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Cobalt−Alkyne Complexes with Imidazoline Ligands as Estrogenic Carriers: Synthesis and Pharmacological Investigations

Miriam Schlenk, Ingo Ott, and Ronald Gust*

Institute of Pharmacy, Freie Universität Berlin, Königin Luise Strasse 2 + 4, 14195 Berlin, Germany

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We synthesized (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxy/hydroxyphenyl)-2-imidazolines (**1** and **2**) as estrogenic carriers for the dicobalthexacarbonyl fragment. The OH-substituted ligand **2** and its related complex **2-**$Co_2(CO)_6$ showed estrogenic activity in a transcriptional assay in ERα-containing MCF-7-2a cells. The cytotoxicity against breast cancer cell lines was more pronounced for the related *O*-methyl derivative **1-**$Co_2(CO)_6$. Further pharmacological investigations were performed on the cellular uptake into MCF-7 and MDA-MB-231 cells, the ability of binding to DNA, and the inactivation by HSA.

Introduction

Dicobalthexacarbonyl complexes with alkyne ligands (cobalt−alkyne complexes) are now accepted as a new class of cytotoxics. These compounds indicated obvious cytotoxic effects in a variety of human tumor cell lines.[1−4] It is of particular importance that some of these complexes were most effective in human breast cancer cells.

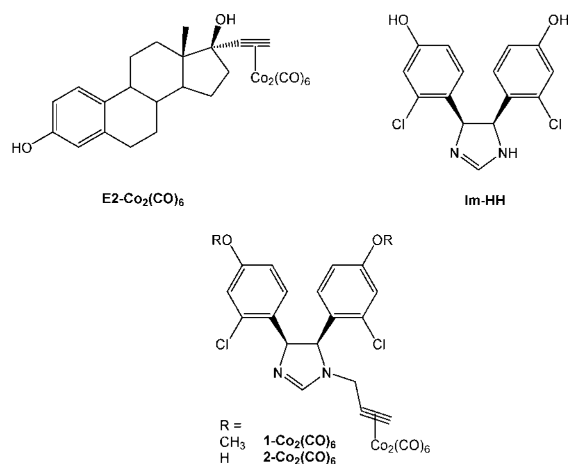
Besides the function as potential cytostatics, cobalt−alkyne complexes gained interest as labeling agents of bioactive molecules. In a previous work,[5] estradiol (E2[a]) was derived with a 17β-alkynyl substituent for coordination to $Co_2(CO)_6$ to yield the complex **E2-**$Co_2(CO)_6$ (Scheme 1). This compound was originally developed as a marker of the estrogen receptor (ER). Despite its voluminous $Co_2(CO)_6$ moiety, the complex exhibited a considerable relative binding affinity to the ER. Analysis of the drug−receptor interaction indicated an inactivation of the ER because of a covalent bond of the cobalt−alkyne complex.[5−7]

These results prompted us to use the $Co_2(CO)_6$ cluster to mediate cytotoxicity to (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline (**Im-HH**), which is an unusual ER ligand (Scheme 1). In prior investigations **Im-HH** indicated only marginal relative binding affinity but remarkable estrogen mediated gene activation. This pharmacological profile as well as molecular modeling studies suggested different binding modes to the ER for **Im-HH** and E2 derivatives.[8−10]

The orientation in the ligand binding domain (LBD)[11] allows the introduction of a propargyl substituent at position N1 of the imidazoline ring to form (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxy/hydroxyphenyl)-2-imidazolines as alkyne ligands for the preparation of cobalt−alkyne complexes (Scheme 1).

The complexes were tested for estrogenic and antiestrogenic activity in comparison to their ligands in ERα-positive MCF-7 cells stably transfected with the reporter plasmid $ERE_{wtc}luc$ (MCF-7-2a cells) for cytotoxicity and cellular uptake in hormone dependent MCF-7 and hormone independent MDA-MB-231 breast cancer cells, while studies on the reactivity toward biomolecules were performed using isolated desoxyribonucleic acid (DNA) as well as isolated human serum albumin (HSA).

Scheme 1[a]



[a] [17β-Alkenylestradiol]dicobalthexacarbonyl (**E2-**$Co_2(CO)_6$) and (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline (**Im-HH**) as lead structures for the development of the novel cobalt−alkyne complexes **1-**$Co_2(CO)_6$ and **2-**$Co_2(CO)_6$.

Results and Discussion

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-chloro-4-methoxyphenyl)-2-imidazoline synthesized as already described[9,10] was converted to the corresponding imidazolinide using NaH in absolute THF. Subsequent reaction with propargyl bromide yielded the *N*-substituted derivative **1** (Scheme 2), which could easily be transformed to the hydroxyl derivative **2** by ether cleavage under mild conditions with BBr_3 at −70 °C. An excess of BBr_3 was methanolized, and the formed trimethoxyborane was removed under reduced pressure. Purification was carried out using column chromatography. In a last step, reaction of the corresponding alkyne ligands **1** and **2** with $Co_2(CO)_8$ in absolute THF (Scheme 2) resulted in the cobalt−alkyne complexes **1-**$Co_2(CO)_6$ and **2-**$Co_2(CO)_6$.[2]

Estrogenic and antiestrogenic activity was measured using MCF-7-2a breast cancer cells.[12] The OH-substituted derivative **2** induced luciferase activation as a measure for ER binding (Figure 1) with $EC_{50} = 0.010$ μM and a relative transcription

* To whom correspondence should be addressed. Phone: (030)83853272. Fax: (030)83856906. E-mail: rgust@zedat.fu-berlin.de.

[a] Abbreviations: HSA, human serum albumin; DNA, desoxyribonucleic acid; ER, estrogen receptor; RTP, relative transcription potency; EC_{50}, half-maximal effective concentration; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; PBS, phosphate buffered saline; RLU, relative light units; AAS, atomic absorption spectroscopy, E2, estradiol.

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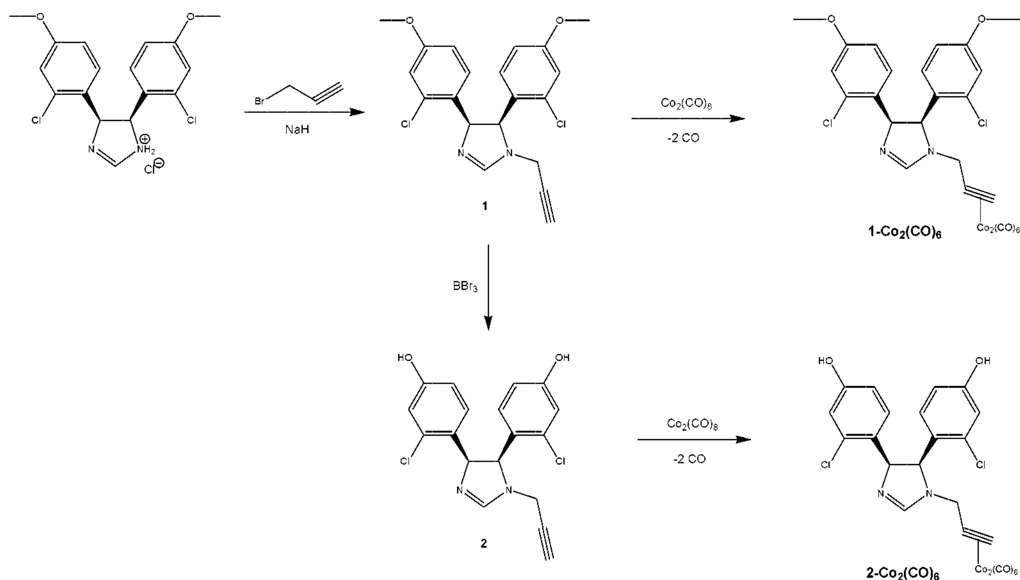
Scheme 2[a]



[a] Synthesis of (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline **1** and (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline **2** and their cobalt−alkyne complexes **1-Co$_2$(CO)$_6$** and **2-Co$_2$(CO)$_6$**.

potency RTP = 0.8%. Despite its $Co_2(CO)_6$ cluster, the complex **2-Co$_2$(CO)$_6$** possessed only marginally lower hormonal potency with EC_{50} = 0.025 μM and RTP = 0.32%. For comparison, the lead structure **Im-HH** showed luciferase activity with EC_{50} = 0.380 μM and RTP = 0.021%.[9] As well-known for **Im-HH** and related compounds, the *O*-methylation abrogated the gene activation. The OMe-substituted alkyne ligand **1** (relative activation at 1 μM: 13%) and its complex **1-Co$_2$(CO)$_6$** (relative activation at 1 μM: 8%) were inactive.[8]

Significant antiestrogenic effects could not be detected for any of the synthesized compounds (Figure 1). At exposure to higher concentrations, the concentration−response curve of **1-Co$_2$(CO)$_6$** significantly decreased. However, this indicated cytotoxic rather than antiestrogenic effects. Therefore, the antiproliferative activity was determined in hormone-dependent MCF-7 and hormone independent MDA-MB-231 breast cancer cells (Figure 2). The ligands **1** and **2** were tested at the maximal applicable concentration of 10 μM (see Figure 5, Supporting Information) and the complexes **1-Co$_2$(CO)$_6$** and **2-Co$_2$(CO)$_6$** in a concentration dependent manner (1, 5, and 10 μM).

Only marginal inhibition of cell proliferation was detected for the free ligands **1** and **2** (see Figure 5, Supporting Information). Attachment to $Co_2(CO)_6$ increased the cytotoxicity. Characteristically, cytotoxic effects were most noticeable for the OMe-substituted complex. **1-Co$_2$(CO)$_6$** even showed cytocidal effects on MDA-MB-231 cells with a T/C_{corr} of −25%. For all tested compounds, cytotoxic activity was stronger in MDA-MB-231 cells than in MCF-7 cells.

Because these effects might be the result of different cellular uptake and accumulation, the intracellular drug level was studied in a concentration and time dependent manner using atomic absorption spectroscopy.

The accumulation of **1-Co$_2$(CO)$_6$** and **2-Co$_2$(CO)$_6$** was determined after 4 h of drug exposure (conc: 1, 2.5, 5, and 10 μM; see Figure 3). Both complexes reached high cellular levels dependent on the substituent in the aromatic ring. The concentrations of the OH-substituted complex **2-Co$_2$(CO)$_6$** were about 3 times higher in both the MCF-7 and MDA-MB 231 cells compared to its more lipophilic OMe derivative **1-Co$_2$(CO)$_6$**. Furthermore, MDA-MB-231 cells accumulated both complexes in higher quantities (**1-Co$_2$(CO)$_6$**: 1.25 fold; **2-Co$_2$(CO)$_6$**: 1.18 fold) than MCF-7 cells. Furthermore, cellular uptake studies revealed a directly proportional correlation of the respective test concentration to the detected intracellular concentration ($r^2 \geq$ 0.99). Several mechanisms for the cellular uptake of **1-Co$_2$(CO)$_6$** and **2-Co$_2$(CO)$_6$** are possible, e.g., the complexes could reach the intracellular area via an active transport without a saturation of the transport system or the cellular uptake could be mediated by different transport mechanisms for each complex.

Time dependent experiments depicted in Figure 4 indicated an increase of cellular uptake within the first 5 h. After this period of time, the intracellular concentration was hold nearly on a constant level.

Unfortunately, cellular uptake, cytotoxicity, and hormonal potency showed no clear correlation. The unexpectedly high estrogenic activity of **2-Co$_2$(CO)$_6$** could be considered as a consequence of the enhanced cellular accumulation in MCF-7 cells. The antiproliferative effects, however, are low ($T/C_{corr} \approx$ 50% at 10 μM after an incubation time of 72 h). *O*-Methylation completely terminated ER binding and lowered the intracellular level but increased the cytotoxicity to $T/C_{corr} \approx 20\%$ (at 10 μM after incubation time of 72 h).

The influence on cell proliferation was more pronounced in the MDA-MB-231 cell line, which is in good agreement with the results from the cellular uptake experiments. Already after 48 h, the maximum of activity was reached. **1-Co$_2$(CO)$_6$** showed even cytocidal effects and reduced the cell mass to $\tau = -25\%$ at a concentration of 10 μM. During the following incubation time (up to 168 h), the cells recuperated, which can be interpreted as development of resistance.

In both cell lines, the cytotoxicity depended on the presence of the $Co_2(CO)_6$ cluster. The ligands were inactive. Therefore, it was necessary to study the binding of **1-Co$_2$(CO)$_6$** and **2-Co$_2$(CO)$_6$** to DNA and HSA to evaluate possible inactivation reactions or preferred target interaction (DNA binding).[13,14]

Interestingly, both complexes showed a comparable strong binding to DNA (**1-Co$_2$(CO)$_6$**: 6.31 ± 0.31 pmol/μg and **2-Co$_2$(CO)$_6$**: 7.51 ± 0.47 pmol/μg) after 4 h. This result documents that DNA binding might be due to a reaction of the cobalt cluster with nucleobases dependent on the presence of a

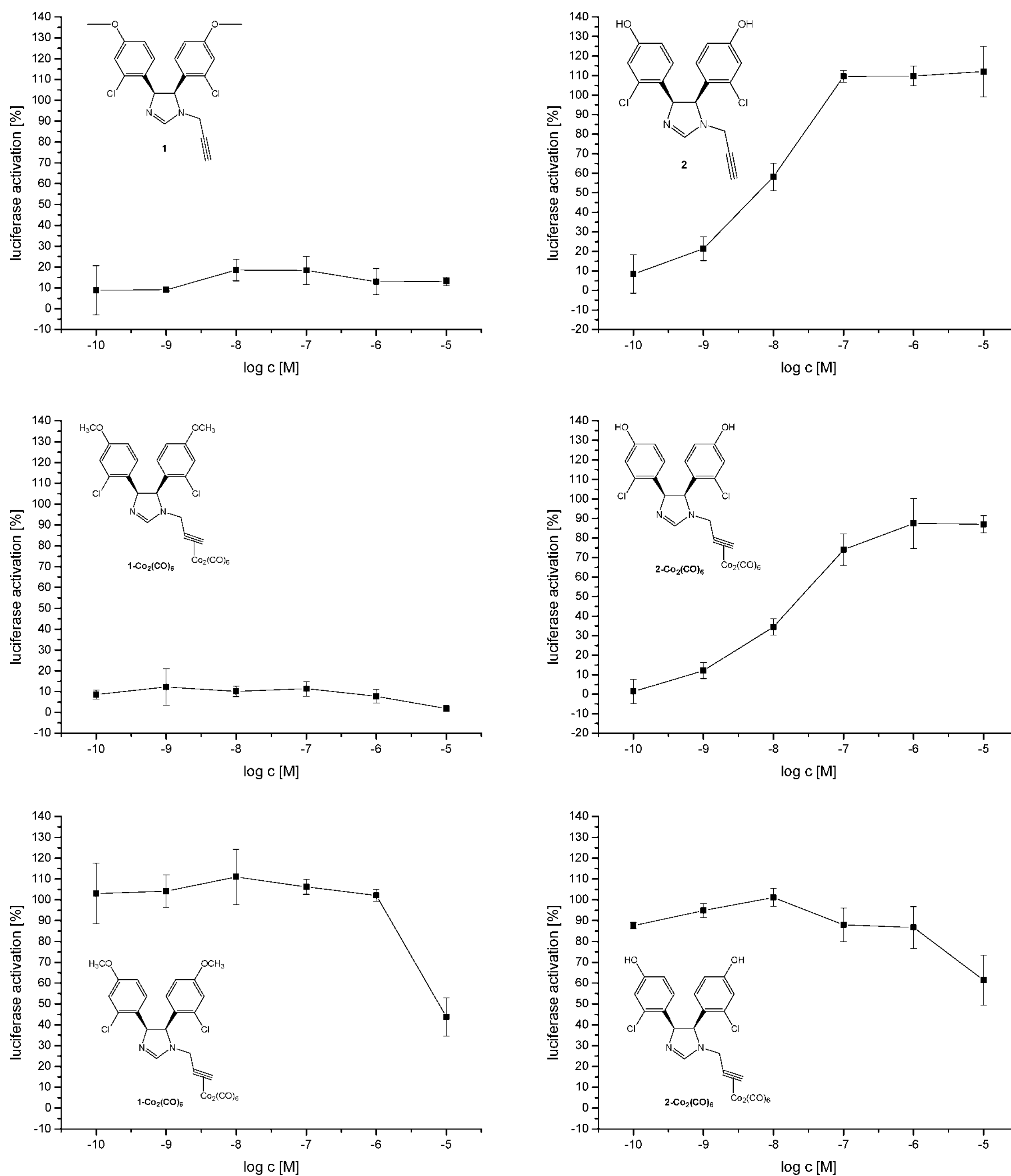


Figure 1. Luciferase expression in ER_{α}-containing MCF-7-2a cells (estrogenic activity: above; antiestrogenic activity: below). For antiestrogenic activity, the compounds were coincubated with 10^{-9} M of estradiol.

alkyne ligand but independent of the substituents at the imidazoline moiety.

After 4 h of incubation with the complexes, HSA was precipitated using ethanol and the bound complex was quantified by AAS measurement of cobalt. The results demonstrated a strong binding to HSA for both complexes (**1-$Co_2(CO)_6$**: 42 ± 6% and **2-$Co_2(CO)_6$**: 68 ± 14%), with a somewhat higher binding tendency of the OH-substituted complex **2-$Co_2(CO)_6$**.

Thus, HSA binding is likely to be caused by interactions of HSA with the $Co_2(CO)_6$ moiety and the imidazoline ligand. These results also implicate that covalent bonds of $Co_2(CO)_6$ and reversible adduct formation is involved in HSA/complex binding.

Despite binding to HSA, there still remains a sufficient concentration of unbound complex (**1-$Co_2(CO)_6$**: 58%; **2-$Co_2(CO)_6$**: 32%) for the interaction with DNA. As the complexes showed comparable binding to DNA but remarkable difference in cytotoxic activity, cytotoxicity of the complexes cannot be solely mediated via DNA interaction.

Conclusion

(4*R*,5*S*)/(4*S*,5*R*)-*N*-Propargyl-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline **2** is a compound with high estrogenic activity. After attachment to dicobalthexacarbonyl, the resulting cobalt−alkyne complex **2-$Co_2(CO)_6$** still showed a remarkable estrogenic activity but low cytotoxic effects. On the contrary, its *O*-methyl derivative **1-$Co_2(CO)_6$** showed no estrogenic activity but strong cytotoxicity. Cytotoxic effects were more distinctive in MDA-MB-231 cells than in MCF-7 cells. Despite the potential function as ER ligands, the cobalt−alkyne complexes enriched in hormone dependent MCF-7 and hormone-independent MDA-MB-231 cells in comparable amount. Therefore, an ER-mediated mode of action can be excluded. Further

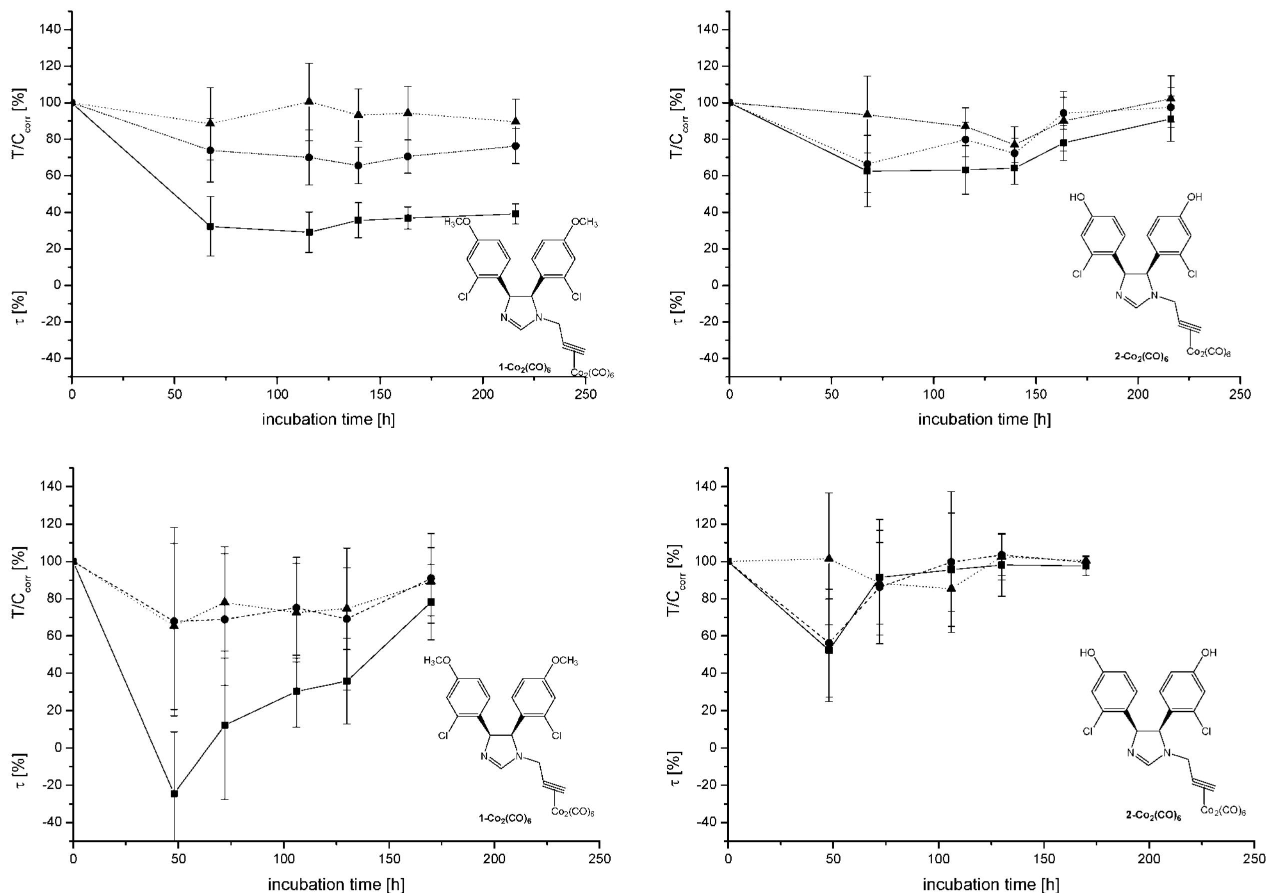


Figure 2. Antiproliferative effects against MCF-7 cells (above) and MDA-MB-231 cells (below): ▲ 1 μM; ● 5 μM; ■ 10 μM.

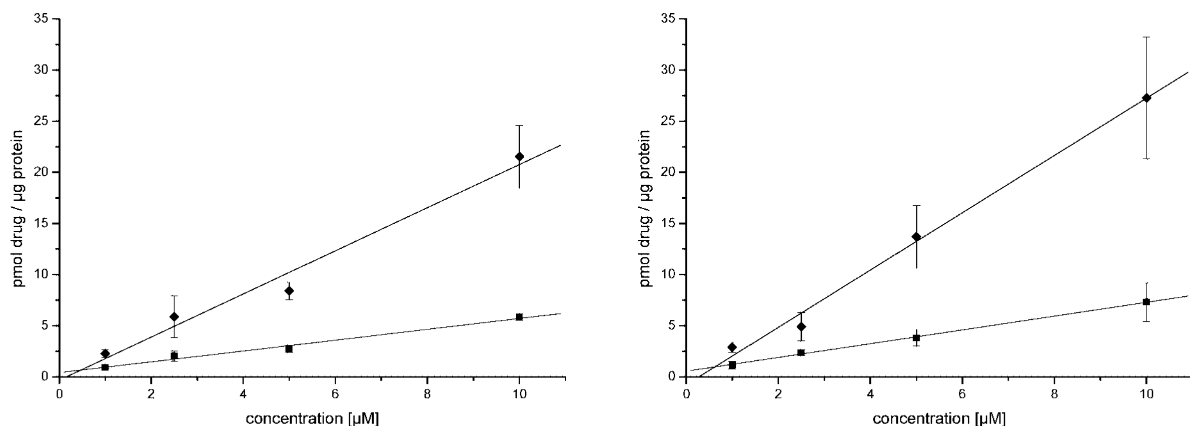


Figure 3. Concentration-dependent cellular uptake of cobalt−alkyne complexes into MCF-7 cells (left) and MDA-MB-231 cells (right): ■ **1-Co$_2$(CO)$_6$**; ◆ **2-Co$_2$(CO)$_6$**.

studies are necessary to determine the real target of [(4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxy/hydroxyphenyl)-2-imidazoline]hexacarbonyldicobalt complexes.

Experimental Section

Synthesis. (4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-chloro-4-methoxyphenyl)-2-imidazoline were synthesized as described earlier.[9,10]

(4*R*,5*S*)/(4*S*,5*R*)-*N*-Propargyl-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline (1). An amount of 3.48 mmol (84 mg) sodium hydride was added to a cooled solution of 1.70 mmol (388 mg) (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline hydrochloride in 25 mL of dry tetrahydrofuran. The mixture was stirred for 15 min and 2.21 mmol of an 80% (m/m) propargyl bromide solution in toluene (328 mg, 238 μL) was added dropwise. The solution was stirred and allowed to warm to room temperature. After 6 h, the reaction was quenched by the slow addition of 15 mL of water. The product was extracted three times with chloroform, the combined organic layers were dried over sodium sulfate, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (mobile phase: chloroform:methanol = 9:1). Yield: 0.98 mmol (382 mg), 58%, yellow oil.

(4*R*,5*S*)/(4*S*,5*R*)-*N*-Propargyl-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline (2). A solution of 0.60 mmol (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline in 20 mL of dry methylene chloride was cooled to −70 °C. At this temperature, 15 mmol (376 mg, 1.41 mL) BBr_3 was added under argon atmosphere. Then the reaction mixture was allowed to warm to room temperature and was stirred for further 48 h. After the reaction was cooled in an ice bath, the surplus of BBr_3 was

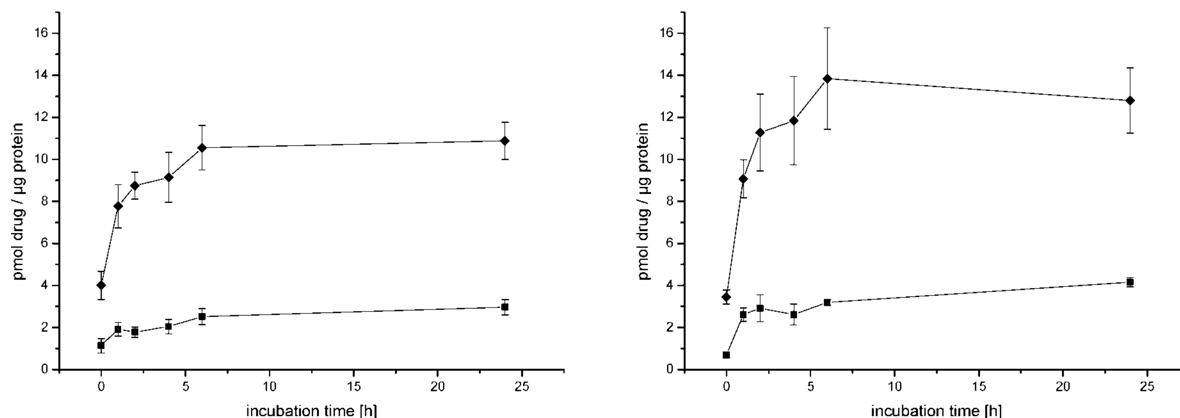


Figure 4. Time-dependent cellular uptake of 5 μM cobalt−alkyne complexes into MCF-7 cells (left) and MDA-MB-231 cells (right): ■ **1-Co$_2$(CO)$_6$**; ◆ **2-Co$_2$(CO)$_6$**.

hydrolyzed three times with dry methanol. The resulting crude product was purified by column chromatography on silica gel (mobile phase: chloroform:methanol = 9:1). Yield: 0.55 mmol (199 mg), 92%, pale-yellow powder (mp: 130 °C).

General Method for the Preparation of Cobalt−Alkyne Complexes. An amount of 0.10 mmol of the respective alkyne derivative was dissolved in 10 mL of dry tetrahydrofuran. The solution was cooled to 0 °C, and 5.00 mmol dicobaltoctcarbonyl was added. The reaction was stirred for 1 h under an argon atmosphere. Subsequently, 1.0 g of silica gel was added and the mixture was evaporated to dryness. The dark-colored products were purified by column chromatography on silica gel.

[(4*R*,5*S*)/(4*S*,5*R*)-*N*-Propargyl-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline]hexacarbonyldicobalt (1-Co$_2$(CO)$_6$). From (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-methoxyphenyl)-2-imidazoline **1** (0.26 mmol, 100 mg). Yield: 0.09 mmol (60 mg), 34%, dark-red oil.

[(4*R*,5*S*)/(4*S*,5*R*)-*N*-Propargyl-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline]hexacarbonyldicobalt (2-Co$_2$(CO)$_6$). From (4*R*,5*S*)/(4*S*,5*R*)-*N*-propargyl-4,5-bis(2-chloro-4-hydroxyphenyl)-2-imidazoline **2** (0.22 mmol, 80 mg). Yield: 0.07 mmol (45 mg), 32%, dark-red oil.

Biological Methods. Estrogenic and antiestrogenic activity,[12,15] cytotoxicity,[16,17] and cellular uptake studies[13,18] as well as DNA- and HSA-binding studies[13,14] were performed according to previously described procedures (for modifications see Supporting Information).

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Supporting Information Available: Elemental and spectroscopic analyses of the target compounds **1**, **2**, **1-Co$_2$(CO)$_6$**, and **2-Co$_2$(CO)$_6$**. Biological Methods: In vitro chemosensitivity assay, transcriptional binding assay; DNA and HSA binding assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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