BioMetals (2005) 18:171–177
DOI 10.1007/s10534-004-6252-z
© Springer 2005

Stability, protein binding and thiol interaction studies on [2-acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt

Ingo Ott & Ronald Gust*
Institute of Pharmacy, Free University of Berlin, Koenigin-Luise-Str. 2+4, 14195 Berlin
**Author for correspondence (E-mail: rgust@zedat.fu-berlin.de)*

Received 8 July 2004; accepted 15 November 2004; Published online: March 2005

Key words: cobalt complexes, glutathione, protein binding, stability

Abstract

Cobalt–alkyne complexes are a new class of potent cytotoxic drugs. The lead compound [2-acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-ASS) showed high effects on several human cancer cell lines. In order to evaluate further the *in-vitro* properties and reactivity of this substance we performed stability and protein binding studies and investigated the interaction of this complex with 1,2-ethanedithiol, L-cysteine and glutathione. UV–Vis, HPLC and AAS studies showed that the compound was sufficiently stable under *in-vitro* conditions. Binding to human serum albumin increased from approximately 25% at the beginning to over 50% after 48 h of incubation as determined by ethanol preciptation and size exclusion chromatography. The interaction with thiols resulted in disulfide bond formation of the thiols.

Introduction

Hexacarbonyldicobalt complexes with alkyne ligands (cobalt–alkyne complexes) are useful reagents in preparative chemistry: In the Pauson–Khand reaction (Pauson 1985) they are used as intermediates for the synthesis of cyclopentenones and the Nicholas reaction (Lockwood & Nicholas 1977) makes use of the complexes ability to form stable propargyl cations, which can be reacted with nucleophiles. Besides their advantages in synthetic chemistry, the complexes have gained attention as labelling agents and new class of cytostatics. Ethinylestradiol was one of the first bioactive molecules coordinated to $Co_2(CO)_6$. This complex can be used as marker in the so-called carbonyl-metallo-immuno-assay and is known to inactivate the estrogen receptor (Vessieres *et al.* 1992; Metzler-Nolte 2001).

Our interests focussed on the use of cobalt–alkyne complexes as cytostatics. Compounds out of this class exhibited strong cytotoxicity. The activity depended strongly on the structure of the alkyne ligand and the kind of cell-line used in the *in-vitro* experiments. [2-Acetoxy-(2-propynyl) benzoate]hexacarbonyldicobalt (Co-ASS, see Figure 1) emerged as the lead compound for this new cytostatic agents. The best effects were observed at human breast cancer cells, where the activity of Co-ASS exceeded the activity of the established anticancer drug Cisplatin (Jung *et al.* 1997; Schmidt *et al.* 2000; Roth *et al.* 2002; Ott *et al.* 2004).

This paper deals with further studies on the lead compound Co-ASS. Experiments were performed to investigate the stability of the complex under *in-vitro* conditions as used in the initial cell culture studies. The purpose of these investigations was to evaluate if an intact complex or formed metabolites can be made responsible for the cytotoxic activity. Protein binding to human serum albumin (HSA) and to protein components of serum containing cell culture medium was measured as the reactivity towards HSA strongly affects the metabolism, efficacy and biodistribution of drugs.

It is a well known fact that the anticancer drug Cisplatin and other platinum containing drugs are

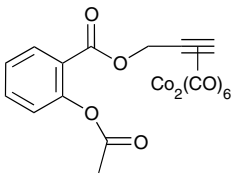

Figure 1. Structural formula of Co-ASS.

very reactive towards the cysteine residue of the tripeptide glutathione (GSH). The levels of GSH in tumor cells reach the low millimolar range and are made responsible for inactivation and drug resistance phenomena (Perquin *et al.* 2001; Boulikas & Vougiouka 2003; Neumann *et al.* 2003). Therefore, the interaction of Co-ASS with 1,2-ethanedithiol, L-cysteine and GSH was evaluated in order to characterize potential adducts formed by e.g. a Nicholas reaction (Lockwood & Nicholas 1977) or a ligand substitution at the dicobalt residue (Verdaguer *et al.* 1999). Furthermore, thiol-induced reduction as described previously for antimony(V) complexes had to be taken into account (Ferreira *et al.* 2003).

Materials and methods

General

Chemicals and reagents were from Sigma, Acros and Fluka, HSA was purchased from Sigma, fetal calf serum (FCS) from PANBiotech, phosphate buffer solution pH 7.0 from Merck. Co-ASS was prepared and analysed as previously described (Jung *et al.* 1997). Protein contents were quantified by the method of Bradford (1976). Cobalt contents were measured by graphite furnace atomic absorption spectroscopy (AAS) at a detection wavelength of 240.7 nm as previously described in detail (Ott *et al.* 2004). HPLC measurements were performed with a Kontron HPLC system equipped with a diode array detector and a Nucleosil C18 (25 cm, 5 μm, 4 mm ID) column. Co-ASS was eluted isocratically with methanol/water 80/20 at a flow rate of 0.6 ml/min. NMR-spectra were recorded on a 400 MHz Avance/DPX 400 (Bruker) and MS-spectra on a CH-/A-Varian MAT (70 eV). EMEM cell culture medium: L-glutamine containing Eagle's MEM (Sigma) supplemented with $NaHCO_3$ (2.2 g/l), sodium pyruvate (110 mg/l), gentamycin (50 mg/l) adjusted to pH 7.4.

Stability studies

UV–Vis absorption spectroscopy

Co-ASS was dissolved in DMF and then immediately mixed with 10% FCS containing EMEM (250 μM Co-ASS, 0.5% V/V DMF). Each 250 μl aliquot ($n = 6$) was pipetted in the wells of a UV 96-well plate (Nunc) and incubated at 37 °C/5% CO_2. After appropriate incubation periods the absorption at 354 nm was read in a microplate reader (Flashscan S12, AnalytikJena AG). Co-ASS concentrations from 50–400 μM were used for calibration purposes ($r^2 \geqslant 0.997$). The drug concentration was calculated as percent of the initial drug concentration (250 μM).

High performance liquid chromatography (HPLC)

Co-ASS was dissolved in DMF, then immediately mixed with phosphate buffer solution pH 7.0 (200 μM Co-ASS, 1.0% V/V DMF) and incubated at 37 °C in a water bath. After appropriate incubation periods samples were analysed by HPLC. Calibration was performed with methanolic solutions of Co-ASS in the range of 50–300 μM ($r^2 \geqslant 0.998$). The concentration was calculated as percent of the initial drug concentration (200 μM) using the absorption data at 354 nm.

Atomic absorption spectroscopy (AAS)

Co-ASS was dissolved in DMF and then immediately mixed with 10% (V/V) FCS containing EMEM (1.0 μM Co-ASS, 0.1% V/V DMF). Each 250 μl aliquot ($n = 6$) was pipetted in the wells of a 96-well plate and incubated at 37 °C/5% CO_2. After appropriate incubation periods a 200 μl aliquot was removed from the surface, stabilized by addition of each 20 μl Triton X-100(1%) and HNO_3(13%) and analysed by AAS.

Protein binding experiments

Ethanol precipitation

The experiments were performed according to a described method with some modifications (Ma *et al.* 1996). A 3.0 mM drug stock solution in DMF was freshly prepared and a 10 μl aliquot was mixed with 10.0 ml EMEM containing 400 mg HSA or with 10.0 ml EMEM containing

10% (V/V) FCS. The mixture was incubated in a gently shaking water bath at 37 °C. After appropriate incubation periods a 250 μl aliquot of the solution was transferred into a 2-ml Eppendorf tube, treated with 500 μl cold (−20 °C) ethanol, stored at −20 °C for 2 h and centrifuged at 2000 U/min (1370 g) for 5 min at 4 °C. A 400 μl aliquot of the supernatant was removed and stabilized with 40 μl HNO_3(13%) for determination of the cobalt content by AAS. The cobalt amount not bound to proteins was determined as the means of three experiments and calculated as percentage of the initial drug amount. The protein binding was obtained by subtracting the unbound percentage from the total (100%).

Size exclusion chromatography

HSA (115 mg) was dissolved in 5.0 ml EMEM and a 1.0 ml aliquot was removed as for determination of the HSA-recovery (total). A 25 mM stock solution of Co-ASS in DMF was freshly prepared, a 4.0 μl aliquot was added to a 4.0 ml aliquot of the HSA-solution and thoroughly mixed. A 100 μl aliquot was removed for the determination of the recovery of Co-ASS (total). The mixture was incubated in a gently shaking water bath at 37 °C. After appropriate incubation periods each 100 μl aliquot of the solution was injected onto a chromatographic column. The stationary phase consisted of 10 ml prequollen 'Sephadex G50 fine' filled in a 10 ml-plastic syringe. Distilled water was used as mobile phase. Fractions of a volume between 0.5 and 1.5 ml were collected until the phenolred of the medium was completely eluted from the column. The fractions were investigated for their protein content and for their cobalt content. To the samples for AAS measurements each 10% (V/V) Triton X-100(1%) and HNO_3(13%) were added. The absorption data from the cobalt and protein measurements were plotted against the elution volume. For this purpose, the highest observed absorption value was set at 1.00 and the absorption values of the other fractions were normed to it (rel. abs.). The area under curve (AUC) of the two peaks was determined by integration over the elution volume. The protein binding to HSA was calculated as percentage of the AUC of the first peak compared to the total AUC. For recovery calculations the sum of eluted protein and the sum of eluted cobalt were compared with the corresponding total amounts.

Interaction with thiols

The reactions were performed under argon atmosphere at room temperature. The product formation of simultaneously processed blank samples (solutions containing the thiols but not containing Co-ASS) was subtracted from the corresponding yields.

1,2-Ethanedithiol

Co-ASS (4.42 mg, 8.77 μmol) and 1,2-ethanedithiol (500 μl, 5.96 mmol) were stirred in 40 ml of MeOH for 5 days. The precipitate was isolated by filtration, washed with methanol and dried over P_2O_5. Yield: 4.0 mg (–S–CH_2–CH_2–S–, MG = 92 g/mol, 43.5 μmol, <1%); MS(EI, 230 °C): m/z (%) = 368(2) [4 × 92], 276(6) [3 × 92], 184(50) [2 × 92], 124(100); ^{1}H-NMR ($CDCl_3$): δ = 3.11(s), 3.16(s), 3.20(s).

L-*Cysteine*

Co-ASS (3.86 mg, 7.66 μmol) and L-cysteine (124.13 mg, 1.03 mmol) were stirred in 20 ml of MeOH/water (3/1) for 5 days. The precipitate was isolated by centrifugation (685× *g* for 10 min) and dried at 85–90 °C. Yield: 69.1 mg (0.24 mmol, 24%); MS(EI, 280 °C): m/z(%) = 256(2) [S_8], 224(3) [S_7], 192(12)[S_6], 160(6) [S_5].

Reduced glutathione (GSH)

Co-ASS (4.61 mg, 9.15 μmol) and GSH (51.09 mg, 160 μmol) were stirred in 20 ml of MeOH/water (3/1) for 7 days. The formation of oxidized glutathione (GSSG) was quantified by HPLC using the absorption data at 215 nm. The mobile phase was composed of MeOH/water/trifluoroacetic acid (7/93/0.01). Other chromatographic conditions were applied as decribed above. Commercially available (Sigma) GSSG was used for calibration purposes. Yield: 3.91 mg (6.4 μmol, 4%) GSSG.

Results and discussion

Stability studies

The stability of Co-ASS was investigated by UV–Vis spectroscopy and HPLC. Figure 2a shows the UV–Vis spectra of Co-ASS, dicobaltoctacarbonyl

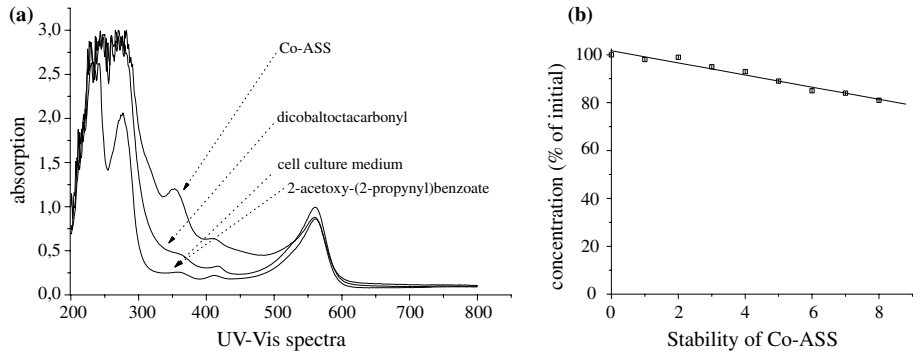

Figure 2. UV–Vis spectra and stability testing of Co-ASS recorded in EMEM cell culture medium supplemented with 10% (V/V) FCS at 37 °C.

and 2-acetoxy-(2-propynyl)benzoate recorded in EMEM cell culture medium (pH 7.4) containing 10% (V/V) FCS. Co-ASS exhibited a strong maximum at 354 nm. Uncomplexed 2-acetoxy-(2-propynyl)benzoate could not be distinguished from the absorption of the medium and dicobaltoctacarbonyl showed only weak absorption. Therefore, the absorption maximum at 354 nm could be used to estimate the stability of the dicobalt–alkyne partial structure. UV–Vis stability studies in cell culture medium showed a slight decrease of the complex concentration over the time (81% after 8 h, see Figure 2b). HPLC experiments in phosphate buffer pH 7.0 confirmed these results: after 1 h 96% (98% in the UV–Vis experiment) and after 6 h 83% (85% in the UV–Vis experiment) intact complex were quantified. After 24 h of incubation 63% intact Co-ASS were determined by HPLC, indicating that the concentration of Co-ASS continuously decreased over longer incubation periods. The potential products of hydrolysis or complex decomposition (2-acetoxybenzoate: $t_{ret} = 4.4$ min, 2-acetoxy-(2-propynyl)benzoate: $t_{ret} = 5.2$ min, hexacarbonyl[2-propynol]dicobalt: $t_{ret} = 8.5$ min, hexacarbonyl[2-hydroxy-(2-propynyl)benzoate]dicobalt: $t_{ret} = 25.1$ min) could be properly separated from Co-ASS ($t_{ret} = 15.5$ min) using HPLC. However, none of these products or others could be detected besides Co-ASS within 24 h of incubation.

As Co-ASS is a highly lipophilic compound (Ott *et al.* 2004) sedimentation or adsorption to the walls caused by the insufficient solubility in aqueous media could be the reason for the loss of absorbance. In order to allow appropriate detection of the complex concentrations of 250 μM (UV–Vis studies) and 200 μM (HPLC studies) were used. These concentrations are much higher than the ones routinely used for *in-vitro* experiments. Therefore, a 1.0 μM solution was investigated by AAS. Over a period of 8 days 99–102% cobalt were recovered from the liquid surface, showing that the compound did not precipitate or adsorb at low concentration over a long period of time.

The above described results indicate that Co-ASS is sufficiently stable under *in-vitro* conditions. It can be concluded that the previously observed cytotoxic activities were caused by an intact complex.

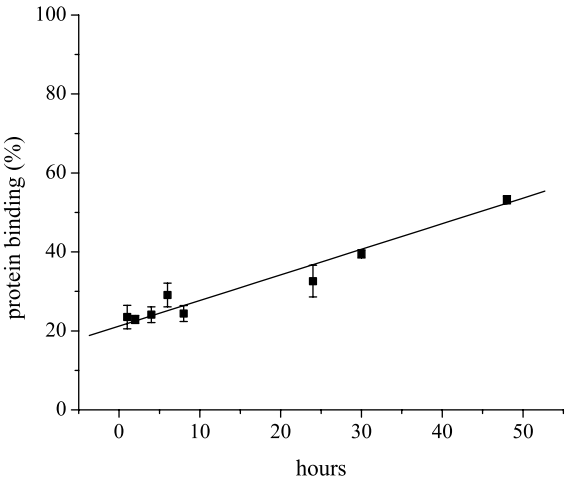

Figure 3. Binding of Co-ASS to HSA in EMEM cell culture medium at 37 °C determined by ethanol precipitation. The results are expressed as means ± standard deviations ($n = 3$).

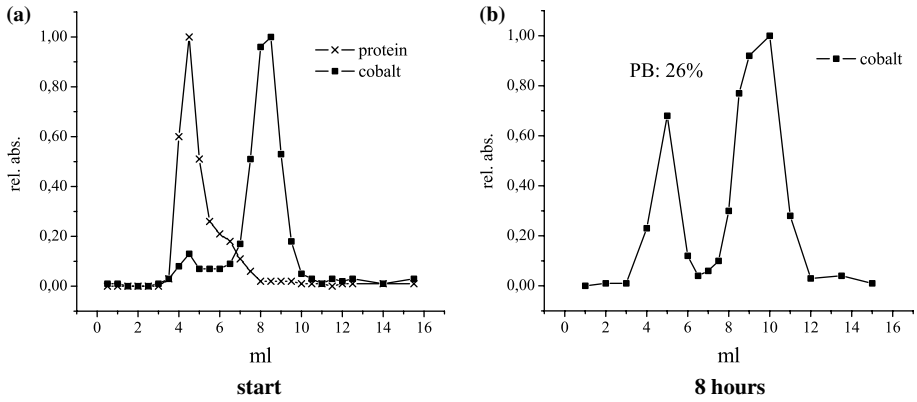

Figure 4. Protein binding (PB) of Co-ASS to HSA in EMEM cell culture medium at 37 °C determined by size exclusion chromatography.

Protein binding

Protein binding was studied by ethanol precipitation and size exclusion chromatography. Both methods have been shown to be useful for the measurement of the binding of metal complexes to proteins (Ma *et al*. 1996; Amann *et al*. 2001). Using the former procedure the unbound cobalt amount of the samples was quantified by AAS and the amount bound to the protein could be calculated. The latter chromatographic procedure was experimentally more complicated but offered the possibility of directly measuring the protein binding. All experiments were performed under *in-vitro* conditions similar to those used in the initial studies. For this purpose EMEM cell culture medium (adjusted to pH 7.4) was used as solvent and all experiments were performed at a temperature of 37 °C. Figure 3 shows the amount of the complex bound to HSA over a period of 48 h determined by the precipitation method. The binding took place very fast. After 1 h of incubation, 24% of Co-ASS were bound to HSA. During the incubation period this high initial binding slightly increased, resulting in 53% of Co-ASS attached to HSA after 48 h. Size exclusion chromatography experiments were performed after 0, 4, 8 and 30 h of incubation. Figure 4a shows the chromatogram obtained at the start of the incubation period. Protein measurements verified that the first peak was HSA. Cobalt determination by AAS resulted in the detection of two peaks: a fast eluting small peak, which corresponded to bound Co-ASS, and a slower moving second peak due to unbound Co-ASS or other low molecular weight cobalt compounds. Recovery rates (cobalt: 104% and HSA: 95%) showed that the sample was completely eluted from the column. Chromatograms obtained after longer incubation showed an increase of the first peak and a decrease of the second peak (shown exemplarily in Figure 4b). Protein binding was calculated by integration (4 h: 22%, 8 h: 26% and 30 h: 40%) and was in excellent agreement with the data from the ethanol precipitation method (4 h: 24%, 8 h: 24% and 30 h: 40%).

The binding behaviour observed by the two methods indicated a fast reversible binding followed by a slower occurring irreversible binding. Similar results were described previously for aryl-substituted [ethylenediamine]platinum(II) complexes (Schertl *et al*. 2004).

HSA was used at 0.59 mM for the ethanol precipitation method, corresponding to physiological plasma conditions. In the size exclusion experiments a lower concentration of 0.34 mM was used to avoid overloading of the chromatographic column. In cell culture experiments medium containing 10% (V/V) FCS (approximately 0.06 mM HSA) was routinely used. After 2 h of incubation in serum containing EMEM 13% binding was determined by ethanol precipitation. Compared with 23% obtained in the HSA-experiment, the lower protein content caused slightly lower protein binding of Co-ASS. This decreased binding at lower protein content was previously described for platinum complexes like Cisplatin. Furthermore, the HSA-binding of Cisplatin (>95% after 24 h) is

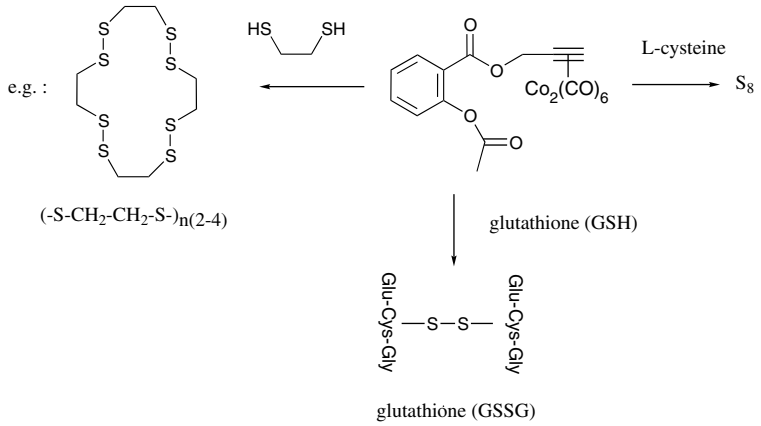

Figure 5. Reaction of Co-ASS with 1,2-ethanedithiol, L-cysteine and GSH.

in general much higher than the HSA-binding of Co-ASS (Bernhardt *et al*. 1999; Schertl *et al*. 2004).

The above described results indicate that sufficiently high plasma levels of unbound Co-ASS can be expected in future *in-vivo* studies and that albumin caused inactivation of the drug may only be of minor relevance.

Interaction with thiols

The interaction with thiols was studied by reacting Co-ASS with an excess of 1,2-ethanedithiol, L-cysteine and glutathione in methanol or methanol/water mixtures. Figure 5 summarizes the results. The expected products formed by a Nicholas reaction (Lockwood & Nicholas 1977) or ligand substitution at the cobalt-cluster (Verdaguer *et al*. 1999) were not observed. Interestingly, the thiols reacted by disulfide bond formation. In the case of 1,2-ethanedithiol a mixture of dark coloured cyclic disulfides of the general formula $[–S–CH_2–CH_2–S–]_{(2–4)}$ precipitated. The reaction with L-cysteine resulted in formation of S_8 and GSH was oxidized (GSSG). These results can be explained by the ability of cobalt–alkyne complexes to be reduced under complex decomposition (Osella *et al*. 1991; Osella & Fiedler 1992). The reductive decomposition of Co-ASS was confirmed by monitoring the decrease of the Co-ASS concentration and the formation of the free alkyne ligand by HPLC. At the end of the reaction the recovery of intact complex was 74% for the reaction with 1,2-ethanedithiol, 17% for the reaction with glutathione and less than 1% for the reaction with L-cysteine, due to the different conditions and different turnover rates. Metal catalysed formation of thiacrowns as observed with 1,2-ethanedithiol was also described previously (Adams *et al*. 1996; Arterburn *et al*. 1997). Besides a direct oxidation of the thiols caused by Co-ASS, an indirect oxidation after formation of reactive oxygen species (ROS) must be considered as a possible mechanism. In aqueous solution ROS are generated in presence of metallic cobalt. This reaction can be enhanced by GSH. (Leonard *et al*. 1998).

Metal drug induced formation of disulfide bonds must be also considered for biologically relevant macromolecules. The formation of dimers and polymers of albumin in the presence of the anticancer drug Cisplatin has been described (Ivanov *et al*. 1998).

Conclusion

[2-Acetoxy-(2-propynyl)benzoate]hexacarbonyldicobalt (Co-ASS) showed good stability under *in-vitro* conditions. Protein binding to serum albumin increased from initial 24% to over 50% after 48 h of incubation, indicating that high plasma levels of unbound drug can be obtained. The reaction with thiols resulted in formation of disulfides.

References

Adams RD, Queisser JA, Yamamoto JH. 1996 Catalytic transformations of thiiranes by the $W(CO)_5$ grouping. A

new rote to cyclic polydisulfides. *J Am Chem Soc* **118**, 10674–10675.

Amann A, Decristoforo C, Ott I *et al*. 2001 Surfactant protein B labelled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ retains biological activity *in vitro*. *Nucl Med Biol* **28**, 243–250.

Arterburn JB, Perry MC, Nelson SL *et al*. 1997 Rhenium-catalyzed oxidation of thiols and disulfides with sulfoxides. *J Am Chem Soc* **119**, 9309–9310.

Bernhardt G, Koch M, Spruss T *et al*. 1999 [Meso-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]sulfatoplatinum (II)- Pharmacokinetic studies. *Arch Pharm Pharm Med Chem* **332**, 195–200.

Boulikas T, Vougiouka M. 2003 Cisplatin and platinum drugs at the molecular level (review). *Oncol Rep* **10**, 1663–1682.

Bradford MM. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Ferreira CdS, Martins PS, Demicheli C *et al*. 2003 Thiol induced reduction of antimony(V) into antimony(III): a comparative study with tryptanothione, cysteinyl-glycine, cysteine and glutathione. *Biometals* **16**, 441–446.

Ivanov AI, Christodoulou J, Parkinson JA *et al*. 1998 Cisplatin binding sites on human albumin. *J Biol Chem* **273**(24), 14721–14730.

Jung M, Kerr DE, Senter PD. 1997 Bioorganometallic chemistry – synthesis and antitumor activity of cobalt carbonyl complexes. *Arch Pharm Pharm Med Chem* **330**, 173–176.

Leonard S, Gannett PM, Rojanasakul Y *et al*. 1998 Cobalt-mediated generation of reactive oxygen species and its possible mechanism. *J Inorg Biochem* **70**, 239–244.

Lockwood RF, Nicholas KM. 1977 Transition metal-stabilized carbenium ions as synthetic intermediates. I. α-[(alkynyl)dicobalt hexacarbonyl] carbenium ions as propargylating agents. *Tetrahedron Lett* **48**, 4163–4166.

Ma J, Stoter G, Verweij J, Schellens JHM. 1996 Comparison of ethanol plasma-protein precipitation with plasma ultrafiltration and trichloroacetic acid protein precipitation for the measurement of unbound platinum concentrations. *Cancer Chemother Pharmacol* **38**, 391–394.

Metzler-Nolte N. 2001 Labeling of biomolecules for medicinal applications – bioorganometallic chemistry at its best. *Angew Chem Int Ed Engl* **40**(6), 1040–1043.

Neumann C, Boubakari, Gruenert R *et al*. 2003 Nicotinamide adenine dinucleotide phosphate-regenerating system coupled to a glutathione-reductase microtiter method for determination of total glutathione concentrations in adherent growing cancer cell lines. *Anal Biochem* **320**, 170–178.

Osella D, Fiedler J. 1992 Reinvestigation of the electrochemical behavior of the $Co_2(CO)_6$(ethynylestradiol) complex. Evidence of efficient recombination of the electrogenerated fragments. *Organometallics* **11**, 3875–3878.

Osella D, Jaouen G, Zanello P. 1991 Transition metal complexes as electrochemical markers for steroids. *Organomet Chem* **401**(1–2), 37–47.

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

Pauson PL. 1985 The Khand reaction. *Tetrahedron* **41**(24), 5855–5860.

Perquin M, Oster T, Maul A *et al*. 2001 The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features. *J Cancer Res Clin Oncol* **127**, 368–374.

Roth T, Eckert C, Fiebig HH *et al*. 2002 Comparative action of cobalt carbonyl complexes on cancer cells using human tumor xenografts. *Anticancer Res* **22**, 2281–2284.

Schertl S, Hartmann RW, Batzl-Hartmann C *et al*. 2004 [1,2-Bis(2,6-difluoro-3-hydroxyphenyl)ethylenediamine]platinum (II) complexes, compounds for the endocrine therapy of breast cancer – mode of action II: contribution of drug inactivation, cellular drug uptake and sterical factors in the drug–target interaction to the antitumor activity. *Arch Pharm Pharm Med Chem* **337**, 349–359.

Schmidt K, Jung M, Keilitz R *et al*. 2000 Acetylenehexa carbonyldicobalt complexes, a novel class of antitumor drugs. *Inorg Chim Acta* **306**, 6–16.

Verdaguer X, Moyano A, Pericas MA *et al*. 1999 Alkyne dicobalt carbonyl complexes with sulfide ligands. Synthesis, crystal structure, and dynamic behavior. *Organometallics* **18**, 4275–4285.

Vessieres A, Top S, Vaillant C *et al*. 1992 Estradiols modified by metal carbonyl clusters as suicide substrates for the study of receptor proteins: application to the estradiol receptor. *Angew Chem Int Ed Engl* **31**, 754–755.