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A tiered approach to investigate the inhalation toxicity of cobalt substances. Tier 2 b: Reactive cobalt substances induce oxidative stress in ToxTracker and activate hypoxia target genes

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ABSTRACT

Cobalt metal and cobalt sulfate are carcinogenic in rodents following inhalation exposure. The pre-carcinogenic effects associated with exposure to these cobalt substances include oxidative stress and genotoxicity. Some, but not all, cobalt substances induce *in vitro* clastogenicity or an increase in micronuclei. As a result, these substances are classified genotoxic carcinogens, having major impacts on their risk assessment, e.g. assumption of a non-thresholded dose response. Here, we investigated the potential of nine cobalt substances to cause genotoxicity and oxidative stress using the ToxTracker assay, with an extension to measure biomarkers of hypoxia. None of the nine tested substances activated the DNA damage markers in ToxTracker, and five substances activated the oxidative stress response reporters. The same five substances also activated the expression of several hypoxia target genes. Consistent with the lower tier of testing found in the preceding paper of this series, these compounds can be grouped based on their ability to release bioavailable cobalt ion and to trigger subsequent key events.

1. Introduction

Inhalation carcinogenicity studies in rodents have shown that cobalt (Co) and Co sulfate cause increased incidences of neoplasms. This has led to a classification as a category 1B carcinogen under EU CLP, which includes overall six Co compounds. The most recent publications (Kirkland et al., 2015) and reviews (Lison et al., 2018; OECD, 2014) conclude that cobalt compounds are not mutagenic; they do, however, induce genotoxic effects *in vitro*, mainly manifest as DNA strand or chromosome breaks, which are consistent with a reactive oxygen mechanism (Christova et al., 2002; Kadiiska et al., 1989; Moorhouse et al., 1985).

The initiating event of the mode-of-action (MoA) of Co is considered to be the release of the bioavailable cobalt ion. In cells, the presence of cobalt ions leads to the generation of reactive oxygen species (ROS) and subsequent oxidative stress (Leonard, 1998). Additionally, exposure to cobalt substances can mimic hypoxia during normoxic conditions by stabilizing members of a family of transcription factors called “hypoxia inducible factors” (HIFs), in particular HIF1α. Cobalt ions are known to interfere with the interaction between the von Hippel-Lindau protein (pVHL) and HIF1α. This interaction is required for the ubiquitination and subsequent degradation of HIF1α under normal oxygen conditions (Yuan et al., 2003), and interference by cobalt results in stabilization of the HIF1α protein. Elevated ROS levels also lead to an inhibition of the hydroxylation of HIF1α, resulting in an increase in active HIF1α. Cobalt dichloride exposure also increases ROS generation via a mitochondria-independent mechanism, leading to HIF1α stabilization (Chandel et al., 1998). Oxidative stress, eventually leading to local inflammation, and HIF1α stabilization, inducing changes in cellular metabolism, inflammation and angiogenesis, together can contribute to carcinogenesis (Lison et al., 2018). Another potential pre-carcinogenic effect, that could contribute to the carcinogenicity of cobalt substances, is genotoxicity, defined as the ability of a substance to induce mutations (mutagenicity) and whether the substance has aneugenic or clastogenic properties. However, the role of genotoxicity in the MoA is not yet clear.

The ToxTracker assay can be used to investigate genotoxicity and the ability of test substances to induce oxidative stress, two important aspects of the possible MoA of cobalt substances. ToxTracker is a mammalian stem cell-based reporter assay that can identify genotoxic compounds with a high accuracy (Fig. 1A) (Hendriks et al., 2012, 2016). Mouse embryonic stem cells (mES cells) with stable integration of

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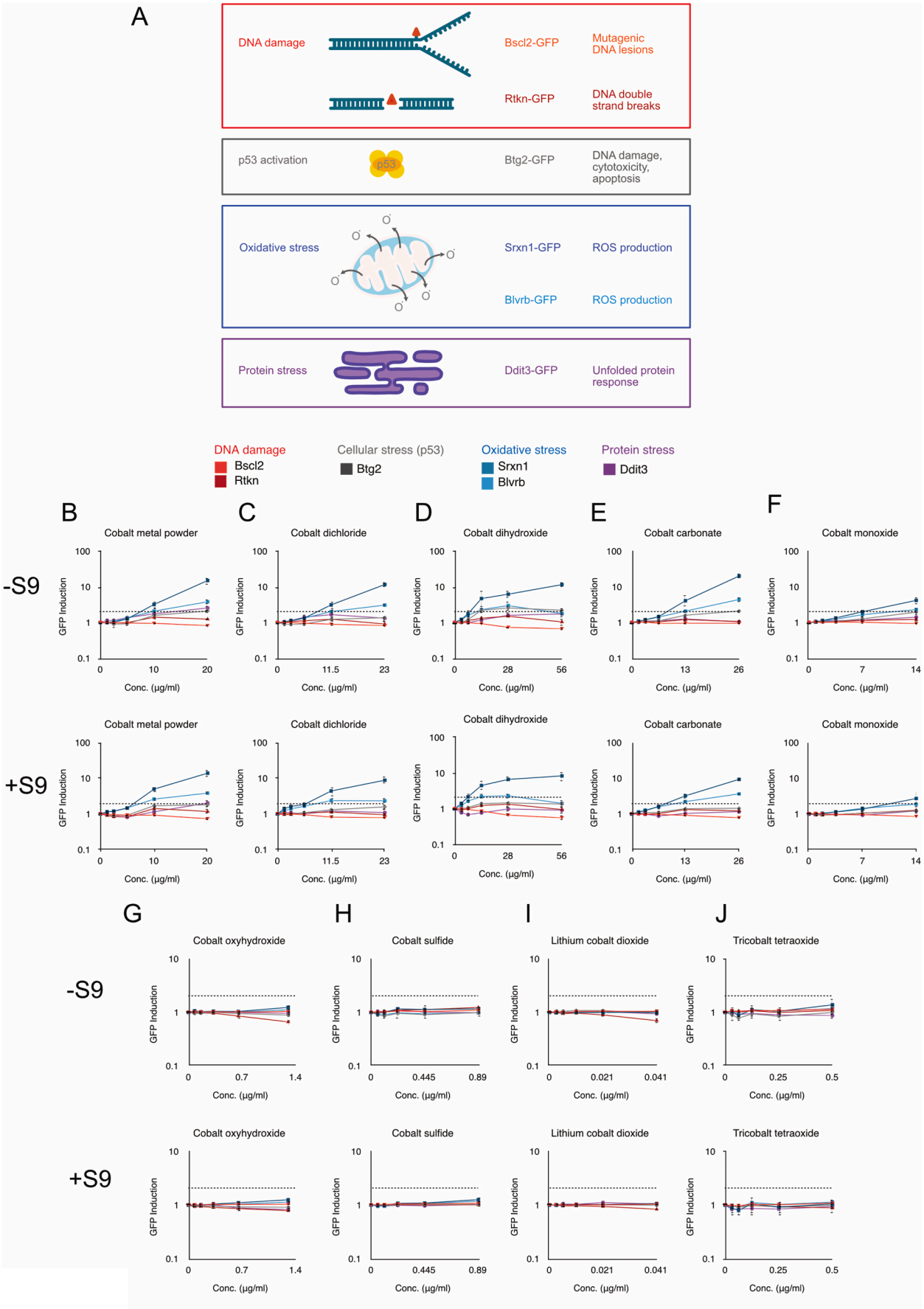

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Fig. 1. ToxTracker analysis of cobalt containing substances
(A) The ToxTracker assay is a panel of six validated GFP-based mouse embryonic stem (mES) reporter cell lines that can be used to identify the biological reactivity and potential carcinogenic properties of newly developed compounds. Activation of specific cellular signaling pathways is used to detect the biological reactivity of compounds. To allow easy assessment of the activation status of the biomarker genes using flow cytometry, green fluorescent protein (GFP) mES reporter cell lines have been generated and extensively validated (Hendriks et al., 2016). (B-J)ToxTracker GFP induction plots for cobalt substances in absence and presence of S9. Cell survival graphs are shown in Fig. S1. GFP induction levels in intact cells were determined by flow cytometry at 24 h after initiation of the exposure. Dashed line for reporter activation indicates threshold for GFP induction (2-fold). n = 3, error bars show SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GFP-tagged biomarkers are used to detect the activation of cellular stress pathways that have been linked to carcinogenicity. In ToxTracker, genotoxicity is readily detected by two independent reporters. The Bscl2-GFP reporter is activated upon formation of bulky DNA adducts and subsequent inhibition of DNA replication. Activation of the Rtkn-GFP genotoxicity reporter is associated with induction of DNA double strand breaks (Hendriks et al., 2016). The ToxTracker assay also contains two reporters for oxidative stress: induction of the Srxn1-GFP reporter is associated with activation of the Nrf2 antioxidant response (Soriano et al., 2009) and activation of the Blvrb-GFP reporter is associated with the Hmox1 antioxidant response (Smith et al., 2008). Ddit3 (Chop) is a transcription factor that is associated with the endoplasmic reticulum (ER) stress response (Tabas and Ron, 2011). The Btg2-GFP reporter in ToxTracker is activated by p53 in response to DNA damage (Rouault et al., 1996) as well as other types of cellular stress.

Another important aspect of the MoA of cobalt is the stabilization of HIF1α, which leads to the transcriptional activation of genes involved in cancer development and progression. To compare the effect of cobalt substance exposure on the hypoxia response to the effect on oxidative stress, the analysis of HIF1α target genes was added to the ToxTracker assay. Since only the protein levels of HIF1α change upon hypoxia and not transcription of the HIF1α gene, we opted to look at the activation of HIF1α target genes by qPCR to assess the hypoxic response. The following four mouse HIF1α target genes were selected: Hmox1 (encoding heme oxygenase 1) (Czibik et al., 2011), Slc2a1 (solute carrier family 2, facilitated glucose transporter member 1 (Glut1)) (McClendon et al., 2017), Bnip3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) (Vengellur and LaPres, 2004) and Ddit4 (DNA damage-inducible transcript 4 protein, Redd1) (DeYoung et al., 2008).

Here we used the ToxTracker assay to assess genotoxicity and oxidative stress upon exposure to nine cobalt containing substances in mammalian cells. Additionally, we investigated the expression of HIF1α target genes as a measure for the activation of the hypoxia response. The aim of this study was to evaluate whether the results of the extended ToxTracker assay can be used to place Co compounds into a group which either activates the endpoints measured by the assay, or into a group which does not elicit such a response. It was of further interest to see whether the results obtained by the extended ToxTracker assay would correlate with findings obtained in lower tier studies of bioaccessibility of the same Co compounds and with another *in vitro* tier of tests in human alveolar and bronchiolar cells as described in the previous two papers of this issue (Tier 1, Verougstraete et al., 2022) and (Tier 2a, van den Brule et al., 2022).

2. Materials and methods

2.1. Test substances

The following substances were tested: Aflatoxin B1 (CAS: 1162-65-8), Cisplatin (CAS: 15663-27-1), Cobalt metal powder (CAS: 7440-48-4), Cobalt carbonate (CAS: 513-79-1), Cobalt dichloride (CAS: 7791-13-1), Cobalt dihydroxide (CAS: 21041-93), Cobalt monoxide (CAS: 1307-96-6), Cobalt oxyhydroxide (CAS: 12016-80-7), Cobalt Sulfide (CAS: 1317-42-6), Diethyl maleate (CAS: 141-05-9), Lithium cobalt dioxide (CAS: 12190-79-3), Rosuvastatin (CAS: 147098-20-02), Sodium (meta)arsenite (CAS: 7784-46-5), Tricobalt tetraoxide (CAS: 1308-06-1) and Tunicamycin (CAS: 11089-65-9).

2.2. Sample preparation

Based on lower tier testing of the solubility of the cobalt test items in biologically relevant lung fluids (Tier 1, Verougstraete et al., 2022), the different cobalt containing substances were expected to dissolve or disperse in the medium to different extents.

For the 9 cobalt containing substances, a stock solution of 100 μg/ml was prepared in cell culture medium and this was mixed at 37 °C for 24h. After 24h, the medium was filtered with a 0.22 μm filter to avoid non-specific effects caused by the presence of particulate matter. A single stock solution, containing the maximum soluble cobalt concentration in mES cell culture medium was prepared and used for all experiments. The cobalt concentration was quantified by ICP-MS (analysis performed by external laboratory WLN, Glimmen, NL). As expected, and presented in Table S1, the cobalt concentration varied from less than 0.5 μg/ml for lithium cobalt dioxide and tricobalt tetraoxide to 80 μg/ml for cobalt metal powder.

It is important to emphasize that the sample preparation represents a fundamental difference between the two *in vitro* testing tiers of this publication series. In the method by van den Brule et al. (2022) (Tier 2 a), poorly soluble Co compounds were suspended and cells were exposed to a solution or suspension with a known nominal Co concentration and without measurement of the concentration of soluble Co ion content. In the ToxTracker system presented here, cells were exposed to known concentrations of soluble Co ion in the absence of particulate matter.

2.3. Cytotoxicity testing/dose range finding

For chemical testing, first a dose range finding was performed using parental mouse embryonic stem (mES) cells (strain B4418). Parental mES cells were exposed to 20 different concentrations (2-fold dilutions, concentration was halved at every step) of the filtered fraction of the test substances. Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and was expressed as fraction of viable cells after 24 h exposure compared to vehicle control exposed cells. If no cytotoxicity was observed in the dose finding, the maximum concentration was selected. If cytotoxicity was observed, the highest cobalt concentration in medium that led to a cell survival of more than 0.25 was selected (See results).

2.4. ToxTracker assay

The six independent mES reporter cell lines were seeded in gelatin-coated 96-well cell culture plates in 200 μl mES cell medium (50,000 cells per well). 24 h after seeding, medium was aspirated and fresh mES cell medium and the diluted chemicals were added to the cells. For each tested compound, five concentrations were tested in 2-fold dilutions based on the results of the dose range finding study. Induction of the green fluorescent protein (GFP) reporters was determined after 24 h exposure using a flow cytometer, using a Guava EasyCyte HT Flow Cytometer (Millipore) or MACSQuant X Flow Cytometer (Miltenyi) equipped with a 488 nm laser. Only GFP expression in intact single cells was determined. Mean GFP fluorescence was measured and used to calculate GFP reporter induction compared to a vehicle control treatment. Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and was expressed as fraction of intact cells after 24 h exposure compared to vehicle exposed controls. For cytotoxicity

assessment in the ToxTracker assay, the relative cell survival for the six different reporter cell lines was averaged. Metabolic activation was included in the ToxTracker assay by addition of S9 liver extract from aroclor1254-induced rats (Moltox). Cells were exposed to five concentrations of the test compounds in the presence of 0.25% S9 and required co-factors (RegenSysA + B, Moltox) for 24 h. Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), sodium arsenite (oxidative stress), tunicamycin (unfolded protein response), rosuvastatin (cytotoxicity) and aflatoxin B1 (metabolic activation of progenotoxins by S9) were included in all experiments.

2.5. Hypoxia assessment

To assess the induction of cellular hypoxia following exposure to the

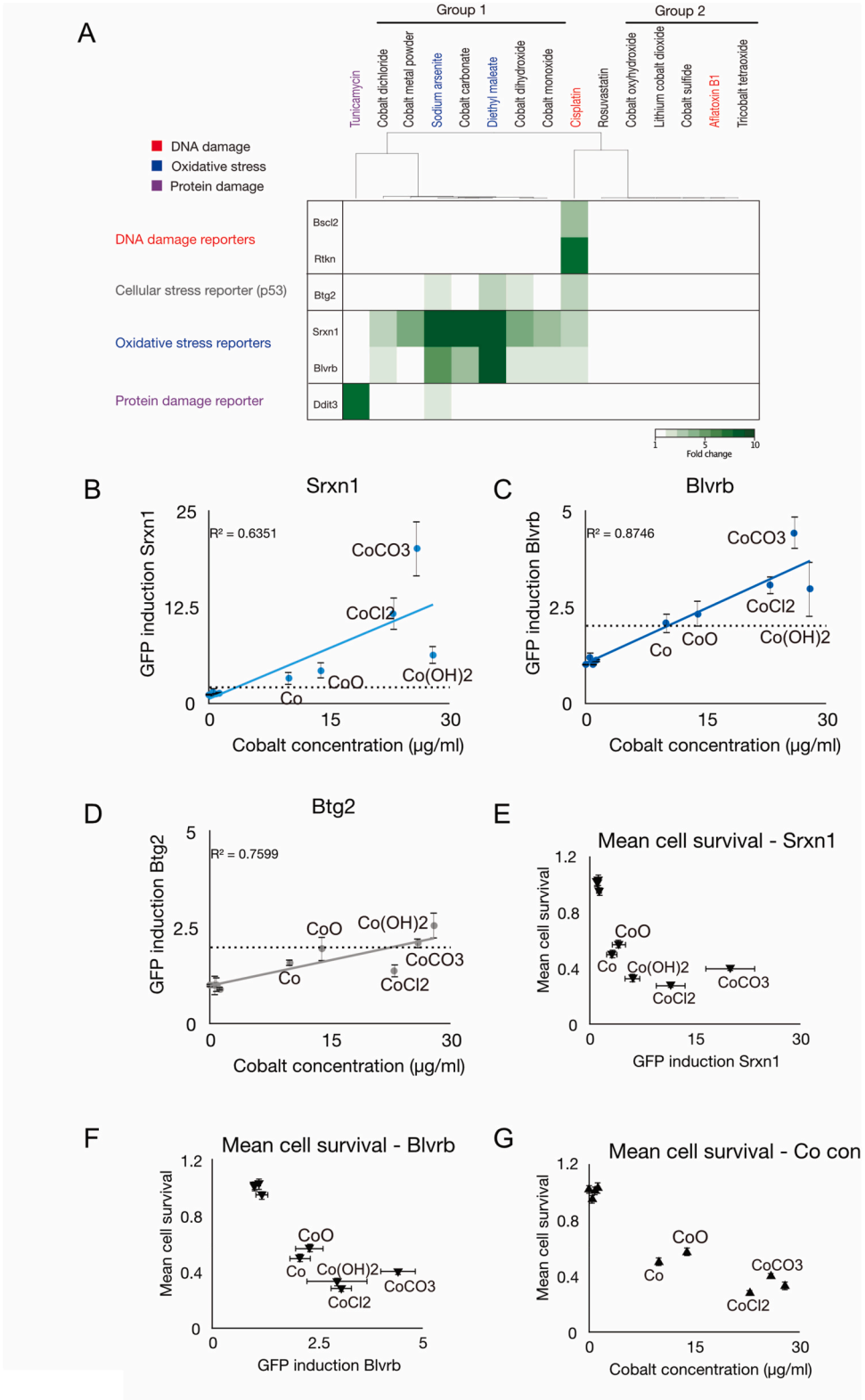

Fig. 2. Relationship between ToxTracker reporter expression and cobalt concentration
(A) Heatmap showing the differential ToxTracker reporter activation for cobalt containing and control substances in ToxTracker in absence of S9. Clustering based on hierarchical clustering at 0.5 cell survival. (B-D) Graphs showing the relationship between cobalt concentration in filtered medium and the reporter induction at the highest tested concentration, with each dot representing the average ± SEM (n = 3) for a different cobalt containing substance. (B) Relationship Co concentration in medium and induction of the Srxn1-GFP reporter for oxidative stress. (C) Relationship Co concentration in medium and induction of the Blvrb-GFP reporter for oxidative stress. (D) Relationship Co concentration in medium and induction of the Btg2-GFP reporter for p53 activation. Co: Cobalt, Co(OH)2: cobalt dihydroxide, CoCO3: cobalt carbonate, CoCl2: cobalt dichloride, CoO: cobalt monoxide. R^2 shown for linear relationship. Dashed line shows threshold for a positive ToxTracker response. (E-G) Graphs showing the relationship between mean cell survival and Srxn1-GFP reporter induction (E), mean cell survival and Blvrb-GFP reporter induction (F) and mean cell survival and cobalt concentration in filtered medium (G). n = 3, error bars show SEM.

cobalt substances, induction of a selection of HIF1α target genes was determined using quantitative real-time PCR (qPCR). Parental mES were treated with two doses of the test materials for 8 hours in triplicate. After treatment, cells were lysed in Trizol, RNA was isolated and after cDNA synthesis, a qPCR was performed using primers for Hmox1, Slc2A1, Ddit4 and Bnip3, using two technical replicates. HPRT and GAPDH were included as reference genes.

2.6. qPCR primer sequences

Gene Target	Forward primer	Reverse primer
Bnip3	GACACCACAAGATACCAACAGAGC	GTCGACTTGACCAATCCCATATCC
Ddit4	AGAGAAGAGGGCCTTGACCG	GGGACACCCCATCCAGGTAT
Hmox1	CCCCAGGATTTGTCTGAGGC	TATGGTACAAGGAAGCCATCACC
Slc2A1	AGGAGCAGTGCTCGGATCAC	CTCCGTAGCGGTGGTTCCAT
Gapdh	GTGTTCCTACCCCCAATGTGT	ATTGTCATACCAGGAAATGAGCTT
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG

2.7. Statistical analysis

The ToxTracker assay was considered to have a positive response when a compound induced at least a 2-fold increase in GFP expression in any of the reporters. This 2-fold threshold is based on extensive validation of the ToxTracker assay using various libraries of reference compounds. Statistically, this threshold corresponds to an increase of at least three standard deviations of background fluorescence in DMSO-exposed cells (99.7% confidence). Only GFP inductions at compound concentrations that showed $\leq$75% cytotoxicity (expressed as survival ratio $\geq$0.25) were used for the ToxTracker analysis. Data from measurements >0.75 cytotoxicity cannot be interpreted in a meaningful way and were therefore discarded. To create the heatmaps, ToxPlot software was used (Hendriks et al., 2016). ToxPlot software used agglomerative hierarchical clustering to visualize the ToxTracker data. To compare the induction of the six GFP reporters, ToxPlot calculated for each compound the level of GFP induction for every individual reporter at a specified level of cytotoxicity. GFP induction levels were calculated by linear regression between two data points around the specified cytotoxicity level. In case the specified level of cytotoxicity cannot be reached at the highest tested compound concentration, ToxPlot displays the GFP induction level at this top concentration.

For the analysis of the qPCR data, an induction more than 2-fold compared to the vehicle treated control sample was considered to be positive. A 2-fold increase corresponds to approximately one standard deviation above the untreated control samples. A *t*-test was applied to assess whether the expression of the gene in the treated sample was significantly different ($p < 0.05$) from the vehicle treated control.

3. Results

3.1. Cytotoxicity of cobalt substances

To select suitable test concentrations for the ToxTracker assay and the analysis of hypoxia response genes, we first assessed the cytotoxicity of the filtered Co containing medium on wild-type mES cells. Cell survival was expressed as fraction of intact cells after 24 h exposure compared to vehicle exposed controls. In the dose finding experiment, a cell survival of less than 0.5 was observed for cobalt monoxide, cobalt dichloride, cobalt carbonate, cobalt dihydroxide and cobalt metal powder at the highest test concentration (Table S1). The other substances had little effect on cell survival. In presence of S9, similar levels of cytotoxicity were observed and a cell survival of less than 0.75 was also observed for lithium cobalt dioxide (Table S1). Generally, the observed cytotoxicity increased with increasing Co concentration in medium, irrespective of the form of the Co compound. For all test substances, except for cobalt metal powder, the undiluted Co containing filtered medium was used as the highest test concentration for the ToxTracker and qPCR analysis. A maximum test concentration of 20 μg/ml was selected for cobalt metal powder, because a survival of 0.125 was observed -S9 and 0.105 +S9 in the dose finding at that concentration.

3.2. ToxTracker analysis

Next, the six ToxTracker reporter cell lines were exposed to the filtered medium for 24 h in absence and presence of S9, to assess activation of the reporters for DNA damage, oxidative damage, p53 activation and protein damage. After 24h of exposure, the cell numbers and GFP signal were quantified using flow cytometry. Cell survival was expressed as above (Table S1). For cytotoxicity assessment, the relative cell survival for the six different reporter cell lines was averaged (mean cell survival).

A mean cell survival below 0.25 was observed at the highest of the five tested concentrations for cobalt metal powder in absence and presence of S9, cobalt dihydroxide in absence of S9 and cobalt dichloride in presence of S9 (Fig. S1). Exposure to cobalt oxyhydroxide, tricobalt tetraoxide, lithium cobalt dioxide and cobalt sulfide had little effect on cell survival. For these substances the lowest cobalt concentrations were measured in the filtered medium.

In ToxTracker, substances are classified as genotoxic when either the Bscl2-GFP or the Rtkn-GFP reporter is activated more than 2-fold compared to the vehicle control. None of the cobalt substances induced more than 2-fold activation of the ToxTracker DNA damage reporters in absence or presence of S9 (Fig. 1B–J) at any of the 5 concentrations tested. In contrast, exposure to the positive control substance cisplatin activated the Bscl2-GFP reporter 4.5-fold and the Rtkn-GFP reporter 12.3-fold in absence of S9 (Figs. 2A and S2). Aflatoxin B1 activated the DNA damage reporters only in presence of S9 (Fig. S2). The non-genotoxic substances diethyl maleate (DEM) and tunicamycin did not activate the ToxTracker DNA damage reporters (Figs. 2A and S2). Exposure to the positive control substances cisplatin, DEM, tunicamycin and aflatoxin B1 showed GFP induction levels compliant with historical data and demonstrated the functionality of the mES reporter cell lines.

The Btg2-GFP reporter is associated with p53 activation (Hendriks et al., 2016). p53 activation occurs upon DNA damage and cell cycle arrest, but also activation of other cellular stress pathways can lead to p53 activation (Kastenhuber and Lowe, 2017). More than 2-fold activation of the Btg2-GFP reporter was observed for cobalt dihydroxide (2.2-fold), and cobalt carbonate (2.1-fold) in absence of S9, but not in presence of S9 (Figs. 1, 2A). However, these substances were not classified as genotoxic in ToxTracker, as no activation of the DNA damage reporters was observed.

Oxidative stress is detected in the ToxTracker assay using the Srxn1-GFP and Blvrb-GFP reporters. Exposure to cobalt monoxide, cobalt metal powder, cobalt carbonate, cobalt dihydroxide and cobalt dichloride activated both reporters (Figs. 1, 2A). For all five substances, the activation of the Srxn1-GFP reporter was stronger than the Blvrb-GFP reporter. These five substances all induced cytotoxicity in

Table 1
NOEL/LOEL/NOGEL/LOGEL concentrations (μg/ml as cobalt).

Compound	NOEL	LOEL	NOGEL	LOGEL
Tricobalt tetraoxide	<0.50	n.a.	<0.50	n.a.
Lithium cobalt dioxide	0.041	n.a.	0.041	n.a.
Cobalt sulfide	0.89	n.a.	0.89	n.a.
Cobalt oxyhydroxide	1.3	n.a.	1.3	n.a.
Cobalt monoxide	3.5	7.0	14.0	n.a.
Cobalt dichloride	5.8	11.5	23.0	n.a.
Cobalt carbonate	6.5	13.0	26.0	n.a.
Cobalt dihydroxide	3.5	14.0	14.0	n.a.
Cobalt metal powder	5.0	10.0	20.0	n.a.

n.a. = not applicable.

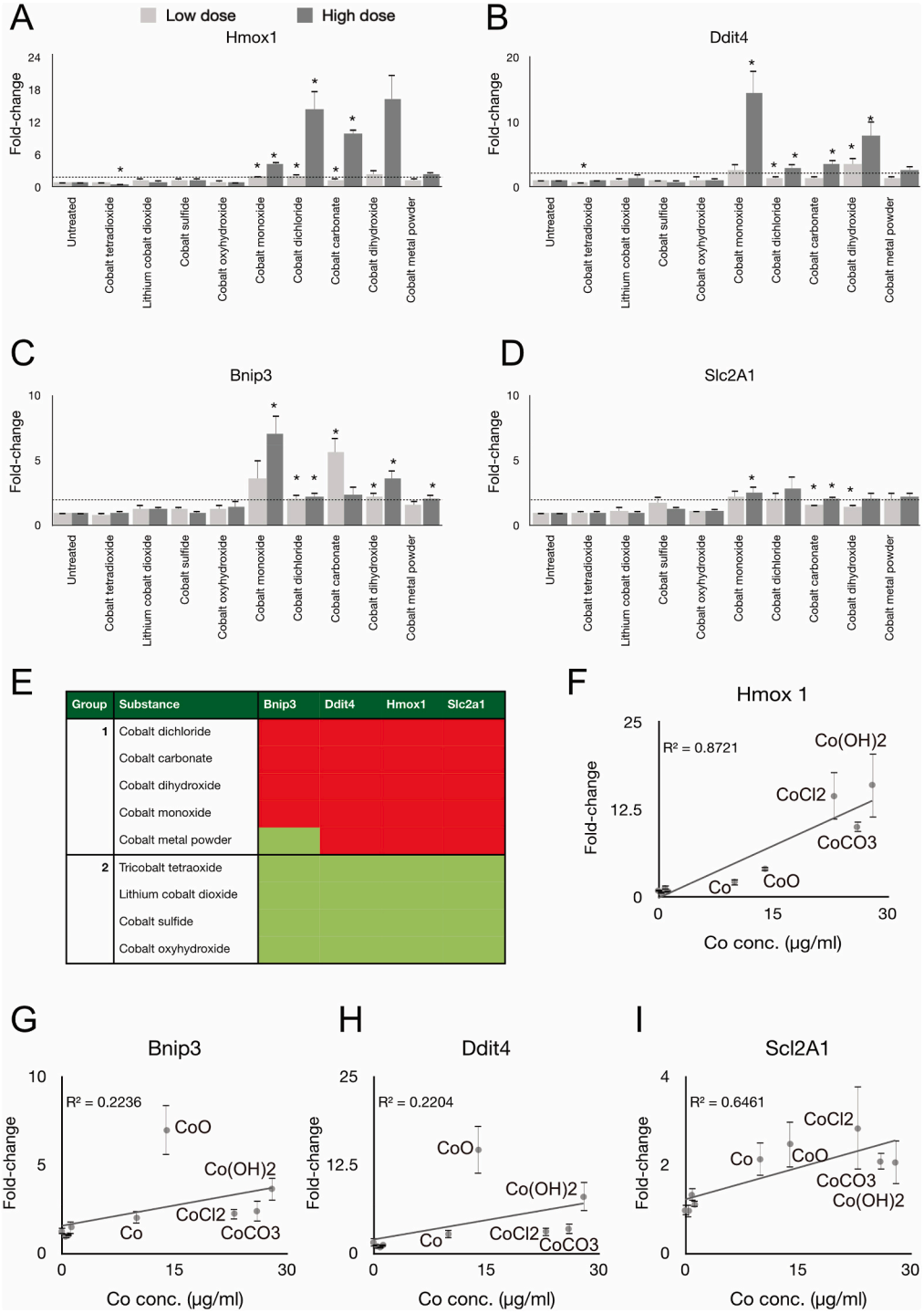

Fig. 3. HIF1α target gene expression upon exposure to cobalt substances
(A-D) Response of HIF1α target genes upon exposure to cobalt containing substances as quantified by qPCR. mES cells were exposed to two concentrations (1:4 dilution) of the cobalt containing substances for 8 h, after which RNA samples were collected. Gene expression is shown as fold-change compared to vehicle control (medium only: untreated). Bar graphs display average ± SEM, n = 3. Dashed line represents 2-fold increase compared to vehicle control. *: $p < 0.05$ (*t*-test). E) Table showing the summarised qPCR data for all 9 cobalt containing substances. Red indicates more than 2-fold increase in expression compared to vehicle control at high dose, green indicates no increase. F-I) Graphs showing the relationship between cobalt concentration in filtered medium and the average (F) Hmox1, (G) Bnip3, (H) Ddit4 and (I) Scl2A1 fold-change for the highest tested concentration, with each dot representing the average ± SEM (n = 3) for a different cobalt containing substance. Co: Cobalt, Co(OH)2: cobalt dihydroxide, CoCO3: cobalt carbonate, CoCl2: cobalt dichloride, CoO: cobalt monoxide. R^2 shown for linear relationship. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ToxTracker (Fig. S1). However, cytotoxicity or cell death is not generally associated with activation of the oxidative stress reporters. For example, exposure to the statin rosuvastatin resulted in clear cytotoxicity, but no activation of the ToxTracker reporters was observed (Fig. S2). Tricobalt tetraoxide, cobalt sulfide, lithium cobalt dioxide and cobalt oxyhydroxide induced no cytotoxicity and no activation of any of the ToxTracker reporters (Figs. 1 and S1).

Based on the ToxTracker data, the no observed effect level (NOEL), lowest observed effect level (LOEL), no observed genotoxic effect level (NOGEL), lowest observed genotoxic effect level (LOGEL) were calculated for the tested cobalt containing substances (Table 1). The NOEL concentration is the concentration at which the activation of all reporters is below <1.5-fold. The LOEL is the concentration at which at least one reporter is induced more than 2-fold. For the NOGEL and LOGEL, only the Bscl2-GFP and Rtkn-GFP genotoxicity reporters were taken into account. Since no genotoxicity was observed, the LOGEL is not applicable, and the highest concentration tested is the NOGEL. Tricobalt tetraoxide, lithium cobalt dioxide, cobalt sulfide and cobalt oxyhydroxide did not activate any ToxTracker reporters and therefore the NOEL and NOGEL are equal to the maximum tested concentration. The LOEL concentration for the other five cobalt substances were determined based on the activation of the Srxn1-GFP oxidative stress reporter.

When comparing the reporter activation profiles using hierarchical

clustering, the reactive cobalt substances (i.e. the substances that activate ToxTracker reporters and cause cytotoxicity) all cluster together with the known oxidants sodium arsenite (Kirkland et al., 2016) and DEM (Bauman et al., 1992) (Fig. 2A). For the substances that activate the oxidative stress reporters, the LOEL is at a cobalt concentration of approximately 7–14 μg/ml in filtered medium (Table 1), even though the maximum cobalt concentration in filtered medium varied from 12 to 80 μg/ml.

In the heatmap in Fig. 2A, two groups of cobalt containing substances can be distinguished. In the first (reactive) group, activation of the ToxTracker oxidative stress reporters is observed, while for the second (non-reactive) group, a lack of activation of more than 2-fold of any of the ToxTracker reporters was observed. When comparing the cobalt concentrations in the filtered medium between these two groups, all substances in the group with ToxTracker reporter activation have a higher cobalt concentration than the substances in the negative group. The induction level of the oxidative stress reports was also generally stronger for substances with a higher cobalt concentration. For example, the activation of the Srxn1-GFP reporter was 11.6-fold for cobalt dichloride at 23 μg/ml and 1.38-fold for tricobalt tetraoxide at 0.5 μg/ml (Fig. 1). Srxn1-GFP and the Blvrb-GFP expression largely increased with increasing cobalt concentration in the filtered medium (Fig. 2B and C). This was also the case for the Btg2-GFP reporter (R^2 Srxn1: 0.6351, R^2 Blvrb: 0.8746, R^2 Btg2: 0.7599). One substance, cobalt dihydroxide, had a lower Srxn1-GFP expression than expected based on this trend, while for cobalt carbonate the Srxn1-GFP expression was higher than expected.

For the three other ToxTracker reporters, Bscl2-GFP, Rtkn-GFP, and Ddit3-GFP, expression did not increase with increasing cobalt concentration (Fig. S3). As was observed in the dose finding experiments (Table S1), also in the ToxTracker assay a higher cobalt concentration corresponded to increased cytotoxicity (Fig. 2G). Substances for which the cobalt concentration in filtered medium was below 2 μg/ml were less cytotoxic than substances with a higher cobalt concentration (Fig. 2G). The expression of the Srxn1-GFP reporter and the Blvrb-GFP reporter increased with decreasing survival (Fig. 2E and F).

3.3. Hypoxia qPCR analysis

Cobalt chloride is known to induce a hypoxic cellular response in normoxic conditions (Yuan et al., 2003). To see whether expression of HIF1α target genes was also affected in mES cells after exposure to cobalt dichloride or other cobalt containing substances, their activation was assessed by qPCR as a measure for the hypoxia response. Parental mES cells were exposed to two concentrations (4-fold dilution) of the filtered medium of the test substances for 8 h in absence of S9.

Exposure to cobalt dichloride increased the expression of Hmox1, Slc2A1, Bnip3 and Ddit4 more than 2-fold compared to vehicle control (Fig. 3A–E). Cobalt carbonate, cobalt monoxide and cobalt dihydroxide exposure also increased the expression of all four HIF1α target genes. Exposure to cobalt metal powder only affected the expression of Hmox1, Slc2A1 and Ddit4. Two groups of reactive and non-reactive substances can also be separated when comparing the expression of HIF1α target genes (Fig. 3E). The first group contains the reactive cobalt substances with a cobalt concentration of more than 14 μg/ml. Substances in the second group, had low cobalt concentrations in the cell culture medium and no change in expression of the HIF1α target genes was observed.

Activation of the HIF1α target genes generally increased with increasing cobalt concentration (Fig. 3F–I). A notable exception is cobalt monoxide, where an intermediate cobalt concentration in medium led to a strong increase in the expression of Ddit4 and Bnip3. Hmox1 expression clearly increases with increasing cobalt concentrations and exposure to the substance with the highest cobalt concentration in medium, cobalt dihydroxide, also resulted in the strongest increase in the expression of the HIF1α target genes. For Slc2a1, the change in expression was very similar for cobalt dihydroxide, cobalt monoxide, cobalt carbonate, cobalt chloride and cobalt metal powder.

4. Discussion

Within the series of tests presented in a preceding study (Tier 2a, van den Brule et al., 2022) Co compounds were tested at “Co equivalent” concentrations. In van den Brule’s approach, the different compounds, with a Co content from 25% to 99.9%, were added to cell medium at such concentrations that they provided an equal concentration of Co. The compounds were dissolved or suspended and added to the cells without further steps to remove undissolved matter.

Since it is known that cellular uptake of metal nanoparticles can generate responses in the ToxTracker system independent of the release of soluble metal ions (Karlsson et al., 2014), Co substances in our study were mixed and incubated in cell culture medium that was filtered before exposing the mES cells. As expected, the cobalt concentration varied between the different substances. The lowest cobalt solubility in medium observed for tricobalt tetraoxide and lithium cobalt dioxide. These substances also had a low solubility in lung fluids. Substances with a high Co concentration in ToxTracker medium generally also had a higher solubility in artificial lung fluids (Tier 1, Verougstraete et al., 2022).

The initiating event in the MoA of Co compounds is thought to be the release of bioavailable Co ion. In line with this hypothesis, the data generated with the ToxTracker system show that the magnitude of response correlates with the concentration of soluble Co ion, independent of the original substance from which the Co ion was released. This is a strong indication that release of bioavailable Co ion is indeed the first initiating event, as has been stated by many authors before. What is currently less clear is the role of the different physiologically relevant fluids and the solubility of Co compounds therein. Given the role of the toxicokinetics and toxicodynamics of each compound, this question can likely only be resolved in higher tier testing.

In addition to corroborating the role of Co ion release as initiating event, the present series of results also helped in identifying relevant key events of Co. In the past, mutagenicity has been reported for some cobalt substances, such as cobalt chloride (Wong, 1988), but in recent GLP studies these effects were not consistently observed (Kirkland et al., 2015) with cobalt chloride or any other of the tested Co compounds, which ranged from highly to poorly water soluble. In the ToxTracker assay, compounds that are known to be mutagenic, activate the Bscl2-GFP reporter (Hendriks et al., 2012). However, exposure to nine cobalt substances did not result in any activation of the Bscl2-GFP reporter, which is consistent with the latest GLP studies, in which all compounds in scope of this paper were tested. Activation of the Rtkn-GFP reporter is often observed for substances that also cause micronuclei and chromosomal aberrations. In the GLP studies by Kirkland et al. (2015), exposure of V79 cells to cobalt oxyhydroxide resulted in an increase in chromosomal aberrations (CA) only following 20 hours of continuous exposure at 200 mg test item/ml in the absence of S9. In these experiments, no other exposure condition or time, or test item concentration resulted in an increase in CA. In ToxTracker, no activation of the Rtkn-GFP reporter was observed either.

Generally, exposure in absence or presence of an exogenous metabolization system (S9) did not influence the effect of cobalt exposure on the ToxTracker mES cells, as very similar reporter activation profiles and cell survival were observed with and without S9 (Fig. 1B–J). An exception was the activation of the Btg2-GFP reporter for cobalt carbonate and cobalt dihydroxide, which was only observed in absence of S9. There is thus no indication that the presence of S9 would act as a ROS scavenger.

The ToxTracker assay contains a reporter for p53 activation: Btg2-GFP. Activation of this reporter is observed upon DNA damage and other types of cellular events such as apoptosis or oxidative stress (Hendriks et al., 2016). Several cobalt containing substances activated the oxidative stress reporters. However, the Btg2-GFP reporter was

Table 2
Summary ToxTracker and hypoxia qPCR data.

		ToxTracker					qPCR
Group	Compound	DNA damage	Oxidative stress	p53	Protein stress	cytotoxicity	Hypoxia
1	Cobalt dichloride	–	+	–	–	+	+
	Cobalt carbonate	–	+	+	–	+	+
	Cobalt dihydroxide	–	+	+	–	+	+
	Cobalt metal powder	–	+	–	–	+	+
	Cobalt monoxide	–	+	–	–	+	+
2	Tricobalt tetraoxide	–	–	–	–	–	–
	Lithium cobalt dioxide	–	–	–	–	–	–
	Cobalt sulfide	–	–	–	–	–	–
	Cobalt oxyhydroxide	–	–	–	–	–	–

activated only upon exposure to cobalt dihydroxide and cobalt carbonate, with no direct correlation to cytotoxicity. Furthermore, activation of the Btg2-GFP reporter was not an indication of genotoxicity, as no activation of the DNA damage reporters was observed upon exposure to any of the cobalt containing substances and all substances were all classified as non-genotoxic in the ToxTracker assay. This strongly indicates that mutagenicity or genotoxicity are not early or key events towards the carcinogenic outcome in rodents as observed in the NTP studies with Co metal and Co sulfate. Genotoxicity can therefore not be used as a marker with scientific confidence in any MoA-based testing paradigm to predict inhalation carcinogenicity of Co compounds.

In general, the cobalt concentration measured in the filtered medium correlated with cytotoxicity and induction of the oxidative stress reporters. Two groups could be separated based on their ToxTracker response and cobalt concentration (Table 1, Table 2). Substances that belonged to the reactive group (group 1 in Table 2) also activated the expression of HIF1α target genes in mES cells. Generally, the results obtained by qPCR of those target genes in mES cells were similar to those obtained by measuring HIF1α stability using ELISA in alveolar epithelial cells (A549) (Tier 2a, van den Brule et al., 2022).

The effects detected in ToxTracker and on the expression of HIF1α target genes, are thought to be triggered by "free" and bioavailable Co ion. The bioavailability of Co ion from each of these substances has been measured and reported by Verougstraete et al. (2022), obtaining an estimation of the magnitude of release of Co ion in three biologically relevant fluids. Using these measurements, a preliminary grouping has been outlined for the lung toxicity of cobalt. This preliminary grouping (Tier 1, Verougstraete et al., 2022) has been corroborated so far by the *in vitro* tests in human alveolar and bronchiolar, as well as in mouse embryonic stem cells.

The *in vitro* systems have many advantages, such as the possibility to test many substances in direct comparison, to conduct dose response studies and generate mechanistic data. These types of studies are ideal to explore and possibly predict the MoAs of different substances by comparing the early steps in a pathway, and by grouping substances based on which cellular early events are triggered. *In vitro* experiments cannot, however, give information on other aspects that are relevant to inhalation toxicity, such as the influence of deposition and potential retention of these substances in the lung, or the influence of neighboring cells on the response to a toxicant by an individual cell type in culture. This information can only be obtained from advanced *in vitro* ("organ on a chip" or Precision Cut Lung Slices (PCLS)) or abbreviated *in vivo* testing, meaning that either fewer substances are tested, or that the *in vivo* testing is only of an abbreviated duration. These kinds of studies are required not only to confirm the grouping but also to elucidate the whole organ and whole animal responses for a limited set of source substances.

In conclusion, the present studies have confirmed the earlier findings on the lack of genotoxicity by Co compounds and the ability of soluble Co compounds to generate ROS and induce a hypoxia response. The data presented here have corroborated the preliminary grouping of the compounds as predicted in the earlier tiers of testing (*in vitro*). Higher tiers of testing are now needed to draw conclusions on the *in vivo* effects of the different groups of compounds, by testing representatives of the groups. These tests are presented in the following manuscripts.

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CRediT authorship contribution statement

Remco Derr: Writing – original draft, Investigation, Formal analysis. **Nynke Moelijker:** Investigation, Formal analysis. **Giel Hendriks:** Conceptualization, Writing – review & editing. **Inger Brandsma:** Writing – original draft, Investigation, Formal analysis.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2022.105120.

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