR spectrum. Furthermore, it should be noted that upon metal binding the ^1H NMR and ^{13}C NMR signals of ethyl, benzyl and phenyl are nearly unaffected.

All novel silver complexes are sufficiently stable in solid state and solution. If the complexes were dissolved in $CDCl_3$, methanol-d_4 or DMSO-d_6 no change of the spectra was observed during the storage at room temperature.

The formation of the complexes could be supported by positive mode ESI mass spectrometry. The spectra indicated a base peak corresponding to the $[2M - AgX_2]^+$ fragment for **5a**–**c**, **5f** and the $[M - AgX]^+$ fragment for **5a**–**f**.

To evaluate the structure of the silver complexes, **5c** was crystallized from a MeOH/CH_2Cl_2 solution and characterized by X-ray diffraction. Its molecular structure is depicted in Fig. 1. Selected bond distances and bond angles are given in the figure caption. The crystallographic experimental data of **5c** are presented in Table 1.

The complexes are arranged in the crystal in dimeric units. Each metal binds one NHC and two bridging bromides (see Fig. 1). The Ag–Br distances are 2.525(2) and 2.809(2) Å, respectively for Ag(1)–Br(2) and Ag(1)–Br(1). The bond angle of C(1)–Ag(1)–Br(2)



Compound	R	R'	X
1a, 2a, 3a, 4a, 5a	2-F	Et	Br
1b, 2b, 3b, 4b, 5b	3-F	Et	Br
1c, 2c, 3c, 4c, 5c	4-F	Et	Br
1d, 2d, 3d, 4d, 5d	4-MeO	Et	Br
3e, 4e, 5e	4-OH	Et	Br
4f, 5f	4-F	$PhCH_2$	Cl

Scheme 2. Synthesis routes and structures of intermediates and silver NHC complexes **5a**–**f**. Reagents and conditions: (I) thiamine hydrochloride, water/ethanol 1:2, room temperture (rt), 2–7 days, 52–59%; (II) formamide, reflux, 3 h, 61–80%; (III) 95% NaH, ethyl bromide, absolute THF, reflux, 2 h, 82–89%; (IV) BBr_3, CH_2Cl_2, 48h, 75%; (V) ethyl bromide or benzyl chloride, CH_3CN, reflux, 48–72 h, 76–85% (VI) Ag_2O, CH_3OH/CH_2Cl_2 1:1.2, rt, 12 h, 51–82%.

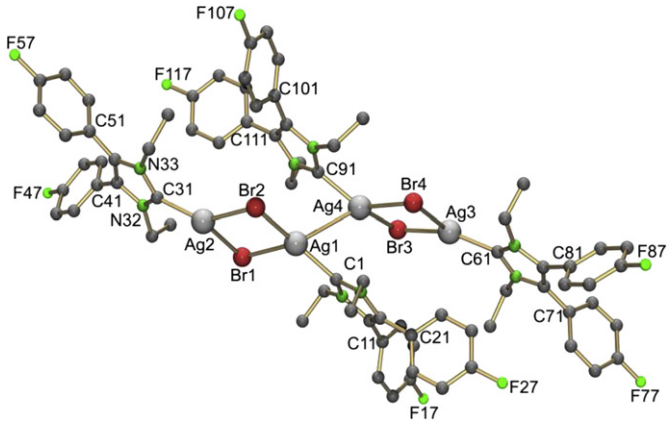


Fig. 1. X-ray molecular structure of **5c**. Selected bond lengths [Å] and angles [°] Ag(1)–C(1) 2.12(1), Ag(1)–Br(2) 2.525(2), Ag(1)–Br(1) 2.809(2), Ag(1)–Ag(4) 3.060(1), C(1)–Ag(1)–Br(2) 153.6(3), Br(2)–Ag(1)–Br(1) 88.90(5), C(1)–Ag(1)–Br(1) 111.0(3), C(1)–Ag(1)–Ag(4) 66.0(3), Br(2)–Ag(1)–Ag(4) 119.35(5), Br(1)–Ag(1)–Ag(4) 120.79(6), N(2)–C(1)–Ag(1) 126.9(7), N(5)–C(1)–Ag(1) 127.7(8), Ag(2)–Br(1)–Ag(1) 86.62(6), Ag(1)–Br(2)–Ag(2) 94.01(6), C(91)–Ag(4)–Ag(1) 67.2(2), Br(4)–Ag(4)–Ag(1) 117.62(9), Br(3)–Ag(4)–Ag(1) 127.89(6).

amounts to 153.6(3)° that of C(1)–Ag(1)–Br(1) to 111.0(3)°. The C(1)–Ag(1) distance is 2.12(1) Å, which is in accordance with published NHC–silver complexes [5,6,18]. Ag(1) and Ag(4) are arranged in a distance of 3.060(1) Å which indicates weak interaction comparable with metal–metal distance in metallic silver(0) (2.88 Å) [6].

3. Results and discussion

All complexes as well as the established anticancer drug cisplatin were screened for cytotoxicity against MCF-7, MDA-MB-231 breast cancer cells and HT-29 colon cancer cells. The experiments were performed according to established procedures [19,20]. IC_{50} values of the silver complexes **5a**–**f** and cisplatin are listed in Table 2.

Cisplatin characteristically reduced the cell growth of MCF-7 (IC_{50} = 1.6 μM), MDA-MB-231 (IC_{50} = 7.8 μM) and HT-29 (IC_{50} = 4.1 μM) cells. The substituents at the 4,5-standing phenyl rings of the silver complexes determined the cytotoxicity. The 4-hydroxyl-substituted compound **5e** showed relatively low antiproliferative activities with IC_{50} values of 9.2–16.2 μM, while the fluoro- and methoxy-substituted compounds **5a**–**d**, **5f** showed strong activities in the three cancer cell lines with IC_{50} values below 10 μM. These results are in accordance with previous results that the methoxy substituents induced higher cytotoxicity than hydroxyl substituents [19].

In addition, **5a**–**c, 5f** showed comparable activities against mammary carcinoma cells (IC_{50} = 3–4 μM). Compared to cisplatin they were half as active at the MCF-7 cell line and two fold more active at the MDA-MB-231 cancer cell line. At HT-29 cells only **5c** achieved an activity comparable to cisplatin. All other complexes (**5a**–**b, 5f**) were less active (IC_{50} = 7–10 μM). Compound **5d** demonstrated a preference for MCF-7 cells, with an IC_{50} = 3.7 μM compared to 8.5 and 9.9 μM determined for the other cell lines.

Time-activity curves of **5c** for concentrations ranged from 0.5 to 10 μM are presented in Fig. 2. Compound **5c** caused cytocidal effects ($T/C_{corr} < 0\%$) at 10 μM and cytostatic effects ($T/C_{corr} \approx 0\%$) at 5 μM, while cisplatin did not reach cytocidal activity. The curves of **5c** at higher concentrations are characterized by a fast onset of activity with maximal effects already after an incubation time of 48 h. Cisplatin achieved its maximal effect at the end of the experiment. Furthermore, Fig. 2 shows in the case of **5c** a recuperation of the tumor cells after a prolonged exposition time. Because exponential cell growth is guaranteed for at least 140 h of incubation, the rise of the growth curve can be explained by development of drug resistance [19]. This effect of **5c** is more pronounced at MDA-MB-231 and HT-29 cells compared to MCF-7 cells.

It is well accepted that the DNA represents the major target of antitumor metal complexes [1–4], so we studied the interaction of **5c** with calf thymus (CT) DNA by using circular dichroism (CD) spectroscopy [21]. However, the missing changes of absorbance (data not showed) exclude a binding of the complex.

During the past years cyclooxygenases (COXs), especially COX-2 became very interesting targets in cancer chemotherapy. We could already demonstrate that the derivation of the nonsteroidal anti-inflammatory drug (NSAID) aspirin (e.g. to (prop-2-ynyl)-2-acetoxybenzoate) and coordination to metals (e.g. [(prop-2-yn)-2-acetoxy]dicobalthexacarbonyl (Co-ASS)) increased the COX inhibitory properties as well as the cytotoxic potency [22–25]. Because the NHC ligands designed in this paper are derivatives of 2,4,5-triarylimidazoles (see Scheme 1), which inactivate the COX enzymes dependent on the substituents in the aromatic rings [26], it makes sense to take this target into consideration as well.

Table 1
Crystallographic experimental data of **5c**.

Empirical formula	$C_{77}\ H_{74}\ Ag_4\ Br_4\ Cl_2\ F_8\ N_8$
Wavelength	0.71073 Å
Radiation	Mo Kα
Crystal system	Monoclinic
Space group	$P2_1/n$
Unit cell dimensions	$a = 17.325(5)$ Å $\alpha = 90°$. $b = 17.344(5)$ Å $\beta = 95.62(1)°$. $c = 25.972(5)$ Å $\gamma = 90°$.
Volume	7767(4) $Å^3$
Z	4
Density (calculated)	1.784 Mg/m^3
Absorption coefficient	3.188 mm^{-1}
F(000)	4104
Crystal description	Needle
Crystal color	Colorless
Crystal size	0.3 × 0.05 × 0.05 mm^3
No. of reflexions (lattice)	98
Theta range (lattice)	2.29 to 59.53
Theta range for data collection	1.79 to 25.00
Index ranges	−20<= h <= 20,−20 <= k <= 20, −28<=l<=30
Reflections collected	35,447
Independent reflections	13,042 [R(int) = 0.0995]
Completeness to theta = 25.00°	95.4%
Absorption correction	None
Decay	0.5%
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	13,042/0/928
Goodness-of-fit on F^2	0.782
Final *R* indices [I > 2sigma(I)]	$R1 = 0.0694$, $wR2 = 0.1699$
R indices (all data)	$R1 = 0.1488$, $wR2 = 0.2261$
Largest diff. peak and hole	0.859 and −1.609 e.$Å^{-3}$

Table 2
Antiproliferative effects against MCF-7, MDA-MB-231 and HT-29 cells after 72 h incubation.

Compound	Cytotoxicity IC_{50}, [μM]		
	MCF-7	MDA-MB-231	HT-29
5a	3.4 ± 0.7	3.6 ± 0.2	7.5 ± 0.5
5b	3.5 ± 0.1	4.1 ± 0.7	7.4 ± 0.8
5c	3.9 ± 0.2	3.5 ± 0.3	4.4 ± 0.1
5d	3.7 ± 0.3	8.5 ± 0.8	9.9 ± 0.1
5e	9.2 ± 0.2	12.8 ± 0.2	16.2 ± 0.2
5f	3.6 ± 0.1	3.4 ± 0.2	6.8 ± 1.2
Cisplatin	1.6 ± 0.5	7.8 ± 0.8	4.1 ± 0.3

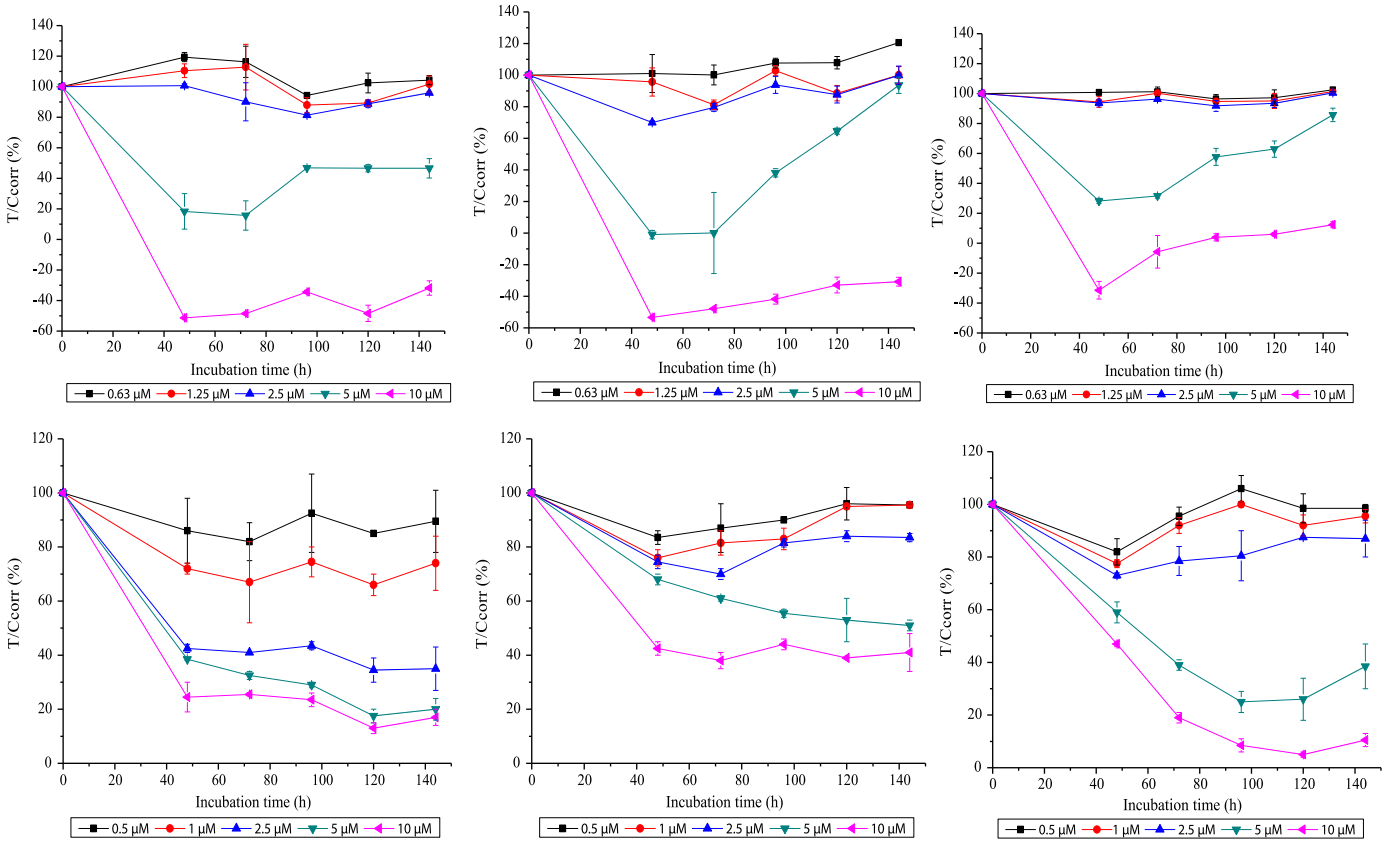


Fig. 2. Time activity curves of **5c** (above) and cisplatin (below) on MCF-7 (left), MDA-MB-231 (middle) and HT-29 (right) cell line lines. Error bars are hidden behind the symbols in some cases.

MCF-7 cells have a basal level of COX-1 and a barely detectable and transient COX-2-inducible expression, whereas MDA-MB-231 cells show a low expression of COX-1 but a constitutive level of COX-2 [27,28]. HT-29 human colon cancer cells constitutively express COX-2 [29]. Therefore, the most cytotoxic complex **5c** was selected for the investigation on isolated COX-1 and COX-2 enzymes by ELISA. Co-ASS and aspirin were used for comparison. At 10 μM Co-ASS inhibited both COX enzymes to about 50% (68% (COX-1) and 63% (COX-2)) so we used this concentration and performed the ELISA without calculation of the IC_{50} values.

Aspirin was only marginally active at COX-1 (29% inhibition) and inactive at COX-2. Unfortunately, at a concentration of 10 μM, **5c** caused no COX inhibition (COX-1 (4.6%) and COX-2 (1.4%)), so it is very unlikely that COX enzymes are involved in the inhibition of the tumor cell growth.

Because the above mentioned 2,4,5-triarylimidazoles were not only COX inhibitors but also ligands of the estrogen receptor (ER) [16,26,30], we investigated if the ER might act as carrier for the complexes. Therefore, ERα-positive MCF-7 breast cancer cells, stably transfected with the plasmid ERE_{wtc}luc (MCF-7-2a cells) and U2–OS cells transiently transfected with the plasmid pSG5-ERα (U2–OS/α) or pSG5-ERβ FL (U2–OS/β) and the reporter plasmid p(ERE)2-luc+ were used to evaluate ER subtype selectivity on molecular level [16,30,31].

Among the complexes only **5f** induced a low luciferase expression (conc. 10 μM: activation 90% ERα and 66% ERβ; conc. 1 μM: activation 36% ERα and 20% ERβ) which is an indication of only slight receptor binding. Therefore, an ER-mediated cytotoxicity can be excluded.

Finally, a number of silver NHC complexes possessing good antibacterial activities [3,4,10–14] which induced us to evaluate the effectiveness of **5c** and **5f** as antibacterial agents *in vitro* through a modified agar diffusion test [32].

Indicator bacteria were grown in Luria broth (LB) at 30 °C (*Erwinia amylovora* Ea 273 and *Erwinia carotovora*) or 37 °C (*Escherichia coli* DH5α, *Bacillus subtilis* 168, *Bacillus megaterium*,

Table 3
Sensitivity test of the silver compounds in inhibiting bacterial growth.

Test Compounds	Concentration (mM)	Diameter of the zone of inhibition (mm)			
		Ervinia amylovora Ea 273	*E. coli* DH5α	*Bacillus subtilis* 168	Bacillus megaterium
5c	5	7	7	9	7
	10	9	8	12	8
5f	5	8	6	15	5
	10	8	7	28	18
$AgNO_3$	5	12	11	9	12
	10	12	11	9	12
H_2O		5	5	5	5

Table 4
Antimicrobial activity of **5c** at 4.2 mM.

Indicator strains	Inhibiting effect (diameter of the zone of inhibition)
Erwinia carotovora	+[a] (**5c** 9.5 mm; H_2O 5 mm)
Pseudomonas aeruginosa	+ (**5c** 8 mm; H_2O 5 mm)
Bacillus cereus ATCC 14579	+ (**5c** 6 mm; H_2O 5 mm)

[a] "+" indicates positive results of **5c.**

Pseudomonas aeruginosa and Bacillus cereus ATCC 14579). When cell concentration of bacteria was up to 4×10^7 CFU/mL 0.5 mL bacteria suspensions were mixed with 20 mL melting LB agar and cooled below 60 °C to prepare the plates. $AgNO_3$ was used as positive control. 50 μL of aqueous solution with increasing complex concentrations or 50 μL of water (containing 0.1% DMSO) was loaded into the wells (diameter: 5 mm) and punched in indicator bacteria plates which were then incubated at 30 °C overnight to observe the growth inhibition effects. The antimicrobial activity of the compounds was determined by measuring the diameter of the inhibiting zone around the wells.

Our assay confirmed that **5c** and **5f** possessed antimicrobial properties at a level comparable to silver nitrate against *E. amylovora* Ea 273, *E. coli* DH5α, *B. subtilis* 168 and *B. megaterium* (see Table 3). The maximum of antimicrobial activity was observed for complexes **5c** and **5f** against the *B. subtilis* 168 and *B. megaterium*. The diameter of the inhibition zone of **5f** against the *B. subtilis* 168 at 10 mM was almost 3-fold than that of $AgNO_3$.

Futhermore, extention testing of **5c** against other bacteria (*E. carotovora, P. aeruginosa and B. cereus* ATCC 14579) also indicated positive results in comparison with the negative control (Table 4).

4. Conclusions

4,5-Diarylimidazole derivatives showed low antiproliferative effects in cancer cells. After coordination to Ag-halides, the antiproliferative effects in cultured tumor cells greatly increased. Preliminary *in vitro* studies showed that **5c** and **5f** exhibited good antibacterial activities. Despite the mode of action of silver NHC complexes remains unclear, these findings indicate that this type of silver NHC complexes may be useful in anticancer and antibacterial chemotherapy. Further studies on the pharmacodynamics and pharmacokinetics as well as antibacterial testing of the complexes are in progress.

5. Experimental section

5.1. Chemistry

5.1.1. General

The following instrumentation was used: IR spectra (KBr pellets): Perkin Elmer Model 580 A (Shelton, USA); ^1H or ^{13}C NMR spectra: Bruker ADX 400 spectrometer operated at 400 MHz or 100 MHz (internal standard, tetramethylsilane); electron impact (EI) MS spectra: Varian CH-7A (70 eV) spectrometer; ESI-TOF spectra: Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA; Elemental C, H, N analysis: Perkin Elmer 240 B and C analyzer. Chemicals were obtained from Sigma–Aldrich (Germany) and used without further purification. Reactions were all monitored by TLC, performed on silica gel plates 60 F254 (Merck, Darmstadt/Germany). Visualization on TLC was achieved by UV light. Column chromatography was performed with Merck silica gel 60H, grain size <0.063 mm, 230 mesh ASTM (Darmstadt/Ger).

5.1.2. General procedure for the synthesis of ***1a–d***

2.00 g (5.94 mmol) of thiamine hydrochloride were dissolved in a mixture of 10 mL of distilled water and 20 mL of ethanol followed by stirring the solution until it is homogeneous. Then 2.0 M NaOH was added dropwise to the stirring solution until the pH reached 9–10. After addition of 74.6 mmol of the substituted benzaldehyde, the reaction was allowed to stand at room temperature. After 2–7 days, the reaction mixture was filtered, the ethanol was evaporated and the remaining water phase was extracted with dichloromethane. After drying the collected organic layers over $MgSO_4$, removal of the solvent under reduced pressure gave the crude product, which was then purified by flash column chromatography (EtOAc/petroleum ether).

*5.1.2.1. 1,2-Bis(2-fluorophenyl)-2-hydroxyethanone (**1a**).* Yield 56.4%; pale yellow solid. MS m/z: 248 $[M]^+$. ^1H NMR ($CDCl_3$): δ 4.53 (s, 1H, OH, exchangeable by D_2O), 6.08 (s, 1H, CH), 6.96–7.08 (m, 4H, ArH), 7.18–7.26 (m, 2H, ArH), 7.45–7.51 (m, 1H, ArH), 7.85–7.89 (m, 1H, ArH).

*5.1.2.2. 1,2-Bis(3-fluorophenyl)-2-hydroxyethanone (**1b**).* Yield 52.3%; pale yellow solid. MS m/z: 248 $[M]^+$. ^1H NMR ($CDCl_3$): δ 4.21 (s, 1H, OH, exchangeable by D_2O), 5.90 (s, 1H, CH), 6.98–7.27 (m, 4H, ArH), 7.28–7.42 (m, 2H, ArH), 7.58–7.67 (m, 2H, ArH).

*5.1.2.3. 1,2-Bis(4-fluorophenyl)-2-hydroxyethanone (**1c**).* Yield 58.1%; pale yellow solid. MS m/z: 248 $[M]^+$. ^1H NMR ($CDCl_3$): δ 4.50 (s, 1H, OH, exchangeable by D_2O), 5.91 (s, 1H, CH), 7.01–7.12 (m, 4H, ArH), 7.30–7.35 (m, 2H, ArH), 7.90–7.95 (m, 2H, ArH).

*5.1.2.4. 2-Hydroxy-1,2-bis(4-methoxyphenyl)ethanone (**1d**).* Yield 58.8%; pale yellow solid. MS m/z: 272 $[M]^+$. ^1H NMR ($CDCl_3$): δ 3.76 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.57 (d, 1H, OH, $J = 5.6$ Hz, exchangeable by D_2O), 5.85 (d, 1H, CH, $J = 4.8$ Hz), 6.83–6.87 (m, 4H, ArH), 7.23–7.26 (m, 2H, ArH), 7.88–7.91 (d, 2H, ArH, $J = 5.6$ Hz).

5.1.3. General procedure for the synthesis of ***2a–d***

A solution of the substituted benzoin **1a–d (**23 mmol) in 30 mL of formamide was heated to reflux for 3 h. The reaction mixture was poured into 100 mL of water and stirred vigorously to dissolve the gummy product. The resulting powder was filtered, washed with water, and suspended in 5% HCl (200 mL). The solution was heated to 80–90 °C and filtered hot. The acidic filtrate was treated with an excess of NH_4OH to form a white precipitate, which was filtered and washed with water and cold acetonitrile.

*5.1.3.1. 4,5-Bis(2-fluorophenyl)-1H-imidazole (**2a**).* Yield 79.5%; white solid. MS m/z: 256 $[M]^+$. ^1H NMR (DMSO-d_6): δ 7.08–7.36 (m, 8H, ArH), 7.59 (s, 1H, N=CH–N), 12.65 (s, 1H, NH, exchangeable by D_2O).

*5.1.3.2. 4,5-Bis(3-fluorophenyl)-1H-imidazole (**2b**).* Yield 80.2%; white solid. MS m/z: 256 $[M]^+$. ^1H NMR (DMSO-d_6): δ 7.07–7.45 (m, 8H, ArH), 7.84 (s, 1H, N=CH–N), 12.66 (s, 1H, NH, exchangeable by D_2O).

*5.1.3.3. 4,5-Bis(4-fluorophenyl)-1H-imidazole (**2c**).* Yield 61.5%; white solid. MS m/z: 256 $[M]^+$. ^1H NMR (DMSO-d_6): δ 7.10–7.14 (t, 2H, ArH, $J = 8.0$ Hz), 7.24–7.28 (t, 2H, ArH, $J = 8.0$ Hz), 7.41–749 (m, 4H, ArH), 7.77 (s, 1H, N=CH–N), 12.50 (s, 1H, NH, exchangeable by D_2O). Anal. calcd. for $C_{15}H_{10}F_2N_2$: C, 70.31; H, 3.93; N, 10.93%. Found C, 70.32; H, 3.95; N, 10.93%.

*5.1.3.4. 4,5-Bis(4-methoxyphenyl)-1H-imidazole (**2d**).* Yield 75.3%; white solid. MS m/z: 280 $[M]^+$. ^1H NMR ($CDCl_3$): δ 3.82 (s, 6H,

OCH_3), 6.82–6.84 (d, 4H, ArH, J = 8.0 Hz), 7.39–7.41 (d, 4H, ArH, J = 8.0 Hz), 7.59 (s, 1H, N=CH–N), 9.67 (1H, NH, exchangeable by D_2O). Anal. calcd. for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99%. Found C, 72.95; H, 5.75; N, 10.08%.

*5.1.4. General procedure for the synthesis of **3a–d***

To a solution of 15.30 mmol of imidazole **2a–d** in 50 mL of dry THF, 16.50 mmol (417 mg, 95% powder) of sodium hydride and 16.50 mmol (1.23 mL, 1782 mg) of ethyl bromide were added and heated to reflux for 2 h. Subsequently, the reaction mixture was hydrolyzed with 50 mL of water and the product was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and the solvent was distilled off to give the crude product, which was then purified by flash column chromatography (diethyl ether/CH_2Cl_2).

*5.1.4.1. 1-Ethyl-4,5-bis(2-fluorophenyl)-1H-imidazole (**3a**).* Yield 82.5%; pale yellow oil. MS m/z: 284 $[M]^+$. ^1H NMR ($CDCl_3$): δ 1.32 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.89 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.89–6.94 (t, 1H, ArH, J = 9.6 Hz), 7.07–7.19 (m, 5H, ArH), 7.36–7.42 (m, 1H, ArH), 7.50–7.54 (m, 1H, ArH), 7.73 (s, 1H, N=CH–N).

*5.1.4.2. 1-Ethyl-4,5-bis(3-fluorophenyl)-1H-imidazole (**3b**).* Yield 88.8%; pale yellow oil. MS m/z: 284 $[M]^+$. ^1H NMR ($CDCl_3$): δ 1.28 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.84 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.81–6.86 (m, 1H, ArH), 7.08–7.22 (m, 6H, ArH), 7.43–7.48 (m, 1H, ArH), 7.63 (s, 1H, N=CH–N).

*5.1.4.3. 1-Ethyl-4,5-bis(4-fluorophenyl)-1H-imidazole (**3c**).* Yield 87.6%; pale yellow oil. MS m/z: 284 $[M]^+$. ^1H NMR ($CDCl_3$): δ 1.27 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.81 (q, 2H, CH_2CH_3, J = 7.2 Hz), 6.89 (t, 2H, ArH, J = 8.8 Hz), 7.17 (t, 2H, ArH, J = 8.8 Hz), 7.29–7.32 (m, 2H, ArH, J = 8.4 Hz), 7.38–7.41 (m, 2H, ArH), 7.61 (s, 1H, N=CH–N). Anal. calcd. for $C_{17}H_{14}F_2N_2$: C, 71.85; H, 4.96; N, 9.85%. Found C, 71.81; H, 4.98; N, 9.88%.

*5.1.4.4. 1-Ethyl-4,5-bis(4-methoxyphenyl)-1H-imidazole (**3d**).* Yield 87.9%; pale yellow oil. MS m/z: 308 $[M]^+$. ^1H NMR ($CDCl_3$): δ 1.27 (t, 3H, CH_2CH_3, J = 7.2 Hz), 3.76 (s, 3H, OCH_3), 3.81 (q, 2H, CH_2CH_3, J = 7.2 Hz), 3.87 (s, 3H, OCH_3), 6.76 (d, 2H, ArH, J = 8.7 Hz), 6.99 (d, 2H, ArH, J = 8.4 Hz), 7.25 (d, 2H, ArH, J = 8.4 Hz), 7.41 (d, 2H, ArH, J = 8.8 Hz), 7.59 (s, 1H, N=CH–N). Anal. calcd. for $C_{19}H_{20}N_2O_2$. 1/6 CH_2Cl_2: C, 71.37; H, 6.35; N, 8.69%. Found C, 71.13; H, 6.39; N, 8.45%.

*5.1.4.5. 1-Ethyl-4,5-bis(4-hydroxyphenyl)-1H-imidazole (**3e**).* A solution of **3d** (10.0 mmol, 3.08 g) in 20 mL of dry CH_2Cl_2 was cooled to −60 °C. At this temperature BBr_3 (45 mmol, 11.2 g) in 5 mL of dry CH_2Cl_2 was added under N_2 atmosphere. Then the reaction mixture was allowed to warm to room temperature and was stirred for further 48 h. After cooling the reaction mixture with an ice bath, the surplus of BBr_3 was hydrolyzed three times with methanol and the phenolic product was dissolved in 0.1 N NaOH. The alkaline water phase was filtered and the pH was adjusted to 8 with 2 N HCl. The precipitate was collected by suction filtration and washed with CH_2Cl_2 and acetone. Yield 75.4%; grey solid. MS m/z: 280 $[M]^+$. ^1H NMR (DMSO-d_6): δ 1.12 (t, 3 H, CH_2CH_3, J = 7.2 Hz), 3.74 (q, 2 H, CH_2CH_3, J = 7.2 Hz), 6.56 (d, 2H, ArH, J = 8.4 Hz), 6.85 (d, 2H, ArH, J = 8.0 Hz), 7.11 (d, 2H, ArH, J = 8.0 Hz), 7.19 (d, 2H, ArH, J = 8.4 Hz), 7.70 (s, 1H, N=C*H*–N), 9.21 (s, 1H, ArOH, exchangeable by D_2O), 9.66 (s, 1H, ArOH, exchangeable by D_2O). Anal. calcd. for $C_{17}H_{16}N_2O_2$. 5/12 CH_2Cl_2: C, 66.26; H, 5.37; N, 8.87%. Found C, 66.09; H, 5.68; N, 8.81%.

*5.1.5. General procedure for the synthesis of **4a–f***

Ethyl bromide or benzyl chloride (25 mmol) and imidazole **3a–e** (5.00 mmol) were combined and refluxed in 50 mL of acetonitrile for 48–72 h. Then removal of the solvent under reduced pressure gave the crude product which was washed with diethyl ether (3 × 30 mL) and dried in vacuo.

*5.1.5.1. 1,3-Diethyl-4,5-bis(2-fluorophenyl)-1H-imidazolium bromide (**4a**).* Yield 78.6%; white solid. ESI-MS m/z: 313 $[M - Br]^+$. IR (KBr, cm^{-1}): 3119, 2990, 1646, 1618, 1560, 1490, 1451, 1228, 1208, 821, 769. ^1H NMR ($CDCl_3$): δ 1.53 (t, 6H, CH_2CH_3, J = 7.2 Hz), 4.30 (q, 4H, CH_2CH_3, J = 7.2 Hz), 7.16–7.22 (m, 6H, ArH), 7.48–7.53 (m, 2H, ArH), 11.24 (s, 1H, N=CH–N). ^{13}C NMR ($CDCl_3$): δ 15.5 (s, CH_3); 43.8 (s, CH_2); 112.3, 112.4, 116.3, 116.5, 125.3, 127.5, 132.4 (s, C_{Ar}); 133.6 (d, C_{Ar}); 137.6 (s, NCHN); 159.0, 161.5 (s, C_{Ar}).

*5.1.5.2. 1,3-Diethyl-4,5-bis(3-fluorophenyl)-1H-imidazolium bromide (**4b**).* Yield 80.7%; white solid. ESI-MS m/z: 313 $[M - Br]^+$. IR (KBr, cm^{-1}): 3114, 3057, 2975, 2910, 1580, 1559, 1465, 1437, 1206, 1150, 885, 804. ^1H NMR ($CDCl_3$): δ 1.52 (t, 6H, CH_2CH_3, J = 7.2 Hz), 4.35 (q, 4H, CH_2CH_3, J = 7.2 Hz), 6.95–6.97 (m, 2H, ArH), 7.06 (d, 2H, ArH, J = 7.6 Hz), 7.18–7.23 (m, 2H, ArH), 7.42–7.47 (m, 2H, ArH), 11.09 (s, 1H, N=CH–N). ^{13}C NMR ($CDCl_3$): δ 15.3 (s, CH_3); 43.3 (s, CH_2); 117.0, 117.3, 117.4, 117.6, 126.1, 126.2, 126.3 (s, C_{Ar}); 130.3 (d, C_{Ar}); 130.9, 131.0 (s, C_{Ar}); 136.6 (s, NCHN); 160.9, 163.4 (s, C_{Ar}).

*5.1.5.3. 1,3-Diethyl-4,5-bis(4-fluorophenyl)-1H-imidazolium bromide (**4c**).* Yield 84.5%; white solid. ESI-MS m/z: 313 $[M - Br]^+$. IR (KBr, cm^{-1}): 3127, 3038, 2984, 2941, 1631, 1597, 1559, 1504, 1226, 1196, 1160, 851, 819. ^1H NMR ($CDCl_3$): δ 1.31 (t, 6H, CH_2CH_3, J = 7.2 Hz), 4.10 (q, 4H, CH_2CH_3, J = 7.2 Hz), 7.33 (t, 4H, ArH, J = 8.8 Hz), 7.50–7.53 (m, 4H, ArH), 9.69 (s, 1H, N=CH–N). ^{13}C NMR ($CDCl_3$): δ 15.7 (s, CH_3); 43.5 (s, CH_2); 116.6, 116.8 (s, C_{Ar}); 120.9 (d, C_{Ar}); 131.1 (s, C_{Ar}); 132.7 (d, $C_{Ar,}$); 136.6 (s, NCHN); 162.4, 164.9 (s, C_{Ar}). Anal. calcd. for $C_{19}H_{19}BrF_2N_2$: C, 58.03; H, 4.87; N, 7.12%. Found C, 58.04; H, 4.93; N, 7.13%.

*5.1.5.4. 1,3-Diethyl-4,5-bis(4-methoxyphenyl)-1H-imidazolium bromide (**4d**).* Yield 80.4%; grey solid. ESI-MS m/z: 337 $[M - Br]^+$. IR (KBr, cm^{-1}): 3136, 3065, 2982, 2937, 2837, 1626, 1611, 1561, 1506, 1251, 1181, 1021, 847, 807. ^1H NMR ($CDCl_3$): δ 1.44 (t, 6H, CH_2CH_3, J = 7.2 Hz), 3.77 (s, 6H, OCH_3), 4.26 (q, 4H, CH_2CH_3, J = 7.2 Hz), 6.87 (d, 4H, ArH, J = 8.8 Hz), 7.10 (d, 4H, ArH, J = 8.4 Hz), 10.77 (s, 1H, N=CH–N). ^{13}C NMR ($CDCl_3$): δ 15.9 (s, CH_3); 43.2 (s, CH_2); 55.4 (s, OCH_3); 114.8, 116.9, 131.5, 131.8 (s, C_{Ar}); 136.2 (s, NCHN); 160.9 (s, C_{Ar}). Anal. calcd. for $C_{21}H_{25}BrN_2O_2$: C, 60.44; H, 6.04; N, 6.71%. Found C, 60.56; H, 6.09; N, 6.75%.

*5.1.5.5. 1,3-Diethyl-4,5-bis(4-hydroxyphenyl)-1H-imidazolium bromide (**4e**).* Yield 76.5%; grey solid. ESI–MS m/z: 309 $[M - Br]^+$. IR (KBr, cm^{-1}): 3600–2300 (OH), 3124, 2997, 1611, 1560, 1508, 1429, 1345, 1270, 1219, 1173, 841. ^1H NMR (DMSO-d_6): δ 1.30 (t, 6H, CH_2CH_3, J = 7.2 Hz), 4.06 (q, 4H, CH_2CH_3, J = 7.2 Hz), 6.82 (d, 4H, ArH, J = 8.4 Hz), 7.19 (m, 4H, ArH), 9.44 (s, 1H, N=CH–N), 9.94 (s, 2H, ArOH, exchangeable by D_2O). Anal. calcd. for $C_{19}H_{21}BrN_2O_2$. 2 CH_2Cl_2: C, 45.11; H, 4.51; N, 5.01%. Found C, 44.97; H, 4.52; N, 5.24%.

*5.1.5.6. 3-Benzyl-1-ethyl-4,5-bis(4-fluorophenyl)-1H-imidazolium chloride (**4f**).* Yield 78.7%; white solid. ESI-MS m/z: 375 $[M - Cl]^+$. IR (KBr, cm^{-1}): 3119, 3032, 3002, 2974, 2941, 1607, 1555, 1506, 1455, 1294, 1253, 1179, 1022, 843. ^1H NMR ($CDCl_3$): δ 1.49 (t, 3H, CH_2CH_3, J = 7.2 Hz), 4.32 (q, 2H, CH_2CH_3, J = 7.2 Hz), 5.55 (s, 2H, CH_2Ar), 7.01–7.14 (m, 8H, ArH), 7.23–7.42 (m, 5H, ArH), 10.97 (s, 1H, N=CH–N). ^{13}C NMR ($CDCl_3$): δ 15.6 (s, CH_3); 43.6 (s, CH_2); 51.6 (s, $ArCH_2$); 116.3 (s, C_{Ar}); 116.5 (d, C_{Ar}); 116.7 (s, C_{Ar}); 120.9 (d, C_{Ar}); 121.0 (d, C_{Ar}); 128.5, 128.8, 129.0 (s, C_{Ar}); 131.3 (d, C_{Ar}); 132.6, 132.7, 133.0, 133.1, 133.7(s, C_{Ar}); 137.5 (s, NCHN); 162.4, 164.9 (s, C_{Ar}).

*5.1.6. General procedure for the synthesis of **5a–f***

To a stirred solution of 1.6 mmol imidazolium salt in CH_2Cl_2/methanol (10:12 v/v) was added silver(I) oxide (203 mg, 0.88 mmol) under N_2 and the reaction mixture was allowed to stir overnight in the dark. The resultant grey precipitate was filtered over a bed of celite, the colorless filtrate was isolated, and the volume of the solution was reduced to 5 mL under vacuum. 30 mL of n-hexane was then added to produce a white solid which was filtered and recrystallized from CH_2Cl_2/n-hexane.

*5.1.6.1. Bromo[1,3-diethyl-4,5-bis(2-fluorophenyl)imidazol-2-ylidene]silver(I) (**5a**).* Yield 79.6%; white solid. ESI-MS m/z: 731 $[2M - AgBr_2]^+$, 313 $[M - AgBr]^+$. IR (KBr, cm^{-1}): 3053, 2978, 2932, 2739, 1616, 1584, 1445, 1235, 1203, 882. ^1H NMR ($CDCl_3$): δ 1.27 (t, 6H, CH_2CH_3, $J = 7.2$ Hz), 4.07 (q, 4H, CH_2CH_3, $J = 7.2$ Hz), 7.10–7.19 (m, 6H, ArH), 7.38–7.44 (m, 2H, ArH). ^{13}C NMR ($CDCl_3$): δ 17.1 (s, CH_3); 45.4 (s, CH_2); 115.4, 115.5, 116.0, 116.2, 124.6, 127.0, 132.1, 132.2, 132.4, 159.2, 161.7 (s, C_{Ar}); signal for Ag–C was not observed. Anal. calcd. for $C_{19}H_{18}AgBrF_2N_2$: C, 45.63; H, 3.63; N, 5.60%. Found C, 45.87; H, 3.84; N, 5.21%.

*5.1.6.2. Bromo[1,3-diethyl-4,5-bis(3-fluorophenyl)imidazol-2-ylidene]silver(I) (**5b**).* Yield 81.2%; white solid. ESI-MS m/z: 731 $[2M - AgBr_2]^+$, 313 $[M - AgBr]^+$. IR (KBr, cm^{-1}): 3057, 2981, 2934, 1630, 1557, 1452, 1345, 1228, 764. ^1H NMR ($CDCl_3$): δ 1.27 (t, 6H, CH_2CH_3, $J = 7.2$ Hz), 4.13 (q, 4H, CH_2CH_3, $J = 7.2$ Hz), 6.89–6.93 (m, 2H, ArH), 6.96–7.00 (m, 2H, ArH), 7.09–7.13 (m, 2H, ArH), 7.34–7.39 (m, 2H, ArH). ^{13}C NMR ($CDCl_3$): δ 17.4 (s, CH_3); 45.1 (s, CH_2); 116.6, 116.8, 117.2, 117.4 (s, C_{Ar}); 126.1 (d, C_{Ar}); 129.5, 129.6 (s, C_{Ar}); 130.7–130.8 (dd, C_{Ar}); 161.3, 163.8 (s, C_{Ar}); signal for Ag–C was not observed. Anal. calcd. for $C_{19}H_{18}AgBrF_2N_2$: C, 45.63; H, 3.63; N, 5.60%. Found C, 45.85; H, 3.98; N, 5.28%.

*5.1.6.3. Bromo[1,3-diethyl-4,5-bis(4-fluorophenyl)imidazol-2-ylidene]silver(I) (**5c**).* Yield 81.3%; white solid. ESI-MS m/z: 731 $[2M - AgBr_2]^+$, 313 $[M - AgBr]^+$. IR (KBr, cm^{-1}): 3053, 2981, 2934, 2874, 1630, 1598, 1505, 1456, 1345, 1227, 847, 820. ^1H NMR ($CDCl_3$): δ 1.27 (t, 6H, CH_2CH_3, $J = 7.2$ Hz), 4.10 (q, 4H, CH_2CH_3, $J = 7.2$ Hz), 7.07 (t, 4H, ArH, $J = 8.4$ Hz), 7.16–7.19 (m, 4H, ArH). ^{13}C NMR ($CDCl_3$): δ 17.4 (s, CH_3); 45.0 (s, CH_2); 116.1, 116.3, 123.7, 123.8, 130.9, 132.2, 132.3, 161.8, 164.3 (s, C_{Ar}); signal for Ag–C was not observed. Anal. calcd. for $C_{19}H_{18}AgBrF_2N_2$. 0.15 n-hexan: C, 46.85; H, 4.05; N, 5.41%. Found C, 47.05; H, 4.15; N, 5.73%.

*5.1.6.4. Bromo[1,3-diethyl-4,5-bis(4-methoxyphenyl)imidazol-2-ylidene]silver(I) (**5d**).* Yield 58.7%; white solid. ESI-MS m/z: 337 $[M - AgBr]^+$. IR (KBr, cm^{-1}): 3036, 2972, 2936, 2838, 1630, 1559, 1519, 1506, 1457, 1345, 1293, 1253, 1180, 1022, 843. ^1H NMR ($CDCl_3$): δ 1.26 (t, 6H, CH_2CH_3, $J = 7.2$ Hz), 3.80 (s, 6H, OCH_3), 4.09 (q, 4H, CH_2CH_3, $J = 7.2$ Hz), 6.86 (d, 4H, ArH, $J = 8.4$ Hz), 7.11 (d, 4H, ArH, $J = 8.4$ Hz). Anal. calcd. for $C_{21}H_{24}AgBrN_2O_2$. 0.3 n-hexane: C, 49.79; H, 5.17; N, 5.09%. Found C, 49.75; H, 4.78; N, 5.42%.

*5.1.6.5. Bromo[1,3-diethyl-4,5-bis(4-hydroxyphenyl) imidazol-2-ylidene] silver(I) (**5e**).* Yield 51.3%; white solid. ESI-MS m/z: 309 $[M - AgBr]^+$. IR (KBr, cm^{-1}): 3600–2300 (OH), 3062, 2983, 2937, 2806, 1630, 1594, 1498, 1277, 1169, 843. ^1H NMR (DMSO-d_6): δ 1.23 (t, 6H, CH_2CH_3, $J = 7.2$ Hz), 4.06 (q, 4H, CH_2CH_3, $J = 7.2$ Hz), 6.75 (d, 4H, ArH, $J = 8.4$ Hz), 7.08 (d, 4H, ArH, $J = 8.4$ Hz), 9.22 (s, 2H, ArOH, exchangeable by D_2O). Anal. calcd. for $C_{19}H_{20}AgBrN_2O_2$. 0.5 n-hexan: C, 49.00; H, 5.05; N, 5.20%. Found C, 48.91; H, 4.77; N, 5.51%.

*5.1.6.6. Chloro[3-benzyl-1-ethyl-4,5-bis(4-fluorophenyl)imidazol-2-ylidene]silver(I) (**5f**).* Yield 76.9%; white solid. ESI-MS m/z: 857 $[2M - AgCl_2]^+$, 375 $[M - AgCl]^+$. IR (KBr, cm^{-1}): 3057, 2983, 2935, 1630, 1502, 1451, 1228, 1159, 840. ^1H NMR ($CDCl_3$): δ 1.29 (t, 3H, CH_2CH_3, $J = 7.2$ Hz), 4.15 (q, 2H, CH_2CH_3, $J = 7.2$ Hz), 5.24 (s, 2H, CH_2Ar), 6.94 (d, 6H, ArH, $J = 6.4$ Hz), 7.05 (t, 2H, ArH, $J = 8.4$ Hz), 7.17–7.26 (m, 5H, ArH). Anal. calcd. for $C_{24}H_{20}AgClF_2N_2$: C, 55.68; H, 3.89; N, 5.41%. Found C, 55.58; H, 3.99; N, 5.40%.

5.2. X-ray crystallography

The intensities for the X-ray determinations were collected on an STOE IPDS 2T instrument with Mo Kα radiation ($\lambda = 0.71073$ Å) at 200 K. Standard procedures were applied for data reduction and absorption correction. Structure solution and refinement were performed with SHELXS97 and SHELXL97 [33]. Hydrogen atom positions were calculated for idealized positions and treated with the "riding model" option of SHELXL. More details on data collections and structure calculations are contained in Table 1 and supporting information.

5.3. Biological activity

5.3.1. Cytotoxicity

The human MCF-7, MDA MB-231 breast cancer and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection. All cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (PAA Laboratories, Austria), supplemented with 5% fetal bovine serum (FBS; Biochrom, Germany) in a humidified atmosphere (5% CO_2) at 37 °C.

The experiments were performed according to established procedures with some modifications [19,20]. In 96 well plates 100 µL of a cell suspension in culture medium at 7500 cells/mL (MCF-7 and MDA-MB-231) or 3000 cells/mL (HT-29) were plated into each well and were incubated for three days under culture conditions. After the addition of various concentrations of the test compounds, cells were incubated for up to appropriate incubation time. Then the medium was removed, the cells were fixed with glutardialdehyde solution 1% and stored under phosphate buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining, followed by extracting of the bound dye with ethanol and a photometric measurement at 590 nm. Mean values were calculated and the effects of the compounds were expressed as % Treated/$Control_{corr}$ values according to the following equations:

$$T/C_{corr}[\%] = \frac{T - C_0}{C - C_0} \cdot 100$$

(C_0 control cells at the time of compound addition; C control cells at the time of test end; T probes/samples at the time of test end).

The IC_{50} value was determined as the concentration causing 50% inhibition of cell proliferation and calculated as mean of at least two or three independent experiments (OriginPro 8).

5.3.2. Intercalation with calf thymus DNA

Intercalation with calf thymus DNA was performed according to previously described procedures without modifications [21].

5.3.3. Estrogen receptor interaction studies

Estrogen receptor interaction studies were performed according to previously described procedures without modifications [16,30,31].

5.3.4. Inhibition of COX enzymes

The inhibition of isolated ovine COX-1 and human recombinant COX-2 was determined with 10 µM of the respective compounds by ELISA ("COX inhibitor screening assay", Cayman Chemicals).

Experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (Victor2, Perkin Elmer). Results were calculated as the means of duplicate determinations.

5.3.5. Antibacterial test

The experiments were performed according to agar diffusion test with some modifications [32]. Indicator bacteria were grown in LB at 30 °C (*E. amylovora* Ea 273 and *E. carotovora*) or 37 °C (*E. coli* DH5α, *B. subtilis* 168, *B. megaterium*, *P. aeruginosa and B. cereus* ATCC 14579). When cell concentration of bacteria was up to 4×10^7 CFU/mL, 0.5 mL bacteria suspensions were mixed with 20 mL melting LB agar and cooled below 60 °C to prepare the plates. 50 μL sterile solution (H_2O:DMSO 999:1 v/v) in various concentrations was loaded into the wells (diameter: 5 mm) and punched in indicator bacteria plates which were then incubated at 30 °C overnight to observe the growth inhibition effects. Equal amount of sterilized water (contain 0.1% DMSO) was also loaded into indicator bacteria plates as a negative control. The inhibition zone diameter was then measured and recorded.

Acknowledgements

This work was supported by the China Scholarship Council (CSC) and the Deutsche Forschungsgemeinschaft (DFG) within FOR 630 "Biological function of organometallic compounds". The authors are also grateful for technical support by Gerhard Rubner, Silke Bergemann, Anja Wellner (Institute of Pharmacy, Freie Universität Berlin); Dr. Xiaohua Chen (Bacterial Genetics, Institute of Biology, Humboldt University of Berlin) and Prof. Jinpei Zhou (Department of Medicinal Chemistry, China Pharmaceutical University).

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.10.002.

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