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A tiered approach to investigate the inhalation toxicity of cobalt substances. Tier 2a: Grouping cobalt compounds based on their capacity to stabilize HIF-1α in human alveolar epithelial cells *in vitro*

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ABSTRACT

Excessive inhalation of cobalt (Co) dust can have harmful effects on the respiratory tract, yet all cobalt substances do not have the same potential for inducing toxicity. The prevalent hypothesis is that the potential of Co substances to release Co^{2+} ions in the organism and in cells drives their toxicity profile. Here, we explored the possibility of grouping Co substances for predicting inhalation toxicity based on *in vitro* data using the stabilization of hypoxia-inducible factor (HIF)-1α as a read out for intracellular Co ion content. We evaluated the potential of 11 inorganic Co compounds and two Co metal powder samples to stabilize intracellular HIF-1α in alveolar epithelial cells (A549) after 24 h exposure to 250–1000 μM Co equivalents. Cytotoxic activity of the substances was assessed in parallel after 72 h at the same doses. Two groups were identified: (1) substances with high intracellular bioavailability (n=9), causing cytotoxicity and stabilizing HIF-1α and (2) substances with low intracellular bioavailability (n = 4), and not inducing these effects. This study provides a link between screening-level data (solubility in artificial lung fluids, Tier 1) and hypothesized biological key events.

1. Introduction

Excessive inhalation of cobalt dust can have harmful effects on the respiratory tract, yet all Co compounds do not have the same potential for inducing this toxicity. The adverse effects of cobalt on the lung include asthma, parenchymal inflammation and fibrosis, and may include cancer (Lison, 2015). The aim of the present study was to explore the possibility of grouping Co compounds for predicting inhalation toxicity based on *in vitro* data, using the stabilization of hypoxia-inducible factor 1α (HIF1α) in epithelial cells as a read out. HIF-1α is an interesting candidate biomarker of cobalt toxicity because (i) its stabilization by intracellular Co^{2+} ions (hypoxia-like response) offers the possibility to assess the cellular bioavailability of Co^{2+} ions from different Co compounds; and (ii) HIF-1α is a pro-inflammatory mediator relevant for the inflammatory and carcinogenic responses induced by some inhaled Co compounds (Maxwell and Salnikow 2004).

HIF-1 is a ubiquitously expressed heterodimeric transcriptional factor composed of a constitutively expressed subunit, HIF-1β, and an oxygen-sensitive subunit, HIF-1α. Under normoxia, ubiquitin and proteasome-dependent pathways continuously degrade HIF-1α. This degradation is mainly controlled by the hydroxylation of two specific prolyl residues by prolyl hydroxylase in the presence of O_2. Under hypoxia, HIF-1α is not degraded, becomes stabilized, heterodimerizes with HIF-1β, recruits coactivators (e.g. p300/CREB binding protein and other tissue-specific proteins), and binds to hypoxia-responsive elements (HREs) in target genes to activate their transcription (Semenza 2001). HIF-1-dependent genes include the pro-inflammatory cytokine interleukin (IL)-6 and the vascular endothelial growth factor (VEGF)-A involved in angiogenesis. HIF-1 also regulates the expression of genes involved in erythropoiesis, glucose metabolism, extracellular matrix formation, epithelial to mesenchymal transition, genome maintenance, metastasis, chemotaxis, or cell proliferation/survival. Several of these genes can promote cancer development, progression, and metastasis (Semenza 2003).

Mediators other than hypoxia can also regulate the stabilization of HIF-1α. Ions such as Co^{2+} mimic hypoxia and stabilize HIF-1α in normoxic conditions by blocking the iron binding site of prolyl hydroxylase (Epstein et al., 2001), disrupting iron homeostasis (Kang et al., 2006), or by directly binding to HIF-1α (Yuan et al., 2003), thus preventing its degradation. In addition to its role in tumor development, HIF-1α also

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Table 1
List of Co compounds tested.

Compound	Chemical composition	CAS number	Batch number	Source	MW (g/mole)	Co %[a]	Physical form	Final form[b]
Co dichloride hexahydrate	$CoCl_2.6H_2O$	7791-13-1	2936300009	Umicore	237.93	24.77	powder	S
Cobalt sulphide	CoS	1317-42-6		Vale Nickel limited	91	64.76	powder	P
Cobalt sulfate heptahydrate	$CoSO_4.7H2O$	10026-24-1		Umicore	281.1	20.97	powder	S
Co carbonate	$CoCO_3$	51839-24-8	01/16	Freeport Cobalt	118.94	52.4	powder	P
Co (di)hydroxide	$Co(OH)_2$	21041-93-0	20035–0002551849	Umicore	92,948	62.1	powder	P
Co diacetate tetrahydrate	$C_2H_4O_2.1/2Co$	6147-53-1	2938000254	Umicore	249.11	23.67	powder	S
Co dinitrate hexahydrate	$Co(NO_3)_2.6H_2O$	10026-22-9	54036	ICoNiChem	118.94	19.91	powder	S
Co hydroxide oxide	CoOOH	12016-80-7	4470	Norilsk Nickel	91.94	28.96	dry/solid	P
Lithium cobalt oxide (LCO)	$LiCoO_2$	12190-79-3	16288	Umicore	97.87	60.4	powder	P
Co monoxide	CoO	1307-96-6		Cixi Feilan	74.93	78.65	powder	P
Tricobalt tetraoxide	Co_3O_4	1308-06-1		Freeport Cobalt	240.8	73.42	powder	P
Co metal 1	Co	7440-48-4		OMG Kokkola	58.93	100	powder	P
Co metal 2	Co	7440-48-4		Eramet, eurotungstene	58.93	100	powder	P

a: as determined by the provider.
b: completely soluble (S), particulates (P) in culture medium.

regulates lung inflammation induced by cobalt. Upon inhalation, cobalt chloride causes an acute inflammatory lung response characterized by Th1-type cytokines and neutrophil infiltration. In the absence of HIF-1α, this inflammatory reaction is shifted from a neutrophilic to an eosinophilic profile associated with a Th2 response (Saini et al., 2010a, 2010b). Thus, HIF-1α appears as an early event triggering the toxic responses to Co^{2+} ions and can serve as a biomarker of cellular response to these ions. Moreover, several biological functions of HIF-1α are relevant for the inflammatory/carcinogenic activity of Co^{2+} ions.

In the present study, we evaluated the potential of several Co compounds to stabilize intracellular HIF-1α in alveolar epithelial cells (A549), as this cell line was previously used to show HIF-1α responses to metal ions (Li et al., 2006) and benzo(a)pyrene (Mavrofrydi et al., 2016). The cytotoxic activity of the Co compounds was assessed in parallel. The Co compounds tested included $CoCl_2$, CoS, $CoSO_4$, $CoCO_3$, $Co(OH)_2$, $Co(NO_3)_2$, CoOOH, Co diacetate, CoO, Co_3O_4, lithium cobalt oxide (LCO) and two Co metal samples (Co metal 1 and Co metal 2). These compounds cover a wide range of solubility, were tested in bioelution tests in tier1 (Verougstraete et al., 2022, this issue), and are all commercially relevant, implying a possibility of human exposure. The main hypothesis for the mechanism of Co toxicity is based on the intracellular Co^{2+} content, i.e. the potential of Co compounds to release Co^{2+} ions in the organism and in the cells drives their capacity to induce adverse effects (Lison et al., 2018). Therefore, Co compounds were tested at equal Co equivalent concentrations (Co μM). Two groups were identified: (1) high bioavailability Co compounds, which stabilized HIF-1α and were cytotoxic and (2) low bioavailability Co compounds, which did not stabilize HIF-1α and were not cytotoxic.

2. Materials and methods

2.1. Cobalt compounds

Thirteen Co compounds were obtained from different sources (Table 1).

2.2. Endotoxin measurement

Endotoxin content was measured with the Pierce LAL chromogenic endotoxin quantitation kit (Thermo Scientific) as recommended by the manufacturer. Co compounds were dissolved or suspended in phosphate-buffered saline (PBS) at a concentration of 2000 μM Co equivalents and then diluted to 1000 μM (maximal concentration tested in the cell experiments). As recommended by the manufacturer, we first verified that the pH of the suspensions was between 6 and 8 with Neutralit pH 5.5–9.0 paper (Millipore). Co compounds were tested as such (unspiked), and spiked with 0.5 EU LPS/ml (1:1 Co compound 2000 μM + endotoxin standard 1 EU LPS/ml) to verify the absence of quenching by the tested compound (false negative). Briefly, 50 μl H_2O, PBS, standards, unspiked or spiked samples were mixed with 50 μl LAL reagent and 100 μl substrate solution in a plate. Negative controls included 50 μl unspiked samples and 150 μl H_2O (see Table 2 for a summary of the procedure). Absorbance was measured at 405 nm after addition of stop reagent (Abs A). The plate was centrifuged 10 min at 250 *g* and 200 μl supernatants were transferred in a new plate. Absorbance was measured again at 405 nm (Abs B). Sample absorbances were calculated by two methods. In method A, Abs A of the negative controls was subtracted from the Abs A of spiked and unspiked samples. In method B, the average Abs B of the PBS blank replicates was subtracted from Abs B of spiked and unspiked samples. Method A takes into account particle absorbance by subtracting negative controls, method B by measuring the absorbance on suspension supernatant after centrifugation. For standards, the average absorbance of the H_2O blank replicates was subtracted from standard absorbances. For each method, a standard curve was plotted and endotoxin concentration of each sample was interpolated from the linear regression.

Table 2
Detailed procedure for the endotoxin assay.

	Sample	50 μl LAL reagent	100 μl substrate	150 μl H_2O	50 μl stop
Blanks	50 μl PBS*	yes	yes	no	yes
Blanks	50 μl H_2O	yes	yes	no	yes
Standard 1 U/ml	50 μl standard 1 U/ml	yes	yes	no	yes
Standard 0.5 U/ml	50 μl standard 0.5 U/ml	yes	yes	no	yes
Standard 0.25 U/ml	50 μl standard 0.25 U/ml	yes	yes	no	yes
Standard 0.1 U/ml	50 μl standard 0.1 U/ml	yes	yes	no	yes
Unspiked samples	50 μl 1000 μM Co equivalent	yes	yes	no	yes
Spiked samples	25 μl 2000 μM Co equivalent +25 μl standard 1 U LPS/ml	yes	yes	no	yes
Negative controls	50 μl 1000 μM Co equivalent	no	no	yes	yes

* PBS = phosphate-buffered saline.

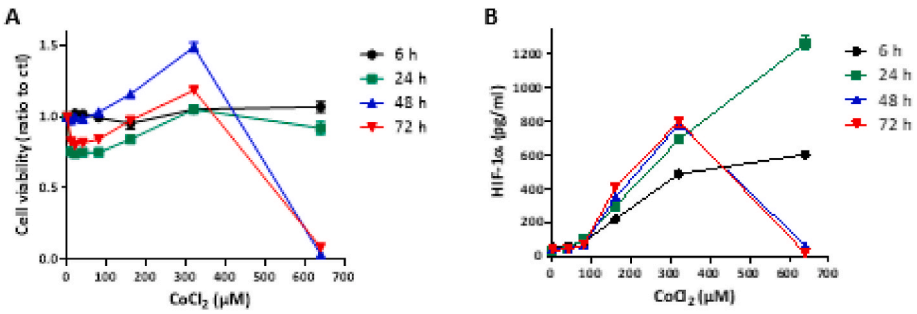

Fig. 1. A549 cell viability and HIF-1a content after exposure to $CoCl_2$.
A549 cells were exposed to 10–640 µM $CoCl_2$. (A) Cell viability (WST-1) expressed as a ratio to untreated cells (ctl) (N = 1, n = 8) and (B) HIF-1a (ELISA on cell lysates) (N = 1, n = 3) were measured after 6, 24, 48 and 72 h. Please note that small error bars are not always visible.

2.3. Particle preparations

$CoCl_2$ was first dissolved in nanopure H_2O at 100 mM and filter-sterilized (0.22 µm) before further dilution in culture medium. This procedure avoided precipitation of Co^{2+} ions with phosphates in the culture medium, and thus possible Co depletion by filtering. Other Co compounds were weighed on the day of exposure in a glass vial covered with aluminum. Prior to weighing, the glass vial was heated for 2 h at 200 °C and left to cool before adding the compounds. Co compounds were then suspended at 10 mM Co equivalents in exposure medium (F12K + 1% antibiotic-antimycotic, Gibco). These stock suspensions were next serially diluted in exposure medium.

2.4. A549 culture and exposure

The A549 type II alveolar epithelial cell line derived from a human lung carcinoma (ATCC, CCL-185) was cultured in F12K supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (aa, Gibco) (complete medium). Cells were passaged every week in 75 cm^2 flasks. After washing with 10 ml phosphate-buffered saline (PBS, Gibco), 3 ml trypsin 0.25 %-EDTA (Gibco) were added to the cells, and flasks were incubated 5–10 min at 37 °C. Trypsin was then neutralized by adding 7 ml of complete medium. Cell suspensions were centrifuged for 10 min at 125 *g*, and resuspended in fresh complete medium. Cell number and viability were determined with Trypan Blue solution (Sigma) in a Bürker cell under a light microscope.

For cell exposure, 1.5×10^5 cells/cm^2 (culture well surface area) were seeded in 96-well plates (50.000 cells/100 µl/well) or 48-well plates (1.5×10^5 cells/300 µl/well) with complete medium and grown at 37 °C. After 24 h, supernatants (SN) were discarded and cells were exposed to Co compounds (concentrations in µM Co equivalents) in serum-free medium (100 µl for 96-well plates or 350 µl for 48-well plates). After exposure (6–72 h), all cell SN were discarded. For HIF-1α (48-well plates), cells were washed once with PBS and lysed in 150 µl lysis buffer as recommended by the HIF-1α ELISA assay (15 min on ice, stored at −80 °C). For cell viability measurements, cells were washed once with PBS and tested with the WST-1 assay (96-well plates).

2.5. Cell viability assay

After exposure in 96-well plates, 100 µl F12K/WST-1 (1:30, Roche Diagnostics, Mannheim, Germany) were added on cells. Cells were incubated at 37 °C and absorbance (A) measured at 480 nm and 680 nm (reference absorbance). When the absorbance ($A_{480\ nm}$ - $A_{680\ nm}$) of control cells (ctl) reached 0.7–1.0 OD, 80 µl SN were transferred in new 96-well plates (to avoid interferences of undissolved Co compounds with the absorbance) and absorbance was measured. The latter absorbance values ($A_{480\ nm}$ - $A_{680\ nm}$) were used to calculate cell viability, and results were expressed as a ratio to non-exposed cells (ctl = 1).

2.6. HIF-1α quantification

HIF-1α levels were quantified by ELISA in cell lysates according to the manufacturer's instructions (R&D Systems, human/mouse total HIF-1 alpha Duoset IC, DYC1935-5). Cell lysates were centrifuged at 4 °C (2000 *g*, 5 min) and testing was performed on the SN.

3. Statistics

Differences between non-treated cells (ctl) and cells exposed to Co compounds were evaluated by a one-way analysis of variance, followed by a Dunnett's multiple comparisons test. Statistical significance was considered at $P < 0.05$. Data were analyzed with GraphPad Prism version 8 (GraphPad Software, San Diego, USA). Number of independent experiments (N) and experimental replicates per experiment (n) are indicated in the figure legends. Graphs present the arithmetic mean (N) of the arithmetic means (n).

4. Results

Endotoxin levels in Co compounds. As endotoxin can upregulate the expression of HIF-1α at the transcriptional level in epithelial cells (Kim et al., 2007), the endotoxin level was measured for all samples. Co suspensions were tested at 1000 µM Co equivalents, i.e. the maximal concentration used in the present study. Both methods (A and B, see methods) revealed no trace of endotoxin in suspensions. Spiked samples (+0.5 U endotoxin/ml) were positive for endotoxin, excluding the possibility of a false negative (table Supplemental 1, S1).

Kinetics of cytotoxicity and HIF-1α stabilization after exposure to $CoCl_2$. $CoCl_2$, which is fully hydrosoluble and thus expected to induce a maximal HIF-1α response, was selected as the reference to calibrate the comparison of the Co compounds. Thus, A549 cells were exposed to $CoCl_2$ (0, 10, 20, 40, 80, 160, 320 or 640 µM) for 6, 24, 48 and 72 h, and we first assessed cell viability to identify doses and exposure durations at which cytotoxicity might confound HIF-1α response.

Fig. 1: *A549 cell viability and HIF-1α content after exposure to $CoCl_2$.*

A549 cells were exposed to 10–640 µM $CoCl_2$. (**A**) Cell viability (WST-1) expressed as a ratio to untreated cells (ctl) (N = 1, n = 8) and (**B**) HIF-1α (ELISA on cell lysates) (N = 1, n = 3) were measured after 6, 24, 48 and 72 h.

WST-1 results indicated that 640 µM $CoCl_2$ was cytotoxic after 48 and 72 h (Fig. 1A). We, therefore, selected 72 h of exposure for comparing the cytotoxic activity of the compounds. $CoCl_2$ concentrations below 640 µM did not appear to affect cell viability strongly, and $CoCl_2$ was not cytotoxic after 6 and 24 h, suggesting that HIF-1α should be evaluated at one of these time points. Intracellular HIF-1α was dose-dependently increased at all time points, except at the cytotoxic concentrations after 48 and 72 h (Fig. 1B). The strongest and completely dose-dependent increase in HIF-1α was observed after 24 h exposure. We therefore selected 24 h of exposure for comparing the capacity of the Co compounds to stabilize HIF-1α.

Cytotoxicity after exposure to the Co samples. Fig. 2 shows that

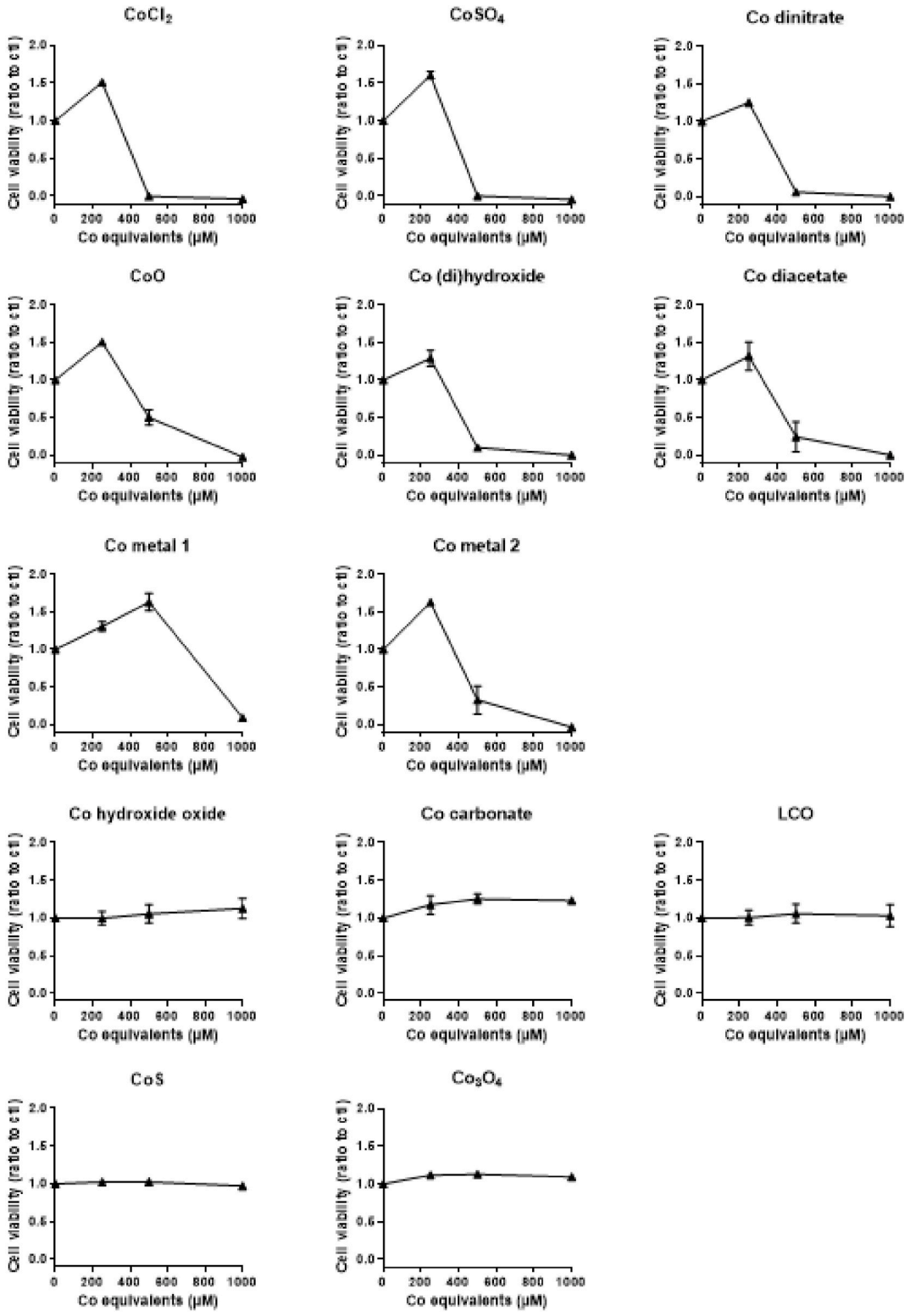

Fig. 2. A549 cell viability after exposure to 12 Co compounds.
A549 cells were exposed to 250, 500 and 1000 µM Co compounds (µM Co equivalents). Cell viability was assessed by the WST-1 assay after 72 h exposure, expressed as a ratio to untreated cells (ctl) (N = 1–3, n = 3–4). Please note that small error bars are not always visible.

CoS, Co_3O_4, Co carbonate, LCO, and Co hydroxide oxide were not cytotoxic at any tested concentration after 72 h. Co metal 1 was cytotoxic at 1000 µM $CoCl_2$, Co metal 2, CoO, $CoSO_4$, Co dihydroxide, Co acetate, and Co nitrate were partially or strongly cytotoxic from 500 µM. An increased WST-1 activity was recorded at low doses for the samples that induced cytotoxicity at higher doses. This phenomenon might reflect increased proliferation of the A549 cells and/or increased dehydrogenase activity induced by the uptake of the compounds and was not induced by non-cytotoxic compounds. Overall, we identified 5 samples that were not cytotoxic at any of the tested concentrations and 8

Table 4
Summary of cytotoxicity results after 72 h exposure.

	250 μM Co equivalents		500 μM Co equivalents		1000 μM Co equivalents	
	Cytotoxicity	p[a]	Cytotoxicity	p[a]	Cytotoxicity	p[a]
$CoCl_2$	–	<0.0001	+	<0.0001	+	<0.0001
$CoSO_4$	–	<0.0001	+	<0.0001	+	<0.0001
Co dinitrate	–	ns	+	<0.0001	+	<0.0001
CoO	–	<0.0001	+	<0.0001	+	<0.0001
Co (di)hydroxide	–	ns	+	<0.0001	+	<0.0001
Co diacetate	–	ns	+	<0.0001	+	<0.0001
Co metal 1	–	<0.0001	–	<0.0001	+	<0.0001
Co metal 2	–	<0.0001	+	<0.0001	+	<0.0001
Co hydroxide oxide	–	ns	–	ns	–	ns
Co carbonate	–	ns	–	ns	–	ns
LCO	–	ns	–	ns	–	ns
CoS	–	ns	–	ns	–	ns
Co_3O_4	–	ns	–	ns	–	ns

[a] Differences relative to non-treated cells (ctl) were evaluated by a one-way analysis of variance, followed by a Dunnett's multiple comparisons test. ns: not significant.

samples that were cytotoxic at 1000 μM. The cytotoxicity results are summarized in Table 4.

HIF-1α stabilization after exposure to the Co samples. After 24 h exposure, Co hydroxide oxide, LCO, CoS and Co_3O_4 and had no effect on intracellular HIF-1α content (Fig. 3). $CoCl_2$, $CoSO_4$, Co dinitrate, Co dihydroxide, Co diacetate, and Co metal 2 stabilized HIF-1α at low doses. This effect on HIF-1α was partially or completely reduced at the highest concentration due to cytotoxicity. Co metal 1, CoO, and Co carbonate dose-dependently stabilized intracellular HIF-1α up to the highest concentration tested. These HIF-1α results are summarized in Table 5. None of the four identified samples that did not stabilize HIF-1α at any of the tested concentrations were cytotoxic. The remaining nine samples increased intracellular HIF-1α cell content at one or more tested concentrations. Eight of these samples were cytotoxic. One of these samples, Co carbonate, was not cytotoxic, yet increased intracellular HIF-1α content.

5. Discussion

Table 6 summarizes the data for the 13 Co samples. Cytotoxicity and HIF-1α stabilization results are shown for the concentrations that best discriminated the Co compounds, i.e. at 1000 μM for cytotoxicity and 500 or 1000 μM for HIF-1α. Based on these results in A549 cells, the Co compounds can be classified in two groups.

Group 1 includes Co compounds which appeared soluble or as particulates in cell culture medium. In the bioaccessibility experiments performed in tier1, these compounds were found either readily or poorly soluble at neutral pH but all highly soluble in acidic lysosomal fluid (Verougstraete et al., 2022, this issue). Group 1 compounds stabilized HIF-1α in the present investigation, irrespective of their solubility at neutral pH, thus reflecting their capacity to generate increased intracellular concentrations of Co^{2+} ions. These compounds can be considered to exert a biological activity through intracellular Co^{2+} ions. With the exception of Co carbonate, these compounds were cytotoxic, which provides additional evidence of the biological activity of intracellular Co^{2+} ions released from these compounds. The particular responses to Co carbonate (likely group 1) could be linked to its low solubility at neutral pH (Verougstraete et al., 2022, this issue), which is further reduced by the high bicarbonate concentration in F12K culture medium (30 mM) used in the present experiment. Thus, low concentrations of Co^{2+} ions released from Co carbonate may be sufficient to induce HIF-1α stabilization in A549 cells, but not to cause cytotoxicity. Higher tier data are needed to confirm whether the somewhat exceptional behavior of Co carbonate *in vitro* is relevant for its potential *in vivo* toxicity.

Group 2, as formed by the present data, includes compounds that appeared as particulates in cell culture medium and were poorly soluble in lysosomal fluid in bioaccessibility experiments (Tier 1, Verougstraete et al., 2022, this issue). These Group 2 compounds are exactly the same as in the third group based on bioaccessibility data (poorly soluble in all fluids) according to Tier 1 (Verougstraete et al., 2022, this issue). The Group 2 compounds did not stabilize HIF-1α in the present investigation and can be considered not to exert a biological activity through Co^{2+} ions. These compounds were not cytotoxic, which again is in line with the low bioavailability of Co^{2+} ions (intra- or extracellularly) from these compounds.

The present study used the A549 alveolar epithelial cell model to discriminate Co compounds according to their cytotoxic activity and ability to stabilize HIF-1α. One advantage of the study design applied here is that any potential "particle toxicity" would be captured in these experiments, as the cells were exposed to the Co compounds "as is", whether fully, partially or incompletely dissolved. A similar approach has already been applied to screen Ni compounds using another human epithelial cell line (H460) (Pietruska et al., 2011).

In the context of particle toxicity, it is worth noting that not all epithelial cell lines react similarly to particles in general, as well as to Co compounds. As a complement to this study, we exposed the bronchial epithelial cell line BEAS-2B to $CoCl_2$ and Co_3O_4. As in A549 cells, $CoCl_2$ was cytotoxic and Co_3O_4 did not affect cell viability at any of the concentrations tested (Suppl Figure 1A). In contrast to A549 cells, $CoCl_2$ but also Co_3O_4 strongly increased HIF-1α stabilization in BEAS-2B (Suppl Figure 1B). This effect of Co_3O_4 on HIF-1α has already been reported in BEAS-2B cells (Ortega et al., 2014). We also observed a HIF-1α stabilizing activity of LCO in the BEAS-2B model (Sironval et al., 2019)) (. In another study, both A549 and BEAS-2B cell lines endocytosed Co_3O_4 micro- or nano-particles (NP), but BEAS-2B were more sensitive to Co_3O_4 NP (in terms of cytotoxicity) than A549 (Cavallo et al., 2015; Ortega et al., 2014; Verstraelen et al., 2014). BEAS-2B also responded to CoO NP at the transcriptional level at a 10-fold lower NP-concentration than A549 cells. Compared to liver and kidney epithelial cells, A549 cells were also shown to be less sensitive (cytotoxicity and ROS production) to WC-Co NP, although NP were taken up by the three cell lines (Paget et al., 2015). Thus, the BEAS-2B model appears more sensitive than A549 to detect the stabilization of HIF-1α upon exposure to poorly soluble Co compounds such as Co_3O_4 and LCO. Further, differences in culture medium composition (defined LHC-9 vs F12K + 10% serum, for BEAS-2B and A549 cells, respectively), as well as the use of a well coating for BEAS-2B cells might also contribute to explaining these varying responses to Co compounds between both cell lines. Differences in iron homeostasis between both cellular models might also contribute to explain different HIF-1α responses (Kang et al., 2006).

It remains, however, to be determined which cellular model (A549 or BEAS-2B) better predicts the *in vivo* lung toxicity of Co compounds. Available *in vivo* data exploring the respiratory toxicity of Co compounds are limited. NTP inhalation studies showed that $CoSO_4$ (0.3–3.0 mg/m^3

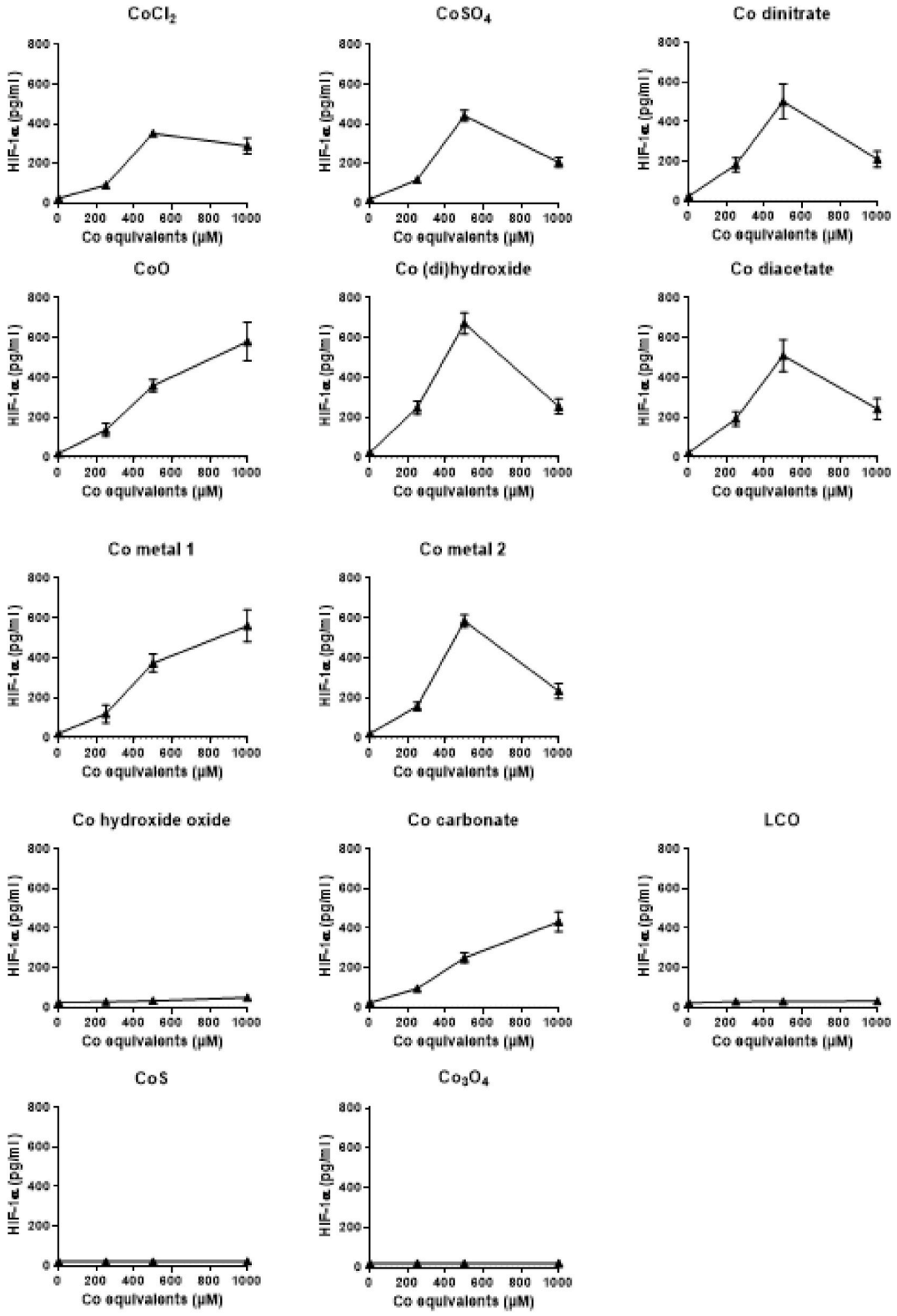

Fig. 3. A549 HIF-1a content after exposure to 12 Co compounds.
A549 cells were exposed to 250, 500 and 1000 μM Co compounds (μM Co equivalents). HIF-1a cell content was measured by ELISA after 24 h exposure (N = 3, n = 3). Please note that small error bars are not always visible.

cobalt sulfate hexahydrate, 6 h/day, 5 days/week, for 104 weeks) induced proteinosis, alveolar epithelial metaplasia, granulomatous alveolar inflammation, and interstitial fibrosis in rats. Inflammatory reactions were less severe in mice than in rats exposed to the same concentrations. Both rats and mice developed alveolar/bronchiolar neoplasms in the same study (Bucher et al., 1999). Rats and mice exposed by inhalation to cobalt metal (1.25–5 mg/m^3, 6 h/day, 5 days/week, for 105 weeks) developed inflammation, fibrosis, and

Table 5
Summary of HIF-1α stabilization results after 24 h exposure.

	250 μM Co equivalents		500 μM Co equivalents		1000 μM Co equivalents	
	HIF-1α	p[a]	HIF-1α	p[a]	HIF-1α	p[a]
$CoCl_2$	+	ns	+	<0.0001	+	<0.0001
$CoSO_4$	+	ns	+	<0.0001	+	<0.0001
Co dinitrate	+	0.0088	+	<0.0001	+	0.0018
CoO	+	0.018	+	<0.0001	+	<0.0001
Co (di)hydroxide	+	0.0002	+	<0.0001	+	0.0001
Co diacetate	+	0.0048	+	<0.0001	+	0.0002
Co metal 1	+	ns	+	<0.0001	+	<0.0001
Co metal 2	+	0.0047	+	<0.0001	+	<0.0001
Co hydroxide oxide	–	ns	–	ns	–	ns
Co carbonate	+	ns	+	0.001	+	<0.0001
LCO	–	ns	–	ns	–	ns
CoS	–	ns	–	ns	–	ns
Co_3O_4	–	ns	–	Ns	–	ns

[a] Differences relative to non-treated cells as evaluated by a one-way analysis of variance, followed by a Dunnett's multiple comparisons test. Ns: not significant.

hyperplasia, as well as alveolar/bronchiolar neoplasms (Behl et al., 2014; Behl et al., 2015). Data from both investigations are consistent with the present results (Table 6) indicating that both compounds are soluble in lysosomal fluid, cytotoxic and stabilize HIF-1α in A549 cells (group 1). Other *in vivo* data are, however, not in line with the grouping presented in Table 6. Two Co compounds, Co_3O_4 and LCO, which did not stabilize HIF-1α in A549 cells, have been reported to induce inflammatory reactions upon acute administration. For example, intratracheal instillation of Co_3O_4 nanoparticles in rats induced acute lung inflammation (Jeong et al., 2015) and pulmonary alveolar proteinosis in rats (Cho et al., 2012). We also found recently that LCO induced lung inflammatory and fibrotic responses in mice after oropharyngeal aspiration (Sironval et al., 2018). Whether these adverse effects are driven by the nanometric size of the compound (Co_3O_4), or due to the high dose rate associated with the mode of administration (Co_3O_4 and LCO) is not known.

Besides advantages of cellular studies presented here, there are also limitations, including the fact that we used isolated cells. *In vivo*, alveolar epithelial cells, modelled here by A549 cells, have close contacts with neighboring alveolar macrophages and other cell types that participate in the response to inhaled respirable particles. The same is relevant for BEAS-2B cells, derived from the bronchial epithelium, which, in situ, would be close to ciliated cells and mucosal glands, or recruited macrophages and neutrophils to eliminate particles. Therefore, in order to interpret the results from the present *in vitro* studies, additional *in vivo* data from well-designed inhalation studies are required to strengthen or challenge the grouping approach proposed here, and to determine for which family of compounds it may be valid. Studies on a whole organ level, e.g. using precision cut lung slices, may also be helpful to distinguish the responses to different Co compounds by the whole lung/respiratory system. If groups of Co compounds with similar responses emerge from these studies, selected candidates from each group could be tested in repeated dose *in vivo* inhalation studies to shed light on the long-term effects of the different groups of Co compounds.

6. Conclusion

In vitro testing in lung-derived A549 cells was shown to generally support a grouping based on bioelution in lung fluids for cobalt substances. In addition, the results are consistent with upstream steps in the mode of action of Co substances associated with lung toxicity. This type of investigation is useful to demonstrate plausibility and relevance of a grouping and read-across approach and gives an indication of tissue markers of interest to differentiate the groups *in vivo*.

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CRediT authorship contribution statement

Sybille van den Brule: Supervision, Writing – original draft. **Saloua Ibouraadaten:** Investigation. **Lisa Brombin:** Investigation. **Dominique Lison:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors Saloua Ibouraadaten, Lisa Brombin, declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

Table 6
Grouping Co compounds according to their capacity to stabilize HIF-1α.

		Form in culture medium[a]	Lysosomal solubility[b]	Cytotoxicity at 1000 μM	HIF-1α 500 or 1000 μM	Group
Inorganic cobalt	$CoCl_2$	S	+	+	+	1
	$CoSO_4$	S	+	+	+	1
	Co dinitrate	S	+	+	+	1
	CoO	P	+	+	+	1
	Co (di)hydroxide	P	+	+	+	1
	Co diacetate	S	+	+	+	1
	Co metal 1	P	+	+	+	1
	Co metal 2	P	+	+	+	1
	Co hydroxide oxide	P	–	–	–	2
	Co carbonate	P	+	–	+	1
	LCO	P	–	–	–	2
	CoS	P	–	–	–	2
	Co_3O_4	P	–	–	–	2

[a] Completely soluble (S), or particulates (P) in culture medium.
[b] Tier 1, Verougstraete et al., 2022, this issue.

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