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Beryllium Metal I. Experimental Results on Acute Oral Toxicity, Local Skin and Eye Effects, and Genotoxicity

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The toxicity of soluble metal compounds is often different from that of the parent metal. Since no reliable data on acute toxicity, local effects, and mutagenicity of beryllium metal have ever been generated, beryllium metal powder was tested according to the respective Organisation for Economical Co-Operation and Development (OECD) guidelines. Acute oral toxicity of beryllium metal was investigated in rats and local effects on skin and eye in rabbits. Skin-sensitizing properties were investigated in guinea pigs (maximization method). Basic knowledge about systemic bioavailability is important for the design of genotoxicity tests on poorly soluble substances. Therefore, it was necessary to experimentally compare the capacities of beryllium chloride and beryllium metal to form ions under simulated human lung conditions. Solubility of beryllium metal in artificial lung fluid was low, while solubility in artificial lysosomal fluid was moderate. Beryllium chloride dissolution kinetics were largely different, and thus, metal extracts were used in the *in vitro* genotoxicity tests. Genotoxicity was investigated *in vitro* in a bacterial reverse mutagenicity assay, a mammalian cell gene mutation assay, a mammalian cell chromosome aberration assay, and an unscheduled DNA synthesis (UDS) assay. In addition, cell transformation was tested in a Syrian hamster embryo cell assay, and potential inhibition of DNA repair was tested by modification of the UDS assay. Beryllium metal was found not to be mutagenic or clastogenic based on the experimental *in vitro* results. Furthermore, treatment with beryllium metal extracts did not induce DNA repair synthesis, indicative of no DNA-damaging potential of beryllium metal. A cell-transforming potential and a tendency to inhibit DNA repair when the cell is severely damaged by an external stimulus were observed. Beryllium metal was also found not to be a skin or eye irritant, not to be a skin sensitizer, and not to have relevant acute oral toxic properties.

Keywords: acute toxicity; beryllium; genotoxicity; mutagenicity; sensitization; solubility

INTRODUCTION

Beryllium metal has physical properties that make its use essential for very specific applications, such as medical diagnostics, nuclear/fusion reactors, and aerospace applications. Only very few articles contain pure beryllium metal or high beryllium-containing composite parts, while most of the beryllium metal is used in copper alloys containing low levels (typically $\leq 2\%$) of the substance. Alloys containing beryllium are used in electronics, energy, automotive, and aerospace applications because of their high strength, elasticity, electrical and thermal conductivity, high melting point, fatigue resistance, and resistance to oxidation. Manufacturers, concerned about the life cycles of their products, use beryllium and beryllium-containing alloys to reduce weight, mass, raw materials, and waste. The impact on fuel consumption (reduction of aeronautic and aerospace vehicle weights) and miniaturization of electrical parts is significant and cannot be attained by any other

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material without negative impacts on performance technology and the environment. High-resolution medical diagnostic X-rays are not possible without the use of beryllium metal.

The hazards of beryllium were mostly assessed and classified in the past by grouping it together with soluble beryllium compounds although intrinsic physical and chemical properties as well as toxicity of soluble metal salts are naturally different from that of the parent metal. It is known from several examples in toxicology that oxidation state and charge can heavily influence metals' toxicity. For examples, the health effects of hexavalent chromium are clearly different from that of chromium metal; the same is true for soluble nickel compounds and nickel metal.

Since no reliable data on acute toxicity, local effects, and mutagenicity of beryllium metal have ever been generated, beryllium metal powder was tested according to the respective Organisation for Economical Co-Operation and Development (OECD) guidelines for inclusion in the technical dossier for beryllium metal as required by the REACH Regulation (EC) No 1907/2006. In addition, it is of practical benefit to test beryllium metal since the insoluble forms of beryllium products comprise almost 100% of the commercial market while commercial application of soluble beryllium salts is extremely rare and confined to laboratory uses.

Conducting *in vitro* genotoxicity tests with soluble metal salts is not considered adequate when the dissolution kinetics between the salt and the metal differs. It is recognized that the complex environment of the lung cannot be easily simulated *in vitro*; however, it is critical to try to at least basically understand what exposes the cells *in vivo* before designing *in vitro* experiments on poorly soluble compounds. Consequently, as a basic approximation, an ion formation test was conducted with the intention to compare the dissolution behavior of beryllium metal and beryllium chloride (as a representative of soluble beryllium compounds) under conditions simulating inhaled beryllium metal in the human lung [non-abrasive shaking for up to 28 days in the dark under normal lung (pH 7.4) and lysosomal (pH 4.5) conditions]. It is important to note that when evaluating solubility of metals, the suspended particulate matter must be removed by centrifugation (Midander *et al.*, 2006). The results obtained from the ion formation test were used to design the exposure in the genotoxicity assays *in vitro*. Comparable assays are classically conducted to test medical devices that contain toxicologically relevant metals like nickel (Wever *et al.*, 1997; Tomakidi *et al.*, 1999; Montanaro *et al.*, 2005). The procedures have been developed by US Pharmacopoeia, have been adapted and laid down in ISO 10993 and genotoxicity assays conducted with extracts are integral part of the safety assessment of medical devices since the mid-1990s. Comparable designs have also been used to investigate cadmium-containing dusts sampled under occupational conditions (Cavallo *et al.*, 2008) but have up to the author's knowledge not been systematically conducted on nickel (Zhao *et al.*, 2009) or other pure metals.

Genotoxicity was addressed by a bacterial gene mutation assay, a mammalian gene mutation and a chromosome aberration assay, and DNA damage by an unscheduled DNA synthesis (UDS) assay. In addition, cell-transforming potential was investigated by a Syrian hamster embryo (SHE) cell transformation assay. Effects on DNA repair were investigated in a modified UDS assay (DNA was damaged to increasing extents by incubation with a known DNA-damaging agent, 2-acetylaminofluorene, and the effect of simultaneous co-incubation with beryllium metal extracts on DNA repair was investigated).

All tests were conducted with extracts of the metal powder generated by extraction of the metal powder under simulated lung conditions (37°C under non-abrasive shaking for 72 h) and the undissolved particles were removed before incubation by centrifugation. This procedure was applied for three reasons: (i) testing of soluble compounds instead of the metal is considered an oversimplification ignoring toxicokinetics and thus not adequate for assessment of the metal, (ii) particles were removed, as the presence of particulate matter can complicate microscopic evaluation, and (iii) the cells validated for use in the assays are not primary cells adapted to contact with particulate matter, which might trigger secondary effects (experimental artifacts) in the test cells. The extraction conditions were designed to achieve the highest technically possible concentration of beryllium ions while maintaining the integrity of the cell culture media.

Since the grouping approach is considered as not adequate for beryllium and its compounds and no reliable data on acute toxicity, local effects, and mutagenicity of beryllium metal have ever been generated, beryllium metal powder was tested according to the respective OECD guidelines for acute oral toxicity, potential skin and eye irritation/corrosion properties, and skin sensitization potential.

The scope of this publication is to present new test data generated on beryllium metal. Interpretation and implementation into the scientific and regulatory

context of existing studies are done in a separate article published in the same issue of this journal (Strupp, 2010).

MATERIALS AND METHODS

All investigations were performed under good laboratory practice at Harlan Laboratories Ltd, Switzerland/Germany. The laboratory is Association for Assessment and Accreditation of Laboratory Animal Care accredited, and all vertebrate studies were conducted under permit of the Kantonales Veterinäramt Basel Land, Switzerland. Full study reports are archived under the study numbers B52108, B52132, C15743, C02024, C31245, C31267, C64286, C24888, C24890, and C24901. Rats and rabbits were obtained from Harlan Laboratories B.V., Horst, the Netherlands; guinea pigs and Syrian golden hamsters were obtained from Charles River, Kissleg, Germany. All animals were allowed to acclimatize to laboratory conditions for at least 5 days before start of treatment and were housed in groups of three (rats)/individually (guinea pigs) in Macrolon type 4 cages on softwood bedding or individually in stainless steel cages (rabbits).

Beryllium metal powder (Batch O-30H) was obtained from Brush Wellman Inc., OH, USA, and the same batch was used in all tests. The metal powder had a purity of 99.4% beryllium with small impurities from beryllium oxide (0.4%) and traces (<0.05%) of other metals. The particles were all <44 μm diameter, with ~90% by weight between 10 and 44 μm and with a substantial number of particles <10 μm (10%) and <4.5 μm (1.5%). Thus, a significant number of particles were small enough to theoretically reach the alveoli. Beryllium chloride (Lot 05987AH, purity 99%) and all other chemicals used, if not indicated otherwise, were obtained from Sigma-Aldrich, Buchs, Switzerland. Artificial lung fluid of pH 7.4 (Gamble's solution) was prepared at Harlan and consisted of 6.6 g l^{-1} NaCl, 2.7 g l^{-1} $NaHCO_3$, 0.0022 g l^{-1} $CaCl_2 \cdot 2\ H_2O$, 0.3580 g l^{-1} Na_2HPO_4, 0.08 g l^{-1} Na_2SO_4, 0.21 g l^{-1} $MgCl_2$, 0.118 g l^{-1} glycine, 0.153 g l^{-1} sodium citrate, 0.18 g l^{-1} L-sodium tartrate, 2 g l^{-1} formaldehyde, 0.171 g l^{-1} pyruvic acid sodium salt, and 0.175 g l^{-1} L-lactic acid sodium salt dissolved in water, pH adjusted to 7.4. The artificial lung fluid of pH 4.5 (artificial lysosomal fluid) was prepared at Harlan and consisted of 3.21 g l^{-1} NaCl, 6.0 g l^{-1} NaOH, 20.8 g l^{-1} citric acid, 0.097 g l^{-1} $CaCl_2$, 0.179 g l^{-1} $Na_3PO_4 \cdot 7\ H_2O$, 0.039 g l^{-1} Na_2SO_4, 0.106 g l^{-1} $MgCl_2 \cdot 6\ H_2O$, 0.059 g l^{-1} glycerol, 0.077 g l^{-1} sodium citrate dihydrate, 0.09 g l^{-1} sodium tartrate dihydrate, 0.085 g l^{-1} L-lactic acid sodium salt, 0.086 g l^{-1} sodium pyruvate, 1 ml l^{-1} formaldehyde, pH adjusted to 4.5. Culture media and cell culture supplements were from Life Technologies, Eggenstein, Germany, and PAA Laboratories, Cölbe, Germany. Although it is recognized that metabolism of beryllium metal by the metabolic activation system (rat liver S9 mix) is unlikely, it was included in the genotoxicity tests to meet the regulatory requirements.

Acute oral toxicity was tested according to OECD guideline 423. Briefly, two groups of each three female RccHan:WIST (SPF) rats (10 weeks and 174–195 g at dosing) were fasted overnight and exposed to a single dose of 2000 mg kg^{-1} body weight (b.w.) beryllium metal powder formulated in polyethylene glycol 300 at a dosing volume of 10 ml kg^{-1} b.w. by oral gavage. The animals were housed in groups of three with *ad libitum* access to pelleted standard rodent diet (except for the fasting as described above) and tap water and observed for mortality, viability, clinical signs, and body weight development for 14 days. At the end of the observation period, all animals were sacrificed and subjected to a detailed macroscopic necropsy.

Skin irritation/corrosion was tested according to OECD guideline 404. Briefly, 0.5 g of beryllium metal powder (moistened with ~0.5 ml of water to intensify skin contact) was applied to the shorn intact skin (2.5 × 2.5 cm) of each of three New Zealand White (SPF) rabbits (one male: 17 weeks and 2.4 kg, two females: 16 weeks and 2.1 and 2.4 kg at dosing) and held in place for 4 h under semi-occlusive conditions (surgical gauze patch held in contact with the skin by means of an adhesive hypoallergenic semi-occlusive dressing and a restrainer bandage wrapped around the abdomen). The test item was removed by washing with lukewarm water after exposure. The animals were housed individually with *ad libitum* access to standard pelleted rabbit diet and tap water. The application site was observed for signs of skin irritation 1, 24, 48, and 72 h after removal of the test substance, and skin reactions were graded according to the scoring system provided in Commission Regulation (EC) 440/2008 B.4.

Eye irritation was tested according to OECD guideline 405. As no signs of irritation were observed in the skin irritation study, the same animals were used in the eye irritation study to reduce the use of vertebrates. Briefly, 0.1 g beryllium metal powder was instilled into the conjunctival sac of the left eye of each of the three rabbits. The untreated eye served as control. The eyelids were held together

for 1 s to prevent loss of test item. The treated eye was not rinsed before evaluation at 1, 24, 48, 72 h, and 7 days after administration. The animals were housed individually with *ad libitum* access to standard pelleted rabbit diet and tap water. Observed signs of irritation were graded according to the scoring system provided in Commission Regulation (EC) 440/2008 B.5.

Skin sensitization was tested according to OECD guideline 406. The local lymph node assay in mice was considered as an alternative, but as beryllium metal cannot be dissolved in a vehicle suitable for this assay to grant systemic exposure and suitability of the assay for metals is limited, the maximization method developed by Magnussen and Kligman was applied. Briefly, 10 male Dunkin-Hartley guinea pigs (5–6 weeks and 344–383 g) were treated by intradermal administration of a beryllium metal powder suspension together with Freund's complete adjuvant into the shorn dorsal skin of the scapular region [three pairs of injections, (i) 1:1 (v/v) mixture of Freund's complete adjuvant and physiological saline, (ii) the test item at 15% in polyethylene glycol 300, and (iii) the test item at 15% in a 1:1 (v/v) mixture of Freund's complete adjuvant and physiological saline]. The 15% suspension was the highest concentration that could technically pass the needle for intradermal injection, while higher concentrations in a pretest did not pass. One week thereafter, this application was followed by an epidermal application on the same site (50% in polyethylene glycol 300, occluded conditions, 48 h) to induce sensitivity. The concentration used was the lowest concentration triggering a slight dermal irritation response in a pretest. Two weeks later, allergic contact reactions were challenged by epidermal application to the skin (10% in polyethylene glycol, occluded, 48 h) of the left flank. The concentration used was the highest non-irritating dose in a pretest. A control group of five male guinea pigs was treated in the same way as the test group, but beryllium was replaced by the respective volume of the vehicle polyethylene glycol. The animals were housed individually with *ad libitum* access to standard pelleted guinea pig diet and tap water. Skin at the application site was depilated by a commercial product (VEET© cream, Reckitt & Colman AG, Switzerland) ~3 h before assessment of skin reactions, flushed with warm water, and blotted dry. Reactions (erythema and edema) were evaluated 24 and 48 h after challenge exposure for local reactions and were graded (0 = no visible change, 1 = discrete or patchy erythema, 2 = moderate and confluent erythema, and 3 = intense erythema and swelling). Animals with a visible reaction (grades 1–3) were considered as positive responders. Correct functionality of the test system in the laboratory was investigated in a positive control study with 3% alpha-cinnamaldehyde (formulated in polyethylene glycol 300) 1 month before the study on beryllium metal.

Ion formation from the beryllium metal particles under simulated lung conditions was tested cell free. Beryllium metal and beryllium chloride were loaded at 100 mg l^{-1} [worst-case loading rate proposed in OECD guidance No. 29 (OECD, 2001)] in Gamble's solution (pH 7.4, simulating normal lung conditions) or artificial lysosomal fluid (pH 4.5, simulating lysosomal environment and worst-case assumption), respectively, and shaken under non-abrasive conditions at 37°C in the dark. Triplicate bottles were incubated for each sampling time point and the pH of the solutions was controlled on days 1, 2, and 3 and later on a weekly basis. The pH was found to be constant with a maximum deviation of ±0.4 pH units and was adjusted if the deviation was >0.1 pH units. Samples were analyzed after 1, 7, 14, and 28 days. Prior to analysis, particulate beryllium metal was removed by centrifugation (3000 x g, 25 min) and the supernatant acidified with nitric acid to prevent precipitation during analysis time. Samples were analyzed by atomic absorption spectrometry (λ = 234.9 nm) after calibration with beryllium chloride standard (J.T. Baker, Phillipsburg, NJ, USA). Results were expressed as percent dissolved of theoretically available for dissolution to allow comparison, as the recommended loading rate is fixed. The mass of beryllium contained in the loaded 100 mg beryllium chloride (~11.2 mg) was set to 100%, while for the pure metal, the theoretically dissolvable beryllium was 100 mg l^{-1}.

Metal extract for exposure in the genotoxicity assays was prepared by extraction of 100 mg l^{-1} beryllium metal powder in either 0.9% aqueous NaCl (bacterial gene mutation) or the culture medium for the respective cell system (mammalian cell chromosome aberration/mammalian cell gene mutation/UDS/cell transformation) by non-abrasive shaking in the dark at 37°C for 72 h. This protocol was developed based on the results of the ion formation test and reflects a compromise between the maximum achievable dissolution and sterility/quality (integrity of supplements, vitamins, etc.) of the culture media used in the genotoxicity assays. Particulate matter was removed by centrifugation (3500 x g, 25 min) and dissolved beryllium was determined by atomic absorption spectrometry in the supernatant. The concentration of dissolved beryllium in the supernatant

was determined as described in the ion formation test.

Bacterial gene mutation (Ames test) was tested according to OECD guideline 471. Briefly, the beryllium metal extract was used to expose the bacteria (*Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and *Escherichia coli* strain WP2 *uvr*A) in the presence and absence of rat liver S9 mix (Ames *et al.*, 1975) to different dilutions of extracts ranging from pure (100%) extract to 10% in physiological saline. Three plates per strain and dose were used in each of the two independent experiments. The first experiment was performed as a plate incorporation assay and the repeat as preincubation assay (60 min at 37°C). Selection of reverse mutants was performed by incubation for 48 h at 37°C on commercially available selection agar plates (E. Merck, Darmstadt, Germany) suitable for the respective strain. Colonies were automatically counted (Petri Viewer Mk2/Ames Study Manager, Perceptive Instruments, Suffolk, UK). Performance of the test system was checked by parallel testing of NaN_3, 4-nitro-*o*-phenylene-diamine, methyl methane sulfonate without metabolic activation, and 2-aminoanthracene with metabolic activation.

Mammalian cell gene mutation at the hypoxanthine-guanine phosphoribosyltransferase locus (HPRT test) was tested according to OECD guideline 476. Briefly, the beryllium metal extract was used to expose the cells (V79 Chinese hamster lung fibroblasts) in the presence and absence of rat liver S9 mix (Ames *et al.*, 1975) to different dilutions of extracts ranging from pure (100%) extract to 12.5% in culture medium (minimum essential medium, supplemented with 10% fetal calf serum and 1% neomycin sulfate). Two independent cultures were exposed per dose level in each of the two independent experiments. Before use, each batch of cells was checked for karyotype stability, mycoplasma contamination, and spontaneous mutant frequency. Cells were exposed for 4 h in the presence or absence of metabolic activation (serum free) and in the second experiment for 24 h in absence of S9 mix (with serum) and for 4 h in the presence of metabolic activation (serum free). After exposure, cells were washed and allowed to express their phenotype for 6 days under normal maintenance conditions (4.5% CO_2, 37°C, subculturing; relative cloning efficiency as a marker of cytotoxicity was determined in parallel). 6-Thioguanine was added to the medium (11 μg ml^{-1}) for 7–10 days, eliminating non-mutated cells by its cytotoxic activity. Colonies formed from surviving cells were stained with methylene blue and counted. Performance of the test system was checked by parallel testing of ethylmethane sulfonate (Acros Organics, Geel, Belgium) without metabolic activation and 7,12-dimethylbenz(*a*)anthracene with metabolic activation.

Mammalian cell chromosome aberration was tested according to OECD guideline 473. Briefly, the beryllium metal extract was used to expose the cells (whole-blood cultures obtained from two healthy female donors, 31 and 35 years of age) in the presence and absence of rat liver S9 mix (Ames *et al.*, 1975) to different dilutions of extracts ranging from pure (100%) extract to 50% in culture medium [Dulbecco's modified eagle medium/Ham's F12 medium; mixture 1:1, supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 3 μg ml^{-1} phytohemagglutinin, 25 000 USP U ml^{-1} heparin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. Two independent cultures were exposed per dose level in each of the two independent experiments. Cells were maintained at 4.5% CO_2, 37°C. Cells were exposed for 4 h in the presence of metabolic activation (serum free) and in absence of metabolic activation (with serum). In the second experiment, cells were incubated for 4 h in the presence (serum free) and for 22 h in absence (with serum) of metabolic activation. Dividing cells were arrested in metaphase by addition of 0.2 μg ml^{-1} colcemid (Fluka, Neu-Ulm, Germany), and erythrocytes were removed by incubation in hypotonic solution (0.0375 M KCl) with subsequent centrifugation. Cells (human peripheral blood lymphocytes) were fixed with methanol/glacial acetic acid, stained on microscope slides with Giemsa solution, and microscopically evaluated (at least 100 well-spread metaphases/cell culture). Performance of the test system was checked by parallel testing of ethylmethane sulfonate (Acros Organics) without metabolic activation and cyclophosphamide with metabolic activation.

Influence of beryllium on UDS was tested according to OECD guideline 483. Briefly, hepatocytes were isolated by *in situ* collagenase perfusion of the liver (0.05% collagenase in Hank's balanced salt solution, 37°C) in anesthetized rats. Livers were excised, minced, and cells isolated by filtering through a stainless steel mesh. Cell viability was determined by trypan blue exclusion, and cells were seeded in six-well plates (5×10^5 viable cells per well) containing a gelatinized round cover slip. Culture conditions were 5% CO_2, humidified, 37°C, in William's medium E supplemented with 2.38 mg ml^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.29 mg ml^{-1} L-glutamine,

100 U ml^{-1} penicillin, 0.10 mg ml^{-1} streptomycin, 0.50 μg ml^{-1} insulin, and 10% fetal calf serum. Non-attached cells were removed after 1.5 h by washing, and adherent cells were exposed to the extracts of beryllium metal containing tritiated thymidine (5 μCi ml^{-1}, specific activity 20 Ci $mmol^{-1}$; New England Nuclear, D-63033 Dreieich) in culture medium. Beryllium metal extracts were applied at 50 and 100%. After an exposure of 18 h, cells were washed, nuclei allowed to swell in a hypotonic solution of 1% sodium citrate for 10 min, cells fixed by three changes of methanol:acetic acid (3:1), rinsed with ethanol, and air-dried. The cover slips were coated with photographic emulsion (KODAK NTB2, Tecnomara, Fernwald, Germany) in the dark, exposed for 7 days at 4°C, developed (KODAK DEKTOL) at room temperature, fixed (TETENAL, Tetenal, Norderstedt, Germany), and stained with hematoxylin/eosin. The number of silver grains above the nucleus and the number of grains of one nuclear-sized cytoplasm area adjacent to the nucleus were counted automatically (Scorecerer UDS device version 2.0 DT3152, Perceptive Instruments) under a NIKON microscope. At least two slides per concentration and 50 cells per slide were evaluated, excluding labeled S-phase cells. Performance of the test system was checked by parallel testing of 2-acetylaminofluorene.

In addition to this standard assay, a modification was introduced to address a potential influence of beryllium metal extracts on repair of an existing DNA damage. To this end, increasing concentrations of 2-acetylaminofluorene were used to damage the DNA in the hepatocytes in a dose-dependent manner. Cells were co-incubated with beryllium metal extracts, and DNA synthesis was determined at the end of treatment as described above.

Cell transformation (SHE assay) was tested according to Commission Regulation (EC) No.440/2008 B.21. Briefly, SHE cells were isolated from Syrian golden hamster [LAK:LVG (SYR)] embryos at day 13 *post coitum*. Embryonic tissue (after decapitation, evisceration, and delimbing) was minced and blood removed from the tissue by several resuspension steps in ice-cold wash buffer (Hank's balanced salt solution calcium/magnesium free, 200 U ml^{-1} penicillin, and 200 μg ml^{-1} streptomycin). The tissue was stirred three times for 10 min at room temperature in dissociation solution (wash buffer supplemented with 0.125% trypsin and 0.0625% pancreatin) and supernatant containing the SHE cells was collected after each interval. Cells were plated in culture dishes and allowed to grow (10% CO_2 at 37°C) antibiotic free until 60–80% confluent, detached by trypsinization, and stored frozen until use. Before use, each batch was checked for plating efficiency, mycoplasma contamination, and spontaneous transformation. To increase plating efficiency, culture dishes were pre-seeded with lethally irradiated cells ('feeder cells'). The beryllium metal extract was used to expose the SHE cells to different dilutions of extracts ranging from pure extract (100%) to 25% of extract in culture medium (Dulbecco's modified eagle medium—LeBoeuf's modification, supplemented with 20% fetal calf serum and 4 mM L-glutamine). Exposure was in one experimental set-up for 24 h with additional substance-free culturing period of 6 days and in a second set-up for 7 days continuously. Five to ten culture dishes were used per exposure condition in each of the two independent experiments. After exposure, cells were fixed with methanol, stained with Giemsa solution, washed with water, and allowed to air-dry before microscopic evaluation. For each test group, >1000 colonies were evaluated for morphological transformation. Performance of the test system was checked by parallel testing of benzo[*a*]pyrene.

RESULTS

Acute oral toxicity study in rats

All animals survived until scheduled necropsy. All animals gained weight in the range expected for rats of this sex, strain, and age. No clinical signs of toxicity were observed; the only observation made was gray staining of the feces on the day after administration (likely to be caused by presence of unchanged test item). No findings indicative of toxicity were identified at macroscopic necropsy (Table 1).

Skin irritation/corrosion

No signs of skin irritation or corrosion were observed at any of the observation time points. No mortality, signs of toxicity, or effects on body weight development were observed throughout the study period (Table 2).

Eye irritation/corrosion

One hour after instillation of beryllium metal powder, slight chemosis and slight to moderate redness of the conjunctiva were observed in all animals. At the next observation time point, 24 h, only slight redness was observed, which persisted in all animals for at least 72 h. At the fourth observation time point, 7 days, no signs of eye irritation were

present in any of the test animals. No mortality, signs of toxicity, or effects on body weight development were observed throughout the study period (Table 3).

Skin sensitization

None of the observed animals demonstrated any skin reaction after challenge procedure (Table 4). Correct functionality of the test system was demonstrated in a positive control study conducted in the same laboratory 1 month before this study on beryllium metal. No mortality, signs of toxicity, or effects on body weight development were observed throughout the study period.

Table 1. Acute oral toxicity study in rats

Rat no.	Dose (mg kg^{-1} b.w.)	Mortality (days 1–15)	Observations	Weight gain (g) (days 1–8/ days 8–15)
1	2000	0/3	Feces stained gray on day 2	22.9/21.5
2			Feces stained gray on day 2	17.7/11.1
3			Feces stained gray on day 2	15.8/9.4
4	2000	0/3	Feces stained gray on day 2	25.5/12
5			Feces stained gray on day 2	14.8/13.4
6			Feces stained gray on day 2	23.3/9.8

Table 2. Skin irritation study in rabbits

Rabbit no.	Erythema (grades 0–4)			Edema (grades 0–4)		
	04	05	06	04	05	06
After 1 h	0	0	0	0	0	0
After 24 h	0	0	0	0	0	0
After 48 h	0	0	0	0	0	0
After 72 h	0	0	0	0	0	0

Ion formation

The maximum solubility of beryllium ions at neutral pH is obviously limited due to reaching the solubility equilibrium, as a maximum of ~7% of the available beryllium of the soluble $BeCl_2$ dissolved, while at lower pH (4.5) $BeCl_2$ dissolved almost completely at the same loading rate. The solubility of beryllium metal differed significantly from the solubility of beryllium chloride, especially under conditions of neutral pH. The percentage of beryllium ions dissolved from metal was found to be factor 3–40 lower than from beryllium chloride (Table 5).

Dissolution kinetics of beryllium metal and beryllium chloride were found to be too different to conduct the *in vitro* tests with beryllium chloride [a more detailed discussion can be found in the same issue of this journal (Strupp, 2010)]. Consequently, the tests were conducted with extracts of the metal as described under Materials and Methods.

Bacterial gene mutation

The Ames test conducted with beryllium metal extracts was negative with and without metabolic activation in both independent experiments (Table 6).

Mammalian cell gene mutation

No mutagenic potential was observed with beryllium metal extracts, neither in the presence nor in the absence of metabolic activation (Table 7).

Chromosome aberration in mammalian cells

The chromosome aberration assay with extracts of beryllium metal conducted with human primary lymphocytes to address cytogenicity did not reveal a genotoxic potential, neither in the presence nor in the absence of metabolic activation (Table 8). The single significant increase in the number of aberrant cells (excluding gaps) upon long-term treatment in absence of metabolic activation is considered not

Table 3. Eye irritation study in rabbits

Rabbit no.	Cornea (grades 0–4)			Iris (grades 0–2)			Conjunctiva					
							Redness (grades 0–3)			Chemosis (grades 0–4)		
	04	05	06	04	05	06	04	05	06	04	05	06
After 1 h	0	0	0	0	0	0	2	1	2	1	1	1
After 24 h	0	0	0	0	0	0	1	1	1	0	0	0
After 48 h	0	0	0	0	0	0	1	1	1	0	0	0
After 72 h	0	0	0	0	0	0	1	1	1	0	0	0
24–72 h mean	0	0	0	0	0	0	1	1	1	0	0	0
After 7 days	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Skin sensitization study in guinea pigs

	Number of animals with skin reactions (no. of animals with skin reactions/total no. of animals in group)	
Time point for scoring (after removal of challenge patch)	Vehicle control	Test item group
Study with beryllium metal		
24 h	0/5	0/10
48 h	0/5	0/10
Positive control study with alpha-hexylcinnamaldehyde		
24 h	0/5	10/10
48 h	0/5	10/10

Table 5. Ion formation test from beryllium metal and beryllium chloride: percent dissolved beryllium of theoretically available for dissolution (±standard deviation)

Extraction period (days)	1	7	14	28
Gamble's solution (pH 7.4)				
Beryllium metal	0.17 ± 0.01	0.31 ± 0.01	0.32 ± 0.02	0.57 ± 0.20
Beryllium chloride	7.01 ± 0.70	6.29 ± 0.50	3.24 ± 0.30	4.48 ± 0.70
Artificial lysosomal fluid (pH 4.5)				
Beryllium metal	1.01 ± 0.04	10.60 ± 0.40	18.90 ± 0.60	32.60 ± 1.40
Beryllium chloride	93.90 ± 5.70	93.90 ± 4.20	87.20 ± 1.90	90.60 ± 1.80

Table 6. Ames test with beryllium metal extracts: mean revertants per plate

Strain		*Salmonella typhimurium*								*Escherichia coli*	
		TA 1535		TA 1537		TA 98		TA 100		WP2 uvrA	
Metabolic activation (S9)		−	+	−	+	−	+	−	+	−	+
Experiment 1 (plate incubation method)[a]											
Beryllium metal extract (%)	0[b]	15	19	12	11	26	30	125	140	58	60
	10	18	19	12	12	25	27	134	148	62	68
	20	16	17	11	11	28	34	115	132	59	65
	40	13	17	10	13	26	31	122	145	57	58
	60	15	18	6	12	31	33	132	141	67	61
	80	15	15	10	11	34	30	130	142	57	62
	100	16	14	10	12	33	32	125	125	61	62
Positive control[c]		1998	373	83	258	352	1662	2125	2418	1492	181
Experiment II (pre-incubation method)[d]											
Beryllium metal extract (%)	0[b]	15	21	17	19	22	31	156	14	52	53
	10	11	19	14	19	24	34	159	12	55	64
	20	13	22	15	14	22	38	157	12	58	56
	40	14	15	16	17	25	36	150	10	53	61
	60	14	19	15	17	21	31	161	11	47	55
	80	13	20	12	18	19	31	163	16	53	63
	100	17	19	19	18	20	34	142	13	53	64
Positive control[c]		1840	237	105	184	377	1324	1906	1550	284	248

[a]Analytical concentration in the 100% extract: 734 μg beryllium per liter = 81 μmol l^{-1}.
[b]Vehicle: 0.9% NaCl.
[c]Without metabolic activation: TA 1535 and 100 = sodium azide, TA 1537 and 98 = 4-nitro-*o*-phenylene-diamine, WP2 uvrA = methyl methane sulfonate; with metabolic activation: 2-aminoanthracene in all strains.
[d]Analytical concentration in the 100% extract: 124 μg Be per liter = 14 μmol l−1.

Table 7. Mammalian cell gene mutation (HPRT) assay in V79 cells with beryllium metal extracts

Exposure period	Expression time/selection time	Beryllium metal extract (%)	Culture I		Culture II	
			Relative cloning efficiency (% control)	Mutant colonies per 10^6 cells	Relative cloning efficiency (% control)	Mutant colonies per 10^6 cells
Without S9 mix						
4 h[a]	6 days/ 7–10 days	0[b]	100.0	22.9	100.0	17.4
		12.5	98.4	18.7	102.6	22.0
		25	102.3	11.8	98.3	16.3
		50	93.8	7.4	102.9	9.1
		75	99.6	21.9	103.7	23.4
		100	93.5	19.7	96.5	20.7
		Positive control[c]	51.4	**125.9**[d]	53.2	**124.8**[d]
24 h[e]	6 days/ 7–10 days	0[b]	100.0	11.0	100.0	9.3
		12.5	93.3	9.6	113.6	16.6
		25	89.9	6.5	86.8	11.8
		50	89.3	9.8	86.3	18.6
		75	83.1	10.2	78.2	10.7
		100	90.8	12.1	86.8	12.3
		Positive control[c]	86.0	**277.3**[d]	73.3	**261.3**[d]
With S9 mix						
4 h[a]	6 days/ 7–10 days	0[b]	100.0	31.0	100.0	7.0
		12.5	99.0	32.4	102.2	21.6
		25	105.2	12.0	101.8	19.0
		50	98.9	16.2	101.4	5.3
		75	102.5	17.3	99.6	22.7
		100	99.2	16.8	99.0	13.3
		Positive control[c]	63.6	**2532.0**[d]	54.7	**554.3**[d]
4 h[a]	6 days/ 7–10 days	0[b]	100.0	21.1	100.0	24.1
		12.5	91.0	48.2	95.5	8.5
		25	97.7	10.3	94.1	12.3
		50	93.0	9.1	97.4	12.1
		75	96.3	29.6	91.0	13.6
		100	89.0	10.2	87.8	8.3
		Positive control[c]	56.1	**1142.9**[d]	56.5	**1171.9**[d]

[a]Analytical concentration in the 100% extract: 4 μg beryllium per liter = 0.4 μmol l^{-1}.
[b]Extraction medium.
[c]Without metabolic activation: ethylmethane sulfonate (150 μg ml^{-1} for 4-h incubation, 1.1 μg ml^{-1} for 24-h incubation); with metabolic activation: 7,12-dimethylbenz(*a*)anthracene (1.1 μg ml^{-1}).
[d]Mutation frequency statistically significant higher than corresponding control values (linear regression, least squares).
[e]Analytical concentration in the 100% extract: 14 μg Be per liter = 1.6 μmol l^{-1}.

to be treatment related because there was no dose-dependent trend (the effect was not observed at the high dose), and no hints from the other parallel experiments (with or without metabolic activation) supported the relevance of this finding.

Unscheduled DNA synthesis

The beryllium metal extract did not induce DNA repair synthesis in the UDS assay in rat primary hepatocytes and is thus not considered to have damaged the DNA. No cytotoxicity was observed

Table 8. Chromosome aberration assay in human lymphocytes with beryllium metal extracts

Exposure period	Preparation interval	Beryllium metal extract (%)	Mitotic index (% control)	Aberrant cells (%)		
				Inclusive gaps[a]	Exclusive gaps[a]	With exchanges
Without S9 mix						
4 h[b]	22 h	0[c]	100.0	1.0	0.5	0.0
		50	86.9	0.5	0.5	0.0
		75	101.6	3.0	2.5	0.0
		100	72.9	1.0	1.0	0.0
		Positive control[d]	77.3	10.5	**9.5**[e]	3.0
22[f]	22 h	0[c]	100.0	0.5	0.5	0.0
		50	73.3	0.0	0.0	0.0
		75	84.0	3.5	**3.0**[e]	0.0
		100	78.7	2.0	1.5	0.0
		Positive control[d]	33.2	19.5	**18.5**[e]	3.5
With S9 mix						
4 h[b]	22 h	0[c]	100.0	4.0	2.5	0.0
		50	76.0	0.5	0.5	0.0
		75	86.2	2.5	2.0	0.0
		100	79.3	0.5	0.5	0.0
		Positive control[d]	37.5	10.0	**10.0**[e]	0.0
4 h[b]	22 h	0[c]	100.0	2.0	1.5	0.5
		50	90.9	2.0	2.0	0.0
		75	86.9	1.5	1.0	0.0
		100	108.8	1.5	1.5	0.5
		Positive control[d]	38.0	17.0	**16.5**[e]	1.5

[a]Inclusive cells carrying exchanges.
[b]Analytical concentration in the 100% extract: 21 μg beryllium per liter = 2.3 μmol l^{-1}.
[c]Extraction medium.
[d]Without metabolic activation: ethylmethane sulfonate (825 μg ml^{-1} for 4-h incubation, 770 μg ml^{-1} for 22-h incubation); with metabolic activation: cyclophosphamide (15 μg ml^{-1}).
[e]Aberration frequency statistically significant higher than corresponding control values (Fisher's exact test).
[f]Analytical concentration in the 100% extract: 4 μg Be per liter = 0.4 μmol l^{-1}.

as determined in parallel by neutral red uptake (Table 9).

Within the same experiment, increasing concentrations of the positive control, 2-acetylaminofluorene, were applied to parallel cultures and co-incubated with beryllium metal extracts. While no effects of beryllium metal extracts on DNA (repair) synthesis were observed under conditions where 0–50% cells were in repair synthesis (=damaged by the 2-acetylaminofluorene treatment), at the highest concentration (2.23 μg ml^{-1} 2-acetylaminofluorene, 96% cells in repair synthesis), an effect on DNA repair synthesis was observed (Table 10).

To verify the observed response, the upper dose range of 2-acetylaminofluorene was investigated by closer dose spacing. DNA damage in the repeat experiment was high in all concentrations of 2-acetylaminofluorene applied (70–96% cells in repair synthesis), but no cytotoxicity was observed as determined in parallel by neutral red uptake (data not shown in detail; neutral red uptake was in all conditions applied between 99 and 113% of the

Table 9. UDS assay in rat primary hepatocytes with beryllium metal extracts

Beryllium metal extract (%)	Cells in repair (%)	Mean net grain counts
0[a]	6	−1.7
50	7	−3.63
100[b]	2	−4.81
Positive control[c]	96	29.93

[a]Extraction medium.
[b]Analytical concentration in the 100% extract: 61 μg beryllium per liter = 6.8 μmol l^{-1} (not determined in the main experiment, but in a range-finding experiment).
[c]2-Acetylaminofluorene (2.23 μg ml^{-1}).

Table 10. Modified UDS assay in rat primary hepatocytes with beryllium metal extracts

Beryllium metal extract (%)	2-Acetylaminofluorene (μg ml^{-1})					
	0	0.018	0.06	0.2	0.67	2.23
Cells in repair (%)						
0[a]	6	11	14	15	48	96
50	7	12	19	22	45	35
100[b]	2	7	15	11	48	60
Mean net grain counts						
0[a]	−1.7	−1.55	−0.45	−0.16	6.72	29.93
50	−3.36	−1.89	−0.38	0.04	5.22	2.62
100[b]	−4.81	−2.58	−1.24	−0.61	5.61	6.8

[a]Extraction medium.
[b]Analytical concentration in the 100% extract: 61 μg beryllium per liter = 6.8 μmol l^{-1} (not determined in the main experiment, but in a range-finding experiment).

Table 11. Modified UDS assay in rat primary hepatocytes with beryllium metal extracts: repeat experiment

Beryllium metal extract (%)	2-Acetylaminofluorene (μg ml^{-1})				
	0	0.67	0.19	1.71	2.23
Cells in repair (%)					
0[a]	5	96	70	78	75
50	6	80	52	90	78
100[b]	4	30	57	28	65
Mean net grain counts					
0[a]	−5.13	31.62	9.02	11.14	18.14
50	−7.42	12.26	6.8	14.37	12.18
100[b]	−6.91	2.22	5.34	1.95	7.06

[a]Extraction medium.
[b]Analytical concentration in the 100% extract: 61 μg beryllium per liter = 6.8 μmol l^{-1} (not determined in the main experiment, but in a range-finding experiment).

untreated control group). The trend to reduced DNA repair was verified in the repeat experiment. The absence of a direct effect of beryllium metal extracts on DNA repair synthesis (=absence of DNA damage) without prior intentional DNA damage was confirmed in the repeat experiment as well (Table 11).

Cell transformation

All concentrations of beryllium metal extract tested in the SHE cell assay had increased transformation rates compared to control (Table 12, Fig. 1).

SUMMARY

An overview of the acute, local, and genotoxicity testing results is presented below: Beryllium metal was found to have no relevant acute oral toxicity and low local irritation properties and was found not to be a skin sensitizer in animal studies conducted in accord with internationally accepted testing guidelines (Table 13). Solubility of beryllium in artificial lung fluids is dependent on the pH and time of extraction and the solubility kinetics and characteristics of beryllium metal differs largely from the features of soluble beryllium chloride under simulated lung conditions. The conducted *in vitro* genotoxicity tests have limitations as they are not ideal for investigating genotoxicity of poorly water soluble test material and relatively artificial procedures have to be applied to simulate the *in vivo* situation but can give a good impression on relevance of the individual endpoints for the *in vivo* situation. A set of genotoxicity tests *in vitro*, covering gene mutation, chromosome aberration, and UDS (repair), did not reveal any primary genotoxic potential for beryllium metal when extracted under simulated

Table 12. SHE cell transformation assay with beryllium metal extracts

Exposure period	Beryllium metal extract (%)	Relative plating efficiency (% control)	Transformation frequency (%)
7 days[a]	0[b]	100.0	0.59
	25	101.0	**1.45**[c]
	37.5	103.8	**1.41**[c]
	50	96.3	**1.59**[c]
	75	96.3	**2.50**[c]
	100	106.9	**1.98**[c]
	Solvent control[d]	100.0	0.26
	Positive control[e]	93.3	**3.12**[c]

[a]Analytical concentration in the 100% extract: 22.95 μg beryllium per liter = 2.5 μmol l^{-1}.
[b]Extraction medium
[c]Transformation frequency statistically significant higher than corresponding control values.
[d]Dimethylsulfoxide (0.2%); only relevant for positive control and not present in test item samples.
[e]Benzo[*a*]pyrene (5.0 μg ml^{-1}).

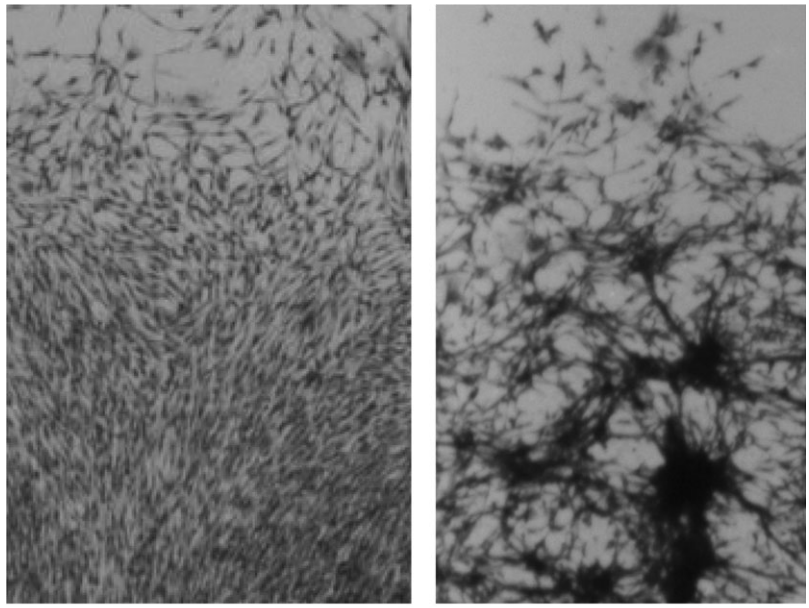

Fig. 1. Typical microscopic pictures of a non-transformed and a transformed colony (outer rim to center) from this experiment are given below.

Table 13. Overview on experimental results obtained with beryllium metal

Study	Species or cell type	Result
Acute oral toxicity	Rat	$LD_{50} > 2000$ mg kg^{-1} b.w.
Skin irritation	Rabbit	No signs of skin irritation at any time point.
Eye irritation	Rabbit	Slight initial conjunctival redness, fully reversible within 7 days. No effects on cornea or iris.
Skin sensitization (maximization method)	Guinea pig	Not sensitizing.
Bacterial gene mutation (Ames test)	*Salmonella typhimurium*/ *Escherichia coli*	Negative
Mammalian cell gene mutation	V79 cell line (*in vitro*)	Negative
Mammalian cell chromosome aberration	Human lymphocytes (*in vitro*)	Negative
Mammalian cell unscheduled DNA repair synthesis (UDS test)	Rat primary hepatocytes (*in vitro*)	Negative (beryllium exposure only); indication of reduced repair of hepatocytes with damaged DNA
Cell transformation (SHE assay)	SHE cells (*in vitro*)	Positive

lung conditions. An *in vitro* cell transformation assay with beryllium metal extracts did indicate a cell-transforming potential of beryllium metal, and a modified UDS test indicated a tendency for inhibition of DNA repair in pre-damaged cells. Beryllium metal is obviously no classical mutagen and causes no classical cytogenetic or DNA damage; an interference of the ions with DNA (repair) synthesis

or genomic regulation is likely more relevant but should be addressed by further investigations for relevance *in vivo*.

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