Antitumor-Active Cobalt−Alkyne Complexes Derived from Acetylsalicylic Acid: Studies on the Mode of Drug Action

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Cobalt−alkyne complexes are drugs with remarkable cytotoxicity. From the complexes tested up to now we selected the aspirin derivative [2-acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-ASS) as the lead compound. To get more insight into the mode of action, we systematically modified the alkyne ligand and determined the cytotoxic properties of the resulting cobalt complexes. Further investigations were performed on the drug lipophilicity, the cellular uptake into MCF-7 and MDA-MB 231 breast cancer cells, the DNA-binding efficacy, and the nuclear drug content. The ability to inhibit glutathione reductase and cyclooxygenase (COX) enzymes, the binding to the estrogen receptor, and the induction of apoptotic processes were examined for selected compounds. Interestingly, the most antitumor active compounds were potent COX inhibitors (COX-1 and COX-2). The presented results indicate that cobalt−alkyne complexes of the Co-ASS type, represent a new class of organometallic cytostatics with a mode of drug action in which COX inhibition probably plays a major role.

Introduction

Platinum-containing anticancer drugs (e.g. cisplatin) are widely used in the treatment of human cancers. Many efforts have been made to establish further metal-containing cytostatics, however, without great success. Only little is known about the antitumor efficacy of trace elements such as copper, zinc, or cobalt.[1] We and others reported recently about cytotoxic cobalt−alkyne complexes.[2−5] These compounds consist of a hexacarbonyldicobalt cluster bound to an alkyne ligand. Some of these complexes were found to be active against a broad variety of human epithelialic tumors as well as lymphomas and leukemias. The best results, however, were obtained on human breast cancer cells lines.

Out of the complexes tested up to now [2-acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-ASS, see Figure 1) emerged as the lead compound. First structure−activity studies showed that the activity depended strongly on the nature of the alkyne ligand and on an intact cobalt−alkyne complex. The mode of drug action remained unclear.

To get more insight into the structural requirements for the high cytotoxicity of Co-ASS, we modified systematically the alkyne ligand: the 2-acetoxy group in the aromatic ring was replaced by H (Co-Benz), OH (Co-SAL), F (Co-2F-Benz) or moved into position 3 (Co-3-Acetbenz), the ester in Co-ASS was cleaved (Co-Prop) or replaced by an amide bond (Co-ASSAM), and Co-Phthal was designed as a phthalimide derivative of alkynehexacarbonyldicobalt (see Figure 1).

The compounds were investigated for cytotoxicity at the MCF-7 and MDA-MB 231 human breast cancer cell lines. We determined the lipophilicity of the complexes as log k_w value by HPLC and the cellular uptake by atomic absorption spectroscopy in order to clarify if drug distribution processes may explain the different drug activities. As the complexes contain metal atoms a "cisplatin-like" mechanism involving covalent DNA modification has to be considered. Therefore, DNA-binding studies were performed, and the drug content of the cell nuclei was quantified. On the basis of these results, we selected compounds for the following studies on the mode of drug action.

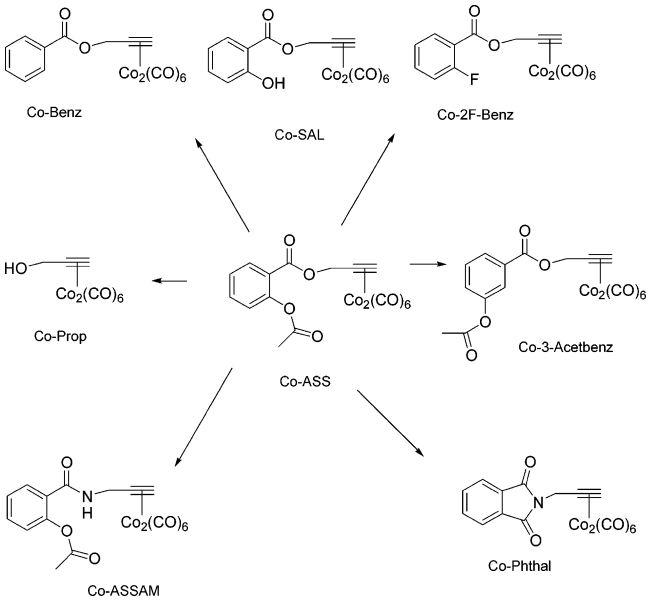

Figure 1. Cobalt−alkyne complexes.

The intracellular concentration of reduced glutathione was found to be in the mM-range in adherent growing cancer cells, and the glutathione reductase system is upregulated in human breast cancers.[6,7] Metal-contain-

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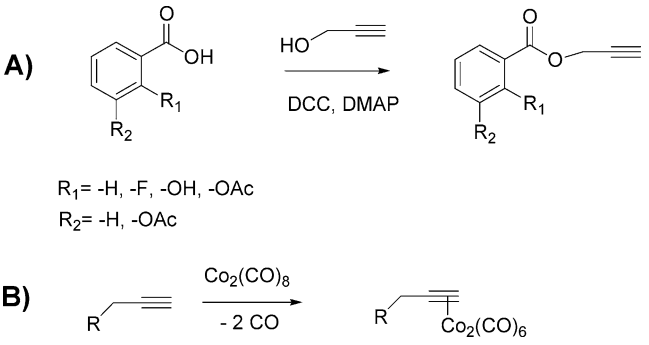
Scheme 1. (A) Preparation of Alkynes. (B) Reaction of the Alkynes with Dicobaltoctacarbonyl



ing drugs disturb this sensible cellular redox system.[8,9] Therefore, we evaluated the influence of cobalt−alkyne complexes on the glutathione reductase activity.

Furthermore, as the lead compound Co-ASS represents a derivative of the nonsteroidal antiinflammatory drug (NSAID) acetylsalicylic acid (aspirin, ASS) the presence of similar pharmacological properties can be expected. NSAIDs are seen as future drugs for the treatment of a broad variety of human tumors, including breast and colon cancer.[10−13] The antitumor activity of NSAIDs involves the inhibition of COX enzymes. Therefore, the ability of the complexes to inhibit COX enzymes was studied by ELISA.

Further investigations include the determination of apoptotic changes in the tumor cells and, due to the selectivity of Co-ASS for breast cancer cells, the proof of a possible interaction with the estrogen receptor.

Chemistry

The synthesis of Co-ASS, Co-SAL,[2] and Co-Prop[14] was described previously. For the synthesis of the cobalt−alkyne complexes Co-Benz, Co-3-Acetbenz, Co-2F-Benz, Co-ASSAM, and Co-Phthal, we used a two-step procedure as depicted in Scheme 1.

In the first step, the alkyne esters (2-propynyl)-benzoate, 3-acetoxy-(2-propynyl)benzoate, and 2-fluoro-(2-propynyl)benzoate were prepared by reaction of the respective benzoic acid with dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), and an excess of propargylic alcohol (see Scheme 1A). The synthesis of the amides was performed as described earlier.[15,16]

In the second step, the alkynes were reacted with dicobaltoctacarbonyl to give the cobalt−alkyne complexes (see Scheme 1B), which were purified by column chromatography on SiO_2 and analyzed by means of IR, NMR, MS, and elemental analysis.

In the ^{1}H NMR spectra of the complexes, the resonances for the C≡CH ($\delta \approx 6.1$ ppm) and C≡C−CH_2 ($\delta = 4.7-5.6$ ppm) protons of the complexes are located in the region of double bonds. This effect is well-known from many studies on cobalt−alkyne complexes and indicates that the triple bond character of the alkynes is lowered toward that of a *Z*-olefin due to the coordination to $Co_2(CO)_6$. The IR spectrum of dicobaltoctacarbonyl shows intensive stretching vibrations of terminal (2000−2100 cm^{-1}) and bridging CO ligands (1830−1850 cm^{-1}). By the coordination reaction the bridging CO ligands are lost and the IR spectra of the complexes exhibit only the signals of the terminal CO ligands.[3]

Table 1. Lipophilicity (log k_w), Cytotoxicity (IC_{50}), and DNA Binding Efficiency

compound	log k_w	IC_{50} (μM) MCF-7	IC_{50} (μM) MDA-MB 231	DNA binding (pmol/μg)
Co-Prop	3.55[a]	7.0 (± 2.0)	>50	33.94 (± 9.29)
Co-ASSAM	3.81	8.8 (± 1.0)	9.1 (± 1.0)	22.63 (± 2.25)
Co-Phthal	4.42	22.2 (± 0.4)	>50	4.57 (± 0.96)
Co-ASS	4.91[a]	1.4 (± 0.3)	1.9 (± 0.3)	9.82 (± 0.20)
Co-3-Acetbenz	4.93	9.1 (± 1.3)	10.3 (± 0.5)	9.18 (± 2.44)
Co-2F-Benz	5.05	7.1 (± 0.9)	9.6 (± 0.7)	9.22 (± 0.28)
Co-Benz	5.22	5.4 (± 0.4)	11.4 (± 0.9)	15.19 (± 0.07)
Co-SAL	5.32	3.9 (± 0.2)	4.4 (± 1.7)	36.97 (± 3.75)
cisplatin		2.0 (± 0.3)	4.0 (± 1.5)	
melphalan		5.7 (± 1.5)	3.9 (± 0.3)	
tamoxifen		2.3 (± 0.4)	10.6 (± 0.6)	
5-fluorouracil		4.8 (± 0.6)	9.6 (± 0.3)	

[a] See ref 5.

Results and Discussion

The antiproliferative activity of the complexes was determined in MCF-7 and MDA-MB 231 human breast cancer cells (see Table 1).

The lead structure Co-ASS showed IC_{50} values lower than 2.0 μM in both cell cultures (1.4 μM in MCF-7 and 1.9 μM in MDA-MB 231 cells). The desacetoxy derivative Co-Benz, the amide derivatives (Co-ASSAM and Co-Phthal), and the structural isomer Co-3-Acetbenz were less active ($IC_{50} > 5$ μM in MCF-7 and $IC_{50} > 9$ μM in MDA-MB 231 cells, see Table 1). Only the 2-hydroxy derivative Co-SAL showed comparable activity with $IC_{50} = 3.9$ μM (MCF-7) and 4.4 μM (MDA-MB 231). The exchange of the 2-OH by fluorine (Co-2F-Benz) again decreased the antiproliferative potency ($IC_{50} > 7$ μM at both cell lines, see Table 1).

These findings clearly indicate the importance of the defined 2-acetoxy-(2-propynyl)benzoate ligand structure for the high cytotoxicity of Co-ASS. Metabolic degradation is only tolerated at the 2-acetoxy group. Cleavage of the propynyl ester led to Co-Prop, which showed effects against MCF-7 cells ($IC_{50} = 7.0$ μM), but was completely inactive at the MDA-MB 231 cell line ($IC_{50} > 50$ μM). Furthermore, the cytotoxic activity strongly depended on the coordination of the alkyne ligand at the $Co_2(CO)_6$ cluster. The free ligand as well as the precursors ASS and $Co_2(CO)_8$ were inactive.[3]

The established antitumor drugs cisplatin, melphalan, tamoxifen, and 5-fluorouracil were used as references and were found to be active in the range of $IC_{50} = 2.0$ to 10.6 μM. It should be mentioned that all structural modifications of the lead compound Co-ASS (see Figure 1) led to a significant decrease in activity, but the compounds (with exception of Co-Prop and Co-Phthal at the MDA-MB 231 cell line) were still active in the range of these drugs.

Various studies correlate the cytotoxicity of metal complexes with the accumulation in tumor cells, which itself depends on the drug lipophilicity (log k_w value) if a passive transport through the cell membrane occurs. The log k_w values of the cobalt−alkyne complexes determined by reversed phase HPLC[5] (see Table 1) were in the range of 3.55 (Co-Prop) to 5.32 (Co-SAL). Co-ASS and its structural isomer Co-3-Acetbenz possessed comparable log k_w values of 4.91 and 4.93, respectively. The amide derivatives Co-Phthal (log $k_w = 4.42$) and Co-ASSAM (log $k_w = 3.81$) exhibited distinctly lower lipophilicity than the benzoic ester derivatives

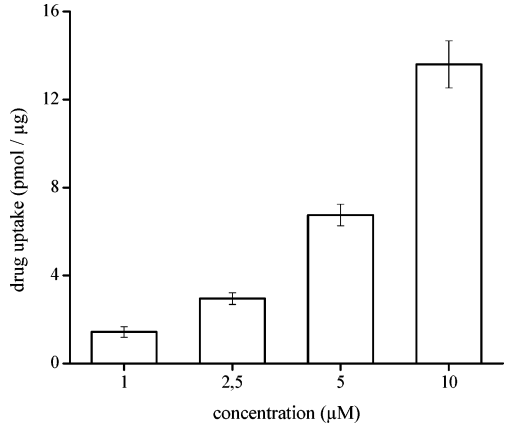


Figure 2. Concentration dependent cellular uptake of Co-ASS into MCF-7 cells.

(log k_w = 4.91−5.32). Interestingly, the high lipophilic character of the cobalt−alkyne complexes was mainly determined by the cobalt cluster as confirmed for Co-ASS. The coordination of the 2-acetoxy-(2-propynyl)-benzoate (Prop-ASS) to $Co_2(CO)_6$ increased the log k_w from 1.98 to 4.91.

The accumulation into tumor cells was studied on both cell lines used for the cytotoxicity experiments. The cells were treated with the respective cobalt−alkyne complex, subsequently lysed by sonification, and the cellular cobalt content was quantified by atomic absorption spectroscopy.

Figure 2 shows the concentration dependent cellular uptake of Co-ASS into MCF-7 cells after 6 h of drug exposure. The high linear correlation ($r^2 > 0.999$) of intra- and extracellular drug concentration indicates that the drug uptake is not saturated within the concentration range (1.0−10 μM) which is important for the cytotoxic action. The cellular molar drug concentration can be estimated from the pmol/μg value as described previously.[17] Dividing by the concentration used in the extracellular medium, the accumulation grade can be calculated. The mean accumulation grade for Co-ASS (using the data shown in Figure 2) was 150.3 (±12.0). This result is of general interest as cisplatin showed accumulation grades lower than 6-fold.[17,18]

The time dependent uptake into MCF-7 and MDA-MB 231 cells was determined over a period of 24 h for Co-ASS, Co-Prop, Co-SAL, and Co-Benz (see Figure 3).

All complexes were taken up to maximum levels within 4−8 h, whereby in MCF-7 cells a higher cobalt content was measured than in MDA-MB 231 cells. Cells treated with 2.0 μM Co-ASS exhibited cobalt content of >2.5 pmol/μg (MCF-7) and >1.7 pmol/μg (MDA-MB 231). The other complexes were taken up in a lower amount. A slight decrease of cellular concentration is observed in most cases after longer drug exposure. Interestingly, cobalt could not be found in comparable concentrations if the cells were incubated with $Co_2(CO)_8$ (<0.15 pmol/μg). Therefore, it can be concluded that the alkyne ligand is inevitably necessary for the uptake into the cells.

The results of the investigations on the drug lipophilicity and the uptake into tumor cells are only in part suitable to explain the differences in cytotoxicity. The highest cellular cobalt levels are achieved with the most active cobalt complex Co-ASS. Co-Benz with distinctly

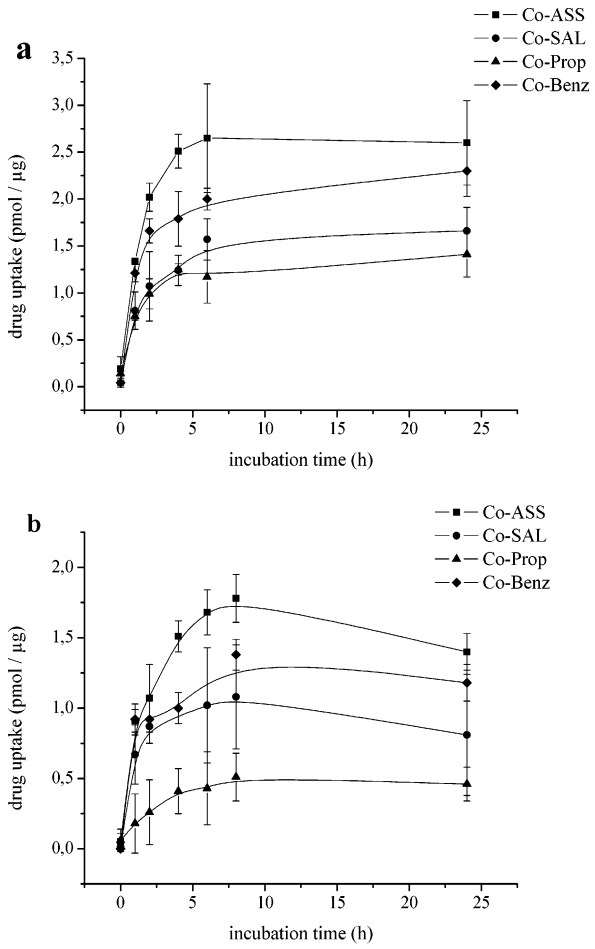


Figure 3. Time-dependend cellular uptake of 2.0 μM cobalt−alkyne complexes into a) MCF-7 cells and b) MDA-MB 231 cells.

lower cytotoxicity is enriched in the tumor cells to nearly the same extent. Furthermore, the attempt failed to correlate the cytotoxicity (IC_{50}) with the lipophilicity (log k_w) (r^2 = 0.31 for MCF-7 cells and r^2 = 0.14 for MDA-MB 231 cells).

These results altogether show that drug distribution processes are only in part responsible for the observed differences in cytotoxic potency. The absence of a correlation between drug activity, lipophilicity, and cellular concentration indicate the involvement of a specific mode of drug action.

A "cisplatin-like" mechanism (covalent binding to the DNA) had to be considered first. Cobalt−alkyne complexes were already designed as labeling agents and used for the so-called carbonyl-metallo-immunoassay (CMIA). Covalent binding to the estrogen receptor was confirmed for derivatives of ethynylestradiol, suggesting that cobalt−alkyne complexes are able to interact covalently with biomolecules.[19,20]

The DNA binding was evaluated by incubation of the complexes with salmon testes DNA. All the compounds bound efficiently to DNA (> 4.5 pmol/μg, see Table 1), distinctly higher than the DNA-interacting platinum drugs cisplatin or carboplatin under similar experimental conditions (<1 pmol/μg [21]).

The lead compound Co-ASS showed only average binding efficiency (9.82 pmol/μg) comparable to Co-3-Acetbenz (9.18 pmol/μg) and Co-2F-Benz (9.22 pmol/μg).

Table 2. Drug Content of the Cell Nuclei (pmol/μg and % of total)

	Co-ASS	Co-SAL	Co-Benz	Co-Prop
MCF-7	0.12 (± 0.03)	0.11 (± 0.01)	0.11 (± 0.05)	0.15 (± 0.02)
	0.8%	1.0%	0.9%	1.4%
MDA-MB 231	0.22 (± 0.13)	0.14 (± 0.05)	0.14 (± 0.05)	0.15 (± 0.05)
	5.0%	1.7%	1.9%	2.0%

Much higher DNA interaction achieved Co-Benz (15.19 pmol/μg), Co-ASSAM (22.63 pmol/μg), Co-Prop (33.94 pmol/μg), and Co-SAL (36.97 pmol/μg). A correlation between the DNA-binding efficiency and the cytotoxicity again could not be found (see data in Table 1). Interestingly, the strongest DNA interaction (105.6 ± 18.3 pmol/μg) was measured for $Co_2(CO)_8$, which allows the assumption that the binding of the cobalt−alkyne complexes to the DNA is due to the interaction with the cobalt cluster.

To get information if drug−DNA binding occurs in the tumor cells, the nuclei were isolated by a short sucrose gradient and investigated for their cobalt content. The results were calculated as pmol drug per μg nuclear protein and as percentage of the total cellular drug amount. Table 2 summarizes the results. Nuclear drug amounts were nearly independent of the kind of alkyne ligand. The observed cobalt content of 0.11 to 0.22 pmol/μg corresponded only to 0.8−1.4% (MCF-7) and 1.7−5.0% (MDA-MB 231) of the total drug amount found in the cells. On the basis of these results, a genomic mode of drug action involving DNA interaction can be excluded for the cobalt−alkyne complexes.

Therefore, we searched for other targets within the tumor cells. First experiments were done with the most active complex Co-ASS to evaluate if apoptotic changes could be achieved in tumor cells. However, neither MCF-7 cells nor MDA-MB 231 cells showed signs of apoptosis after treatment with Co-ASS. These results correlate well with findings that acetylsalicylic acid induces apoptotic changes only at extremely high (millimolar) concentration.[22]

The growth of hormone dependent MCF-7 breast cancer cells cannot be inhibited only by cytostatics but also by estrogen receptor modulating compounds. On the basis of this fact, the relative estrogen receptor binding affinity (RBA) was determined exemplarily for Co-ASS and Co-3-Acetbenz. Both compounds showed extremely low receptor binding (RBA = 0% for Co-ASS and RBA = 0.05% for Co-3-Acetbenz), and as a consequence we exclude the estrogen receptor as target for cobalt−alkyne complexes.

The influence of Co-ASS and Co-Prop as well as the non-cobalt-containing precursors of Co-ASS acetylsalicylic acid (ASS) and its propargylic ester (Prop-ASS) on the glutathione reductase activity was evaluated to estimate if the interaction with this very sensible redox system caused the antiproliferative effects. As listed in Table 3, none of the compounds significantly inhibited or stimulated the glutathione reductase enzyme activity after 6 h of drug incubation. After 24 h similar results were observed (data not shown).

During the last years, cyclooxygenases became very interesting targets in cancer chemotherapy. Presently a few NSAIDS are studied in clinical trials for tumor therapy and prevention. As the most active compounds (Co-ASS, Co-SAL) of the SAR study are derivatives of the NSAIDs acetylsalicylic acid and salicylic acid (SAL), it might be possible that the NSAID-character of the compounds led to the growth inhibitory effects in MCF-7 and MDA-MB 231 cells.

Table 3. Influence on Cellular Glutathione Reductase Activity

compound	glutathione reductase activity (%)
Co-ASS	93 (± 5)
Co-Prop	105 (± 13)
ASS	101 (± 14)
Prop-ASS	97 (± 1)

The COX-inhibitory potential of Co-ASS, Co-SAL, Co-Prop, and Co-Benz was determined for COX-1 and COX-2 by ELISA (see Figure 4). The precursors acetylsalicylic acid and its propargylic ester were used as references.

Acetylsalicylic acid was only active at COX-1 in a concentration of 200 μM (91% inhibition), its propargylic ester showed lower activity at COX-1 (48% inhibition at 200 μM), and no notable activity at COX-2. These results are in agreement with studies of Kalgutkar et al. who reported recently that the formation of esters of NSAIDs favors the interaction with COX-2.[23,24]

Coordination of Prop-ASS at the $Co_2(CO)_6$ cluster enormously increased the inhibitory effects. Co-ASS showed good inhibitory potential at 10 μM (COX-1: 37%, COX-2: 58% inhibition) and terminated the enzyme activity completely at 200 μM. It is worth to note that due to the 150-fold accumulation grade of Co-ASS (see cellular uptake studies) cellular concentrations of 200 μM and higher can be easily achieved.

Co-ASS is by far a more effective COX inhibitor than the parent compound acetylsalicylic acid. The positive effects of metal complexation of NSAIDs have already been described for copper derivatives. These complexes exhibit stronger or similar antiinflammatory potency compared to the parent NSAIDs.[25−27] Interestingly, a copper aspirinate complex showed also higher antiinflammatory effects in vivo than aspirin.[25] Unfortunately, investigations on the cytotoxic properties of these compounds are missing.

The COX inhibitory potency of the cobalt−alkyne complexes strongly depended on the alkyne ligand. Co-SAL completely inhibited both COX enzymes at 200 μM, but was inactive at 10 μM. Co-Benz was more active at COX-1 (40% inhibition at 10 μM and 47% inhibition at 200 μM) than at COX-2 (inactive at 10 μM, 41% inhibition at 200 μM). Co-Prop showed low inhibitory potential only in the highest used concentration of 200 μM (COX-1: 53%, COX-2: 41% inhibition). Interestingly, the order of COX-inhibitory activity (Co-ASS > Co-SAL > Co-Benz > Co-Prop) correlates well with their cytotoxicity. So it is very likely but not confirmed that the inhibition of the COX enzymes is the mode of action of the cobalt−alkyne complexes presented in this paper.

Inhibitors of cyclooxygenase enzymes are seen as anticancer drugs of the future. Up to now only a few COX inhibiting compounds with strong antiproliferative potency are described in the literature. The COX-2 selective aspirin derivative APHS strongly influences the growth of COX-2 positive HCA-7 colon carcinoma cells with IC_{50} values in the 2−5 μM range.[28,29]

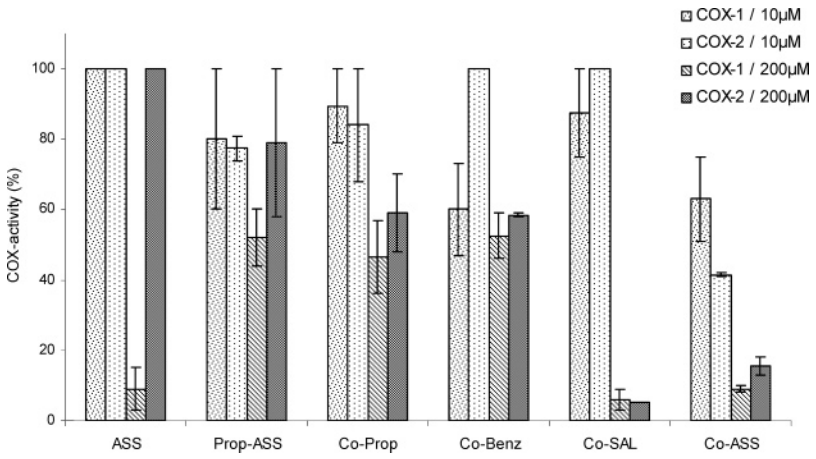


Figure 4. COX activity (COX-1 and COX-2) after treatment with compounds (10 μM and 200 μM).

Many studies deal with another COX-2 selective compound: celecoxib. It showed antiproliferative effects (IC_{50} = 35−65 μM) at both COX-2 positive and COX-2 negative cell lines.[30] The cellular mechanisms causing the antitumor effects of NSAIDS are seen controversially within the scientific community. Besides the inhibition of COX enzymes, effects on lipoxygenase or inhibition of angiogensis, for example, are under discussion.[30,31] However, the anticancer effects are a common effect of many NSAIDs.

Conclusion

To our best knowledge, Co-ASS represents the most cytotoxic drug among the COX-inhibiting anticancer drugs so far. It can be stated that derivatization of aspirin with propargylic alcohol and subsequent complexation to $Co_2(CO)_6$ afforded a compound, which is a more effective COX inhibitor than aspirin and which exhibits elevated cytotoxic activity. The COX inhibitory activity as well as the cytotoxic activity of closely related compounds was definitely lower. Further studies to extend this derivatization concept to other NSAIDs are going on.

Experimental Section

General. Chemicals were purchased from Sigma, Fluka, or Acros. MCF-7 and MDA-MB 231 cells were maintained as described.[3] Drugs were freshly prepared as stock solutions in dimethylformamide (DMF) and diluted with cell culture media or buffer when used for the biochemical experiments (DMF 0.1 V/V). *N*-(2-Propynyl)phthalimide, 2-acetoxy-*N*-(2-propynyl)-benzamide, Prop-ASS, Co-ASS, Co-SAL, and Co-Prop were synthesized and analyzed as described previously.[2,14−16] Protein quantification was performed by the method of Bradford[32] using human serum albumin (Sigma) for calibration purposes. Cobalt amounts were determined by graphite furnace atomic absorption spectroscopy[5] using a detection wavelength of 240.7 nm. Elemental analysis: Perkin-Elmer 240C, IR spectra: ATI Mattson Genesis, NMR spectra: Avance/DPX 400 (Bruker), MS spectra: CH-/A- Varian MAT (70 eV).

General Method for the Preparation of Benzoic Acid Esters. The respective benzoic acid derivative (10−17 mmol), dicyclohexylcarbodiimide (DCC, 12−18 mmol), 4-(dimethylamino)pyridine (DMAP, 2 mmol), and 2-propynol (12−16 mmol) were stirred in 30 mL of dry CH_2Cl_2 until no further product formation was observed by thin-layer chromatography (approximately 2 h). The mixture was filtered, the precipitate was rinsed with CH_2Cl_2, and the combined filtrates were evaporated. The resulting oil was purified by flash column chromatography on silica gel (mobile phase: diethyl ether/petroleum ether).

(2-Propynyl)benzoate. Benzoic acid: 2.00 g (16.3 mmol), DCC: 3.70 g (17.9 mmol), DMAP: 244.0 mg (2.0 mmol), 2-propynol: 1.01 g (17.9 mmol); Yield: 2.11 g (13.2 mmol), 81% yellow oil; IR (KBr, cm^{-1}) 1724 (C=O); MS (EI, 35 °C): *m/z* (%) = 160 (7) [M^+], 105 (100). ^1H NMR ($CDCl_3$): δ = 2.52 (t, 1H, J = 2.5 Hz, CH), 4.93 (d, 2H, J = 2.5 Hz, CH_2), 7.44−7.47 (m, 2H, Ar-H), 7.57−7.60 (m, 1H, Ar-H), 8.06−8.09 (m, 2H, Ar-H); ^{13}C NMR ($CDCl_3$): δ = 52.5 (CH_2), 75.0 (CH), 77.8 (−C≡), 128.4 (2 Ar-C), 129.4 (Ar-C), 129.8 (2 Ar-C), 133.3 (Ar-C), 165.8 (C=O).

3-Acetoxy-(2-propynyl)benzoate. 3-Acetoxybenzoic acid: 2.00 g (11.1 mmol), DCC: 2.52 g (12.2 mmol), DMAP: 244.0 mg (2.0 mmol), 2-propynol: 685.0 mg (12.2 mmol); Yield: 1.58 g (7.23 mmol), 65% colorless oil; IR (KBr, cm^{-1}) 1767 (C=O), 1729 (C=O); MS (EI, 35 °C): *m/z* (%) = 218 (1) [M^+], 105 (100); ^1H NMR ($CDCl_3$): δ = 2.32 (s, 3H, CH_3), 2.52 (t, 1H, J = 2.4 Hz, CH), 4.92 (d, 2H, J = 2.4 Hz, CH_2), 7.31−7.33 (ddd 1H, J = 8.0 Hz, J = 2.2 Hz, J = 0.9 Hz, Ar-H), 7.47 (dd, 1H, J = 8.0 Hz, J = 8.1 Hz, Ar-H), 7.79 (dd, 1H, J = 2.2 Hz, J = 0.9 Hz, Ar-H), 7.94−7.96 (ddd, 1H, J = 8.0 Hz, J = 0.9 Hz, J = 1.0 Hz, Ar-H); ^{13}C NMR ($CDCl_3$): δ = 21.0 (CH_3), 52.7 (CH_2), 75.2 (CH), 77.5 (−C≡), 123.1 (Ar-C), 126.8 (Ar-C), 127.3 (Ar-C), 129.5 (Ar-C), 131.0 (Ar-C), 150.7 (Ar-C), 164.9 (C=O), 169.2 (C=O).

2-Fluoro-(2-propynyl)benzoate. 2-Fluorobenzoic acid: 2.00 g (14.3 mmol), DCC: 3.24 g (15.7 mmol), DMAP: 244.0 mg (2.0 mmol), 2-propynol: 880.0 mg (15.7 mmol); Yield: 2.18 g (12.2 mmol), 86% colorless oil; IR (KBr, cm^{-1}) 1729 (C=O); MS (EI, 35 °C): *m/z* (%) = 178 (5) [M^+], 123 (100); ^1H NMR ($CDCl_3$): δ = 2.52 (t, 1H, J = 2.5 Hz, CH), 4.94 (d, 2H, J = 2.5 Hz, CH_2), 7.13−7.18 (m, 1H, Ar-H), 7.20−7.24 (m, 1H, Ar-H), 7.52−7.57 (m, 1H, Ar-H), 7.95−8.00 (m, 1H, Ar-H); ^{13}C NMR ($CDCl_3$): δ = 52.6 (CH_2), 75.2 (CH), 77.5 (−C≡), 117.1 (J_{CF} = 22.2 Hz, Ar-C), 118.0 (J_{CF} = 9.5 Hz, Ar-C), 124 (J_{CF} = 3.9 Hz, Ar-C), 132.3 (Ar-C), 135.0 (J_{CF} = 9.3 Hz, Ar-C), 162.1 (J_{CF} = 260.8 Hz, Ar-C), 163.5 (C=O).

General Method for Preparation of Cobalt−Alkyne Complexes. Cobalt−alkyne complexes were prepared according to an already described procedure.[3] The alkyne (0.5−1.0 mmol) was dissolved in 10 mL of dry THF or diethyl ether. Dicobaltoctacarbonyl was added in excess, and the solution was stirred at room temperature until no more product formation was observed by thin-layer chromatography. One gram of silica gel was added, and the mixture was evaporated to dryness. The dark colored products were isolated by flash column chromatography on silica gel (mobile phase: diethyl ether/petroleum ether). Yields were not optimized.

[(2-Propynyl)benzoate]hexacarbonyldicobalt (Co-Benz). (2-Propynyl)benzoate: 100.0 mg (0.62 mmol), dicobaltoctacarbonyl: 356.0 mg (0.94 mmol); Yield: 231 mg (0.16 mmol), 83% dark-red crystals (mp: 86−87 °C); IR (KBr, cm^{-1}) 2095, 2058, 2044, 2033, 2002 (Co−CO), 1717 (C=O); MS (EI,

100 °C): *m/z* (%) = 418 (3) [M^+ − CO], 390 (31) [M^+ − 2CO], 362 (34) [M^+ − 3CO], 334 (24) [M^+ − 4CO], 306 (7) [M^+ − 5CO], 278 (98) [M^+ − 6CO], 59 (100) [Co]; ^{1}H NMR ($CDCl_3$): δ = 5.53 (br, 2H, CH_2), 6.13 (br, 1H, CH), 7.45−7.47 (m, 2H, Ar-H), 7.56−7.58 (br, 1H, Ar-H), 8.10−8.12 (m, 2H, Ar-H). Anal. ($C_{16}H_8Co_2O_8$) C, H, N

[3-Acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-3-Acetbenz). 3-Acetoxy-(2-propynyl)benzoate: 107.0 mg (0.49 mmol), dicobaltoctacarbonyl: 280.0 mg (0.74 mmol); Yield: 164 mg (0.33 mmol), 66% brown-red crystals (mp: 51−52 °C); IR (KBr, cm^{-1}) 2097, 2058, 2029 (Co−CO), 1770 (C=O), 1721 (C=O); MS (EI, 100 °C): *m/z* (%) = 476 (4) [M^+ − CO], 448 (24) [M^+ − 2CO], 420 (25) [M^+ − 3CO], 392 (5) [M^+ − 4CO], 365 (35) [M^+ − 5CO], 343 (100), 336 (2) [M^+ − 6CO]; ^{1}H NMR ($CDCl_3$): δ = 2.31 (s, 3H, CH_3), 5.52 (br, 2H, CH_2), 6.13 (br, 1H, CH), 7.26 (br, 1H, Ar-H), 7.40−7.60 (br, 1H, Ar-H), 7.75−7.83 (br, 1H, Ar-H), 7.92−8.07 (br, 1H, Ar-H). Anal. ($C_{18}H_{10}Co_2O_{10}$) C, H, N.

[2-Fluoro-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-2F-Benz). 2-Fluoro-(2-propynyl)benzoate: 100.0 mg (0.56 mmol), dicobaltoctacarbonyl: 320.0 mg (0.842 mmol); Yield: 215 mg (0.432 mmol), 83% red crystals (mp: 88−89 °C); IR (KBr, cm^{-1}) 2097, 2037, 2001 (Co−CO), 1726 (C=O); MS (EI 100 °C): *m/z* (%) = 436 (3) [M^+ − CO], 408 (26) [M^+ − 2CO], 380(28) [M^+ − 3CO], 352(10) [M^+ − 4CO], 324(2) [M^+ − 5CO], 296 (100) [M^+ − 6CO]; ^{1}H NMR ($CDCl_3$): δ = 5.54 (br, 2H, CH_2), 6.12 (br, 1H, CH), 7.13−7.23 (m, 2H, Ar-H), 7.52−7.56 (br, 1H, Ar-H), 7.98−8.02 (br, 1H, Ar-H). Anal. ($C_{16}H_7Co_2FO_8$) C, H, N.

[2-Acetoxy-*N*-(2-propynyl)benzamide]hexacarbonyldicobalt (Co-ASSAM). 2-Acetoxy-*N*-(2-propynyl)benzamide: 100.0 mg (0.46 mmol), dicobaltoctacarbonyl: 260.0 mg (0.76 mmol); Yield: 14 mg (0.03 mmol), 7% red crystals (mp: 94 °C); IR (KBr, cm^{-1}) 2025, 2054, 2094 (Co−CO), 1627 (C=O), 1606 (C=O); MS (EI 100 °C): *m/z* (%) = 447 (12) [M^+ − 2CO], 419 (97) [M^+ − 3CO], 391 (57) [M^+ − 4CO], 363 (18) [M^+ − 5CO], 335 (100) [M^+ − 6CO]; ^{1}H NMR: δ = 2.35 (s, 3H, CH_3), 4.78 (br, 2H, CH_2), 6.10 (br, 1H, CH), 6.60 (s, 1H, NH), 7.14 (br, 1H, Ar-H), 7.20−7.30 (br, 1H, Ar-H), 7.48 (br, 1H, Ar-H), 7.75 (br, 1H, Ar-H). Anal. ($C_{18}H_{11}Co_2NO_9$) C, H, N.

[*N*-(2-Propynyl)phthalimide]hexacarbonyldicobalt (Co-Phthal). *N*-(2-Propynyl)phthalimide: 0.50 g (2.7 mmol), dicobaltoctacarbonyl: 1.03 g (3.0 mmol): Yield: 1.097 g (2.3 mmol), 85% red-brown crystals (mp: 105 °C); IR (KBr, cm^{-1}) 2095, 2054, 2036, 2016 (Co−CO); 1712 (C=O); MS (EI, 100 °C): *m/z* (%) = 443 (12) [M^+ − CO], 415 (48) [M^+ − 2CO], 387 (50) [M^+ − 3CO], 359 (29) [M^+ − 4CO], 331 (67) [M^+ − 5CO], 303 (90) [M^+ − 6CO], 28(100) [CO]; ^{1}H NMR ($CDCl_3$): δ = 5.04 (br, 2H, CH_2), 6.08 (br, 1H, CH), 7.74 (m, 2H, Ar-H), 7.88 (m, 2H, Ar-H). Anal. ($C_{17}H_7Co_2NO_8$) C, H, N.

Cytotoxicity Experiments (IC_{50}). Each 100 μL of 10 000 cells/mL (MCF-7) or 5 000 cells/mL (MDA-MB 231) were incubated in 96-well plates at 37 °C in 5% CO_2/95% air atmosphere for 72 h. One plate was used for the determination of the initial cell biomass and was treated in the following way: the medium was removed, cells were fixed by a 20−30 min incubation with 100 μL of glutardialdehyde solution (0.5 mL glutardialdehyde + 12.5 mL phosphate-buffered saline pH 7.4), the wells were emptied, 180 μL phosphate-buffered saline 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 (six replicates). After further incubation for 72 h (MDA-MB 231) or 96 h (MCF-7), these plates were treated as described above. The cell biomass was determined by crystal violet staining according to the following procedure: the phosphate-buffered saline 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 in a microplate reader at 590 nm (Flashscan AnalytikJena AG). 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 100%. IC_{50} was determined as that concentration causing 50% inhibition of cell proliferation and calculated as mean of at least two independent experiments.

Drug Lipophilicity (log k_w). Log k_w values were determined by an RP-HPLC method as described previously.[5]

Cellular Uptake Studies. MCF-7 and MDA-MB 231 cell cultures were grown in 24-well plates at 37 °C in 5% CO_2 atmosphere until at least 70% confluency. The medium was removed and replaced by a 2.0 μM drug-containing one (500 μL). After a proper incubation time at 37 °C in 5% CO_2 atmosphere, the drug-containing medium was removed and the cell monolayer was washed with 500 μL of phosphate-buffered saline pH 7.4. Distilled water (400 μL) was added, and after 10−15 min of incubation, the cells were lysed by means of a sonotrode. An aliquot of each lysate was removed for protein determination and stored at −20 °C. Probes (200 μL) for cobalt determination by atomic absorption measurement were stabilized by addition of 20 μL of HNO_3 (13%) and 20 μL of Triton X-100 (1%) and stored at 4 °C until measurement. Results were expressed as means of 6−12 wells as pmol drug per μg cellular protein. The cellular drug concentration can be estimated thereof as described by us.[17]

DNA-Binding Studies. Precipitation of drug DNA adducts was performed according to a described method[21] with some modifications: Salmon testes DNA (Sigma) was dissolved in phosphate-buffered saline pH 7.4 and drugs were added as stock solutions in DMF. The final solutions contained 40.6 μM drug, 250 μg/mL salmon testes DNA and 0.1% V/V DMF. After vortexing, the solutions were incubated at 37 °C in a water bath for 4 h. Aliquots of 200 μL were mixed with 100 μL of 0.9 M sodium acetate and three volumes of ice cold ethanol. Samples were stored at −20 °C for 30 min. The pellets were isolated by centrifugation (5000 U/min, 10 min, 4 °C) and resuspended in 300 μL of 0.3 M sodium acetate. 900 μL of ice cold ethanol were added, and the precipitate was collected after centrifugation (5000 U/min, 10 min, 4 °C). Samples were washed twice with ice cold ethanol and were stored at −20 °C. The pellets were dissolved in 500 μL of water (twice distilled), and the DNA content was determined by absorption reading at 260 nm in a UV-microplate reader (Flashscan AnalytikJena AG). Salmon testes DNA dissolved in water (twice distilled) was used for calibration purposes. Samples (200 μL) for cobalt determination by atomic absorption spectroscopy were stabilized by addition of 20 μL of Triton X-100 (1%). The amount of drugs bound to DNA was expressed as pmol of drug per μg of DNA. Results were calculated as means of at least two independent experiments, which were performed with two replicates.

Nuclear Drug Content. The nuclei of the tumor cells were isolated according to a described procedure[33] with some modifications: Cells were grown in 175 cm^2 cell culture flasks until at least 70% confluency. The medium was removed and replaced with 10 mL of medium containing 2.0 μM drug. After 24 h of incubation at 37 °C in humidified atmosphere, the drug-containing medium was removed, cells were trysinized, resuspended in 10 mL of cell culture medium, and isolated by centrifugation (1500 U/min, 5 min), and 0.5−1.0 mL of 0.9% NaCl solution was added. After centrifugation (1500 U/min, 5 min), pellets were resuspended in 300 μL of RSB-1 (0.01 M Tris-HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4) and left for 10 min in an ice-bath. Swollen cells were centrifuged (2000 U/min, 5 min), resuspended in 300 μL of RSB-2 (RSB-1 containing each 0.3% V/V Nonidet-P40 and sodium desoxycholate), and homogenized by 10−15 up/down-pushes in an 1 mL syringe with needle. Aliquots of 50 μL of the homogenisate were removed for determination of the total cobalt content and mixed with 500 μL of a 10 g/L EDTA solution. The homogenisate was centrifuged at 2500 U/min for 5 min and the resulting crude nuclei were taken up in 150 μL of 0.25 M sucrose containing 3 mM $CaCl_2$. The suspension was underlayed with 150 μL of 0.88 M sucrose and centrifuged 10 min at 2500 U/min. The nuclei pellets were stored at −20 °C or immediately

dissolved in 200 μL of 10 g/L EDTA solution and disrupted by use of a sonotrode. The cobalt content of the samples was determined by atomic absorption spectroscopy and the protein content by the method of Bradford.[32] Each experiment consisted of the data obtained from three pellets. Results are expressed as means of two independent experiments as pmol drug per μg nuclear protein. The ratio of drug taken up into the nucleus is calculated as percentage of the total cobalt content of each pellet.

Glutathione Reductase Activity. The influence on cellular glutathione reductase activity was assayed according to literature procedures.[8,9] In short: MCF-7 cells were grown in 24-well plates until at least 70% confluency. The medium was removed, and the cells were washed with phosphate-buffered saline (pH 7.4). Subsequently, 500 μL of cell culture medium containing 8.0 μM of the drugs or only DMF (control) were added. After 6 h of exposure, the drug-containing media were removed and the cells were washed with phosphate-buffered saline (pH 7.4). 250 μL of phosphate buffer (pH 7.0) was added, and the cells were lysed after 15 min by use of a sonotrode. Lysates were centrifuged (4000 U/min, 10 min: room temp) and assayed directly thereafter. Aliquots of 40 μL of each lysate were added to 290 μL of a mixture of NADPH (final concentration: 200 μM) and reduced glutathione (final concentration: 1 mM). The consumption of NADPH was monitored as the decrease in absorbance at 340 nm. Protein concentrations of the lysates were determined by the Bradford method.[32] Enzyme activity was calculated as nmol consumed NADPH/(min × μg protein) and expressed as percentage of the control. Results are calculated as means of at least two independent experiments ($n = 6$).

Apoptosis Detection. Apoptosis was measured after 3 h of drug exposure by use of an "Annexin-V-Fluos Staining Kit" (Roche) and after 5 days of drug exposure by an "ss-DNA Apoptosis ELISA Kit" (Chemicon Int.) according to the manufacturers instructions. Two independent experiments were performed at drug concentrations of 5 μM and 10 μM.

Estrogen Receptor Binding Affinity. The estrogen receptor binding affinity (RBA-value) was monitored as already described by us.[34]

Inhibition of COX Enzymes. The inhibition of isolated ovine COX-1 and human recombinant COX-2 was determined with 10 μM and 200 μM of the respective compounds by ELISA ("COX inhibitor screening assay", Cayman Chemicals). Experiments were performed according to the manufacturer's instructions. Results were calculated as the means of duplicate determinations.

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