

Case Study Summary

Title: A tiered approach to the assessment of inhaled cobalt compounds

Version: 1.1

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Provide a few sentences summarizing the method illustrated by the case study.

The Cobalt REACH Consortium (CoRC) has designed, and is in the process of carrying out, a tiered testing programme for a group of cobalt substances registered under REACH, with the aim of predicting longer-term inhalation effects on the basis of middle or lower tiers such as the measurement of acute in vivo responses and in vitro biomarkers. The results of 12 of the substances is presented here.

Describe the problem formulation(s) the case study is designed to address. How is the method described in the case useful for addressing the problem formulation?

Cobalt sulphate and cobalt metal have been found to cause lung tumours following inhalation by rats and mice in two NTP carcinogenicity studies¹. Based on these data, both substances meet the criteria for classification under the EU CLP (Classification, Labelling and Packing of Chemicals) regulation as carcinogenic Category 1B (presumed to have carcinogenic potential for humans). It is now necessary to evaluate whether further cobalt compounds meet the criteria for classification under the same endpoint. However, it is neither desirable (for animal welfare reasons) nor possible to conduct lifetime inhalation carcinogenicity studies on every cobalt-containing substance for the purpose of determining inhalation carcinogenicity hazard. The CLP Regulation foresees the 'read-across' of the carcinogenicity classification to a structurally similar substance; such an approach "must always be based on a robust and transparent argument" and must take into account other important factors such as physico-chemical, toxicokinetic and any other available relevant information (CLP Annex I sections 3.6.2.2.7 to 3.6.2.2.9 and Guidance on the Application of the CLP Criteria).

¹ Bucher, J. R. (1998). Toxicology and carcinogenesis studies of cobalt sulfate heptahydrate (CAS NO. 10026-24-1) in F344/N rats and B6C3F1 mice (inhalation studies) National Toxicology Program. NTP TR 471. Behl, M. and M. J. Hooth (2014).

Toxicology studies of cobalt metal (CAS NO. 7440-48-4) in F344/N rats and B6C3F1/N mice and toxicology and carcinogenesis studies of cobalt metal in F344/NTac rats and B6C3F1/N mice (inhalation studies), National Toxicology Program. NTP TR 581.

The method presented here is useful as it measures the important factors in a robust and transparent way: as a first tier, solubility in relevant biological media (physico-chemical parameter) is measured. The method then provides, step-by-step, further relevant information following a mode of action-based tiered testing scheme.

The method is useful as it may accurately predict hazardous properties on a mode of action-based argumentation, with a significant reduction in at least repeated dose, and perhaps even acute exposure animal testing.

Comment on whether the method is general enough to be used directly, or if it can be extrapolated, for application to other chemicals and/or problem formulations. Please explain why or why not.

Figure 1 describes in general terms the method employed through a series of tiered tests. Each tier is ranked from left to right in terms of increasing power of information, with the cancer tier ranked as the highest. In this particular example, cobalt substances are separated out into two distinct groups based on reactivity and severity or type of local lung effects. Substances that are non-reactive/poorly-reactive across the different tiers are placed in the 'non-cancer' group, and the substances that are reactive across the tiers are placed in a chronic inhalation toxicity (cancer) grouping with cobalt sulphate and cobalt metal.

This general approach is applicable to other groupings of chemicals that have a similar diversity in reactivity. Starting with tests of solubility or in vitro measurements of bioavailability, other cellular biomarkers may be relevant for other compounds, depending on their mode of action. Equally, other organ-level markers for lung responses may be relevant for other groups of chemicals. The principle of this approach can however be extrapolated easily to any group of compounds for which the common "toxic unit" and some key steps in the mode of action are known.

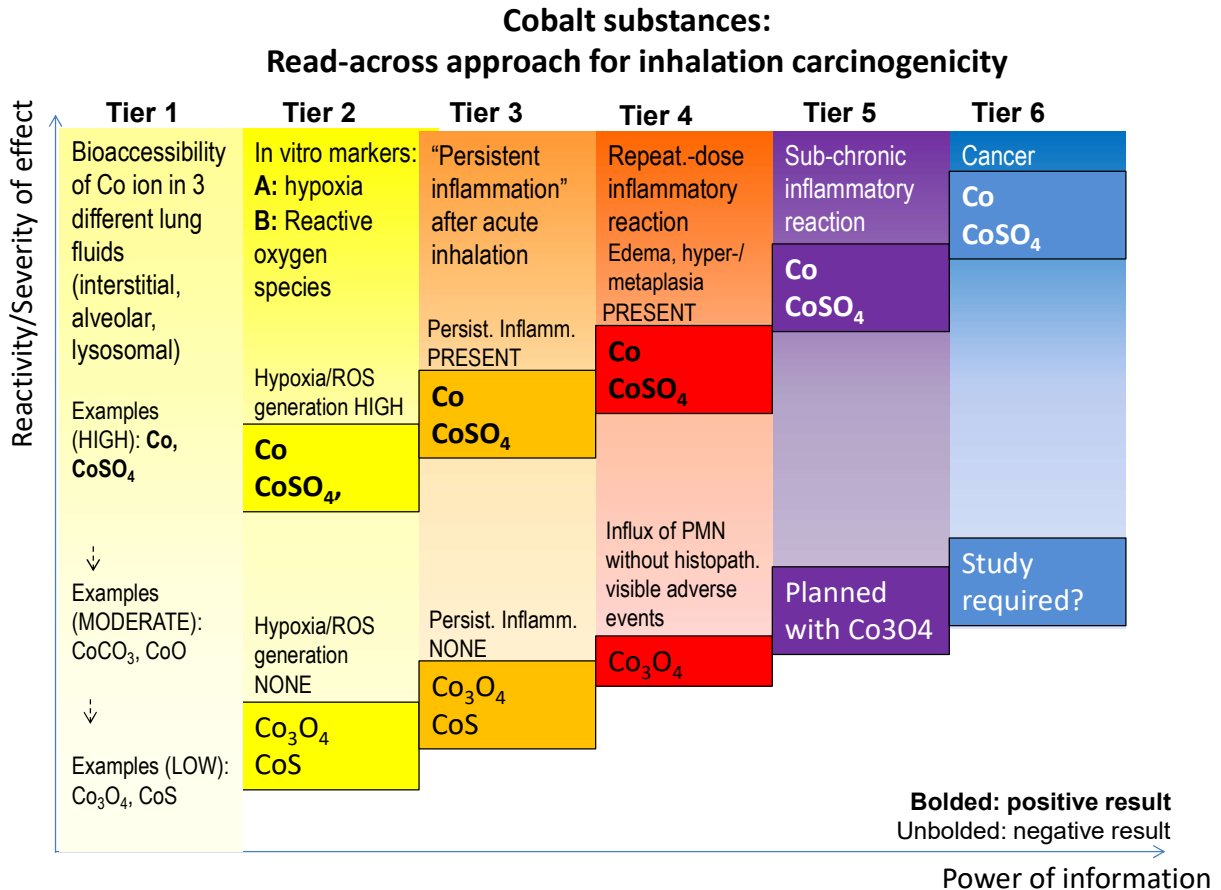


Figure 1. Cobalt inhalation read-across and grouping approach: Tiered strategy with examples for each group of compounds

Discuss the overall strengths and weaknesses of the method.

The predominant mode of action of cobalt-related lung cancer is local inflammation, which in turn is caused by several pre-inflammatory events. The extent to which a cobalt-containing substance causes these events can be measured, and these ‘markers’ are used in a Weight of Evidence approach to group cobalt-containing substances based on their predicted potential to cause chronic inhalation effects.

Several tiers of testing have been defined. They range from the lower (less weighted in terms of relevance) tiers of in vitro testing, up to mid-level tiers of in vivo acute and repeated-dose inhalation testing and conclude with the highest tier, a two-year rodent carcinogenicity study. The lower tiers are aimed at making distinctions between the various cobalt compounds based on common toxicological drivers (e.g. hypoxia, cytotoxicity, oxidative stress), whereas the higher tier testing is aimed at “proving the concept” of the existence of different groups of compounds regarding chronic inhalation toxicity endpoints (e.g. chronic pulmonary inflammation, metaplasia).

A strength of the lower tiers is that each tier is aimed at measuring a known event in the mode of action of a “reactive” cobalt compound. The lower tiers are suitable for application to many compounds, in that these are animal free methods, as well as inexpensive and relatively fast to carry out. One strength of the method is therefore the possibility to screen many substances and put them into groups of similar properties. The higher tiers are more precise in determining the exact biological response (e.g. type of inflammatory cells being recruited to lung, lack or presence and type of histopathological damage to epithelium), but cannot be carried out with a large number of compounds, as these are animal intensive methods. The higher tiers are meant to confirm the predicted mode of action of a certain chemical group with just one or a few examples.

A weakness of the method is that there is the possibility of diverging responses between tiers. In those cases, it may become difficult to judge which responses are the most reliable indicators of the longer-term response. Another weakness of the method is that incomplete knowledge of the mode of action may result in missing endpoints included in the lower tiers, and responses at higher tiers that are not detected in the lower tier testing.

As might perhaps be expected, the variability among biological responses among cobalt compounds tested within these various tiers can sometimes make overall judgments difficult. Despite this occasional difficulty, however, the CoRC has made some determinations and welcomes comments from the Science Panel during this ARA workshop.

As an aside, mutagenic responses have not been detected in guideline-compliant studies with any cobalt substance (also with those substances that are positive for cancer by inhalation exposure in rodent studies). See for example, data generated under REACH for the endpoint mutagenicity, including ToxTracker data², a recent OECD CoCAM conclusion on four soluble cobalt salts³, and an extensive cobalt-related genotoxicity database publication by D. Kirkland (2015).⁴ It has therefore been concluded by the CoRC that mutagenicity/genotoxicity is not a relevant driver of cobalt-related carcinogenicity, and it is not considered by the CoRC as a possible early marker of cancer in this cobalt-specific read across paradigm.

Outline the minimum data requirements and describe the types of data sets that are needed.

² The ToxTracker assay (Toxys) is a stem cell-based reporter assay that provides mechanistic insights into the genotoxic properties of chemicals, contributing to a mechanism-based, animal-free, cancer hazard and risk assessment of chemicals. It is currently undergoing review to become an OECD guideline testing method.

³ OECD (2014). "SIDS Initial Assessment Profile Soluble Co salts CoCAM 6." <http://webnet.oecd.org/hpv/ui/handler.axd?id=e5e60085-1f3f-4df5-92f6-8f32c26c3082>

⁴ Kirkland, D., T. Brock, H. Haddouk, V. Hargeaves, M. Lloyd, S. M. Garry, R. Proudlock, S. Sarlang, K. Sewald, G. Sire, A. Sokolowski and C. Ziemann (2015). "New investigations into the genotoxicity of cobalt compounds and their impact on overall assessment of genotoxic risk." *Regul Toxicol Pharmacol* **73**(1): 311–338.

The minimum requirements for the grouping of a cobalt substance are

- 1 – knowledge on the solubility of the compound in relevant biological fluids (each substance)
- 2 – measurement of substance's ability to induce cytotoxicity, oxidative stress and hypoxia in in vitro cellular systems (each substance)
- 3 - Persistent inflammation and/or upper respiratory tract reactivity upon acute exposure (it is debatable if step 3 is a strict data requirement for each substance)

The minimum requirements for proving the concept of the predictive power of the tiered approach are

- higher tier testing in repeated dose toxicity studies for at least one example (source substance) of each group with similarity in the lower tiers (above)

Description of the individual tiers

Tier 1: Bioaccessibility (artificial lung fluids)

Hypothesis: The toxic unit for inhalation effects is the bioaccessible Co^{2+} ion.

Test-system: Bioelution testing in artificial interstitial (pH 7.4), alveolar (pH 7.4 with phosphatidyl choline) and lysosomal (pH 4.5-5) fluid at 5 hours or longer for cobalt compounds; presented here are data for cobalt metal powder and cobalt substances with inorganic ligand.

Status and Results: Significant differences in solubility between 12 tested cobalt compounds and between lung fluids were found, indicating that the substances should display significant differences in their inhalation toxicology. However, there is no clear distinction into different groups. Instead, the Co substances vary in their solubility, spanning about 2-3 orders of magnitude. The compounds were labelled as highly, medium or poorly soluble in each of the three fluids, based on a comparison with Co sulphate⁵, which is highly water-soluble and carcinogenic in rodents. This resulted in compounds being labelled as highly, medium or poorly soluble in each of the three fluids. The most soluble compounds were labelled as High-High-High (e.g. Co sulfate) and the least soluble compounds as Low-Low-Medium (e.g. Co_3O_4), reflecting solubility in interstitial, alveolar and lysosomal fluid, respectively.

Conclusion: There is not enough evidence to conclude that solubility on its own can be used as a reliable predictor of inhalation toxicity for the following reasons: (i) Cobalt-related inhalation

⁵ High = solubility within one order of magnitude (OM), Medium = solubility below one OM, Poor = solubility below 2 OM; all solubility comparisons with CoSO_4 solubility in the respective fluid. The most soluble compounds are labelled as High, High, High (e.g. Co sulfate) and the least soluble compounds as Low, Low, Medium (e.g. Co_3O_4), reflecting solubility in interstitial, alveolar and lysosomal fluid, respectively.

toxicity is a portal-of-entry (local) effect, and, in addition to bioaccessibility of a substance, other effects may also play a role (e.g. clearance from the lung, binding to proteins and other biological targets), (ii) Based on the initial dataset, no robust correlation is observed between the bioelution profile of a substance and its acute inhalation toxicity (e.g. CoO and CoCO₃ display exactly the same solubility profile, both Medium-Medium-Low, yet one is acutely toxic and the other is not).

Tier 2a – In vitro biomarkers (hypoxia and cytotoxicity)

Introduction: Cobalt is known to be a potent trigger of a 'hypoxia-like' response and local damage in cells. Hypoxia and cytotoxicity are contributors to the development of cancer.

Hypothesis: Hypoxia and cytotoxicity can lead to an inflammatory reaction and cobalt-related acute and/or chronic inhalation toxicity (if this environment is maintained). Chronic inflammation, in turn, is a precursor to cobalt-related cancer. Substances that are positive for both cytotoxicity and hypoxia are thought to induce inflammation in vivo.

Test-system: Testing 12 cobalt compounds, including 2 grades of cobalt metal powder⁶, in vitro (human lung cells; A549) for two markers: cytotoxicity (WST-1) and hypoxia (HIF-1alpha).

Status and Results: Twelve compounds were tested. Results demonstrate significant differences in cytotoxicity and induction of hypoxia between the different test items. Eight compounds induced hypoxia and were considered "reactive"; four compounds did not induce hypoxia and were considered "non-reactive". There was generally an overlap between ability to cause cytotoxicity and hypoxia, with the exception of Co carbonate, which induced hypoxia in the absence of cytotoxicity.

Conclusions: Cytotoxicity and HIF-1alpha may be good indicators of longer-term cobalt-related inhalation toxicity effects.

Tier 2b – In vitro gene reporter assay (p53, protein damage, oxidative stress, DNA damage, hypoxia)

Introduction: Analysis of the genotoxicity database of cobalt and cobalt substances demonstrates that the Co²⁺ ion has 'secondary' or 'indirect' genotoxic modes-of-action (e.g. chromosomal aberrations, cell cytotoxicity, hypoxia etc.). The 'ToxTracker' assay was conducted to further support secondary/indirect genotoxic modes-of-action by adding data for

⁶ Counted as one compound

these effects into the weight-of-evidence for the overall database. Where possible, thresholds for effects were also identified (e.g. NOELs, LOELs, NOGELs and LOGELs).⁷

Hypothesis: 'Reactive' cobalt substances will induce secondary/indirect genotoxicity upon reaching a certain in vitro concentration (i.e. threshold). These effects will be measured by upregulation of common genes associated with each effect. Non-reactive cobalt substances will not show an upregulation of genetic markers associated with the reactive group.

Test-system: ToxTracker is a panel of mammalian stem cell lines (mouse embryonic stem cells) that contain different fluorescent reporters representing four distinct biological responses that are associated with carcinogenesis, i.e. general cellular stress, DNA damage, oxidative stress and the unfolded protein response induction of DNA damage, oxidative stress and protein damage.⁸ The differential induction of the Green Fluorescent Protein (GFP) reporters as well as cytotoxicity of the tested compounds was determined by flow cytometry. Upregulation of hypoxia genetic markers was determined by quantitative Polymerase Chain Reaction (qPCR).

Status and Results: Eight substances have been tested in the ToxTracker assay. All were negative for the markers of direct DNA damage (mutagenicity). Four were identified as upregulating the markers for oxidative stress, cytotoxicity and hypoxia. Four were negative for upregulation of any of the biomarkers tested.

Conclusions: Direct DNA damage is not a hallmark effect of any cobalt compounds tested. An increase in ROS and hypoxia at low cytotoxic concentrations of a compound was observed in eleven cases and may be a good marker for "reactivity" and long-term inhalation toxicity of a cobalt compound.

Overall conclusion (tier 2):

The in vitro systems in tier 2 are suitable to separate cobalt compounds into groups based on cellular responses. Two groups emerged: one group of "reactive compounds"⁹ which triggered responses of hypoxia, usually in combination with cytotoxicity, in tier 2 a as well as hypoxia and increased ROS in tier 2 b. Another group of compounds was non-reactive¹⁰. All compounds

7 No observed effect level (NOEL) = where fluorescent reporters showed < 1.5-fold increase; low observed effect level (LOEL) = fluorescent reporters showed > 2-fold increase; no observed genotoxicity effect level (NOGEL) = where fluorescent reporters showed < 1.5-fold increase and low observed genotoxicity effect level (LOGEL) = where fluorescent reporters showed > 2-fold increase.

8 Hendriks et al. (2016). The Extended ToxTracker Assay Discriminates Between Induction of DNA Damage, Oxidative Stress, and Protein Misfolding. *Toxicol Sci.* 150, 190–203.

9 CoCl₂, CoSO₄, Co(NO₃)₂, Co acetate, Co carbonate, CoO, Co metal powder, Co(OH)₂

10 Co₃O₄, CoS, CoLiO₂, CoOOH

displayed the same reactivity in tier 2 a and b; there was no disparity between the results of the two in vitro methods with the exception of cobalt carbonate (not cytotoxic).

The comparison with tier 1 shows that the reactive group had a solubility of at least Medium-Medium-High (interstitial, alveolar, lysosomal fluid, respectively). The non-reactive group had a solubility of Low-Low-High or Low-Low-Medium.

The first two tiers may result in an imprecise estimation of the toxic response, due to e.g. absence of certain defense mechanisms (e.g. antioxidant capacity, clearance mechanisms). Also, inhalation toxicity involves many cell types, including immune cells and phagocytes; further, particle effects may play a role that cannot be predicted by in chemico and in vitro cellular responses.

In order to consider the effect of a Co substance in terms of its effects on a whole organ, each "feasible"¹¹ cobalt substance will be tested in tier 3 (abbreviated in vivo testing) in order to better understand the correlation between cytotoxicity, hypoxia, oxidative stress and the inflammatory response in the complete organ system.

Tier 3 – In vivo persistent inflammation or upper respiratory tract meta- and hyperplasia (acute inhalation testing)

Introduction: The potential for cobalt and cobalt compounds to induce respiratory inflammation is investigated in an acute in vivo inhalation study, using the identical substances tested in tier 2. Cobalt is known to be a strong inducer of inflammation, and chronic inflammation is a known precursor of cancer¹², therefore several markers of inflammation were investigated.

Hypothesis: Acute inhalation exposure to reactive Co substances can lead to either 'persistent' inflammation or upper respiratory tract metaplasia or hyperplasia, both of which are still present two weeks post-exposure, whereas some Co substances have no toxic effect after acute inhalation. It is hypothesised that the aforementioned effects are either precursors developing into sub-chronic and chronic inhalation effects, or they may be biomarkers of an ability to cause long-term effects. Therefore, the finding of the acute effects can be used to form two read-across groups: (1) reactive Co substances which cause an inflammatory or metaplastic response after acute exposure, indicative of the potential to cause long-term inhalation toxicity and (2) non-reactive Co substances which do not cause any adverse effect after acute inhalation exposure, indicative of a lack of reactivity in terms of their inhalation toxicity behaviour.

¹¹ "Feasible" in this context means that a substance generates an inhalation atmosphere without prior major manipulation.

¹² Allavena, P., C. Garlanda, M. G. Borrello, A. Sica, and A. Mantovani. 2008. "Pathways connecting inflammation and cancer." *Curr Opin Genet Dev* 18 (1):3-10.. Coussens, L. M., and Z. Werb. 2002. "Inflammation and cancer." *Nature* 420 (6917):860-7. Shacter, E., and S. A. Weitzman. 2002. "Chronic inflammation and cancer." *Oncology (Williston Park)* 16 (2):217-26, 229; discussion 230-2.

Test-system and method: 'Persistent' inflammation was defined as histopathologically visible markers of inflammation (inflammatory oedema (perivascular), alveolar pulmonary oedema and pneumonia) present two weeks after an acute exposure (4 hours). Upper respiratory tract findings were defined as a change in the larynx present two weeks after an acute exposure (4 hours) leading to epithelial hyper- or metaplasia, with scoring mirroring the system used for markers of persistent inflammation. If the sum of all severity scores is above 5, this corresponds to a "+" (strong) or a "++" (very strong) response. Any score below 5 was marked as "-" (minimal / not present).

Status and Results: Acute inhalation tests have been completed on a total of 8 cobalt substances. Substances rated with "++" or "+" are grouped with Co metal and CoSO₄, substances with a "-" are placed in either an 'unreactive' group or a group still yet to be determined.

For the Co substances not feasible for acute inhalation toxicity testing, due to physical form or inability to generate an inhalation atmosphere, the potential need for substance manipulation (for feasibility purposes) will be carefully considered.

Conclusions: A significant difference in inflammatory response was observed between the Co substances that were reactive (all with severity scores of > 30) and those Co substances which were non-reactive (all measured to date with a severity score of < 1). It should be noted that Co carbonate with a score around 1 carries a harmonised classification as Carc 1B (based on read across from Co sulphate) and its classification is therefore outside of the framework of the grouping presented here.

All cobalt substances with inorganic ligands showed a good correlation between solubility and reactivity across the first three tiers.

Tier 4 – 28-day RDT inhalation testing (tricobalt tetraoxide)

Introduction: The higher-tier dataset is currently limited to 2 sub-chronic and chronic inhalation studies (both positive). Acute inflammation, sub-chronic and chronic effects correlate in both cases (Co powder and Co sulphate)¹³. Based on this limited dataset, there is a clear indication that "persistent inflammation" or "upper respiratory tract meta- or hyperplasia", as well as the lower tier data, are predictive of repeated dose inhalation toxicity. It is now being investigated whether a lack of the lower tier effects is predictive of a lack of repeated dose inhalation toxicity, or a different quality of effect.

¹³ Data from NTP TR 471 (cobalt sulfate) and TR 581 (cobalt metal)

Hypothesis: A lack of persistent inflammation after acute inhalation exposure is predictive of a lack of repeated-dose inhalation toxicity, or predictive of a different quality of effect. By conducting higher-tier testing on one Co substance with an inorganic ligand (Co₃O₄) that is negative (i.e. unreactive) in lower tiers, we intend to provide further evidence for the predictive power of the lower tier tests in indicating whether a substance can cause chronic inflammatory changes, potentially leading to cancer. Absence of lower tier toxicity would then allow identification of a non-reactive group of substances that would not be expected to induce repeated-dose inhalation toxicity or carcinogenicity.

Test system and method: A 28-day repeated dose inhalation toxicity study (nose-only) in rats (Sprague-Dawley) with one reactive Co substance (CoSO₄, positive for cytotoxicity and all tier 2 markers, positive in acute, subacute, subchronic and chronic in vivo inhalation toxicity tests) and an unreactive Co substance (Co₃O₄, negative for cytotoxicity and all tier 2 markers, negative for acute in vivo inhalation toxicity) with a 90-day recovery period. Investigations of haematology, histopathology, immunohistochemistry (8-OH-dG) and bronchoalveolar lavage (BAL; total cell count, differential cell count, β-glucuronidase, total protein, LDH, HIF-1α, IL-8, MCP-1) were undertaken.

Status and results: Testing completed; NOAEC (Males and Females): 5 mg Co₃O₄/m³; LOAEC (Males and Females): 20 mg Co₃O₄/m³ (corresponding to 14 mg Co/m³), based on a statistically significant increase in PMN cell %.¹⁴

Repeated exposure to tricobalt tetraoxide over 28 days resulted in a statistically significant increase in polymorphonuclear (PMN) cell % that persisted after 90 days of recovery. The increase in PMN cell % alongside fibrosis, increased lung weights (increased fibrotic tissue), upregulation of CINC-1 and MCP-1, decrease in macrophage percentages and an increase in lymphocyte percentage are attributed to a poorly soluble particle (PSP) or poorly soluble low toxicity particle effect (PSLT).

Conclusions: The testing lab stated that 28 days of exposure to 5 mg/m³ of Co₃O₄ led to effects that were toxicologically similar to a 90 day exposure to 5 mg/m³ of TiO₂ (known and characterised PSLT). After consultation with an expert of PSP/PSLT effects, it was recommended to perform a longer-term repeated-dose inhalations study to conclusively attribute this effect to tricobalt tetraoxide (representing the 'non-reactive' read-across group)

The HIF1- alpha results were inconclusive due to an extremely high standard deviation with the mid- and high-dose tricobalt tetraoxide groups. Due to this variation, an alternative measurement for hypoxia will be proposed for the 90-day RDT inhalation study.

¹⁴ There are no directly comparable RDT studies with Co sulfate or Co metal. In the 13-week study with Co sulfate, pulmonary effects were observed at all concentrations, the lowest being 0.3 mg Co sulfate hexahydrate corresponding to approx. 0.067 mg Co/m³. In the 13-week study with Co metal, no NOAEC was achieved. The Co metal inhalation LOAEC was at 1.25 mg Co/m³ (13-week RDT).

Tier 5 – 90-day RDT inhalation testing (tricobalt tetraoxide)

Introduction: Based on the 28-day RDT study results with tricobalt tetraoxide, a conclusion of a poorly soluble particle effect was recorded by the testing laboratory. An independent expert has stated that in order to conclusively determine if the inhalation effects associated with tricobalt tetraoxide exposure are due to a PSP or PSLT effect (and subsequent lung overload), a longer duration RDT study should be conducted. Tricobalt tetraoxide is the source substance for the poorly reactive group of substances in the inhalation read-across approach. To further protect this group of substances from a more conservative classification, longer duration data should be generated to compare against results from the reactive Co substances.

Hypothesis: Tricobalt tetraoxide has been negative for all markers associated with longer term inhalation toxicity as observed with the reactive substance group (source substances: Co sulphate and Co metal powder). It is hypothesised, based on the 'overall toxicological profile' of this substance, that a PSP/PSLT effect will be seen in a 90-day RDT study.

Test system and method: A 90-day repeated dose inhalation toxicity study (nose-only) in male and female Wistar rats. One poorly reactive Co substance (Co₃O₄, negative for cytotoxicity and all hypoxia, negative for acute in vivo inhalation toxicity) is proposed for testing at the following concentration levels: 2 (low), 8 (medium), 32 (high) mg/m³ with clean-air control and a 90-day recovery period. Aerosol to be generated by dispersing dry powder. Investigations of haematology, histopathology, immunohistochemistry (8-OH-dG) and bronchoalveolar lavage (BAL; total cell count, differential cell count, β-glucuronidase, total protein, LDH, HIF-1α, IL-8, MCP-1) should be undertaken.

Status and results: Awaiting decision on testing proposal, submitted to EU authorities (ECHA).

Does the research case study?

Describe the dose-response relationship in the dose range relevant to human exposure?

This method is more related to determine potential hazards associated with different cobalt compounds and thus, has not been matched to anticipate human exposures in any particular case. However, if human exposures can be seen to invoke either or both of the key events, or perhaps not, then a judgment can be made as to how serious the particular cobalt compound exposure might be.

Address human variability and sensitive populations?

This method is not designed to determine within-human variability in response to various cobalt compounds. Any judgment of hazard or lack of hazard by any cobalt compound run through this scheme would need additional characterization of potential human sensitive subgroups.

Address background exposures or responses?

This method is not designed to determine exposures to cobalt compounds, nor address background cobalt exposures. However, cobalt is an essential component of vitamin B12.

Address incorporation of existing biological understanding of the likely mode of action?

This method specifically incorporates several key events in the development of toxicity due to inhaled cobalt compound exposure, in particular:

Release of Co ion, cytotoxicity, upregulation of reactive oxygen species (ROS) and oxidative stress; and hypoxia

Persistent inflammation and/or upper respiratory tract reactivity upon acute exposure.

These key events are tied into a hypothesized mode of action to determine the appropriate category placement, either cancer causing or not, for various cobalt compounds that have not been tested in traditional experimental animal cancer bioassays.

Address other extrapolations, if relevant – insufficient data, including duration extrapolations, interspecies extrapolation?

The proposed tiered testing system is to anticipate the likely cancer causation potential of less studied cobalt compounds based on a read across from other, better-studied cobalt compounds. As such, this is best characterized as a hazard identification method. It does use shorter term effects, e.g. toxicity following acute inhalation, to predict longer term effects, e.g. cancer. However, duration extrapolation in terms of dose-adjustment and species extrapolations are not addressed in this method per se.

Address uncertainty?

As in any read-across scheme designed to forgo testing in experimental animals, uncertainties may exist in the use of in vitro data as a stand in for in vivo data. Part of this potential uncertainty is mollified by the use of interlocking tiered approaches that can readily be tied into the hypothesized two key events. However, judgment is still needed when the available data are at odds with expectations. In this case the development of a scheme weighting the various uncertainties might be useful.

Allow the calculation of risk (probability of response for the endpoint of interest) in the exposed human population?

This method does not allow the development of a probabilistic statement. Rather, it is a hazard identification scheme developed to forgo the need of extensive experimental animal testing.

Work practically? If the method still requires development, how close is it to practical implementation?

This method appears to work as intended. Two groups of cobalt compounds were distinguished with albeit, some uncertainty in the grouping of at least one compound. Extensive experimental animal testing appears to not be needed (see Tables 1 and 2). General applicability to other groups of compounds, especially metal compounds, might be envisioned.

Table 1. Summary of Co compounds and presence/absence of data in tiers.

Name	Formula	Bioelution data (12 compnds)	HIF1a, A549 cells (12 compnds)	ToxTracker (8 compnds)	Acute inhalation (8 compnds)	RDT inhalation (3 compnds)	Chronic inhalation (2 compnds)
Cobalt dichloride	CoCl ₂	Yes	Yes	Yes	x	x	x
Cobalt dinitrate	Co(NO ₃) ₂	Yes	Yes	x	x	x	x
Cobalt sulphate	CoSO ₄	Yes	Yes	x	Yes	Yes	Yes
Cobalt di(acetate)	Co(CH ₃ COO) ₂	Yes	Yes	x	x	x	x
Cobalt carbonate	CoCO ₃	Yes	Yes	Yes	Yes	x	x
Cobalt	Co	Yes	Yes (two types of Co powder)	Yes	Yes	Yes	Yes
Cobalt dihydroxide	Co(OH) ₂	Yes	Yes	Yes	Yes	x	x
Cobalt oxide	CoO	Yes	Yes	x	Yes	x	x
Tricobalt tetraoxide	Co ₃ O ₄	Yes	Yes	Yes	Yes	Yes	x
Cobalt sulfide	CoS	Yes	Yes	Yes	Yes	x	x
Cobalt hydroxide oxide	CoOOH	Yes	Yes	Yes	x	x	x
Lithium cobalt dioxide	CoLiO ₂	Yes	Yes	Yes	Yes	x	x

Table 2. Details of Co compounds and presence/absence of data in tiers.

Substance	TIER 1			TIER 2a		TIER 2b, ToxTracker					TIER 3	TIER 4	TIER 5	TIER 6	Classification
	Cobalt Release ($\mu\text{g Co/ml}$) ¹			Cyto-tox	HIF-1 α	DNA damage	P53 up-reg	Protein Damage	Oxid. Stress	HIF-1 α up-reg	Upper Resp. or Persist. Inflamm. ²	RDT (28 days)	RDT (90 days)	Rodent Cancer Study	
	Int.	Alv.	Lys.												
CoCl ₂ #	228	340	446*	+	+	-	-	-	+	+	Not feasible	Not planned		1B; H350i (harmonised classification)	
Co(NO ₃) ₂ #	84	64	408	+	+	Read-across from CoCl ₂					Not feasible	Not planned		1B; H350i (harmonised classification)	
CoSO ₄	278	216	331*	+	+	Read-across from CoCl ₂					+	+	+	+	1B; H350i (harmonised classification)
Co di(acetate)#	46	49	363	+	+	Read-across from CoCl ₂					Not feasible	Not planned		1B; H350i (harmonised classification)	
CoCO ₃ #	19	6.7	1078	-	+	-	-	-	+	+	+/-	Not planned		1B; H350i (self-classification)	
Co	75	40	1907	+	+	-	-	-	+	+	++	+	+	+	1B; H350 all routes (harmonised classification)
Co(OH) ₂	3.6	2.9	1214	+	+	-	-	-	+	+	+	Not planned		Grouped with CoSO ₄ based on tier 3 results 1B; H350i (self-classification)	
CoO	25	9.1	1343	+	+	Testing ongoing					+	Not planned		Grouped with CoSO ₄ based on tier 3 results 1B; H350i (self-classification)	
Co ₃ O ₄	0.05	0.08	22	-	-	-	-	-	-	-	-	-	TBC Testing proposal	Insufficient data for classification	

Substance	TIER 1			TIER 2a		TIER 2b, ToxTracker					TIER 3	TIER 4	TIER 5	TIER 6	Classification
	Cobalt Release ($\mu\text{g Co/ml}$) ¹			Cyto-tox	HIF-1 α	DNA damage	P53 up-reg	Protein Damage	Oxid. Stress	HIF-1 α up-reg	Upper Resp. or Persist. Inflamm. ²	RDT (28 days)	RDT (90 days)	Rodent Cancer Study	
Int.	Alv.	Lys.													
CoS	15	15	82	-	-	-	-	-	-	-	-	tbd	tbd	tbd	Grouped with Co3O4 based on tier 3 results
CoOOH	0.19	0.1	68	-	-	-	-	-	-	-	Not feasible	tbd	tbd	tbd	Grouped with Co3O4 based on tier 3 results
CoLiO ₂	0.05	0.05	15	-	-	-	-	-	-	-	-	tbd	tbd	tbd	Grouped with Co3O4 based on tier 3 results