Aspirin Derivatives

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Modulation of the Biological Properties of Aspirin by Formation of a Bioorganometallic Derivative**

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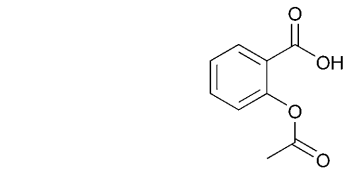
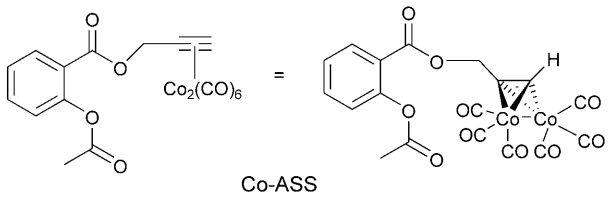
Despite recent advances in modern tumor therapy the development of effective drugs remain a challenge for medicinal chemists. The demand for innovative agents triggers interest in novel chemical strategies and new concepts for modern drug design.

The vast majority of drugs used to date are purely "organic" compounds. However, stimulated by the tremendous success of the inorganic compound cisplatin in modern tumor therapy, interest in the development of other metal complexes has been rapidly growing.[1–5] Bioorganometallic chemistry is a novel emerging field in medicinal chemistry, which aims at probing the biological (and therapeutic) potential of organometallic compounds.[6–9] As a result of their different coordination geometries, chemical properties, and reactivities, metal complexes offer a wide spectrum of functional groups more or less unexplored in modern drug design and development.

The hexacarbonyldicobalt moiety $Co_2(CO)_6$ bound to an alkyne, is one such functional group, for which promising results on medical applications have been reported.[10] For example, $Co_2(CO)_6$ derivatives of antiepileptic drugs (e.g. carbamazepine) were used as diagnostic tools in the so-called carbonyl metallo immuno assay (CMIA), and complexes with fructopyranose, nucleoside, and neuropeptide ligands displayed interesting bioactivities.[11–14]

We have recently reported on alkyne hexacarbonyldicobalt species with promising antiproliferative properties.[15] Interestingly, the cell growth inhibitory activity of the complexes depended strongly on the chemical structure of the alkyne ligand. Weakly active and inactive derivatives showed that the cobalt cluster does not cause general (unspecific) cytotoxic effects. In further studies the $Co_2(CO)_6$ complex of the aspirin (*o*-acetylsalicylic acid, ASS) derivative prop-2-ynyl-2-acetoxybenzoate (Co-ASS) emerged as a lead compound for this class of antiproliferative agents.





Initial studies on the mode of action of Co-ASS indicated that the inhibition of the cyclooxygenase enzymes COX-1 and COX-2 might play an important role: Co-ASS inhibited isolated COX-1 and COX-2 more efficiently than the parent compound aspirin. The preferential inhibition of COX-1 by aspirin was not observed with the metal complex, which inhibited both isoenzymes approximately to the same extent.[16] Studies on human platelets further confirmed the COX-1 inhibitory potential of Co-ASS. Interestingly, no inhibition of the related enzyme 12-LOX could be observed.[12]

Aspirin belongs to the class of nonsteroidal anti-inflammatory drugs (NSAIDs), whose pharmacological effects (analgetic, antipyretic, and anti-inflammatory) 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.[17] However, the exact mode of action of NSAIDs as antitumor drugs is the subject of debate.

It was found that the COX-2 isoenzyme is overexpressed in various tumors, and elevated levels of the products of cyclooxygenase (prostaglandins) were detected. The link between COX-2 activity and antiproliferative properties of

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NSAIDs could not be proved so far, and NSAID effects on different pathways independent or downstream from COX-2 activity (e.g. apoptosis, angiogenesis, interaction with matrix metalloproteinases) are also considered to be important for the observed antitumoral effects.[18] This suggests that a single mechanism for NSAID antitumor activity does not exist and multiple pathways might be relevant.

In this report we present evidence for the modulation of antitumor-related biochemical activities of aspirin as a consequence of its derivatization as the hexacarbonyldicobalt complex Co-ASS.

To study the influence of Co-ASS and ASS on cellular cyclooxygenase activity, the levels of the major COX metabolite prostaglandin E_2 (PGE_2) and COX-2 gene expression from arachidonic acid stimulated MDA-MB-231 breast tumor cells were measured by enzyme-linked immunoabsorbant assay (ELISA) and real-time polymerase chain reaction (RT-PCR), respectively (Table 1). Both Co-ASS and ASS significantly reduced cellular PGE_2 formation at 10 μM ($p < 0.001$ for each), which indicates that the cyclooxygenase activity was suppressed by both compounds. The MDA-MB-231 cells used have been reported to exhibit low levels of the constitutively expressed isoenzyme COX-1 but high levels of the inducible isoenzyme COX-2.[19] Thus the reduced PGE_2 concentrations can be mainly attributed to the activity of COX-2.

Table 1: Influence of Co-ASS and ASS on various COX-related parameters.[a]

	PGE_2	COX-2	Bcl-2	TGFα	IL-10	MMP-7
Co-ASS 1 μM	81 ± 30	73 ± 16	97 ± 1	82 ± 14	82 ± 17	77 ± 18
Co-ASS 10 μM	34 ± 14	79 ± 19	129 ± 49	83 ± 24	73 ± 16	53 ± 11
aspirin 1 μM	60 ± 27	125 ± 12	135 ± 55	93 ± 14	90 ± 8	86 ± 14
aspirin 10 μM	44 ± 29	122 ± 23	127 ± 23	87 ± 26	83 ± 7	73 ± 12

[a] After 24 h exposure; values are presented as percentage of the untreated control ± standard deviation.

COX-2 expression levels were slightly lowered by Co-ASS ($p < 0.05$). In contrast, a small increase after exposure to aspirin could be noted ($p < 0.05$ for 1 μM). Based on these results for both compounds a mechanism involving the direct interaction with the enzyme as well as a perturbation of its expression is likely.

The mechanism of action of aspirin is based on the acetylation of a serine residue in the active site of cyclooxygenase enzymes and a subsequent blockade of the oxidation of the physiological COX substrate arachidonic acid. To evaluate the molecular interaction with COX-2, peptide fragments generated by trypsin digestion after incubation of the enzyme with either aspirin or Co-ASS were examined by LC–ESI tandem mass spectrometry.[20] The enzyme was also analyzed alone for comparison purposes. As expected, COX-2 incubated with aspirin showed exclusive acetylation of Ser516, which is in excellent agreement with literature reports on the mode of action of this drug.[21] In contrast, exposure to Co-ASS did not lead to detectable acetylation of this residue. Interestingly, the lysine residues 166, 346, 432, and 598 were acetylated in this case (see Figure 1 and Table 2), as confirmed by their highly significant SEQUEST parameters ($X_{corr} \geq 3.13$, $\Delta C_n \geq 0.65$, observed/expected ions ≥ 57%; see Table 2).[22] The lysine residues are not acetylated in COX-2 alone or in the enzyme following treatment with aspirin.

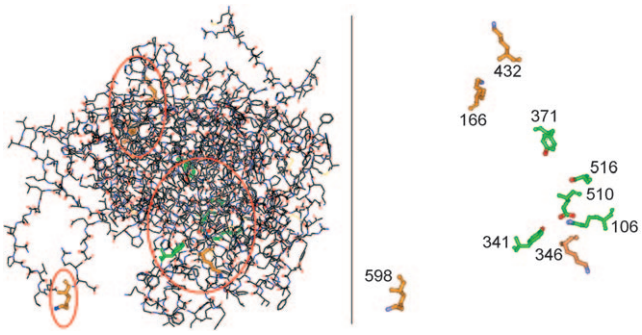

Figure 1. Interaction of Co-ASS with COX-2. The carbon atoms of amino acids relevant for the catalytic activity and those that are Co-ASS acetylation sites are in color (catalytic activity: green, Co-ASS acetylation: orange). Left: Model of human COX-2, full view. Right: Close-up of relevant amino acid residues (other amino acid residues and the backbone of the enzyme in this area are omitted for clarity; the heme is not depicted as it is not available in the model used). Pictures were generated using ViewerLight 4.2 (Accelrys) and were based on RCSB protein database entry 1v0x[31] (www.pdb.org).

Of these residues the side chain of Lys346 is positioned close to the entrance channel of the active site of the enzyme. Thus, the distance from the side-chain nitrogen atom of Lys346 to the side-chain nitrogen atoms of Arg106, which forms the entrance channel together with Arg499 and Glu510,

Table 2: Acetylation sites (@) in COX-2 as determined by ESI tandem mass spectrometry.

Peptide sequence	Site	Charge	SEQUEST parameters			Ions[a]
			X_{corr}	ΔC_n	Sp	
COX-2 + aspirin						
K.PRPDAIFGETMVEVGAPFS@LK.G	S516	3	4.75	0.73	1257.9	32/80
COX-2 + Co-ASS						
K.QLPDSNEIVEK@LLLR.R	K166	2	4.80	0.79	1284.7	21/28
L.PDSNEIVEK@LLLR.R	K166	2	3.13	0.75	1253.7	17/24
K.LK@FDPELLFNK.Q	K346	2	3.80	0.65	658.2	15/20
V.PPAVQK@VSQASIDQSR.Q	K432	2	3.13	0.72	771.6	17/30
R.SGLDDINPTVLLK@ER.S	K598	2	4.27	0.70	822.7	18/28

[a] Observed/possible b^+ and y^+ ions resulting from cleavage of peptide C(O)–N(H) bonds.[32]

is only 16.5 Å. While Lys589 is positioned quite distant from the active site, Lys166 and Lys432 are located near a heme prosthetic group above Tyr371, which itself is involved in the electron transfer between the physiological substrate arachidonic acid and the heme group.

In analogy to the established pharmacology of aspirin (acetylation of Ser516 of the enzyme) these results indicate that the mode of drug action of Co-ASS might be based on the acetylation of multiple lysine side chains. It can be speculated that a blockade of the entrance to the active site, as known from other NSAIDs, or a interference with the electron-transfer mechanism of the enzyme may be of high relevance.[21] It should be noted that covalent modifications of the enzyme supposedly lead to an irreversible inhibition of its function (in analogy to the irreversible inhibition of COX activity by ASS related to serine acetylation).

NSAIDs trigger many effects downstream or independent of COX-2 activity in tumor cells which are supposed to be important for the antitumor properties of this class of compounds. Among these activities, the regulation of the anti-apoptotic protein bcl-2, caspases, the tumor growth factor (TGF)α, interleukin-10 (IL-10), and matrix metalloproteinases (MMPs) plays a major role.[18]

We found no significant influence of Co-ASS and aspirin on the bcl-2 and TGFα pathways. Exposure to 10 μM of either agent significantly lowered the IL-10 levels ($p < 0.05$). Interestingly, caspase-3 activity was found to be strongly induced by Co-ASS. In contrast ASS led to no activity at all concentrations tested (Figure 2). This indicates that apoptotic

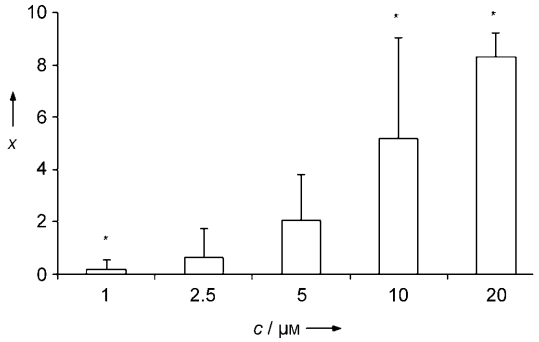

Figure 2. Influence of Co-ASS on caspase-3 activity after exposure for 24 h in MDA-MB-231 cells ($n = 3$). n = the number of experiments. The x-fold caspase induction of treated cells relative to that of an untreated control is depicted.

events related to caspase activation (but not the bcl-2 pathway) are of relevance for the bioactivity of the $Co_2(CO)_6$ complex.

Both drugs significantly decreased the levels of MMP-7, whereby the effect was more marked for Co-ASS ($p < 0.05$ for Co-ASS at both concentrations and $p < 0.05$ for ASS at 10 μM). This result is of special interest as MMP-7 is considered to be relevant for metastases formation of gastric and endometrial carcinomas.[23] The impact of NSAIDs on metastasis and tumor blood vessel formation is an established fact and for several NSAIDs antiangiogenic effects have been reported. PGE_2 is considered to be an important downstream mediator regulating angiogenesis, and the inhibition of its formation was related to antiangiogenic effects.[18]

In order to evaluate the effects of Co-ASS and aspirin as angiogenesis inhibitors we chose the zebrafish (*Danio rerio*), an established model organism for the study of angiogenesis and vascular development in vivo (Figure 3).[24,25] The small

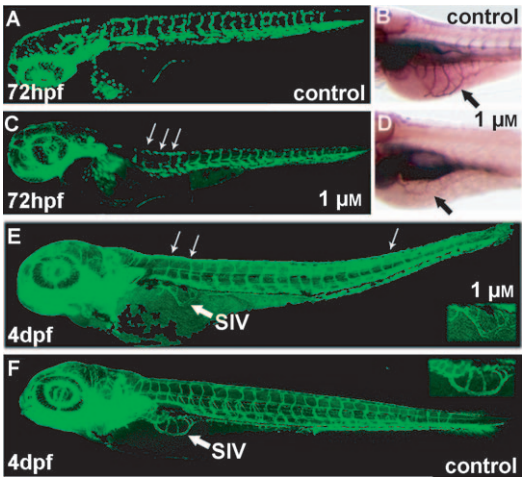

Figure 3. Effect of Co-ASS treatment on blood vessel formation in zebrafish embryos. Lateral views of embryos at 72 hours post fertilization (hpf) and 4 days post fertilization (dpf) are depicted. A) Untreated control embryo at 72 hpf and C) corresponding Co-ASS treated embryo. B, D) Close-ups of alkaline phosphatase stained blood vessels of control (B) and treated (D) embryos. E, F) Control (E) and treated (F) embryos at 4 dpf; the inset shows close-ups of the subintestinal vein; thin white arrows in C and E indicate damaged or missing dorsal longitudinal anastomotic vessels. ASS (1 μM) was used as a control. Fluorescence images of the Tg:fli1/eGFP zebrafish (A, C, E, and F) were recorded by laser scanning confocal microscopy. Alkaline phosphatase stained embryos were depicted by stereomicroscopy (B and D). For more details see the Supporting Information.

size of the fish, the fecundity, the development external to its mother, and the optical clarity of the embryo provide great advantages over other animal models. For observing vascular development albino strains are particularly useful. Because of its small size, the embryo can receive enough oxygen by passive diffusion. Its development can be monitored for several days even in complete absence of blood circulation. Besides these advantages, the vascular anatomy in the developing zebrafish has been well characterized.[26]

Cyclooxygenases have been shown to be expressed in the developing zebrafish, and the relevance of these enzymes for vascular tube formation was demonstrated.[27–29] We treated zebrafish embryos and larvae with different concentrations of either Co-ASS or ASS. For these experiments two independent methods were used. Method 1 makes use of the lack of pigmentation in developing albino zebrafish embryos.[24] After drug treatment, vascularization is visualized by alkaline phosphatase staining and stereomicroscopy.[24] Method 2 uses the transgenic zebrafish line Tg:fli1/eGFP, which allows for live imaging of angiogenesis in zebrafish.[25] The Tg:fli1/eGFP embryos and larvae express the green fluorescent protein

(GFP) under an early endothelial promoter and therefore exhibit a fluorescent green vasculature. The results presented here for method 2 were obtained by using either laser scanning confocal microscopy or high-resolution fluorescence stereomicroscopy.

Our data show that developing zebrafish embryos treated with Co-ASS display severe defects in vascularization and angiogenesis (impaired formation or lack of intersegmental vessels and of dorsal longitudinal anastomotic vessels, and reduced subintestinal veins). Several of these connective vessels were missing in the Co-ASS treated embryos but not in the control groups. Details on the induced vascular damages as well as general statements on the zebrafish vascular anatomy are given in the Supporting Information. In contrast to the impact of Co-ASS on zebrafish vascularization ASS at the same concentration did not show any notable effects.

Modification of the established NSAID aspirin as an organometallic derivative resulted in a significant modulation of the known biological properties of the parent drug. While basic pharmacological properties remained essentially unchanged (e.g. reduction of cellular PGE_2 formation) certain pathways downstream of COX activity were modified significantly. Thus, the organometallic derivative exhibited additional effects involving anti-angiogenic properties (as observed by reduction of blood vessel formation in the developing zebrafish embryo), inhibited the activity of MMP-7 more strongly than aspirin, and significantly induced caspase-3 activity.

In this context it is of interest to note that anti-angiogenic and anti-metastatic properties have also been demonstrated for other metallodrugs such as NAMI-A, a ruthenium complex currently undergoing clinical trials. These complexes have high potential in the development of novel antitumor drugs that stop tumor growth by interruption of the essential blood supply.[30] Furthermore, it can be speculated that some of the observed COX-independent effects might be also observed with other $Co_2(CO)_6$ derivatives, for which anti-proliferative effects have been reported.[12–14]

The differing pharmacological properties of Co-ASS and ASS might be the consequence of an altered interaction with the target enzyme COX-2 based on the acetylation of various lysine side chains. Of those, Lys346 might probably be the most relevant acetylation site for the inhibition of the enzyme.

The results presented here illustrate a major concept in drug development in bioorganometallic medicinal chemistry: The pharmacological properties of established bioactive compounds can be modulated as a result of an altered receptor interaction, which itself is the consequence of the presence of an organometallic fragment.

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