Full Paper

Synthesis, Characterisation and Biological Evaluation of Copper and Silver Complexes based on Acetylsalicylic Acid

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Metalcarbonyl complexes with ligands derived from acetylsalicylic acid demonstrated high cytotoxic potential against various tumor cell lines and strong inhibition of the cyclooxygenase enzymes COX-1 and 2. In this study we tried to achieve comparable effects with [alkyne]silver or copper trifluoromethanesulfonate complexes which are more hydrophilic then the uncharged metalcarbonyl derivatives. All compounds were evaluated for growth inhibition against breast (MCF-7, MDA-MB 231) and colon cancer (HT-29) cell lines and for COX-1 and COX-2 inhibitory effects at isolated isoenzymes. Pure ligands showed neither cytotoxic nor COX-inhibitory effects. While the silver complexes of (but-2-ynyl)-2-acetoxybenzoate (**But-ASS-Ag**) and (but-2-yne-1,4-diyl)-bis(2-acetoxybenzoate) (**Di-ASS-But-Ag**) were strong cytostatics, only the copper complex **Di-ASS-But-Cu** was active. At the COX enzymes the complexes were more effective than their ligands and aspirin.

Keywords: Acetylsalicylic acid / Copper / Cyclooxygenase / Cytotoxicity / Silver / Transition metal complexes

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Introduction

Despite the success of cisplatin and closely related platinum antitumor agents in tumor therapy [1, 2], there is an increasing interest in new transition metal antitumor drugs for the second line therapy. In our group, we focused our attention on the design of multi-target complexes. A specifically acting ligand was combined with a cytotoxic metal moiety. This drug design was successful regarding hormone-dependent tumors [3–8].

Our present drug design based on the observation that various tumor cells e.g. MCF-7 and MDA-MB 231 mammary carcinoma cells contain permanent or inducible amounts of cyclooxygenase enzymes 1 and 2 (COX-1, COX-2). Their growth can therefore be influenced by non-steroidal anti-inflammtory drugs (NSAIDs). While indometacin exerts high cytotoxicity acetylsalicylic acid (aspirin, ASS) was nearly inactive. The cell growth inhibitory effects can strongly be increased by esterification of ASS with propargylic alcohol and coordination of the alkyne to a $Co_2(CO)_6$ cluster (Co-ASS) [9]. Co-ASS showed high COX inhibitory effects and another acetylation profile of COX enzymes than ASS. Structure activity relationship studies demonstrated a high relevance of an aspirin partial structure and the presence of a metal-carbonyl cluster for these effects [10, 11].

Using a cyclopentadienyl (Cp) moiety for the design of organometal complexes only the thallium complexes showed high cytotoxicity. Related Cp-metalcarbonyls were distinctly less active [12]. These finding induced us to synthesize further carbonyl-free organometal complexes and to evaluate their growth inhibitory potency. We decided to use in this preliminary study silver and copper alkynyl complexes with (but-2-ynyl)-2-acetoxybenzoate (**But-ASS**) and (but-2-yne-1,4-diyl)-bis(2-acetoxybenzoate) (**Di-ASS-But**) already proven as suitable ligands. The new complexes were tested for growth inhibition against MCF-7, MDA-MB 231 and HT-29 cells as well as for COX inhibitory effects at isolated enzymes (COX-1 and COX-2).

Results and discussion

Synthesis and Structural Characterisation

The synthesis of **But-ASS** and **Di-ASS-But** were described elsewhere [11]. All organometalic syntheses were carried out under ''Schlenk'' conditions using an inert, dry atmosphere.

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Complexation of the corresponding alkyne ligands with copper and silver triflate were performed in THF with stirring for 3 h (Scheme 1) [13]. Silver salts immediately precipitated as white salts of the [alkyne]$Ag^{+}CF_3SO_3^{-}$ type in a yield of over 90%. To precipitate the yellow colored copper complexes by addition of diethyl ether, the solution had prior to be concentrated and cooled (yields about 80%).

Both complex types can be best described by ESI-TOF-MS and ^{1}H-NMR. In the MS spectra characteristic mol peaks with significant line pattern confirmed the binding of the ligands to the metals. While in the case of the silver complexes the ^{1}H-NMR signals of the ligands were nearly unaffected upon coordination, copper complex signals are broadened and shifted to lower field. The methylene group of **But-ASS** is shifted from $\delta = 4.84$ to $\delta = 4.97$ and the terminal methyl group from $\delta = 1.88$ even to $\delta = 3.88$. The $-CH_2-$ groups of **Di-ASS-But** remained nearly unchanged (shift from 4.94 to 4.96 ppm).

It should be mentioned that the silver and copper complexes are easily soluble in water, alcohol or DMSO, moderate in THF or chloroform and insoluble in ether or hydrocarbons.

In-vitro Chemosensitivity Assay

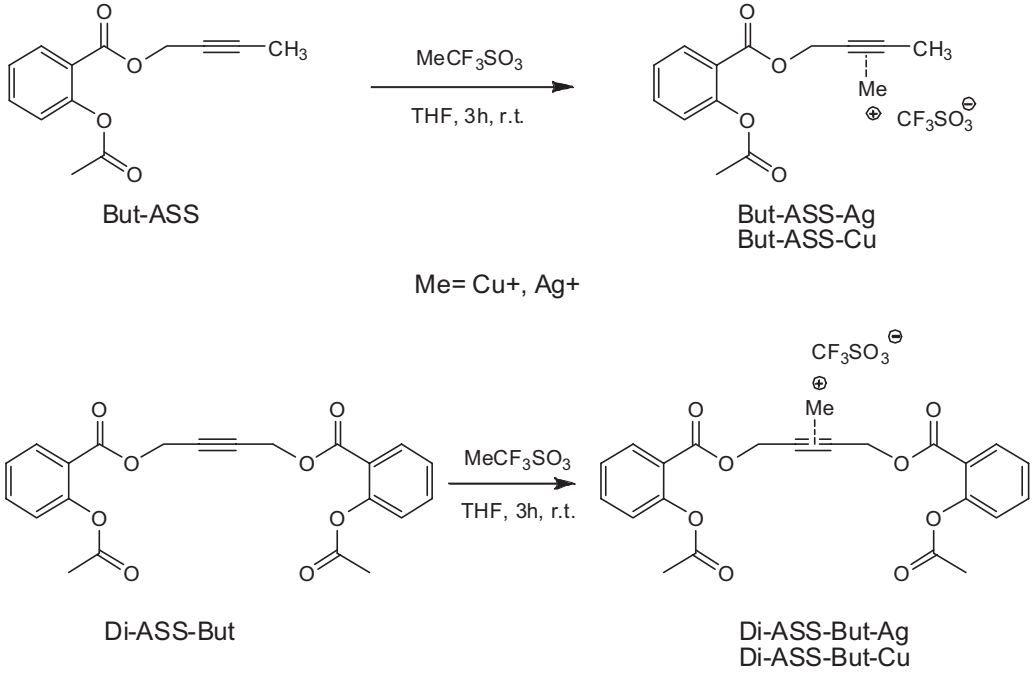
Antitumor activity was determined in vitro using colon (HT-29) as well as breast cancer (MCF-7 and MDA-MB 231) cell lines. Cisplatin was used as reference. All compounds showed their maximum of activity in a time dependent chemosensitivity test (data not shown) after 72–96 h. Therefore, IC_{50} values were determined after an incubation time of 72 h.

Table 1. Cytotoxicity (IC_{50} values [μM]) against breast cancer and colon carcinoma cell lines.

Compound	MCF-7	MDA-MB 231	HT-29
Cisplatin	2.0 ± 0.3	3.3 ± 0.5	2.4 ± 0.4
Aspirin (ASS)	>100	>100	>100
Organic ligands	>50	>50	>50
But-ASS-Ag	7.3 ± 1.1	5.8 ± 0.7	17.9 ± 0.2
Di-ASS-But-Ag	4.9 ± 0.5	5.0 ± 0.3	16.1 ± 0.5
But-ASS-Cu	>100	>100	>100
Di-ASS-But-Cu	27.5 ± 2.2	14.6 ± 0.3	18.5 ± 1.5

All ligands as well as aspirin were tested up to concentrations of 50 and 100 μM, respectively, and demonstrated no inhibitory effects (Table 1).

Silver complexes showed high activity in MCF-7 and MDA-MB 231 breast cancer cell lines and medium activity in HT-29 cell lines. The effects are nearly independent on the ligands used. In contrast, the growth inhibitory effects of copper complexes depended on the ligand. **But-ASS-Cu** was completely inactive even at 100 μM at all three cell lines, whereas **Di-But-ASS-Ag** reduced the cell growth with IC_{50} of 15 to 28 μM. Interestingly, **Di-ASS-But-Cu** showed the lowest effects at MCF-7 cells. This would be an indication of different COX inhibitory potency, since MCF-7 cells have a basal level of COX-1 and a barely detectable and transient COX-2 inducible



Scheme 1. Synthesis of copper and silver complexes.

Table 2. COX inhibition at a concentration of 10 μM

compound	COX-1	COX-2
Aspirin (ASS)	29.2 ± 2.0%	1 ± 0.1%
Organic ligands	<10%	<1%
But-ASS-Ag	11.0 ± 1.2%	27.0 ± 1.5%
Di-ASS-But-Ag	36.6 ± 4.5%	18.8 ± 1.0%
But-ASS-Cu	31.7 ± 2.8%	10.4 ± 0.8%
Di-ASS-But-Cu	28.7 ± 3.7%	15.8 ± 2.2%

expression, whereas MDA-MB 231 cells show a low expression of COX-1 but a constitutive level of COX-2 [14].

COX-Inhibition

At the used concentration of 10 μM **ASS** reduced the activity of COX-1 by 29.2% and did not influence COX-2. Derivation to **But-ASS** and **Di-ASS-But** led to inactive compounds.

The ligands modulated the results of the silver complexes. **But-ASS-Ag** was more active at COX-2, while **Di-ASS-But-Ag** showed higher activity at COX-1 (see Table 2). In contrast, **But-ASS-Cu** and **Di-ASS-But-Cu** caused identical inhibitory effects more effective at COX-1 than at COX-2.

Conclusion

In this study we could demonstrate that silver and copper trifluoromethanesulfonate complexes of (but-2-ynyl)-2-acetoxybenzoate (**But-ASS**) and (but-2-yne-1,4-diyl)-bis(2-acetoxybenzoate) (**Di-ASS-But**) can be used as lead structures for the design of organometallic inhibitors of cyclooxygenase enzymes with cytotoxic potency. Metal binding to the ligands increased the pharmacological properties and make the complexes to interesting drugs for the treatment of mammary and colon carcinoma.

Experimental section

Syntheses

General

Commercially available chemicals were used without further purification. Solvents were purified by distillation from an appropriate drying agent: Tetrahydrofuran and diethyl ether were dried over sodium/potassium alloy and distilled under argon atmosphere. Pyridine was dried over KOH and was stored over 4-Å molecular sieves. Products were purified by flash chromatography on silica gel (230–400 mesh, Merck). Melting points: 510 Büchi (Flawil/Schweiz) capillary melting point apparatus. ^{1}H-NMR: Avance DPX-400 spectrometer (Bruker, Karlsruhe/Germany) at 400 MHz with TMS as internal standard. Elemental analyses: Microlaboratory of the Freie Universität Berlin on Perkin-Elmer 240C. 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/Germany).

But-ASS and **Di-ASS-But** were prepared as already published [11].

General Method for the Preparation of Ag(I) and Cu(I) trifluoromethanesulfonate complexes of alkyne compounds

All preparations and reactions were performed in pure argon atmosphere and absolute THF.

A solution of 1 mmol of the corresponding alkyne in 8 mL of THF was degassed at −80°C in oil pump vacuum and allowed to warm to room temperature. An amount of 1 mmol of the metal salt ($AgSO_3CF_3$: 257 mg; $CuSO_3CF_3 \cdot 0.5\ C_6H_6$: 252 mg) was then added under argon counterflow and stirred for one hour at room temperature. Whereas white silver complexes **But-ASS-Ag** and **Di-ASS-But-Ag** precipitated, copper complexes were easily soluble in THF. Therefore, THF was removed under reduced pressure to 3 mL and 10 mL of diethyl ether were added in small portions. All precipitates were sucked off, washed three times with pentane and afterwards dried for three hours in oil pump vacuum.

*[(But-3-ynyl-2)-2-acetoxybenzoate]-silvertrifluoromethanesulfonate (**But-ASS-Ag**)*

But-ASS: 232 mg (1.0 mmol). Yield: 435 mg (89%). MS (FAB-MS): m/z = 597 [M + Ag] (13), 339 [M-(CF_3SO_3)] (84), 296 [M-(CF_3SO_3)-(Ac)] (5), 120 (100) [HO-(C_6H_4)-CO]. HR-MS (FAB): Calcd. for $C_{13}H_{12}O_4Ag$ [M-(CF_3SO_3)] 338.97810; found 338.97867, calcd. for $C_{14}H_{12}O_7SF_3Ag_2$ [M + Ag] 596.83543; found 596.83555. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 1.87 (t, ^5J = 2.3 Hz, 3H, ≡C–CH_3), 2.38 (s, 3H, O=C–CH_3), 4.85 (q, ^5J = 2.3 Hz, 2H, O–CH_2–C≡), 7.11 (dd, ^3J = 8.1 Hz, ^4J = 0.6 Hz, 1H, 3′-H), 7.32 (ddd, ^3J = 7.9 Hz, ^3J = 7.4 Hz, ^4J = 0.9 Hz, 1H, 5′-H), 7.54 (ddd, ^3J = 7.9 Hz, ^3J = 7.6 Hz, ^4J = 1.2 Hz, 1H, 4′-H), 8.03 (dd, ^3J = 7.8 Hz, ^4J = 1.5 Hz, 1H, 6′-H). Anal. calcd. ($C_{14}H_{12}O_7SF_3Ag$): C, 34.37; H, 2.47. Found: C, 34.44; H, 2.68%.

*[(But-3-ynyl-2)-2-acetoxybenzoate]-coppertrifluoromethanesulfonate (**But-ASS-Cu**)*

But-ASS: 232 mg (1.0 mmol). Yield: 369 mg (83%). MS (FAB-MS): m/z = 507 [M + Cu] (5), 295 [M-(CF_3SO_3)] (22), 253 [M-(CF_3SO_3)-(Ac)] (14), 136 (100) [HO-(C_6H_4)-COO], 120 (46) [HO-(C_6H_4)-CO]. HR-MS (FAB): Calcd. for $C_{13}H_{12}O_4Cu$ [M-(CF_3SO_3)] 295.00261; found 295.00317. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 2.38 (s, 3H, O=C–CH_3), 3.88 (br, 3H, ≡C–CH_3), 4.97 (br, 2H, O–CH_2–C≡), 7.15 (br, 1H, 3′-H), 7.37 (br, 1H, 5′-H), 7.62 (br, 1H, 4′-H), 8.07 (br, 1H, 6′-H). Anal. calcd. ($C_{14}H_{12}O_7SF_3Cu$): C, 37.80; H, 2.72. Found: C, 37.68; H, 2.93%.

*[(But-2-yne-1,4-diyl)-bis(2-acetoxybenzoate)]-silvertrifluoromethanesulfonate (**Di-ASS-But-Ag**)*

Di-ASS-But: 410 mg (1.0 mmol). Yield: 614 mg (92%). MS (FAB-MS): $m/z = 517$ [M-(CF_3SO_3)] (14), 353 [M-(CF_3SO_3)-($C_9H_7O_3$)] (6), 120 (100) [HO-(C_6H_4)-CO]. HR-MS (FAB): Calcd. for $C_{22}H_{18}O_8Ag$ [M-(CF_3SO_3)] 517.00525; found 517.00516. ^{1}H-NMR ($CDCl_3$, 400 MHz): $\delta = 2.37$ (s, 6H, O=C–CH_3), 4.97 (s, 4H, O–CH_2–C≡), 7.12 (dd, $^3J = 7.9$ Hz, 2H, 3′-H), 7.33 (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), 8.08 (dd, $^3J = 7.9$ Hz, $^4J = 1.5$ Hz, 2H, 6′-H). Anal. calcd. ($C_{23}H_{18}O_{11}SF_3Ag$): C, 41.40; H, 2.72. Found: C, 41.27; H, 2.90%.

*[(But-2-yne-1,4-diyl)-bis(2-acetoxybenzoate)]-coppertrifluoromethanesulfonate (**Di-ASS-But-Cu**)*

Di-ASS-But: 410 mg (1.0 mmol). Yield: 542 mg (87%). MS (FAB-MS): $m/z = 473$ [M-(CF_3SO_3)] (54), 310 [M-(CF_3SO_3)-($C_9H_7O_3$)] (11), 120 (100) [HO-(C_6H_4)-CO]. HR-MS (FAB): Calcd. for $C_{22}H_{18}O_8Cu$ [M-(CF_3SO_3)] 473.02979; found 473.02922. ^{1}H-NMR ($CDCl_3$, 400 MHz): $\delta = 2.36$ (s, 6H, O=C–CH_3), 4.96 (s, 4H, O–CH_2–C≡), 7.13 (br, 2H, 3′-H), 7.33 (br, 2H, 5′-H), 7.59 (br, 2H, 4′-H), 8.07 (br, 2H, 6′-H). Anal. calcd. ($C_{23}H_{18}O_{11}SF_3Cu$): C, 44.34; H, 2.91. Found: C, 44.26; H, 3.05%.

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 [15]. All three cell lines were maintained in L-glutamine and 4.5 g/L D-glucose containing Dulbecco's Modified Eagle Medium DMEM (Sigma, Germany), supplemented with 5% fetal calf serum (FCS; Gibco, Germany) using 25-cm^2 culture flasks (Sarstedt, Nürnberg) in a humidified atmosphere at 37°C. MCF-7 cell line was passaged weekly after treatment with trypsin (0.05%) and ethylenediaminetetraacetic acid (0.02%; EDTA; Boehringer, Germany) whereas HT-29 and MDA-MB 231 were 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 5% CO_2 (MDA-MB 231: 6% CO_2) 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 20–30 min incubation with 100 μL of glutardialdehyde solution (0.5 mL glutardialdehyde + 12.5 mL phosphate-buffered saline pH 7.4). The solution in the wells was sucked off, 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 (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 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 at 590 nm using a microplate reader (Flashscan S12, 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} value was determined as that concentration causing 50% inhibition of cell proliferation and calculated as mean of at least two independent experiments.

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). The experiments were performed according to the manufacturer's instructions. Absorption was measured at 415 nm (Flashscan S12, Analytikjena AG).

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