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New investigations into the genotoxicity of cobalt compounds and their impact on overall assessment of genotoxic risk

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ABSTRACT

The genotoxicity of cobalt metal and cobalt compounds has been widely studied. Several publications show induction of chromosomal aberrations, micronuclei or DNA damage in mammalian cells *in vitro* in the absence of S9. Mixed results were seen in gene mutation studies in bacteria and mammalian cells *in vitro*, and in chromosomal aberration or micronucleus assays *in vivo*. To resolve these inconsistencies, new studies were performed with soluble and poorly soluble cobalt compounds according to OECD-recommended protocols. Induction of chromosomal damage was confirmed *in vitro*, but data suggest this may be due to oxidative stress. No biologically significant mutagenic responses were obtained in bacteria, $Tk^{+/-}$ or *Hprt* mutation tests. Negative results were also obtained for chromosomal aberrations (in bone marrow and spermatogonia) and micronuclei at maximum tolerated doses *in vivo*. Poorly soluble cobalt compounds do not appear to be genotoxic. Soluble compounds do induce some DNA and chromosomal damage *in vitro*, probably due to reactive oxygen. The absence of chromosome damage in robust GLP studies *in vivo* suggests that effective protective processes are sufficient to prevent oxidative DNA damage in whole mammals. Overall, there is no evidence of genetic toxicity with relevance for humans of cobalt substances and cobalt metal.

Abbreviations: 2-AA, 2-aminoanthracene; 2NF, 2-nitrofluorene; 4-NOPD, nitro-*o*-phenylenediamine; 6 TG, 6-thioguanine; 8-OH-dG, 8-hydroxy-2-deoxyguanosine; 9AA, 9-aminoacridine; AAF, artificial alveolar fluid; B[a]P, benzo(a)pyrene; CA, chromosomal aberrations; CE, cloning efficiency; $CoSO_4$, cobalt sulphate heptahydrate; CoOct, cobalt octoate (more correctly known as cobalt bis(2-ethylhexanoate)); CPA, cyclophosphamide; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's minimal essential medium; DMH, 1,2-dimethylhydrazine monohydrate; EDX, energy dispersive X-ray spectroscopy; EMS, ethyl methanesulfonate; GEF, global evaluation factor; GLP, Good Laboratory Practice; *gpt*, glutamic-pyruvate transaminase gene; HBSS, Hank's balanced salts solution; hOGG1, human 8-hydroxyguanine DNA-glycosylase 1; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase gene; ICP-MS, inductively coupled plasma mass spectrometry; IWGT, International Workshops on Genotoxicity Testing; LMF, log mutant frequency; MC, methyl cellulose; MF, mutant frequency; MI, mitotic index; MMC, mitomycin C; MMS, methyl methanesulfonate; MN, micronucleus or micronuclei; MTD, maximum tolerated dose; NA, nuclear anomalies; NADP, nicotinamide adenine dinucleotide phosphate; NCE, normochromatic erythrocytes; NQO, 4-nitroquinoline-1-oxide; NTP, National Toxicology Program; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; PE, plating efficiency; PHA, phytohaemagglutinin; PSD, particle size distribution; ROS, reactive oxygen species; RPMI 5, RPMI 10 and RPMI 20, RPMI medium containing 5%, 10% and 20% heat-inactivated horse serum, respectively; RS, relative survival; RTG, relative total growth; S9, 9000 *g* supernatant from livers of rats induced either with Aroclor-1254 or phenobarbital and β-naphthoflavone; SD, standard deviation; SEM, scanning electron microscopy; SHE, Syrian hamster embryo; SIN-1, 3-morpholinosydnonimine hydrochloride; TI, tail intensity; TFT, trifluorothymidine; *Tk*, thymidine kinase gene.

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1. Introduction

Cobalt metal has many important uses in industry. The most important use of cobalt metal is as an alloying element in superalloys, magnetic and hard-metal alloys, such as stellite and cemented carbides, cobalt-containing high-strength steels, electrodeposited alloys and alloys with special properties. Cobalt salts and oxides are used as pigments in the glass and ceramics industries, as catalysts in the oil and chemical industries, as paint and printing ink driers and as trace metal additives for agricultural and medical uses. Cobalt, in the form of cobalamin, is also an essential trace element in human nutrition, as cobalt is found as the active centre in cobalamin coenzymes. Given the widespread use of and exposure to cobalt and cobalt compounds, its safety for humans is of prime importance. The potential health hazards resulting from exposure to cobalt compounds have recently been reviewed (Paustenbach et al., 2013), but mainly focussed on the chemistry, pharmacokinetics and systemic toxicity of cobalt. In this paper we review the existing published genotoxicity data for cobalt substances and present diverse new experimental data from studies with a variety of different cobalt substances that should be considered in the overall assessment of cobalt and its genotoxic potential.

2. Review of published data

The genotoxic potential of soluble cobalt compounds and cobalt metal itself has been widely studied and reported.

2.1. Studies with bacteria

In terms of mutagenic activity in bacteria, Zeiger et al. (1992) reported that **cobalt sulphate heptahydrate** was a weak mutagen in *Salmonella typhimurium* strain TA100 when using the pre-incubation method, and Pagano and Zeiger (1992) reported a stronger mutagenic response for **cobalt chloride** in TA97, again using the pre-incubation method with different aqueous solvents. **Cobalt chloride** was also reported to be mutagenic in the absence of S9 in strains TA98 and TA1537 using the plate incorporation method (Wong, 1988). However, the range of concentrations used was very toxic, no data from individual treatments were presented, and the control revertant numbers for strains TA1535 and TA1537 were unusually high. For these reasons these findings are less convincing. **Cobalt metal** itself was tested in *S. typhimurium* strains TA98 and TA100, and in *E. coli* WP2*uvrA*/pKM101 as part of the National Toxicology Program (NTP, 2013). No mutagenic responses were observed in *E. coli*, either in the absence or presence of S9 using the pre-incubation method. An equivocal response was observed in TA100 in the absence of S9, but mutagenic responses that were weak and not well correlated with dose level were observed in strain TA98 in the absence of S9. In a meeting abstract, Turoczi et al. (1987) reported very weak to weak mutagenic effects for **cobalt acetate** in 3 out of 5 *Salmonella* strains at a high dose (10 mg/plate), but no details were given.

2.2. Studies in mammalian cells in vitro

Mixed results have been reported for mutagenic activity of **cobalt chloride** in mammalian cells. Amacher and Paillet (1980) found no induction of *Tk* mutations in mouse lymphoma cells following 3-hrs treatment in the absence of metabolic activation. However, there was no indication what levels of toxicity (if any) these treatments induced, and the assay was not optimised to detect small colony mutants. By contrast, Hartwig et al. (1990, 1991) and Miyaki et al. (1979) reported that **cobalt chloride** induced *Hprt* mutations in V79 cells after longer (20 or 24 h) treatment in the absence of S9. Kitahara et al. (1996) reported that mutations were induced in Chinese hamster ovary cells, transfected with a bacterial *gpt* gene, by **cobalt chloride** and, to a lesser extent, by **cobalt sulfide**.

There are surprisingly few published studies on induction of chromosomal aberrations (CA) in mammalian cells with cobalt compounds. Olivero et al. (1995) found a weak, but insignificant induction of CA in human lymphocytes treated for 48 h in the absence of S9 with **cobalt chloride**, but not with **cobalt sulphate** or **nitrate**. Paton and Allison (1972) also reported no induction of CA by **cobalt nitrate** in two human cell lines, WI38 and MRC5, or in human lymphocytes when treated in the absence of metabolic activation for periods ranging from 2 to 24 h in the cell lines, and for 48 h in lymphocytes. However, the methods are far from standard and the negative result must be viewed with caution. Figgitt et al. (2010) used multicolour fluorescence *in situ* hybridisation (M-FISH) to show that divalent cobalt (**cobalt chloride hexahydrate**) was a weak inducer of aneuploidy, but a very weak inducer of chromosomal breaks in cultured human fibroblasts.

There are, in contrast, many more publications on induction of micronuclei (MN) in mammalian cells, most of which reported positive results for ultrafine cobalt metal and soluble cobalt salts. Daley et al. (2004) investigated MN induction in human lymphocytes by wear debris from hip or knee joints. The test material was therefore not a pure chemical (titanium, vanadium, aluminium, chromium, nickel and molybdenum were present) and therefore the reported increases in MN frequency cannot be exclusively attributed to cobalt. Van Goethem et al. (1997) reported significant induction of MN in human lymphocytes treated for short periods (15 min) with low concentrations of **ultrafine cobalt metal** in the absence of metabolic activation. These results were confirmed by De Boeck et al. (2003a) and Miller et al. (2001). As with the CA study described above Olivero et al. (1995) reported weak induction of MN in human lymphocytes treated with **cobalt chloride**, but not with **cobalt sulphate** or **nitrate**. However, the MN response with cobalt chloride was flat (a plateau) whilst toxicity (mitotic inhibition) increased with concentration, making it difficult to evaluate the results. On the other hand, Ponti et al. (2009) did not detect induction of MN in Balb/3T3 cells by concentrations of **cobalt chloride** up to 10 μM, while Gibson et al. (1997) reported dose-related induction of MN in Syrian hamster embryo (SHE) cells treated with **cobalt sulphate** for 24 h in the absence of metabolic activation.

There are several publications describing induction of direct DNA damage in mammalian cells, mainly using the comet assay as detection method. Caicedo et al. (2008), De Boeck et al. (1998), Hartwig et al. (1990, 1991) and Ponti et al. (2009) all reported induction of DNA damage by **cobalt chloride** in human Jurkat cells by the neutral comet assay, in isolated human lymphocytes using the alkaline comet assay, in HeLa cells by nucleoid sedimentation in sucrose density gradients, and in Balb/3T3 cells by the comet assay, respectively. De Boeck et al. (1998) and Van Goethem et al. (1997) also reported induction of direct DNA damage with low concentrations of **ultrafine cobalt metal** in human leukocytes and blood lymphocytes from three different donors using the alkaline version of the comet assay, although in a later paper, De Boeck et al. (2003a) were unable to reproduce the DNA damage responses. The differences did not appear to be due to particle size, cytotoxicity or oxidised bases.

A publication by Ponti et al. (2009) describes the induction of MN and DNA strand breaks (comet assay) in Balb/3T3 cells by cobalt nanoparticles. The induction of DNA damage appears to be due to oxidative stress caused by the nanoparticles according to Alarifi et al. (2013). However, nanosized cobalt is not commonly

produced, is used in negligible quantities in industry, and the new studies presented here did not include testing of cobalt nanoparticles.

2.3. In vivo studies

A small number of publications have reported on investigations of genotoxicity in somatic cells *in vivo* and have given mixed results. Palit et al. (1991) reported dose- and time-related increases in CA frequency in the bone marrow of groups of mice dosed orally with **cobalt chloride hexahydrate**. Different sampling times (6, 12, 18 and 24 h) were included in this study to allow for the different kinetics and metabolism of other different chemicals included in the study. It is most unusual for genotoxins to produce dose-related responses at all sampling times tested, and the results are therefore difficult to interpret. Farah (1983) reported increased hyperdiploid and pseudodiploid cells in the bone marrow of male Syrian hamsters dosed intraperitoneally on five consecutive days with **cobalt chloride**. Since control levels of hyperdiploid cells were high, the definition of pseudodiploid cells is unclear, and there was no measure of bone marrow toxicity, these results are difficult to interpret.

By contrast, high oral doses (probably above the maximum tolerated dose) of **cobalt chloride hexahydrate** gave negative results at 2 different sampling times when tested for induction of CA and MN in rat bone marrow (Gudi and Ritter, 1998). Some reduction in mitotic index and marked reduction in percent polychromatic erythrocytes (PCE) indicated systemic (and therefore bone marrow) exposure. Although the extent of bone marrow toxicity may have prevented some damaged cells from dividing and producing MN at the later (42 h) sampling time, the negative MN responses at 18 h and the negative CA responses at both sampling times indicate a robust assessment.

Also, lethal doses of **cobalt 2-ethyl hexanoate** gave negative results when tested for MN induction in CD-1 mice that were dosed orally on 2 consecutive days, 24 h apart (Richold et al., 1981). Although there was no clear evidence of bone marrow toxicity, signs of systemic exposure (hypopnoea, lethargy and piloerection) were seen at several doses.

In an abstract, Turoczi et al. (1987) reported that **cobalt acetate** did not induce MN in mouse bone marrow after intraperitoneal dosing. No details of numbers of animals, sampling times or cells scored are given, and therefore this negative result has to be viewed with considerable caution. At the end of a 13-week (5 days per week) inhalation study with five different doses of **cobalt metal powder**, NTP (NTP, 2013) reported no increases in MN in normochromatic erythrocytes (NCE) of mice. There was no evidence of bone marrow toxicity. However, at the same time, toxicokinetic experiments demonstrated systemic exposure. By contrast, a recent paper by Rasgele et al. (2013) reported significant induction of MN in bone marrow of mice treated intraperitoneally with **cobalt chloride**. Mice were sacrificed 24 and 48 h after injection and there were statistically significant increases in MN frequency at the top two doses of cobalt chloride at 24 h, and at all three doses (but not dose-related) at 48 h. There was no evidence of bone marrow toxicity (based on PCE:NCE ratio) at any dose level, although the ratio in control animals was higher than usually experienced. Also, a single intraperitoneal injection of **cobalt chloride** in BALB/c mice caused an increase in MN formation (Suzuki et al., 1993). However, in both of these studies with **cobalt chloride**, the increased MN frequencies may be due to stimulation of erythropoiesis, as has been described by Goldwasser et al. (1958).

Only one publication reported relevant results on genotoxic effects in germ cells. Farah (1983) studied the effects of **cobalt chloride** for induction of numerical chromosomal aberrations in metaphase I and II meiotic testicular cells of male Syrian hamsters using the same protocol as for measurement of CA in bone marrow, discussed above. An increase in cells with at least 23 bivalents (instead of the normal 22) was seen in metaphase I preparations from the treated group. Based on the shortcomings mentioned above, the relevance of these results is difficult to assess.

There is one relevant study on humans. De Boeck et al. (2000) integrated different methods to assess both initial DNA damage (urinary excretion of 8-hydroxyguanosine and DNA migration in lymphocytes) and definitive chromosome breakage or loss (micronuclei in lymphocytes) in 35 workers from cobalt refineries and in 29 workers of hard metal production sites. The workers were exposed to an average cobalt dust concentration of 20 μg/m^3 (estimated by the urinary concentration of 20 μg Co/g creatinine), which is equivalent to the current ACGIH threshold limit value (TLV-TWA). No significant increase in any genotoxicity biomarker was found in exposed workers when compared with matched controls nor in hard metal dust exposed workers when compared to cobalt exposed workers.

Thus, the published studies on genotoxicity of soluble cobalt compounds (such as cobalt chloride, cobalt sulphate) and cobalt metal show some inconsistent results. Both positive and negative findings have been reported for mutation in bacterial and mammalian cells, and for induction of CA and MN both *in vitro* and *in vivo*, although the increased MN frequencies in bone marrow cells *in vivo* may be due to stimulation of erythropoiesis. In order to try to resolve these inconsistencies, new studies for these endpoints have been performed using well-characterised samples of cobalt test substances, including poorly soluble cobalt compounds, and carried out according to current (OECD-recommended) protocols. The results are presented here and are discussed in the light of the published findings.

3. Materials and methods

3.1. Test chemicals

In the materials and studies described below we refer to **cobalt metal powder** or **ultrafine cobalt metal powder**. The cobalt metal powders or particles used in these studies cannot be considered to be nanoparticles. In all studies, a “fine cobalt powder” by Umicore was used. There were slight, batch-dependent, differences in the particle size distributions (PSDs) between the powders used for the *Hprt* and for the bacterial mutagenicity (Ames) studies. Below, for each batch, the PSDs are given by specifying the D50, the median, defined as the diameter where half of the particle population lies below this value (that is, half of the particle population is smaller than the median sized particle). Similarly, 90 percent of the particle population is smaller than the D90, and 10 percent of the population lies below the D10.

Further, the total particle population is characterised below expressed as the distributions by % volume. For example, 47.6% of the particles (*Hprt* Studies), are in the size range of between 2 and 4 μm. Only 0.2% and 0.5% of the particles in the *Hprt* and in the Ames tests, respectively, are in the size range of smaller than 1 μm. Whilst it is possible that the “smaller than 1 μm fraction” contains particles smaller than 100 nm (“nanoparticles”), a powder with the below PSD characteristics is not considered a “nanoparticle” powder, since the vast majority (99.8% and 99.5%) of the particles constituting these powders are in the micron range.

Laser diffraction analysis of Umicore fine cobalt powder – ultrafine, as used in Ames tests:

- D10 – 1.6 μm
- D50 – 2.9 μm

- D90 – 5.1 μm
- +10 μm fraction – 0.0 volume%
- −10 +8 μm fraction – 0.0 volume%
- −8 +6 μm fraction – 4.2 volume%
- −6 +4 μm fraction – 19.9 volume%
- −4 +2 μm fraction – 52.3 volume%
- −2 +1 μm fraction – 23.1 volume%
- −1 μm fraction – 0.5 volume%

Laser diffraction analysis of Umicore fine cobalt powder – ultrafine, as used in *Hprt* studies:

- D10 – 1.8 μm
- D50 – 3.4 μm
- D90 – 6.2 μm
- +10 μm fraction – 0.0 volume%
- −10 +8 μm fraction – 2.0 volume%
- −8 +6 μm fraction – 9.6 volume%
- −6 +4 μm fraction – 25.8 volume%
- −4 +2 μm fraction – 47.6 volume%
- −2 +1 μm fraction – 14.8 volume%
- −1 μm fraction – 0.2 volume%

For the bacterial mutation tests **ultrafine cobalt metal powder** (99.94% pure), **cobalt chloride** (>99% pure) and **cobalt sulphate** (>99.9% pure) were obtained from Umicore Cobalt and Specialty Materials (CSM), Watertorenstraat 33, 2250 Olen, Belgium.

For Ames, mouse lymphoma *Tk* mutation and *in vitro* CA assays in human lymphocytes, **cobalt resinate** (83.7%, 83.1% and 83.7% pure for the 3 assays respectively) and **cobalt acetyl acetonate** (98.9%, 99.4% and 98.9% pure for the 3 assays respectively) were obtained from Michelin, Clermont-Ferrand, France.

For the mammalian cell *Hprt* gene mutation studies, **tricobalt tetraoxide** (cobalt content 73.43%), **cobalt sulphate** (99.9% pure), **cobalt oxalate** (100% pure) and **cobalt metal powder** (99.98% pure) were obtained from Umicore Cobalt and Specialty Materials (CSM), Watertorenstraat 33, 2250 Olen, Belgium. **Lithium cobalt dioxide** (cobalt content 60.2%) was obtained from Umicore Korea Limited, Chunan-City, Chungnam, 330-200, Korea. **Cobalt 2-ethyl hexanoate** (cobalt content 16.6%) was obtained from Rockwood Pigments UK Ltd., County Durham DH3 1QX, England. **Cobalt oxide hydroxide** (cobalt content 60.49%) was obtained from QNI Technology, Greenvale Street, Yabulu Via Townsville, Queensland 4818 Australia. **Cobalt sulphide** (100% pure) was obtained from Vale Inco Ltd., Port Colborne, Canada. **Cobalt monoxide** (99.998% pure) was obtained from Alfa Aesar, Johnson Matthey, Ward Hill, MA 01835-8099, USA. **Cobalt borate neodecanoate** (100% pure) was obtained from OMG OM Group, OMG Borchers GmbH, Berghausener Str. 100, 40764 Langenfeld, Germany. **Cobalt dihydroxide** (100% pure, cobalt content 62.3%) was obtained from OM Group, OMG Kokkola Chemicals OY, 67101 Kokkola, Finland.

For the *in vitro* CA study in V79 cells, **cobalt oxyhydroxide** (cobalt content 60.49%) was obtained from QNI Technology, Greenvale Street, Yabulu Via Townsville, Queensland 4818, Australia.

For the *in vitro* studies on oxidative damage (induction of reactive oxygen species and modified comet assay), **cobalt octoate** (cobalt content 17%) was obtained from Umicore Specialty Materials Brugge, Kleine Pathoekeweg 82, 8000 Brugge, Belgium and **cobalt sulphate heptahydrate** (cobalt content 21%) was obtained from Umicore Cobalt & Specialty Materials, Watertorenstraat 33, 2250 Olen, Belgium.

For the *in vivo* MN studies, **cobalt acetyl acetonate** (98.9% pure) and **cobalt resinate** (83.7% pure) were obtained from Michelin, Clermont-Ferrand, France.

For the *in vivo* CA assays, **cobalt sulphate** (cobalt content 21%) and **tricobalt tetraoxide** (cobalt content 73.43%) were obtained from Umicore Leemanslaan 36, Olen, Belgium 2250, and **cobalt monoxide** (cobalt content 78.2%), was obtained from OMG Kokkola Chemicals Oy, Kokkola, Finland.

For the *in vivo* spermatogonial CA study, **cobalt dichloride hexahydrate** (cobalt content 24.74%) was obtained from Umicore Cobalt and Specialty Materials (CSM), Watertorenstraat 33, 2250 Olen, Belgium.

Positive control and other reference chemicals of the highest purity available were obtained as follows:

- 4-nitro-o-phenylenediamine (4NOPD) and ethyl methanesulfonate (EMS) from Sigma–Aldrich, 82024 Taufkirchen, Germany
- 2-aminoanthracene (2-AA) from Sigma–Aldrich, 82024 Taufkirchen, Germany, from Sigma–Aldrich, Poole, Dorset, UK and also from Trinova Biochem GmbH, 35394 Gieβen, Germany
- Sodium azide (NaN_3), 2-nitrofluorene (2NF), benzo(a)pyrene (B[a]P) and 9-aminoacridine (9AA) from Trinova Biochem GmbH, 35394 Gieβen, Germany and from Sigma–Aldrich, Poole, Dorset, UK.
- Methyl methanesulfonate (MMS) and mitomycin C (MMC) from Sigma–Aldrich, Saint-Quentin Fallavier, France
- Cyclophosphamide (CPA) from Sigma–Aldrich, Saint-Quentin Fallavier, France, from Sigma–Aldrich, 82024 Taufkirchen, Germany and also from Sigma–Aldrich, Oakville, Ontario, Canada
- 4-nitroquinoline-1-oxide (NQO) from Sigma–Aldrich, Poole, Dorset, UK
- 3-morpholinosydnonimine hydrochloride (SIN-1) and triton X-100 from Sigma–Aldrich, Germany
- Quartz, type DQ12, mid-size Dörentrup, from Bergbauforschung Essen, Germany, geometric mean diameter, weighting by mass: 3.01 ± 1.53 μm, 87% α quartz, 13% amorphous
- Potassium bromate ($KBrO_3$) from Merck, Darmstadt, Germany
- Aluminium oxide (Al_2O_3), fused, from Sigma–Aldrich, 82024 Taufkirchen, Germany, sized in a water column, resulting in a geometrical mean diameter, weighting by mass: 4.09 ± 1.77 μm
- 1,2-dimethylhydrazine monohydrate (DMH) from Sigma–Aldrich, Oakville, Ontario, Canada

For the mammalian cell gene mutation studies trifluorothymidine (TFT) was obtained from Sigma–Aldrich, Saint-Quentin Fallavier, France and 6-thioguanine (6 TG) was obtained from Sigma–Aldrich, Poole, Dorset, UK.

For the production of reactive oxygen species (ROS) *in vitro*, 2′,7'-dichlorofluorescein diacetate (DCFH-DA) was obtained from Sigma–Aldrich, 82024 Taufkirchen, Germany. Hank's salt solution (HBSS), without phenol red was obtained from Biochrom GmbH, 12247 Berlin, Germany.

3.2. Rat liver S9

The rat liver S9 used in the bacterial mutation studies was either prepared in-house from Aroclor-1254 or phenobarbital/β-naphthoflavone pre-treated rats according to Maron and Ames (1983), with a minimum protein content of 33 mg/mL, or was obtained from Molecular Toxicology Inc. (Boone, North Carolina, USA). The S9 was mixed with standard cofactors for use in the individual assays as described below.

For the mouse lymphoma *Tk* and *Hprt* mutation studies, and for the *in vitro* CA tests in human lymphocytes, Aroclor-induced S9 was purchased from Molecular Toxicology Inc. (Boone, North Carolina, USA). It was mixed with standard cofactors (glucose-6-phosphate, NADP and KCl) to give a final concentration in the treatment medium of 2% for the gene mutation studies and a final concentration

of 1.5% for the CA tests.

For the *in vitro* CA study in V79 cells, S9 was prepared at BSL BioService GmbH, Germany, from male Wistar rats that had been induced with phenobarbital (80 mg/kg) and β-naphthoflavone (100 mg/kg). The protein content was 33 mg/mL. After mixing with co-factors the final concentration of S9 in the treated cultures was 2.27%.

3.3. Ames tests

3.3.1. Single strain tests

In light of the published positive results with **cobalt metal, cobalt chloride and cobalt sulphate**, a series of GLP studies were performed in order to investigate the bacterial strains that had shown evidence of potential mutagenic effects. Cobalt metal was tested in *S. typhimurium* strain TA98, cobalt chloride was tested in strain TA97a and cobalt sulphate was tested in strain TA100, and each substance was tested in three different laboratories.

The bacteria used were obtained from different sources as follows:

- For Flügge (2013a,b,c) and Laboratory 3, the 3 bacterial strains were all obtained from Trinova Biochem GmbH, 35394 Gießen, Germany, who originally obtained the strains from Prof. B.N. Ames.
- For Laboratory 2, strain TA100 was obtained from Covance Laboratories Inc., Vienna, Virginia, USA, strain TA97a was obtained from Glaxo Group Research, Ware, Hertfordshire, UK, and strain TA98 was obtained from the National Collection of Type Cultures, Public Health England, Porton Down, Salisbury, Wiltshire, UK. In each case the strains were originally obtained from Prof. B.N. Ames.

All strains were regularly checked for membrane permeability (*rfa* mutation), ampicillin resistance, UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates.

The following positive control chemicals were used by the participating laboratories:

- For strain TA98, 2NF at 5 or 10 μg/plate or 4NOPD at 10 μg/plate in the absence of S9, and B[a]P at 10 μg/plate or 2-AA at 2.5 μg/plate in the presence of S9
- For strain TA97a, 9AA at 100 μg/plate or 4NOPD at 10 μg/plate in the absence of S9, and 2-AA at 2, 2.5 or 5 μg/plate in the presence of S9
- For strain TA100, NaN_3 at 2 or 10 μg/plate in the absence of S9, and 2-AA at 2, 2.5 or 5 μg/plate in the presence of S9

Some details of the methods used by the 3 laboratories were slightly but not dramatically different. The different approaches may be summarised as follows:

- All 3 laboratories corrected for the water content of cobalt chloride hexahydrate and cobalt sulphate heptahydrate in determining the concentrations tested on each plate. However, although all 3 laboratories suspended cobalt powder in anhydrous analytical grade DMSO, cobalt sulphate and cobalt chloride were dissolved in deionised water in one laboratory but were dissolved in DMSO in the other 2 laboratories.
- All 3 laboratories performed 2 independent experiments in the absence and presence of S9, but Laboratory 3 also performed additional experiments with cobalt chloride and cobalt sulphate.
- Induced rat liver S9 was mixed with standard co-factors ($MgCl_2$, KCl, glucose-6-phosphate and NADP in phosphate buffer) for use. Flügge (2013a,b,c) used 5% S9 mix whereas the other two laboratories used 10% S9 mix.
- All 3 laboratories performed the first experiment using the plate incorporation method (Maron and Ames, 1983). Flügge (2013a,b,c) and Laboratory 3 performed the whole of the second experiment using the pre-incubation method (Yahagi et al., 1975), whereas Laboratory 2 performed the treatments in the absence of S9 using the plate incorporation method and only used the pre-incubation method for the treatments in the presence of S9. The additional experiments performed by Laboratory 3 included both plate incorporation and pre-incubation methods in the absence and presence of S9.
- Each laboratory tested at least 6 concentrations of each test chemical in each part of the study, and each treatment was carried out at least in triplicate. The top concentration was usually 5000 μg/plate, but in some cases precipitation dictated that a slightly lower top concentration was used.
- Flügge (2013a,b,c) tested the same range of concentrations in each of the 2 experiments. Laboratory 3 tested similar ranges of concentrations in all experiments, although the top concentration was lower for one of the pre-incubation experiments. Laboratory 2 chose a narrower range of concentrations for the second experiment.

Following incubation for 2–3 days at 37 °C in the dark, the plates were examined for signs of toxicity to the background lawn and revertant colonies were counted electronically using a Petri Viewer Mk2 or Sorcerer Colony Counter (Perceptive Instruments ltd, UK), or with a Biocount 5000γ (Bio-Sys GmbH, Kiefernweg 10, 61184 Karben, Germany). Where confounding factors affected the accuracy of the automated counter, plates were counted manually. The means and standard deviations were determined for each treatment concentration.

The 3 laboratories used slightly different approaches to the evaluation of the data in terms of defining a positive response. In summary they were:

- Flügge (2013a,b,c) concluded a positive response if the number of revertants was significantly increased ($p \leq 0.05$, U-test according to Mann and Whitney) compared with the solvent control to at least 2-fold of the solvent control in both independent experiments; a significant ($p \leq 0.05$) concentration (log value)-related effect (Spearman's rank correlation coefficient) was observed; and positive results were reproducible and the histidine independence of the revertants was confirmed by streaking random samples on histidine-free agar plates.
- Laboratory 2 concluded a positive if there was a concentration related increase in revertant numbers to ≥2.0-fold the concurrent vehicle control values and positive trend/effects were reproducible.
- Laboratory 3 concluded a positive response if a biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control was observed, or if a dose-dependent increase above the 2-fold threshold was seen at more than one concentration, or if an increase exceeding the 2-fold threshold at only one concentration was reproduced in an independent second experiment.

Thus, the common factors which have been applied in the evaluation of data for these single strain experiments were that a test substance was judged to be mutagenic if it produced, for at least one concentration, a response equal to at least 2x the control incidence of revertants, a dose-related trend was observed, and the effects were reproducible.

3.3.2. Five strain tests

GLP studies in 5 strains of *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537, obtained from Prof. B.N. Ames, Berkeley, California) were performed with **cobalt resinate** and **cobalt acetyl acetonate**. Strain-specific positive control chemicals were included in the absence of S9 and 2-AA was used as positive control in the presence of S9.

Cobalt resinate was dissolved in tetrahydrofuran, and cobalt acetyl acetonate was suspended in tetrahydrofuran (Carlo Erba, Val de Reuil, France). Both were tested in the absence and presence of induced rat liver S9 (10% final concentration) in 2 independent experiments. The plate incorporation method was used for treatments in the absence and presence of S9 in Experiment 1, and for treatments in the absence of S9 in Experiment 2, but treatments in the presence of S9 in Experiment 2 were performed using a 60 min pre-incubation prior to plating. At least 5 concentrations of each chemical were evaluated in triplicate plates in each part of the study. Both compounds produced precipitate, and so the top concentration tested for both compounds was 1000 μg/plate. Some precipitate was seen either in the tubes or on the plates at concentrations of 250 or 500 μg/plate for cobalt resinate and cobalt acetyl acetonate respectively.

A positive result was defined by a reproducible 2-fold (for strains TA98, TA100 and TA102) or 3-fold increase (for strains TA1535 and TA1537) in the number of revertants compared with the vehicle controls at any dose-level, and/or evidence of a dose-response.

3.4. Mammalian cell gene mutation tests

3.4.1. Mouse lymphoma tk mutation tests

L5178Y $Tk^{+/-}$ cells were obtained from ATCC (American Type Culture Collection, Manassas, USA), through Biovalley (Marne-La-Vallée, France). The cells were stored at −80 °C in cryoprotective medium containing 10% horse serum and 10% dimethylsulfoxide (DMSO). Each batch of frozen cells was purged of spontaneous $Tk^{-/-}$ mutants and checked for the absence of mycoplasma. The cells were maintained in flasks as suspension cultures in RPMI 1640 culture medium containing L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and sodium pyruvate (200 μg/mL) and supplemented with heat inactivated horse serum in a 37 °C, 5% CO_2 humidified incubator. For suspension growth and for the 24 h treatments the medium was supplemented with heat inactivated horse serum at 10%, v/v, for the 3 h treatments it was 5%, v/v, and for plating for viability and mutant selection it was 20%, v/v.

Cobalt resinate was dissolved in tetrahydrofuran and tested using the microwell method. Treatments were for 3 h and 24 h in the absence of S9, and for 3 h (2 experiments) in the presence of S9. The compound precipitated in culture medium and persisted to the end of treatment at concentrations of 25 μg/mL and higher for the 3 h treatments and at 50 μg/mL and higher for the 24 h treatment. However, 100 μg/mL was chosen as the top concentration in all of the main mutation experiments in order to try to achieve acceptable levels of toxicity.

Cobalt acetyl acetonate was suspended in tetrahydrofuran, but the maximum concentration that produced a homogeneous suspension gave, after dilution in culture medium, a top concentration of 91.625 μg/mL. This concentration did not produce precipitate in the final treatment medium, but it did induce toxicity (at least 60% reduction in relative total growth, RTG). The microwell method was again used, and treatments were for 3 h in the absence or presence of S9 in 2 independent experiments. In light of the results after 3 h it was not necessary to also treat for an extended 24 h in the absence of S9.

MMS (25 μg/mL for 3 h treatments and 5 μg/mL for 24 h treatments) and CPA (3 μg/mL) were used as positive control treatments in the absence and presence of S9 respectively.

For the 3-hr treatments, 20 mL cultures (5×10^5 cells/mL) were treated in 50 mL tubes. For the 24-hr treatments, 50 mL cultures (2×10^5 cells/mL) were treated in flasks which were gently shaken at least once. At the end of the treatment period, the cells were rinsed, centrifuged, the supernatant removed, the pellets suspended in medium and the cells counted using a haemocytometer. The cell concentrations were adjusted to 2×10^5 cells/mL and maintained close to this density during the 48 h expression period, at the end of which cells were counted and seeded into microwell plates as follows:

- Viability plates – to define the number of viable cells (CE_2: Cloning Efficiency at the end of the expression period), an average of 1.6 cells/well were seeded in two 96-well plates/culture (4 plates/dose-level). After at least 7 days of incubation, in a 37 °C, 5% CO_2 humidified incubator, the clones were counted.
- Mutant selection plates – to select the TFT^R (trifluorothymidine resistant) mutant cells (for the determination of CE_{mutant}), 2000 cells/well were seeded in four 96-well plates/culture (8 plates/dose-level). After 11–12 days of incubation in a 37 °C, 5% CO_2 humidified incubator in the presence of 4 μg TFT/mL of culture medium, the clones were counted, differentiating small and large colonies as follows:
  - Size of small colonies: < 25% of the diameter of the well,
  - Size of large colonies: > 25% of the diameter of the well.

For scoring of colonies in mutant plates, the following parameters were considered:

- Well containing mutant colony (small or large),
- Well not containing mutant colony.

When both small and large colonies were present in the same well, both mutant colonies were counted (one small and one large).

Cloning efficiency, suspension growth, adjusted relative total growth and mutant frequency were calculated using standard formulae as described by Mei et al. (2014).

IWGT recommendations (Moore et al., 2003, 2006, 2007) were followed for the determination of a positive result, namely:

- For at least one concentration the induced mutant frequency (mutant frequency treated minus mutant frequency of the vehicle control) equalled or exceeded the Global Evaluation Factor (GEF) of 126×10^{-6},
- A dose-response was demonstrated by a statistically significant trend test (in this case using the Kendall rank correlation coefficient).

3.4.2. Hprt mutation tests in mouse lymphoma cells

Ten different cobalt salts/compounds, plus cobalt metal powder and an extract of cobalt metal powder, were tested for induction of *Hprt* mutations in mouse lymphoma L5178Y cells. Concentrations for the mutation experiments were selected after range-finding experiments and top concentrations were based on solubility limits or cytotoxicity as described below. In each mutation study the chemicals were tested for 3 h in the absence and presence of S9. At least 2 independent experiments were performed, but occasionally additional experiments were performed for clarification of results. Although not required according to OECD guideline 476, an extended 24 h treatment in the absence of S9 was performed with 5 of the test substances in order to detect any mutagenic effects that

might only be manifest after being in contact with the cells for a full cell cycle.

The 7 substances that were tested only for 3 h in the absence and presence of S9 were prepared and tested as follows:

- **Cobalt dihydroxide** was suspended in 0.5% methyl cellulose (MC) and diluted 10-fold into culture medium. It was toxic at low concentrations, inducing >80% reduction in relative survival in the range 19–35 μg/mL where no precipitation was seen.
- **Lithium cobalt dioxide** was suspended in 0.5% MC and diluted 10-fold into culture medium where it precipitated and persisted to the end of treatment at concentrations of 50–60 μg/mL (the top concentrations chosen for the mutation experiments) and higher. Due to a formulation error, concentrations up to 600 μg/mL were tested in the presence of S9 in the first experiment, but although no precipitate was visible at lower concentrations the cell pellets were discoloured when treatment medium was removed after 3 h. Therefore the solubility limit was almost certainly exceeded in this experiment. None of the treatments induced significant toxicity (maximum 22% reduction in relative survival).
- **Cobalt oxide hydroxide** was suspended in 0.5% MC and diluted into culture medium. The top concentrations in each experiment were the lowest that produced persistent precipitate through the treatment period, namely 15 and 12.5 μg/mL in both the absence and presence of S9 in the 2 main experiments respectively. None of the treatments induced significant toxicity (maximum 18% reduction in relative survival).
- **Cobalt oxalate** was suspended in 0.5% MC and diluted 10-fold into culture medium where it precipitated and persisted to the end of treatment at concentrations of 70 μg/mL and higher. It was toxic at this and lower concentrations, inducing >80% reduction in relative survival at 55 μg/mL in the absence of S9, and at 60 or 70 μg/mL in the presence of S9. Therefore most concentrations evaluated for mutations were soluble.
- **Cobalt metal powder** was suspended in 0.5% MC at a series of concentrations which were each diluted 10-fold into the cultures for treatment. Undissolved cobalt metal powder was present at many lower concentrations. It was also toxic at lower concentrations, inducing >80% reduction in relative survival at 37.5–40 μg/mL in the absence of S9, and at 40–200 μg/mL in the presence of S9, and so these lower concentrations were evaluated for mutations. There was marked variability in the toxic responses in the presence of S9 between the different experiments which may be a reflection of the fact that undissolved cobalt metal powder was present, and may have exerted toxic effects on the cells due to its physical presence.
- **Tricobalt tetraoxide** was dissolved in DMSO at a series of concentrations which were diluted 100-fold into the cultures for treatment. The top concentrations in each experiment were observed to be the lowest producing persistent precipitate through the treatment period, namely 2000 and 2408 μg/mL in the absence of S9, and 600 and 750 μg/mL in the presence of S9. None of these induced significant toxicity (maximum 34% reduction in relative survival).
- **Cobalt 2-ethyl hexanoate** was dissolved in DMSO at a series of concentrations which were diluted 100-fold in culture medium. It was toxic at low concentrations, inducing >80% reduction in relative survival in the range 80–120 μg/mL, and therefore all concentrations evaluated for mutations were soluble.

The 5 substances that were tested both for 3 h in the absence and presence of S9, and for 24 h in the absence of S9, were prepared as follows:

- **Cobalt sulphate** was dissolved in water at a series of concentrations and diluted in culture medium. It was toxic at low concentrations, inducing >80% reduction in relative survival at 50–100 μg/mL for the 3 h treatments, and at 35 μg/mL for the 24 h treatment. Therefore all concentrations evaluated for mutations were soluble.
- **Cobalt sulphide** was suspended in 1.0% carboxymethyl cellulose at a series of concentrations and diluted 10-fold in culture medium. For the 3 h treatments it was possible to achieve a top concentration of 10 mM (922 μg/mL), which was not toxic, and is the maximum required for testing of non–toxic chemicals. It exhibited some toxicity following 24 h treatments, and so the maximum concentration (that reduced relative survival to 11% of controls) in this part of the study was 800 μg/mL.
- **Cobalt monoxide** was suspended in 0.5% MC and diluted 10-fold into culture medium where it precipitated and persisted to the end of treatment at concentrations of 60–80 μg/mL and higher. Following 3 h treatments, significant (86%) toxicity was induced at 80 μg/mL in the absence of S9 in the first experiment, but at 60 μg/mL (the top concentration for the second experiment in the absence of S9 and both experiments in the presence of S9) only slight to moderate (15–41%) toxicity was induced. It was also toxic following 24 h treatments in the absence of S9, and the top concentration tested for mutation induction was 60 μg/mL, which induced 90% reduction in relative survival.
- **Cobalt borate neodecanoate** was dissolved in tetrahydrofuran and diluted 200-fold into the cultures for treatment. Based on range-finding data the top concentrations used in the mutation experiments were 100 μg/mL for the 3 h treatments and 7.5 μg/mL for the 24 h treatment.
- An **extract of cobalt metal powder** was prepared by incubating the powder in RPMI 5 medium (i.e. RPMI 1640 medium containing 5% heat-inactivated horse serum, plus 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 0.5 mg/mL pluronic) for approximately 72 h at 37 °C, with shaking. The resultant suspension was then centrifuged at 1000 *g* for 10 min and the supernatant decanted for treatment of the cells (see below). Concentrations were expressed according to the amounts of cobalt metal powder added to the extraction medium and the top concentration was therefore 589.3 μg/mL (equivalent to 10 mM). Tyndall analysis (measuring LASER light scattering) was performed for 60 min at 5 min intervals during the range-finding experiment to ensure the level of any residual particulate matter was at an acceptable level, indicating that the formulation sample was suitable for testing.

L5178Y $Tk^{+/-}$ 3.7.2C mouse lymphoma cells originated from Dr Donald Clive, Burroughs Wellcome Co., USA. Cells were stored as frozen stocks in liquid nitrogen. Each batch of frozen cells was purged of mutants and confirmed to be mycoplasma free. For each experiment, at least one vial was thawed rapidly, the cells diluted in RPMI 10 culture medium (i.e. RPMI 1640 medium containing 10% heat-inactivated horse serum, plus 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B and 0.5 mg/mL pluronic) and incubated in a humidified atmosphere of 5% CO_2 in air. When the cells were growing well, subcultures were established in an appropriate number of flasks.

Treatment and post treatment dilution of cell cultures for the cytotoxicity range-finding experiments was as described below for the mutation experiments. However, single cultures only were used and positive controls were not included. Osmolality and pH measurements were made on post-treatment media. Cell concentrations were adjusted to 8 cells/mL and, for each concentration,

0.2 mL was plated into each well of a 96-well microtitre plate for determination of relative survival. The plates were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air for 7 days. Wells containing viable clones were identified by eye using background illumination and counted.

For all mutation experiments each treatment was in duplicate except for positive controls where single cultures only were used. Vehicle and positive control treatments were included throughout, but since some less common vehicles were used, untreated controls were included in many experiments to confirm no adverse effects from the vehicle.

- For 3 h treatments with most of the test substances (cobalt dihydroxide, cobalt metal powder, cobalt monoxide, cobalt oxalate, lithium cobalt dioxide, cobalt oxide hydroxide, cobalt sulphate and cobalt sulphide), 10^7 cells were suspended in 17.0 mL of RPMI 5. For cultures prepared in this way, treatment consisted of adding 2 mL vehicle or test chemical solution (or 0.2 mL positive control solution plus 1.8 mL vehicle), together with S9 mix or 150 mM KCl, to give a final volume of 20 mL. For the remaining substances (except extract of cobalt metal powder, described below), at least 10^7 cells were suspended in 18.9 mL of RPMI 5, or in 18.8 mL RPMI 5 for tricobalt tetraoxide, cobalt 2-ethyl hexanoate and for positive controls, in a series of sterile disposable 50 mL centrifuge tubes. For all treatments of cultures prepared in this way, 0.1 mL vehicle or test chemical solution (or 0.2 mL tricobalt tetraoxide, cobalt 2-ethyl hexanoate or positive control solution) was added. S9 mix or 150 mM KCl (1.0 mL per culture) was added for treatments in the presence and absence of metabolic activation, respectively, giving a final treatment volume of 20 mL.
- For 24 h treatments in the absence of S9, at least 4×10^6 cells in a volume of 19.9 mL RPMI 10 (19.8 mL for positive controls) were used. The cell suspensions were placed in a series of appropriate sterile disposable containers which were gassed with 5% CO_2 in air. For all treatments of cultures prepared in this way 0.1 mL of vehicle or test chemical solution (or 0.2 mL of positive control solution) was added giving a final treatment volume of 20 mL. For some test substances (cobalt monoxide, cobalt sulphate and cobalt sulphide) 4×10^6 cells were suspended in 18.0 mL of RPMI 10. Treatment consisted of adding 2 mL vehicle or test chemical solution (or 0.2 mL positive control solution plus 1.8 mL vehicle) to give a final volume of 20 mL.

The treatments with the extract of cobalt metal powder were different:

- For the 3 h treatments, at least 10^7 cells in a volume of 3 mL RPMI 5 were placed in a series of sterile disposable 50 mL centrifuge tubes. Then 16 mL vehicle (RPMI 5) or supernatant from extraction as described above (0.2 mL positive control solution plus 15.8 mL vehicle) was added. S9 mix or 150 mM KCl (1.0 mL per culture) was added to give a final volume of 20 mL.
- For the 24 h treatment in the absence of S9, at least 4×10^6 cells in a volume of 4 mL RPMI 5 were placed in a series of sterile disposable 75 cm^2 tissue culture flasks. Then 16 mL vehicle (RPMI 5) or supernatant from extraction as described above (or 0.2 mL positive control solution plus 15.8 mL vehicle) was added. Thus the final volume was 20 mL.

After 3 h incubation at 37 °C with gentle agitation (short treatments), or after 24 h static incubation (long treatments), cultures were centrifuged (200 *g*) for 5 min, washed and resuspended in 20 mL RPMI 10. Cell densities were determined using a Coulter counter and the concentrations adjusted to 2×10^5 cells/mL. For assessment of survival, samples from these cultures were diluted to 8 cells/mL. Using a multichannel pipette, 0.2 mL of each of these diluted cultures was placed into each well of 2×96-well microtitre plates (192 wells, averaging 1.6 cells/well). The plates were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air for 7 days. Wells containing viable clones were identified by eye using background illumination and counted.

For the expression of *Hprt*$^-$ mutations, post-treatment cultures that had been adjusted to 2×10^5 cells/mL were maintained in flasks for a period of 7 days. Sub-culturing was performed as required with the aim of not exceeding 1×10^6 cells/mL and, where possible, retaining at least 6×10^6 cells/flask. From observations on recovery and growth of the cultures during the expression period, appropriate cultures were selected to be plated for viability and 6TG resistance.

To determine viability at the end of the expression period, cell densities in the selected cultures were determined using a Coulter counter and adjusted to give 1×10^5 cells/mL. Samples from these were further diluted in RPMI 20 (similar to RPMI 10 but with 20% heat-inactivated horse serum and without pluronic) to 8 cells/mL. Using a multichannel pipette, 0.2 mL of each diluted culture was placed into each well of 2×96-well microtitre plates (192 wells averaging 1.6 cells/well). The plates were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air for 8 days. Wells containing viable clones were identified by eye using background illumination and counted.

To select for mutation at the end of the expression period, 6 TG (1.5 mg/mL) was diluted 100-fold into the 1×10^5 cells/mL cultures to give a final concentration of 15 μg/mL. Using a multichannel pipette, 0.2 mL of each suspension was placed into each well of 4×96-well microtitre plates (384 wells at 2×10^4 cells/well). Plates were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air for 12–13 days and wells containing clones were identified as above and counted.

Cloning (plating) efficiency and mutant frequency were calculated in the same way as for *Tk* mutants (Mei et al., 2014). Relative survival was used to determine cytotoxicity and was calculated by comparing plating efficiencies in test and control cultures thus:

$$\%RS = \left[PE_{(test)} \Big/ PE_{(control)}\right] \times 100$$

To take into account any loss of cells during the 3 or 24 h treatment period, percentage relative survival values for each concentration of test article were adjusted as follows:

$$\text{Adjusted } \%RS = \%RS \times \frac{\text{Post} - \text{treatment cell concentration in treated culture}}{\text{Post} - \text{treatment cell concentration in vehicle control}}$$

Statistical analysis of mutant frequencies was carried out according to Robinson et al. (1990). The control log mutant frequency (LMF) was compared with the LMF from each treatment concentration and the data were checked for a linear trend in mutant frequency with test chemical treatment. These tests require the calculation of the heterogeneity factor to obtain a modified estimate of variance. The LMF for each treatment was compared with control using Dunnett's test (one-sided). Linear trend was assessed by weighted rank sum analysis and the level of significance was determined by chi-squared (one-sided) analysis.

A chemical was considered to be mutagenic in this assay if:

1. The mutant frequency at one or more concentrations was significantly greater than that of the negative control ($p \leq 0.05$).
2. There was a significant concentration-relationship as indicated by the linear trend analysis ($p \leq 0.05$).
3. The effects described above were reproducible.

For biological significance the fold increase in mutant frequency relative to vehicle controls was also taken into account, and in general a 3-fold increase was considered biologically significant. Results that only partially satisfied the assessment criteria described above or did not achieve biological significance, were considered on a case-by-case basis. Positive responses seen only at high levels of cytotoxicity required careful interpretation when assessing their biological relevance. Extreme caution was exercised with positive results obtained at levels of RS lower than 10%.

3.5. Chromosomal aberration (CA) tests in mammalian cells

Cobalt acetyl acetonate was suspended in tetrahydrofuran and tested for CA induction in cultures of human lymphocytes. To prepare each culture, 0.5 mL of heparinised whole blood from 2 healthy donors was added to 5 mL of RPMI 1640 medium containing 20% foetal calf serum, L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and phytohaemagglutinin (PHA: a mitogen to stimulate lymphocyte division). The cultures were then placed in an incubator at 37 °C for 48 h, when treatment was begun. Two independent experiments were performed, and in each case cells were treated with cobalt acetyl acetonate for 3 h in the absence or presence of S9, and harvested 20 h after the start of treatment, corresponding to approximately 1.5 cell cycles. One and a half hours before harvest, each culture was treated with a colcemid solution (10 μg/mL) to block cells at the metaphase-stage of mitosis. Harvested cells were gently swollen by hypotonic treatment (KCl, 0.075 M), fixed in a methanol/acetic acid mixture (3/1; v/v), spread on glass slides and stained with Giemsa. All the slides were coded, so that the scorer was unaware of the treatment group of the slide under evaluation ("blind" scoring).

Positive control chemicals were MMC at 3 μg/mL in the absence of S9, and CPA at 12.5 or 25 μg/mL for treatments in the presence of S9.

At least 5 concentrations of cobalt acetyl acetonate were tested, in duplicate, in each part of the study. Although added to the cultures as a suspension in tetrahydrofuran, no precipitate was seen at the end of treatment. Cytotoxicity was evaluated by mitotic index (number of cells in mitosis/1000 cells examined). Where possible, 100 metaphases with 44–46 chromosomes were analysed for CA from each replicate culture (200 metaphases/dose level) from the 3 highest concentrations producing acceptable levels of cytotoxicity (target at least 50% mitotic inhibition).

For each test concentration and for each harvest time, the frequency of cells with structural CA (excluding gaps) in treated cultures was compared to that of the vehicle control cultures. If necessary, the results were analysed using the χ^2 test, in which $p = 0.05$ was used as the lowest level of significance. A reproducible and statistically significant increase in the frequency of cells with structural CA for at least one of the dose-levels was considered as a positive result. Reference to historical data or other considerations of biological relevance, was also taken into account in the evaluation of the findings.

Cobalt resinate was dissolved in tetrahydrofuran and tested for CA induction in cultures of human lymphocytes according to a protocol similar to that used for cobalt acetyl acetonate. However, in the second experiment in the absence of S9 mix, cells were exposed continuously to the test or control items until harvest, whereas in the presence of S9 mix, cells were exposed for 3 h and then rinsed. In both cases cells were harvested 20 and 44 h after the beginning of treatment, corresponding to approximately 1.5 normal cell cycles and 24 h later, respectively. In the third confirmatory experiment, lymphocyte cultures were exposed to cobalt resinate in the presence of S9 mix and harvested 20 h after the start of treatment. Harvesting and slide preparation were the same as for cobalt acetyl acetonate.

Positive control chemicals were MMC at 3 μg/mL (3 h treatment) or 0.2 μg/mL (20 or 44 h treatment) in the absence of S9, and CPA at 12.5 or 25 μg/mL for treatments in the presence of S9.

At least 5 concentrations of cobalt resinate were tested, in duplicate, in each part of the study. A slight to marked precipitate was seen at the end of treatment at concentrations of 37.5 μg/mL and above. Cytotoxicity, scoring of CA and evaluation of results were the same as for cobalt acetyl acetonate.

Another CA study was performed on V79 cells treated with **cobalt oxyhydroxide**. V79 cells were obtained from the American Type Culture Collection (ATCC CCL-93) and checked for mycoplasma contamination before stocks were prepared and stored frozen in liquid nitrogen. Thawed cultures were set up in 75 cm^2 flasks (5×10^5 cells per flask in 15 mL minimal essential medium containing 10% foetal calf serum) at 37 °C in an atmosphere of 5% CO_2 in air, and subcultured every 3–4 days. For the experiments, cells were seeded into Quadriperm dishes containing microscope slides (at least 2 chambers per dish and test group). Into each chamber 1×10^4–5×10^4 cells were seeded dependent on preparation time. Two days after seeding, the cells were treated either in serum-free medium (short treatments) or in medium containing 10% foetal calf serum.

Cobalt oxyhydroxide was dissolved in cell culture medium prior to treatment, and all treatments, including solvent and positive controls (EMS in the absence of S9 and CPA in the presence of S9) were in duplicate. Two independent experiments were performed. In the first experiment cells were treated with cobalt oxyhydroxide for 4 h in the absence or presence of S9, and harvested 20 h after the start of treatment. In the second experiment, cells were again treated for 4 h in the presence of S9 and harvested at 20 h, but in the absence of S9 treatment was continuous for 20 h until harvest. Cytotoxicity was measured both by mitotic index (based on 1000 cells scored) and by cell density (mean of cell counts from 20 visual fields at 400x magnification). The concentrations chosen for analysis were based on mitotic index (MI), which declined with treatment concentration more steeply than cell density. Thus, top concentrations were scored at which MI was reduced by >50%, as required by current OECD guidelines, but in some cases exceeded 60%. Precipitation was present at all concentrations of cobalt oxyhydroxide tested.

Cells were arrested in metaphase by addition of Colcemid (0.2 μg/mL for the final 2.5 h) and the cells were gently swollen on the slides with 0.4% KCl for 20 min at 37 °C. Cells were fixed with methanol/glacial acetic acid (3:1, v/v), and stained with Giemsa. Slides were coded before analysis. Only cells with 22 ± 1 chromosomes were scored, and 100 cells per replicate (200 cells per concentration) were scored for CA. No statistical analysis was performed. CA frequencies were judged as biologically significant based on reference to historical negative control ranges.

3.6. Studies on production of reactive oxygen species (ROS) and induction of oxidative damage

As an *in vitro* model system relevant for the assessment of damage to the lungs, A549 lung epithelial cells were used for investigations on ROS production and induction of oxidative DNA damage by **cobalt octoate** compared with the inorganic, water soluble cobalt compound **cobalt sulphate heptahydrate** as a positive control for high cobalt solubility and cobalt-dependent biological effects. Cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, 38124 Braunschweig, Germany). The cell lot used was tested and found free of mycoplasma contamination.

Before cell exposure cobalt octoate and cobalt sulphate

heptahydrate were extracted in artificial interstitial fluid (Gamble's solution, pH 7.4) or artificial alveolar fluid (AAF, pH 7.4) to simulate dissolution in the physiological lung environment. The salts used for preparation of the artificial fluids were purchased from different providers in highest purity. Gamble's solution was composed of magnesium chloride hexahydrate (0.2033 g/L), sodium chloride (6.0193 g/L), potassium chloride (0.2982 g/L), dibasic sodium phosphate (0.1420 g/L), sodium sulphate (0.0710 g/L), calcium chloride dihydrate (0.3676 g/L), sodium acetate trihydrate (0.9526 g/L), sodium bicarbonate (2.6043 g/L), sodium citrate dihydrate (0.0970 g/L). Salts were successively dissolved under stirring in 950 mL of double distilled water in the given order. Finally pH was adjusted to pH 7.4 using HCl/NaOH and the solution was filled up to 1 L. AAF was prepared like the Gamble's solution, but contained, in addition, phosphatidylcholine (0.1000 g/L; Sigma–Aldrich, 82024 Taufkirchen, Germany). For supplementation of phosphatidylcholine the solution was heated to approximately 60 °C after adjustment of pH, and phosphatidylcholine was added under stirring. Finally pH was checked and AAF was filtered to remove any potentially undissolved material.

Directly before use cobalt octoate and cobalt sulphate heptahydrate were pulverised with a pestle and mortar under liquid nitrogen. From each cobalt compound 0.2 g were then suspended in 10 mL of either artificial interstitial fluid (Gamble's solution) or artificial alveolar fluid (AAF) to simulate dissolution in physiological environments. Solutions were subsequently shaken for 48 h. After 48 h the extracts were divided into two equal aliquots. One of the aliquots was first filtered through a 0.45 μm filter, followed by filtration through a 0.1 μm filter, to remove any solids. The second aliquot was used without a filtration step. To evaluate the impact of particles on the biological activity of cobalt octoate both the filtered (soluble) and unfiltered fractions were used for measurement of ROS production (see 3.6.1) and for the hOGG1-modified comet assay (see 3.6.2). The concentrations given in the results section (50–800 μg/mL) refer to the originally weighted substance mass of 0.2 g/10 mL with subsequent dilutions. The concentrations don't represent real substance concentration in the different fractions after extraction and filtration. The extraction and filtration procedure was repeated for each of the various runs of the different experiments. In each run, each chemical was extracted once.

Aliquots of two different fractions were also used for determination of cobalt solubility of the two test chemicals in every experiment after shaking for 48 h in Gamble's or AAF. The soluble cobalt content was analysed by ICP-MS using the prepared samples after recommended dilution with water. For all solvent ratios and final sample volumes, blank solutions were also prepared. Cobalt ion concentrations were finally expressed as μg cobalt per ml of the extract.

3.6.1. Production of reactive oxygen species (ROS)

For ROS production, A549 cells were seeded into 96-well plates (2×10^4 cells/well) and cultivated overnight. On the next day, the medium was replaced by 100 μL of Hanks' Balanced Salt Solution (HBSS, Sigma–Aldrich, Germany) with 50 μM HDCF-DA and was kept in the dark for one hour. Cells were then washed and exposed to 100 μL chemical suspensions which were previously diluted in HBSS to be able to achieve final dilutions that spanned a range from non-cytotoxic to slightly cytotoxic. Five concentrations (100, 200, 400, 600 and 800 μg/mL) of each preparation of **cobalt octoate** and **cobalt sulphate heptahydrate** were tested. At least two replicate experiments were performed with each preparation of the cobalt compounds. The amount of generated DCF was determined after 4 h by measurement of fluorescence intensity at 528 nm using excitation at 485 nm (Molecular Devices). The mean values of triplicates were recorded.

Cytotoxicity was measured after 4 h by Hoechst and WST-1 assay (described below). Cell cultures containing only HBSS and no chemicals were used as a reference control. For the Hoechst cytotoxicity assay, cells were fixed and incubated with staining reagent (Hoechst 33,258·10 μg/mL) for 10 min at room temperature. Cells were washed and fluorescence was analysed using a microtiter plate reader (Molecular Devices, Spectramax Gemini) at 352 nm (excitation) and 461 nm (emission). The WST-1 assay (Roche, Mannheim, Germany) was used as another test for cytotoxicity. Cells were therefore incubated with and without chemicals as described above. Supernatant was removed and cells were incubated with 100 μL WST-1 solution per well as described by the manufacturer. The absorbance was determined at 420–480 nm with a reference wavelength of 690 nm.

All data were calculated as means ±SD. Statistical analyses were performed by the non-parametric Dunnett's test (Software: GraphPad Prism 4, version 4.03). Differences between exposed samples and controls were considered as statistically significant at the level of $p < 0.05$.

3.6.2. Detection of DNA strand breaks and oxidative DNA lesions using the hOGG1-modified comet assay

To investigate induction of DNA-strand breaks and oxidative DNA lesions **cobalt octoate** and **cobalt sulphate heptahydrate** were prepared in Gamble's solution and AAF and cobalt solubility was measured as described above. The extracts prepared at concentrations of 20 mg/mL were finally diluted with HBSS to the desired concentrations, i.e. 50, 200 and 800 μg/mL for cobalt octoate, and 800 μg/mL for the inorganic, water soluble positive control cobalt compound, cobalt sulphate heptahydrate. Both extracts, in Gamble's and AAF, were tested in the first set of comet assay experiments. Two independent additional experiments were performed with AAF as extraction fluid. In the first set of experiments particle size distribution was determined for the particle fractions of cobalt octoate. In the comet assay experiments cobalt octoate was compared with HBSS buffer as negative control, aluminium oxide particles (Al_2O_3, 200 μg/cm^2, 4 h) as particulate negative control, with cobalt sulphate heptahydrate as cobalt positive control, with ethyl methanesulfonate (EMS, 0.75 μL/mL, 1 h) as clastogenic positive control and potassium bromate ($KBrO_3$, 1 mM, 4 h) as positive control chemical that induces oxidative DNA lesions, in particular 8-hydroxy-2-deoxy-guanosine (8-OH-dG). Notably, in the comet assay oxidative DNA-base modifications can only be detected if lesion-specific repair enzymes like human 8-hydroxyguanine DNA-glycosylase 1 (hOGG1) are used.

For the comet assay experiments A549 cells were seeded in DMEM culture medium (Biochrom GmbH, Berlin, Germany), supplemented with 10% FCS and antibiotics (gentamicin, final concentration 5 μg/mL in DMEM, Life Technologies GmbH Invitrogen, Darmstadt, Germany) onto 12-well plates (1.75×10^5 cells per well). Before incubation with the test chemical or the reference items, the cells were grown overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 using an incubator. Cytotoxicity was determined by cell counting using an enhanced Neubauer counting chamber.

DNA-strand breaks and oxidative DNA lesions (8-OH-dG) were analysed in A549 cells after 4 h of incubation (1 h only for the positive control EMS) with 1 mL of the different extracts, using the hOGG1-modified alkaline comet assay, originally described by Smith et al. (2006), based on the alkaline version of the comet assay (Singh et al., 1988; *Tice* et al., 2000). In brief, after detachment from the culture surface, two aliquots of A549 cells per treatment condition were centrifuged for 5 min at 900 rpm (Heraeus Biofuge® 15, Thermo Scientific, Germany), re-suspended in pre-heated 0.75% low melting agarose (peqlab, Erlangen, Germany), applied to

agarose pre-coated slides, using an agarose sandwich technique, and lysed overnight at 4 °C to liberate the DNA. One of the two slides per treatment condition was subsequently incubated for 12 min with 0.16 U/mL of hOGG1 (New England Biolabs, Ontario, Canada) to detect oxidative DNA-damage. The other slide received enzyme buffer only. In both cases, DNA-unwinding and electrophoresis were done on ice, in 4 °C cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). DNA was finally stained with ethidium bromide and analysed using the Comet Assay III software from Perceptive Instruments (Steeple Bumpstead, Haverhill, UK). All methodological steps, following cell detachment, were performed under red light to avoid unspecific DNA damage due to UV-irradiation. As the main endpoint the tail intensity (TI) of 100 nuclei per slide (if available) and treatment (with or without hOGG1 incubation) were determined per experiment. The TI is a direct measure for the amount of damaged DNA that can be standardised among various studies. An increase in TI on the hOGG1-treated slides, as compared to the slides treated with enzyme buffer only, was indicative for the occurrence of the oxidative DNA base lesion 8-OH-dG. The comet assay analyses were all performed in a blinded manner, without knowledge of the identity of the test chemicals.

The comet assay data were calculated as means ±SD. Student's t-test was used to evaluate differences between the negative controls and the treated cells or the slides without and with hOGG1-treatment (unpaired and paired comparisons, respectively).

As the particle size is crucial for uptake of particles by cells and subsequent intracellular ROS-liberation by cobalt ions in the cytoplasm, the lysosomal compartment, or for very small particles even in the nucleus, particle size distribution in the incubation media (particle fraction) was determined for **cobalt octoate** in both Gamble's and AAF and correlated with data on DNA damage and cytotoxicity. For determination of the particle size distribution the particle fractions of cobalt octoate in Gamble's solution and AAF were used directly after extraction and were diluted with HBSS to get suspensions of assumed 50 μg material per 10 mL of HBSS. The diluted suspensions (about 10 mL) were sucked onto Nucleopore® filters (diameter 25 mm). Filters were then rinsed once with 5 mL of water to remove salt crystals from the extraction solutions and from HBSS which can disturb analysis. Part of the filter was then mounted on an aluminium stub and prepared for scanning electron microscopy (SEM) analysis. Determination of particle size distribution was performed at 2000 x (AAF) to 3000× magnification (Gamble's) using a scanning electron microscope (SEM) and an FE-SEM Supra 55 with image analysis software Scandium (Zeiss Co.). The samples were checked first by energy dispersive X-ray spectroscopy (EDX) in the SEM for their cobalt content. As particles are not spheres, the length (highest diameter) and diameter (extension perpendicular to the length) of about 200 particles per extract were then measured. Finally, the geometric diameter Dg was calculated as mean value of length and diameter for each particle and mass weighting was used for analysis of the diameter distribution. The resulting diameters of the cobalt octoate samples were presented as geometric means with standard deviations. All data were recorded electronically and evaluated using the SAS software package (Version SAS Institute, Cary, NC, USA, Release 9.1 on Windows XP Computer).

3.7. Bone marrow micronucleus (MN) tests in vivo

Cobalt resinate and **cobalt acetyl acetonate** were tested for induction of MN in mouse bone marrow cells. The studies were conducted in compliance with the following Animal Health regulation: Council Directive No. 86/609/EEC of 24th November 1986 on the harmonisation of laws, regulations or administrative provisions relating to the protection of animals used for experimental or other scientific purposes. *Swiss* Ico: OF1 (IOPS Caw) mice, 6 weeks old, were obtained from Charles River Laboratories, l'Arbresle, France. They were housed in cages in a room maintaining a temperature of 22 ± 2 °C, with relative humidity of 30–70% and a 12 h light/dark cycle. Ventilation achieved at least 12 cycles/hour of filtered non-recycled fresh air. All animals had free access to SsniffR/M-H pelleted maintenance diet (SSNIFF Spezialdiäten GmbH, Soest, Germany), and drinking water filtered by an FG Millipore membrane (0.22μ) was provided *ad libitum*. Mice were acclimatised for at least 5 days prior to treatment.

Both substances were suspended in 0.5% MC and administered orally by gavage to groups of 5 male and 5 female mice on 2 occasions, 24 h apart (additional animals were included in the top dose groups to allow for any unexpected mortalities). Range-finding studies were conducted to select the dose levels for the main studies. In each case doses above the selected top dose induced mortalities, and clinical signs (hypoactivity, piloerection) were observed at the selected top dose, which was therefore considered the maximum tolerated dose (MTD). The MTD plus 2 other dose levels were used for the main studies. The doses given were:

- Cobalt resinate to males at 375, 750 and 1500 mg/kg/day
- Cobalt resinate to females at 187.5, 375 and 750 mg/kg/day
- Cobalt acetyl acetonate to males and females at 125, 250 and 500 mg/kg/day.

Negative control groups were dosed with the vehicle (0.5% MC) on 2 occasions. Positive control animals were treated with 50 mg/kg cyclophosphamide (CPA) on a single occasion. All mice were killed by asphyxiation with CO_2 24 h after the second or only dose. The femurs were removed and the bone marrow was flushed out using foetal calf serum. After centrifugation, the supernatant was removed and the sedimented cells were resuspended by shaking. A drop of this cell suspension was placed and spread on a slide. The slides were air-dried, stained with Giemsa, and coded before scoring “blind”. For each animal, 2000 polychromatic erythrocytes (PCE) were counted to determine the MN frequency, and a total of 1000 erythrocytes were counted to determine the ratio of PCE to normochromatic erythrocytes (NCE).

The MN data were analysed for homogeneity within each group using the heterogeneity chi-square test. When there was no significant within-group heterogeneity, the frequencies of MN PCE in each treated group were compared with those in the concurrent vehicle control group by using a 2×2 contingency table to determine the χ^2 value. When there was significant within-group heterogeneity, then that group was compared with the control group using a non-parametric analysis, the Mann–Whitney test. Student's t-test was used for the PCE/NCE ratio comparison. Probability values of $p \leq 0.05$ were considered as significant.

Blood samples were taken from small groups of animals 2 h after the second treatment but could not be analysed for plasma levels of each test chemical due to technical problems.

3.8. Bone marrow chromosomal aberration (CA) test in vivo

A non-GLP bone marrow chromosomal aberration (CA) study was performed with **cobalt sulphate, cobalt monoxide and tricobalt tetraoxide** following oral administration to rats. The study protocol was approved by the Charles River institutional animal care and use committee. The test facility is regularly assessed and certified by the Canadian Council on Animal Care (CCAC). Weanling Sprague–Dawley SD® (Hsd:SD®) albino outbred rats were obtained from Harlan, USA, and were approximately 14 days old upon dispatch. A minimum acclimation period of 5 days was allowed between animal receipt and the start of treatment. Animals were

weighed, and males and females were separately randomised to treatment groups. Animals in poor health or at the extremes of the body weight range were not assigned to groups.

Animals remained with their respective dams throughout acclimation and exposure periods. Each dam and litter were housed (groups of 13 pups in the single dose phase study and 9 to 11 pups in the multi-dose phase study) on corn-cob bedding (Bed'O-Cob®) in solid-bottomed cages equipped with an automatic watering valve. Each dam and weanling animal was uniquely identified using the AIMS® tail tattoo system. During the study, temperature was maintained at 22 ± 3 °C, relative humidity was 50 ± 20%, and the light cycle was 12 h light and 12 h dark (except during designated procedures). All animals had free access to a standard certified pelleted commercial laboratory diet (PMI Certified Rodent 5002, PMI Nutrition International Inc.) except during designated procedures. Maximum allowable concentrations of contaminants in the diet were controlled and routinely analysed by the manufacturers. Municipal tap water, suitable for human consumption, was freely available except during designated procedures.

The study included a preliminary single dose phase using groups of 2 male and 2 female rats, which received the following doses (dose volume 10 mL/kg):

- Cobalt sulphate – 80, 160 and 320 mg/kg
- Cobalt monoxide – 100, 300 and 1000 mg/kg
- Tricobalt tetraoxide – 500, 1000 and 2000 mg/kg.

The vehicle control was aqueous 1% MC. An additional vehicle control group was included in this phase to confirm that administration of colchicine did not cause any substantial increase in micronuclei or other nuclear anomalies (see later). This phase was intended primarily to determine acute toxicity and to provide preliminary genotoxicity information. The results were used to help determine the maximum tolerated doses, and to select additional dose levels and tissues for examination in the subsequent multi-dose phase, which was designed in accordance with appropriate guidelines for *in vivo* genotoxicity tests. In the multi-dose phase, groups of 5 male and 5 female rats were treated daily with the vehicle control or each cobalt compound for up to 5 consecutive days. The dose levels were:

- Cobalt sulphate – 100, 300 and 1000 mg/kg/day (equivalent to cobalt doses of 21, 63 and 210 mg/kg/day)
- Cobalt monoxide – 200, 600 and 2000 mg/kg/day (equivalent to cobalt doses of 157, 472 and 1573 mg/kg/day)
- Tricobalt tetraoxide – 200, 600 and 2000 mg/kg/day (equivalent to cobalt doses of 47, 141 and 470 mg/kg/day).

Positive control animals in both phases of the experiment were treated with a single low dose of CPA or DMH. All animals were dosed orally by intra-gastric gavage and were sampled 16 h after the last, or only, treatment, having been injected, where appropriate, with colchicine (4 μg/kg) 1 h earlier.

The bone marrow from both femurs of each animal was pooled/eluted in 5 mL Hanks' Balance Salts Solution by aspiration with a syringe and needle. The resulting cell suspensions were centrifuged at *ca*. 1000 rpm for 5 min. The pellets were resuspended in 10 mL aqueous 0.075M (hypotonic) KCl and incubated for approximately 12 min at *ca*. 37 °C. The cells were fixed (methanol: acetic acid, 3:1), centrifuged, washed with 3 changes of neat fixative, and resuspended in a small volume of fixative. The fixed cells were dropped onto clean slides in a humid atmosphere and air-dried before staining. At least two slides were prepared from each animal. Slides were washed with 3 changes of purified water, stained with 10% v/v Giemsa for 15 min, rinsed, air-dried and mounted with coverslips. Metaphases containing between 40 and 44 chromosomes were accepted for scoring and 100 cells/animal scored for CA. Mitotic index was determined from counting 1000 cells/animal. Histological sections of appropriate organs (potential sites of genotoxicity and carcinogenicity) were quantitatively examined for nuclear anomalies (NA) produced as a result of apoptosis. These NA were described as follows:

- "Nuclear anomalies/aberrations are bizarre nuclear forms easily recognised in histological sections; they are formed by condensation and degradation of the nucleus as a result of single cell necrosis. Single cell necrosis (also referred to as apoptosis or cytotoxicity) is characteristically seen in tissues containing dividing cells following treatment of animals or people with DNA damaging agents including radiation and cytotoxic drugs. No good methods exist for examining chromosome breakage in solid tissues. Therefore, examination for nuclear anomalies represents a relatively sensitive and practical alternative method of detection of DNA damage in these tissues following exposure to a chemical. Note that micronuclei also form in the same tissues following treatment with DNA damaging agents and are classified as nuclear anomalies; they generally form as a result of chromosome damage rather than cell necrosis."

Increases in NA can be a useful screen for genotoxic damage in solid tissues of whole animals where conventional cytogenetic examination is impractical, although the possibility remains that NA may be induced by non-genotoxic mechanisms. Mitotic index was also recorded.

The toxicity of the cobalt compounds was much more pronounced following multiple administrations so that, in the case of sulphate and the monoxide, many animals could not be dosed for the planned 5 days, due to severity of clinical signs and mortalities. Therefore, some animals were sampled earlier than planned and group sizes were sometimes substantially reduced, which may have impacted the sensitivity of the study. Only the low dose sulphate group (100 mg/kg/day) survived until the end of the 5-day dosing, and no CA data could be obtained from the mid or high dose group animals. Also, no CA data were obtained from females in the high dose monoxide group. Thus, although the initial design of the study complied with OECD guidelines, deaths and early sacrifices compromised the compliance. Tricobalt tetraoxide was less toxic and animals survived dosing with 200, 600 or 2000 mg/kg/day for 5 days. However, the vehicle and positive control groups were all dosed and sampled as planned.

Due to the limited group size, no formal statistical analysis was performed on results from the single dose experiment. The mitotic indices (MI), incidences of CA, and NA (corrected for number of cells examined if variable) for the multi-dose phase were subjected to non-parametric statistical analysis using StatXact (Cytel Inc, Mass.). Exact two-sided p-values were calculated for MI, and one-sided values for other parameters using permutation tests (Gibbons, 1985; Agresti et al., 1990) based on absolute values and rank dose level with the individual animal being considered as the unit of variance. Comparison of different dose levels was not made because, for the sulphate and the monoxide, the animals in each group were not treated for the same period. MIs for animals not treated with colchicine were excluded from the analysis.

3.9. Spermatogonial chromosomal aberration (CA) test in vivo

Cobalt dichloride hexahydrate was investigated for induction of CA in spermatogonial cells of male Sprague–Dawley CD rats obtained from Charles River Laboratories Germany GmbH. The rats were housed, fed, and handled as all other laboratory animals in

compliance with EU Directive 86/609/EEC. Following an initial health check approximately one week before commencement of treatment, the animals were weighed and randomly allocated to the test groups. All animals were healthy at the start of the study, and on the first day of dosing animals were 35 days old and weighed 140.5–164.1 g. Commercial Ssniff® R/M-H V1530 diet (Ssniff Spezialdiäten GmbH, 59494 Soest, Germany) and drinking water were provided *ad libitum*.

Fresh preparations of cobalt chloride dissolved in water were made each day and administered orally by gavage once daily for 28 days. Dose levels that were initially selected, based on prior knowledge, were 3, 10, 30, 100 and 300 mg/kg/day. However, since deaths occurred at both 100 and 300 mg/kg/day, the maximum tolerated dose was deemed to be 30 mg/kg/day. Data were therefore obtained from animals dosed with cobalt chloride at 3, 10 and 30 mg/kg/day for 28 days. The vehicle control group received water daily by gavage. No concurrent positive control group was included in this study, but the laboratory had obtained consistent significant positive responses with mitomycin C.

Animals were sacrificed 24 h after the last treatment. Three hours prior to sampling, the animals received 4 mg colchicine/kg b.w. i.p. The animals were sacrificed by ether. After having removed the tunica albuginea, the seminiferous tubules of both testicles were exposed to hypotonic 1% sodium citrate solution for 20 min. Afterwards, still *in toto*, the seminiferous tubules were fixed in freshly prepared methanol/glacial acetic acid (3 + 1) fixative and the samples were left overnight (about 16 h) at low temperature (0–4 °C). In order to prepare the slides, the samples were centrifuged and the fixative was completely removed with a Pasteur pipette. The pellet was gently resuspended by adding 60% acetic acid. Approx. 50 μL of each cell suspension was dropped onto prewarmed slides at 48 °C and spreads were made. The air-dried cells were stained with 10% Giemsa (in buffered phosphate solution, pH 7.2) for 45 min. Afterwards the slides were mounted. At least two slides were prepared per animal.

Analysis of CA was performed on all 3 cobalt chloride dose groups, and on vehicle (water) controls. Slides for evaluation were blind coded before microscopic analysis and examined at a magnification of 1000× (Planapochromat 100/1.25). The mitotic index was determined by counting the number of metaphases per 1000 cells in each cell preparation. The frequencies of structural CA (chromosome- and chromatid type) were determined from 200 well-spread metaphases per animal. Since fixation procedures often result in breakage of some metaphases, with a concomitant loss of chromosomes, only cells with $2n \pm 2$ centromeres were scored. Cells with an incomplete number of centromeres or insufficient spreading were not used for analysis.

Statistical evaluation was carried out by comparing frequencies of aberrant cells in treated groups with the vehicle controls, using a chi-square test corrected for continuity according to Yates, as recommended by Lovell et al. (1989).

4. Results

4.1. Ames tests

4.1.1. Single strain tests

The results of the tests on cobalt metal in strain TA98, cobalt chloride in TA97a and cobalt sulphate in TA100 are summarised in Table 1.

Some slight differences in toxicity and precipitation were seen across the 3 laboratories, and are not easy to explain, but this made no impact on the robustness of the studies. The differences noted and their effects on choice of concentrations can be summarised as follows:

- All 3 laboratories obtained numbers of revertants/plate in negative control cultures that were normal, except for 2 experiments in Laboratory 3 (Table 1B) where the revertant counts for TA97a were in one case considerably lower and in another considerably higher than the historical control range, and the experiments rejected. However, each laboratory demonstrated clear positive responses with the positive control chemicals (data not shown).
- For **cobalt metal**, which was suspended in DMSO by each laboratory prior to addition to the plates, Flügge (2013a) observed insoluble material at all concentrations tested, Laboratory 3 observed precipitation at concentrations of 1000 μg/plate and above, whereas Laboratory 2 did not observe any precipitation. Each laboratory saw toxic effects which led to slightly different selection of top concentration, as can be seen from the table.
- For **cobalt chloride**, all 3 laboratories observed some toxic effects (reduced background lawn of growth or reduced numbers of revertants) in some experiments at the higher concentrations, although in the studies from Laboratory 3 these observations were not consistent between repeat experiments, and in one of the experiments using plate incorporation the laboratory observed widespread bacterial colony growth in the absence of S9 which necessitated manual counting. Laboratory 3 also observed some precipitation at 2500 μg/plate and above in all experiments. This led to slightly different selection of top concentrations, as can be seen from the table.
- For **cobalt sulphate**, each laboratory selected a top concentration of 5000 μg/plate, but whereas Flügge (2013c) and Laboratory 3 observed some precipitate but no toxicity at this concentration, Laboratory 2 did not see any precipitate but did note some toxicity.

Although Laboratory 3 had some inconsistent results and needed to repeat some experiments, there were no clear or reproducible positive responses even for this laboratory alone. When the responses seen in the other 2 laboratories are considered, where maximum increases in revertant numbers were always low and there was no evidence of dose responses (peak revertant counts occurred randomly across the concentration range), it is clear that these 3 substances have not produced the mutagenic responses seen with these strains in published papers (see Introduction) either in the absence or presence of S9, using either plate incorporation or pre-incubation methodology.

4.1.2. Five strain tests

Cobalt acetyl acetonate (Supplementary Table 1) induced slight toxicity (slightly reduced revertant counts) at the top concentration in some strains. **Cobalt resinate** (Supplementary Table 2) was not toxic at the concentrations tested but did produce precipitate at concentrations of 250 μg/plate and above. Negative control revertant frequencies for all strains in both experiments were normal, and were significantly increased by positive control treatments. Cobalt resinate induced small but biologically significant (i.e. >2-fold) increases in TA98 revertants at the lowest and highest concentrations in the absence of S9 in Experiment 1. The increase at the top concentration could be considered to be dose-related, but the responses were not reproduced under the same conditions in Experiment 2. They may be due to chance or technical error and are not considered biologically relevant. All other strains treated with cobalt resinate, both in the absence and presence of S9, exhibited revertant frequencies that were similar to controls and there were no biologically significant or dose-related increases. All strains treated with cobalt acetyl acetonate, both in the absence and presence of S9, exhibited revertant frequencies that were similar to controls and there were no biologically significant (maximum

increase in any strain was in TA1537 and was only 1.8-fold) or dose-related increases. Therefore it is concluded that neither cobalt resinate nor cobalt acetyl acetonate induced reverse mutations in Ames bacteria.

4.2. Mammalian cell gene mutation tests

4.2.1. Mouse lymphoma Tk mutation tests

For both studies described below, negative control mutant frequencies fell within acceptable ranges, and the positive control chemicals induced significant increases in mutant frequency.

The results for **cobalt resinate** are summarised in Supplementary Table 3. It produced precipitate at the end of treatment at the top 2 or 3 concentrations, which could have been problematical, as it is difficult to separate the suspended cells from the precipitate by centrifugation at the end of treatment, but appears not to have interfered with the study. In the absence of S9 the top concentration induced approximately 50% toxicity (reduction in relative total growth, RTG) in both 3 and 24 h treatments. In the presence of S9 the top concentration was less toxic and induced only 15–25% reduction in RTG. Although target levels of toxicity (>80%) were not reached, the inclusion of several precipitating concentrations constitutes a valid assay. Mutant frequencies in all treated cultures were very similar to concurrent controls, and there were no increases that approached a biologically significant level, i.e. did not approach the Global Evaluation Factor (GEF) of +126 mutants per 10^6 cells as recommended by Moore et al. (2006). Therefore cobalt resinate did not induce *Tk* mutations in mouse lymphoma cells in a robust test up to precipitating concentrations.

The results for **cobalt acetyl acetonate** are summarised in Table 2.

It can be seen that increases in mutant frequency that exceeded the GEF were induced in cultures treated in the absence of S9 in both experiments. These increases were only seen at relatively high levels of toxicity (reductions in RTG of 70% or more), and were not dose-related in the second experiment, but the toxic effects were not dose-related either. Biologically significant increases in mutant frequency were also seen in the first experiment in the presence of S9. The result at the top concentration (91.625 μg/mL) should be ignored because excessive toxicity (>90%) was induced and artefacts can occur under such highly toxic conditions. A significant increase was also seen at a lower level (36%) of toxicity, but this was not clearly reproduced in the second experiment at similar concentrations and levels of toxicity – mutant frequencies did not

Table 1
Summary of results with single Ames strains.

A. Cobalt metal powder in TA98

Experiment and condition	Flügge (2013a)		Laboratory 2		Laboratory 3	
	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)
Expt. 1, −S9	1000^{PL}	1.4 (31.6)	5000^{*PL}	1.6 (158.1)	5000^{*PL}	1.4 (333)
Expt. 1, +S9	1000^{PL}	1.3 (31.6)	5000^{*PL}	1.0 (158.1)	5000^{*PL}	1.1 (333)
Expt. 2, −S9	1000^{PR}	1.3 (3.16)	5000^{*PL}	1.2 (156.3)	5000^{*PR}	1.3 (33)
Expt. 2, +S9	1000^{PR}	1.0 (100)	5000^{*PR}	1.0 (625)	5000^{*PR}	1.2 (333)

* Extensive toxicity seen. PL = plate incorporation; PR = pre-incubation.

B. Cobalt chloride in TA97a

Experiment and condition	Flügge (2013b)		Laboratory 2		Laboratory 3	
	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)
Expt. 1, −S9	3160^{*PL}	1.1 (100)	5000^{*PL}	1.0 (15.81)	5000^{*PL}	2.7 (2500)[a]
Expt. 1, +S9	3160^{*PL}	1.0 (31.6)	5000^{*PL}	1.2 (15.81)	5000^{PL}	1.7 (2500)
Expt. 2, −S9	3160^{*PR}	1.0 (1000)	5000^{*PL}	1.0 (625)	5000^{*PR}	1.6 (3750)
Expt. 2, +S9	3160^{*PR}	1.0 (316)	5000^{*PR}	0.9 (312.5)	5000^{*PR}	0.9 (5000)[a]
Expt. 3, −S9					5000^{PL}	1.5 (100)
Expt. 3, +S9					5000^{*PL}	0.8 (3)
Expt. 4, −S9					5000^{*PR}	1.1 (333)
Expt. 4, +S9					5000^{*PR}	0.9 (1)

* Slight toxicity seen. PL = plate incorporation; PR = pre-incubation.
[a] Experiments were considered invalid because solvent control counts were outside historical range.

C. Cobalt sulphate in TA100

Experiment and condition	Flügge (2013c)		Laboratory 2		Laboratory 3	
	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)	Maximum concentration tested (μg/plate)	Maximum fold increase in revertants (at concentration, μg/plate)
Expt. 1, −S9	5000^{PL}	1.0 (1000)	5000^{*PL}	1.3 (5000)	5000^{PL}	1.1 (333)
Expt. 1, +S9	5000^{PL}	1.2 (100)	5000^{*PL}	1.2 (1581)	5000^{PL}	1.3 (1000)
Expt. 2, −S9	5000^{PR}	1.1 (5000)	5000^{*PL}	1.1 (1250)	5000^{PR}	*2.1 (100 & 333)*
Expt. 2, +S9	5000^{PR}	1.1 (5000)	5000^{*PR}	1.2 (5000)	5000^{PR}	1.4 (5000)
Expt. 3, −S9					1000^{PR}	*2.0 (125)*
Expt. 4, −S9					5000^{PL}	1.9 (5000)
Expt. 4, +S9					5000^{PL}	1.4 (2500)
Expt. 5, −S9					5000^{PR}	1.7 (2500)
Expt. 5, +S9					5000^{PR}	1.5 (5000)

* Slight toxicity seen. PL = plate incorporation; PR = pre-incubation.
Italics indicate results questionable due to “plateau” effect across all test concentrations.

quite reach the GEF but a dose-response was evident. In the positive cultures, both large and small colony mutants were induced, but the predominant response was induction of small colony mutants, indicative of a clastogenic (chromosome damaging) effect. Therefore, cobalt acetyl acetonate did induce *Tk* mutations in mouse lymphoma cells, particularly in the absence of S9, and the effect was predominantly clastogenic.

4.2.2. Hprt mutation studies

In all studies negative control mutant frequencies (MF) were within the historical range established by the laboratory, and positive control chemicals induced significant increases in MF (data not shown).

The results for the 7 salts/compounds and cobalt metal powder tested only for 3 h in the absence and presence of S9 are summarised as follows:

- **Cobalt dihydroxide** precipitated in culture medium and persisted to the end of treatment at concentrations of 116.1 μg/mL and higher. However, as shown in Supplementary Table 4, it was toxic at much lower concentrations. It did not induce any statistically or biologically significant increases in mutant frequency (MF) when tested up to toxic concentrations in the absence of S9. Statistically significant increases in mutant frequency (MF) were seen at 1 or 2 concentrations in the presence of S9 in two out of three experiments, but the MF only exceeded 3x the historical control mean (8.34 per 10^6 cells, considered biologically significant) at 1 concentration in 1 experiment. Also, a significant dose-response trend was only seen in a different experiment and was therefore not reproducible. The increased MF are therefore of questionable biological relevance. Overall it is concluded that cobalt dihydroxide did not induce biologically relevant *Hprt* mutations when tested up to toxic concentrations in the absence or presence of S9.
- The results for **lithium cobalt dioxide** are summarised in Supplementary Table 5. It can be seen that in each part of the study little or no toxicity was induced, but the top concentration was limited by solubility. In none of the treated cultures were the MFs statistically significant when compared to concurrent controls, no MFs exceeded 3x the historical control means (9.42–12.36 mutants per 10^6 cells for the different treatment conditions), and there were no dose-related trends. It is therefore concluded that lithium cobalt dioxide did not induce *Hprt* mutations when tested to toxic concentrations in the absence or presence of S9.
- The results for **cobalt oxide hydroxide** are summarised in Supplementary Table 6. It can be seen that the top concentration in each part of each experiment was limited by solubility and induced little or no toxicity. MFs in all treated cultures were similar to those in controls, not statistically different, did not exceed 3x the historical control means (11.04–13.14 mutants per 10^6 cells for the different treatment conditions), and there were no dose-related trends. It is therefore concluded that cobalt oxide hydroxide did not induce *Hprt* mutations when tested to precipitating concentrations in the absence or presence of S9.
- **Cobalt oxalate** precipitated in culture medium and persisted to the end of treatment at concentrations of 70 μg/mL and higher. However, as discussed earlier and as can be seen from Supplementary Table 7, it was toxic at this and lower concentrations, inducing >80% reduction in relative survival at the top concentrations evaluated for mutations. It did not induce any statistically or biologically significant increases in MF when tested up to toxic concentrations in the absence of S9. A statistically significant increase in MF was seen at 1 intermediate concentration in the second experiment in the presence of S9, and did exceed 3x the historical control mean (8.19 mutants per 10^6 cells). However, the isolated increase in MF was not seen at a similar concentration and similar level of toxicity in the first experiment, and there was no dose-response. The increase in MF is therefore of questionable biological relevance and probably due to chance. Overall it is concluded that cobalt oxalate did not induce biologically relevant *Hprt* mutations when tested up to toxic concentrations in the absence or presence of S9.
- As mentioned earlier, **cobalt metal powder** clearly precipitated in culture medium and undissolved cobalt metal powder was present at many lower concentrations. As can be seen from Table 3 it was toxic at low concentrations, particularly in the absence of S9. The marked variability in the toxic responses in the presence of S9 between the different experiments may be a reflection of the fact that undissolved cobalt metal powder was present, and may have exerted toxic effects on the cells due to its physical presence. It did not induce any statistically or biologically significant increases in MF when tested up to toxic concentrations in the absence of S9, although a weak dose-response was seen in the second experiment. Statistically significant increases in MF were seen at single intermediate concentrations in each of the experiments in the presence of S9, and a dose-response was seen in the second experiment. However, the MFs did not exceed 3x historical control means (6.69 or 9.0 mutants per 10^6 cells for treatments in the absence and presence of S9 respectively). A third experiment was performed in the presence of S9 in order to investigate these findings further. In this experiment, significant increases in MF were seen at 30 and 40 μg/mL accompanied by a significant dose-response. The MFs at these concentrations were close to or exceeded 3x the historical control mean, and may therefore be considered biologically relevant. Overall it is concluded that cobalt metal powder did not induce biologically relevant *Hprt* mutations when tested up to toxic concentrations in the absence of S9, but did induce weak, reproducible mutagenic effects in the presence of S9. The influence of undissolved cobalt metal powder on the toxic and mutagenic effects seen is unclear.
- The results for **tricobalt tetraoxide** are summarised in Supplementary Table 8. It can be seen that the top concentration in each experiment was the lowest producing persistent precipitate through the treatment period, but did not induce significant toxicity (maximum 34% reduction in relative survival). Although some MFs exceeded concurrent negative control values, none were statistically significant, did not exceed 3x historical control means (9.6–11.79 mutants per 10^6 cells for the different treatment conditions), and there were no dose-related trends. It is therefore concluded that tricobalt tetraoxide did not induce *Hprt* mutations when tested to precipitating concentrations in the absence or presence of S9.
- The results for **cobalt 2-ethyl hexanoate** are summarised in Supplementary Table 9. It can be seen that the top concentration in each part of the study produced >80% reduction in relative survival. Although some MFs exceeded concurrent control values, none were statistically significant, did not exceed 3x historical control means (10.35–12.69 mutants per 10^6 cells for the different treatment conditions), and there were no dose-related trends. It is therefore concluded that cobalt 2-ethyl hexanoate did not induce *Hprt* mutations when tested to toxic concentrations in the absence or presence of S9.

The results for the 5 substances tested both for 3 h in the absence and presence of S9, and for 24 h in the absence of S9, are summarised as follows:

- The results for **cobalt sulphate** are summarised in Supplementary Table 10. It can be seen that the top

Table 2
Mouse lymphoma *Tk* mutation results with cobalt acetyl acetonate.

Expt.	S9	Treatment period (hrs)	Conc. of cobalt acetyl acetonate (μg/mL)	Mutant frequency ($\times 10^{-6}$)	% Toxicity (reduction in RTG)
1	–	3	0	105	–
			2.93	91	2
			5.86	87	5
			11.46	86	13
			22.90	116	6
			45.83	144	15
			91.625	***383***	83
2	–	3	0	160	–
			11.43	168	26
			22.87	184	19
			45.74	239	48
			60.99	***471***	70
			76.23	275	58
			91.77	***415***	72
1	+	3	0	139	–
			2.93	135	–
			5.86	119	–
			11.46	94	–
			22.90	132	2
			45.83	***307***	36
			91.625	***1222***[a]	98[a]
2	+	3	0	107	–
			11.43	73	–
			22.87	96	17
			45.74	113	20
			60.99	185	40
			76.23	222	52
			91.77	222	62

Bold italics indicate biologically significant responses (increase in mutant frequency exceeds GEF).
[a] Values are considered unreliable and should be rejected due to excessive (>90%) toxicity.

concentration in each part of the study induced >80% reduction in relative survival. Cobalt sulphate did not induce any statistically or biologically significant increases in MF when tested up to toxic concentrations in the absence of S9 using either 3 or 24 h treatments. Statistically significant increases in MF were seen at the 2 highest concentrations in the second experiment in the presence of S9 leading to a dose-response, but the concurrent control MF was unusually low, and the MFs in the treated cultures did not exceed 3x the historical control mean (8.7 mutants per 10^6 cells). Moreover, these increases in MF were not seen at even higher concentrations and similar levels of toxicity in the first experiment. The increases in MF are therefore of questionable biological relevance. Overall it is concluded that cobalt sulphate did not induce biologically relevant *Hprt* mutations when tested up to toxic concentrations in the absence or presence of S9, even when extended treatment periods were used in the absence of S9.

- The results for **cobalt sulphide** are summarised in Supplementary Table 11. It can be seen that for the 3 h treatments the maximum concentration was 10 mM (922 μg/mL). However, following 24 h treatment, >80% reduction in relative survival was induced at 800 μg/mL. Although some MFs exceeded concurrent control values, none were statistically significant, did not exceed 3x historical control means (6.9, 7.8 and 6.06 mutants per 10^6 cells for 3 h in the absence and presence of S9, and 24 h in the absence of S9, respectively), and there were no dose-related trends. It is therefore concluded that cobalt sulphide did not induce *Hprt* mutations when tested either to 10 mM or toxic concentrations in the absence or presence of S9.
- **Cobalt monoxide** precipitated in culture medium and persisted to the end of treatment at concentrations of 60–80 μg/mL and higher. However, as mentioned previously and as can be seen from Supplementary Table 12, it was toxic in this concentration range particularly in the absence of S9. Although MFs were increased in some of the cultures treated in the presence of S9, they did not exceed control MFs seen elsewhere in this series of studies. A 3-fold increase in MF was seen at one of the intermediate concentrations in the 24 h treatment protocol, but it was not significantly different from the concurrent control and there was no dose response. In fact none of the MFs in any part of the study, either in the absence or presence of S9, were statistically significant when compared to concurrent controls, and none exceeded 3x the historical control means (7.5–8 mutants per 10^6 cells for the different treatment conditions) which would be required for biological significance. It is therefore concluded that cobalt monoxide did not induce *Hprt* mutations when tested to precipitating concentrations in the absence or presence of S9.
- **Cobalt borate neodecanoate** precipitated in culture medium and persisted to the end of treatment at concentrations of 100 μg/mL and higher. However, as can be seen from Supplementary Table 13, it was toxic at lower concentrations, inducing >80% reduction in relative survival at 2.75–3.0 μg/mL in the absence of S9, and at 50–60 μg/mL in the presence of S9 following short (3 h) treatments. It was also toxic following 24 h treatments, and the maximum concentration used was 2.0 μg/mL, which induced 90% reduction in relative survival. Therefore all concentrations evaluated for mutations were soluble. A statistically significant increase in MF was seen at the highest concentration in the second experiment with 3 h treatment in the absence of S9, and it did exceed 3x the historical control mean (6.72 mutants per 10^6 cells), although it did not exceed control MFs observed elsewhere in this series of studies, and there was no dose-response. Moreover, the isolated increase in MF was not seen at similar or higher concentrations and similar levels of toxicity in the first experiment with 3 h treatment. Dose-responses were seen in the first experiment with 3 h treatment in the absence of S9 and in both experiments with 3 h

Table 3
Hprt mutation data with cobalt metal powder.

Expt.	S9	Treatment time (hr)	Treatment (μg/mL)	Mutant frequency (per 10^6 cells)	Cytotoxicity (reduction in % relative survival)
1	−	3	0	2.66	0
			1.172	2.38	5
			2.344	1.31	11
			4.688	1.44	2
			9.375	2.10	22
			18.75	0.95	59
			37.5	4.70	92
1	+	3	0	2.69	0
			50	3.70	74
			100	4.39	78
			150	2.12	56
			200	**6.99**	89
			250	4.14	81
2*	−	3	0	1.72	0
			5	1.26	19
			10	1.62	1
			15	1.48	14
			20	1.14	29
			30	2.36	25
			35	2.87	32
			40	3.39	92
			50	3.30	78
2**	+	3	0	1.60	0
			10	1.11	4
			20	0.82	0
			40	**5.75**	53
			60	3.65	97
3***	+	3	0	2.28	0
			5	3.73	9
			10	4.71	24
			30	**9.46**	68
			40	**8.82**	84

Bold figures indicate statistically significant from vehicle control (Dunnett's test, $p < 0.05$).
Asterisks indicate significant linear trend in this data set (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

treatment in the presence of S9, but these were strongly influenced by low negative control MFs, and, apart from the exception described earlier, none of the MFs in these treated cultures were statistically significant when compared to concurrent controls. Following 24 h treatment in the absence of S9 all MFs were similar to control and there were no significant differences or dose response. Overall it is concluded that cobalt borate neodecanoate did not induce biologically relevant *Hprt* mutations when tested up to toxic concentrations in the absence or presence of S9.

- For the **extract of cobalt metal powder** Tyndall analysis showed that no undissolved solid was found in the supernatant after the 72 h extraction and centrifugation, and therefore the preparation was suitable for testing.
  As can be seen from the summary data in Table 4, at the top concentrations of the extract tested, relative survival was reduced to <20% under all treatment conditions, indicating that divalent cobalt cations were liberated during the extraction process and induced toxic effects in the cells. In Experiment 1 (3 h treatments in the absence and presence of S9 with top concentrations of 290 and 320 μg/mL respectively) no statistically significant increases in MF were observed following treatment with extract at any concentration tested and there were no significant linear trends. In Experiment 2, statistically significant increases in MF, compared to the concurrent vehicle controls, were observed at concentrations of 105 μg/mL in the absence of S9 (24 h treatment) and 290 μg/mL in the presence of S9 (3 h treatment). However, the mean MFs in these cultures were only 4.99 and 6.21 mutants/10^6 viable cells. These frequencies did not exceed 3x the historical control mean MFs (5.61 and 6.78 mutants per 10^6 cells respectively) and were therefore considered not to constitute a biologically relevant positive response. Furthermore, although a statistically significant linear trend was seen in the absence of S9 (but not in the presence of S9) in Experiment 2, there appeared to be no clear concentration-related effects in the presence of S9 in Experiment 2 and no evidence of reproducibility between Experiments 1 and 2 in the absence or presence of S9. Overall, these increases in MF were considered sporadic and of no biological relevance. Thus, the weak responses seen with cobalt metal powder in the earlier study were not reproduced with extracts of the powder, when tested to toxic concentrations.

4.3. Chromosomal aberration (CA) tests in vitro

A summary of the key results for **cobalt acetyl acetonate** is given in Table 5. Although some statistically significant increases in % cells with CA were seen at low and middle concentrations, frequencies below 5% would probably not be considered biologically significant, although they do exceed the upper limits of the historical control ranges, namely 2.5 and 3% in the absence and presence of S9 respectively. At first sight it appears that biologically significant induction of CA is only seen at concentrations of cobalt acetyl acetonate inducing >50% toxicity. The second experiment in the absence of S9 gave a most unusual toxicity response (not dose-related) and therefore the reported 18% mitotic inhibition at 150 μg/mL is probably not reliable, but higher concentrations could not be scored as they were very cytotoxic. However, biologically significant increases in CA were clearly seen at modest levels of mitotic inhibition in the second experiment in the presence of S9. Therefore it does not seem likely that the increased levels of CA are an indirect result of excessive toxicity.

Given that:

- There is a consistent pattern of increased CA frequencies in both the presence and absence of S9
- The effects are reproducible across independent experiments
- The increased CA are not exclusively associated with high (>50%) levels of cytotoxicity

it is concluded that cobalt acetyl acetonate did induce a biologically meaningful clastogenic response in human lymphocytes both in the absence and presence of S9.

A summary of the key results for **cobalt resinate** is given in Table 6.

The data from this study are not easy to interpret, possibly because of the impact of treating suspension cultures with precipitating concentrations. The following inconsistencies arise:

- In Experiment 1 in the absence of S9 it is not clear why cytotoxicity increased through the precipitating range.
- In Experiment 2 with 20 h treatment in the absence of S9 there is less (in fact no) toxicity at the same concentrations that induced increasing toxicity after only 3 h treatment. Longer treatments at the same concentration would be expected to be more toxic. On the other hand, 44 h treatment in the absence of S9 is much more toxic.
- In the 3 h treatment in the presence of S9 in Experiment 1 the toxicity profile exhibited a plateau across the precipitating range (which would be expected), yet in Experiment 2 there was neither a plateau nor a dose response, and in Experiment 3 there was some evidence of a dose-related toxic response. On the other hand, after 41 h recovery, significant toxicity was induced.

Some of the inconsistencies in toxicity probably reflect the difficulties in removing precipitate from suspension cultures following 3 h treatments. However, the inconsistencies following continuous treatments are not readily explained.

The small increase in CA frequency (4.5%) at the lowest concentration in Experiment 1 in the absence of S9 is probably due to chance and not biologically significant. The increases in CA frequency in Experiment 1 in the presence of S9 were not reproduced in Experiment 2 (but then neither was the toxicity); however they were reproduced in Experiment 3 where toxicity similar to that seen in Experiment 1 was induced. Overall it can be concluded that cobalt resinate does induce CA in the presence of S9, although the impact of cytotoxicity on these responses is unclear because of the confounding factor of precipitation.

The results of the CA assay with **cobalt oxyhydroxide** in V79 cells are summarised in Table 7.

According to the mitotic index measures, the top concentrations induced at least 58% cytotoxicity, which is in the target range specified in OECD guidelines. However, in some cases >60% mitotic inhibition was induced, and results at such high levels of cytotoxicity need to be interpreted with caution. In fact, the latest revision to OECD guideline 473 (OECD, 2014) states that care should be taken even when interpreting positive results found only at the upper end of the target cytotoxicity range. The only treatment that induced a biologically significant increase in CA frequency at less than 50% reduction in mitotic index was the 20 h continuous treatment at 200 μg/mL in the absence of S9. All other increases were associated with at least 59% reduction in mitotic index.

Current guidelines for genotoxicity testing in mammalian cells recommend that only 1 concentration should be included at which precipitate is present at the end of treatment. This is to demonstrate that an acceptable upper concentration was evaluated but without creating artefacts. The impact of precipitation at every concentration tested in this study is unclear. As mentioned earlier, it is curious how mitotic index and cell density continued to decline with increasing concentrations of cobalt oxyhydroxide through the insoluble range. Precipitate can "blanket" cells growing in monolayer, and inhibit nutrient uptake and gas exchange, leading to physiological stress and cytotoxicity. This would be a particular problem in cultures treated for long periods of time (e.g. the 20 h treatments in the absence of S9). Thus, it is highly likely that the presence of persistent precipitate in the 20 h treatments resulted in physiological stress leading to both cytotoxic and genotoxic effects. All of the observed increases in CA frequency, including at 200 μg/mL in the 20 h treatment, may well be an indirect consequence of such physiological stress.

In addition, V79 cells have been shown to be particularly susceptible to giving positive results with substances that are not genotoxic *in vivo* or carcinogenic– so-called "misleading" or "false" positive results (Fowler et al., 2011). Hamster cells such as V79 that are p53-deficient, genomically altered, and potentially genomically unstable, are much more likely to express genotoxic changes resulting from effects on non-DNA targets, oxidative damage, or metabolic overload than p53-competent, normal diploid cells. Thus, the biological relevance of the CA results with cobalt oxyhydroxide is unclear.

4.4. Tests for oxidative damage

4.4.1. Production of reactive oxygen species (ROS)

From the analysis of cobalt in the extraction media, it was shown that significant concentrations of cobalt ions could be recovered (around 40–50 μg/mL for **cobalt octoate** and around 60–70 μg/mL for **cobalt sulphate heptahydrate** in Gamble's solution), and were therefore bioavailable to the cells. These concentrations were stable over 24 h.

Following a 4 h exposure of A549 cells to both AAF and Gamble's solution extracts of **cobalt octoate**, DCFH was oxidised resulting in a strong increase of DCF fluorescence (up to 949% in AAF and 725% in Gamble's solution, see Fig. 1). Metabolic activity and DNA content were reduced after incubation with high concentrations of cobalt octoate (400 and 800 μg/mL, particulate fractions). Here, ROS formation was already observed at non-cytotoxic concentrations (<400 μg/mL) of cobalt octoate. Both filtered and unfiltered extracts of cobalt octoate induced ROS formation, indicating that the cobalt released from the particulate fraction gave rise to the same ROS levels as medium directly prepared with the corresponding transition metal ion (Fig. 1).

Exposure of A549 cells to highly soluble **cobalt sulphate heptahydrate** extracted in either AAF or Gamble's solution also resulted in increases in DCF fluorescence after 4 h (increase up to 1090% in AAF and 859% in Gamble's solution, Fig. 2). Again both filtered and unfiltered extracts of cobalt sulphate heptahydrate induced ROS formation. Both cobalt compounds are therefore highly comparable for their effects on ROS formation. However, ROS production by cobalt octoate was associated with marked cytotoxicity (measured both by Hoechst and WST-1), whereas for cobalt sulphate ROS production was not associated with cytotoxicity (cf. Figs. 1 and 2).

4.4.2. hOGG1-modified comet assay

To enable a better understanding and comparison of the biological effects of **cobalt octoate** and the inorganic, water soluble **cobalt sulphate heptahydrate**, as a positive reference item, cobalt solubility was determined in all soluble fractions. Cobalt octoate and cobalt sulphate heptahydrate exhibited different cobalt mass contents of 17 and 21%, and thus a theoretical cobalt net weight per ml at extraction start of 3.40 and 4.19 mg/mL, respectively. In AAF, cobalt sulphate heptahydrate exhibited the higher cobalt solubility,

Table 4
Hprt mutation data with an extract of cobalt metal powder.

Expt.	S9	Treatment time (hr)	Treatment (μg of cobalt used for extraction/mL)	Mutant frequency (per 10^6 cells)	Cytotoxicity (reduction in % relative survival)
1	−	3	0	4.68	0
			120	1.16	0
			160	3.34	0
			200	4.14	4
			230	5.20	43
			260	3.81	66
			290	3.57	90
1	+	3	0	1.34	0
			120	2.73	8
			160	2.98	2
			200	1.64	0
			230	3.38	48
			260	1.83	40
			290	2.62	77
			320	1.66	89
2***	−	24	0	1.26	0
			40	0.79	20
			60	1.71	25
			75	4.29	31
			90	2.55	33
			105	**4.99**	65
			120	3.57	47
			135	3.88	72
			150	4.02	83
2	+	3	0	2.10	0
			100	1.99	0
			150	2.29	7
			200	1.82	23
			230	1.90	27
			260	1.59	53
			290	**6.21**	73
			320	4.80	76
			350	2.57	85

Bold figures indicate statistically significant from vehicle control (Dunnett's test, $p < 0.05$).
Asterisks indicate significant linear trend in this data set (***$p < 0.001$).

Table 5
Chromosomal aberration (CA) results for cobalt acetyl acetonate in human lymphocytes.

Expt.	S9	Treat + Recovery (hr)	Conc. of cobalt acetyl acetonate (μg/mL)	% cells with CA (excl. gaps)	% toxicity (reduction in mitotic index)
1	−	3 + 17	0	0	0
			34	**3.5**	31
			68	**4.7**	47
			136	***18.0***	56
2	−	3 + 17	0	0	0
			75	**3.5**	0
			100	**3.5**	43
			150	***12.0***	18
1	+	3 + 17	0	0.5	0
			17	**4.5**	16
			34	**4.5**	31
			68	***32.0***	68
2	+	3 + 17	0	0.5	0
			50	***8.0***	35
			75	***12.0***	46
			100	***26.0***	64

Bold figures indicate statistical significance. Bold italics indicate both statistical and clear biological significance.

with mean cobalt concentrations amounting to 2.97 ± 0.051 mg/mL (=70.8% of the theoretical cobalt net weight; n = 6 independent fractions), as compared to 2.06 ± 0.065 mg/mL (=60.7 ± 1.90% of the theoretical cobalt net weight; n = 3 independent fractions) for cobalt octoate. Extraction with Gamble's led to comparable results (data not shown).

In addition, **cobalt octoate** particle size distribution was determined in the unfiltered incubation media. By using energy dispersive X-ray spectroscopy in SEM, most of the particles present were shown to contain no cobalt, which was in line with the high cobalt solubility of cobalt octoate in AAF of about 61%. The analysed fractions were composed of particles of a wide size range (data not shown) with in part very small, respirable particles, up to larger agglomerates. But, irrespective of the extraction fluid, the particles in the cobalt octoate unfiltered fractions exhibited geometric mean values (weighting by mass) which exceeded respirable dimensions. Geometric mean values of the cobalt octoate fractions in Gamble's solution (7.54 ± 1.95 μm) were more close to respirable dimensions than the cobalt octoate fractions in AAF (10.26 ± 1.79 μm).

Induction of DNA strand breaks and oxidative DNA lesions by the AAF-extracted cobalt compounds, as determined using the hOGG1-modified comet assay, are shown in Fig. 3. Negative

Table 6
Chromosomal aberration (CA) results for cobalt resinate in human lymphocytes.

Expt.	S9	Treat + Recovery (hr)	Conc. of cobalt resinate (μg/mL)	% cells with CA (excl. gaps)	% toxicity (reduction in mitotic index)
1	−	3 + 17	0	1.0	0
			75[P]	**4.5**	39
			150[P]	2.0	49
			300[P]	2.0	61
2	−	20 + 0	0	0.5	0
			75[P]	0.5	0
			150[P]	2.0	0
			300[P]	1.5	0
2	−	44 + 0	0	1.5	0
			18.8	2.0	61
1	+	3 + 17	0	1.5	0
			75[P]	***6.5***	44
			150[P]	***18.0***	44
			300[P]	***16.0***	49
2	+	3 + 17	0	0	0
			75[P]	0.5	15
			150[P]	1.5	21
			300[P]	2.5	0
2	+	3 + 41	0	0.5	0
			75[P]	3.0	59
3	+	3 + 17	0	0.5	0
			37.5	**4.0**	47
			75[P]	***7.3***	34
			150[P]	***8.7***	69

[P] = precipitate observed at harvest.
Bold figures indicate statistical significance. Bold italics indicate both statistical and clear biological significance.

Table 7
CA results for cobalt oxyhydroxide in V79 cells.

Expt.	S9	Treat + Recovery (hr)	Conc. of cobalt oxyhydroxide (μg/mL)	% cells with CA (excl. gaps)	% toxicity (reduction in mitotic index)	% toxicity (reduction in cell density)
1	−	4 + 16	0	1.5	–	–
			100[P]	1.5	10	10
			200[P]	3.0	45	15
			300[P]	2.0	67[a]	11
2	−	20 + 0	0	2.5	–	–
			100[P]	2.5	18	9
			200[P]	***7.0***	16	22
			400[P]	***7.0***	61[a]	18
			500[P]	***8.0***	70[a]	12
1	+	4 + 16	0	2.0	–	–
			250[P]	3.5	21	23
			500[P]	1.0	16	2
			1000[P]	1.5	58	15
2	+	4 + 16	0	3.5	–	–
			600[P]	1.5	21	25
			800[P]	3.5	13	24
			1000[P]	2.7	34	24
			1200[P]	3.7	60[a]	34
			1500[P]	***8.0***	59	42

Bold italics indicate clear CA frequencies that exceed historical control range.
[P] = precipitate observed at harvest.
[a] Cytotoxicity reaches or exceeds the current recommended upper limit of 60%.

controls which received HBSS only demonstrated acceptably low mean tail intensity (TI) of 0.86 ± 0.081%. The clastogenic positive control EMS, a direct alkylating compound, induced a sufficiently high, significant increase in TI (mean TI: 6.59 ± 1.382%, $p \leq 0.01$), indicating that the comet assay method was performed correctly and that the cells were sensitive towards directly DNA-damaging substances. In cells treated with $KBrO_3$ there was no increase in TI without hOGG1 incubation (mean TI: 0.76 ± 0.070%) and thus no direct DNA-strand break induction. In contrast, after incubation with hOGG1, a marked statistically significant increase in TI after $KBrO_3$ exposure was noted, if compared to the respective slides without enzyme treatment (mean TI: 31.72 ± 8.641%, $p \leq 0.05$). Human OGG1-induced increase in TI indicated sufficient activity of the positive control and the hOGG1 enzyme batch used and also sensitivity of the cell system towards oxidative DNA-base lesions.

Cobalt octoate, after extraction in AAF, induced a significant increase in mean TI, compared to the HBSS control, which was statistically significant at the highest concentration of the soluble fraction (mean TI at 800 μg/mL: 7.95 ± 1.959%, $p \leq 0.01$) and the two highest concentrations of the unfiltered fraction (mean TI at 200 μg/mL: 2.68 ± 0.497%, $p \leq 0.01$; mean TI at 800 μg/mL: 9.73 ± 1.966%, $p \leq 0.001$). At all concentrations tested, mean TI was further enhanced in the presence of hOGG1, indicating induction of oxidative DNA-base lesions. Induction of oxidative DNA-base lesions was significant at the highest concentration used (800 μg/mL) for both the filtered (soluble) and the unfiltered fractions, with

mean TI in the presence of hOGG1 amounting to 14.70 ± 1.955 ($p \leq 0.01$) and 16.70 ± 3.477% ($p \leq 0.05$), respectively. The inorganic, water soluble positive control **cobalt sulphate heptahydrate** induced a significant increase in TI by using both the filtered (soluble) (mean TI: 6.41 ± 3.389%, $p \leq 0.01$) and unfiltered fractions (mean TI: 5.32 ± 1.537%, $p \leq 0.01$), which was further enhanced in the presence of hOGG1 (mean TI soluble fraction: 11.54 ± 6.438%; mean TI unfiltered fraction: 11.93 ± 6.064%), indicating oxidative damage. Despite some differences in cytotoxicity (see below) the TI values were not statistically different for the soluble and the unfiltered fraction, indicating that mainly cobalt solubility and the cobalt cations are important determinants of the observed DNA damaging effect and that the observed particles seemed to be of minor relevance.

In pre-experiments with the positive control cobalt sulphate heptahydrate, extracted in Gamble's, very high variability was observed for TI values of the unfiltered fractions. For this reason, the main experiments were performed with AAF extracts only, because of better reproducibility. Nevertheless there was one initial experiment with direct comparison of AAF and Gamble's extracts from cobalt octoate and cobalt sulphate heptahydrate. This experiment indicated similar results, but with a tendency towards slightly higher TI values for the Gamble's extracts.

In parallel to the hOGG1-modified comet assay experiments with AAF extracts of cobalt sulphate heptahydrate and cobalt octoate, manual cell counting was performed to estimate cytotoxicity (see Fig. 4). The positive control **cobalt sulphate heptahydrate** (800 μg/mL) clearly reduced mean cell counts in A549 cell cultures. This was detected for both the soluble (33% reduction) and the unfiltered fraction (63% reduction, $p \leq 0.05$). Thereby, higher cytotoxicity of the unfiltered fraction might indicate an additional, particle-like effect. The soluble fractions also exhibited a cytotoxic potential with reductions in cell number by 37 (50 μg/mL), 65 (200 μg/mL), and 53% (800 μg/mL), respectively. For the unfiltered **cobalt octoate** extracts, significant reduction in mean cell counts (by 78%, $p \leq 0.05$) was only observed for the highest concentration used (800 μg/mL). At 200 μg/mL there was also marked cytotoxicity in two out of three experiments, but the first experiment demonstrated unexpectedly high cell counts. Respective cytotoxicity of the cobalt compounds was also observed for the extracts in Gamble's (data not shown). In every case, marked induction of DNA-strand breaks and oxidative DNA-base lesions seemed to coincide with significant reduction in cell number and thus cytotoxic activity of the cobalt compounds.

4.5. Bone marrow micronucleus (MN) tests in vivo

The results of the mouse bone marrow MN tests are shown in Tables 8 and 9 for **cobalt acetyl acetonate** and **cobalt resinate** respectively. At the top dose (500 mg/kg/day) of cobalt acetyl acetonate, two females out of eight were found dead 24 h following the second treatment. At the top dose (1500 mg/kg/day) of cobalt resinate, 1/5 males died just after the second treatment. In both cases the dead animals were replaced by supplementary animals.

It can be seen that MN frequencies in vehicle control groups were normal, and were significantly increased by treatment with the positive control, CPA. There was no evidence of bone marrow toxicity (no significant decrease in PCE:NCE ratio) in animals treated with cobalt acetyl acetonate. However, there was a significant decrease in PCE:NCE ratio in the top dose males and females treated with cobalt resinate. There were no significant increases in MN frequency in any of the groups treated with either cobalt compound.

Blood samples were taken from treated animals 2 h after the final dose. Since cobalt resinate induced bone marrow toxicity, and therefore demonstrated that systemic exposure to the chemical

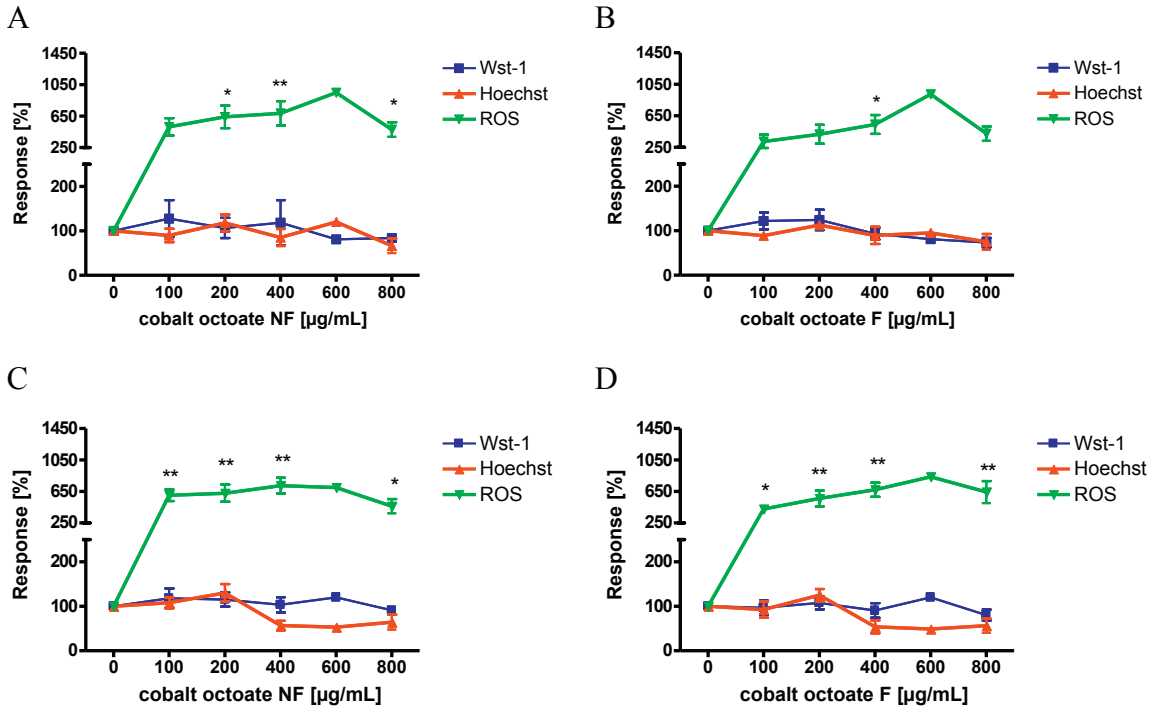

Fig. 1. Oxidation of DCFH to fluorescent DCF in A549 cells after 4 h exposure to cobalt octoate, given as fluorescence intensity relative to reference cultures without chemical exposure. Hoechst and Wst-1 fluorescence intensity corresponding to DNA content and metabolic activity, respectively, are also given relative to reference cultures without chemical exposure. The chemical was extracted 48 h in AAF (A and B) or Gamble's solution (C and D) and exposed to cells as non-filtered (NF) fraction (A and C) and filtered (F) fraction (B and D). Data are given as mean ± standard error of mean. **$p \leq 0.01$. WST-1 assay: n = 3, Hoechst and ROS assay: n = 4, each run was measured in triplicate.

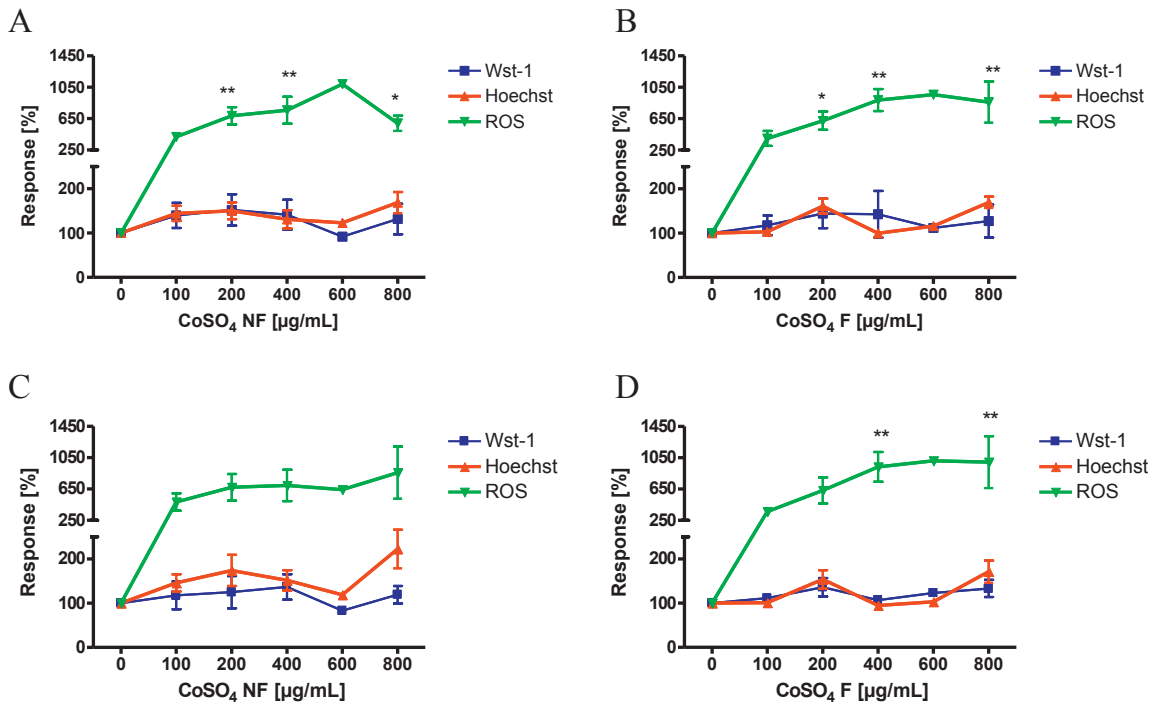

Fig. 2. Oxidation of DCFH to fluorescent DCF in A549 cells after 4 h exposure to cobalt sulphate heptahydrate, given as fluorescence intensity relative to reference cultures without chemical exposure. Hoechst and WST-1 fluorescence intensity corresponding to DNA content and metabolic activity, respectively, are also given relative to reference cultures without chemical exposure. The chemical was extracted 48 h in AAF (A and B) or Gamble's solution (C and D) and exposed to cells as non-filtered (NF) fraction (A and C) and filtered (F) fraction (B and D). Data are given as mean ± standard error of mean. $^{*}p < 0.05$, $^{**}p \leq 0.01$. Wst-1 assay: n = 3, Hoechst and ROS assay: n = 4, each run was measured in triplicate.

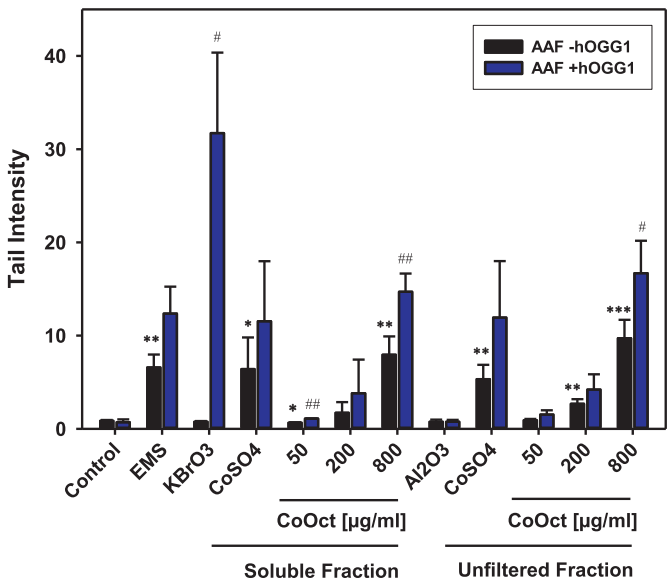

Fig. 3. Induction of DNA-strand breaks and oxidative DNA-damage by cobalt octoate and cobalt sulphate heptahydrate extracts in AAF. Human OGG1-modified comet assay. A549 cells were incubated for 4 h with the unfiltered or soluble fractions of the extracts in AAF (extraction 48 h at room temperature). Increase in TI without hOGG1-incubation indicates occurrence of DNA-damage, in particular DNA single- and double-strand breaks and alkali labile sites. Increase in TI with hOGG1-incubation, as compared to the respective hOGG1-untreated slide, indicates oxidative DNA-damage. Data represent mean values ±SD of three independent experiments. Significantly different from control cells (*) or from the respective hOGG1-untreated sample (#): $^{*}/^{\#}p \leq 0.05$, $^{**}/^{\#\#}p \leq 0.01$, $^{***}p \leq 0.001$, one-sided Student's t-test for unpaired (*)/paired (#) values. EMS = 0.75 µL/ml, 1 h; $KBrO_3$ = 1 mM, 4 h; $CoSO_4$ = 800 µg/mL, 4 h; CoOct = 50, 200, or 800 µg/mL, 4 h; Al_2O_3 = 200 µg/cm^2, 4 h.

must have occurred, the blood samples were not analysed for cobalt concentrations. Cobalt acetyl acetonate did not induce bone marrow toxicity, and therefore it would have been useful to analyse the blood samples in order to demonstrate systemic exposure. However, validation of the analytical method was prevented by interferences with the biological matrix. Therefore analysis of the plasma samples could not be undertaken. Thus, there was no direct proof that cobalt acetyl acetonate was absorbed and achieved systemic exposure. However, since:

- Notable clinical signs (half-closed eyes, hypoactivity, and piloerection) were observed in both the range-finder and main studies,
- Cobalt acetyl acetonate is more soluble than cobalt resinate, which was clearly absorbed,

it is reasonable to conclude that cobalt acetyl acetonate was absorbed and that the bone marrow was exposed.

Thus, neither cobalt acetyl acetonate nor cobalt resinate induced MN in mouse bone marrow at doses up to the maximum tolerated.

4.6. Bone marrow chromosomal aberration (CA) test in vivo

Although no mortalities occurred in the single dose phase of the study with any of the cobalt compounds, mortalities did occur at similar doses in the multi-dose phase (see Table 10).

Most chemicals show overt toxic effects (in terms of clinical signs and mortalities) within a few hours of administration, at the time when blood levels are highest. In comparison, the effects seen later on histological examination and in the multi-dose phase were consistent with delayed toxicity and indicated that dose levels for **cobalt sulphate** and **cobalt monoxide** may well have exceeded the maximum tolerable, where any genotoxic effects would need to be

interpreted with caution. However, the exaggerated toxicity seen in the multi-dose phase was likely due to cumulative toxic and cytotoxic effects in the gastrointestinal tract and possible accumulation of the chemicals in the animals. No mortalities occurred in the vehicle, positive control or **cobalt tetraoxide** groups in the multi-dose phase. Therefore, the appropriate numbers of animals were sampled at the planned times and examined as planned. Two animals sampled in the sulphate group, and one in the monoxide group, were not dosed with colchicine due to the severity of the clinical signs. Although two of these animals showed slightly altered chromosome morphology compared with other animals in the same groups, the incidence of structural and numerical aberrations in the bone marrow and NA in other tissues was comparable to other animals in the relevant groups, so these results were included in the statistical analysis although mitotic index results were necessarily excluded.

Mortalities in the multi-dose phase meant that mid-level sulphate and monoxide groups, as well as males in the high level monoxide group, could not be sampled. In addition, the severity of the clinical signs meant that other animals treated with sulphate and monoxide were necessarily sampled after receiving fewer than the 5 planned daily doses, although all animals received the minimum 2 doses necessary to comply with the guidelines. This impacted the statistical power of the experiment, partly because animals did not receive a comparable number of doses and (more importantly) because the number of groups/animals sampled was reduced below normally recommended levels. However, both sulphate and monoxide were evaluated at levels around the maximum tolerated dose, where genotoxic effects (if seen) would be expected to be most pronounced. Although, theoretically, severe cytostatic or cytotoxic effects in the bone marrow could result in a downturn in response in the chromosome aberration test, neither of these were evident in the present study.

(i) CA and NA results for the single dose phase

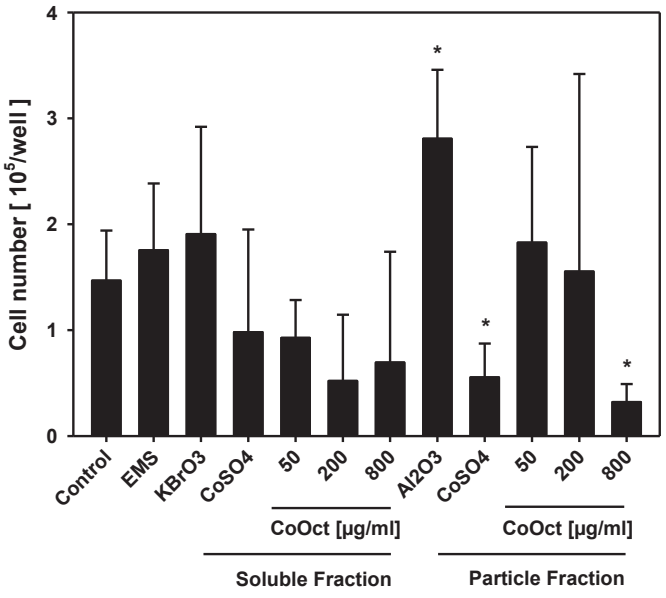

Fig. 4. Manual cell counts after incubation of A549 cells with cobalt octoate and cobalt sulphate heptahydrate extracts in AAF. Cells were incubated for 4 h with the non-filtered or soluble fractions of the extracts in AAF (extraction 48 h at room temperature). Data represent mean values ±SD of three independent experiments. Significantly different from control cells: $^*p \leq 0.05$, two-sided Student's t-test for unpaired values. EMS = 0.75 µL/mL, 1 h; $KBrO_3$ = 1 mM, 4 h; $CoSO_4$ = 800 µg/mL, 4 h; CoOct = 50, 200, or 800 µg/mL, 4 h; Al_2O_3 = 200 µg/cm^2, 4 h.

No treatment had any apparent effect on mitotic index in the bone marrow. CA frequencies in vehicle control animals were normal and were significantly increased by treatment with the positive control chemicals (data not shown). No notable increases in the % cells with CA were seen in the bone marrow with any of the cobalt compound in this phase (data not shown).

No notable increases in NA were seen in the non-glandular stomach, lungs or urinary bladder with any cobalt compound in this phase (data not shown). Animals treated with cobalt sulphate and cobalt monoxide showed dose-related increases in NA in all regions of the intestine and, in the case of cobalt monoxide, in the glandular stomach. Animals treated with tricobalt tetraoxide showed weak or marginal increases in NA in the duodenum, ileum and colon at the high dose level only (data not shown). Only cobalt monoxide showed any clear evidence of inhibiting mitosis, with the reduction in mitotic activity being perhaps most marked in the rectum (data not shown).

(ii) CA and NA results for the multi-dose phase

The results of the bone marrow CA analyses are summarised in Table 11. There was evidence of bone marrow toxicity with both cobalt sulphate and cobalt monoxide, but mitotic index (MI) increased at the low and mid-doses of cobalt tetraoxide. The reason for this is not known. However, the effects on MI indicate that the test chemicals were absorbed and exposure of the bone marrow occurred.

It can be seen from Table 11 that CA frequencies in vehicle control animals were very low (0.2%). Animals treated with the positive control CPA showed a significant increase in % cells with CA. A marginal increase in chromosome aberrations was obtained with DMH in the single but not the multiple dose phase. These borderline effects are consistent with similar borderline effects seen at somewhat higher doses in the rat and mouse bone marrow micronucleus test with this compound (e.g. Hamada et al., 2001). It should be noted that DMH is subject to first pass metabolism therefore substantial systemic genotoxic effects were not expected with this agent which was, however, expected to cause genotoxic effects in the gut. The clear positive responses with CPA in both phases of the study indicate the test system was sensitive to a known clastogen.

No increased CA frequencies were seen in animals treated with tricobalt tetraoxide. Some increased CA frequencies were seen in the top dose groups treated with cobalt sulphate and cobalt monoxide. With such low CA frequencies in vehicle control animals in this phase, the finding of 1.8% cells with CA in the high dose sulphate and monoxide groups of males could be indicative of a clastogenic response. Mean CA frequencies of 1% had been seen historically in control animals in the testing laboratory. However, frequencies of 0–2% are quite normal, and frequencies similar to this (1.5%) were seen in control rats in the single-dose phase. The marginal increases could also have been an indirect result of the severity of clinical signs at dose levels that proved to be above the MTD. Thus, these slightly increased CA frequencies are probably the result of chance variation and not biologically significant; it is considered that none of the cobalt salts induced CA in rat bone marrow at high doses (at or above the MTD) in the multi-dose phase.

The results for analysis of NA and effects on mitotic rate are summarised in Table 12. Since the NA endpoint is not widely used, there are no reference control data against which to judge the frequencies of NA cells in vehicle control animals. Induction of NA by CPA was quite weak (only a slight effect in the rectum), but DMH induced marked increases in NA in the rectum and slight increases in the liver. Increases in NA in the large intestine and liver are consistent with the known distribution and sites of carcinogenicity for this compound. Marginal increases in NA were obtained in the

Table 8
Mouse bone marrow MN data for cobalt acetyl acetonate.

Group	Doses (mg/kg/day)	MN/1000 PCE[a] (group mean ±SD)	PCE/NCE ratio (group mean ±SD)
Males			
Vehicle	–	0.7 ± 0.8	0.4 ± 0.1
Cobalt acetyl acetonate	125	0.8 ± 0.8	0.7 ± 0.2
	250	1.4 ± 0.7	0.7 ± 0.2
	500	1.6 ± 0.4	0.6 ± 0.1
CPA[b]	50	**36.9 ± 11.6**	0.7 ± 0.0
Females			
Vehicle	–	1.2 ± 0.3	1.0 ± 0.4
Cobalt acetyl acetonate	125	1.2 ± 0.8	0.8 ± 0.2
	250	1.5 ± 0.9	0.7 ± 0.3
	500	1.5 ± 0.6	0.6 ± 0.1
CPA[b]	50	**30.0 ± 8.8**	0.6 ± 0.1

Bold figures indicate statistically significant ($p < 0.05$) from concurrent control.
[a] Based on 2000 PCE/animal scored.
[b] Positive control only administered once, 24 h prior to sacrifice.

Table 9
Mouse bone marrow MN data for cobalt resinate.

Group	Doses (mg/kg/day)	MN/1000 PCE[a] (group mean ±SD)	PCE/NCE ratio (group mean ±SD)
Males			
Vehicle	–	0.6 ± 0.2	0.6 ± 0.1
Cobalt resinate	375	1.4 ± 1.1	0.7 ± 0.1
	750	1.3 ± 1.1	0.4 ± 0.1
	1500	0.9 ± 0.8	**0.3 ± 0.1**
CPA[b]	50	**35.0 ± 17.8**	0.8 ± 0.3
Females			
Vehicle	–	0.5 ± 0.4	0.9 ± 0.2
Cobalt resinate	187.5	0.9 ± 0.8	0.8 ± 0.1
	375	1.1 ± 1.1	0.7 ± 0.2
	750	0.9 ± 0.4	**0.4 ± 0.2**
CPA[b]	50	**17.7 ± 3.2**	0.7 ± 0.2

Bold figures indicate statistically significant ($p < 0.05$) from concurrent control.
[a] Based on 2000 PCE/animal scored.
[b] Positive control only administered once, 24 h prior to sacrifice.

Table 10
Mortalities in the multi-dose phase of the *in vivo* chromosomal aberration/nuclear anomaly study.

Treatment	Dose (mg/kg/day)	No. of dosing days	Mortality	
			Males	Females
Vehicle control		5	0/5	0/5
Cobalt sulphate	100	5	0/5	1/5
	300	3	1/5	2/5
		4	4/5	3/5
	1000	2	1/5	0/5
Cobalt monoxide	200	3	1/5	2/5
		4	4/5	3/5
	600	2	0/5	0/5
	2000	2	0/5	5/5
Cobalt tetraoxide	200	5	0/5	0/5
	600	5	0/5	0/5
	2000	5	0/5	0/5
CPA	10[a]	1	0/5	0/5
DMH	10[a]	1	0/5	0/5

[a] Single dose given on the day prior to euthanasia.

urinary bladder of animals treated with both positive control agents. However, the mitotic rate (and therefore the number of inducible anomalies) in this organ is low and variable. It is not therefore clear whether the apparent increases in NA are due to positive control treatments or due to normal variance. Again, since there are no reference positive control data against which to compare, it is unclear whether these positive control indicate that the test system was appropriately sensitive, or not.

Animals treated with cobalt sulphate showed clear increases in NA in all regions of the intestine and, at the high dose only, a small increase in anomalies in the liver. Slight increases in NA were also seen in lung and bladder. However, as these tissues show a very low and variable mitotic index, the apparent increases were not considered conclusive. A slight increase in NA in the testes of animals tested with high dose cobalt sulphate could be due to indirect toxic effects or normal variation, given the relatively low number of animals involved.

Animals treated with cobalt monoxide showed clear increases in NA in both regions of the gastrointestinal tract examined and in the glandular stomach. Effects were seen to a lesser extent in the liver.

Animals treated with tricobalt tetraoxide showed marked or slight increases in NA in the regions of the gut that were analysed, but for the high dose group only.

Reductions in mitotic rate (consistent with a cytotoxic effect) were generally evident in those tissues showing an increase in NA, with the exception that an increase in mitotic rate in the glandular stomach was obtained at the low dose of the sulphate. However, reductions in mitotic rate were also seen in tissues where there were no increases in NA. Reduced mitotic rates in the intestine of animals treated with the sulphate and the monoxide were, in part, explained by erosion of the mucosal layer in these tissues, as well as the direct cytotoxic effect.

4.7. Spermatogonial chromosomal aberration (CA) test in vivo

No changes in behaviour, external appearance of the animals or

the faeces were noted for control animals or those treated with cobalt chloride at any of the 3 dose levels. Although no obvious clinical signs were observed at the doses used, some small but statistically insignificant reductions in body weight were seen in animals treated at 30 mg/kg/day (by 5.0% at day 8 and by 14.1% at day 29). Reductions in absolute (25.0%) and relative liver weights (12.3%) were seen in the 30 mg/kg/day group, but these were also not statistically significant. Since it would not have been possible to use higher doses due to deaths at 100 and 300 mg/kg, the top dose used in this study (30 mg/kg/day) is considered an acceptable maximum tolerated dose.

The results of the CA analysis in spermatogonia are summarised in Table 13. It can be seen that there was no bone marrow toxicity as measured by mitotic index, and there were also no increases in the frequency of CA. In the cobalt chloride groups all group mean structural CA frequencies fell within the historical control range. Also, there were no polyploid cells found from 1000 metaphases scored in each of the groups. Although no concurrent positive control was employed, the testing laboratory has consistently observed significantly increased CA frequencies (in the range 9–13%) in animals treated with mitomycin C. Although plasma was not analysed for presence of cobalt chloride, Nation et al. (1983) demonstrated significant exposure of multiple tissues after oral dosing of cobalt chloride to rats.

5. Discussion

The published studies on the genotoxicity of soluble cobalt compounds (such as cobalt chloride, cobalt sulphate) and cobalt metal show some inconsistent results. Both positive and negative findings have been reported for mutation in bacterial and mammalian cells, and for induction of CA and MN both *in vitro* and *in vivo*. The results from the new GLP studies also show some variable responses, but with relatively clear evidence for chromosomal aberrations *in vitro*, no clear evidence for chromosomal effects *in vivo* and generally negative findings for gene mutation *in vitro*.

The published positive Ames results for cobalt chloride in strain TA97a (Pagano and Zeiger, 1992), cobalt sulphate in TA100 (Zeiger et al., 1992) and cobalt metal in TA98 (NTP, 2013) have not been reproduced in three new GLP studies evaluating the relevant strains. Also, cobalt resinate and cobalt acetyl acetonate were negative in robust 5-strain Ames tests.

Earlier publications (Hartwig et al., 1990; Miyaki et al., 1979; Kitahara et al., 1996) suggested soluble cobalt compounds could induce gene mutation in mammalian cells *in vitro*. However, the predominantly negative results in recent GLP *Hprt* mutation studies with 10 different cobalt salts/compounds have not confirmed this. Cobalt chloride has not been reinvestigated for gene mutation induction in mammalian cells, however negative *Hprt* mutation results with cobalt sulphate (which would equally expose the cells to the cobalt cation) indicate that cobalt chloride would also not be mutagenic in mammalian cells. Although cobalt resinate did not induce biologically relevant increases in $Tk^{+/-}$ mutations in L5178Y cells, cobalt acetyl acetonate induced small colony mutants, which is indicative of a clastogenic rather than a point mutational effect. Thus, there is still no convincing or consistent evidence of induction of gene (point) mutations in either bacteria or mammalian cells *in vitro*.

There are several published papers all indicating induction of MN (Van Goethem et al., 1997; De Boeck et al., 2003a; Miller et al., 2001; Olivero et al., 1995; Gibson et al., 1997), and possibly also CA (Olivero et al., 1995), by soluble cobalt salts and ultrafine metal cobalt. These effects were often seen at moderate or low levels of cytotoxicity and do not appear to be artefacts of excessive toxicity. No new GLP studies on MN induction *in vitro* have been performed. However, the consistency of effects across several publications indicates a real potential to induce genotoxic effects in mammalian cells. None of these studies investigated whether the MN were due to a chromosome breakage (clastogenic) or chromosome loss/gain (aneuploidy) mechanism, although the review by Beyersmann and Hartwig (1992) mentions that cobalt chloride induced aneuploidy in human lymphocytes. It is not clear from the summarised results in which paper these data were originally published. If cobalt salts are able to induce aneuploidy, then this may contribute to the positive MN results. However, in the new studies reported here, CAs were induced by cobalt acetyl acetonate at modest levels of cytotoxicity, by cobalt resinate in the presence of S9 (although the impact of cytotoxicity is unclear because of precipitation), and by cobalt oxyhydroxide at a single concentration in the absence of S9, in the current studies. It is therefore unclear whether the published positive MN results are due to clastogenic or aneugenic modes of action, or both. If aneuploidy is involved, such a mode of action is accepted as exhibiting a threshold (Parry et al., 1994).

There are several published reports of induction of DNA damage in mammalian cells (mainly using the comet assay) by cobalt metal and cobalt chloride (Caicedo et al., 2008; Hartwig et al., 1990, 1991; De Boeck et al., 1998; Van Goethem et al., 1997). However, De Boeck et al. (2003a) were unable to reproduce the DNA damage responses

Table 11
Bone marrow CA results for the multi-dose phase.

Treatment	Dose (mg/kg/day)	Relative MI	No. of cells examined (M+F)	% cells with CA (excluding gaps, polyploidy and endoreduplication)		
				M	F	M+F
Vehicle control	0	100	1000	0.2	0.2	0.2
Cobalt sulphate	100	97	900	0.0	0.3	0.1
	1000	**65**	900	***1.8***	0.8	**1.2**
Cobalt monoxide	600	**73**	1000	0.8	0.6	0.7
	2000	**39**	500	***1.8***	ND	**1.8**
Cobalt tetraoxide	200	**139**	1000	0.2	0.8	0.5
	600	**154**	1000	0.2	0.8	0.5
	2000	113	1000	0.6	0.0	0.3
CPA	10*	117	1000	***7.2***	***11.2***	**9.2**
DMH	10*	123	900	0.3	0.2	0.2

M = male.
F = female.
ND = no data due to mortalities.
* Single dose given on day prior to euthanasia.
Bold figures indicate statistical significance ($p < 0.05$).
Bold italics indicate values exceed laboratory historical control range.

Table 12
Results of analysis of NA and mitotic rate in various tissues during the multi-dose phase of the *in vivo* study in rats.

Tissue	Cobalt sulphate		Cobalt monoxide		Tricobalt tetraoxide		CPA		DMH	
	Increased NA cells	Decreased mitotic rate	Increased NA cells	Decreased mitotic rate	Increased NA cells	Decreased mitotic rate	Increased NA cells	Decreased mitotic rate	Increased NA cells	Decreased mitotic rate
Non-glandular stomach	0	++	0	++	0	0	0	**	0	0
Glandular stomach	+++	++*	+++	++	+/−	0	0	**	0	0
Duodenum	+++	+	+++	+++	+/−	0	0	0	0	0
Ileum	+++	+++	ND	ND	ND	ND	0	0	0	+/−
Caecum	+++	+++	ND	ND	ND	ND	0	0	0	0
Colon	+++	++	+++	+++	+	0	+/−	0	0	0
Rectum	+++	+	ND	ND	ND	ND	+	0	++	0
Liver	+	+++	++	+++	0	+	0	**	+/−	+/−
Lungs	+	ND	ND	ND	ND	ND	0	ND	0	ND
Urinary bladder	+	ND	ND	ND	ND	ND	+/−	ND	+/−	ND
Testis	+/−	0	ND	ND	ND	ND	0	0	0	0

0 = no change.
+/− = possible increase in NA or decrease in mitotic rate.
+ = slight increase in NA or decrease in mitotic rate.
++ = substantial increase in NA or decrease in mitotic rate.
+++ = very substantial increase in NA or decrease in mitotic rate.
ND = no data.
* = possible stimulation of mitosis at low dose, but decrease at high dose.
** = possible stimulation of mitosis at only dose tested.

with ultrafine cobalt metal. In the new data presented here, cobalt sulphate heptahydrate and cobalt octoate also induced DNA strand breakage, but the damage appeared to be explained by oxidative damage resulting from reactive oxygen species, probably caused by the cobalt cation. Such oxidative damage would also be consistent with the induction of CA, and several authors (e.g. De Boeck et al., 2003b; Simonsen et al., 2012; Patel et al., 2012) have concluded that oxidative damage is the main cause of the observed DNA and chromosomal breakage. However, it is unclear whether the oxidative damage is a primary effect of cobalt, or a secondary effect of cytotoxicity. In addition, there is evidence from *in vitro* studies that cobalt inhibits base and nucleotide excision repair (Hartwig and Schwerdtle, 2002; Hartwig et al., 2003), damages zinc fingers in DNA repair proteins (Kopera et al., 2004; Witkiewicz-Kucharczyk and Bal, 2006) and can also enhance DNA cleavage mediated by topoisomerase II (Baldwin et al., 2004), which could also contribute to DNA strand breakage.

The MN, CA and DNA damage-inducing potential of cobalt and cobalt compounds *in vitro* has not been confirmed *in vivo* in the new studies. No biologically relevant induction of MN or CA has been found in bone marrow of rodents treated with cobalt sulphate, cobalt monoxide, tricobalt tetraoxide, cobalt resinate or cobalt acetyl acetonate in the present studies. Negative results were also found with cobalt chloride (Gudi and Ritter, 1998), cobalt 2-ethyl hexanoate (Richold et al., 1981) and cobalt metal (NTP, 2013). Some of these studies did not include analysis of plasma for presence of the cobalt compounds under test or cobalt cations. However, Nation et al. (1983) demonstrated that cobalt chloride administered orally to rats achieved significant exposure levels in multiple tissues, and, therefore, the *in vivo* MN and CA studies reported here would have achieved target organ exposures at least with the soluble compounds. The negative result with cobalt chloride is interesting since it has been shown to stimulate erythropoietin production (Goldwasser et al., 1958) which can lead to increases in spontaneous MN levels (Yajima et al., 1993). If the DNA and chromosome breaking effects of cobalt and cobalt compounds *in vitro* is due to oxidative stress, the more effective anti-oxidant defences of normal mammalian tissues *in vivo* may explain the lack of effects in whole animals. Thus, the genotoxic activity of cobalt substances, whether it might be based on aneuploidy or clastogenicity due to reactive oxygen species, appears to have a threshold which can be exceeded at high concentrations and extreme conditions *in vitro* but seems not to be exceeded at high doses *in vivo*. A mode of action based on oxidative stress at toxic exposures may well be important in fully assessing the potential human risk of exposure to cobalt compounds, and the role of oxidative stress in carcinogenesis has been elegantly discussed by Klaunig et al. (2010).

Given the lack of *in vivo* clastogenicity in somatic cells it would be expected that cobalt compounds would not produce any genotoxic effects in germ cells. The findings of increased numerical aberrations in germ cells of Syrian hamsters by Farah (1983) are therefore unexpected. In contrast to those findings, in the recent GLP rat spermatogonial CA study, no polyploid cells were found in the control or any of the cobalt chloride treated groups from 1000 metaphases per animal scored. In addition, the absence of any structural aberrations in spermatogonia indicates no clastogenic activity in germ cells as well as in somatic cells.

Table 13
Chromosomal aberration frequencies in spermatogonia of rats treated with cobalt dichloride hexahydrate.

Dose (mg/kg/day)	MI	No. of cells examined (M+F)	Group mean % cells with CA (range)		% polyploid cells
			Including gaps	Excluding gaps[a]	
0	1.00	1000	2.8 (1.5–4.0)	1.1 (1.0–1.5)	0.0
3	1.48	1000	1.3 (0.5–3.0)	0.7 (0.5–1.5)	0.0
10	1.26	1000	2.2 (1.5–2.5)	0.7 (0.5–1.0)	0.0
30	1.11	1000	2.2 (1.5–2.5)	0.9 (0.5–1.5)	0.0

[a] Historical control range = 0.7–1.5% for group mean CA, excluding gaps.

In summary, poorly soluble cobalt salts/compounds do not appear to be genotoxic *in vitro* or *in vivo*, at least in tests where the results can be reliably evaluated. On the other hand, soluble cobalt salts/compounds and ultrafine cobalt metal have produced several positive genotoxicity results across a range of different *in vitro* systems, including chromosomal aberrations, micronuclei, small colony *Tk* mutations and DNA damage in mammalian cells, although they do not convincingly or consistently induce gene (or point) mutations in either bacteria or mammalian cells. These differences are probably due to the availability of free cobalt cations that can penetrate the cells when soluble compounds are tested. There is evidence that cobalt salts/compounds induce formation of reactive oxygen species (ROS), inhibit DNA repair, and enhance DNA cleavage by topoisomerase, and the fact that the most reproducible effects seem to be for DNA and chromosomal breakage is consistent with this. Such a mode of action would be expected to exhibit a threshold, and the absence of such chromosome damage in robust GLP studies *in vivo* suggests that the more effective protective processes that exist in whole mammals compared to single cells are sufficient to prevent DNA damage resulting from reactive oxygen, even at the high doses tested. Overall, there is no evidence of genetic toxicity with relevance for humans of cobalt substances and cobalt metal.

Conflicts of interest

D. Kirkland received funding from the CDI and CoRC to review the data and co-ordinate the preparation of the manuscript.

T. Brock was consulting toxicologist to the CDI and CoRC at the time the new studies reported here were carried out or initiated.

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Appendix A. Supplementary data

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