Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 6889–6898 **6889**
DOI: 10.1021/jm101019j

Synthesis and Biological Activities of Transition Metal Complexes Based on Acetylsalicylic Acid as Neo-Anticancer Agents

Gerhard Rubner,[†] Kerstin Bensdorf,[†] Anja Wellner,[†] Brigitte Kircher,[‡] Silke Bergemann,[†] Ingo Ott,[§] and Ronald Gust*[,†,∥]

[†]*Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Strasse 2 + 4, 14195 Berlin, Germany,* [‡]*Immunobiology and Stem Cell Laboratory, Innsbruck Medical University, Department of Internal Medicine V—Hematology & Oncology, Anichstrasse 35, A-6020 Innsbruck, Austria,* [§]*Institute of Pharmacy, TU Braunschweig, Beethovenstrasse 55, 38106 Braunschweig, Germany, and* [∥]*Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria*

Received February 19, 2010

[(μ^4-η^2)-(Prop-2-ynyl)-2-acetoxybenzoate]dicobalthexacarbonyl (**Co-ASS**), a derivative of aspirin (**ASS**), demonstrated high growth-inhibitory potential against various tumor cells with interference in the arachidonic acid cascade as probable mode of action. The significance of the kind of metal and cluster was verified in this structure−activity study: $Co_2(CO)_6$ was respectively exchanged by a tetrameric cobalt-, trimeric ruthenium-, or trimeric ironcarbonyl cluster. Furthermore, the metal binding motif was changed from alkyne to 1,3-butadiene. Compounds were evaluated for growth inhibition, antiproliferative effects, and apoptosis induction in breast (MCF-7, MDA-MB 231) and colon cancer (HT-29) cell lines and for COX-1/2 inhibitory effects at isolated isoenzymes. Additionally, the major COX metabolite prostaglandin E2 (PGE_2) was quantified in arachidonic acid-stimulated MDA-MB 231 breast tumor cells. It was demonstrated that the metal cluster was of minor importance for effects on cellular activity if an alkyne was used as ligand. Generally, no correlation existed between growth inhibition and COX activity. Cellular growth inhibition and antiproliferative activity at higher concentrations of the most active compounds **Prop-ASS-Co$_4$** and **Prop-ASS-Ru$_3$** correlated well with apoptosis induction.

Introduction

Platinum-based drugs such as cisplatin or oxaliplatin are widely used in the treatment of cancer although they induce strong side effects (e.g., nausea or alopecia) and develop resistance during therapy.[1] Therefore, many groups searched for nonplatinum complexes with another mode of action usable in second-line therapy.[2]

In our group, we intensively investigated **Co-ASS** (formula see Scheme 2), a derivative of aspirin (**ASS**).[3−8] We assumed a selective interference in the arachidonic acid cascade leading to a growth inhibition of cyclooxygenase (COX[a]) dependent tumor cells. Indeed, this organometallic drug was an effective COX inhibitor and showed strong growth-inhibitory effects at mammary, colon, and leukemia cell lines.[3,4] For the induction of strong effects, however, the ligand has to be a derivative of aspirin. The exchange of the 2-acetoxybenzoyl substructure in **Co-ASS** by other nonsteroidal anti-inflammatory drugs (NSAID) strongly reduced the activity.[4] In continuation of this structure−activity relationship study, we tried to consider the significance of the [alkyne]dicobalthexacarbonyl cluster for antitumor activity.

The mode of action of **Co-ASS** is not completely resolved but might involve the inhibition of the COX enzymes probably by CO poisoning the haem iron center.[9] Therefore, the dicobalthexacarbonyl moiety was exchanged in a first step by a higher cobalt- ($Co_4(CO)_{10}$)[10] and related ruthenium- and ironcarbonyl cluster ($Ru_3(CO)_9$; $Fe_3(CO)_9$) to increase the CO proportion compared to the **ASS** ligand.[11,12]

In the next step, the influence of the alkyne bonding was studied on the example of the $Ru_3(CO)_9$ and $Fe_3(CO)_9$ complexes. For this purpose, the alkyne was exchanged by an 1,3-butadiene moiety. Further modifications were the use of but-2-yne-1,4-diyl-bis(2-acetoxybenzoate) (**Di-ASS**) as ligand bearing two ASS and the use of ferrocene as metal unit.

For comparison with **Co-ASS**, all ligands and complexes were tested for growth inhibition of MCF-7, MDA-MB 231, and HT-29 cells for COX inhibitory effects at isolated enzymes (COX-1 and COX-2) as well as arachidonic acid-stimulated breast cancer cells (MDA-MB 231) using the intracellular content of the major metabolite prostaglandin E2 as parameter. Antiproliferative and proapoptotic effects were studied with the most impressive compounds.

Results and Discussion

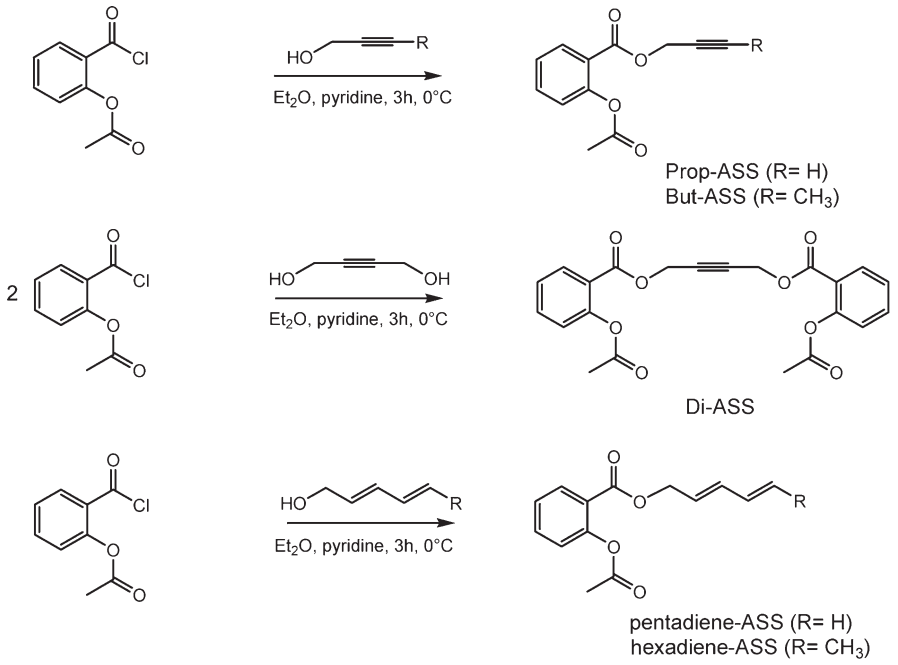
Synthesis and Molecular Characterization. The reaction of alkynoles or alkadienoles and 2-aetoxybenzoyl chloride[13] (ASSCl) at 0 °C gave the respective ester. Pyridine as base bound liberated HCl.

*To whom correspondence should be addressed. Phone: +43 512 507 5245. Fax: +43 512 507 2940. E-mail: ronald.gust@uibk.ac.at. Address: Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria.

[a] Abbreviations: COX, cyclooxygenase; NSAID, nonsteroidal anti-Inflammatory drug; HR-MS, high resolution mass spectrometry; FAB-MS, fast atomic bombardment mass spectrometry; EI-MS, electron ionization mass spectrometry; ESI-TOF MS, electrospray ionization time-of-flight mass spectrometry; TLC, thin layer chromatography; ELISA, enzyme-linked immunoabsorbant assay; PGE_2 prostaglandin E2; DMEM, Dulbecco's Modified Eagle Medium; OD, optical density; ssDNA, single-stranded DNA; PBS, phosphate-buffered saline.

Published on Web 09/21/2010

Scheme 1. Synthesis of Ligands



Penta-2,4-dien-1-ol, which was not commercially available, was synthesized as *E*/*Z* mixture from bromoethylacetate and acroleine via Wittig reaction[14] and subsequent reduction[15] with $LiAlH_4$. Esterification with ASSCl yielded **pentadiene-ASS**.

All metal carbonyl syntheses were carried out under Schlenk conditions using an inert, dry atmosphere. The progress of the reactions could be followed by CO release (Scheme 1).

$Co_4(CO)_{12}$ was freshly prepared by heating an *n*-hexane solution of $Co_2(CO)_8$ for 5 h to reflux.[16] After addition of the appropriate ligand, the dark-blue complexes were formed at room temperature within three days with yields from 48% to 69% (Scheme 2). The building of **Co-ASS** was avoided by using $Co_4(CO)_{12}$ and the ligands in a molar ratio of 1:1 and a reaction temperature not higher than room temperature.[10] Degradation or side products were separated by column chromatography on silica gel.

Tetracobaltdecacarbonyl complexes can be best characterized by IR spectroscopy.[10,16] Gervasio et al. solved the structure of [alkyne]tetracobaltdecacarbonyl in 1984 and demonstrated the direct linkage of the alkyne to two neighboring $Co(CO)_2$ centers. Each Co atom build further bonds to the metals of a $Co_2(CO)_6$ unit with four terminal and two bridged CO ligands. In accordance with this structure, CO bands appeared in the spectra at 1750−1880 cm^{-1} (bridged CO) and about 2000−2100 cm^{-1} (terminal CO).[17]

In the IR spectrum of **Prop-ASS-Co$_4$**, the characteristic C≡C−H vibrations at 3240 (ν(C−H)) and 2126 cm^{-1} (ν(C≡C)) of the unbound ligand are replaced by ethylene-like absorptions at 2958 and 1628 cm^{-1} in the complex. The carbonyl bands appear at 1869 cm^{-1} (bridged) as well as 2033, 2055, and 2097 cm^{-1} (terminal).

Because of the magnetic properties of the cobalt cluster, NMR spectra can only be used for structural characterization with restrictions. The ^{1}H NMR signals of the alkynol or the alkyndiol moiety are broadened, but characteristically shifted to lower field upon complexation (e.g., **Prop-ASS-Co$_4$**: −O-CH_2− from δ = 4.88 to δ = 5.03; ≡C−H from δ = 2.53 to δ = 6.10).

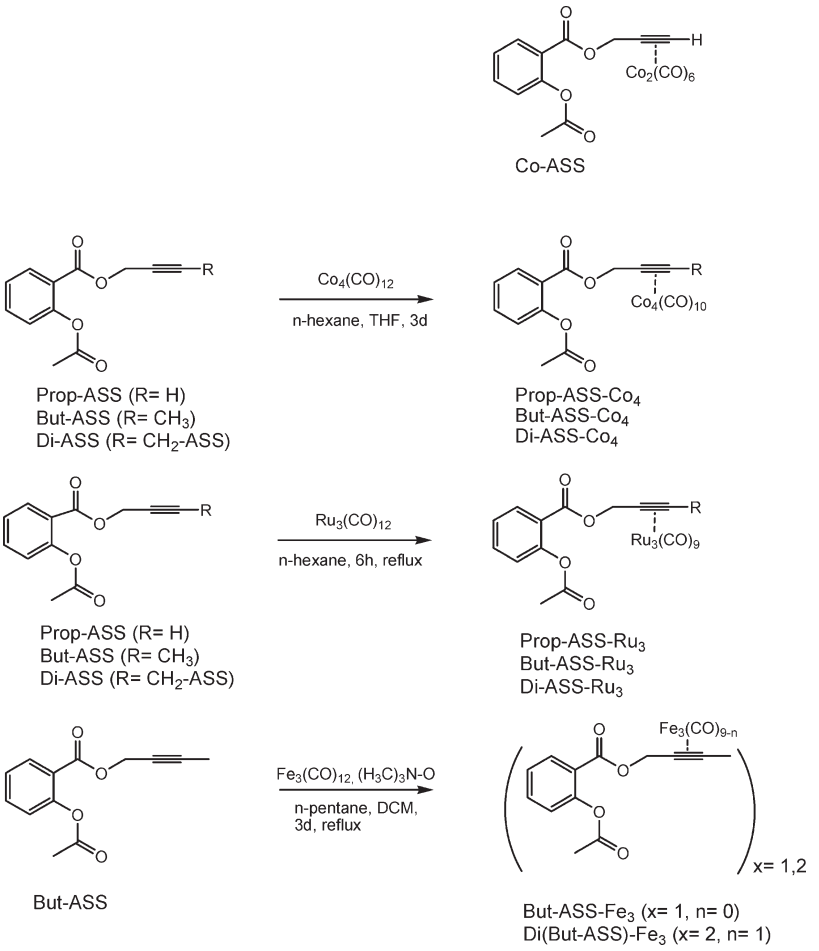
In the ^{13}C NMR spectra of the cobalt complexes, the terminal and bridged carbonyls showed resonances at about 200 ppm, comparable to those described by Lang et al.[18] The O-CH_2 group of Prop-ASS is shifted from 52.73 to 65.5 ppm and the alkyne carbons from 77.52 and 75.44 to 72.2 and 74.9 ppm, respectively. The NMR resonances allow the distinction of **Prop-ASS-Co$_4$** from **Co-ASS** (^{1}H NMR: δ (−O-CH_2−) = 5.47; ^{13}C NMR: δ (−C≡C−H))= 72.88 and 88.42) and excluded a contamination of **Prop-ASS-Co$_4$** with its dicobalthexacarbonyl derivative.

Whereas the formation of [alkyne]tetracobaltdecacarbonyl cluster was accessible at room temperature, $Ru_3(CO)_{12}$ and the respective ligands had to be refluxed in *n*-hexane under argon atmosphere for at least 6 h to obtain the trinuclear ruthenium complexes. The separation of **Prop-ASS-Ru$_3$** and **But-ASS-Ru$_3$** from their reaction mixtures (yields from 6% to 17%) was successful using column chromatography on silica gel.

Their structural assignment to [alkyne]triruthenium nonacarbonyl complexes based on publications from 1970s[19,20] but also on actual studies (e.g., on dicobalt/triruthenium carbonyl clusters[21]). These papers describe the crystal structures of complexes, which consist of a triangular array of ruthenium atoms with an alkyne ligand bound to one face of the cluster in a μ^3,η^2-coordination mode and with the carbon−carbon bond positioned perpendicular to one Ru−Ru edge. At each metal, three CO are terminally bound. In accordance with this structure, the IR spectra exhibited carbonyl bands in the range of 1960−2095 cm^{-1}. No band of a bridged CO near 1878 cm^{-1} as observed in the $Ru_3(CO)_{10}$ cluster was present.[22,23] The latter can be obtained using activated ruthenium cluster (e.g., $Ru_3(CO)_{10}(MeCN)_2$).[23]

In accordance with a $Ru_3(CO)_9$ structure, the IR band of **Prop-ASS-Ru$_3$** and **But-ASS-Ru$_3$** showed bands at 2005−2080 cm^{-1} without a resonance of bridged CO. The ^{1}H NMR spectra of the ruthenium complexes followed the trend of the cobalt complexes. The methylene group in e.g. **Prop-ASS-Ru$_3$** is shifted from 4.88 ppm to 5.47 ppm and the alkyne proton from 2.53 to 6.11 ppm. The resonance

Scheme 2. Synthesis of Alkyne Complexes



of the methyl group in **But-ASS-Ru$_3$** is not as strongly influenced and appeared at 2.66 ppm.

These spectra pointed to the presence of a nonhydrido triruthenium clusters (**Prop-ASS-Ru$_3$**: one proton singlet resonance at 6.11 ppm). It is well-known that the heating of $Ru_3(CO)_{12}$ with alkynes giving oxidative addition of the terminal or an internal hydrogen to the Ru cluster.[22] Therefore, the building of the hydrido-alkynyl complexes **Prop-ASS-Ru$_3$H** and **But-ASS-Ru$_3$H** or the hydrido-allyl complex **allyl-But-ASS-Ru$_3$H** during the synthesis of **Prop-ASS-Ru$_3$** and **But-ASS-Ru$_3$** cannot be excluded (separation as side products by column chromatography is very likely).

If the alkyne in **Prop-ASS** is replaced by a diene moiety (**pentadiene-ASS**), the coordination to $Ru_3(CO)_{12}$ is possible by a η^4-bond.[19] Besides a trinuclear complex, the presence of its mononuclear similarity **pentadiene-ASS-Ru** is possible.[24]

Pentadiene-ASS-Ru$_3$ (Scheme 3) was separated from the reaction mixture by column chromatography. The light-yellow powder which was obtained in low yield (8%) was characterized by IR spectroscopy and high resolution mass spectrometry (HR-MS). The two characteristic bands at 2034 and 1948 cm^{-1} in the IR spectra indicated terminal CO bands but did not allow a differentiation from **pentadiene-ASS-Ru**. However, the MS studies (FAB- and EI-MS) clearly point to a trinuclear complex. Unfortunately, attempts failed to gain structural information by NMR spectroscopy, so a possible rearrangement building a hydrido complex[22] cannot be excluded.

It should be noted that it was impossible to coordinate **hexadiene-ASS** to $Ru_3(CO)_{12}$. Neither decomposition products nor the target compound were detected by thin layer chromatography (TLC).

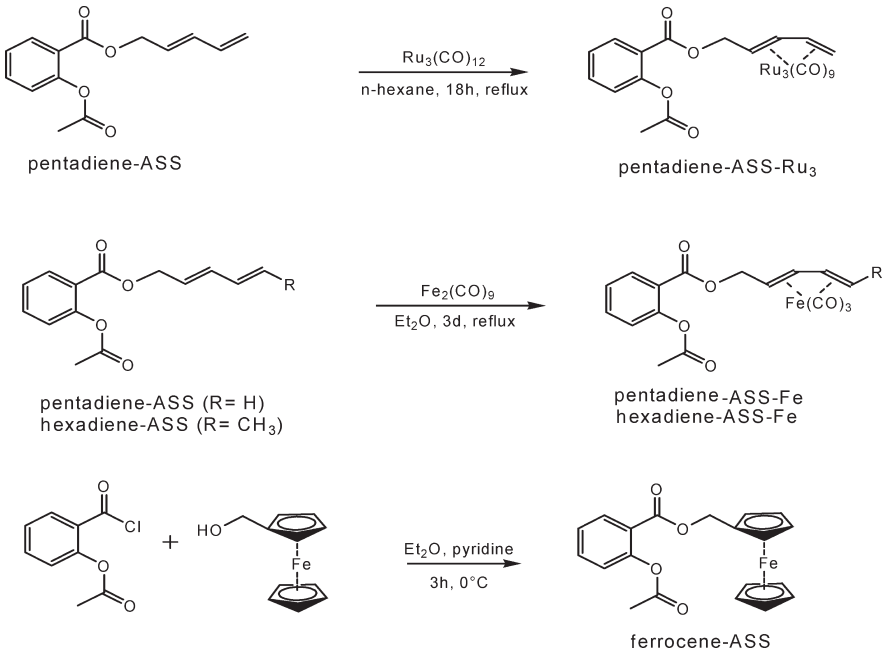
The best route for the synthesis of [alkyne]triironnonacarbonyl complexes was the reaction of $Fe_3(CO)_{12}$ and the appropriate alkyne in low boiling solvents (Scheme 2). However, from the title compounds only **But-ASS-Fe$_3$** could be obtained in small amounts, although trimethylamine-*N*-oxide as catalyst already increased the yields.[25] Furthermore, the attempt failed to optimize the synthesis by variation of the solvent. Only *n*-pentane offered satisfying yields. Nevertheless, it was impossible to get **Prop-ASS-Fe$_3$** and **Di-ASS-Fe$_3$**.

Interestingly, in the case of **But-ASS-Fe$_3$**, a further orange colored complex (**Di(But-ASS)-Fe$_3$**) was isolated from the reaction mixture. MS spectra clearly indicated the presence of two But-ASS ligands at a triironoctacarbonyl cluster.

Triironnonacarbonyl clusters with one alkyne ligand are well-known in the literature.[25] We propose a comparable structure of **But-ASS-Fe$_3$** in which the acetylene moiety of the ligand is perpendicularly oriented over the Fe_3 triangle. In accordance with this structure, the IR spectra exhibited ν(CO) bands at 2061, 2026, and 1986 cm^{-1}.

[η^4-Alkadienyl]irontricarbonyl complexes were accessible via two synthetic routes: They can be obtained by UV irradiation of an *n*-hexane solution of the ligand and $Fe(CO)_5$ in a cooled quartz glass tube[26] or by refluxing the ligand together with $Fe_2(CO)_9$ in diethyl ether.[27] $Fe_2(CO)_9$ was previously produced in glacial acetic acid from $Fe(CO)_5$ by irradiation with UV light.[28] It turned out that the second

Scheme 3. Synthesis of Alkadiene and Ferrocene Complexes



route led to much higher yields and was therefore preferably used.

The Fe coordination can be confirmed by ^{1}H NMR spectroscopy.[29,30] The O-CH_2 group in **hexadiene-ASS** is high field shifted from 5.78 to 4.26 ppm, the methyl group from 1.79 to 1.43 ppm. The most characteristic effects occurred at C2 and C5, with a shift of the isochronic protons from 4.79 ppm to 1.30 ppm (C5−H) and 1.08 ppm (C2−H), and in a much lower extent at C3−H (6.08 to 5.08 ppm) and C4−H (6.29 to 5.27 ppm).

As already described above, **pentadiene-ASS** existed as an *E*/*Z*-mixture and was reacted with $Fe(CO)_5$. From the reaction mixture only the complex of the *E*-isomer was isolated as indicated by the NMR resonances, which were very similar to those of **hexadiene-ASS-Fe** from which the *E*-isomer was used for coordination.

The IR spectra showed two characteristic bands at about 1970 and 2050 cm^{-1} of [η^4-*s*-*cis*-1,3-butadiene]$Fe(CO)_3$.[26] In the case of the [η^4-*s*-*trans*-1,3-butadiene]$Fe(CO)_3$ isomer, these vibrations would appear at about 30 cm^{-1} lower frequencies.[26]

To study the relevance of the carbonyl cluster for biological effects, we combined ASS with a ferrocene moiety by reaction of 2-acetoxybenzoyl chloride with ferrocenyl carbinol (**ferrocene-ASS**) (Scheme 3). Ferrocenyl carbinol can either be produced from ferrocene with formaldehyde and sulphuric acid or by reduction of the commercially available ferrocene carboxaldehyde with $LiAlH_4$.[31]

In Vitro Chemosensitivity Assay. Antitumor activity was determined in vitro using mammary carcinoma (MCF-7 and MDA-MB 231) as well as colon (HT-29) cell lines. Cisplatin and **Co-ASS** were used as reference.

Because all compounds showed their maximum of activity in a time-dependent growth-inhibition test after 72−96 h (data not shown), the IC_{50} value was determined after an incubation time of 72 h.

Co-ASS[5] caused comparable effects to cisplatin against MCF-7 cells (IC_{50} = 1.4 and 2.0 μM, respectively) and was slightly more active against MDA-MB 231 cells (IC_{50} = 1.9 and 3.3 μM, respectively) but less active at the HT-29 cell line (IC_{50} = 9.8 and 2.4 μM, respectively; Table 1).

Table 1. Growth-Inhibitory Effects (IC50 Values [μM]) on Breast Cancer and Colon Carcinoma Cell Lines

compd	MCF-7	MDA-MB 231	HT-29
cisplatin	2.0 ± 0.3	3.3 ± 0.5	2.4 ± 0.4
Co-ASS	1.4 ± 0.3	1.9 ± 0.3	9.8 ± 3.0
aspirin	> 50	> 50	> 50
Prop-ASS-Co_4	1.9 ± 0.2	3.5 ± 0.2	2.7 ± 0.7
But-ASS-Co_4	11.4 ± 0.4	15.0 ± 1.5	11.1 ± 0.0
Di-ASS-Co_4	4.7 ± 0.2	2.1 ± 1.3	8.0 ± 1.3
Prop-ASS-Ru_3	1.4 ± 0.2	2.4 ± 0.0	2.2 ± 0.2
But-ASS-Ru_3	4.1 ± 0.6	17.8 ± 0.0	6.4 ± 0.0
Di-ASS-Ru_3	5.5 ± 0.5	7.6 ± 0.7	10.1 ± 0.1
pentadiene-ASS-Ru_3	10.0 ± 1.7	7.5 ± 0.4	19.8 ± 1.9
pentadiene-ASS-Fe	> 20	> 20	> 20
hexadiene-ASS-Fe	> 20	> 20	> 20
But-ASS-Fe_3	> 20	> 20	> 20
ferrocene-ASS	> 20	> 20	> 20

All ligands as well as aspirin, $Co_4(CO)_{12}$, $CoCl_2$, $Fe(CO)_5$, $FeCl_2$, $Ru_3(CO)_{12}$, and $RuCl_3$ were separately tested up to concentrations of 20−50 μM and demonstrated no inhibitory effects (data not shown).

Prop-ASS-Co_4 and **Co-ASS** induced comparable growth inhibition at the breast cancer cell lines. The stability of the $Co_4(CO)_{10}$ cluster in solution was already described,[10] so a break down into **Co-ASS** can be excluded. This assumption confirmed the results with HT-29 cells at which **Prop-ASS-Co_4** was distinctly more active than **Co-ASS** and even reached the IC_{50} values of cisplatin (Table 1).

Elongation of the spacer length between the [alkyne]cobalt cluster and the ASS moiety drastically reduced the cellular growth. **But-ASS-Co_4** possessed IC_{50} values at all cell lines higher than 10 μM. The related dicobalthexacarbonyl compounds

Table 2. Antiproliferative Activity of **Prop-ASS-Ru$_3$** and **Prop-ASS-Co$_4$** against MDA-MB 231 Cells

compd	conc (μM)	cell proliferation after an incubation time of 24 h (%)	48 h (%)	72 h (%)
control		100	100	100
Prop-ASS-Ru$_3$	5	52.9 ± 8.1	41.9 ± 17.7	39.0 ± 29.0
Prop-ASS-Ru$_3$	10	17.2 ± 1.9	0.6 ± 0.2	0.4 ± 0.3
Prop-ASS-Co$_4$	5	56.1 ± 5.3	57.1 ± 13.2	44.3 ± 2.5
Prop-ASS-Co$_4$	10	37.7 ± 5.9	24.2 ± 39.2	0.9 ± 0.6

were nearly inactive (IC_{50} values of **But-ASS-Co$_2$** and **Di-ASS-Co$_2$** > 20 μM).

A second ASS at the Prop-ASS ligand (**Di-ASS-Co$_4$**) did not increase the cell growth-inhibitory capacity. **Di-ASS-Co$_4$** was comparably active as **Prop-ASS-Co$_4$** at MDA-MB 231 cells but was less active against MCF-7 (IC_{50} = 4.7 μM) and HT 29 cells (IC_{50} = 8.0 μM).

The exchange of the $Co_4(CO)_{10}$ cluster in **Prop-ASS-Co$_4$** by $Ru_3(CO)_9$ (**Prop-ASS-Ru$_3$**) did not change the cell growth-inhibitory properties. For both compounds, nearly identical IC_{50} values were calculated (Table 1). If the same modification was done at **But-ASS-Co$_4$**, the growth inhibition of MCF-7 and HT-29 cells ($IC_{50} \approx 5$ μM) increased. Interestingly, in the case of **Di-ASS-Ru$_3$**, the activity at the MDA-MB 231 cell line was reduced (IC_{50} = 7.6 μM) compared to **Di-ASS-Co$_4$** (IC_{50} = 2.1 μM). The growth inhibition of MCF-7 and MDA-MB 231 cells, however, remained mainly unchanged.

From the data listed in Table 1, it is obvious that an alkyne ligand seems to be essential for high growth-inhibitory activity. **Pentadiene-ASS-Ru$_3$** reduced the growth of breast cancer cells only marginally and was nearly inactive at HT-29 cells. The related iron complexes **pentadiene-ASS-Fe** and **hexadiene-ASS-Fe** and **ferrocene-ASS** were completely inactive.

Generally, the ruthenium and cobalt complexes induced stronger effects than aspirin. Therefore, their effect on the proliferation of MDA-MB 231 and MCF-7 cells was studied on the example of **Prop-ASS-Ru$_3$** and **Prop-ASS-Co$_4$** by thymidine uptake. As shown in Table 2, both compounds time-dependently inhibited the proliferative activity of MDA-MB 231 cells. After a 48 h incubation, 10 μM of **Prop-ASS-Ru$_3$** even completely blocked proliferation. Similar results were observed with MCF-7 cells (data not shown).

COX and PGE$_2$ Assay. Aspirin belongs to the class of NSAIDs, whose pharmacological effects are based on their ability to inhibit cyclooxygenase enzymes. NSAIDs have also attracted attention as novel cytostatics, as clinical studies proved positive therapeutic effects for cancer patients.[32] Furthermore, it was found that the COX-2 isoenzyme is overexpressed in various tumors, and elevated levels of the products of cyclooxygenase (prostaglandins) were detected.

Therefore, we studied the influence of the organometallic compounds for their COX-1 and COX-2 inhibiting profile and quantified the levels of the major COX metabolite prostaglandin E2 (PGE_2) in arachidonic acid-stimulated MDA-MB 231 breast tumor cells by enzyme-linked immunoabsorbant assay (ELISA) (Table 3).

As already described, **Co-ASS** inhibited isolated COX-1 and COX-2 more efficiently than the parent compound aspirin.[4] The preferential inhibition of COX-1 by aspirin was not observed with the metal complex, which inhibited both isoenzymes approximately to the same extent.[4] Modification at the ligand reduced the COX-inhibitory effects as well as the cellular growth-inhibiting capacity.[4] Therefore, it was assumed that interference in the arachidonic acid cascade is part of the mode of action.

Table 3. COX-Inhibitory Effects and PGE_2 Inhibition at a Concentration of 10 μM

compd	COX-1 (%)	COX-2 (%)	PGE_2 (%)
aspirin	29.2 ± 2.0	1.0 ± 0.1	0
Co-ASS	68.0 ± 5.4	63.0 ± 5.0	40.0 ± 5.0
Prop-ASS-Co$_4$	38.8 ± 1.9	39.9 ± 1.6	6.7 ± 0.1
But-ASS-Co$_4$	20.3 ± 1.9	22.0 ± 1.2	5.1 ± 0.3
Di-ASS-Co$_4$	22.0 ± 4.3	22.4 ± 5.5	0
Prop-ASS-Ru$_3$	42.7 ± 0.9	33.0 ± 1.2	*a*
But-ASS-Ru$_3$	35.1 ± 1.1	13.8 ± 2.3	0
Di-ASS-Ru$_3$	67.3 ± 9.4	23.6 ± 1.8	29.5 ± 1.8
pentadiene-ASS-Ru$_3$	87.7 ± 1.6	56.3 ± 10.7	*a*
pentadiene-ASS-Fe	33.7 ± 3.3	5.2 ± 0.4	28.5 ± 1.7
hexadiene-ASS-Fe	16.1 ± 1.3	0	24.6 ± 4.2
But-ASS-Fe	11.5 ± 1.3	2.5 ± 0.1	21.9 ± 1.5
ferrocene-ASS	25.5 ± 1.3	0	0

[a] Not evaluable because of too high growth-inhibitory effects.

To confirm this hypothesis, the COX-1 and COX-2 inhibitory potential of all organometallic compounds synthesized in this study was determined by ELISA at isolated enzymes and in a cellular system using MDA-MB 231 cells (COX-1) by PGE_2 assay.

At 10 μM, **Co-ASS** inhibited both cyclooxygenase enzymes to 68% (COX-1) and 63% (COX-2), respectively. Therefore, 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. The missing activity of all ligands at both isoenzymes documented the relevance of the metal cluster for COX-inhibitory effects.

The kind of cluster is also of greater importance than the number of CO ligands. The [alkyne]$Co_4(CO)_{10}$ or [alkyne]-$Ru_3(CO)_9$ complexes mediated lower COX inhibition than Co-ASS. Only Di-ASS-Ru$_3$ reached at COX-1 the same efficacy (67.3%) as **Co-ASS**.

It might be possible that the size of the higher cluster hindered the attachment of the drug to the active site so poisoning of the haem inside the enzyme did not occur. Nevertheless, acetylation of amino acids at the entrance channel comparable to **Co-ASS** (Lys 346) might be possible[33] but was not yet investigated in detail.

Pentadiene-ASS-Ru$_3$ was identified as most COX-active compound among the new complexes. It was comparably active at COX-2 and even more active then **Co-ASS** at COX-1 (87.7% at 10 μM). Exchange of the $Ru_3(CO)_9$ cluster by $Fe(CO)_3$ drastically reduced the inhibitory effects (see Table 3). The same is true if ferrocene (**ferrocene-ASS**) is used as metal moiety.

The investigations on the PGE_2 level in MDA-MB 231 cells, however, has to be interpreted with caution because the compounds reduced the cell number at a concentration of 10 μM and in some cases even no cellular growth was detected after 24 h anymore. Only **Di-ASS-Ru$_3$**, **pentadiene-ASS-Fe**, and **hexadiene-ASS-Fe** suppressed the PGE_2 level by about 20−30%.

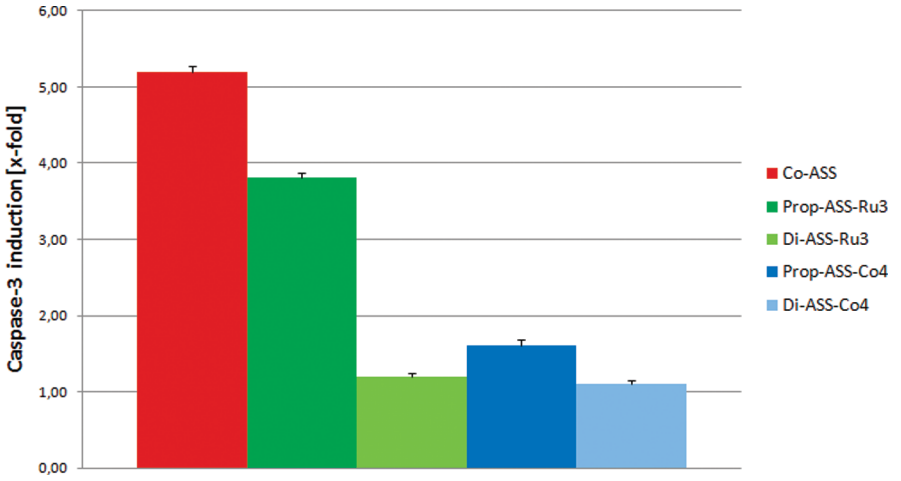


Figure 1. Caspase-3 activity compared to untreated control (drug concentration 10 μM) of selected compounds.

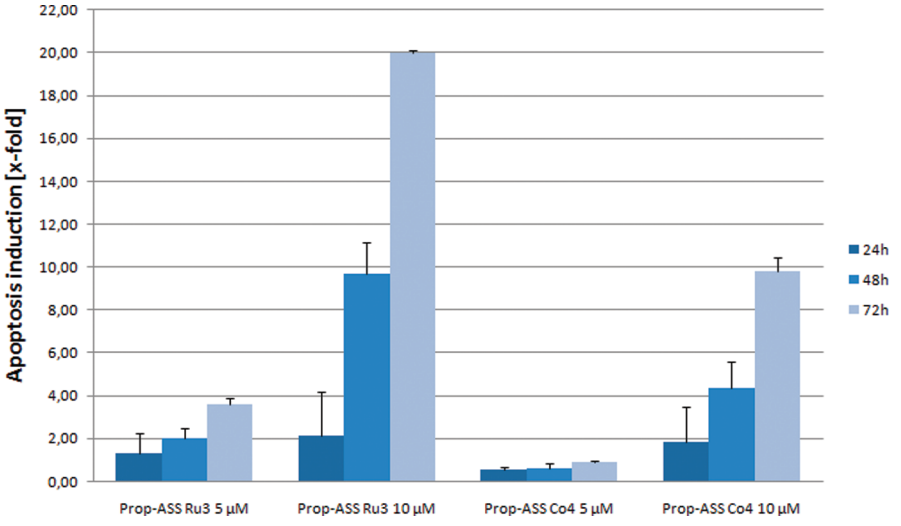


Figure 2. Time-dependent apoptosis induction by **Prop-ASS-Ru$_3$** and **Prop-ASS Co$_4$**. Apoptosis in the absence of the compounds was set at 1.

Antiapoptotic Activity. Since it is well-known that COX inhibition can induce cell cycle arrest and apoptosis,[34] we studied apoptosis induction by activation of caspase-3 in MDA-MB 231 and by single-stranded DNA (ssDNA) formation as well as acridine orange staining in MDA-MB 231 and MCF-7 cells.

The reference **Co-ASS** strongly activated programmed cell death indicated by a 5.19 times higher caspase-3 level after a 24 h incubation compared to untreated cells.[8] This effect depended on the metal cluster. The $Co_4(CO)_{10}$ and $Ru_3(CO)_9$ analogues were less effective. Interestingly, **Prop-ASS-Ru$_3$** showed medium effectiveness compared to **Co-ASS** and **Prop-ASS-Co$_4$** (see Figure 1). The attachment of a second ASS moiety at the Prop-ASS did not increase the apoptosis induction of the Co and Ru complexes.

Apoptosis induction was also studied with the most active compounds **Prop-ASS-Ru$_3$** and **Prop-ASS** by formation of ssDNA in MDA-MB 231 and MCF-7 cells. As shown in Figure 2, both compounds induced apoptosis time- and dose-dependently, which was more pronounced, and in good agreement with the proliferation experiments, after treatment with **Prop-ASS-Ru$_3$**. Similar effects were induced in MCF-7 cells (data not shown).

An acridine orange-staining method was used for visualization of apoptotic processes in MDA-MB 231 and MCF-7 cells. Monolayers of the cells incubated for 24 h with 10 μM of the complexes were stained with the dye and thereafter immediately analyzed under a fluorescence microscope. In accordance with the results shown above, both compounds induced growth inhibition and caused morphological changes (cell blebbing and formation of apoptotic bodies), whereas **Prop-ASS-Ru$_3$** was again more effective than **Prop-ASS Co$_4$** at MDA-MB 231 (Figure 3) and MCF-7 (Figure S1, Supporting Information) cells.

Summary and Outlook

[(μ^4-η^2)-(Prop-2-ynyl)-2-acetoxybenzoate]dicobalthexacarbonyl (**Co-ASS**) was synthesized in 1997 by Jung et al. among a series of organometallic compounds and documented outstanding in vitro activity. The molecule contains on the one hand a organometal cluster and on the other hand in the organic component aspirin, an effective inhibitor of cyclooxygenases. Both parts can interfere with intracellular pathways leading to growth inhibition of tumor and leukemia cells. In this study, we demonstrated that **Co-ASS** represents the ideal composition of ASS and the [alkyne]$Co_2(CO)_6$ moiety. Higher carbonyl cluster of cobalt or ruthenium did not increase the activity. The use of iron cluster even completely terminated the growth-inhibitory capacity.

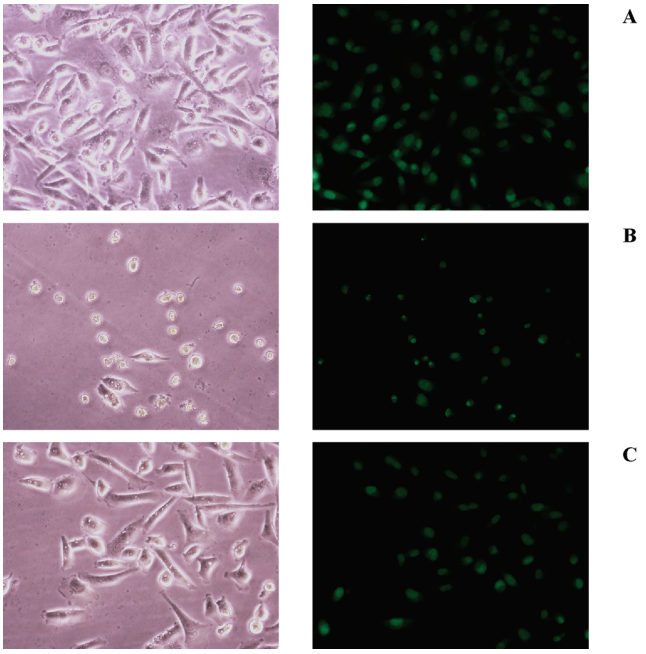


Figure 3. MDA-MB 231 cells stained by acridine orange and observed under fluorescence microscopy (left, phase contrast microscopy; right, fluorescence microscopy; 20× magnification). (A) Cells without treatment (control: DMSO). (B) Cells incubated with 10 μM of **Prop-ASS-Ru$_3$**. (C) Cells incubated with 10 μM of **Prop-ASS-Co$_4$**.

Furthermore, the assumed mode of action, the interference in the arachidonic acid cascade, might be of minor relevance. **Prop-ASS-Ru$_3$** and **Prop-ASS-Co$_4$** showed lower COX-inhibitory effects but inhibited tumor-cell growth comparable to **Co-ASS**. Vice versa, **pentadiene-ASS-Ru** as most potent COX-inhibitor was only marginally less active. In this context, it is of great interest to look for the mode of action at the COX enzymes and the acetylation profile compared to **Co-ASS** and aspirin. These investigations are in progress and part of a forthcoming paper.

Experimental Section

Syntheses. General. Commercially available chemicals were used without further purification. Solvents are purified by distillation from an appropriate drying agent: tetrahydrofuran, diethyl ether, toluene, *n*-pentane, and *n*-hexane were dried over sodium/potassium alloy and distilled under argon atmosphere. Pyridine was dried over KOH and dichloromethane over P_2O_5, and both were stored over molecular sieves (4 and 3 Å, respectively). Products were purified by flash chromatography on silica gel (230−400 mesh, Merck). Melting points: 510 Büchi (Flawil, Swiss) capillary melting point apparatus. IR spectra (KBr pellets): Perkin-Elmer model 580 A (Rodgau-Jügesheim, Germany). ^{1}H and ^{13}C NMR: Avance DPX-400 spectrometer (Bruker; Karlsruhe, Germany) at 400 MHz (^{1}H) or 100 MHz (^{13}C), Avance III-700 spectrometer (Bruker) at 700 MHz (^{1}H) or 175 MHz (^{13}C) with TMS as internal standard. Elemental analyses: Microlaboratory of the Freie Universität Berlin on Perkin-Elmer 240C. Purity of all synthesized substances was confirmed by elemental analysis or HR-MS. All complexes reported in the manuscript have a purity >95%. MS and HR-MS spectra: Finnigan MAT 711 (EI, 70 eV), MAT CH7A (EI, 80 eV, 3 kV), CH5DF (FAB, 80 eV, 3 kV), and Agilent ESI-TOF 6210 (4 μL/min, 1 bar, 4000 V). Microplate reader: FLASHscan S12 (AnalytikJena AG, Jena, Germany).

Synthesis. $Co_4(CO)_{12}$,[16] $Fe_3(CO)_{12}$,[35] trimethylamine-*N*-oxide,[36] 2-acetoxybenzoyl chloride (ASSCl),[37] ferrocenylcarbinol,[31] and *E*/*Z*-penta-2,4-dien-1-ol[14,15] were prepared by literature procedures.

$Fe_2(CO)_9$ was prepared by a slightly modified literature procedure[28] using a quartz Schlenk tube, starting with 25 mL of $Fe(CO)_5$, 150 mL of glacial acetic acid, and 10 mL of acetic anhydride (the latter was added to prevent the mixture containing too much water). A mercury-vapor lamp Philips HPL-N 250W was used for irradiation under cooling with a continuous stream of air. Filtration, washing with water, ethanol, diethyl ether, and subsequently drying in vacuo gave $Fe_2(CO)_9$ usually in more than 90% yield. All complexations were carried out in dry and degassed solvents under pure argon atmosphere.

General Method for the Preparation of 2-Acetoxybenzoic Acid Esters. 2-Acetoxybenzoyl chloride (ASSCl, 10−20 mmol) dissolved in diethyl ether was added dropwise at 0 °C to an etherous solution containing the respective alcohol (10−25 mmol) and 10 mL of pyridine as base. To ensure the completion of the reaction, the solution was stirred for 2−3 h at room temperature. In a separating funnel, the organic layer was washed with 1 N HCl and afterward with saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4, and the solvent was removed in vacuo. Purification was done by flash column chromatography on silica gel.

(Prop-2-ynyl)-2-acetoxybenzoate (Prop-ASS). Prop-2-yn-1-ol, 1.30 g (23.2 mmol); ASSCl, 3.98 g (20.0 mmol). Column chromatography: petroleum ether/diethyl ether = 3:1. Yield: 3.66 g (84%) as white crystals (mp 75.4 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.35 (s, 3H, O=C-C**H$_3$**), 2.53 (t, ^4J = 2.4 Hz, 1H, ≡C-**H**), 4.88 (d, ^4J = 2.4 Hz, 2H, C**H$_2$**-C≡C), 7.11 (d, ^3J = 8.1 Hz, 1H, 3′-H), 7.33 (ddd, ^3J = 7.6 Hz, ^3J = 7.6 Hz, ^4J = 0.8 Hz, 1H, 5′-H), 7.56 (ddd, ^3J = 7.8 Hz, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H, 4′-H), 8.01 (dd, ^3J = 7.8 Hz, ^4J = 1.4 Hz, 1H, 6′-H).

(But-2-ynyl)-2-acetoxybenzoate (But-ASS). But-2-yn-1-ol, 1.55 g (22.1 mmol); ASSCl, 4.01 g (20.2 mmol). Column chromatography: petroleum ether/diethyl ether = 5:1. Yield: 4.18 g (89%) as white crystals (mp 45.3 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.88 (t, ^5J = 2.4 Hz, 3H, ≡C−C**H$_3$**), 2.36 (s, 3H, O=C−C**H$_3$**), 4.84 (q, ^5J = 2.4 Hz, 2H, O−C**H$_2$**-C≡), 7.11 (dd, ^3J = 8.1 Hz, ^4J = 0.9 Hz, 1H, 3′-H), 7.33 (ddd, ^3J = 7.8 Hz, ^3J = 7.6 Hz, ^4J = 0.9 Hz, 1H, 5′-H), 7.54 (ddd, ^3J = 7.9 Hz, ^3J = 7.6 Hz, ^4J = 1.5 Hz, 1H, 4′-H), 8.03 (dd, ^3J = 7.9 Hz, ^4J = 1.7 Hz, 1H, 6′-H).

(But-2-yne-1,4-diyl)-bis(2-acetoxybenzoate) (Di-ASS). But-2-yne-1,4-diol, 0.88 g (10.2 mmol); ASSCl, 4.21 g (21.2 mmol). Column chromatography: *n*-hexane/ethyl acetate = 5:1. Yield: 3.86 g (92%) as white crystals (mp 108.2 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.37 (s, 6H, O=C−C**H$_3$**), 4.94 (s, 4H, O−C**H$_2$**-C≡), 7.12 (dd, ^3J = 8.1 Hz, 2H, 3′-H), 7.30 (ddd, ^3J = 7.7 Hz, ^3J = 7.5 Hz, 2H, 5′-H), 7.59 (ddd, ^3J = 7.9 Hz, ^3J = 7.6 Hz, ^4J = 1.5 Hz, 2H, 4′-H), 8.07 (dd, ^3J = 7.9 Hz, ^4J = 1.5 Hz, 2H, 6′-H).

((*E*,*Z*)-Penta-2,4-dienyl)-2-acetoxybenzoate (pentadiene-ASS). Penta-2,4-dien-1-ol, 1.69 g (20.1 mmol); ASSCl, 4.12 g (20.7 mmol). Column chromatography: petroleum ether/diethyl ether (5:1). Yield: 2.4 g (48%) cis and 1.8 g (37%) trans as mixture (colorless oil). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.65−2.50 (m, 2H, −C=C**H$_2$**), 2.32 (s, 3H, O=C-C**H$_3$**), 4.28−4.80 (d, 2H, ^3J = 6.0 Hz, −O-C**H$_2$**-CH=), 5.00−6.36 (m, 3H, −**H**C=C**H**-C**H**=C), 7.09 (dd, ^3J = 8.1 Hz, ^4J = 1.0 Hz, 1H, 3′-H), 7.30 (ddd, ^3J = 7.7 Hz, ^3J = 7.5 Hz, ^4J = 1.0 Hz, 1H, 5′-H), 7.54 (ddd, ^3J = 7.8 Hz, ^3J = 7.7 Hz, ^4J = 1.5 Hz, 1H, 4′-H), 8.05 (dd, ^3J = 7.8 Hz, ^4J = 1.6 Hz, 1H, 6′-H).

((2*E*,4*E*)-Hexa-2,4-dienyl)-2-acetoxybenzoate (hexadiene-ASS). (2*E*,4*E*)-Hexa-2,4-dien-1-ol, 1.82 g (18.6 mmol);ASSCl, 3.98 g (20.0 mmol). Column chromatography: petroleum ether/diethyl ether = 5:1. Yield: 3.77 g (78%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.78 (d, ^3J = 6.8 Hz, 3H, =CH-C**H$_3$**), 2.32 (s, 3H, CO−O-C**H$_3$**), 4.80 (d, ^3J = 6.7 Hz, 2H, −O-C**H$_2$**-CH=), 5.74 (m, 2H, -C=C**H**-C**H**=C), 6.08 (m, 1H, =C**H**-CH_3), 6.28 (m, 1H, CH_2-C**H**=C−), 7.11 (dd, ^3J = 8.1 Hz, 1H, 3′-H), 7.30 (ddd, ^3J = 7.5 Hz, ^3J = 7.7, ^4J = 0.9 Hz, 1H, 5′-H), 7.54 (ddd, ^3J = 7.9 Hz, ^3J = 7.6 Hz, ^4J = 1.7 Hz, 1H, 4′-H), 8.02 (dd, ^3J = 7.7 Hz, ^4J = 1.7 Hz, 1H, 6′-H).

General Method for the Preparation of (μ^4-η^2)-(Alkynyl)-tetracobaltdecacarbonyl. In a typical preparation, 410 mg of dicobaltoctacarbonyl ($Co_2(CO)_8$, 1.2 mmol) were dissolved in 50 mL of dry *n*-hexane in a 100 mL Schlenk tube. The solution was degassed to remove any oxygen and afterward fitted with a bubble counter. The solution was refluxed for 5 h to produce $Co_4(CO)_{12}$. After cooling to room temperature, 0.6 mmol of the corresponding alkyne were added. Then the solution was allowed to stir for three days with TLC control. The solvent was removed under vaccuo and purified by flash chromatography on silica gel.

[(μ^4-η^2)-(Prop-2-ynyl)-2-acetoxybenzoate]tetracobaltdecacarbonyl (Prop-ASS-Co$_4$). Prop-ASS: 130 mg (0.6 mmol). Column chromatography: petroleum ether/diethyl ether = 10:1. Yield: 280 mg (64%) of dark-blue oil. IR (KBr, cm^{-1}): 2097, 2055, 2033 (terminal Co-CO), 1870 (bridged Co-CO), 1727, 1768 (C=O), 1629 (C=C), 1250, 1200 (C−O). ^{1}H NMR ($CDCl_3$, 700 MHz): δ = 2.38 (br, 3H, O=C-C**H$_3$**), 5.03 (br, 2H, −O-C**H$_2$**—), 6.10 (br, 1H, C≡C**H**), 7.32 (br, 1H, 3′-H), 7.63 (br, 1H, 5′-H), 8.05 (br, 1H, 4′-H), 8.42 (br, 1H, 6′-H).

[(μ^4-η^2)-(But-3-ynyl)-2-acetoxybenzoate]tetracobaltdecacarbonyl (But-ASS-Co$_4$). But-ASS: 140 mg (0.6 mmol). Column chromatography: petroleum ether/diethyl ether = 15:1. Yield: 310 mg (69%) of blue crystals. IR (KBr, cm^{-1}): 2093, 2053, 2027 (terminal Co-CO), 1871 (bridged Co-CO), 1727 (C=O), 1629 (C=C), 1255, 1199 (C−O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.28 (br, 3H, O=C-C**H$_3$**), 2.76 (s, 3H, ≡C-C**H$_3$**), 5.02 (br, 2H, −O-C**H$_2$**-C≡C), 7.12 (br, 1H, 3′-H), 7.30 (br, 1H, 5′-H), 7.57 (br, 1H, 4′-H), 8.00 (br, 1H, 6′-H).

[(μ^4-η^2)-(But-2-yne-1,4-diyl)-bis(2-acetoxybenzoate)]tetracobaltdecacarbonyl (Di-ASS-Co$_4$). Di-ASS: 240 mg (0.6 mmol). Column chromatography: petroleum ether/diethyl ether = 5:1. Yield: 260 mg (48%), blue crystals. IR (KBr, cm^{-1}): 2097, 2058, 2035 (terminal Co-CO), 1868 (bridged Co−CO), 1769, 1725 (C=O), 1607 (C=C), 1249, 1197 (C−O). ^{1}H NMR ($CDCl_3$): δ = 2.35 (br, 6H, O=C-C**H$_3$**), 5.49 (br, 4H, −O-C**H$_2$**-C≡), 7.16 (br, 2H, 3′-H), 7.35 (br, 2H, 5′-H), 7.60 (br, 2H, 4′-H), 8.05 (br, 2H, 6′-H).

General Method for the Preparation of [(μ^3-η^2)-(Alkynyl)]-tririutheniumnonacarbonyl. An excess of 0.2 mol of the corresponding alkyne was treated with $Ru_3(CO)_{12}$ in 100 mL of dry *n*-hexane in a 250 mL Schlenk tube. The solution was degassed to remove any oxygen and afterward fitted with a bubble counter. The solution was refluxed for 3 h. After cooling to room temperature, *n*-hexane was removed under vaccuo and the residue was purified by flash chromatography on silica gel.

[(μ^3-η^2)-(Prop-2-ynyl)-2-acetoxybenzoate]tririutheniumnonacarbonyl (Prop-ASS-Ru$_3$). Prop-ASS, 130 mg (0.6 mmol); $Ru_3(CO)_{12}$, 260 mg (0.41 mmol). Column chromatography: petroleum ether/diethyl ether = 5:1. Yield: 45 mg (14%) of orange oil. IR (KBr, cm^{-1}): 3071 (C≡C-H); 2080, 2049, 2011 (Ru−CO); 1729 (C=O); 1609 (C=C); 1286, 1198 (C−O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.36 (s, 3H, O=C-C**H$_3$**), 5.47 (br, 2H, −O-C**H$_2$**-C≡), 6.11 (br, 1H, C≡C-**H**), 7.13 (d, ^3J = 8.1 Hz, 1H, 3′-H), 7.31 (br, 1H, 5′-H), 7.58 (br, 1H, 4′-H), 8.10 (br, 1H, 6′-H). Anal. ($C_{21}H_{10}O_{13}Ru_3$) C, H.

[(μ^3-η^2)-(But-3-ynyl)-2-acetoxybenzoate]tririutheniumnonacarbonyl (But-ASS-Ru$_3$). But-ASS, 150 mg (0.65 mmol); $Ru_3(CO)_{12}$, 280 mg (0.44 mmol). Column chromatography: petroleum ether/diethyl ether = 10:1. Yield: 60 mg (17%) of orange powder. IR (KBr, cm^{-1}): 2080, 2050, 2006 (Ru−CO); 1769, 1725 (C=O); 1608 (C=C); 1253, 1197 (C−O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.36 (s, 3H, O=C-C**H$_3$**), 2.66 (s, 3H, C≡C-C**H$_3$**), 5.49 (s, 2H, −O-C**H$_2$**-C≡), 7.14 (d, ^3J = 8.0 Hz, 1H, 3′-H), 7.31 (dd, ^3J = 7.6 Hz, ^3J = 7.6 Hz, 1H, 5′-H), 7.58 (dd, ^3J = 7.5 Hz, ^3J = 7.1 Hz, 1H, 4′-H), 8.08 (d, ^3J = 7.6 Hz, 1H, 6′-H). Anal. ($C_{22}H_{12}O_{13}Ru_3$) C, H.

[(μ^3-η^2)-(But-2-yne-1,4-diyl)-bis(2-acetoxybenzoate)]tririutheniumnonacarbonyl (Di-ASS-Ru). Di-ASS, 410 mg (1.0 mmol); $Ru_3(CO)_{12}$, 420 mg (0.66 mmol). Column chromatography: petroleum ether/diethyl ether = 3:1. Yield: 35 mg (5.5%) of orange crystals. IR (KBr, cm^{-1}): 2086, 2058, 2023, 1947 (Ru−CO); 1767, 1727 (C=O); 1608 (C=C); 1251, 1198 (C−O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.37 (s, 6H, O=C-C**H$_3$**), 5.49 (s, 4H, −O-C**H$_2$**-C≡), 7.12 (d, ^3J = 8.0 Hz, 2H, 3′-H), 7.28 (dd, ^3J = 8.0 Hz, ^3J = 7.5 Hz, 2H, 5′-H), 7.57 (ddd, ^3J = 7.9 Hz, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 2H, 4′-H), 8.07 (dd, ^3J = 7.9 Hz, ^4J = 1.5 Hz, 2H, 6′-H). Anal. ($C_{31}H_{18}O_{17}Ru$) C, H.

[(μ^3-η^4)-(Penta-2,4-dienyl)-2-acetoxybenzoate]tririutheniumnonacarbonyl (Pentadiene-ASS-Ru$_3$). In a 500 mL Schlenk tube, 400 mg (0.63 mmol) of $Ru_3(CO)_{12}$ and 250 mg (1.0 mmol) of **pentadiene-ASS** were dissolved in 350 mL of dry *n*-hexane. The solution was degassed as described above and subsequently refluxed for 18 h. TLC was used to control the progress of the reaction. After cooling to room temperature, *n*-hexane was removed in vacuo and the residue was purified by column chromatrography on silica gel using petroleum ether/diethyl ether = 3:1. Yield: 40 mg (8%) of light-yellow powder. IR (KBr, cm^{-1}): 3061, 3019 (C=C-H); 2034, 1948 (Ru−CO); 1744 (C=O); 1610, 1592 (C=C); 1296, 1258 (C−O). Anal. ($C_{23}H_{14}O_{13}Ru_3$) C, H.

General Method for the Preparation of [η^4-Alkadienyl]-irontricarbonyl. In a typical preparation 1.1 g of $Fe_2(CO)_9$ (3 mmol) and the corresponding diene (2 mmol) were dissolved in 50 mL of dry diethyl ether in a 100 mL Schlenk tube. The solution was degassed as described above and fitted with a bubble counter. The solution was refluxed for 3 days with TLC control. After cooling to room temperature, the solvent was removed in vaccuo and purified by flash chromatography on silica gel to receive light-yellow powders.

[η^4-(Penta-2,4-dienyl)-2-acetoxybenzoate]irontricarbonyl (Pentadiene-ASS-Fe). Pentadiene-ASS, 500 mg (2.0 mmol); $Fe_2(CO)_9$, 1.0 g (2.8 mmol). Column chromatrography: petroleum ether/diethyl ether = 10:1. Yield: 60 mg (10%) of light-yellow powder. IR (KBr, cm^{-1}): 3069, 3011 (C=C-H); 2051, 1978 (Fe−CO); 1769, 1722 (C=O); 1609, 1630 (C=C); 1292, 1198 (C−O). ^{1}H NMR ($CDCl_3$): δ = 0.45 (ddd, ^2J = 9.4 Hz, ^3J = 2.6 Hz, ^4J = 0.9 Hz, 1H, R-C=C**H$_2$**), 1.06 (tdd, ^3J = 8.2 Hz, ^3J = 5.8 Hz, ^4J = 0.9 Hz, 1H, −CH$_2$-C**H**=), 1.81 (ddd, ^2J = 7.0 Hz, ^3J = 2.6 Hz, ^4J = 1.0 Hz, 1H, −C**H**=CH$_2$), 2.30 (s, 3H, O=C-C**H$_3$**), 4.27 (m, 2H, −O-C**H$_2$**-C=), 5.26 (m, 1H, HC=CH-**CH**=CH$_2$), 5.42 (dd, ^3J = 8.2 Hz, ^3J = 4.8 Hz, 1H, HC=**CH**-CH=CH$_2$), 7.06 (dd, ^3J = 8.1 Hz, ^4J = 1.1 Hz, 1H, 3′-H), 7.28 (ddd, ^3J = 7.7 Hz, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H, 5′-H), 7.53 (ddd, ^3J = 7.8 Hz, ^3J = 7.7 Hz, ^4J = 1.7 Hz, 1H, 4′-H), 8.05 (dd, ^3J = 7.9 Hz, ^4J = 1.7 Hz, 1H, 6′-H). Anal. ($C_{17}H_{14}O_7Fe$) C, H.

[η^4-(Hexa-2,4-dienyl)-2-acetoxybenzoate]irontricarbonyl (Hexadiene-ASS-Fe). Hexadiene-ASS, 520 mg (2.0 mmol); $Fe_2(CO)_9$, 1.0 g (2.8 mmol). Column chromatrography: petroleum ether/diethyl ether = 10:1. Yield: 100 mg (13%) of light-yellow powder. IR (KBr, cm^{-1}): 3061, 3019 (C=C-H); 2046, 1969 (Fe−CO); 1768, 1706 (C=O); 1605 (C=C); 1295, 1196 (C−O). ^{1}H NMR ($CDCl_3$): δ = 1.08 (ddq, ^3J = 5.8 Hz, ^3J = 2.6 Hz, ^4J = 0.7 Hz, 1H, C=C**H**-CH$_3$), 1.30 (m, 1H, CH$_2$−C**H**=), 1.43 (d, ^3J = 3.6 Hz, 3H, C=CH-C**H$_3$**), 2.33 (s, 3H, CO-O-CH$_3$), 4.26 (m, 2H, O-C**H$_2$**-C=C), 5.08 (dd, ^3J = 8.4 Hz, ^3J = 4.9 Hz, 1H, HC=CH-**CH**=CH-CH$_3$), 5.27 (dd, ^3J = 8.4 Hz, ^3J = 5.3 Hz, 1H, HC=**CH**-CH=CH$_2$), 7.11 (dd, ^3J = 8.1 Hz, ^4J = 1.1 Hz, 1H, 3′-H), 7.31 (ddd, ^3J = 7.7 Hz, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H, 5′-H), 7.56 (ddd, ^3J = 7.8 Hz, ^3J = 7.7 Hz, ^4J = 1.7 Hz, 1H, 4′-H), 8.05 (dd, ^3J = 7.9 Hz, ^4J = 1.7 Hz, 1H, 6′-H). Anal. ($C_{18}H_{16}O_7Fe$) C, H.

[(μ^3-η^2)-(But-3-ynyl)-2-acetoxybenzoate]triironnonacarbonyl (But-ASS-Fe$_3$) and Di-[(μ^4-η^4)-(But-3-ynyl)-2-acetoxybenzoate]-triironoctacarbonyl (Di-(But-ASS)-Fe$_3$). A 500 mL Schlenk flask was charged with 1.5 g of $Fe_3(CO)_{12}$ (3 mmol) and 460 mg of **But-ASS** (2 mmol). About 150 mL of dry *n*-pentane and 80 mL of dry CH_2Cl_2 were added. This solution was cooled to −80 °C, and a solution of 230 mg (3 mmol) of dehydrated trimethylamine-*N*-oxide as a catalyst in 100 mL of CH_2Cl_2 was added. The solution was degassed as described above and fitted with a bubble counter. The solution was refluxed for 4 days. Solvents were removed under vaccuo, and the residue was purified by column chromatography with petroleum ether/diethyl ether (15:1).

But-ASS-Fe$_3$. Yield: 20 mg (1.5%) as brown crystals. IR (KBr, cm^{-1}): 2959, 2927, 2858 (C−H); 2061, 2026, 1986 (Fe-CO); 1730 (C═O); 1636 (C═C); 1261, 1203 (C−O).

Di-(But-ASS)-Fe$_3$. Yield: 15 mg (1.7%) of **Di-(But-ASS)-Fe** as orange crystals. IR (KBr, cm^{-1}): 2091, 2058, 2017, 1989 (Fe-CO); 1734 (C═O); 1633 (C═C); 1261, 1200 (C−O).

Preparation of 1-((2-Acetoxybenzoyloxy)methyl)ferrocene (Ferrocene-ASS). Synthesis was performed as described for **Prop-ASS**. Ferrocenylcarbinol: 1.1 g (5.1 mmol), ASSCl: 1.07 g (5.4 mmol). Column chromatrography: petroleum ether/diethyl ether = 5:1. Yield: 1.67 g (87%) as dark-yellow crystals (mp 118.2 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.17 (s, 3H, O═C-C**H$_3$**), 4.18 (s, 5H, Fe−C_5H_5), 4.21 (t, ^3J = 1.8 Hz, 2H, Fe(C_5H_4), 3′4′-H), 4.33 (t, ^3J = 1.8 Hz, 2H, Fe(C_5H_4), 2′5′-H), 5.10 (s, 2H, Fe(C_5H_4)-C**H$_2$**-O), 7.07 (dd, ^3J = 8.1 Hz, ^4J = 1.1 Hz, 1H, 3′-H), 7.29 (ddd, ^3J = 7.7 Hz, ^3J = 7.6 Hz, ^4J = 1.1 Hz, 1H, 5′-H), 7.54 (ddd, ^3J = 7.8 Hz, ^3J = 7.6 Hz, ^4J = 1.6 Hz, 1H, 4′-H), 8.02 (dd, ^3J = 7.9 Hz, ^4J = 1.7 Hz, 1H, 6′-H). Anal. ($C_{20}H_{18}O_4Fe$) C, H.

Biological Methods. Cytotoxicity Experiments. The human MCF-7 and MDA-MB 231 breast cancer cell lines as well as the HT-29 colon cancer cell line were obtained from the American Type Culture Collection (ATCC, USA). Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay.[38] All three cell lines were maintained in L-glutamine and 4.5 g/L D-glucose containing Dulbecco's Modified Eagle Medium (DMEM; PAA, Pasching, Austria) without phenol red, supplemented with 5% fetal calf serum (FCS; Biochrom, Berlin, Germany) using 25 cm^2 culture flasks (Sarstedt, Nürnberg, Germany) in a humidified atmosphere at 37 °C. The MCF-7 cell line was passaged weekly after treatment with trypsin (0.05%) and ethylenediaminetetraacetic acid (0.02%; EDTA; Boehringer, Mannheim, Germany), whereas HT-29 and MDA-MB-231 where passaged twice per week.

Each 100 μL of 7500 cells/mL (MCF-7), 2850 cells/mL (HT-29), or 7500 cells/mL (MDA-MB 231) were incubated in 96-well plates at 37 °C in a 5% CO_2/95% air atmosphere for 72 h with the test compound of various concentration. One plate was used for the determination of the initial cell biomass. The medium was removed and the cells were fixed by a 20−30 min incubation with 100 μL of glutardialdehyde solution (0.5 mL of glutardialdehyde +12.5 mL of phosphate-buffered saline (PBS), pH 7.4). The solution in the wells was sucked off, 180 μL of PBS, pH 7.4, were added, and the plate was stored at 4 °C until further treatment. In the "experimental" plates, the medium was replaced with medium containing the drugs in graded concentrations (eight replicates). After further incubation for 96 h, these plates were treated as described above. The cell biomass was determined by crystal violet staining according to the following procedure: the PBS, pH 7.4 was removed, 100 μL of a 0.02 M crystal-violet solution were added, and plates were incubated for 30 min at room temperature, washed three times with water, and incubated on a softly rocking rotary shaker with 180 μL of ethanol (70%) for further 3−4 h. Absorption was recorded at 590 nm using a microplate reader (FLASHscan S12). The mean absorption of the initial cell biomass plate was subtracted from the mean absorption of each experiment and control. The corrected control was set at 100%. IC_{50} value was determined as that concentration causing 50% inhibition of cell growth and calculated as mean of at least two independent experiments.

Analysis of Proliferation and Apoptosis. In each experiment, 100 μL of 7500 cells/mL (MCF-7 and MDA-MB 231) were plated in 96-well flat bottom plates at 37 °C in a 5% CO_2/95% air atmosphere. After a three-day culture various concentrations (5 and 10 μM) of test compounds were added in 100 μL and further incubated for 24, 48, and 72 h. Thereafter, cultures were analyzed for cellular proliferation using [^{3}H-methyl]-thymidine uptake and apoptosis induction using the ssDNA apoptosis kit (Chemicon International, Hofheim, Germany) according to the manufacturer's instructions. Apoptosis rate was evaluated by dividing the optical density (OD) of the wells measured in the plates for ssDNA apoptosis by the OD of the wells analyzed with a modified MTT assay (EZ4U kit; Biomedica, Vienna, Austria) as control for cellular biomass which was set up in parallel. Apoptosis in the absence of compounds was set at 1.

For proliferation analysis, each well was exposed for the last 22 h of culture to 2 μCi of [^{3}H-methyl]-thymidine (specific activity 74 GBq/mmol, Hartmann Analytic, Braunschweig, Germany). Cells were harvested using a semiautomated device and [^{3}H-methyl]-thymidine uptake expressed in counts per minute was measured in a liquid scintillation counter (Microbeta Trilux; Perkin-Elmer Life Sciences, Boston, MA). Proliferation in the absence of the compounds was set at 100%.

Caspase-3 Activity Assay. Cells were cultured in black 96-well microplates for 24 h (plating density 50000 cells/well) in a 5% CO_2/95% air atmosphere. Medium was then removed and replaced by substance containing DMEM; control cells were maintained in the absence of any compound. After 24 h of treatment, cells were lysed (Cell Culture Lysis Reagent, Promega) and incubated with 30 μM Ac-DEVD-AMC (biomol), a specific caspase-3 substrate, which upon cleavage by active caspase-3 generates a highly fluorescent product, for 2 h at 37 °C. Fluorescence was assayed at excitation/emission wavelengths of 380/460 nm. Caspase-3 activity in the absence of the compounds was set at 1.

Acridine Orange Staining. Each 620 μL of 7500 cells/mL (MCF-7 and MDA-MB 231) were plated in 24-well flat bottom plates at 37 °C in a 5% CO_2/95% air atmosphere. After a three-day culture, 10 μM of the test compounds were added in fresh 620 μL of medium and incubated for further 24 h. Thereafter, cells were fixed with 200 μL of 1% glutardialdehyde/PBS and stained with 200 μL of 2 μg/mL acridine orange/PBS (biomol) for 2 min. Photos were taken with Zeiss Axiovert 40 CFL (λ_{ex} 470/40 nM, λ_{em} 525/50 nm) with 20× magnification.

Inhibition of COX-Enzymes. The inhibition of isolated ovine COX-1 and human recombinant COX-2 at 10 μM of the respective compounds was determined by ELISA (COX Inhibitor Screening Assay; Cayman Chemical Company, Ann Arbor, MI). The experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (FLASHscan S12).

PGE$_2$-Assay. MDA-MB 231 cells were grown in 24-well microplates in a 5% CO_2/95% air atmosphere until at least 70% confluency. The medium was then removed and replaced by fresh DMEM containing the respective substances (10 μM); control cells were maintained in the absence of any compound. The cells were incubated for 23 h followed by the addition of 100 μM of arachidonic acid. Twenty-four h after the addition of the corresponding substances, the drug-containing medium with the including prostaglandin E2 was removed and tested by ELISA (Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemicals). Experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (FLASHscan S12).

Acknowledgment. The financial support of DFG—Deutsche Forschungsgemeinschaft (project: FOR 630) is greatly appreciated. We especially thank Evelina Fogelström for reviewing this paper.

Supporting Information Available: Additional spectroscopic data of title compounds; elemental analyses of all complexes; acridine orange staining of MCF-7 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Go, R. S.; Adjei, A. A. Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *J. Clin. Oncol.* **1999**, *17*, 409–422.
(2) Reedijk, J. Medicinal applications of heavy-metal compound. *Curr. Opin. Chem. Biol.* **1999**, *3*, 236–240.

(3) Ott, I.; Abraham, A.; Schumacher, P.; Shorafa, H.; Gastl, G.; Gust, R.; Kircher, B. Synergistic and additive antiproliferative effects on human leukemia cell lines induced by combining acetylenehexacarbonyldicobalt complexes with the tyrosine kinase inhibitor imatinib. *J. Inorg. Biochem.* **2006**, *100*, 1903–1906.

(4) Ott, I.; Schmidt, K.; Kircher, B.; Schumacher, P.; Wiglenda, T.; Gust, R. Antitumor-active cobalt−alkyne complexes derived from acetylsalicylic acid: studies on the mode of drug action. *J. Med. Chem.* **2005**, *48*, 622–629.

(5) Schmidt, K.; Jung, M.; Keilitz, R.; Schnurr, B.; Gust, R. Acetylenehexacarbonyldicobalt complexes, a novel class of antitumor drugs. *Inorg. Chim. Acta* **2000**, *306*, 6–16.

(6) Ott, I.; Kircher, B.; Gust, R. Investigations on the effects of cobalt−alkyne complexes on leukemia and lymphoma cells: cytotoxicity and cellular uptake. *J. Inorg. Biochem.* **2004**, *98*, 485–489.

(7) Ott, I.; Gust, R. Stability, protein binding and thiol interaction studies on [2-acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt. *BioMetals* **2005**, *18*, 171–177.

(8) Ott, I.; Kircher, B.; Bagowski, C. P.; Vlecken, D. H. W.; Ott, E., B.; Will, J.; Bensdorf, K.; Sheldrick, W. S.; Gust, R. Modulation of the biological properties of aspirin by formation of a bioorganometallic derivative. *Angew. Chem., Int. Ed.* **2009**, *48*, 1160–1163.

(9) Niesel, J.; Pinto, A.; N'Dongo, H. W. P.; Merz, K.; Ott, I.; Gust, R.; Schatzschneider, U. Photoinduced CO release, cellular uptake and cytotoxicity of a tris(pyrazolyl)methane (tpm) manganese tricarbonyl complex. *Chem. Commun.* **2008**, 1798–1800.

(10) Krüerke, U.; Hübel, W. Uber Organometall-Komplexe, VIII. Reaktionen von Kobaltcarbonyl-Verbindungen mit Alkinen. *Chem. Ber.* **1961**, *94*, 2829–2856.

(11) Ma, D.-L.; Che, C.-M.; Siu, F.-M.; Yang, M.; Wong, K.-Y. DNA Binding and cytotoxicity of ruthenium(II) and rhenium(I) complexes of 2-amino-4-phenylamino-6-(2-pyridyl)-1,3,5-triazine. *Inorg. Chem.* **2007**, *46*, 740–749.

(12) Schlawe, D.; Majdalani, A.; Velcicky, J.; Hessler, E.; Wieder, T.; Prokop, A.; Schmalz, H.-G. Iron-containing nucleoside analogues with pronounced apoptosis-inducing activity. *Angew. Chem., Int. Ed.* **2004**, *43*, 1731–1734.

(13) Weizmann, C.; Bergmann, E. D.; Sulzbacher, M. Derivatives of salicylic acid. *J. Org. Chem.* **1948**, *13*, 796–799.

(14) Isler, O.; Gutmann, H.; Montavon, M.; Rüegg, R.; Ryser, G.; Zeller, P. Synthesen in der Carotinoid-Reihe. 10. Mitteilung. Anwendung der Wittig-Reaktion zur Synthese von Estern des Bixins und Crocetins. *Helv. Chim. Acta* **1957**, *40*, 1242–1249.

(15) Mizawa, T.; Takenaka, K.; Shiomi, T. Synthesis of polymers containing conjugated dienyl end-groups by means of anionic living polymerization. *J. Polym. Sci., Polym. Chem.* **1999**, *37*, 3464–3472.

(16) Gervasio, G.; Rossetti, R.; Stanghellini, P. L. Vibrational study and crystal structure of (μ^4-η^2-acetylene)decacarbonyltetracobalt, (μ^4-η^2-C_2H_2)$Co_4(CO)_8$(μ-CO)$_2$. *Organometallics* **1985**, *4*, 1612–1619.

(17) Greenfield, H.; Sternberg, H. W.; Friedel, R. A.; Wotiz, J. H.; Markby, R.; Wender, I. Acetylenic dicobalt hexacarbonyls. Organometallic compounds derived from alkynes and dicobalt octacarbonyl. *J. Am. Chem. Soc.* **1956**, *78*, 120–124.

(18) Lang, H.; Rheinwald, G.; Lay, U.; Zsolnai, L.; Huttner, G. Synthese und Reaktionsverhalten von Synthese und Reaktionsverhalten von (η^2-BrC≡CR)$Co_2(CO)_6$. Die Festkörperstruktur von Pentacarbonyl-[μ-(1,2,3,4-η:1,4-η)-1,4-diphenyl-1,3-butadien-1,4-diyl]dicobalt. *J. Organomet. Chem.* **2001**, *634*, 74–82.

(19) Sappa, E.; Gambino, O.; Milone, L.; Cetini, G. Reactions of $Ru_3(CO)_{12}$ with asymmetrically substituted acetylenes. A new type of interaction between acetylenes and a metal-atom cluster. *J. Organomet. Chem.* **1972**, *39*, 169–172.

(20) Evans, M.; Hursthouse, M.; Randall, E. W.; Rosenberg, E.; Milone, L.; Valle, M. Proton and carbon-13 nuclear magnetic resonance spectra and X-ray crystal structure of [1-methyl-3-ethyl-(σ-1,3-h2,π-1,2,3-h3)-allyl]nonacarbonyltriruthenium: the allyl group as a five-electron donor. *J. Chem. Soc.: Chem. Commun.* **1972**, *9*, 545–546.

(21) Moreno, C.; Marcos, M.-L.; Macazaga, M.-J.; Gomez-Gonzalez, J.; Gracia, R.; Benito-Lopez, F.; Martinez-Gimeno, E.; Arnanz, A.; Medina, M.-E.; Pastor, C.; Gonzalez-Velasco, J.; Medina, R.-M. Synthesis, characterization, and redox behavior of mixed 1,3-diyne dicobalt/triosmium and dicobalt/triruthenium carbonyl clusters. *Organometallics* **2007**, *26*, 5199–5208.

(22) Bruce, M. I.; Skelton, B. W.; White, A. H.; Zaitseva, N. N. Preparation and structural characterisation of some ruthenium cluster carbonyls containing allenylidene ligands. *Dalton* **2000**, *6*, 881–890.

(23) Vessieres, A.; Top, S.; Vaillant, C.; Osella, D.; Mornon, J. P.; Jaouen, G. Transition metal cluster-modified estradiols for the investigation of proteins: use at the estradiol receptor. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 754–755.

(24) Deeming, A. J.; Ullah, S. S.; Domingos, A. J. P.; Johnson, B. F. G.; Lewis, J. Reactivity of coordinated ligands. XX. Preparation and reactions of cyclooctadiene complexes of iron, ruthenium, and osmium. *J. Chem. Soc., Dalton Trans.* **1974**, *19*, 2093–2104.

(25) Calderón, R.; Vahrenkamp, H. Reactivity of $Fe_3(CO)_9$(μ^3-C_2Et_2). Synthesis and structures of $Fe_2(CO)_6${C_2Et_2C[CH_2N(Me)CH_2Ph]CH} and $Fe_3(CO)_8${C_2Et_2C(H)C[CH_2N(Me)CH_2Ph]}. *J. Organomet. Chem.* **1998**, *555*, 113–118.

(26) Bachler, V.; Grevels, F.-W.; Kerpen, K.; Olbrich, G.; Schaffner, K. A novel facet of carbonyliron-diene photochemistry: The η^4-*s-trans* isomer of the classical $Fe(CO)_3$(η^4-*s-cis*-1,3-butadiene) discovered by time-resolved IR spectroscopy and theoretically examined by density functional methods. *Organometallics* **2003**, *22*, 1696–1711.

(27) Siebenlist, R.; Frühauf, H.-W.; Vrieze, K.; Smeets, W. J. J.; Spek, A. L. Preparation and properties of carbonyliron complexes of 1-aza-4-oxa-1,3-butadiene (α-imino ketone)—X-ray crystal structures of $Fe(CO)_3$[MeN=C(Ph)C(Ph)=O] and $Fe_2(CO)_4$[*t*BuN=C(H)(Me)C(O)(O)C(Me)C(H)=N*t*Bu]. *Eur. J. Inorg. Chem.* **2000**, 907–919.

(28) Braye, E. H.; Hübel, W. Diiron enneacarbonyl. *Inorg. Synth.* **1966**, *8*, 178–181.

(29) DePuy, C. H.; Parton, R. L.; Jones, T. Synthesis of substituted hydroxybutadiene tricarbonyliron complexes. *J. Am. Chem. Soc.* **1977**, *99*, 4070–4075.

(30) Kongsaeree, P.; Tanboriphan, S.; Tarnchompoo, B.; Thebtaranonth, Y. Tricarbonyliron complexes derived from dimethyl 1,3-butadiene-2,3-dicarboxylate: Formation of [$Fe(CO)_3$]$_2$-dimethyl 1,3-butadiene-2,3-dicarboxylate. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1999**, *55*, 2007–2010.

(31) Misterkiewicz, B.; Dabard, R.; Patin, H. Substitution electrophile du ferrocene et du dimethylferrocene par les derives carbonyles protones: II. Influence des effets stériques et conformationnels lors de la synthèse des α-ferrocénylcarbinols. *Tetrahedron* **1985**, *41*, 1685–1692.

(32) Ulrich, C. M.; Bigler, J.; Potter, J. D. Nonsteroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nature Rev. Cancer* **2006**, *6*, 130–140.

(33) Wennogle, L. P.; Liang, H.; Quintavalla, J. C.; Bowen, B. R.; Wasvary, J.; Miller, D. B.; Allentoff, A.; Boyer, W.; Kelly, M.; Marshall, P. Comparison of recombinant cyclooxygenase-2 to native isoforms: aspirin labeling of the active site. *FEBS Lett.* **1995**, *371*, 315–320.

(34) Chan, T. A. Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol.* **2002**, *3*, 166–174.

(35) Mcfarlane, W.; Wilkinson, G.; Hübel, W. Triiron dodecacarbonyl. In *Inorganic Syntheses*; Henry, F. H., Jr., Ed.; Wiley: New York, 2007; Vol. 8, pp 181−183.

(36) Meisenheimer, J. Uber die Ungleichartigkeit der fünf Valenzen des Stickstoffs. *Justus Liebig's Ann. Chem.* **1913**, *397*, 273–300.

(37) Riegel, B.; Wittcoff, H. The Preparation of Acetylsalicylyl Disulfide and Salicylyl Disulfide. *J. Am. Chem. Soc.* **1942**, *64*, 1486–1487.

(38) Hay, R. J. The seed stock concept and quality control for cell lines. *Anal. Biochem.* **1988**, *171*, 225–237.