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Anne van der Meeren, Karine Devilliers, Sylvie Coudert, Agnès Moureau, Martine Defrance, et al.. Determination of drug efficacy to dissolve cobalt oxide particles in cellular models: Towards a therapeutic approach to decrease pulmonary retention. *Toxicology in Vitro*, 2022, 84, pp.105448. 10.1016/j.tiv.2022.105448 . hal-03749151

HAL Id: hal-03749151

<https://hal.science/hal-03749151>

Submitted on 10 Aug 2022

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Determination of drug efficacy to dissolve cobalt oxide particles in cellular models: towards a therapeutic approach to decrease pulmonary retention.

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The authors declare that they have no conflict of interest.

Acknowledgements

The authors acknowledge Pascale Delangle (CEA/DRF/IRIG/SYMMES) and Laurence Lebaron-Jacobs (CEA/DRF/PROSITON) for fruitful discussions and constant support, Mélody Laporte and Célia Gouzerh for technical assistance. This work was supported by the CEA and Electricité De France (EDF).

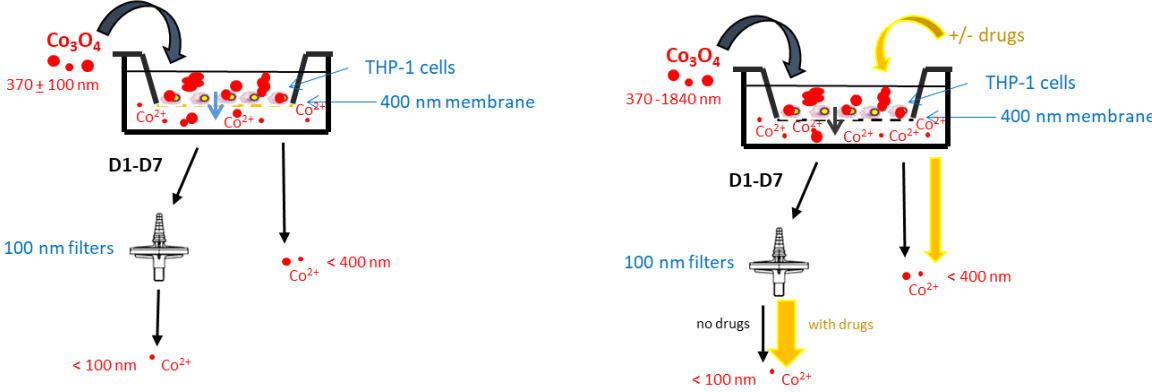
Key words: macrophages, epithelial barrier, cobalt particles, dissolution, decorporating agent

Highlights:

- Ascorbic acid and chelators enhance intracellular cobalt oxide particles dissolution
- Cobalt translocation across an epithelial barrier is not compromised by these drugs
- A new decorporating strategy following radioactive cobalt particle inhalation is proposed

Graphical abstract

Effect of drugs on uptake and release of cobalt particles by THP-1 cells



Abstract

Following accidental inhalation of radioactive cobalt particles, the poorly soluble and highly radioactive Co_3O_4 particles are retained for long periods in lungs. To decrease their retention time is of crucial importance to minimize radiation-induced damage. As dissolved cobalt is quickly transferred to blood and eliminated by urinary excretion, enhancing the dissolution of particles would favor ^{60}Co elimination. We evaluated the ability of ascorbic acid alone or associated with the chelating agents DTPA¹, DFOB² or EDTA³ to enhance dissolution of cobalt particles after macrophage engulfment, and the drug effects on the translocation of the soluble species CoCl_2 through an epithelial barrier. We exposed differentiated THP-1 macrophage-like cells and Calu-3 lung epithelial cells cultured in a bicameral system to cobalt and selected molecules up to 7 days. DTPA, the recommended treatment in man, used alone showed no effect, whereas ascorbic acid significantly increased dissolution of Co_3O_4 particles. An additional efficacy in intracellular particles dissolution was observed for combinations of ascorbic acid with DTPA and EDTA. Except for DFOB, treatments did not significantly modify translocation of dissolved cobalt across the epithelial lung barrier. Our study provides new insights for decorporating strategies following radioactive cobalt particle intake.

Introduction

Cobalt (Co) is widely used in industrial processes for its magnetic properties, corrosion resistance, wear resistance, and/or its strength at elevated temperatures (Barceloux, 1999). The γ -emitter ^{60}Co is a neutron activation product formed from structural materials in nuclear reactors. During procedures such as maintenance, ^{60}Co can be released as highly mobile and highly radiant submicronic cobalt oxide particles ($\text{Co}_3\text{O}_4\text{P}$). Accidental internal or cutaneous contamination of

¹ Diethylenetriamine-pentaacetic acid

² Desferrioxamine B

³ Ethylenediamine-tetraacetic acid

workers must be considered, as cases of internal contamination have been reported (Beleznay and Osvay, 1994; Davis et al., 2007; Gupton and Brown, 1972; Hegde et al., 1979; Le Guen and Ansoborlo, 2005; Newton and Rundo, 1971; Raghavendran et al., 1978; Vrba et al., 2007) . $\text{Co}_3\text{O}_4\text{P}$ are poorly soluble, leading to long retention times in lungs (ICRP, 2016; Kreyling et al., 1991) . In case of inhalation of $^{60}\text{Co}_3\text{O}_4\text{P}$ and because cobalt radioisotopes are highly penetrating gamma emitters, the long-time retention of particles in the lungs may cause pulmonary damage as a consequence of chronic radiation exposure (Davis et al., 2007) . In contrast to $\text{Co}_3\text{O}_4\text{P}$, soluble forms of cobalt (Co^{2+}) have been shown to be rapidly eliminated from the body by urinary excretion with 68% of cobalt eliminated with a half-life of 1.9 h following intravenous administration in rats (ICRP, 2016; Weber et al., 2012) . However, a long-term component of retention has been described for cobalt in different studies (ICRP, 2016) .

Currently, whatever the route of internal contamination and the physicochemical form of incorporated cobalt, the recommendation for the management of contamination is the intravenous administration of Ca-DTPA or EDTA (REMM) . In addition, various decorporating drugs have been proposed to enhance urinary excretion of cobalt following intravenous administration of cobalt chloride (CoCl_2) in animal studies. Treatments with DTPA (Diethylenetriamine-pentaacetic acid calcium trisodium salt hydrate) or EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate) significantly increased cobalt excretion and decreased cobalt retention in several tissues (Fisher and Dunavant, 1978; Llobet et al., 1986, 1988; Zylitz et al., 1975) . Some success has also been obtained with molecules selected for their Co^{2+} -binding properties (Levitskaia et al., 2010a; Levitskaia et al., 2011; Levitskaia et al., 2010b; Llobet et al., 1986, 1988) . However, following inhalation of poorly soluble $\text{Co}_3\text{O}_4\text{P}$, the most likely scenario at the work place, only a small fraction of soluble cobalt is available to chelating agents. Similarly to actinides, chelation by DTPA is expected to concern mainly the ionized form of cobalt, Co^{2+} , suggesting that DTPA treatment would have limited efficacy in case of $\text{Co}_3\text{O}_4\text{P}$ inhalation (Gremy et al., 2010) . It is expected to access to the soluble fraction of cobalt

resulting from the degradation of Co_3O_4 particulate matter (free cations or complexes of small sizes) whether retained in lung fluids or in the blood after crossing the epithelial lung barrier.

Thus, to decrease the retention time of $^{60}\text{Co}_3\text{O}_4\text{P}$ in the lungs by facilitating the cobalt dissolution into Co^{2+} seems the most effective strategy for the treatment of contaminated workers following inhalation of radioactive submicronic $^{60}\text{Co}_3\text{O}_4\text{P}$.

After deposition in the deep lung, absorption of insoluble compounds is a two-stage process: first, dissociation of the particles into chemical forms that can be absorbed into body fluids (dissolution) and second, absorption into body fluids of these soluble materials across the epithelial lung barrier (uptake). These processes result respectively from the phagocytosis by alveolar macrophages after recognition of the contaminant as a foreign body, and the intracellular dissolution of the contaminant followed by the translocation into the systemic compartment after crossing the epithelial barrier. The critical role for macrophages following $\text{Co}_3\text{O}_4\text{P}$ inhalation has been widely described (Collier et al., 1992; Kreyling, 1992; Kreyling et al., 1990; Lundborg et al., 1992) . Intracellular dissolution within macrophages represents the initial step of the lung clearance mechanism before translocation and the rate-determining step of absorption (Kreyling, 1992; Kreyling et al., 1990) . In addition to physicochemical properties of the particles (size, specific surface area, surface properties, etc...), other parameters such as the pH of phagolysosomes, or the presence of intracellular chelating agents are likely to be involved in the uptake and intracellular dissolution (Kreyling et al., 1991; Kreyling et al., 1990) .

With the ultimate goal of proposing decorporation strategies based on increasing the dissolution of Co_3O_4 particles, we undertook a multi-stage study. In the first step, we used acellular *in vitro* models to screen and select various drugs, such as cobalt ligands and reductants, able to increase the dissolution of $\text{Co}_3\text{O}_4\text{P}$ in lung fluid surrogates (Van Der Meeren et al., 2020) . Our results showed that the most efficient effect was obtained by combining the use of a reductant, ascorbic acid, to alter the redox state of Co and destabilize the surface of the $\text{Co}_3\text{O}_4\text{P}$, with Co chelating agents

such as DTPA, EDTA or DFOB. Our choice of ascorbic acid, was based on a similar approach reported on iron oxide particles. Ascorbic acid increased the dissolution of Fe₃O₄ particles (Afonso et al., 1990) with a more prominent effect when used in combination with efficient Fe-chelating molecules such as EDTA (Ananthan et al., 2003) . The proposed mechanism of action was ascorbic acid-induced reduction of Fe(III) into Fe(II) at the particle surface destabilizing the surface structure, and reduction of the Fe(III)-EDTA complexes formed in solution, with the resulting Fe(II)-EDTA complex proposed to accelerate the reductive particle dissolution. We have shown the combined effect of ascorbate and DTPA, EDTA or DFOB to enhance Co₃O₄P dissolution in lung fluids surrogates (Van Der Meeren et al., 2020) .

The present work is the second step of the study, and aims at evaluating the effect of ascorbic acid, in association with Co-high affinity chelating agents to increase dissolution of cobalt particles in cellular models. We chose *in vitro* cellular models representative of the two crucial steps in lung retention and clearance of Co₃O₄P: macrophages and lung epithelial barrier. We determined the efficacy of the previously selected molecules to enhance dissolution of Co₃O₄P after their engulfment by macrophage-like cells and evaluated the consequences on the transfer of dissolved cobalt across an *in vitro* model of functional epithelial lung barrier. To target the fraction of dissolved cobalt potentially remaining in the lungs or in secondary organs, and increase its excretion, we tested Co-high affinity chelating agents (DTPA, EDTA and DFOB) together with acid ascorbic. It has been widely described for Pu and Am, that Pu/Am-DTPA complexes are rapidly excreted through urinary excretion and not retained in secondary organs. Knowing the high affinity of DTPA towards cobalt, similar effect is expected.

Finally, postulating that similar effects of treatments can be expected *in vitro* whatever the isotope, the present studies were conducted with the stable isotope, ⁵⁹Co, using Co₃O₄ submicronic particles, similar in size to those involved in an accidental inhalation at the work place (Davis et al., 2007) .

1. Materials and Methods

2.1 Cobalt compounds

Cobalt particles ($\text{Co}_3\text{O}_4\text{P}$) were obtained from Sigma-Aldrich. Transmission electron microscopy (TEM) images show that particles are mainly aggregated, and exhibit a polyhedral structure of heterogeneous size, in the 100 to 400 nm range (Darolles et al., 2013). Using the Brunauer–Emmett–Teller method, their mean specific surface area was found to be $5 \text{ m}^2/\text{g}$. Once this parameter had been evaluated, the mean diameter of the particles was calculated to be 200 nm (Darolles et al., 2013). This result is in accordance with the size range measured by electron microscopy and is representative of particles possibly released during an accident at the work place in a nuclear power plant (Davis et al., 2007). In culture medium, dynamic light scattering measurements showed the detection of aggregates ranging from 600 to 2000 nm (Darolles et al., 2013).

Cobalt particles were suspended in deionized water to prepare stock solutions with a cobalt concentration of 7.9 mg/ml. Suspensions were then sonicated for 15 min with an Autotune sonicator (Fisher Scientific; Illkirch, France) operated at 750 W and stored at -20°C until use, as previously described (Ortega et al., 2014; Uboldi et al., 2016).

Cobalt chloride (CoCl_2) was used to mimic the dissolved fraction of cobalt oxide particles, as it has been shown that CoCl_2 remains in a completely dissolved state even in complex media such as lung surrogates (Stopford et al., 2003). $^{57}\text{CoCl}_2$ was obtained from Perkin Elmer (Courtaboeuf, France). A stock solution of approximately 2500 MBq/ml ($3.12 \cdot 10^{11} \text{ Bq/mg}$) prepared in HCl 0.1M was further diluted in water and used at 10 MBq/ml. To correct from the decay half-life (271 days), a reference date was used. Cells were exposed to activity levels ranging from 10 to 40 kBq/ml, corresponding to 0.1 - 5 nM ^{57}Co .

2.2 Reagents

Diethylenetriamine-pentaacetic acid (Ca-DTPA) was purchased from Pharmacie centrale des Armées (France). Ethylenediamine-tetraacetic acid (EDTA), Ascorbic acid (Asc) and Desferrioxamine B (DFOB) were obtained from Sigma-Aldrich (France). One hundred mM solutions freshly prepared with deionized ultrapure water were further diluted in the culture medium immediately before treatment to obtain various concentrations (5-500 μ M). The term “drug” will be used in the present manuscript to refer to the reducing agent L- ascorbic acid and to metal chelators DTPA, DFOB and EDTA.

2.3 Cell culture

The THP-1 cell line (ATCC, France) is derived from the blood of a boy with acute monocytic leukemia (Tsuchiya et al., 1980) . THP-1 cells were routinely maintained at 37° C in 5 % CO₂ atmosphere in complete RPMI medium (Fisher Scientific) supplemented with penicillin-streptomycin (1%) and fetal bovine serum (FBS, Pan Biotech, 10 %), at a seeding concentration of 2-3 10⁵ cells/ml. THP-1 cells in suspension were seeded onto polyester membranes of inserts (0.33 cm² Transwell®, Corning) at a density of 1.0 x 10⁵ cells/well and differentiated into macrophage-like cells by adding 50 nM PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) for 3 days.

Human lung adenocarcinoma cells (Calu-3, ATCC, France) were cultured in DMEM medium (Fisher scientific) supplemented with penicillin-streptomycin (1%) and fetal bovine serum (FBS, Pan Biotech, 10 %). Cells were seeded onto polyester membranes of inserts (Transwell®, Corning) at a density of 2.0 x 10⁵ cells/well. Inserts had a growth area of 0.33 cm² and porosity of 0.4 μ m. Calu-3 cells were cultured for approximately 10 days under liquid-covered conditions with culture medium changed both in the apical and basal compartments every 2-3 days. Cell monolayer formation was evaluated by measuring transepithelial electrical resistance (TEER) using an epithelial Voltohmmeter (EVOM). TEER values >400 Ω .cm² are usually taken as indicative of a tight epithelium formation (Kreft et al., 2015) . TEER readings were obtained by subtracting the resistance in ohms of the blank insert (no cells) from the recorded resistance of the cell monolayer, and subsequently multiplying the resulting

value by the effective membrane surface area of the insert to yield $\Omega \cdot \text{cm}^2$. Inserts with TEER > 1000 $\Omega \cdot \text{cm}^2$ were then used for the translocation studies.

2.4 Toxicity evaluation

To detect loss of membrane integrity associated with cell death, the lactate dehydrogenase (LDH) release from cells was evaluated using the Homogeneous Membrane Integrity Assay (CytoTox-ONE™, Promega) and the potential disturbances of cell metabolism were evaluated using a resazurin assay (Presto blue, In Vitrogen).

Cells were seeded in 96-well plates (3×10^4 cells/well, 100 μl /well). Twenty four hours after plating, for Calu-3 cells, or 3 days after PMA treatment for THP-1 cells, culture medium was removed and replaced by fresh medium containing drugs (5-500 μM) or $\text{Co}_3\text{O}_4\text{P}$ (5-500 $\mu\text{g}/\text{ml}$) for THP-1 cells. At each time of toxicity evaluation (days 1, 2, 3 and 7), 50 μl of culture medium were taken and placed into a 96-well plate. After 20 min incubation at room temperature, 50 μl of CytoTox-ONE™ reagent were added to each sample. The amount of fluorescence was measured, after addition of a stop solution, using a microtiter plate reader (TriStar 2S, Berthold) with excitation at 560 nm and emission at 600 nm. For the resazurin assay, 5 μl Presto Blue reagent were added to the remaining medium in culture plates containing cells. Absorbance (570 nm) was measured 10 min thereafter using a microtiter plate reader (TriStar 2S, Berthold).

Toxicity of drugs (respectively 500 μM or 10 μM) in the presence of Cobalt was assessed on the two cell models in parallel to the transfer studies. For such determination, 50 μl were collected in the basolateral chamber before collection of medium for Cobalt measurement. LDH release was measured in the 50 μl aliquots as described above.

Results are expressed as percentage of fluorescence (or absorbance) values in the sample/mean fluorescence (or absorbance) values from triplicate of non-treated control cells.

2.5 Dissolution and transfer studies (THP-1 differentiated cells)

At Day 0, three days following PMA addition on THP-1 cells, the medium was replaced by fresh medium and $^{59}\text{Co}_3\text{O}_4\text{P}$ (50 $\mu\text{g}/\text{ml}$) were added to the apical chamber. Sampling of medium in the basolateral chamber was done 1, 2, 3 and 7 days after cobalt addition. At each collection time, the total volume of medium in the basolateral chamber was collected and replaced by an equivalent volume of fresh medium (750 μl). Cells were then returned to 37 °C incubation for the indicated periods of time (1- 7 days). At Day 7, medium from apical chambers as well as cell lysates were also collected. Cells were thoroughly rinsed using DPBS (Gibco) before lysis with Triton X100 0.5 % (Sigma).

To determine the effects of the various treatments on $\text{Co}_3\text{O}_4\text{P}$ dissolution and on transfer of dissolved material, the drugs were added in both apical and basolateral chambers at the initiation of the experiment, and in the basolateral medium at each collection time.

In control experiments, transfer was measured in the medium in the absence of macrophage-like cells, to evaluate the amount of cobalt able to cross the insert membrane.

To assess the proportion of dissolved cobalt or nanoparticles initially present in cobalt oxide suspensions, freshly prepared dilutions in culture medium were filtered through 100 nm syringe filters (Acrodisc, Pall). This proportion was found to be below 0.1 % in each experiment. Specific filtration studies were carried out in parallel of transfer studies. For this purpose, samples from dedicated wells (basolateral chamber at each collection time, and cell lysate and apical medium at day 7) treated similarly to others were collected and filtered using 100 nm filter units.

2.6 Transfer across epithelial cell layer (Calu-3 cells)

At Day 0, when epithelial monolayers had reached satisfying properties (TEER > 1000 $\Omega\cdot\text{cm}^2$) $^{57}\text{CoCl}_2$ (10-40 kBq/ml) was added to the epithelial monolayer.

Sampling of medium in the basolateral chamber was done 1, 2, 3 and 7 days after cobalt addition. At each collection time, the total volume of medium in the basolateral chamber was collected and replaced by an equivalent volume of fresh medium (750 μl). Cells were then returned to 37 °C

incubation for the indicated periods of time (1- 7 days). At Day 7, the insert content was collected in addition to the medium from basolateral chamber as for the other time points. Cells were thoroughly rinsed using DPBS and lysed with triton X100 0.5%.

To determine the effects of the various treatments on CoCl_2 transfer, the drugs were added in both apical and basolateral chambers at the initiation of the experiment, and in the basolateral medium at each collection time.

2.7 Cobalt measurement using ICP-MS and gamma spectrometry

For $^{59}\text{Co}_3\text{O}_4\text{P}$ studies, all samples (medium from apical and basal chambers and cell lysates) were collected in polypropylene vials (Nalgen) and digested with concentrated ultrapure 30 % HCl and 65 % HNO_3 as previously described (Ortega et al., 2014) . Digested samples were diluted in HNO_3 1% and analyzed using ICP-MS (NexION 350X, PerkinElmer or ICAPO Thermo). The calibration was conducted with 0.1, 0.5, 2, 10, 20, 50 $\mu\text{g/L}$ standards prepared in HNO_3 1%. Gallium (Analab) was used as internal standard.

For radioactive studies (^{57}Co), all samples (medium from apical and basal chambers and cell lysates) were deposited in Petri dishes and were further covered with non-woven swab to obtain an homogeneous deposit all over the surface. Activity was measured using an HPGe low background γ counter (Canberra). Samples were counted for 10 minutes to 1 h depending on the level of activity.

The total cobalt (mass or activity) recovered over 7 days in basolateral chambers at each collection time, and at day 7 in cell lysate and apical chambers, is considered to represent the initial cobalt added to the apical chamber at D0. The percentage of transfer is expressed as the cumulated cobalt measured in the basolateral chamber at each sampling time divided by the initial deposited cobalt.

2.8 Data processing

All experiments were carried out using triplicate cell samples and were repeated 1-3 times depending on experimental conditions. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.) or Sigma plot (sigma plot v12.5). Differences between groups were

determined with one way ANOVA, set at 95 % confidence interval as indicated in the table or figure legends. Data are presented as mean \pm SD.

3.Results

3.1 THP-1 cell model

3.1.1 Toxicity evaluation of cobalt particles and drugs

To determine the concentration of cobalt particles at which transfer experiments could be performed with no interference from cell toxicity, we evaluated the toxicity of $\text{Co}_3\text{O}_4\text{P}$ on differentiated THP-1 macrophage-like cells using two complementary assays evaluating cell membrane integrity (LDH release) or metabolism (resazurin assay). No major membrane damage was observed over 7 days with concentrations ranging from 10 to 500 $\mu\text{g}/\text{ml}$ $\text{Co}_3\text{O}_4\text{P}$ (Figure 1A). However, disturbances in cell metabolism were evidenced as early as 24 h post exposure to the two higher $\text{Co}_3\text{O}_4\text{P}$ concentrations of 250 and 500 $\mu\text{g}/\text{mL}$ (Figure 1B). These results confirm the low toxicity of $\text{Co}_3\text{O}_4\text{P}$ as compared to that of soluble cobalt ions observed on epithelial cells (Ortega et al., 2014) .

For the subsequent dissolution experiments, the concentration of 50 $\mu\text{g}/\text{ml}$ of $\text{Co}_3\text{O}_4\text{P}$ was used to avoid cell and membrane cytotoxicity. This concentration is higher than what can be found in an inhalation case, even accidental. However, due to the very low solubility of particles, this concentration was chosen to allow the detection of soluble cobalt in the basolateral compartments with good accuracy and reproducibility.

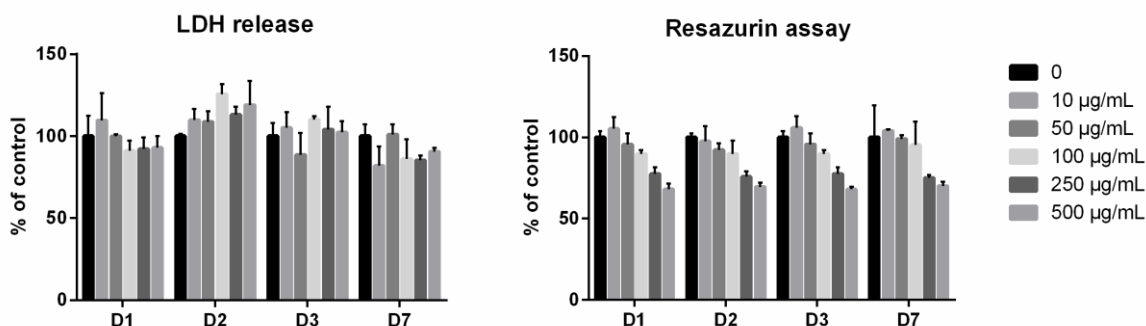


Figure 1: Toxicity of $\text{Co}_3\text{O}_4\text{P}$ in THP-1 cells. $\text{Co}_3\text{O}_4\text{P}$ (10-500 $\mu\text{g}/\text{ml}$) were added on differentiated cells plated in 96 well plates. One, 2, 3 and 7 days post treatment, the toxicity of Cobalt was assessed using LDH release measurement (CytoTox One) and resazurin assay (Presto Blue). Data are normalized to control conditions (untreated cells) at the same time point. Results are mean percentages/control \pm SD from 1 experiment in triplicate.

The toxicity of the different drugs was assessed using the same cytotoxicity assays, from 1 to 7 days post exposure. Concentrations tested ranged from 5 μM to 500 μM . No effect was observed for the addition of up to 500 μM of ascorbic acid (Asc). A decrease in cell viability was measured in our cell model for concentrations above 50 μM for DTPA, or for DTPA, DFOB and EDTA associated with Asc (supplementary data Tables S1 and S2). Nevertheless, the highest concentration of 500 μM was used in our transfer experiments. This concentration was based on a rough concentration calculation deduced from the recommended dosage for DTPA administered intravenously in case of contamination (30 $\mu\text{mole}/\text{kg}$, <http://agence-prd.ansm.sante.fr/php/ecodex/frames.php>, access date Dec 8th, 2021). A similar concentration was used for all other treatments. Table 1 reports data obtained after treatment with 500 μM drugs in one specific experiment. The full set of data is available in Table S1 and S2 in supplementary material. It has to be noted that, when used in combination with Asc, DTPA toxicity was slightly less than when used alone. The combination Asc/EDTA is slightly less toxic than the other conditions (Table 1). Our viability tests were conducted over 7 days with no medium replacement, which is not the case when cells are grown in the

bicameral system, in which the basal medium is replaced by fresh medium at each collection time. A lower toxicity is therefore expected in these later experimental conditions.

Cobalt concentrations of 50 µg/ml and drugs concentrations of 500 µM correspond to a drug/Co ratio of approximately 3.4.

The higher chemical toxicity of soluble cobalt as compared to particulate forms has been demonstrated (Danzeisen et al., 2020; Ortega et al., 2014) . Because treatments are expected to increase solubility and thus the concentration of soluble cobalt, LDH release was measured over 7 days in the presence of both Co₃O₄P and drugs. The toxicity of drugs (500 µM) in the presence of cobalt particles (50 µg/ml) was evaluated at days 1, 2, 3 and 7 post-exposure using the LDH release assay (Figure 2).

Table 1: Toxicity of drugs on THP-1 cells. Differentiated THP-1 cells plated in 96-well plates were exposed to various treatments at 500 µM. Viability was evaluated by LDH release assay (Cytotox one assay) and by resazurin assay (Presto blue assay) at D1, D2, D3 and D7 after treatment. Data are normalized to control conditions (untreated cells) at the same time point. Results are mean percentages/control ± SD from one representative experiment in triplicate.

		None	DTPA	Asc	Asc + DTPA	Asc + DFOB	Asc + EDTA
Resazurin assay	D1	100 ± 2.1	92.8 ± 2.6	90.3 ± 5.5	92.2 ± 6.6	81.1 ± 4.5	80.7 ± 4.6
	D2	100 ± 12.2	95.7 ± 1.6	87.8 ± 2.9	105 ± 2.3	83.2 ± 8.9	77.6 ± 3.1
	D3	100 ± 1.3	90.7 ± 0.3	86.9 ± 3.1	77.1 ± 1.2	88.8 ± 1.4	88.5 ± 3.4
	D7	100 ± 1.5	93.6 ± 4.4	93.8 ± 5.0	91.7 ± 1.7	90.4 ± 4.4	82.2 ± 6.1
LDH assay	D1	100 ± 0.5	209 ± 4.1	102 ± 0.9	242 ± 19.2	165 ± 23.1	189 ± 23.0
	D2	100 ± 1.5	181 ± 5.1	90.7 ± 2.0	145 ± 1.3	134 ± 23.4	182 ± 3.6
	D3	100 ± 0.8	188 ± 7.4	91.4 ± 2.8	179 ± 2.4	115 ± 1.9	139 ± 21.7
	D7	100 ± 2.0	229 ± 9.0	72.7 ± 17.3	135 ± 36.2	114 ± 6.6	121 ± 8.4

The addition of Ascorbic acid alone did not induce any toxicity in the presence of cobalt, and only a modest toxicity was observed upon addition of Asc and EDTA or DFOB. In the presence of cobalt, the DTPA-induced toxicity seems to be slightly attenuated, notably in the presence of Asc. This may be due to a direct interaction of DTPA with cobalt, and/or to the medium renewal over the 7-day period.

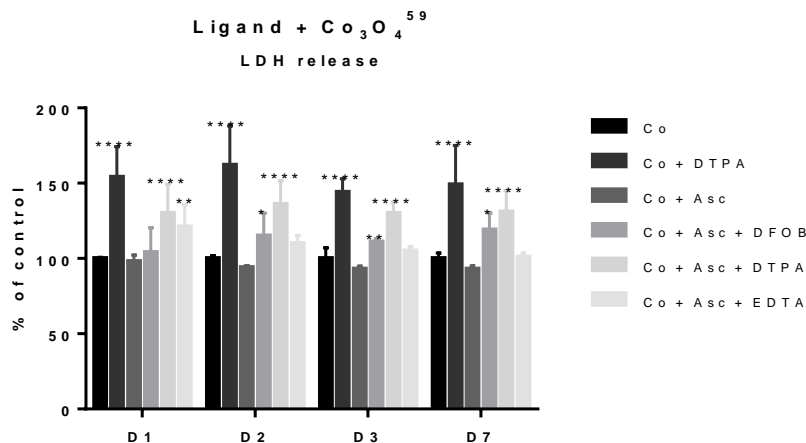


Figure 2: Toxicity of drugs on cobalt oxide treated THP-1 cells. $\text{Co}_3\text{O}_4\text{P}$ ($50 \mu\text{g}/\text{ml}$) were added on differentiated cells plated in insert wells in the absence or presence of various drugs ($500 \mu\text{M}$). $50 \mu\text{l}$ were taken from basolateral chambers 1, 2, 3 and 7 days after treatment. LDH release was measured using the CytoToxOne assay. Data are normalized to control conditions (cobalt treated cells, no drug) at the same time point. Results are means percentages/control \pm SD from 2 to 4 independent experiments each performed in triplicate. Statistically significant differences from the control were determined by one-way ANOVA followed by Dunnett's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.1.2 Dissolution of cobalt particles and transfer across an artificial membrane in the absence of treatments: cell-free versus THP-1 model

Before evaluating the efficacy of the selected drugs, i.e Asc alone or associated with DTPA, EDTA or DFOB, we first measured the dissolution of $\text{Co}_3\text{O}_4\text{P}$ in RPMI medium supplemented with 10% SVF and the subsequent ability of cobalt fractions to cross the synthetic membrane without treatments. For

this purpose, $\text{Co}_3\text{O}_4\text{P}$ ($50\mu\text{g}/\text{ml}$) were added in the upper compartment of a bicameral system in a cell free system. Cobalt translocation in these conditions was then compared to that obtained in the presence of THP-1 cells. In this experimental model using inserts with membranes of 400 nm pores, aggregates and particles over 400 nm are retained in the apical chamber, while soluble cobalt and smaller particles will be transferred to the basolateral chamber. The removal and replacement of the medium in the basolateral chamber at each collection time is expected to favor the continuous transfer of cobalt across the insert membrane, by contrast with a static model in which the medium is not renewed.

In the culture medium before incubation, less than 0.1% of cobalt was filterable through 100 nm filters. This is consistent with previous observation that native particles have an approximately 370 nm diameter and rapidly form aggregates in the culture medium (Ortega et al., 2014) . Accordingly, in the cell free system, the vast majority of cobalt ($\approx 89\%$) remained in the apical chamber over the 7-day incubation period (Table 2). At day 1, 0.33% of the initially deposited cobalt was found in the basolateral chamber. This proportion increased with time approximately three fold to 0.98% between day 1 and 7. Similar values are obtained in the presence of cells (0.9 % at Day 7) despite the internalization of about half of initially deposited cobalt in THP-1 cells. The other half remains in the medium of the apical chamber, from where it undergoes changes leading to cobalt transfer. The low transfer is in accordance with the low solubility of particles particularly in the complex lung surrogate medium Gambles (Van Der Meeren et al., 2020) .

Table 2: Cumulative transfer of cobalt in the presence or absence of THP-1 cells. Cobalt particles ($50\mu\text{g}/\text{ml}$) were added on differentiated cells plated in insert wells or in a cell-free system. Cobalt concentrations were measured by ICP-MS in the basolateral chambers 1, 2, 3 and 7 days after cobalt addition. Results are expressed as percentages of cumulative cobalt in basolateral chambers divided by initially deposited cobalt. Values are means \pm SD from 3 independent experiments in triplicate.

Basolateral chamber (%/initial)

Apical chamber

Cell lysate

	D1	D2	D3	D7	(%/initial)	(%/initial)
<i>THP-1 cells</i>	0.25 ± 0.07	0.46 ± 0.10	0.60 ± 0.10	0.90 ± 0.14	52.7 ± 9.5	46.4 ± 9.6
<i>No cells</i>	0.33 ± 0.12	0.60 ± 0.16	0.77 ± 0.19	0.98 ± 0.22	89.3 ± 11.22	-

3.1.3 Effect of drugs on the dissolution of $\text{Co}_3\text{O}_4\text{P}$ after macrophage-like cell engulfment and transfer across an artificial membrane:

We have shown in a previous work that some molecules, alone or in association, were able to increase $\text{Co}_3\text{O}_4\text{P}$ dissolution in acellular models (Van Der Meeren et al., 2020). Drugs showing the highest efficacy in these acellular models were tested on $\text{Co}_3\text{O}_4\text{P}$ -loaded THP-1 cells representing the main phagocytic cells in lungs, as well as in the cell free model to mimic the fraction of particles that was not internalized.

In both systems, all treatments but DTPA increased transfer of cobalt as compared to untreated controls (Figure 3). The most prominent effect of treatments occurs at D1 after treatment, with a decreasing effect over time, although the drugs were re-added in the culture medium at each collection time. An explanation for this observation is that the percentage of cobalt transferred in the basolateral compartment increases to a smaller extent in the presence of treatments (1.6-2 fold) than in their absence (3 fold). It has to be noted that the time-dependent effect is more pronounced in the cell free system than in the presence of macrophage-like cells. These results suggest that less cobalt is available to chelating agents over time and/or that the spontaneous dissolution of particles occurring over time apparently limits the effect of treatments. At D1, a higher increase in transfer of cobalt in the presence of drugs as compared to untreated controls is measured in the cell free system (approximately 3.5-4 times, Figure 3B) than in the presence of cells (around three times, Figure 3A). Thereafter, an enhancement factor of cobalt transfer of approximately 2.5 is observed in the cell free system for the three other time-points.

Only small differences are observed between the transfer obtained after Asc treatment alone or Asc combined with chelators. Nonetheless, at day 7, the highest percentage of Co transferred is obtained

with the Asc + DTPA combination. It is of 2.35 % in the cell-free system and of 1.6 % in the presence of cells, to be compared respectively to 0.98 and 0.9 % in the absence of drugs (Table 2).

The differences in cobalt transfer between setups containing or not THP-1 cells could be explained by a less direct action of treatments when $\text{Co}_3\text{O}_4\text{P}$ are engulfed by cells, which concerns about 50 % of the initially deposited particles (Table 2).

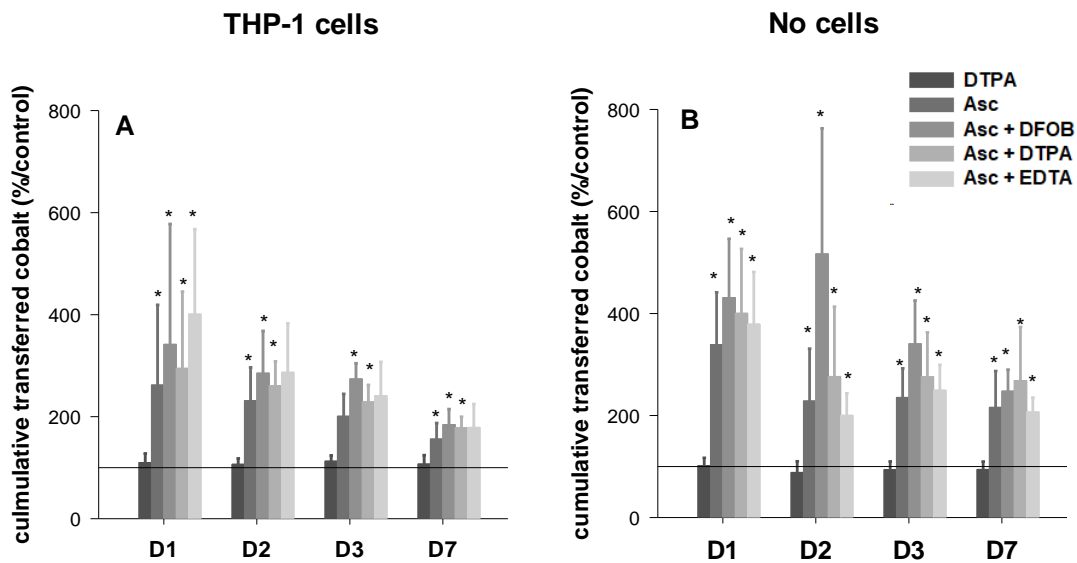


Figure 3: Cumulative transfer of cobalt in the presence (A) of THP-1 cells or in absence of cells (B). $\text{Co}_3\text{O}_4\text{P}$ ($50 \mu\text{g/ml}$) were added on differentiated cells plated in insert wells (A) or in a cell-free system (B). The transfer was measured without drugs (control), as well as in the presence of DTPA, Asc, or combinations of Asc with DFOB, DTPA or EDTA. Each drug was used at $500 \mu\text{M}$. Cobalt concentrations were measured by ICP-MS in the basolateral chambers 1, 2, 3 and 7 days after $\text{Co}_3\text{O}_4\text{P}$ addition. Results are expressed as percentages of cobalt in basolateral chambers in treated wells divided by cobalt in basolateral chambers in wells without drugs. Values are means \pm SD from 3 independent experiments each performed in triplicate. Statistically significant differences from the control (no drug) were determined by one-way ANOVA followed by pairwise multiple comparison (Dunn's method): * $p < 0.05$. The horizontal line is 100% (no effect versus control).

3.1.4 Intracellular cobalt content in THP-1 cells following treatments

Determining the effect of treatments on particle retention is an important feature, as the long-term retention in macrophages might induce biological consequences such as modification of the inflammatory status of these immunocompetent cells (Laskin et al., 2011) as well as radiation induced damage in the neighboring cells. Measurement of cobalt concentration in lysates from macrophage-like cells at Day 7 showed that whereas Asc alone or associated with EDTA does not significantly change the cell content in cobalt, DTPA, Asc with DTPA and Asc with DFOB decrease intracellular cobalt content. Further investigation would be needed to evaluate whether this results from an initial compromised phagocytosis in the presence of these drugs or to an enhanced release of dissolved cobalt from the intracellular compartment over the 7-day period. Caution has to be brought in the interpretation of the results due to the altered cell viability in the presence of these drugs. Thus, the decreased cell retention might be, at least partly, a consequence of cell membrane damage, as indicated by the LDH release at day 7 (Figure 2).

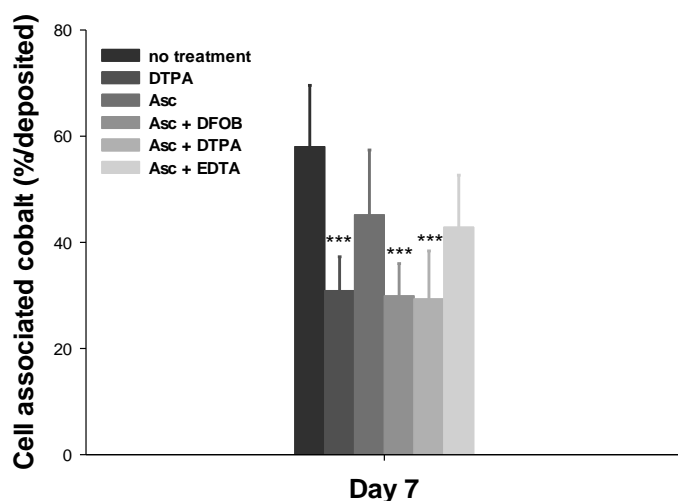


Figure 4: Intracellular cobalt content in THP-1 cells. $\text{Co}_3\text{O}_4\text{P}$ ($50 \mu\text{g}/\text{ml}$) were added on differentiated cells plated in insert wells in the presence or not of DTPA, Asc, Asc with DFOB, DTPA or EDTA ($500 \mu\text{M}$). Cobalt concentrations were measured by ICP-MS in cell lysates 7 days after cobalt addition. Results are expressed as

*percentages of cobalt in cell lysates divided by deposited. Values are means \pm SD from 2-4 independent experiments each performed in triplicate. Statistically significant differences from the control (no treatment) were determined by one-way ANOVA followed by pairwise multiple comparison (Dunn's method). *** $p < 0.005$*

3.1.5 Characterization of the transferred compounds

The cobalt present in the basolateral chamber ($< 1\%$ /initial) may not solely result from dissolution of the $\text{Co}_3\text{O}_4\text{P}$. The size of the pores separating the two chambers being of 400 nm, we cannot exclude the possibility for particles smaller than 400 nm to have crossed the membrane. Thus, changes resulting in cobalt transfer may include, in addition to dissolution, dislocation of aggregates or degradation of the pristine particles generating particles of smaller size. To verify this possibility, the medium harvested at the different collection times in the basolateral compartment of dedicated wells was filtered through 100 nm filters. Cobalt concentrations measured in the filtrates were compared to those of non-filtered samples, obtained in the same experimental conditions. Both THP-1 and cell-free systems were considered (Table 3).

In the absence of treatment, the comparison of filtered and non-filtered samples drives important statements. First, at day 7, half of the initially deposited cobalt is found in cell lysates. However, this proportion drops to $0.57\% \pm 0.47$ when cell lysates are filtered (data not shown) indicating that the vast majority of cobalt retained in cells is in particulate form. These particles, trapped in the intracellular compartment, are not able to cross the membrane of the insert. In contrast, particles remaining “free” in the apical medium may be transferred in the basolateral chamber according to their size, as THP-1 cells do not form a confluent monolayer. Second, cobalt concentrations in the basolateral chambers are lower after filtration, indicating the existence of a fraction of $\text{Co}_3\text{O}_4\text{P}$ having a size between 100 and 400 nm. These particles are likely individualized non-agglomerated particles. Third, whereas in the non-filtered samples no difference exists between the fraction of cobalt transferred in the absence or in the presence of cells, the fraction of cobalt in the filtered fraction in

the basolateral chamber is larger by 2-fold in the presence of cells. At day 7, the proportion of particulate cobalt is 2/3rd in the absence of cells and 1/3rd in the presence of THP-1 cells (Table 3). This result confirms the existence of a macrophage-assisted dissolution of cobalt particles following phagocytosis in our macrophage-like cell model, as previously described by Kreyling et al. for alveolar macrophages (Kreyling, 1992) . However, the presence of particles smaller than 100 nm in the filtered samples as well cannot be excluded.

Table 3: Cumulative transfer of cobalt at D7 in the different compartments of the bicameral system. Co_3O_4P (50 $\mu g/ml$) were added on differentiated cells plated in insert wells or in a cell-free system. Content from the apical chamber, cell lysate and basolateral chamber were either filtered (100 nm) or not and cobalt concentrations were measured by ICP-MS. Results are expressed as percentages of cumulative cobalt values at D7 divided by deposited cobalt. Values are means \pm SD from 3 independent experiments in triplicate.

	Apical (> 400 nm)		Basolateral non-filtered (<400 nm)	Basolateral filtered (< 100 nm)
	medium	Cell associated	medium	medium
<i>Initial- D0</i>	> 99%	-	-	-
<i>THP-1 - D7</i>	52.7 % \pm 9.5	46.4 % \pm 9.6	0.90 % \pm 0.14	0.72 % \pm 0.05
<i>Cell free - D7</i>	89.3 % \pm 11.2	-	0.98 % \pm 0.22	0.32 % \pm 0.13

As suggested in our previous work in acellular assays, the mechanism by which treatments increase transfer of cobalt might consist in an increased dissolution of particles. The drugs may also limit the aggregation of particles as a possible consequence of modifications of the particle surface properties. To explore further the mechanisms by which ascorbic acid and the cobalt chelators enhance the transfer of cobalt in the basolateral compartment, a dedicated experiment was carried out in which cobalt was measured in basolateral chambers after filtration of samples (100 nm) or in

non- filtered samples. Medium from treated wells were compared to non-treated ones. In addition, both cell free-system and THP-1 models were considered. Since DTPA had no effect in enhancing transfer of cobalt, wells treated with DTPA alone were not studied.

In the analysis provided in Table 4, results were expressed as ratios of cobalt concentrations measured in non-filtered samples divided by cobalt concentrations in filtered samples to get read of the influence of the time-dependent increase in dissolution. The smaller is the ratio, the larger is the proportion of cobalt in forms < 100 nm. The full set of raw data, expressed as % of transferred cobalt/deposited, is given in supplementary data (Figure S1).

At D1, in the absence of cells, treatments decrease the proportion of 100-400 nm cobalt particles in the basolateral chamber by more than 3 fold, with ratios of 3.87 and 0.91-1.18, respectively (Table 4). In the presence of macrophage-like cells, the ratio is already smaller without treatment, at 1.92. The effect of treatments is thus more limited than in absence of cells by about 1.5 fold. A similar trend is observed at the other time-points. Another interesting difference between the THP-1 model and the cell free system is that in the presence of treatments, the ratio values are small, showing that the vast majority of transferred cobalt is dissolved or in the form of nanoparticles. This is significantly different from ratios in the absence of cells. This indicates that although more than half of particles are in the intracellular compartment, a dissolution or important degradation of $\text{Co}_3\text{O}_4\text{P}$ is occurring. This is in favor of an intracellular action of treatments.

Altogether, these results suggest that in the absence of cells, the increased transfer over time is due mainly to the passage of individualized particles (de-aggregation) through the membrane. In contrast, in the presence of cells, the transfer is mainly the result of a dissolution and/or high degradation of particles, leading to a decrease in their size.

It is also of interest to note that differences are observed between treatments. Analysis of non-filtered samples indicates that all treatments are equally effective to increase cobalt transfer (Figure 3). However, when considering the filtered fraction, i.e. the proportion of Cobalt in forms < 100 nm,

the association of a chelating agent to Asc is more efficient than Asc alone (Table 4). As illustrated by the smaller ratio values observed for the combined treatment, the presence of chelators favors the higher degradation of the particles. Such an observation can be made not only on the medium from basolateral chamber, but even more interestingly on the cell lysates. In the cell lysates at D7, the non-filtered/filtered ratio is of 51.3 without treatments indicating the particulate form of the cobalt in the intracellular compartment. This ratio is not changed in the presence of Asc alone, while it is diminished by a factor of 3.5 with the combination of Asc + DTPA and by a factor of 2.2 by Asc + DFOB. This indicates an important intracellular dissolution of cobalt mediated by the two treatments (Table 4).

Table 4: Effect of treatments on the dissolution of cobalt particles: non-filtered/filtered ratios. Co_3O_4P (50 $\mu g/ml$) were added on differentiated cells plated in insert wells or in a cell-free system in the presence or not of treatments. Cobalt concentrations were measured by ICP-MS in the basolateral chambers 1, 2, 3 and 7 days after cobalt addition, and in cell lysates and apical chambers at D7. Separate wells were used for non-filtered samples and for samples with a filtration through 100 nm filters. Cumulative cobalt in basolateral chambers/initially deposited from non-filtered samples were divided by the mean from the corresponding filtered samples. A ratio of 1 corresponds to the full amount of cobalt being <100 nm. Values are means \pm SD from 1 experiment in triplicate.

Non-filtered/filtered ratios (< 400 nm / < 100 nm)

	<i>Basolateral chamber</i>				<i>Cell lysate</i>	
		D1	D2	D3	D7	
<i>THP-1 cells</i>	none	1.62 \pm 0.04	1.63 \pm 0.11	1.22 \pm 0.13	1.17 \pm 0.07	51.3 \pm 15.1
	Asc	1.22 \pm 0.08	1.1 \pm 0.07	1.23 \pm 0.16	1.17 \pm 0.06	50.3 \pm 2.7
	Asc/DTPA	0.92 \pm 0.05	0.84 \pm 0.04	0.85 \pm 0.09	0.86 \pm 0.12	14.4 \pm 6.1
	Asc/DFOB	1.09 \pm 0.12	0.96 \pm 0.15	1.43 \pm 0.22	1.17 \pm 0.15	23.1 \pm 4.4
<i>No cells</i>	none	3.87 \pm 0.48	5.59 \pm 2.45	7.73 \pm 0.49	3.26 \pm 0.65	-
	Asc	1.14 \pm 0.17	2.39 \pm 0.18	2.0 \pm 0.51	1.51 \pm 0.17	-

Asc/DTPA	1.18 ± 0.06	2.64 ± 0.19	2.16 ± 0.37	2.54 ± 0.76	-
Asc/DFOB	0.91 ± 0.08	1.83 ± 0.14	1.58 ± 0.16	1.89 ± 0.3	-

To summarize, our results on THP-1 cells suggest that treatments do not act solely on disaggregation of particles, but on dissolution as well, although we cannot exclude the presence of particles smaller than 100 nm in the basolateral compartment. Our results demonstrate the ability of ascorbic acid to favor the transfer of cobalt from the apical to the basolateral chambers, whether or not particles were engulfed in macrophages. A slightly higher but not significant efficacy was observed when ascorbic acid was associated with chelating agents in the absence of cells, but a significant increase in cobalt dissolution was observed notably for the Asc DTPA combination in the presence of the cells, and particularly in the cell lysates. The higher proportion of dissolved cobalt and/or of particles <100 nm in the THP-1 model as compared to the cell-free system clearly indicates that drugs can access to the intracellular compartment and contribute to intracellular dissolution. Of note is the absence of effect of DTPA alone regarding dissolution and/or disaggregation of particles.

3.2 Epithelial lung barrier Calu-3 cells

After macrophage internalisation of $\text{Co}_3\text{O}_4\text{P}$ and subsequent release of dissolved Co, the dissolved fraction of cobalt will cross the epithelial barrier to reach blood. To study the effect of the drugs on this transfer, we used the radioisotope ^{57}Co , in a soluble form of cobalt, CoCl_2 . Cobalt salts have been shown to be highly toxic on lung epithelial cells ($\text{IC}_{50} = 4.4 \mu\text{g}/\text{ml}$ after a 72h exposure (Ortega et al., 2014)). The use of the gamma emitter ^{57}Co isotope allowed us to use very low concentrations of Co, in the pg/ml range, far below the toxic doses.

3.2.1 Toxicity evaluation of drugs

Similarly to THP-1 cells, toxicity of drugs on Calu-3 cells was evaluated using LDH release and the resazurin assay.

For concentrations of 10 μ M, we did not observed major effects of Asc and the drugs whether alone or in combination, on cell viability (Table 5) using the resazurin and LDH assays. At higher concentrations, DTPA and DFOB associated with Asc decreased cell viability after 3 days in culture. Data obtained for the whole range of concentrations are given in supplementary Table S2. For subsequent translocation experiments, a drug concentration of 10 μ M was chosen, due to the slight and transient toxicity. For this concentration, the drug to cobalt ratio was of 10^5 to 10^6 , depending on the experiment.

Table 5: Toxicity of drugs on Calu-3 cells. Calu-3 cells were exposed to various treatments (each drug was at 10 μ M), 24 h after plating in 96-well plates. Viability was evaluated by LDH release assay (Cytotox one assay) and by resazurin assay (Presto blue assay) at D1, D2, D3 and D7 after treatment. Data are normalized to control conditions (non-treated cells) at the same time point. Results are mean percentages/control from one experiment in triplicate.

		None	DTPA	Asc	Asc + DTPA	Asc + DFOB	Asc + EDTA
Resazurin assay	D1	100 \pm 3.5	94.7 \pm 3.0	97.1 \pm 3.1	92.4 \pm 3.6	82.1 \pm 2.7	86.8 \pm 4.2
	D2	100 \pm 1.8	92.2 \pm 3.2	88.7 \pm 4.2	91.1 \pm 2.4	85.5 \pm 0.5	100.0 \pm 2.5
	D3	100 \pm 5.6	93.0 \pm 2.6	89.0 \pm 4.4	85.4 \pm 2.5	75.0 \pm 2.8	73.1 \pm 11.4
	D7	100 \pm 1.8	99.9 \pm 4.2	98.6 \pm 1.5	98.4 \pm 3.1	97.2 \pm 3.0	98.2 \pm 3.6
LDH assay	D1	100 \pm 2.7	104.9 \pm 1.5	102.1 \pm 4.5	131.0 \pm 25.3	137.8 \pm 12.8	175.0 \pm 26.6
	D2	100 \pm 3.7	106.0 \pm 3.8	97.6 \pm 3.6	115.7 \pm 9.5	95.3 \pm 5.2	101.5 \pm 5.6
	D3	100 \pm 3.0	105.9 \pm 2.4	104.4 \pm 4.5	161.7 \pm 25.7	157.4 \pm 15.1	207.7 \pm 49.8
	D7	100 \pm 32.4	97.4 \pm 14.2	124.0 \pm 19.6	107.9 \pm 15.1	101.4 \pm 17.5	95.2 \pm 7.0

3.2.2 Assessment of the epithelial barrier integrity

The preservation of a functional barrier (TEER) is of great importance when evaluating translocation across an epithelial monolayer. This is particularly true since CoCl_2 has been shown to alter epithelial barrier integrity on the Caco-2 model (DiGuilio et al., 2018). In addition, alteration of epithelial barrier properties could result from Ca depletion induced by the use of chelators. However, it has to be noted that the affinity for Co(II) is several orders of magnitude larger than that for calcium for DTPA (9 orders of magnitude), EDTA (6 orders of magnitude) and DFOB (8 orders of magnitude) (Farkas et al., 1999; Smith, 1976). Complexes formed with Co(III) are expected to be even stronger.

To ensure that the barrier properties were not affected by the chronic exposure to gamma rays, CoCl_2 and/or by the various drugs, we measured trans-epithelial resistance (TEER), as an index of tight junction integrity at the end of the translocation experiments. No significant differences in TEER values were observed 7 days after contamination and treatment, indicating the maintained performance of the epithelial monolayer whatever the culture conditions (Figure 5).

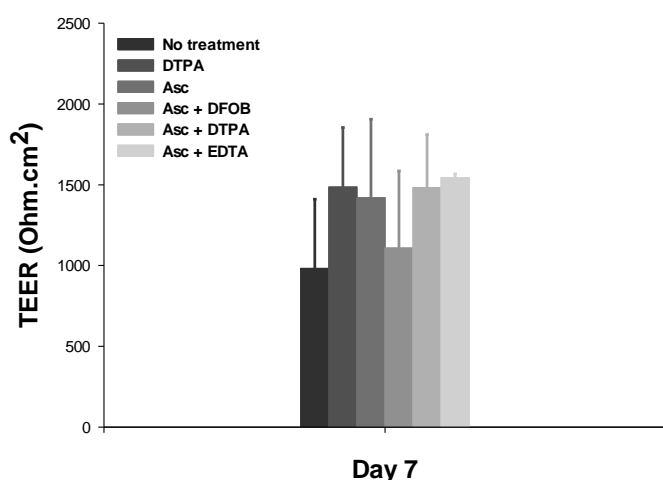


Figure 5: Transepithelial resistance of Calu-3 cells 7 days after treatment. $^{57}\text{CoCl}_2$ (10-40 kBq/ml) was added on confluent Calu-3 cells plated in insert wells in the presence or not of various drugs (10 μM). TEER was measured 7 days after treatment. Values are means \pm SD from 2-3 independent experiments each performed in triplicate. Statistically significant differences from the control were determined by one-way ANOVA followed by Dunn's multiple comparisons test.

3.2.3 Transfer of cobalt chloride across the epithelial cell layer in the presence of the various drugs

Calu-3 monolayer grown on filter devices has been widely used to mimic translocation from lungs to blood of various substances (Florea et al., 2003; Foster et al., 2000; George et al., 2015; Haghi et al., 2010; Van der Meeren et al., 2021) . We measured the transfer of the soluble cobalt compound $^{57}\text{CoCl}_2$ across Calu-3 monolayers over 7 days to mimic the behavior of the dissolved fraction of cobalt particles.

In the absence of treatments, the percentage of $^{57}\text{CoCl}_2$ into the basolateral chamber increased from 1.3 % of the deposited activity at Day 1, to 8.9 % at Day 7 (data not shown). Figure 6 shows the effect of the various treatments on this basal transfer. No major differences on the ability of Cobalt to cross the epithelial monolayer were detected. Nevertheless, a small but significant decrease in the transfer was evidenced when cells were treated with Asc + DFOB.

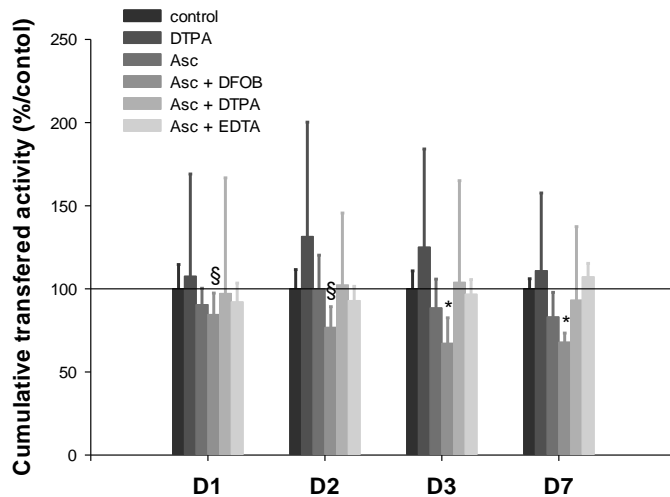


Figure 6: Cumulative transfer of cobalt across a Calu-3 cell monolayer in the presence of various treatments (10 μM). Activity was measured by gamma spectrometry in medium collected in basolateral chambers at the indicated time following treatment. Results are expressed as percentages of the ^{57}Co activity measured in basolateral chambers in treated wells versus the activity measured in basolateral chambers from “not treated” wells. Values are means \pm SD from 2-3 independent experiments each performed in triplicate. Statistically

significant differences from the control were determined by one-way ANOVA followed by pairwise multiple comparison (Dunn's method) ξ : $p < 0.05$, *: $p < 0.001$. The horizontal line is 100% (no effect of treatment).

3.2.4 Intracellular Co content in Calu-3 cells in the presence of various treatments

To evaluate whether the presence of the various treatments influences the cell-associated ^{57}Co activity, i.e. the accumulation of Cobalt by the Calu-3 cells, cells were thoroughly rinsed and lysed at day 7. The ^{57}Co activity was then measured in the cell lysate. Figure 7 indicates that approximately 0.7% of the initial activity is either intracellular or strongly bound to the membrane in the absence of treatment. Cell-associated cobalt significantly decreases in the presence of DTPA either alone or with Asc, as well as in the presence of EDTA. Asc alone or associated with DFOB does not result in a significant difference in intracellular cobalt accumulation. Interestingly, such a decrease in cell-associated activity in the presence of DTPA has been also observed following Calu-3 contamination with americium (Drouet et al., 2021; Van der Meeren et al., 2021).

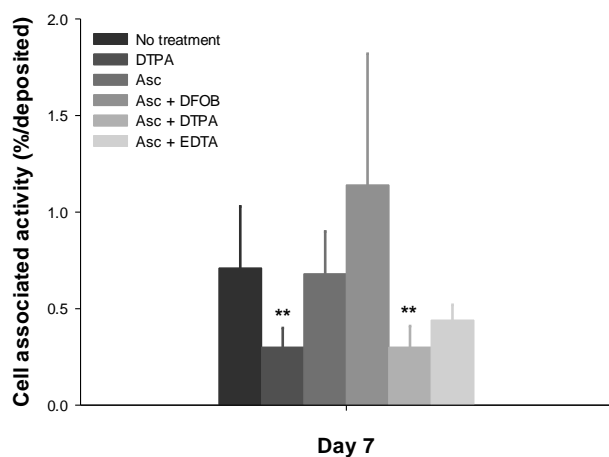


Figure 7: Intracellular cobalt content in Calu-3 cells. ^{57}Co Cobalt chloride (10-40 kBq/ml) was added on confluent Calu-3 cells plated in insert wells in the presence or not of various drugs (10 μM). Activity was measured by gamma spectrometry in cell lysates 7 days after treatment. Results are expressed as percentages of radioactivity in cell lysates divided by deposited activity. Values are means \pm SD from 3 independent experiments each performed in triplicate. Statistically significant differences from the control were determined

by one-way ANOVA followed by pairwise multiple comparison (Dunn's method) **:p<0.01. The horizontal line is 100% (no effect of treatment).

Discussion

With the long-term goal of providing new treatments for decorporation of the gamma ray emitters $^{60}\text{Co}_3\text{O}_4\text{P}$, we evaluated in the present study the efficacy of potential decorporation candidates to increase dissolution of submicronic cobalt oxide particles following macrophage-like cell uptake. Transfer of dissolved cobalt species across the pulmonary epithelial barrier in the presence of the various drugs was also measured.

Following inhalation, absorption to blood depends on the physical and chemical forms of the deposited material. For all elements, the international commission on radiological protection (ICRP) classifies inhaled compounds according to their solubility properties. For dose calculation using mathematical models, ICRP proposes parameters according to whether the absorption of the contaminant is considered to be fast (Type F), moderate (M) or slow (S). Fast or moderate absorption kinetics are believed less problematic than slow absorption, due to the reduced exposure time to radioactive substances before final elimination.

Various chemical forms and cobalt isotopes can be encountered and involved in accidental occupational exposure. Cobalt oxide ($^{60}\text{Co}_3\text{O}_4$) is of major concern, as it is assigned by ICRP to type S, meaning that the exposure time will be much longer than for soluble cobalt, which is of type F (ICRP, 2016) . For similar radioactive intake, the calculated committed effective dose is approximately 10 times lower for soluble cobalt as compared to oxide compounds (ICRP, 2016) . ^{60}Co is an important activation product produced in nuclear industry and is a highly penetrating gamma emitter. Therefore, when inhaled under the form of $^{60}\text{Co}_3\text{O}_4$ particles, the reduction of the pulmonary

retention of these poorly soluble compounds represents an important strategy to limit the long-term exposure to radiation. In previous *in vitro* acellular assays (Van Der Meeren et al., 2020) , we demonstrated that combining molecules with different properties, such as reducing agents and Co-chelating molecules, improved the efficacy of each drug used alone, to dissolve $\text{Co}_3\text{O}_4\text{P}$. In the present work, we used two cellular models previously developed to study the uptake and release of actinides after macrophage phagocytosis (Van der Meeren et al., 2016) and the subsequent translocation across an epithelial monolayer (Van der Meeren et al., 2021) to explore the therapeutic potential of ascorbic acid associated or not with the chelators DTPA, EDTA or DFOB. These molecules present possible medical applications in human, given their market authorization. Although DTPA alone did not enhance dissolution of $\text{Co}_3\text{O}_4\text{P}$ in acellular models (Van Der Meeren et al., 2020) , this chelator was considered as a reference and used in the present study, as it is the currently recommended treatment of contaminated victims (REMM) . Intracellular particle dissolution in alveolar macrophages is a critical step, as it will influence how long the particles will be retained in the lungs and, for radioactive compounds, for how long neighboring cells will be exposed to radiation. To mimic uptake of $\text{Co}_3\text{O}_4\text{P}$ by alveolar macrophages, their release from cells and their accessibility to chelating drugs, the macrophage-like differentiated THP-1 cells were chosen. This model was validated in a previous work, as we showed that, in these culture conditions, similar dissolution of plutonium compounds was observed with THP-1 differentiated cells and rat alveolar macrophages (Van der Meeren et al., 2016) . Using a bicameral system, in which the two compartments are separated by a synthetic membrane of 400 nm pores, we confirmed the slow and progressive dissolution of cobalt particles in culture medium over 7 days, due to the intrinsic low solubility of cobalt oxide accentuated by the particle aggregation in culture medium (Table 2). Such an aggregation of particles is likely to occur as well in the lung fluids (Kendall et al., 2002) . When cobalt particles were added on macrophage-like cells, approximately half of the particles were retained in the cells 7 days after contamination. No difference was observed in the transfer of cobalt from the apical to the basolateral chamber whether in the presence or absence of cells, suggesting

that a cobalt cell-mediated transfer is negligible during the 7 days of exposure. Only a small fraction (< 1%) is transferred in the basolateral chamber (Table 2). However, in the presence of the macrophage-like cells, the proportion of cobalt in the <100nm fraction increased by about two fold as compared to the cell-free model (0.72% and 0.32% respectively, Table 3). This result indicates a higher degradation of internalized particles, leading either to the release of dissolved species or to the generation of nanoparticles. This is in accordance with the existence of a macrophage-assisted dissolution of metal particles previously published by others (Kreyling, 1992; McCarrick et al., 2021) .

In animal studies, soluble cobalt compounds, such as CoCl_2 have been shown to be rapidly translocated to the systemic compartment following pulmonary administration (Kreyling et al., 1986; Menzel et al., 1989) . However, a small fraction, estimated between 1 and 5% of the initial lung deposit is cleared much more slowly, suggesting that some dissolved cobalt remains present in the lung fluids, bound to biomolecules (ICRP, 2016) . Thus, we hypothesized that combining drugs enhancing the dissolution of particles to molecules able to complex the ionic form of cobalt could contribute to an efficient effect of treatments, favoring translocation across epithelial barrier by limiting tissue deposit. Indeed, ascorbic acid increased by 2-4 fold the transfer of cobalt in the bicameral system in culture medium as well as in the presence of macrophage-like cells (Figure 3). The association of chelators to ascorbic acid did not increase further the total cobalt transfer. DTPA alone did not increase cobalt transfer, confirming the absence of efficacy of DTPA alone on particle dissolution. A continuous and significant action of all the treatments is observed over 7 days, suggesting that a chronic treatment *in vivo* would be more effective than a unique treatment, as previously demonstrated for actinide decorporation (Stradling et al., 1991) . Although half of the cobalt particles are internalized, a comparable efficacy of treatments in the cell-free and cellular models is observed, suggesting the intracellular action of the various drugs. In addition and interestingly, as indicated by the results from filtered samples, in the presence of treatments and at all time-points tested, the cobalt in the basolateral chamber consists almost exclusively in dissolved cobalt or cobalt in the form of < 100 nm particles, as compared to the situation without treatment.

Hence, treatments are able to access the intracellular compartment and increase the dissolution of cobalt particles. This intracellular particle dissolution seems higher with the Asc and DTPA combination (Table 4).

The access of treatments to the intracellular compartment is an important finding, as the engulfed particles will be the highest contributors to the delivered radiation dose. However, it is also important to note that the efficacy of treatments is also observed on the extracellular cobalt particles. This might indicate that whatever the size of particles and their localization in the body, an enhanced dissolution of particles is expected to occur.

Considering the high toxicity of cobalt salts towards lung epithelial cells, and more particularly their genotoxicity (Uboldi et al., 2016; Xie et al., 2016) , our concern was to ensure that the treatments would not limit translocation from lungs into the systemic compartment. To simulate translocation of cobalt across the air-blood barrier, in the presence or not of treatments, we have chosen human airway epithelial (Calu-3) cells that have been shown to provide a suitable model for pulmonary translocation studies (Mathia et al., 2002) . To mimic the transportable fraction of cobalt, i. e. dissolved species from particles, cobalt chloride was used. In the absence of treatments, the epithelial monolayer formed by Calu-3 cells is an effective barrier in controlling soluble cobalt translocation, with less than 10% of initially deposited cobalt able to cross the epithelial barrier over 7 days. As illustrated by TEER measurements, the treatments do not compromise barrier properties (Figure 5). Treatments do not enhance cobalt translocation either (Figure 6). A progressive decrease in translocation is observed in the presence of Asc/DFOB (Figure 7). Furthermore, DTPA, alone or with Asc decreased the intracellular cobalt content, and thus could limit the associated toxicity. Whereas Asc in the presence of DFOB enhances dissolution of particles, the decreased cobalt translocation does not indicate a suitable use for this combined treatment for decorporation *in vivo*.

Administration of Asc might represent a good strategy following accidental pulmonary intake of radioactive cobalt particles. In association with this drug, the use of DTPA, able to chelate the dissolved species generated by Asc action, should enhance cobalt urinary excretion. DTPA can be

administered in rats by inhalation (Miccoli et al., 2019) and Asc either via oral or intraperitoneal route as it has been previously shown that those two administration modes decrease lung damage in rats (Fanucchi et al., 2012; Mohamed et al., 2019) .

Conclusion

Our results indicate that ascorbic acid significantly enhances dissolution of $\text{Co}_3\text{O}_4\text{P}$ internalized in THP-1 differentiated cells, whereas DTPA alone has no effect. Combination of ascorbic acid with metal chelators (DTPA, EDTA) significantly increases the proportion of soluble cobalt species in the intracellular compartment. In addition, none of the treatments induced disruption of an epithelial layer formed by Calu-3 cells nor major changes in the translocation of the soluble cobalt species CoCl_2 . DFOB (Desferrioxamine B) only slightly decreased this translocation. These promising results obtained with well-tolerated drugs in man, provide a good hope for the development of treatments allowing the increased lung clearance following inhalation of radioactive $\text{Co}_3\text{O}_4\text{P}$ and a subsequent decrease in the radiation dose received following occupational exposure. The next phase in this multi-step approach will be the *in vivo* evaluation in rodent models of the efficacy of ascorbic acid combined with DTPA as a decorporating agent after exposure to radioactive $\text{Co}_3\text{O}_4\text{P}$.

Supplementary data

Table S1. Effect of drugs on THP-1 viability via the analysis of the Cytotox one assay.

		day 1	day 2	day 3-4	day 7
Asc	0 μ M	100 \pm 6	100 \pm 3.7	100 \pm 4.4	100 \pm 3.9
	5 μ M	106.1 \pm 7.4	108.4 \pm 2.7	101.4 \pm 5.3	108.2 \pm 3.8
	10 μ M	110.9 \pm 5.2	107.4 \pm 5.9	102.3 \pm 4.9	101.4 \pm 10.8
	50 μ M	106 \pm 10	106.3 \pm 7.4	102 \pm 5.4	81.8 \pm 16.3
	100 μ M	107.9 \pm 4.2	101.7 \pm 9.7	103.3 \pm 7.6	89.5 \pm 25.9
	500 μ M	109.9 \pm 7.3	99.1 \pm 12.2	95.4 \pm 10	85 \pm 17.7
DTPA	0 μ M	100 \pm 6	100 \pm 3.7	100 \pm 4.4	100 \pm 3.9
	5 μ M	105 \pm 2.6	106.9 \pm 9.8	101.9 \pm 9.9	111.9 \pm 15.7
	10 μ M	121.2 \pm 5.2	128.9 \pm 20.4	116.3 \pm 8.8	116.8 \pm 21.9
	50 μ M	153 \pm 38	139.4 \pm 25.6	136.2 \pm 22.6	159.3 \pm 49.4
	100 μ M	155.6 \pm 43.3	146 \pm 22	145.1 \pm 25.9	145.9 \pm 39.1
	500 μ M	166.2 \pm 46.7	155 \pm 22.2	156.2 \pm 25.8	179.4 \pm 54.7
Asc + DFOB	0 μ M	100 \pm 2.1	100 \pm 6.5	100 \pm 3	100 \pm 2.5
	5 μ M	111.2 \pm 10.8	108.3 \pm 9.3	101.2 \pm 2	101.3 \pm 13.7
	10 μ M	112.9 \pm 11.7	109.7 \pm 11.5	100.3 \pm 3.5	106.8 \pm 12.9
	50 μ M	153.8 \pm 53.6	134.9 \pm 13.3	142.4 \pm 43.3	129 \pm 26.1
	100 μ M	148.8 \pm 49.5	137.9 \pm 15.7	144.2 \pm 31	154.7 \pm 43.3
	500 μ M	130.8 \pm 28.2	135.7 \pm 22.1	139.4 \pm 36.6	142.1 \pm 24.7
Asc + DTPA	0 μ M	100 \pm 0.5	100 \pm 1.7	100 \pm 0.8	100 \pm 2
	5 μ M	106 \pm 1.7	99.3 \pm 2.1	101 \pm 4.4	91.7 \pm 17
	10 μ M	112 \pm 3.6	100.7 \pm 0.6	107 \pm 4	95 \pm 50.2
	50 μ M	176.7 \pm 25.5	165 \pm 2.6	165.3 \pm 7.1	198.3 \pm 12.2
	100 μ M	188.7 \pm 15.6	164 \pm 4.6	162 \pm 7.5	195 \pm 14.5
	500 μ M	241.7 \pm 19.1	145.3 \pm 1.5	179.7 \pm 2.5	135 \pm 36.3
Asc + EDTA	0 μ M	100 \pm 6	100 \pm 3.7	100 \pm 4.4	100 \pm 3.9
	5 μ M	108.6 \pm 12.7	104 \pm 10.4	105.7 \pm 4.9	97.1 \pm 17.9
	10 μ M	109.6 \pm 22.4	118.4 \pm 5.7	105.5 \pm 13.1	110.6 \pm 9
	50 μ M	136.2 \pm 40.7	129.8 \pm 7.6	127.4 \pm 13.5	127.1 \pm 9.9
	100 μ M	152.7 \pm 46.1	130.4 \pm 13.1	116.7 \pm 10.2	140.8 \pm 18.2
	500 μ M	145.1 \pm 51.6	143.2 \pm 31.1	115.9 \pm 21.1	107.8 \pm 13.3

The viability of THP-1 cells exposed to ligands was evaluated by the LDH release assay. Assays were performed 24h after exposure (day 1), and then at regular time points (day 2, 3-4, 7).

Results are means percentages/control \pm SD of cell viability from 1 to 3 independent experiments each performed in triplicate.

Table S2. Effect of drugs on THP-1 viability via the analysis of the Presto Blue assay.

		day 1	day 2	day 3-4	day 7
Asc	0 μ M	100 \pm 7.1	100 \pm 8.5	100 \pm 6.1	100 \pm 1.5
	5 μ M	130.3 \pm 3.6	98.2 \pm 2.7	93.9 \pm 4.5	96 \pm 4.6
	10 μ M	106.2 \pm 15.8	99.8 \pm 1.3	98.4 \pm 3.6	96.3 \pm 4.2
	50 μ M	100.5 \pm 8.2	98.5 \pm 3	92.6 \pm 1.3	93.8 \pm 3.9
	100 μ M	98.4 \pm 8	93.9 \pm 4.2	92.9 \pm 3	96.2 \pm 4.9
	500 μ M	97.5 \pm 10	97.1 \pm 9	92.9 \pm 7.4	91.5 \pm 5.6
DTPA	0 μ M	100 \pm 7.1	100 \pm 8.5	100 \pm 6.1	100 \pm 1.5
	5 μ M	92.3 \pm 0.6	98.6 \pm 2.2	94.7 \pm 3.8	98 \pm 4.9
	10 μ M	105.4 \pm 14.7	95.2 \pm 4.1	98.5 \pm 5	94.9 \pm 5.2
	50 μ M	101.9 \pm 16.1	93.3 \pm 3	90.3 \pm 3.9	89.5 \pm 4.1
	100 μ M	98.5 \pm 12.3	94.2 \pm 3.9	89.2 \pm 2.1	88.6 \pm 2.5
	500 μ M	99.6 \pm 14.7	93 \pm 6.6	88.8 \pm 2.3	87.8 \pm 7.1
Asc + DFOB	0 μ M	100 \pm 10.6	100 \pm 10.6	100 \pm 12.1	100 \pm 3.8
	5 μ M	97 \pm 23.3	101.2 \pm 20.3	95.7 \pm 5.6	93.2 \pm 14.7
	10 μ M	93.9 \pm 11.9	98.5 \pm 11.4	88.3 \pm 10.6	72.1 \pm 21
	50 μ M	88.2 \pm 13.2	79.2 \pm 5.7	58.4 \pm 22.2	52.7 \pm 30.6
	100 μ M	89.2 \pm 18.4	74 \pm 5.5	55.8 \pm 21.3	53.7 \pm 27.5
	500 μ M	88.9 \pm 18.9	74.6 \pm 8	59.2 \pm 26.3	70.4 \pm 22.6
Asc + DTPA	0 μ M	100 \pm 2	100 \pm 12.3	100 \pm 1.3	100 \pm 1.5
	5 μ M	91 \pm 6.2	89 \pm 6.1	88 \pm 5.3	91.7 \pm 4.5
	10 μ M	92.7 \pm 4.9	91.7 \pm 2.1	90.3 \pm 1.2	93 \pm 1.7
	50 μ M	88.7 \pm 3.2	91.3 \pm 6.5	86.3 \pm 3.8	91.3 \pm 6.1
	100 μ M	80.7 \pm 1.5	98.3 \pm 6.8	91.3 \pm 4.7	93.7 \pm 2.1
	500 μ M	92.3 \pm 6.4	105.3 \pm 2.5	77 \pm 1	91.7 \pm 1.5
Asc + EDTA	0 μ M	100 \pm 7.1	100 \pm 8.5	100 \pm 6.1	100 \pm 1.5
	5 μ M	92.8 \pm 8.4	93.1 \pm 3.6	88.9 \pm 6.5	88 \pm 1.8
	10 μ M	87 \pm 9.6	90.6 \pm 7.4	93.2 \pm 9.9	90 \pm 4.3
	50 μ M	96.6 \pm 19	98.8 \pm 7.2	96.2 \pm 9.6	86 \pm 12.6
	100 μ M	96.7 \pm 25.2	99.7 \pm 7.5	99.8 \pm 4.8	97.9 \pm 7.2
	500 μ M	90.2 \pm 11.6	97.9 \pm 15.6	95.6 \pm 8.3	89 \pm 4

The viability of THP-1 cells exposed to ligands was evaluated by the resazurin assay. Assays were performed 24h after exposure (day 1), and then at regular time points (day 2, 3-4, 7).

Results are means percentages/control \pm SD of cell viability from 1 to 3 independent experiments each performed in triplicate.

Table S3. Effect of drugs on Calu-3 viability via the analysis of the Cytotox one assay.

		day 1	day 2	day 3	day 7-8
Asc	0 μ M	100 \pm 7.8	100 \pm 8.5	100 \pm 5.9	100 \pm 22.4
	5 μ M	94.9 \pm 13.4	97.5 \pm 5.8	98.5 \pm 2.6	130.4 \pm 9.9
	10 μ M	92.7 \pm 10.7	98.9 \pm 3.1	103.6 \pm 7.8	123.6 \pm 14
	50 μ M	95 \pm 7	98.4 \pm 2.7	97.8 \pm 2	139.9 \pm 8.8
	100 μ M	103.7 \pm 6.3	89.7 \pm 1.4	103.8 \pm 5.9	143.6 \pm 11.4
	500 μ M	99.4 \pm 3.3	98.7 \pm 7.6	103.4 \pm 5.6	126 \pm 4.7
DTPA	0 μ M	100 \pm 7.8	100 \pm 8.5	100 \pm 5.9	100 \pm 22.4
	5 μ M	93.6 \pm 10.4	88.6 \pm 5.9	91.7 \pm 4.6	105.9 \pm 25
	10 μ M	97.1 \pm 8.7	102.7 \pm 5.6	106.8 \pm 4.5	105.3 \pm 18.6
	50 μ M	120.9 \pm 11.6	131.3 \pm 15.8	154.6 \pm 6.3	176.9 \pm 13.4
	100 μ M	110.6 \pm 8.7	170.2 \pm 51.6	156.1 \pm 8.7	148.1 \pm 14.4
	500 μ M	127.4 \pm 4.1	127.9 \pm 4.5	176.8 \pm 15.5	242.1 \pm 24.9
Asc + DFOB	0 μ M	100 \pm 7.8	100 \pm 8.5	100 \pm 5.9	100 \pm 22.4
	5 μ M	89.7 \pm 20.6	98.5 \pm 16.9	109.9 \pm 11	104.6 \pm 22.2
	10 μ M	106.5 \pm 26.3	96.9 \pm 12.9	152.2 \pm 38.3	137.5 \pm 44.9
	50 μ M	108.8 \pm 10.5	102.9 \pm 6.4	188.7 \pm 41.3	497 \pm 126.7
	100 μ M	120.1 \pm 29.9	119.9 \pm 15.7	200.2 \pm 64.3	495.7 \pm 109.6
	500 μ M	101.1 \pm 14.8	135.2 \pm 33.8	331.5 \pm 117.1	578.5 \pm 92.9
Asc + DTPA	0 μ M	100 \pm 7.8	100 \pm 8.5	100 \pm 5.9	100 \pm 22.4
	5 μ M	99.5 \pm 9.1	78 \pm 16.6	147.4 \pm 40.5	150.3 \pm 46.1
	10 μ M	110.6 \pm 28.8	110.5 \pm 9.6	186.8 \pm 45.1	144.2 \pm 67.3
	50 μ M	134.5 \pm 70.7	147.7 \pm 23.6	269.9 \pm 69.7	301.3 \pm 227.9
	100 μ M	136.3 \pm 24.7	224.6 \pm 45	264.7 \pm 132.7	292.1 \pm 178.2
	500 μ M	108.1 \pm 12.6	151 \pm 42.8	279.7 \pm 66.7	340 \pm 85.2
Asc + EDTA	0 μ M	100 \pm 7.8	100 \pm 8.5	100 \pm 5.9	100 \pm 22.4
	5 μ M	88 \pm 7	100.9 \pm 25.2	99.4 \pm 10.6	176.1 \pm 119
	10 μ M	140.1 \pm 42.2	93.6 \pm 9.9	156.1 \pm 64.9	110.5 \pm 15.9
	50 μ M	98.8 \pm 11.6	90.3 \pm 3.4	102.3 \pm 16.2	122.5 \pm 29.2
	100 μ M	91.9 \pm 13.4	93.5 \pm 0.7	98.8 \pm 1	153.9 \pm 41
	500 μ M	115.9 \pm 14.8	104.9 \pm 11	105.8 \pm 3.6	131.4 \pm 21.1

The viability of Calu-3 cells exposed to ligands was evaluated by LDH release assay. Assays were performed 24h after exposure (day 1), and then at regular time points (day 2-3-7-8).

Results are means percentages/control \pm SD of cell viability from 1 to 3 independent experiments each performed in triplicate.

Table S4. Effect of drugs on Calu-3 viability via the analysis of the Presto blue assay.

		day 1	day 2	day 3	day 7-8
Asc	0 μ M	100 \pm 26.3	100 \pm 11.3	100 \pm 14.1	100 \pm 7.1
	5 μ M	81.5 \pm 2.4	105 \pm 5.5	118.8 \pm 17.6	123.6 \pm 6.4
	10 μ M	90.5 \pm 8.1	93.5 \pm 6.9	97.7 \pm 10	106.2 \pm 10.4
	50 μ M	81.3 \pm 5.9	106.8 \pm 5.5	124.5 \pm 20.4	115.6 \pm 8.9
	100 μ M	78.3 \pm 1.7	111 \pm 7.2	117.6 \pm 5.1	114.4 \pm 8.4
	500 μ M	81.6 \pm 2.7	114 \pm 13.6	107 \pm 4.4	58.8 \pm 7.1
DTPA	0 μ M	100 \pm 5.2	100 \pm 10	100 \pm 6.7	100 \pm 36.4
	5 μ M	96.4 \pm 14.5	97.6 \pm 5.5	94.7 \pm 11.3	62 \pm 54.6
	10 μ M	89.5 \pm 10.9	102.2 \pm 21.9	111 \pm 56.9	71.5 \pm 39.2
	50 μ M	114.6 \pm 37.4	99.6 \pm 7.6	89 \pm 7.9	37.9 \pm 6.3
	100 μ M	107.9 \pm 5.3	152.5 \pm 70.1	118.8 \pm 13.3	101.1 \pm 9.6
	500 μ M	99.9 \pm 3.2	98.3 \pm 15.1	126.6 \pm 4.1	122.3 \pm 7.4
Asc + DFOB	0 μ M	100 \pm 21.1	100 \pm 11.9	100 \pm 12	100 \pm 31.2
	5 μ M	85.3 \pm 38.7	95.4 \pm 4.7	98.5 \pm 5.3	106.6 \pm 5.5
	10 μ M	80.4 \pm 19	92.4 \pm 5.1	90.7 \pm 15.1	117.1 \pm 22.6
	50 μ M	82 \pm 15.8	101.9 \pm 5.4	103.1 \pm 12.5	38.2 \pm 7
	100 μ M	90.5 \pm 22.2	113.1 \pm 25.6	90.9 \pm 31.9	18.4 \pm 13.8
	500 μ M	91.2 \pm 19.3	103.3 \pm 12.5	98.6 \pm 36.5	85.1 \pm 35.2
Asc + DTPA	0 μ M	100 \pm 5.2	100 \pm 10	100 \pm 6.7	100 \pm 36.4
	5 μ M	105.4 \pm 8	85.6 \pm 14	91.1 \pm 51.3	112 \pm 1
	10 μ M	102.6 \pm 16.5	90.6 \pm 7.3	80.3 \pm 25.9	101.5 \pm 4.8
	50 μ M	100.5 \pm 9.3	82.8 \pm 15.8	74 \pm 39.4	98.7 \pm 2.2
	100 μ M	98.6 \pm 9.4	108.9 \pm 26.8	88.3 \pm 31.7	89.6 \pm 12.8
	500 μ M	122.2 \pm 37.5	100.6 \pm 31.9	83.9 \pm 27.2	90.5 \pm 49.7
Asc + EDTA	0 μ M	100 \pm 5.2	100 \pm 10	100 \pm 6.7	100 \pm 36.4
	5 μ M	120.3 \pm 38.8	99.9 \pm 1.5	118.4 \pm 8.1	122.7 \pm 9.7
	10 μ M	93.5 \pm 8.4	101.7 \pm 5	95.9 \pm 26.8	116.4 \pm 21.5
	50 μ M	102.6 \pm 6.3	103.3 \pm 8.6	113.6 \pm 1.7	125.4 \pm 5.4
	100 μ M	100.9 \pm 18.3	106.6 \pm 10.7	111.2 \pm 5.5	116 \pm 10.7
	500 μ M	106 \pm 7.8	111.2 \pm 12.7	108.6 \pm 35.3	38 \pm 9.8

The viability of Calu-3 cells exposed to ligands was evaluated by resazurin assay. Assays were performed 24h after exposure (day 1), and then at regular time points (day 2, 3-4, 7).

Results are means percentages/control \pm SD of cell viability from 1 to 3 independent experiments each performed in triplicate.

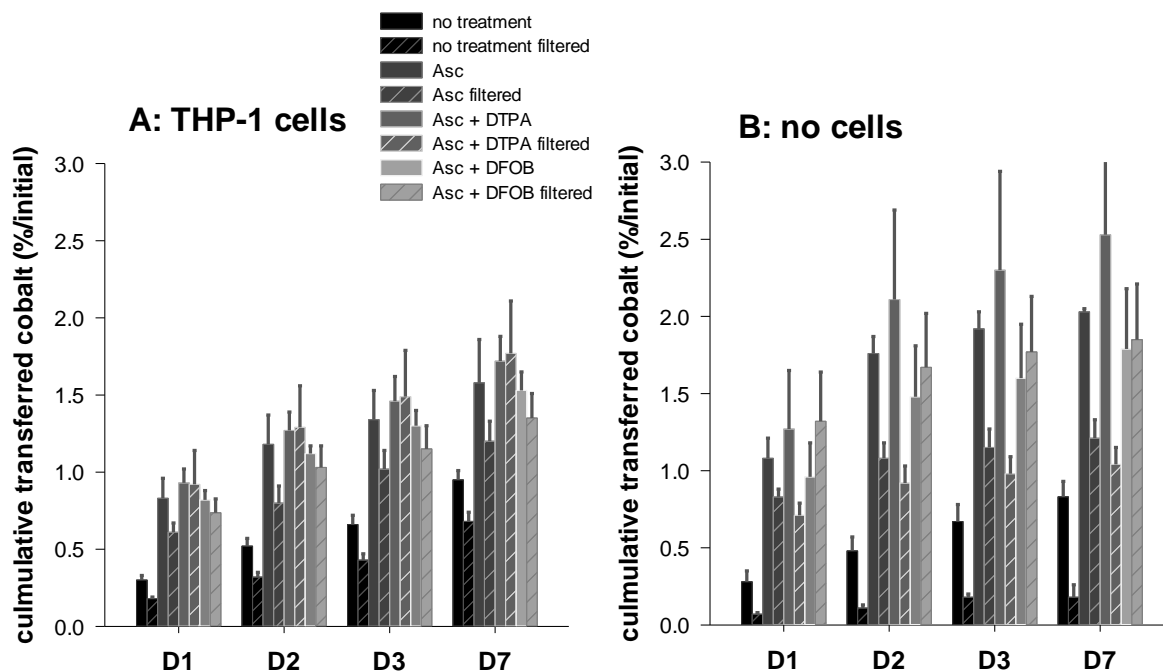


Figure S1: Cumulative transfer of cobalt in the presence (A) of THP-1 cells or in absence of cells (B). $\text{Co}_3\text{O}_4\text{P}$ ($50 \mu\text{g}/\text{ml}$) were added on differentiated cells plated in insert wells (A) or in a cell-free system (B). The transfer was measured without drugs (no treatment), as well as in the presence of Asc, or combinations of Asc with DTPA or DFOB ($500 \mu\text{M}$). Cobalt concentrations were measured by ICP-MS in the basolateral chambers 1, 2, 3 and 7 days after $\text{Co}_3\text{O}_4\text{P}$ addition in non-filtered or filtered (100 nm) samples. Results are expressed as percentages of cobalt in basolateral chambers divided by deposited cobalt. Values are means \pm SD from 1 experiment in triplicate.

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