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## ***In vitro* and *in vivo* genotoxicity assessment of ferric ferrocyanide and potassium-cobalt ferrocyanide**

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### **HIGHLIGHTS**

- Fe-ferrocyanide and K-Co-ferrocyanide are cesium decontaminant agents in human
- In vitro genotoxic profiles of both agents are similar with positive responses
- In vivo, Fe-ferrocyanide induced genotoxicity
- It is expected that K-Co-ferrocyanide is an in vivo genotoxicant
- The use of both decontaminants must follow a benefit/risk approach

### **Abstract**

Ferric hexacyanoferrate(II) ( $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ ), *i.e.* Prussian blue (PB) has been used for many years to remove from the body the two toxic isotopes of cesium and thallium following irradiation.

Recently, potassium cobalt hexacyanoferrate(II) ( $\text{K}_2\text{CoFe}(\text{CN})_6$ ), which has shown a better efficacy for decontamination, is also being considered for use to enhance the elimination of cesium isotopes. In

view to its preclinical and clinical development, *in vitro* and *in vivo* GLP-compliant genotoxicity studies were carried out on this product as well as on PB for comparison. Several tests dissecting the main events leading to genotoxicity, *i.e.* mutagenicity and chromosomal aberrations, both structural and quantitative were implemented.

*In vitro*, no mutagenic effect was observed in the Ames test but both compounds were positive in the mouse lymphoma assay on TK locus and induced clastogenic effects in the *in vitro* chromosomal aberrations test on human lymphocytes, either in absence or in presence of metabolic activation.

K-Co-ferrocyanide was also assayed *in vivo* in the mouse bone marrow micronucleus assay and PB was assessed for DNA fragmentation in the rodent Comet assay in both glandular stomach and colon. In the *in vivo* micronucleus mouse bone marrow, K-Co-ferrocyanide did not display any genotoxic activity up to 2000 mg/kg/d (x2) by oral route. In opposite, PB induced a significant increase in DNA fragmentation both in the glandular stomach and in the colon of rat treated 3 times with intake ranging from 2000 to 500 mg/kg. PB should be considered as an *in vivo* mutagen as well as Potassium cobalt hexacyanoferrate(II) since the *in vitro* genotoxicity profiles of both ferrocyanides are quite similar.

Their use as cesium/ thallium decontamination agents in human should be assessed following a benefit/risk approach to enable a robust decision-making.

**Abbreviations:** CC, Cytoplasmic grain count; DMSO, Dimethylsulfoxide; K-Co-ferrocyanide, Potassium-cobalt ferrocyanide; MMC, mitomycin C; MMS, methyl methanesulfonate; NC, Nuclear grain count; NNG, Net nuclear grains; PB, Prussian blue; TFT, trifluorothymidine; UDS, Unscheduled DNA Synthesis

**Keywords:** Genotoxicity; mutagenicity; ferrocyanides; Prussian blue; cesium poisoning

## 1. Introduction

Ferric hexacyanoferrate(II), known as Prussian blue (PB), has the empirical formula  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ . It was probably synthesized for the first time by the paint maker Diesbach in Berlin in 1704, and it was one of the first synthetic pigments. Prussian blue is available as Radiogardase® in Germany and France. The use of Prussian blue against thallium poisoning and as a decorporation agent for  $^{134}\text{cesium}$  and  $^{137}\text{cesium}$  has been investigated since the 1960s. It was recommended in the treatment of thallium intoxication in the 1970s and it is now normally used. The disaster at the Chernobyl nuclear reactor in 1986 led to further studies on the elimination of radioactive cesium. In 1987, following Goiania accident in Brazil, Prussian blue was used in the management of a large scale radiation disaster [1]. It is used as an orally ingested drug to enhance the excretion of isotopes of cesium and thallium from the body by means of ion exchange: thallium ions are excreted into the intestine and reabsorbed mainly in the colon into blood to be excreted again into the intestinal tract while cesium is excreted into the intestinal tract in the bile to be reabsorbed into portal blood and transported to the liver to again be excreted via bile (enterohepatic circulation). Therefore, orally administered Prussian blue is able to take over these two toxic metal ions in the intestine, stopping the reabsorption from the gastrointestinal tract and favoring their fecal excretion. Prussian blue is not intestinally absorbed in significant amounts, and can be considered safe and effective for the treatment of internal contamination with radioactive or nonradioactive thallium, and with radioactive cesium.

Potassium cobalt hexacyanoferrate(II), formula  $\text{K}_2\text{COFe}(\text{CN})_6$ , has also been investigated as a cesium decontamination agent. *In vivo*, on rats, this compound proved to be at least as efficient as PB and even more efficient according some studies [2] to enhance the excretion of isotopes of cesium from the body. This result, associated with a total insolubility, meaning no absorption in gastrointestinal tract, made Potassium cobalt hexacyanoferrate(II) suitable for a potential clinical use.

To investigate the opportunity of further preclinical and clinical development of this compound, it was then necessary to assess its genotoxicity profile and to compare it with the reference clinical standard PB. For this, the bacterial reverse mutation assay (Ames test), the mutation assay at the TK locus in L5178Y mouse lymphoma cells and the *in vitro* chromosomal aberrations test on peripheral blood lymphocytes were carried out. Moreover, the *in vitro* Unscheduled DNA Synthesis (UDS) assay was performed with potassium-cobalt ferrocyanide salts. Furthermore, the *in vivo* mouse bone marrow micronucleus test and the Comet Assay in rat were implemented for Ferric ferrocyanide exclusively.

## 2. Material and methods

### 2.1. Reagents and Chemicals

The reagents and chemicals used in the experiments were purchased from the following suppliers: Dimethylsulfoxide from Acros Organics, Noisy le Grand, France; normal melting point agarose, low melting point agarose from Biorad, Marnes la Coquette, France; RPMI 1640 medium, horse serum, fetal calf serum, phosphate buffered saline from GIBCO Invitrogen SARL, Cergy-Pontoise, France; sterile water from Fresenius, Bad Homburg, Germany; HCl, L-glutamine, tryptophan, NaOH, Na<sub>2</sub>HPO<sub>4</sub>, Na H<sub>2</sub>PO<sub>4</sub>, H<sub>2</sub>O, absolute ethanol, 2-nitro fluorene (CAS Registry Number: 607-57-8) from Merck, Darmstadt, Germany; NaH<sub>2</sub>PO<sub>4</sub>, MgCl<sub>2</sub> from Merck VWR, Fontenay-sous-Bois, France; Oxoid N°2, Agar Oxoid from Basingstoke, United Kingdom; glucose-6-phosphate, NADP from Roche, Mannheim, Germany; Harris hemalun, Trifluorothymidine, collagenase Ia, CaCl<sub>2</sub>, sodium bicarbonate, potassium dichromate, histidine, biotin, sodium pyruvate, penicillin, streptomycin, pluronic acid, fungizone, Trypan blue, carboxymethylcellulose, May Grunwald, Triton X-100, EDTA, trizma base, HEPES, propidium iodide, KCl, NaCl, 2-acetaminofluorene (2-AAF CAS Registry Number: 53-96-3), benzo[a]pyrene (CAS Registry Number: 50-32-8), sodium azide (CAS Registry Number: 26628-22-8), 9-amino-acridine (CAS Registry Number: 90-45-9), mitomycin C (MMC, CAS Registry Number: 50-07-7), methyl methanesulfonate (MMS, CAS No. 66-27-3), cyclophosphamide (CPA, CAS Registry Number: 6055-19-2) from Sigma-Aldrich, Saint-Quentin Fallavier, France; DMSO, 2-anthramine (CAS Registry Number: 613-13-8) from Sigma-Aldrich GmbH, Steinheim, Germany; Giemsa reagent from Sigma-Aldrich, St Louis MO, USA; (3H)thymidine 40-60 Ci/mmol from Perkin Elmer, phytohaemagglutinin A from Thermo Fisher, Dardilly, France; cyclophosphamide (CPA, CAS Registry Number: 6055-19-2) from Baxter, Guyancourt, France; Colcemide from Fluka, Steinheim, Germany; non-essential aminoacids from Gibco, Bleiswijk, the Netherlands; methanol from Fisher Chemical, Loughborough, UK; acetic acid from Chemlab, Zedelgem, Germany.

Potassium-cobalt ferrocyanide (K-Co-ferrocyanide, batch 10204/005) was provided by Pharmacie Centrale des Armées. Prussian Blue or Ferric ferrocyanide (Fe-ferrocyanide, CAS Registry Number 14038-43-8) was also provided by Pharmacie Centrale des Armées. The batch used in the bacterial mutagenicity test, the mutation assay at the TK locus in L5178Y mouse lymphoma cells, the *in vitro* metaphase analysis on peripheral blood lymphocytes and the *in vivo* micronucleus test in rat bone marrow was 1817/N0110. In the *in vivo* rodent Comet Assay in rat, the batch 10205/007 was used.

### 2.2. Metabolic activation system

After enzymatic induction with Aroclor-1254, rat liver S9 was prepared according to Ames *et al.* [3] and Maron and Ames [4].

In the Ames test, each S9-mix contained per mL: 0.1 mL of S9, 0.02 mL of MgCl<sub>2</sub> 0.4M, 0.02 mL of KCl 1.65M, 0.5 mL of phosphate buffer 0.2M (pH 7.4), 0.04 mL of NADP 0.1M, 0.005 mL of glucose-6-phosphate 1M and 0.315 mL of H<sub>2</sub>O and was used at 500 µL/plate.

In the *in vitro* test using mammalian cell cultures (L5178Y and human lymphocytes), S9-mix contained per mL: 0.4 mL of S9, 0.2 mL of KCl 150 mM, 0.2 mL of NADP 25 mg/mL and 0.2 mL of glucose-6-phosphate 180 mg/mL and was used at 5 %, giving 2 % of S9 in final concentration.

Except S9, all cofactors were filtered through a sterilizing membrane before use.

All the studies were performed according to OECD Principles of Good Laboratory Practice [5].

### 2.3. Bacterial reverse mutation assay (Ames test)

K-Co-ferrocyanide and Fe-ferrocyanide were assayed via the Ames test performed according to the OECD Guideline No. 471 (1997) for gene mutation capability in 5 histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102). The tests were performed both in the absence and in the presence of the metabolic activation system, in two separate experiments. With metabolic activation, the first experiment was performed using agar incorporation method and the second assay using pre-incubation method while the experiments without metabolic activation were both conducted without pre-incubation.

K-Co-ferrocyanide was formulated in a mix of gelose/sterile water (50/50 v/v) and Fe-ferrocyanide formulated in sterile water at a concentration of 50 mg/mL giving a final top dose of 5000 µg/plate when used at 100 µL/plate, corresponding to the highest dose recommended by the OECD guideline. Test doses for the dose range-finding experiment (data not shown) and for both independent experiments for all strains were 50, 150, 500, 1500 and 5000 µg/plate with and without metabolic activation.

The plate incorporation assay was carried out as described by Ames *et al.* [3], Maron and Ames [4] and OECD guidelines No. 471 [6].

The pre-incubation assay with metabolic activation (assay 2) was carried out as described by Bartsch *et al.* [7], Bridges *et al.* [8] and Yamanaka *et al.* [9].

Appropriate positive reference controls were also used (without S9: TA1537: 9-amino-acridine 50 µg/plate, TA98: 2-nitrofluorene 2 µg/plate, TA1535 and TA100: sodium azide 1 µg/plate, E. coli WP2 (pKM101): mitomycin C 0.125 µg/plate, TA102: mitomycin C 0.125 µg/plate) - with liver S9: TA1535, TA1537, TA98 and TA100 2-anthramine 2 µg/plate (without pre-incubation) or 1 µg/plate (with pre-incubation), TA102 benzo(a)pyrene 2 µg/plate (with and without pre-incubation). Revertants were counted 48 hours after treatment.

A response is considered positive in the assay if a dose-response relationship is observed on 3 consecutive doses with, for the highest increase, an induction ratio greater than or equal to 3 (strains TA1535 and TA1537) or 2 (strains TA98, TA100 and TA102). In parallel, data were analysed by means of Dunnett's method [10] allowing the comparison of the values for each dose to the values for the corresponding solvent control.

#### 2.4. Mutation assay at the TK locus in L5178Y mouse lymphoma cells using a microtiter cloning technique (Trifluorothymidine Resistance)

K-Co-ferrocyanide and Fe-ferrocyanide were assayed in the mouse lymphoma TK gene mutation assay according to the OECD Guideline No. 476 (1997, the test guideline No. 490 (2016) had not yet entered into force). L5178Y mouse lymphoma cells were obtained from the American Type Culture Collection (Sophia-Antipolis, France).

K-Co-ferrocyanide and Fe-ferrocyanide were prepared at a maximum concentration of 50 mg/mL in culture medium and used at 10%, to a final concentration of 5000 µg/mL. At this concentration, we do not observe precipitation. It presents neither an increase in the osmolality superior to 50 mOsm/kg in respect to the solvent control [11] nor a significant pH variation [12]. Therefore, it was selected as the maximum concentration for the cytotoxicity assays (data not shown). However, in the study of K-Co-ferrocyanide in absence of metabolic activation this concentration induced a strong toxicity (data not shown). In consequence, the highest concentration in this assay was lowered to 4000 µg/mL. The concentration was maintained at 5000 µg/mL in presence of metabolic activation for K-Co-ferrocyanide, and for Fe-ferrocyanide both in absence and in presence of metabolic activation.

Both ferrocyanides were tested with a 3-hour short treatment with and without metabolic activation and with a 24-h continuous treatment in the absence of S9 in compliance with the current OECD Guideline No. 476 at the time of the experiments.

The criteria for acceptance were the ones recommended by Moore *et al.* [14].

Each treatment was compared with the control using the one-sided Dunnett's test for multiple comparisons with the same control.

A response is considered positive in the assay if the induced mutation frequency for at least one tested concentration is higher than the mutation frequency in the vehicle control cultures by at least the global evaluation factor of  $126 \times 10^{-6}$  [14] with a dose-response relationship at concentrations inducing no less than 10 % RTG is observed. This effect must be reproducible in an independent experiment.

## 2.5. *In vitro* chromosomal aberrations test on peripheral blood lymphocytes

Both ferrocyanides were assayed in the *in vitro* chromosomal aberrations test on peripheral blood lymphocytes assay performed according to the OECD Guideline No. 473 [15].

Both ferrocyanides were formulated in culture medium and were used at 10%. Top test concentrations for the independent experiments were determined after a preliminary toxicity assay during which cytotoxicity was assessed through mitotic index calculation. Concentrations ranged from 571.43 to 1142.86 mg/mL for K-Co-ferrocyanide and from 2307.7 to 3000 µg/mL for Fe-ferrocyanide. The tests were thus performed up to the highest dose compatible with the toxicity induced by the substances.

Gaps and breaks are analysed using Student's t-test. Although the standard deviations found do not demonstrate that the distribution is normal, this test can be used because of the large number of samples. The total number of damaged cells with and without gaps was analysed statistically using the  $\chi^2$  test. From the comments already made regarding the significance of the presence of gaps, it results that greater weight is given to change in the number of cells presenting aberrations other than gaps in assessing the genotoxicity of the test item. All numerical aberrations (polyploidy) were analysed statistically using the  $\chi^2$  test.

A response is considered positive in the assay if at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, with a dose-related response and if any of the results are outside the distribution of the historical negative control data.

Historical controls of the laboratory were 0.010 – 0.063, 0.015 – 0.025 and 0.015 and 0.065 breaks per cell in the 4-hour, 20-hour or 44-hour continuous treatments without metabolic activation and 0.010 – 0.045 and 0.015-0.050 in the treatment with metabolic activation for. The values for the number of aberrant cells/200 were of 3 – 4, 3 – 10 and 2 - 9 in the 4-hour, 20-hour or 44-hour continuous treatments without metabolic activation and 2 – 9 and 3 – 8 in the treatment with metabolic activation.

## 2.6. *In vitro* UDS assay

The potential genotoxic activity of K-Co-ferrocyanide was also investigated using the *in vitro* Unscheduled DNA Synthesis (UDS) assay performed according to the OECD guideline 482 [17]. It should be stressed that this assay was performed before OECD considered this guideline obsolete.

K-Co-ferrocyanide was prepared at a maximum concentration of 50 mg/mL in culture medium and used at 10%, giving a final concentration of 5000 µg/mL. This concentration inducing no precipitate and presenting no increase in the osmolality superior to 50 mOsm/kg in respect to the solvent control and no significant pH variation, it was retained as the maximum concentration for the cytotoxicity assays (data not shown). However, this concentration induced strong toxicity (data not shown), the highest concentration was thus decreased for the genotoxicity assay to 150 µg/mL and the maximum concentration analyzed was set to 18.75 µg/mL. In a second experiment, the maximum concentration used was 40 µg/mL.

A response is considered positive in this test system at any dose tested when the group mean NNG value is greater than 0 NNG and 20% or more of cells are in repair (NNG value  $\geq 5$ ) or if an increase is observed in both NNG and the percentage of cells in repair compared with the concurrent control group. Historical controls of the laboratory for solvent controls were -5.57 - -0.13 for NNG and 5.96 – 9.5 for NNG of cells in repair and 2 - 12% of cells in repair.

## 2.7. *In vivo* mouse erythrocyte bone marrow micronucleus test

The potential genotoxic activity of K-Co-ferrocyanide was tested in the mouse using the *in vivo* bone marrow micronucleus test in the mouse, in compliance with the OECD Guideline 474 [18].

K-Co-ferrocyanide was solubilised in Carboxymethylcellulose 0.5% in sterile water at the concentration of 80 mg/mL, giving the final dose of 2000 mg/kg b.w., as recommended by the OECD guideline in force, when administered at 25 mL/kg b.w. (this dose was determined after a preliminary toxicity assay, the data are not shown).

The statistical comparison for the polychromatic/normochromatic erythrocyte ratio was performed for each sex using the Student's t test. For micronucleus number, statistical analysis was performed on males and females separately and on two sexes combined using the Mann Whitney U rank test [20].

For a substance to be considered positive in the micronucleus test, a statistically significant increase in the number of micronuclei must be observed in any dose tested, with at least a doubling in

the number of micronuclei when compared with the negative control animals. The statistical comparison for the polychromatic/normochromatic erythrocyte ratio and for the weight homogeneity within the sex of each group was performed using the Student's t-test. Historical controls of the laboratory were 0 – 1.5 micronuclei for 1000 polychromatic in males and females.

## 2.8. *In vivo* Comet Assay in rat

The *in vivo* genotoxic activity of Fe-ferrocyanide was investigated by using the *in vivo* comet assay. OFA Sprague Dawley male rats (5 per group) were treated by oral route (gavage) using 3 daily treatments at 24-hour intervals followed by one sampling time of 3-4.5 hours after the third treatment. The OECD guideline No. 489 had not yet entered in force.

Fe-ferrocyanide was solubilised in methylcellulose 1% in sterile water at the concentrations of 200, 100 and 50 mg/mL, giving the final doses of 2000, 1000 and 500 mg/kg b.w. when administered at 10 mL/kg b.w. (this dose and the sex actually used were determined from a preliminary toxicity assay, data not shown). The top dose was in line with international recommendations. Furthermore, positive control animals were treated orally twice at 24-hour interval with MMS at 100 mg/kg b.w. and once 4 to 4.5 hours before sacrifice with MMS 70 mg/kg b.w..

The percentage of DNA in tail per animal was the parameter used to evaluate DNA damage [20,21]. The non-parametric Kruskal-Wallis test was used to display a possible dose-effect relationship. Moreover, the statistical significance of differences in the median values between each group versus control was determined with the non-parametric Mann-Whitney U-test.

All statistical analyses were performed with StatView® Software (version 5.0, SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, USA).

A positive response is defined as a statistically significant change in the % of tail DNA in at least one group in comparison with the vehicle control value. The positive control should produce a statistically significant increase. Cytotoxicity was evaluated by the enumeration of hedgehogs. Indeed, important fragmentation of the DNA can be induced not only by genotoxicity but also during the process of cell death, *i.e.* apoptosis and necrosis.

The range of negative historical controls of the laboratory was 1.98 – 7.83% and 2.26 – 6.45 % DNA in tail in glandular stomach and colon, respectively.

## 2.9. Histopathology

OFA Sprague Dawley male rats (5 per group) were treated with the same experimental protocol than in the comet assay (see section 2.8), by oral route (gavage) using 3 daily treatments at 24-hour intervals followed by one sampling time of 3-4.5 hours after the third treatment. A small piece of stomach of each treatment groups including the negative control was sampled and preserved in 4% buffered formalin pending sending to ProPath UK Limited (United Kingdom). After fixation, pieces of stomachs were dehydrated then embedded in blocks of paraffin wax. Sections (thickness about 5 µm) were stained by the haematoxylin and eosin method (one slide per stomach). Qualitative analysis with quotation of necrosis and apoptosis was carried out.

# 3. Results

## 3.1. Bacterial reverse mutation assay (Ames test)

Results of the second main assay carried out with the more sensitive pre-incubation protocol in presence of metabolic activation are summarized in Table 1 for K-Co-ferrocyanide and in Tables 2 for Fe-ferrocyanide.

In two independent assays in absence and in presence of S9-mix, no significant increase in the mean number of revertants was observed in the 5 *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 in the presence of either K-Co-ferrocyanide or Fe-ferrocyanide.

In these experimental conditions, both ferrocyanides presented no mutagenic effects.

## 3.2. Mutation assay at the *TK* locus in L5178Y mouse lymphoma cells

Results are summarized in Figures 1 and 2 for K-Co-ferrocyanide and in Figures 3 and 4 for Fe-ferrocyanide.

### 3.2.1. Experiments with K-Co-ferrocyanide

During the first test in absence of metabolic activation, a significant increase in the number of total induced mutants was observed in the presence of K-Co-ferrocyanide at the 2 maximum concentrations studied of 4000 and 2000 µg/mL with induced mutations frequencies of +220.1 and +306.4 x 10<sup>-6</sup> cells, respectively and a clear dose-response relationship. These significant increases were observed at acceptable levels of cytotoxicity, *i.e.* 25.1 and 44% relative total growth or RTG, respectively. In addition, the mutagenic activity was confirmed during the second test performed with the same conditions, *i.e.* 3 hours of treatment, and during which the maximum concentration of 4000 µg/mL caused an increase in the mutant frequency of +269.5 x 10<sup>-6</sup> cells.

Regarding the first assay in presence of metabolic activation, a biologically significant increase in the number of total induced mutants was observed in the presence of K-Co-ferrocyanide at the 3 top concentrations studied ranging from 5000 to 1730.1 µg/mL with mutation frequencies ranging from +172.1 to +534.9 x 10<sup>-6</sup> cells. These significant and dose-related increases were observed at acceptable cytotoxicity levels, ranging from 14% to 62.8% RTG. In addition, this mutagenic activity was confirmed in the second experiment. During the course of this study the maximum concentration of 5000 µg/mL proved to be too cytotoxic (RTG of 6.9% compared to the solvent control). However, its mutation rate was not evaluated; In contrast, the following 2 concentrations of 2941.2 and 1730.1 induced acceptable cytotoxicity levels with 33.6 and 67.2 % RTG, respectively, and resulted in biologically significant increases with induced mutations frequencies of +343.3 and +149.4 x 10<sup>-6</sup> cells, respectively.

Noteworthy, dose-related and statistically significant increases in the proportion of small colonies compared to that of large colonies were also observed in both assays in the absence and in the presence of metabolic activation.

### *3.2.2. Experiments with Fe-ferrocyanide*

In the first trial using a 3-hour treatment without metabolic activation, Fe-ferrocyanide (Prussian Blue) did not induce biologically significant increases in induced mutation frequency (large and small colonies) at the concentrations ranging from 1481.5 to 5000 µg/mL and the second test was thus carried out with a 24-hour treatment with a modified range of concentrations from 2048 to 5000 µg/mL. In this 24-hour trial, a biologically significant increase in the frequency of induced mutants (large and small colonies) was noted only at the highest tested concentration of 5000 µg/mL with an induced mutations frequency of 191.8 x 10<sup>-6</sup> cells. This concentration presented an acceptable level of cytotoxicity with a RTG of 34.7% compared to the corresponding negative control. In addition, during this treatment, the product tested induced a statistically and biologically significant increase in the number of small colonies and the mutation frequency of small colonies at the 3 highest studied concentrations of 3200, 4000, and 5000 µg/mL relative to the corresponding negative control, with mean induction ratios of 2.2 - 2.1 and 3.4, respectively. These observations are in favor of a clastogenic effect.

In the first trial with metabolic activation, statistically but not biologically significant increases in the mutation frequency (large and small colonies) were observed at all tested concentrations. Indeed, the values for induced mutant frequency were below the threshold of +126 x 10<sup>-6</sup> cells. However, the values for the mutation frequency at 3333.3 and 5000 µg/mL were over the upper level of historical data for solvent controls in the laboratory, *i.e.* 162.5 and 148.4 x 10<sup>-6</sup> cells vs. 137.20 x 10<sup>-6</sup> cells. Moreover, during this experiment, these concentrations induced statistically significant increases in the number of small colonies and in the mutation frequency of small colonies when compared to the corresponding negative control, with induction ratios of 2.4 and 2.9. In the second trial carried out with concentrations ranging from 2048 to 5000 µg/mL for which slight to no cytotoxicity was noted, only a statistically significant increase in mutation frequency (large and small colonies) was noted at the top concentration, but no biologically significant increase was noted with values for induced mutant frequency below the threshold of +126 x 10<sup>-6</sup> cells. However, at 4000 and 5000 µg/mL of Fe-ferrocyanide, the values for the mutation frequency were over the upper bound of historical data for solvent controls in the laboratory, *i.e.* 146.1 and 141 x 10<sup>-6</sup> cells, respectively vs. 137.20 x 10<sup>-6</sup> cells. Even if it was non-statistically significant, an increase in the number of small colonies or the mutation rate of small colonies was observed.

From the results obtained in 2 independent experiments, Fe-ferrocyanide was concluded as slightly clastogenic in presence of metabolic activation.

### *3.3. In vitro chromosomal aberrations test on peripheral blood lymphocytes*

Results are summarized in Tables 3 and 4 for K-Co-ferrocyanide and in Tables 5 and 6 for Fe-ferrocyanide.

### 3.3.1. Experiments with K-Co-ferrocyanide

In the three experiments in the absence of metabolic activation using 4, 20 or 44 hours of treatment the concentrations analysed ranging from 408.16 to 800 µg/mL, 186.59 to 571.43 µg/mL and from 98.77 to 222.22 µg/mL, respectively, K-Co-ferrocyanide induced statistically significant and dose-related increases in the number of breaks per cell and in the number of cells carrying aberrations when compared with to the relative solvent control.

In the first assay in presence of metabolic activation (4-hour treatment) using 2% of S9, K-Co-ferrocyanide induced, at the three tested doses of 444.44 - 666.67 and 1000 µg/mL, statistically significant and dose related increases compared to the control in the number of breaks per cell and the number of cells carrying aberration with 0.085 to 0.29 breaks per cell vs. 0.03 in the control and 17 to 36 aberrant cells vs. 5 in the control. Moreover, in the second assay in the presence of metabolic activation using 4% of S9, K-Co-ferrocyanide induced at the highest test concentration of 1142.86 µg/mL, a statistically significant increase compared to the control of the number of breaks per cell and the number of cells carrying aberrations (excluding gaps only) with 18 aberrant cells vs. 3 in the solvent control.

K-Co-ferrocyanide did not induce any polyploidy whatever the condition tested (data not shown).

Therefore, K-Co-ferrocyanide presents an *in vitro* clastogenic effect both in absence and in presence of metabolic activation.

### 3.3.2. Experiments with Fe-ferrocyanide

In the 4-h short-term treatment in absence of metabolic activation, Fe-ferrocyanide (Prussian Blue) and at the 2 highest concentrations studied of 1000 and 2500 µg/mL induced statistically significant increases in the number of cells carrying aberrations with 24 and 22 cells with aberrations / 200, respectively vs 9 / 200 in the solvent control.

In the 20-hour treatment, Fe-ferrocyanide caused, at the three test concentrations of 1775.2, 2307.7 and 3000 µg/mL, statistically significant increases in the number of cells with aberrations with 21 - 16 and 13 cells / 200, respectively, vs. 3/200 in the solvent control.

In the 44-hour treatment, Fe-ferrocyanide caused statistically significant increases in the number of cells with aberrations at the three test concentrations of 1365.5 - 1775.2 and 2307.7 µg/mL with 10 - 18 and 14 aberrant cells / 200, respectively, vs. 1/200 in the solvent control. Moreover, at the intermediate concentration of 1775.2 µg/mL, one of the cells showed a chromatid exchange in the form of a tetra-radial, which represents a significant marker of clastogenic activity whereas this type of rearrangement was not observed in the solvent control.

In both experiments in the presence of metabolic activation, using either 2% or 4 of S9, Fe-ferrocyanide did not induce any statistically significant increase in the number of breaks per cell or in the number of cells carrying aberrations at any of the concentrations analysed ranging from 1333.3 to 3000 µg/mL.

Fe-ferrocyanide did not induce any polyploidy whatever the test condition (data not shown).

Therefore, Fe-ferrocyanide presents an *in vitro* clastogenic effect in absence of metabolic activation only.

### 3.4. *In vitro* UDS assay

In the two experiments performed with Fe-ferrocyanide, group mean net nuclear grain count (NNG) values at the concentrations range tested from 1.17 to 18.75 µg/mL in the first assay, and from 14 to 40 µg/mL in the second assay were less than zero (*i.e.* -2.80 to -3.44 vs. -3.77 in the solvent control in the first UDS assay, and -5.14 to -6.70 vs. -6.76 in the solvent control in the second UDS assay), that is to say below the threshold value of 0 NNG for a positive response.

Furthermore, no significant increase was observed in the mean net nuclear grain count values of cells in repair (NNG ≥5) at any concentrations of Fe-ferrocyanide tested when compared with the respective controls (7.53 to 7.37 vs. 6.53 in the solvent control in the first assay, and 7.85 to 5.45 vs. 6.73 in the solvent control in the second assay). Regarding the percentage of cells in repair in the first assay, an increase was observed only at the highest analysable concentration of 18.75 µg/mL when

compared with the solvent control with 11.35% vs. 5.09%. Nevertheless, no increase in the percentage of cells in repair was observed at the lower concentration with values ranging from 6.51% at 9.37 µg/mL to 6.31% at 1.17 µg/mL vs. 5.09% in the solvent control in the first assay). As no dose-related increase was observed in the percentage of cells in repair and as no increase was seen in NNG at any concentrations tested, this effect could not be attributed to an expression of a genotoxic activity.

Furthermore, the percentage of cells in repair at the highest concentration tested in the second assay was increased when compared with the respective solvent control with 4.19% at 40 µg/mL vs. 2.31%; however, no increase in the percentage of cells in repair was observed at the immediately lower concentration of 30.77 µg/mL with 3.42% vs. 2.31% in the solvent control. No dose-related increase was seen in both NNG and the percentage of cells in repair; this effect could not be attributed to an expression of a genotoxic activity.

In addition, no cell in S-phase was observed in both assays (Table 7). Therefore, it was concluded that Fe-ferrocyanide did not induce any *in vitro* proliferative effect in primary culture of hepatocytes under these experimental conditions.

### 3.5. *In vivo* Mammalian erythrocyte bone marrow micronucleus test with K-Co-ferrocyanide

No statistically or biologically significant increase in the frequency of micronuclei was observed in animals treated with K-Co-ferrocyanide at the single studied dose of 2000 mg/kg b.w. (x2), considering animals of both sexes separately or by grouping results of both genders (Table 8). On the other hand, the ratio of the number of polychromatic erythrocytes (PCE) to the number of normochromatic erythrocytes (NCE) determined for the treated groups of animals and the control groups demonstrated no statistically significant change in the PCE/NCE ratio in the groups treated with K-Co-ferrocyanide. Under these conditions, K-Co-ferrocyanide does not exhibit any clastogenic activity or aneugenic as demonstrated by the micronucleus test performed at bone marrow level in mouse, but the question of the systemic exposure may be raised, and negative results should be considered with caution.

### 3.6. *In vivo* Comet Assay in rat with Fe-ferrocyanide

Statistically and or biologically significant increases in the percentage of DNA in tail were observed at all the doses tested doses ranging from 500 to 2000 mg/kg b.w./day (x3) in the stomach cells from male OFA Sprague Dawley rats, with 8.52 to 47.65% for the values of the mean of medians of DNA in tail vs. 6.64 in the vehicle control group. This effect was considered as biologically significant as the mean percentages of tail DNA values were over the intervals of historical data for negative control (*i.e.* 1.98 – 7.83%), with only a slight increase in the percentage of ghost cells, up to 1.67 fold increase when compared to the vehicle control at the intermediary dose of 1000 mg/kg b.w./day (x3), corresponding to 10.33% of ghost cells. Moreover, a dose-effect relationship was noted. To complete the genotoxic assessment, a histopathological analysis on the pieces of stomachs sampled on satellite animals treated with the same doses under the same experimental conditions as in the main assay was implemented in order to check whether a high level of toxicity and/or apoptosis could be responsible for the observed effects. Such analysis failed at demonstrating any implication of necrosis and/or apoptosis in the increase in DNA damage as measured by the tail intensity (data not shown). This indicates that the increase in the percentage of DNA in tail observed in the main assay is related to an intrinsic genotoxic activity of the test item toward stomach.

All of the criteria for a test item to be considered positive in the comet assay were met. Therefore, Fe-ferrocyanide is considered to be able to induce DNA strand breakage in the stomach after oral exposure and should be considered as an *in vivo* genotoxin in this organ.

Regarding the results obtained in the colon, first, we noted that the value for the mean of median in the vehicle control was above the upper limit of our historical data, *i.e.* 16.29% vs. 6.45%. However, statistically significant increases in the percentage of DNA in tail were observed at the highest and lowest tested doses of 2000 and 500 mg/kg b.w./day (x3) in the colon cells from male OFA Sprague Dawley rats, with 33.11 and 30.22% for the values of the mean of medians of DNA in tail. No clear dose-effect relationship was noted, however, despite the value for the mean of median in the solvent control was above the intervals of historical data, a doubling in the value of the percentage of DNA in tail was noted when compared to the solvent control and Fe-ferrocyanide was also considered as genotoxic in colon cells from rats. Otherwise, only slight increases in the percentage of ghost cells were noted, with up to 1.73-fold increase when compared to the vehicle control at the intermediary dose of 1000 mg/kg b.w./day (x3), corresponding to 23.49% of ghost cells. Given the clear genotoxic

activity of Fe-ferrocyanide in the stomach, it was not deemed necessary to implement further histology study in the colon.

#### 4. Discussion/Conclusion

*In vivo*, on rats, Potassium cobalt hexacyanoferrate(II) proved to be at least as efficient as PB and even more efficient according to some studies, to enhance the excretion of isotopes of cesium from the body [2]. This result, associated with a total insolubility meaning no absorption in gastrointestinal tract, made potassium cobalt hexacyanoferrate(II) suitable for a potential use in Human. To test the opportunity of a further preclinical and clinical development of this compound, it was then necessary to assess its genotoxicity profile and to compare it with the reference clinical standard PB.

To adequately cover all the genetic endpoints, because no individual assay can provide information on all genetic events leading to genotoxicity, several tests were implemented. For this, both ferrocyanides were assayed *in vitro* using the Ames' test, the mouse lymphoma assay on TK locus and the *in vitro* chromosomal aberrations test on peripheral blood human lymphocytes. The *in vitro* UDS assay on rat hepatocytes was also carried out for K-Co-ferrocyanide only. Finally, *in vivo* investigations were also implemented.

Ferric and potassium-cobalt ferrocyanides displayed no mutagenic effects in the Ames test. These results are in line with the lack of genotoxic effect in *Bacillus subtilis* in the Rec-assay [22].

In contrast, both compounds were positive in the mouse lymphoma assay on TK locus both in absence and in presence of metabolic activation. Noteworthy, these effects were mainly due to increases in the proportion of small colonies which reflect chromosomal events induced by a clastogenic activity. For K-Co-ferrocyanide, the clastogenic effect was confirmed in the *in vitro* chromosomal aberrations test on peripheral blood human lymphocytes both in absence and in presence of metabolic activation while Fe-ferrocyanide presented a significant effect in absence of metabolic activation only. K-Co-ferrocyanide was negative in the *in vitro* UDS assay on rat hepatocytes. However, it should be noted that, since its implementation, the unscheduled DNA synthesis in mammalian cells *in vitro* [17] was officially deleted by OECD on 2<sup>nd</sup> April 2014 due to its lack of sensitivity.

Overall, on the basis of the *in vitro* tests, the genotoxicity profile of both ferrocyanides is qualitatively similar. To determine whether the genotoxicity observed *in vitro* occurs *in vivo*, follow-up studies were carried out. K-Co-ferrocyanide was tested in the *in vivo* bone marrow micronucleus assay in order to complete the standard genotoxicity battery, while the *in vivo* Comet assay was implemented with Fe-ferrocyanide.

Following oral administration, a small amount (approx. 2%) of the hexacyanoferrate ion is absorbed [23]. More recently, Besse-Bardot *et al.* [24] demonstrated that following oral gavage of [<sup>14</sup>C] Prussian blue, radioactivity was mostly eliminated in the feces (96.9% of the dose), whilst 0.305% was in the urine. If the oral route was preferred as it is the anticipated route of human exposure, taking into account the expected low absorption following an oral exposure, target organs specific from the gastro-intestinal tract were selected for the Comet assay.

In the *in vivo* micronucleus in mouse bone marrow, K-Co-ferrocyanide did not display any genotoxic activity up to 2000 mg/kg b.w./d (x2).

In contrast, in the *in vivo* Comet assay, Fe-ferrocyanide (PB) induced statistically significant increases in DNA fragmentation in both the glandular stomach and the colon of rats treated 3 times with 2000, 1000 or 500 mg/kg b.w.. Even if no clear dose-effect relationship was observed in colon, it was concluded that Fe-ferrocyanide is able to induce DNA strand breaks in both these local organs. It is known that under alkaline conditions (> pH 13), the comet assay can detect single and double strand breaks in eukaryotic cells, resulting, for example, from direct interactions with DNA alkali labile sites, or as a consequence of transient DNA strand discontinuities resulting from DNA excision repair. These strand breaks may be: 1) repaired, resulting in no persistent effect; 2) lethal to the cell; or 3) fixed as a mutation resulting in a permanent heritable change. Therefore, the alkaline comet assay detects primary DNA damage that does not always lead to gene mutations and/or chromosomal aberrations. Indeed, fragmentation of the DNA can be caused not only by chemically-induced genotoxicity, but also during the process of cell death, *i.e.*, apoptosis and necrosis. It is difficult to

distinguish between genotoxicity and apoptosis/necrosis by the shape of the nucleus and comet tail after electrophoresis, e.g., by scoring “hedgehogs” [25,26].

Therefore, the primary DNA damage measured in the comet assay may lead to cell death, or it may initiate DNA repair, which can return the DNA either to its original state or result in mutation. In order to determine whether DNA lesions observed *in vivo* in both glandular stomach and colon are converted into gene mutations, the transgenic rodent gene mutations could be performed in those tissues. Without such data, Fe-ferrocyanide should be considered as an *in vivo* mutagen but an *in vivo* genotoxin.

Taking into account the similarity of the *in vitro* genotoxicity pattern for both ferrocyanides, the same conclusions may be drawn for K-Co-ferrocyanide. Even if clearly negative, the lonely *in vivo* micronucleus in mouse bone marrow is not sufficient to rule out any *in vivo* genotoxic potential particularly on gastro-intestinal tract organs after oral exposure. Indeed, in case of relevant positive effects in *in vitro* tests, two *in vivo* tests are generally called for, with appropriate endpoints and usually on different appropriate tissues (EMA, 2013). Therefore, unless specific data using for instance the Comet assay on gastro-intestinal tract organs after oral exposure are produced, it is not excluded that K-Co-ferrocyanide is an *in vivo* genotoxic substance and in any case, *in vitro* data indicating clastogenic activity are sufficient to warrant caution in the use of these compounds in patients.

## Declarations – Conflict of interests

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**Table 1: Second mutagenicity assay with K-Co-ferrocyanide using the Ames test**

	Doses in µg/plate	TA 1535		TA 1537		TA 98		TA 100		TA102	
		rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd
Positive control	(a)	<b>333.3</b>	25.7	<b>301.7</b>	47.4	<b>360.0</b>	18.5	<b>305.7</b>	9.1	<b>1569.3</b>	121.1
Second assay w/o S9 mix w/o pre-incubation	0	<b>9.0</b>	2.8	<b>3.2</b>	2.9	<b>11.8</b>	6.1	<b>60.0</b>	3.8	<b>240.2</b>	13.6
	50	<b>8.7</b>	2.3	<b>3.0</b>	2.0	<b>14.0</b>	2.0	<b>60.0</b>	3.6	<b>201.0</b>	15.7
	150	<b>7.7</b>	6.4	<b>3.0</b>	1.0	<b>14.0</b>	1.0	<b>64.7</b>	10.1	<b>219.3</b>	9.3
	500	<b>9.3</b>	0.6	<b>2.0</b>	1.0	<b>11.7</b>	3.2	<b>59.7</b>	4.2	<b>215.0</b>	27.5
	1500	<b>7.3</b>	1.5	<b>6.3</b>	0.6	<b>15.0</b>	1.0	<b>70.7</b>	6.4	<b>185.7</b>	19.3
	5000	<b>4.7</b>	0.6	<b>3.3</b>	1.5	<b>11.3</b>	4.5	<b>59.0</b>	1.0	<b>181.3</b>	14.2
Positive control	(b)	<b>107.3</b>	10.2	<b>126.7</b>	17.6	<b>988.0</b>	60.5	<b>956.7</b>	104.2	<b>722.3</b>	55.8
Second assay with S9 mix with pre- incubation	0	<b>8.3</b>	2.5	<b>5.5</b>	2.3	<b>17.7</b>	4.3	<b>64.2</b>	5.9	<b>214.5</b>	22.2
	50	<b>8.0</b>	2.6	<b>6.7</b>	1.2	<b>21.7</b>	3.2	<b>59.0</b>	2.6	<b>220.0</b>	17.4
	150	<b>11.0</b>	2.6	<b>9.3</b>	3.5	<b>21.3</b>	4.7	<b>61.7</b>	1.5	<b>234.7</b>	2.5
	500	<b>14.7</b>	5.8	<b>5.7</b>	0.6	<b>23.7</b>	2.1	<b>68.0</b>	4.6	<b>265.0</b>	18.1
	1500	<b>14.7</b>	3.2	<b>7.3</b>	1.5	<b>27.7</b>	10.1	<b>59.7</b>	8.7	<b>287.7</b>	15.4
	5000	<b>12.7</b>	3.2	<b>9.7</b>	0.6	<b>15.3</b>	2.1	<b>47.0</b>	4.6	<b>223.7</b>	65.8

\*: 3 plates /dose, 6 for solvent control

Reference substances (µg/plate)

(a) TA1535 et TA100 : Azide de sodium 1 ; TA1537 : 9-amino-acridine 50 ; TA98 : 2-nitrofluorène 2; TA102: Mitomycin C 0.125

(b) TA 1535, TA1537, TA98, TA100 : 2-anthramine 1 ; TA102: benzo(a)pyrene 2

sd: standard deviation

**Table 2: Second mutagenicity assay with Fe-ferrocyanide using the Ames test**

	Doses in µg/plate	TA 1535		TA 1537		TA 98		TA 100		TA102	
		rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd	rev. /plate*	sd
Positive control	(a)	<b>387.0</b>	47.3	<b>532.3</b>	52.2	<b>614.0</b>	96.5	<b>466.3</b>	34.6	<b>1130.7</b>	105.4
Assay w/o S9 mix w/o pre-incubation	0	<b>11.8</b>	3.4	<b>3.0</b>	2.0	<b>9.8</b>	1.2	<b>75.8</b>	9.2	<b>168.5</b>	28.4
	50	<b>9.0</b>	4.6	<b>2.3</b>	1.5	<b>6.7</b>	1.2	<b>58.7</b>	5.1	<b>160.3</b>	9.3
	150	<b>12.0</b>	4.6	<b>3.0</b>	1.7	<b>5.0</b>	0.0	<b>57.7</b>	5.8	<b>148.7</b>	18.6
	500	<b>9.3</b>	1.2	<b>2.0</b>	1.0	<b>8.0</b>	2.0	<b>62.7</b>	4.2	<b>163.3</b>	12.7
	1500	<b>10.0</b>	2.6	<b>2.3</b>	1.5	<b>7.0</b>	1.0	<b>65.0</b>	4.4	<b>169.0</b>	9.8
	5000	<b>12.3</b>	3.5	<b>2.0</b>	1.0	<b>5.3</b>	1.2	<b>64.7</b>	7.5	<b>167.3</b>	9.1
Positive control	(b)	<b>185.7</b>	22.5	<b>147.3</b>	20.3	<b>1893.3</b>	80.1	<b>2913.3</b>	95.8	<b>1109.3</b>	198.3
Assay with S9 mix with pre- incubation	0	<b>9.5</b>	1.6	<b>5.0</b>	2.4	<b>23.8</b>	4.1	<b>98.7</b>	11.8	<b>217.7</b>	29.2
	50	<b>9.0</b>	2.6	<b>3.3</b>	1.2	<b>20.3</b>	2.5	<b>100.0</b>	8.7	<b>180.7</b>	18.0
	150	<b>8.7</b>	3.1	<b>4.0</b>	1.0	<b>25.0</b>	1.7	<b>100.3</b>	1.2	<b>179.3</b>	23.2
	500	<b>8.0</b>	1.0	<b>4.0</b>	2.0	<b>25.0</b>	6.2	<b>95.3</b>	16.1	<b>210.7</b>	41.2
	1500	<b>10.3</b>	2.5	<b>5.0</b>	3.0	<b>19.3</b>	1.5	<b>91.3</b>	12.0	<b>186.0</b>	15.1
	5000	<b>6.3</b>	1.5	<b>3.3</b>	0.6	<b>22.0</b>	1.7	<b>92.7</b>	10.5	<b>187.3</b>	9.9

\*: 3 plates /dose, 6 for solvent control

Reference substances (µg/plate)

(a) TA1535 et TA100 : Sodium Azide 1 ; TA1537 : 9-amino-acridine 50 ; TA98 : 2-nitrofluorene 2; TA102: Mitomycin C 0.125

(b) TA 1535, TA1537, TA98, TA100 : 2-anthramine 1 ; TA102: benzo(a)pyrene 2

sd: standard deviation

**Table 3: Results of the *In vitro* chromosomal aberrations test on peripheral blood lymphocytes exposed to K-Co-ferrocyanide without metabolic activation**

4 hour treatment			
Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
Mitomycin C	97.6	0.510**	38**
Solvent control	-	0.015	3
408.16	74.7	0.110**	20**
571.42	57.1	0.100**	18**
800	40.4	0.160**	25**

20 hour treatment				44 hour treatment			
Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200	Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
Mitomycin C	20.6	0.800**	44**				
Solvent control	-	0.025	4	Solvent control	-	0.065	10
186.59	82.2	0.175**	30**	98.77	75.2	0.185**	27**
326.53	56.6	0.255**	29**	148.15	57.5	0.275**	36**
571.43	45.7	0.320**	39**	222.22	49.0	0.260**	41**

\*: Excluding cells with gap only

\*\*: Statistically significant  $p < 0.05$  (Dunnett's test for break per cell,  $\chi^2$  for aberrant cells).

Positive control: Mitomycin C: 0.25 µg/mL

**Table 4: Results of the *In vitro* chromosomal aberrations test on peripheral blood lymphocytes exposed to K-Co-ferrocyanide with metabolic activation**

4 hour treatment with 5% S9-mix				4 hour treatment with 10% S9-mix			
Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200	Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
CPA	49.1	1.130**	49**	CPA	-	0.710**	46**
Solvent control	-	0.030	5	Solvent control	-	0.015	3
444.44	71.9	0.085**	17**	373.18	76.6	0.045**	8
666.67	65.6	0.290**	37**	653.06	60.2	0.055**	8
1000	42.4	0.220**	36**	1142.86	42.0	0.100**	18**

\*: Excluding cells with gap only

\*\*: Statistically significant  $p < 0.05$  (Dunnett's test for break per cell,  $\chi^2$  for aberrant cells).

Positive control: CPA: Cyclophosphamide 10 µg/mL

**Table 5: Results of the *In vitro* chromosomal aberrations test on peripheral blood lymphocytes exposed to Fe-ferrocyanide without metabolic activation**

Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
Mitomycin C	76.8	0.82**	49**
Solvent control	-	0.050	9
408.16	72.4	0.070	14
1000	88.8	0.150**	24**
2500	66.4	0.150**	22**

20 hour treatment				44 hour treatment			
Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200	Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
Mitomycin C	57.7	1.070**	62**	Solvent control	-	0.005	1
Solvent control	-	0.015	3	1365.5	82.77	0.065**	10**
1775.2	81.5	0.130**	21**	1775.2	63.7	0.105**	18**
2307.7	68.7	0.115**	16**	2307.7	54.2	0.080**	14**
3000	51.3	0.085**	13**				

\*: Excluding cells with gap only

\*\*: Statistically significant  $p < 0.05$  (Dunnett's test for break per cell,  $\chi^2$  for aberrant cells).

Positive control: Mitomycin C: 0.25 µg/mL

**Table 6: Results of the *In vitro* chromosomal aberrations test on peripheral blood lymphocytes exposed to Fe-ferrocyanide with metabolic activation**

4 hour treatment with 5% S9-mix				4 hour treatment with 10% S9-mix			
Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200	Conc. in µg/mL	Relative Mitotic index	Break per cell	Aberrant cells* /200
CPA	35.9	0.81	54	CPA	70.4	0.79	54
Solvent control	-	0.035	7	Solvent control	-	0.05	6
1333.3	90.1	0.065	13	1333.3	72.3	0.075	12
2000	70.3	0.075	13	2000	63.9	0.08	11
3000	44.1	0.06	10	3000	48.8	0.085	13

\*: Excluding cells with gap only

Positive control: CPA: Cyclophosphamide 10 µg/mL

**Table 7: Results of the *in vitro* UDS assay with K-Co-ferrocyanide**

Concentration in $\mu\text{g/mL}$	Viability relative to solvent control (%)	Net Nuclear Grain Count NNG	Net Nuclear Grain Count of cells in repair NNG $\geq 5$	Cells in repair NNG $\geq 5$ (%)	Cells in S-Phase per group (%)
<b>Experiment 1</b>					
2-AAF 6.25 $\mu\text{M}$	94.52	18.02	25.37	71.92	0.0
Solvent control	-	-3.77	6.53	5.09	0.0
1.17	115.94	-3.44	7.37	6.31	0.0
2.34	107.53	-3.25	6.43	7.14	0.0
4.69	110.46	-6.13	7.87	4.13	0.0
9.37	112.63	-4.85	7.37	6.51	0.0
18.75	103.70	-2.80	7.53	11.35	0.0
<b>Experiment 2</b>					
2-AAF 6.25 $\mu\text{M}$	115.48	14.34	16.00	87.42	0.0
Solvent control	-	-6.76	6.73	2.31	0.0
14	95.82	-6.70	5.45	1.16	0.0
18.21	100.79	-5.42	6.60	3.85	0.0
23.67	108.81	-5.54	7.43	4.88	0.0
30.77	110.17	-4.26	5.90	3.42	0.2
40	103.05	-5.14	7.85	4.19	0.2

Positive control: 2 acetaminofluorene 6.25  $\mu\text{M}$

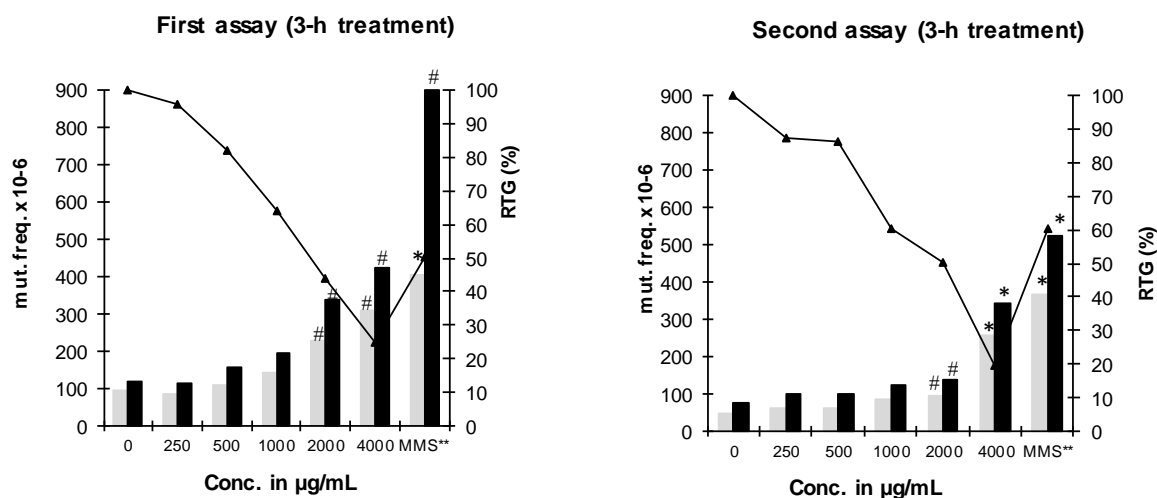
**Table 8: Results of the *in vivo* micronucleus test in the bone marrow of OF1 male and female mouse treated with K-Co-ferrocyanide**

Groups	SEX	PCE/NCE Ratio Mean $\pm$ Sd	Micronucleates/1000 PCE Mean $\pm$ Sd
CPA**	Male	3.43 $\pm$ 0.59	22.70* $\pm$ 9.83
	Female	3.09* $\pm$ 0.21	19.90* $\pm$ 3.91
	Male + Female	3.26 $\pm$ 0.45	21.30* $\pm$ 7.21
Vehicle groups	Male	3.58 $\pm$ 0.87	0.70 $\pm$ 0.27
	Female	3.72 $\pm$ 0.49	0.40 $\pm$ 0.22
	Male + Female	3.65 $\pm$ 0.67	0.55 $\pm$ 0.28
2000 mg/kg (bw) (x2)	Male	3.73 $\pm$ 0.49	0.50 $\pm$ 0.50
	Female	4.26 $\pm$ 1.28	0.40 $\pm$ 0.42
	Male + Female	4.00 $\pm$ 0.95	0.45 $\pm$ 0.44

\*: Statistically significant  $p < 0.05$  (Student's test for PCE/NCE ratio, Mann-Whitney for micronucleated cells).

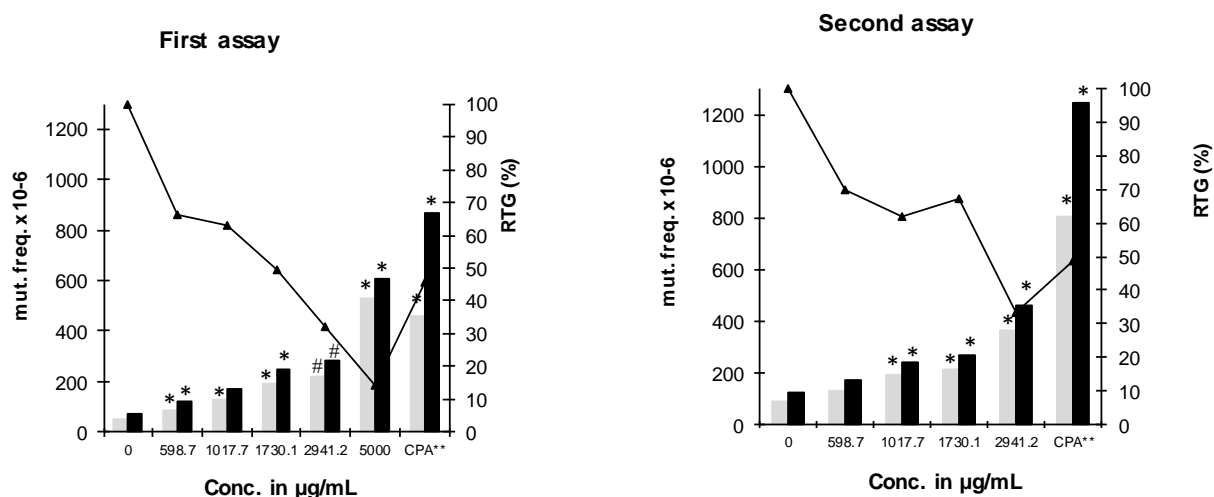
\*\*Positive control: CPA: Cyclophosphamide 50 mg/kg b.w. (Intraperitoneal)

**Figure 1: Results of the Mouse lymphoma TK+/- gene mutation assay in L5178Y cells exposed to K-Co-ferrocyanide after 3-hour of treatment without metabolic activation**



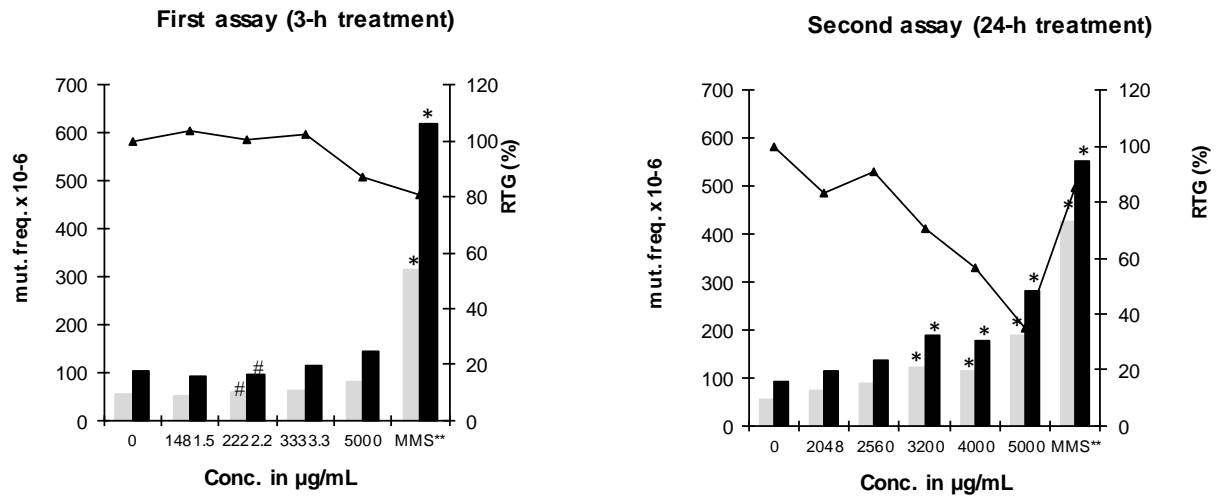
Cytotoxicity is expressed as percentage of relative total growth (RTG). Line: RTG; Grey bars : Mutation frequency of small colonies mutants; Black bars: Mutation frequency of Total induced mutants; \*: statistically significant  $p < 0.05$  (one-sided Dunnett's test). \*\* Positive control: Methyl methanesulfonate 10 µg/mL; #: because of heterogeneity between cultures, statistical analysis could not be performed

**Figure 2: Results of the Mouse lymphoma TK+/- gene mutation assay in L5178Y cells exposed to K-Co-ferrocyanide after 3-hour of treatment with metabolic activation**



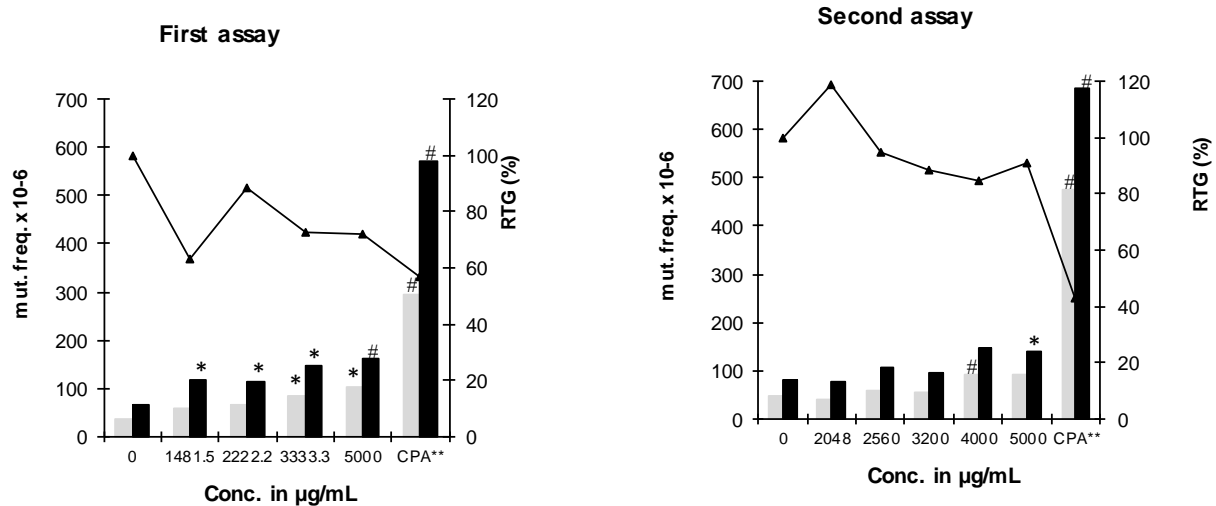
Cytotoxicity is expressed as percentage of relative total growth (RTG). Line: RTG; Grey bars : Mutation frequency of small colonies mutants; Black bars: Mutation frequency of Total induced mutants; \*: statistically significant  $p < 0.05$  (one-sided Dunnett's test). \*\* Positive control: Cyclophosphamide 2 µg/mL; #: because of heterogeneity between cultures, statistical analysis could not be performed

**Figure 3: Results of the Mouse Lymphoma TK+/- gene mutation Assay in L5178Y cells exposed to Ferrocyanide after 3 or 24-hour of treatment without metabolic activation**



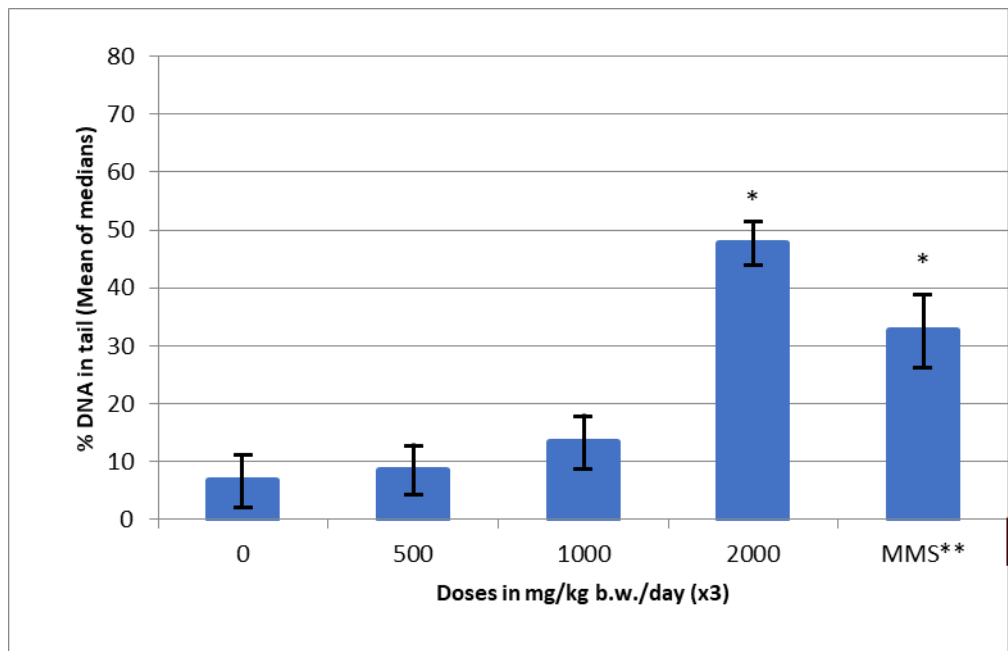
Cytotoxicity is expressed as percentage of relative total growth (RTG). Line: RTG; Grey bars : Mutation frequency of small colonies mutants; Black bars: Mutation frequency of Total induced mutants; \*: statistically significant  $p < 0.05$  (one-sided Dunnett's test). \*\* Positive control: Methyl methanesulfonate 10 µg/mL; #: because of heterogeneity between cultures, statistical analysis could not be performed

**Figure 4: Results of the Mouse Lymphoma TK+/- gene mutation Assay in L5178Y cells exposed to Ferrocyanide after 3-hour of treatment with metabolic activation**



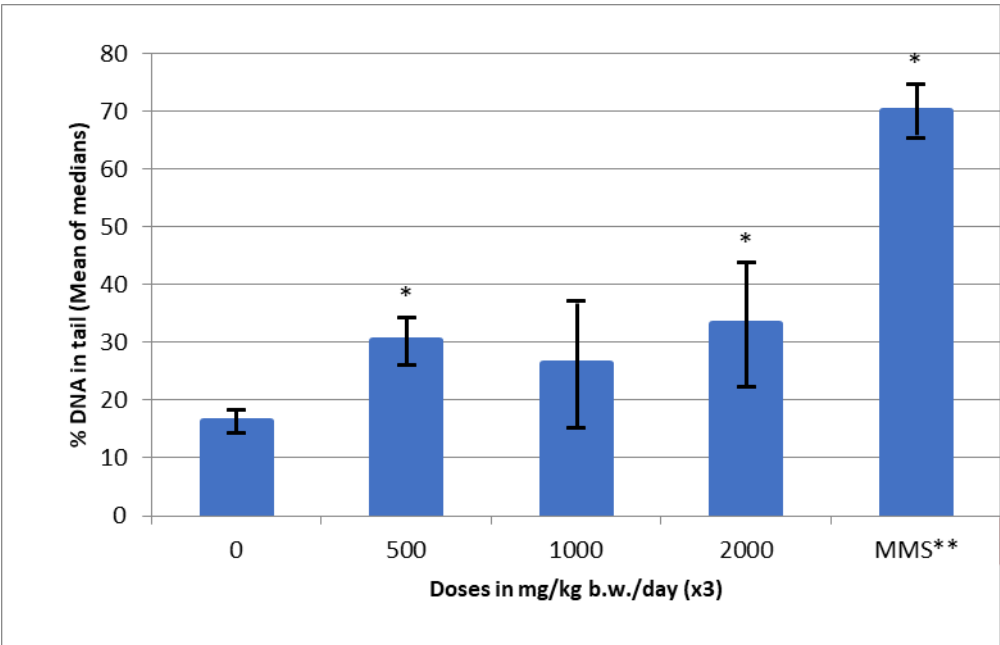
Cytotoxicity is expressed as percentage of relative total growth (RTG). Line: RTG; Grey bars : Mutation frequency of small colonies mutants; Black bars: Mutation frequency of Total induced mutants; \*: statistically significant  $p < 0.05$  (one-sided Dunnett's test). \*\* Positive control: Cyclophosphamide 2 µg/mL; #: because of heterogeneity between cultures, statistical analysis could not be performed

**Figure 5: Results of the *in vivo* Comet Assay in the glandular stomach of OFA Sprague Dawley male rat treated with Fe-ferrocyanide**



Results expressed as mean of medians  $\pm$  sd for 5 animals per group  
 sd: standard deviation  
 \*: statistically significant  $p < 0.05$  (Mann-Whitney). \*\* Positive control: MMS: Methyl methanesulfonate 100 mg/kg b.w./day (x2) and 70 mg/kg b.w./day (x1)

**Figure 6: Results of the *in vivo* Comet Assay in the colon of OFA Sprague Dawley male rat treated with Fe-ferrocyanide**



Results expressed as mean of medians  $\pm$  sd for 5 animals per group (4 animals in positive control group)  
 sd: standard deviation  
 \*: statistically significant  $p < 0.05$  (Mann-Whitney). \*\* Positive control: MMS: Methyl methanesulfonate 100 mg/kg b.w./day (x2) and 70 mg/kg b.w./day (x1)