

TITLE: Methods for testing active compounds in models with elevated heavy metal accumulation

FIELD OF THE INVENTION

The present invention relates to the field of pharmaceutical test studies. Particularly, the present invention relates to the field of pharmaceutical test studies in view of heavy metal accumulation in humans. More particularly, the invention relates
5 to the production of *in vitro* and/or *in vivo* test models for testing pharmaceuticals under conditions of elevated heavy metal accumulation.

BACKGROUND OF THE INVENTION

Since the industrial revolution environmental exposure of modern man to
10 heavy/toxic metals has accelerated significantly. People gradually accumulate toxic metals over a lifetime. However, little recognition is given to the chronic accumulation of heavy metals in humans since, most often, the accumulation does not show overt medical symptoms recognizable as derived from the metal load.

Nevertheless, the heavy metals may have profound influence at the biochemical
15 level and substantially affect aspects of cell function. The effects of chronic accumulation of specific heavy metals on cellular or biochemical function have been demonstrated. In the context of cancer – single metals, particularly copper, have been shown to affect the tertiary structure and overall function of the tumor suppressor protein p53 and excessive levels of multiple metals have shown to be associated with
20 inferior clinical outcomes (Ohanian, M. et al., A heavy metal baseline score predicts outcome in acute myeloid leukemia. Am J Hematol. 2020:422–434).

The inventors of the invention have demonstrated that loading of multiple heavy metals in cells and animals results in chemoresistance. In consequence, studies of emergence of diseases and disorders, such as cancers, as well as their treatment,
25 including the testing of novel approaches and active compounds in a research environment which is meticulously kept clean of heavy metals may be inherently misleading.

Therefore, there is an urgent need in the art for methods and test models which facilitate the study of disease emergence as well as testing of pharmaceuticals, such as

cancer drugs, under conditions mimicking the chronic heavy metal accumulation in human patients to be treated.

SUMMARY OF THE INVENTION

5 The inventors of the present invention have recognized that the animal models that are used for preclinical testing of drugs, fall short because they do not mimic the chronic heavy metal accumulation in human patients that are to be treated with the drugs. To analyze the effects of chronic heavy metal accumulation on the efficacy of an active compound, the inventors have developed *in vitro* and/or *in vivo* test models
10 having elevated multi-metal accumulation. Said test models resemble the chronic elevated heavy metal accumulation and heavy metal profiles of modern humans, i.e., patients to be treated. As metal profiles in humans differ by location, profession, age and multiple other parameters of individual nature, only an approximation can be made. However, it has been shown that, for example, in the context of cancer (such as
15 in the case p53 function), the overall metal burden may be important for the efficacy of a drug, whereas less importance is ascribed to the accumulation or removal of specific metals. It is thus possible to generate a viable and relevant model without matching an exact metal profile of a specific patient or patient group.

 In a first aspect, the invention provides a method for testing the effects of
20 elevated heavy metal levels on the efficacy of an active compound for cancer treatment, comprising (i) providing an *in vivo* non-human test model having elevated heavy metal levels, (ii) providing the model with a tumor graft, and (iii) testing the efficacy of the active compound for cancer treatment by providing the active compound to the model, optionally wherein at least one chelating agent is applied in combination
25 with the active compound. For comparison, the steps of tumor grafting, providing the active compound and, optionally, the application of a chelating agent, may also be applied to a naïve model without elevated heavy metal levels.

 The *in vivo* non-human test model may be obtained by a method comprising (a) providing at least one heavy metal mixture, (b) loading an *in vivo* non-human model
30 with the at least one heavy metal mixture, (c) equilibrating the model and, optionally, (d) confirming the elevated heavy metal levels and/or distribution thereof in the model. A resulting test model has increased levels of heavy metals, when compared to an unloaded control and/or when compared to the levels of the model prior to the loading step.

A method of the invention may further comprise a step of confirming that the heavy metal accumulation is sub-toxic, meaning that the model does not show symptoms of toxicity. Said step may comprise determining parameters selected from mortality, weight, weight gain, behavior, appearance, parameters of a standard
5 clinical blood biochemistry or hematology panel, or a combination thereof. For example, the sub-toxicity of the heavy metal accumulation in the test model is confirmed when the test model does not show a clinically significant difference in parameters tested after loading and equilibrating, when compared to the parameters of the model before loading and/or compared to an unloaded control.

10 In methods of the invention, the tumor graft having elevated heavy metal levels prior to the step of tumor grafting.

In methods of the invention, the loading step is performed over a certain period of time, preferably consecutively over a certain period of time, such as once a day, several times a day, or once a week, preferably once a day for 10 days.

15 In methods of the invention, the equilibrating step is performed for at least one week, preferably wherein the equilibrating step is performed for at least 3 to 6 weeks.

In methods of the invention, testing the efficacy of the active compound may further provide knowledge whether the active compound is safe in a metal-contaminated model.

20 The invention further provides a method of producing a test model having elevated metal levels comprising the steps of

- (i) providing at least one heavy metal mixture,
- (ii) loading a model with the at least one heavy metal mixture, (iii) equilibrating the model, and optionally
- 25 (iv) confirming the elevated heavy metal levels and/or distribution thereof in the model.

In a preferred embodiment of the method according to the invention the test model has increased levels of heavy metals loaded by the at least one heavy metal mixture after the loading step and/or equilibrating step compared to an unloaded
30 control and/or compared to the levels of the model prior to the loading step.

Methods of the invention for producing a test model may further comprise a step of confirming that the heavy metal accumulation is sub-toxic, meaning that the model does not show symptoms of toxicity. Said step of confirming the sub-toxicity of the heavy metal accumulation may comprise determining general parameters selected

from mortality, viability, growth rate, morphology, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof.

The test model produced by methods of the invention is an *in vitro* or *in vivo* test model. The *in vivo* test model is non-human animal model. The *in vitro* model may be any cell, cell line, or combination thereof, of interest. For example, the *in vitro* model is selected from a cell culturing system, a tissue culture system, a coculturing system, a spheroid culture, or an organoid culture. For example, the *in vivo* model is selected from rat, mouse, rabbit, guinea pig, zebrafish, cat, dog, chicken, non-human primate such as rhesus macaque or chimpanzee, or a combination thereof.

In methods of the invention for producing a test model, the confirmation of sub-toxicity of the heavy metal accumulation for an *in vitro* test model may comprise determining parameters selected from viability, growth rate, morphology, or a combination thereof. The sub-toxicity of the heavy metal accumulation for an *in vitro* test model is confirmed when the test model does not show biologically significant differences in parameters selected from viability, growth rate, morphology, or a combination thereof. Confirming sub-toxicity of the heavy metal accumulation for an *in vivo* test model may comprise determining parameters selected from mortality, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof. The sub-toxicity of the heavy metal accumulation for an *in vivo* test model is confirmed when the test model does not show biologically or clinically significant differences in parameters selected from mortality, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof.

In embodiments, the heavy metals of the at least one heavy metal mixture of the invention are selected from manganese, nickel, cadmium, lead, mercury, arsenic, chromium, iron, zinc, cobalt, copper, vanadium, platinum, titanium, antimony, tin, or a combination thereof. For example, the heavy metals of the at least one heavy metal mixture are applied as heavy metal salts. For example, the heavy metal salts are selected from MnCl_2 , CdCl_2 , $(\text{CH}_3\text{COO})_2\text{Pb}$, HgCl_2 , NaAsO_2 , $\text{K}_2\text{Cr}_2\text{O}_7$, $(\text{NH}_4)_5\text{Fe}_x(\text{C}_6\text{H}_8\text{O}_7)$, NiCl_2 , ZnCl_2 , ZnSO_4 , CoCl_2 , CuSO_4 , Na_3VO_4 , or a combination thereof.

In embodiments, the *in vivo* loading of the at least one heavy metal mixture is performed over a certain period of time, preferably consecutively over a certain period

of time. Preferably, the loading step is performed once a day, several times a day, or once a week. More preferably the loading is performed at least 2 days. Most preferably, the loading is performed once a day for 10 consecutive days, or once a day for 5 consecutive days, two days of rest without treatment and once a day for another 5 consecutive days. For *in vitro* models, the loading of the at least one heavy metal mixture may occur in a time span of between 30 minutes and four days, such as 4 hours, 1 day, 2 days or 3 days.

In embodiments, the equilibrating step is performed for *in vivo* models, for at least one week, preferably, the equilibrating step of the method according to the invention is performed for at least 3 to 6 weeks; for *in vitro* models, for a time span of between 5 minutes and 4 days, preferably wherein the equilibrating step is performed for 30 minutes, 1 hour, 2 hours, 6 hours 12 hours or 24 hours.

In methods of producing a test model according to the invention the method further comprises a tumor grafting step onto an *in vivo* model. Said step of tumor grafting may be performed prior to the loading step or after the equilibrating step. Preferably the tumor grafting is performed after the equilibrating step of the method, wherein the tumor graft has a sub-toxic heavy metal accumulation prior to the step of tumor grafting.

In another preferred embodiment of the method according to the invention, the elevated heavy metal levels and/or distribution is confirmed by measuring the heavy metal levels via mass spectrometry (MS), such as inductively coupled plasma (ICP) MS, total reflection X-rays fluorescence (TXRF), such as TXRF using Bruker S2 PICOFAF, optical emission spectroscopy (OES), such as ICP-OES, atomic emission spectroscopy (AES), such as ICP-AES, atomic absorption spectroscopy (AAS), HPLC, or any other method suitable to the skilled person or a combination thereof.

In embodiments, the sub-toxicity of the heavy metal accumulation is confirmed by determining the absence of overt toxicity as reflected in general parameters such as mortality, viability, growth rate, morphology, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof. For example, for *in vitro* models confirming the sub-toxicity of the heavy metal accumulation comprises determining parameters selected from viability, growth rate, morphology, or a combination thereof. For example, for *in vivo* test models confirming the sub-toxicity of the heavy metal accumulation comprises determining parameters selected from mortality, weight, weight gain, behavior,

appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof to rule out toxicity. For example, for *in vivo* test models, determining the sub-toxicity of heavy metal accumulation comprises determining the presence or absence of clinically significant differences in parameters of a standard clinical blood biochemistry or hematology panel. For example, the sub-toxicity of heavy metal accumulation in a test model is confirmed when the test model does not show any difference, preferably no significant difference, in one or more of the parameters tested, preferably all of the parameters tested, when compared to the test model's performance before loading and/or compared to an unloaded control. For example, the sub-toxicity of the heavy metal accumulation in the *in vitro* test model is confirmed when the test model does not show a difference of more than 20%, more than 15%, more than 10 % in one or more of said parameters tested, when compared to the parameters of the model before loading and/or compared to an unloaded control. For example, the sub-toxicity of heavy metal accumulation in an *in vivo* test model is confirmed when the test model does not show a clinically significant difference in one or more of the parameters tested, preferably all of the parameters tested, when compared to the test model's performance before loading and/or compared to an unloaded control. For example, the sub-toxicity of the heavy metal accumulation is determined, after confirmation of the heavy metal accumulation and/or distribution, by analyzing one or more, preferably all, general parameters that are applicable for the *in vivo* or *in vitro* model, as indicated herein above.

In another aspect, the invention provides a heavy metal mixture for producing a test model having a sub-toxic heavy metal accumulation comprising heavy metals selected from manganese, cadmium, lead, mercury, arsenic, chromium, iron, nickel, zinc, cobalt, copper, vanadium, platinum, titanium, antimony, tin, or a combination thereof, preferably the heavy metals of the heavy metal mixture are applied as metal salts selected from MnCl_2 , CdCl_2 , $(\text{CH}_3\text{COO})_2\text{Pb}$, HgCl_2 , NaAsO_2 , $\text{K}_2\text{Cr}_2\text{O}_7$, $(\text{NH}_4)_y\text{Fe}_x(\text{C}_6\text{H}_8\text{O}_7)$, NiCl_2 , ZnCl_2 , ZnSO_4 , CoCl_2 , CuSO_4 , Na_3VO_4 , or a combination thereof.

In another aspect, the invention provides a use of the heavy metal mixture according to the invention for producing a test model having a sub-toxic heavy metal accumulation.

In another aspect, the invention provides a test model which is obtainable by the methods of producing a test model according to the invention. Said test model may be

an *in vitro* or *in vivo* test model, wherein the *in vivo* test model is non-human. In a preferred embodiment of the invention the test model is an *in vivo* test model having a chronic sub-toxic heavy metal accumulation.

5 The invention further provides a use of the test model which is obtainable by the methods of the invention, for studying of emergence and/or treatment of diseases and/or disorders. Said use of the test model for studying of emergence and/or treatment of diseases and/or disorders may comprise studying of emergence and/or treatment of cancer, tumors, infectious diseases, inflammatory diseases and/or neurodegenerative disorders. In embodiments, said disease or disorder is a cancer.

10 The invention further provides a test model which is obtainable by the method of producing a test model of the invention and/or its use for studying of emergence and/or treatment of diseases and/or disorders, wherein an active compound is applied to the test model, optionally wherein at least one chelating agent is applied in combination with the active compound.

15 The invention further provides a test model which is obtainable by the method of the invention and/or its use for studying of emergence and/or treatment of diseases and/or disorders, wherein the active compound which is applied to the test model is an anti-cancer drug.

20 The invention further provides a method for determining the influence of heavy metal accumulation on the toxicity and/or efficacy of an active compound comprising applying the active compound to a test model according to the invention, and comparing the toxicity and/or efficacy of the active compound obtained in the test model with the toxicity and/or efficacy obtained in a naïve model. The invention further provides a method for determining the influence of elevated heavy metal levels
25 on the toxicity and/or efficacy of an active compound, comprising applying the active compound to a test model according to the invention, and further comprising applying a chelating agent in combination with the active compound to the test model and optionally, also to the naïve model.

30 The invention further provides a use of an *in vitro* test model, that is grafted on an *in vivo* test model, for studying of emergence and/or treatment of diseases and/or disorders, whereby the *in vitro* test model and *in vivo* test model are obtainable by the methods of the invention, preferably for studying of emergence and/or treatment of cancer, tumors, infectious diseases, inflammatory diseases and/or neurodegenerative disorders. In embodiments, said disease or disorder is a cancer.

The methods of the invention will help to predict whether an active compound is likely to cross the gap between preclinical testing of drugs in metal-free animals and use of the drugs in humans having chronic metal accumulation.

- The invention also provides a kit of parts for producing a test model having a
- 5 sub-toxic heavy metal accumulation comprising
- (i) a heavy metal mixture
 - (ii) instructions for producing the test model, and optionally
 - (iii) an unloaded model.

10 DESCRIPTION OF DRAWINGS

Figure 1: Experimental workflow of *in vivo* studies using the chronic multi-metal accumulation model. The example depicts a rat study. It depicts the three phases of the model: Metal loading; equilibration; and treatment. In this example, rats were loaded by oral gavage daily for 10 days, followed by five weeks of

15 equilibration during which no further treatment was provided. Experimental treatments started at the end of the equilibration period. In the case depicted, chelator treatments were conducted daily for nine consecutive days. Control animals, that were not metal loaded, went through the same schedule, but without the oral gavage treatments. Samples were collected after 1, 5, and 9 treatments and again 11 days

20 after the ninth chelator treatment. Urine and feces were collected for 24 hours using metabolic cages at the timepoints indicated. Blood samples for serum metal analysis were obtained immediately after collection of the excretion samples. Animals were sacrificed 72 hours after the indicated chelator treatment or upon completion of the holding period. Blood was collected for serum metals as well as for a full hematology

25 and biochemistry panel and organs were collected for metal analysis. The numbers depict the number of chelator treatments each group has undergone prior to the test performed.

Figure 2: Excretion and serum levels of Mg and Ca (not supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal

30 excretion (B) and serum levels (C) of Mg and Ca (not supplemented) through the loading phase and equilibration. It is seen that excretion was affected by the metal loading phase but was stable during equilibration, whereas the serum levels for both metals remained stable.

Figure 3: Excretion and serum levels of Se and Rb (not supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Se and Rb (not supplemented) through the loading phase and equilibration. It is seen that excretion of Se but not of Rb was
5 affected by the metal loading phase, whereas the serum levels for both metals remained stable.

Figure 4: Excretion and serum levels of Mn (not supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Mn (not supplemented in this example) through the loading phase
10 and equilibration. It is seen that excretion of Mn was strongly affected by the metal loading phase, whereas its serum levels remained overall stable with only a mild fluctuation.

Figure 5: Excretion and serum levels of Fe (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and
15 serum levels (C) of Fe (supplemented) through the loading phase and equilibration. It is seen that excretion of Fe was affected by the metal loading phase, whereas its serum levels remained overall stable.

Figure 6: Excretion and serum levels of Zn (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and
20 serum levels (C) of Zn (supplemented) through the loading phase and equilibration. It is seen that both excretion of Zn and its serum levels were affected by the metal loading phase, all increasing following metal loading and then stabilizing.

Figure 7: Excretion and serum levels of V (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and
25 serum levels (C) of V (supplemented) through the loading phase and equilibration. It is seen that both excretion of V and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level.

Figure 8: Excretion and serum levels of Cr (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and
30 serum levels (C) of Cr (supplemented) through the loading phase and equilibration. It is seen that both excretion of Cr and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level.

Figure 9: Excretion and serum levels of Co (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Co (supplemented) through the loading phase and equilibration. It is seen that both excretion of Co and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level.

Figure 10: Excretion and serum levels of Ni (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Ni (supplemented) through the loading phase and equilibration. It is seen that both excretion of Ni and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level.

Figure 11: Excretion and serum levels of Cu (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Cu (supplemented) through the loading phase and equilibration. It is seen that excretion of Cu markedly increased after metal loading and then declined to a stable level, whereas serum levels remained overall stable.

Figure 12: Excretion and serum levels of As (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of As (supplemented) through the loading phase and equilibration. It is seen that both excretion of As and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level. Both urinary excretion and serum levels of As remained markedly elevated throughout the equilibration phase, as compared to pre-loading values.

Figure 13: Excretion and serum levels of Cd (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Cd (supplemented) through the loading phase and equilibration. It is seen that both excretion of Cd and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level. Both urinary excretion and serum levels of Cd remained elevated throughout the equilibration phase, as compared to pre-loading values.

Figure 14: Excretion and serum levels of Hg (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and

serum levels (C) of Hg (supplemented) through the loading phase and equilibration. It is seen that both excretion of Hg and its serum levels were affected by the metal loading, all increasing sharply following the metal loading phase. Fecal excretion and serum levels declined rapidly to a stable level, whereas urinary excretion remained elevated as compared to pre-loading values and declined slowly during equilibration.

Figure 15: Excretion and serum levels of Pb (supplemented) through the loading phase and equilibration. Urinary excretion (A), fecal excretion (B) and serum levels (C) of Pb (supplemented) through the loading phase and equilibration. It is seen that both excretion of Pb and its serum levels were affected by the metal loading phase, all increasing sharply following metal loading and then rapidly declining to a stable level. Both urinary excretion and serum levels of Pb remained elevated throughout the equilibration phase, as compared to pre-loading values.

Figure 16: Organ levels of Mg (not supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Mg levels in organs are not affected by supplementation of other metals, except for a small elevation in the heart. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 17: Organ levels of Ca (not supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include bone marrow levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Ca levels in organs are only mildly affected by supplementation of other metals. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 18: Organ levels of Mn (not supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Mn levels in organs are not affected by supplementation of other metals. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 19: Organ levels of Se (not supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Se levels in
5 organs are not affected by supplementation of other metals, except for a small elevation in the kidney. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 20: Organ levels of Rb (not supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by
10 oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Rb levels in organs are largely unaffected by supplementation of other metals, except for a small elevation in the spleen and heart. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 21: Organ levels of V (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include spleen and
15 kidney levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal
20 analysis. It is seen that V levels in organs are strongly elevated by metal supplementation, except for pancreas and brain in which the elevation is less pronounced. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 22: Organ levels of Cr (supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by
25 oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Cr levels are specifically elevated in spleen and kidney and mildly in bone marrow, while other organs are largely unaffected by supplementation. Naïve – before metal loading;
30 Loaded – after metal loading and equilibration.

Figure 23: Organ levels of Fe (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include spleen levels
and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks.

They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Fe levels in organs are not affected by metal supplementation, in line with the tight regulation of iron in the body. Naïve – before metal loading; Loaded – after metal loading and equilibration.

5 **Figure 24: Organ levels of Co (supplemented) before (Naïve) and after metal loading and equilibration (loaded).** Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Co levels are moderately elevated across organs except the lung.

10 **Figure 25: Organ levels of Ni (supplemented) before and after metal loading and equilibration.** Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Ni levels are only slightly affected by supplementation. Naïve – before metal loading; Loaded – after
15 metal loading and equilibration.

Figure 26. Organ levels of Cu (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include kidney levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks.
20 They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Cu levels in organs are not affected by metal supplementation, with the exception of the kidney, in line with the tight regulation of copper in the body. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 27: Organ levels of Zn (supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Zn levels are only slightly perturbed by supplementation, in line with the tight regulation of zinc in the body. Naïve – before metal loading; Loaded – after metal loading and
25 equilibration.
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Figure 28: Organ levels of As (supplemented) before and after metal loading and equilibration. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that As levels are

strongly elevated in all organs after supplementation. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 29. Organ levels of Cd (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include kidney and liver levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Cd levels in all organs are substantially elevated by metal supplementation. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 30. Organ levels of Hg (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include kidney levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Hg levels were particularly high in kidneys and clearly elevated in liver and heart, while only mildly affected in other organs. Naïve – before metal loading; Loaded – after metal loading and equilibration.

Figure 31: Organ levels of Pb (supplemented) before and after metal loading and equilibration. Shown at a low resolution (A) to include kidney levels and at a higher resolution (B) to show all the rest. Rats were loaded with a metal mixture for ten days by oral gavage and allowed to equilibrate for at least 5 weeks. They were subsequently sacrificed, and organs were harvested for metal analysis. It is seen that Pb levels were particularly high in kidneys and markedly elevated in bone marrow, spleen, liver and brain, while only mildly elevated in other organs.

Figure 32: Growth of rats during loading and equilibration. The non-loaded group serves as reference for normal growth of rats over the experimental period. The loaded group received a metal mix by oral gavage for 10 days and had no further treatment during the 40-day equilibration period. It is seen that the growth of the rats is slowed down during the acute phase of metal administrations, followed by a period of catch-up growth. Growth in the metal-loaded group has caught up fully four weeks after completion of the loading.

Figure 33: Pb levels (as an example of metals provided in the metal mix) in organs, serum and urine over time in metal supplemented rats. Rats were

loaded with a metal mix for 7 consecutive days and allowed 4 weeks for equilibration. Urine and serum were collected from time to time. Groups of animals were sacrificed at different time points and organs were collected for metal analysis. The results are depicted as relative concentrations (multiples of the preloading concentration in Naïve animals). Tissue levels are presented at low resolution for organs with high Pb content (A), including bone marrow, liver and kidney, and at high resolution for organs with lower Pb content (B). Urinary excretion and serum levels are depicted in a separate panel (C). It is seen that Pb levels and excretion increase sharply after loading and then decrease to a near steady state level. In some organs Pb levels remain significantly elevated at the end-levels, whereas others return to their preloading level, testifying to the inherent selectivity of organs in accumulating contaminating metals.

Figure 34: The effect of chelator treatment on Mg in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Mg (not supplemented) was slightly perturbed, whereas fecal excretion (B) and serum levels (C) remained largely stable.

Figure 35: The effect of chelator treatment on Ca in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Ca (not supplemented) was slightly depressed by DMSA and slightly elevated by miaDMSA, whereas fecal excretion (B) was unchanged and serum levels (C) dipped after one treatment with all chelators but recovered subsequently.

Figure 36: The effect of chelator treatment on Se in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of

at least 2 experiments. It is seen that urinary excretion (A) of Se (not supplemented), fecal excretion (B) and serum levels (C) remained stable.

Figure 37: The effect of chelator treatment on Rb in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Rb (not supplemented), fecal excretion (B) and serum levels (C) were only minimally perturbed by the treatment.

Figure 38: The effect of chelator treatment on Mn in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Mn (not supplemented) is markedly decreased by DMPS and DMSA, but only moderately by miaDMSA, while fecal excretion (B) is unaffected and serum levels (C) are mildly decreased by all chelators.

Figure 39: The effect of chelator treatment on Fe in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Fe (supplemented) is transiently decreased by DMPS and DMSA, but slightly elevated by miaDMSA, while fecal excretion (B) is decreased by DMSA and miaDMSA, and serum levels (C) are mildly affected by DMSA and miaDMSA.

Figure 40: The effect of chelator treatment on Co in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Co (supplemented) is

mildly affected by chelators with a mild excess excretion by miaDMSA, whereas fecal excretion (B) is not affected, and serum levels (C) are slightly elevated by miaDMSA.

Figure 41: The effect of chelator treatment on Ni in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Ni (supplemented) is mildly decreased by DMPS and DMSA, while fecal excretion (B) is not affected, and serum levels (C) are slightly decreased by DMPS and elevated by DMSA and miaDMSA.

Figure 42: The effect of chelator treatment on Cu in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Cu (supplemented) is increased by all chelators with the strongest effect seen with DMPS, whereas fecal excretion (B) is not affected, and serum levels (C) are minimally affected by chelators.

Figure 43: The effect of chelator treatment on Zn in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Zn (supplemented), like Cu, is increased by all chelators with the strongest effect seen with DMPS, whereas fecal excretion (B) and serum levels (C) are not affected by chelators.

Figure 44: The effect of chelator treatment on As in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of As (supplemented) is not affected by DMSA but is increased slightly by DMPS and substantially by miaDMSA,

whereas fecal excretion (B) is slightly increased and serum levels (C) are mildly perturbed by chelators.

Figure 45: The effect of chelator treatment on V in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of V (supplemented) is mildly decreased by DMPS and DMSA but increased slightly by miaDMSA, whereas fecal excretion (B) is unaffected and serum levels (C) are slightly decreased by chelators.

Figure 46: The effect of chelator treatment on Cr in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Cr (supplemented) is mildly decreased by DMPS and DMSA but not by miaDMSA, whereas fecal excretion (B) is unaffected and serum levels (C) are slightly decreased by DMPS, neutral by DMSA, and increased transiently by miaDMSA.

Figure 47: The effect of chelator treatment on Cd in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Cd (supplemented) is mildly increased by DMPS but not by DMSA, whereas excretion was strongly increased by miaDMSA. Fecal excretion (B) was strongly increased by miaDMSA but not by DMPS and DMSA, whereas serum levels (C) were mildly increased by DMPS (transiently) and DMSA but decreased by miaDMSA.

Figure 48: The effect of chelator treatment on Hg in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was

collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Hg (supplemented) is transiently increased by chelator treatment, followed by decreased excretion after several consecutive treatments. Fecal excretion (B) and serum levels (C) followed a similar pattern with the strongest response being generated by miaDMSA.

Figure 49: The effect of chelator treatment on Pb in rats. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20. Serum was collected 72 hours after the respective chelator treatment. Each result is an average of at least 2 experiments. It is seen that urinary excretion (A) of Pb (supplemented) is substantially increased by chelator treatment, while fecal excretion (B) is slightly increased by DMSA and miaDMSA, and serum levels (C) are decreased by all chelators with the strongest response being generated by miaDMSA.

Figure 50: The effect of chelator treatment on Cd in rat organs. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Animals were sacrificed and organs were collected for metal analysis 72 hours after 1, 5 and 9 treatments and after an additional holding period of 11-20 days (noted as 9 treatments + 16). It is seen that miaDMSA treatment substantially reduced Cd (supplemented) levels in liver (A) and kidney (B) but had little effect in lungs (C), brain (D) and bone marrow (E). DMPS and DMSA had little to no effect on Cd levels in all organs.

Figure 51: The effect of chelator treatment on Hg in rat organs. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Animals were sacrificed and organs were collected for metal analysis 72 hours after 1, 5 and 9 treatments and after an additional holding period of 11-20 days (noted as 9 treatments + 16). It is seen that all chelators substantially reduced Hg (supplemented) levels in liver (A) and kidney (B) whereas only miaDMSA substantially decreased Hg in lungs (C), brain (D) and bone marrow (E). DMSA slightly increased Hg levels in the lung, while both DMSA and DMPS slightly decreased Hg in the brain.

Figure 52: The effect of chelator treatment on As in rat organs. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Animals were sacrificed and organs were collected for metal analysis 72 hours after 1, 5 and 9 treatments and after an additional holding period of 11-20 days (noted as 9 treatments + 16). It is seen that chelators had only a minor effect on As (supplemented) levels in liver (A), kidney (B), lungs (C), brain (D) and bone marrow (E), with DMSA causing a marginal decrease in As levels in liver, kidney and brain.

Figure 53: The effect of chelator treatment on Cu in rat organs. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Animals were sacrificed and organs were collected for metal analysis 72 hours after 1, 5 and 9 treatments and after an additional holding period of 11-20 days (noted as 9 treatments + 16). It is seen that metal supplementation increased Cu (supplemented) levels only in kidneys (B) but not in other organs. All chelators reduced Cu levels in kidneys (B) to restore their original levels, whereas chelators did not affect Cu in liver (A), lungs (C), brain (D) and bone marrow (E), where supplementation did not elevate the preexisting Cu levels.

Figure 54: The effect of chelator treatment on Mg in rat organs. After metal loading and equilibration, rats were treated iv with one of three chelators, DMPS, DMSA and miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Animals were sacrificed and organs were collected for metal analysis 72 hours after 1, 5 and 9 treatments and after an additional holding period of 11-20 days (noted as 9 treatments + 16). It is seen that chelators had no effect on Mg (not supplemented) levels in liver (A), kidney (B), brain (D) and bone marrow (E), with a marginal increase in Mg levels in lungs (C).

Figure 55: Dox and chelator treatment in tumors grafted in loaded and non-loaded mice. Mice were loaded with a metal mix and allowed 3.5 weeks for equilibration. Naïve mice were prepared in parallel without metal loading. Subcutaneous xenografts were induced by injection of A549 cells, using non-loaded cells for naïve mice and metal-loaded cells for metal-loaded mice. Treatment of tumors started 23 days after induction. A) Tumor growth in naïve animals treated with 2 mg/kg doxorubicin twice per week; B) Tumor growth in metal-loaded animals treated with 2 mg/kg doxorubicin twice per week; C) Tumor growth in metal-loaded animals

treated with 2 mg/kg doxorubicin and 0.3 mmol/kg miaDMSA twice per week. It is seen that tumors in naïve animals are sensitive to doxorubicin whereas in metal-loaded animals they are not. A chelator alone has no effect on tumor growth. However, the combination of doxorubicin and miaDMSA in metal-loaded animals is highly efficient in blocking tumor growth.

Figure 56: *In vitro* metal loading in cell lines. Cells were metal loaded by incubating for 24 hours with either of two metal mixes (Table 7) or vehicle (control) in serum reduced (SR) growth medium containing 1% fetal bovine serum (FBS) for 24 hours. Cells were washed, counted and sampled for metal measurement by ICP-MS. (A) Sample metals in MCF7 cells after loading; (B) A sample metal in MCF7 cells after loading; (C) Sample metals in Hec-1a cells after loading; (D) A sample metal in Hec-1a cells after loading; (E) Sample toxic metals in Beas-2B cells after loading; (F) Sample essential metals in Beas-2B cells after loading.

Figure 57: The effect of equilibration (recovery) on metal content in cell lines. Cells were metal loaded by incubating for 24 hours with a “100%” metal mix (Table 7) or vehicle (control) in serum reduced (SR) growth medium containing 1% fetal bovine serum (FBS) for 24 hours. Cells were washed and incubated in serum free (SF) medium without added metals for another 24 hours. Samples were collected for cell count and metal measurement by ICP-MS. (A) Sample metals in MCF7 cells after loading and recovery (equilibration); (B) Sample metals in Hec-1a cells after loading and recovery.

Figure 58: The effect of serum content in growth medium on metal uptake in cells. MCF7 cells were metal loaded by incubating for 24 hours with a “100%” metal mix (MMI, Table 9) in SR medium or with variations of this metal mix (MMI – MMIII) or vehicle (control) in full growth medium. Cells were washed and samples were collected for cell count and metal measurement by ICP-MS. (A) Uptake of vanadium in MCF7 cells; (B) Uptake of arsenic, mercury and lead in MCF7 cells; (C) Uptake of copper, zinc and cadmium in MCF7 cells.

Figure 59: Metal removal from loaded cells by chelators. MCF7 cells were metal-loaded by incubation for 24 hours with a metal mix (Table 10) in SR medium, washed and equilibrated (recovery) for 24 hours. Chelators were added at 100 μ M for 1 hour, whereafter cells were washed and samples were collected for cell count and metal measurement by ICP-MS. (A) Levels of sample toxic metals in MCF7 cells after

chelator treatment; (B) Levels of sample essential metals in MCF7 cells after chelator treatment.

Figure 60: The effect of metals and chelators on Gemcitabine treatment in PDM41 organoids. PDM41 organoids, derived from cells from a primary

5 pancreatic tumor, were grown with or without a metal mix (Table 11) for three days. The resulting organoids were treated with varying concentrations of gemcitabine with or without chelators (at 150 μ M) for 5 days, whereafter proliferation was determined. As may be seen, metals caused resistance to treatment whereas both chelators reversed the resistance.

10 **Figure 61: The effect of metals and chelators on Gemcitabine treatment in PDM106 organoids.** PDM106 organoids, derived from a liver metastasis of a

pancreatic tumor, were grown with or without a metal mix (Table 11) for three days. The resulting organoids were treated with varying concentrations of gemcitabine with or without chelators (at 150 μ M) for 5 days, whereafter proliferation was determined. 15 As may be seen, metals caused resistance to treatment whereas both chelators reversed the resistance.

Figure 62: The effect of metals and chelators on nab-paclitaxel treatment in PDM41 organoids. PDM41 organoids, derived from cells from a primary pancreatic tumor, were grown with or without a metal mix (Table 11) for 20 three days. The resulting organoids were treated with varying concentrations of nab-paclitaxel with or without chelators (at 150 μ M) for 5 days, whereafter proliferation was determined. As may be seen, metals caused resistance to treatment whereas miaDMSA, but not DMSA, reversed the resistance.

Figure 63: The effect of metals and chelators on Gemcitabine treatment 25 in PDM106 organoids. PDM106 organoids, derived from a liver metastasis of a

pancreatic tumor, were grown with or without a metal mix (Table 11) for three days. The resulting organoids were treated with varying concentrations of nab-paclitaxel with or without chelators (at 150 μ M) for 5 days, whereafter proliferation was determined. As may be seen, cells were resistant to treatment from the onset (control) 30 and metals did not cause any further resistance, nor did chelators reverse the resistance.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a test model for determining whether the activity of existing drugs or drugs in development is affected by chronic heavy metal accumulation in an organism, as is commonplace in modern humans.

In one aspect, the invention provides a method for producing a test model having
5 chronic heavy metal accumulation comprising the steps of: providing a heavy metal mixture, loading a model with the heavy metal mixture, and optionally equilibrating the model. Further, the present invention provides a test model produced by the method of the invention for studying the emergence and/or treatment of a disease and/or disorder, such as cancer, tumors, inflammatory diseases, infectious diseases,
10 neurodegenerative disorders etc.. For example, such test model is used for determining the toxicity and/or efficacy of an active compound in an organism that is prone to (chronic) heavy metal accumulation and/or allows to monitor the effects of applying a chelator to the test model. More specifically, the invention provides knowledge whether an active compound that is effective and safe in a heavy metal deficient
15 model, may also be effective and safe in a metal-contaminated model. The model can be used to test existing and future/novel active compounds and/or to novel medical uses of active compounds. Further, the model is able to predict whether an active compound preferably is to be applied together with a chelator, or not. In line with this, the invention refers to a method for determining toxicity and/or efficacy of an active
20 compound comprising applying an active compound to a test model according of the invention. Moreover, the invention refers to a kit of parts comprising the heavy metal mixture according to the invention and optionally an untreated model.

Surprisingly, the invention facilitates the production of a test model having a sub-toxic accumulation of heavy metals which is comparable to the (chronic) sub-toxic
25 heavy metal accumulation in modern human, whereby the term “sub-toxic” indicates that the model does not show any symptoms of toxicity. Thus, the invention provides a test model which allows for a more accurate and meaningful studying of emergence and/or treatment of diseases and/or disorder in humans having (chronic) sub-toxic heavy metal accumulation, such as cancer, tumors, inflammatory diseases, infectious
30 diseases, neurodegenerative disorders etc., when compared to standard laboratory models without any metal accumulation.

In the following, the features of the invention will be described in more detail. It should be understood that embodiments may be combined in any manner and in any number to create additional embodiments. The variously described examples and

embodiments should not be construed to limit the invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed features. Furthermore, any permutations and
5 combinations of all described features in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

Throughout this specification and the claims, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising",
10 will be understood to imply the inclusion of a stated member, integer or step or group of members, integers or steps but not the exclusion of any other member, integer or step or group of members, integers or steps. The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural,
15 unless otherwise indicated herein or clearly contradicted by the context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.

20 All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as", "for example"), provided herein is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the
25 specification should be construed as indicating any non-claimed element essential to the practice of the invention.

All documents cited or referenced herein and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any
30 document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

Definitions

The terms “treatment” and “treating”, as used herein, include reference to the application of a form of therapy to a subject, with the object of e.g. curing the patient
5 from a disease, halting or slowing down the development of a disease, prolonging the life of a subject, or relieving symptoms such as pain in a subject suffering from a disease or injury. For example, a treatment includes chemotherapy, immunotherapy, radiotherapy, performance of surgery, and any combination thereof. Prophylactic treatment, therapy with the aim of preventing induction or onset of a disease, is also
10 to be understood to be part of the term “treatment”.

The term “model”, as used herein, includes reference to any *in vitro* living culture, such as a cell culture or organoid culture, and/or any *in vivo* living organism which is not human, such as a model animal. The model is for example free of any heavy metal accumulation before the method of the invention is performed. A model
15 without heavy metal accumulation, i.e. before the method of producing a test model of the invention is performed, is also denominated as “naïve model” or “unloaded model” or “non-loaded model”. The terms “free of any heavy metal accumulation”, “heavy metal deficient” and “without heavy metal accumulation” are used interchangeably herein and define that the model is essentially free of heavy metals. This means that
20 the heavy metal levels in the model are low and limited to heavy metal levels which are typically present in *in vivo* and/or *in vitro* models under best practice of laboratory husbandry and cleanest conditions for *in vitro* experiments, respectively.

The term “test model”, as used herein, includes reference to any model, i.e. *in vitro* culture (including 2-dimensional (2-D) and 3-D cultures such as spheroids and
25 organoids or cocultures) and/or *in vivo* non-human organism having a heavy metal accumulation produced by the methods of the invention. An alternative term for a test model is “loaded model”. The term “loading” as used herein, includes reference to any form of application of a heavy metal mixture which results in the model’s intake of heavy metals.

30 The term “*in vitro* culture”, as is used herein, includes reference to any 2-dimensional or 3-dimensional culturing system, including an adherent and non-adherent cell culturing system, a tissue culture system, a coculturing system for example of different cell types, a spheroid culture and/or an organoid culture. An *in*

vitro culture may be obtained from patient-derived cells or tissue, from one or more established cell lines, or from a combination thereof.

The term “sub-toxic heavy metal accumulation”, as used herein, includes reference to any form of heavy metal accumulation and/or increased heavy metal levels that do not result in any signs of toxicity to a test model. Said sub-toxic heavy metal levels are apparently non-toxic for the test model, i.e., no overt medical symptoms recognizable as derived from the metal load are observed. For example, the sub-toxic heavy metal accumulation does not impair any readily observable functions, including vital functions, of the test model. The sub-toxicity of the increased heavy metal levels and/or heavy metal accumulation in the test model is for example determined by general parameters selected from mortality, viability, growth rate, morphology, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof. For example, chronic sub-toxic heavy metal accumulation is achieved when heavy metal levels are increased and the test model does not show a difference, preferably not a clinically significant difference, in one or more, preferably all, of the general parameters tested. For example, heavy metal accumulation is toxic, i.e., not sub-toxic, if one or more of the general parameters are altered after loading, for example for *in vitro* models, or after loading and equilibration, for example for *in vivo* models, especially if one or more of the general parameters is significantly altered. As is indicated herein below, for *in vivo* models some signs of mild toxicity may be observed in or during the loading period (termed acute phase), but these should be absent in the test model after the equilibration period. The test model thus is a model for chronic, sub-toxic, heavy metal accumulation.

For example, toxicity of an *in vivo* model is present when one or more of increased mortality; a change in appearance (e.g. ruffled fur); neurological effects (e.g. behavioral changes or defects in gait, balance and similar parameters); a decrease or arrest in weight gain or even weight loss; and clinically significant changes in a standard clinical blood biochemistry and hematology panel, are observed, especially when they are observed after the equilibration period for *in vivo* models. In the absence of any of these changes, an increase of heavy metal levels in a model is defined as sub-toxic. It is noted that mild signs of toxicity, such as reduced weight or weight gain, may be observed for *in vivo* models in the loading period, but preferably should be absent after the equilibration period.

For example, toxicity of an *in vitro* test model is present when one or more of changes in viability, changes in growth rate, and changes in morphology, are observed. In the absence of any of these changes, an increase of heavy metal levels is defined as sub-toxic. Again, mild signs of toxicity, such as temporally decrease of growth rate, or
5 a slight difference in morphology, may be observed in the loading period, but these signs preferably have disappeared when the test model is to be used.

For example, for *in vivo* test models sub-toxicity is achieved when a test model having a heavy metal accumulation does not show a difference of more than 20 %, more than 15 %, more than 10 %, more than 5 %, more than 2 %, more than 1 %
10 difference in weight or in weight gain, or does not show any clinically significant difference, in weight or in weight gain, when compared to a model without said heavy metal accumulation.

For example, for *in vitro* test models sub-toxicity is achieved when less than 20 %, less than 15 %, less than 10 %, less than 5 %, less than 2 %, less than 1 %, of test
15 models, e.g., cells or organoids, having a heavy metal accumulation show a difference in morphology, or show a significant difference in morphology, when compared to a model without said heavy metal accumulation.

The terms “significance” and “significant”, respectively, as used herein refer to biological and/or clinical significant differences for example in the context of
20 determining differences of general parameters. For example, for determining *in vitro* parameters such as morphology “significant” refers to “biologically significant”. For example, for determining *in vivo* parameters such as behavior, appearance, and a standard clinical blood biochemistry or hematology panel “significant” refers to “clinically significant”.

25 The terms “biological significance” and “biologically significant”, respectively, as used herein refer to the biological importance of a specific parameter for the basic functions and/or structure such as morphology, development, viability, growth rate, propagation of an *in vitro* test model. Biological significance is for example determined by a biologist, preferably a cell biologist.

30 The terms “clinical significance” and “clinically significant”, respectively, as used herein refer to the practical importance of a difference of a specific parameter, whether it has a real genuine, palpable, and/or noticeable effect on the basic function and processes of an *in vivo* (test) model. Clinical significance is for example defined as a level of difference compared to a control at which a skilled person, such as a qualified

physician, will start to expand diagnostics, or will start to intervene, for example by starting treatment.

The term “chronic” as used herein, refers to a settlement of heavy metals into a stable heavy metal accumulation and/or distribution within the test model. This means that the heavy metal levels and/or distribution in the test model remain constantly and/or continuously increased compared to the heavy metal levels before loading and/or compared to an unloaded control. For example, chronic sub-toxic heavy metal accumulation is facilitated after the equilibrating step is performed. For example, the loaded heavy metal levels remain increased for a period of at least 1 day to at least 3 years, such as for at least 3 days, for at least 4 days, for at least 5 days, for at least 6 days, for at least 7 days, for at least 8 days, for at least 9 days, for at least 10 days, for at least 14 days, for at least 21 days, or for at least 1 month, for at least 1.5 months, or for at least 3 months after equilibration. For example, the loaded heavy metal levels remain increased for a period of up to 3 years, including a period of up to 2 years, a period of up to 1 year, a period of up to 6 months, a period of up to 5 months, a period of up to 4 months, or a period up to 3 months. For example, the heavy loaded heavy metal levels remain increased for the rest of the life time of the test model. The term “chronic” is used herein as different from an acute heavy metal accumulation that causes a major influx into the body. Acute heavy metal accumulation is characterized by rapid acquisition of the metal(s) that may cause specific symptoms. Typically, during acute heavy metal accumulation a large part of the metal(s) is accessible to chelation therapy by traditional chelators. Once the metals are in the body they start to equilibrate across organs, cells and within cells, moving from initial binding sites to stronger binding sites, including binding to (storage) molecules such as metallothioneins. Over time metals reach an equilibrium, which is termed chronic heavy metal accumulation. As an example, after metal loading, an elevation of many metals may be seen in the lungs, but over a few weeks, much of these metals leak out again and redistribute in the body. Similarly, after treatment with chelators, the equilibrium is disturbed and metals levels will drop in most organs but many metals increase transiently and drop subsequently in lungs.

The terms “control” and “reference”, as used herein, are used interchangeable and refer to an untreated counterpart, such as an unloaded model, or a loaded model to which no active compound and/or chelating agent has been applied to. For example, “control” or “reference” refers to a model having no, or no intentional, heavy metal

accumulation. The control is for example another model organism which has not been loaded with the heavy metal mixture of the invention. Alternatively, a control is for example the same (test) model prior to loading with the heavy metal mixture. An alternative term for a model without heavy metal accumulation is “naïve” such as
5 “naïve model” or “naïve animals”. If the test model is used for determining toxicity and/or efficacy of an active compound, a control, i.e. an untreated counterpart, is for example a unloaded model and/or a test model of the invention having a heavy metal accumulation to which no active compound and/or chelating agent has been applied to.

The term “subject”, as used herein, includes reference to a recipient of an active
10 agent as described herein, i.e. a subject that is suffering, or suspected of suffering, from cancer. Preferably, the subject is a mammal, more preferably a human. The terms “patient” and “subject” can be used interchangeably herein. The subject is preferably a human, more preferably a human having a cancer. The subject can be 50 years of age, or older or younger than 50 years.

The term “tumor”, as used herein, includes reference to an abnormal growth of
15 tissue that may be benign, pre-cancerous, malignant, or metastatic, or to a syndrome such as myelodysplastic syndrome (MDS). The tumor is preferably malignant, i.e. a cancer. Examples of cancers include, but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, spleen cancer,
20 colorectal cancer, renal cancer, liver cancer, brain cancer, leukemia such as acute myeloid leukemia (AML), lymphoma such as Hodgkin lymphoma and non-Hodgkin lymphoma, lymph node cancer, bone marrow cancer, lung cancer such as small cell lung cancer, stomach cancer, eye cancer, brain cancer and the like.

The term “liquid tumor”, as used herein, includes reference to a form of cancer
25 wherein the cancer cells are primarily situated in body fluids, such as blood, bone marrow and lymph.

The term “solid tumor”, as used herein, includes reference to a form of cancer wherein the cancer cells are primarily situated in a solid tissue, such as tissue of the lung, brain, spleen, pancreas, liver, breast, prostate, bowel, stomach, bone, skin and
30 cartilage.

The term “metal”, as used herein, includes reference to a chemical element that may occur in the body of a subject. Preferably, said metal is present in the body in ionic form. Metalloids should also be understood to fall under the definition of ‘metal’ in the context of the invention. Elements that are commonly referred to as metals or

metalloids, are lithium, beryllium, sodium, magnesium, aluminum, potassium, calcium, scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, gallium, rubidium, strontium, yttrium, zirconium, niobium, molybdenum, technetium, ruthenium, rhodium, palladium, silver, cadmium, indium, tin, cesium, 5 barium, lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, lutetium, hafnium, tantalum, tungsten, rhenium, osmium, iridium, platinum, gold, mercury, thallium, lead, bismuth, polonium, francium, radium, actinium, thorium, protactinium, uranium, neptunium, plutonium. Elements that are commonly referred 10 to as metalloids, and thus are also referred to by the term “metal” in this document, include boron, silicon, germanium, arsenic, antimony, and tellurium.

The term “heavy metal”, as used herein, includes metals selected from the group consisting of antimony (Sb), cerium (Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), gallium (Ga), germanium (Ge), holmium (Ho), indium (In), 15 lanthanum (La), lutetium (Lu), neodymium (Nd), niobium (Nb), praseodymium (Pr), samarium (Sm), tantalum (Ta), terbium (Tb), thulium (Tm), tungsten (W), uranium (U), ytterbium (Yb), iridium (Ir), osmium (Os), palladium (Pd), platinum (Pt), rhodium (Rh), ruthenium (Ru), gold (Au), silver (Ag), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), molybdenum (Mo), nickel (Ni), tin (Sn), zinc (Zn), arsenic (As), 20 bismuth (Bi), cadmium (Cd), hafnium (Hf), manganese (Mn), mercury (Hg), protactinium (Pa), rhenium (Re), selenium (Se), tellurium (Te), thallium (Tl), thorium (Th), vanadium (V), zirconium (Zr), actinium (Ac), polonium (Po), promethium (Pm), radium (Ra), technetium (Tc), astatine (At), rubidium (Rb) and a combination thereof.

The terms “active compound”, “therapeutic agent” and “pharmaceutical”, as used 25 herein, are interchangeable and include reference to any agent, drug, compound, drug candidate and/or compositions which is, or may be, used for treating and/or ameliorating any disease or disorder, such as cancer/tumor, inflammatory disease, infectious disease and/or a neurodegenerative disease. For example, the active compound is an anti-cancer drug. For example, the active compound is selected from a 30 chemotherapeutic agent, immunotherapeutic agent, tyrosine kinase inhibitors, check point inhibitors and/or a chelating agent. The term “active compounds” as used herein also includes a combination of at least two active compounds.

Non-limiting examples of anti-cancer therapeutic agents include:

- 1) Inhibitors or modulators of a protein involved in one or more of the DNA damage repair (DDR) pathways such as:
 - a. PARP1 / 2, including, but not limited to: olaparib, niraparib, rucaparib;
 - b. checkpoint kinase 1 (CHK1), including, but not limited to: UCN-01,
 - 5 AZD7762, PF477736, SCH900776, MK-8776, LY2603618, V158411, and EXEL-9844;
 - c. checkpoint kinase 2 (CHK2), including, but not limited to: PV1019, NSC 109555, and VRX0466617;
 - d. dual CHK1 / CHK2, including, but not limited to: XL-844, AZD7762, and PF-473336;
 - 10 e. WEE1, including, but not limited to: MK-1775 and PD0166285;
 - f. ATM, including, but not limited to KU-55933,
 - g. DNA-dependent protein kinase, including, but not limited to NU7441 and M3814; and
 - h. Additional proteins involved in DDR;
- 15 2) Inhibitors or modulators of one or more immune checkpoints, including, but not limited to:
 - a. PD-1 inhibitors such as nivolumab (OPDIVO), pembrolizumab (KEYTRUDA), pidilizumab (CT-011), cemiplimab (LIBTAYO), spartalizumab (PDR001), camrelizumab (SHR1210), sintilimab (IB 1308), tislelizumab (BGB-A317), toripalimab
 - 20 (JS 001), and AMP-224 (AMPLIMMUNE);
 - b. PD-L1 inhibitors such as atezolizumab (TECENTRIQ), avelumab (Bavencio), durvalumab (Imfinzi), MPDL3280A (Tecentriq), BMS-936559, and MEDI4736;
 - 25 c. anti-CTLA-4 antibodies such as ipilimumab (YERVOY) and CP-675,206 (TREMELIMUM AB) ;
 - d. inhibitors of T-cell immunoglobulin and mucin domain 3 (Tim-3);
 - e. inhibitors of V-domain Ig suppressor of T cell activation (Vista);
 - f. inhibitors of band T lymphocyte attenuator (BTLA);
 - 30 g. inhibitors of lymphocyte activation gene 3 (LAG3); and
 - h. inhibitors of T cell immunoglobulin and immunoreceptor tyrosine -based inhibitory motif domain (TIGIT);
- 3) telomerase inhibitors or telomeric DNA binding compounds;

- 4) alkylating agents, including, but not limited to: chlorambucil (LEUKERAN), oxaliplatin (ELOXATIN), streptozocin (ZANOSAR), dacarbazine, ifosfamide, lomustine (CCNU), procarbazine (MATULAN), temozolomide (TEMODAR), and thiotepa;
- 5) DNA crosslinking agents, including, but not limited to: carmustine, chlorambucil (LEUKERAN), carboplatin (PARAPLATIN), cisplatin (PLATIN), busulfan (MYLERAN), melphalan (ALKERAN), mitomycin (MITOSOL), and cyclophosphamide (ENDOXAN);)
- 6) anti-metabolites, including, but not limited to: cladribine (LEUSTATIN), cytarbine, (ARA-C), mercaptopurine (PURINETHOL), thioguanine, pentostatin (NIPENT),
- 10 cytosine arabinoside (cytarabine, ARA-C), gemcitabine (GEMZAR), fluorouracil (5- FU, CARAC), capecitabine (XELODA), leucovorin (FUSILEV), methotrexate (RHEUMATREX), and raltitrexed;
- 7) antimitotics, which are often plant alkaloids and terpenoids, or derivates thereof including but not limited to: taxanes such as docetaxel (TAXITERE), paclitaxel (ABRAXANE, TAXOL), vinca alkaloids such as vincristine (ONCOVIN), vinblastine, vindesine, and vinorelbine (NAVELBINE);
- 15 8) topoisomerase inhibitors, including, but not limited to: amsacrine, camptothecin (CTP), genisten, irinotecan (CAMPTOSAR), topotecan (HYCAMTIN), doxorubicin (ADRIAMYCIN), daunorubicin (CERUBIDINE), Idarubicin and a combination thereof, such as daunorubicin-cytarabine (VYXEOS), epirubicin (ELLENC), ICRF-193, teniposide (VUMON), mitoxantrone (NOVANTRONE), and etoposide (EPOSIN);
- 20 9)DNA replication inhibitors, including, but not limited to: fludarabine (FLUDARA), aphidicolin, ganciclovir, and cidofovir;
- 10) ribonucleoside diphosphate reductase inhibitors, including, but not limited to:
- 25 hydroxyurea;
- 11) transcription inhibitors, including, but not limited to: actinomycin D (dactinomycin, COSMEGEN) and plicamycin (mithramycin);
- 12) DNA cleaving agents, including, but not limited to: bleomycin (BLENOXANE), idarubicin,
- 30 13) cytotoxic antibiotics, including, but not limited to: actinomycin D (dactinomycin, COSMEGEN),
- 14) aromatase inhibitors, including, but not limited to: aminoglutethimide, anastrozole (ARIMIDEX), letrozole (FEMARA), vorozole (RI VIZOR), and exemestane (AROMASIN);

- 15) angiogenesis inhibitors, including, but not limited to: genistein, sunitinib (SUTENT), and bevacizumab (AVASTIN);
- 16) anti-steroids and anti-androgens, including, but not limited to: aminoglutethimide (CYTADREN), bicalutamide (CASODEX), cyproterone, flutamide (EULEXIN),
- 5 nilutamide (NILANDRON) ;
- 17) tyrosine kinase inhibitors, including, but not limited to: imatinib (GLEEVEC), erlotinib (TARCEVA), lapatinib (TYKERB), sorafenib (NEXAVAR), and axitinib (INLYTA);
- 18) mTOR inhibitors, including, but not limited to: everolimus, temsirolimus
- 10 (TORISEL), and sirolimus;
- 19) monoclonal antibodies, including, but not limited to: trastuzumab (HERCEPTIN) and rituximab (RITUXAN);
- 20) apoptosis inducers such as cordycepin;
- 21) protein synthesis inhibitors, including, but not limited to: clindamycin,
- 15 chloramphenicol, streptomycin, anisomycin, and cycloheximide;
- 22) antidiabetics, including, but not limited to: metformin and phenformin;
- 23) antibiotics, including, but not limited to:
- a. tetracyclines, including, but not limited to: doxycycline;
- b. erythromycins, including, but not limited to: azithromycin;
- 20 c. glycyglycines, including, but not limited to: tigecycline;
- d. antiparasitics, including, but not limited to: pyriminium pamoate;
- e. beta-lactams, including, but not limited to the penicillins and cephalosporins;
- f. anthracycline antibiotics, including, but not limited to: daunorubicin and doxorubicin;
- 25 g. other antibiotics, including, but not limited to: chloramphenicol, mitomycin C, and actinomycin;
- 24) antibody therapeutic agents, including, but not limited to: muromonab-CD3, infliximab (REMICADE), adalimumab (HUMIRA), omalizumab (XOLAIR), daclizumab (ZENAPAX), rituximab (RITUXAN), ibritumomab (ZEVALIN),
- 30 tositumomab (BEXXAR), cetuximab (ERBITUX), trastuzumab (HERCEPTIN), ADCETRIS, alemtuzumab (CAMPATH-1H), Lym-1 (ONCOLYM), ipilimumab (YERVOY), vitaxin, bevacizumab (AVASTIN), and abciximab (REOPRO);
- 25) conjugated antibody therapeutics, such as gemtuzumab ozogamicin (MYLOTARG) and inotuzumab ozogamicin (BESPONSA);

- 26) targeted therapies other than those otherwise classified above, including BCL-2 targeted therapies (e.g., oblimersen, venetoclax, and navitoclax), FLT3 inhibitor (e.g., midostaurin and gilteritinib), a BCR-ABL tyrosine kinase inhibitor (e.g., imatinib, nilotinib, dasartininib, bosutinib, posatinib, and bafetinib), a JAK-2 inhibitor (e.g., fedratinib, gandotinib, and pacritinib), an IDH1 inhibitor (e.g., ivosidenib) or IDH2 inhibitor (e.g., enasidenib);
- 27) other agents, such as Bacillus Calmette-Guerin (B-C-G) vaccine; buserelin (ETILAMIDE); chloroquine (ARALEN); clodronate, pamidronate, and other bisphosphonates; colchicine; demethoxyviridin; dichloroacetate; estramustine; filgrastim (NEUPOGEN); fludrocortisone (FLORINEF); goserelin (ZOLADEX); interferon; leucovorin; leuprolide (LUPRON); levamisole; lonidamine; mesna; metformin; mitotane (o,r'-DDD, LYSODREN); nocodazole; octreotide (SANDOSTATIN); perifosine; porfimer (particularly in combination with photo- and radiotherapy); suramin; tamoxifen; titanocene dichloride; tretinoin; anabolic steroids such as fluoxymesterone (HALOTESTIN); estrogens such as estradiol, diethylstilbestrol (DES), and dienestrol; progestins such as medroxyprogesterone acetate (MPA) and megestrol; and testosterone; and
- 28) hypomethylating agents such as decitabine, azacitidine, guadecitabine, a dinucleotide of decitabine and deoxyguanosine, and compounds such as curcumin, (-)-epigallocatechin-3-gallate (EGCG), mahanine, genistein, quercetin, silibinin, luteolin, kazinol Q, laccaic acid, hypericin, boswellic acid, and lycopene.

The term “chemotherapeutic agent” and “chemotherapeutic”, as used herein, includes reference to an anti-cancer drug that is used as part of a chemotherapy, which is a type of cancer treatment. A chemotherapeutic agent may have a cytotoxic effect and/or a cytostatic effect, and it may be used to cure a subject from cancer, to reduce symptoms in a subject, or to prolong life of a subject. Non-limiting examples of a chemotherapeutic agent include an (i) alkylating agent such as altretamine, bendamustine, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, lomustine, mechlorethamine, melphalan, oxaliplatin, temozolomide, thiotepa or trabectedin, an nitrosourea such as carmustine, lomustine or streptozocin; (ii) an antimetabolite such as azacitidine, 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), capecitabine, cladribine, clofarabine, cytarabine (Ara-C), decitabine, floxuridine, fludarabine, gemcitabine, hydroxyurea, methotrexate, nelarabine, pemetrexed, pentostatin, pralatrexate, thioguanine or trifluridine/tipiracil

combination, (iii) an anthracycline such as daunorubicin, doxorubicin, doxorubicin liposomal, mitoxantrone, epirubicin, idarubicin or valrubicin, (iv) an topoisomerase I or II inhibitor such as irinotecan, irinotecan liposomal, topotecan, etoposide (VP-16), mitoxantrone or teniposide, (v) a taxane such as cabazitaxel, docetaxel, nab-paclitaxel
5 or paclitaxel, (vi) a vinca alkaloid (such as vinblastine, vincristine, vincristine liposomal or vinorelbine, (vii) a corticosteroid such as prednisone, methylprednisolone or dexamethasone; (viii) all-trans-retinoic acid, arsenic trioxide, asparaginase, eribulin, hydroxyurea, ixabepilone, mitotane, omacetaxine, pegaspargase, procarbazine, romidepsin or vorinostat, (ix) an antibiotic such as dactinomycin,
10 mitomycin, gemifloxacin, adriamycin, bleomycin, ciprofloxacin, plicamycin, actinomycin, and salinomycin; or (x) a hormone therapy compound, including an aromatase inhibitor such as anastrozole, letrozole, and exemestane, an estrogen receptor modulator (ERMs), including a selective ERM such as tamoxifen and toremifene, fulvestrant, an androgen depletion therapeutic agent such as a luteinizing
15 hormone-releasing hormone (LHRH) agonist or antagonist including leuprolide, goserelin, triptorelin, histrelin, buserelin, and degarelix, an anti-steroid such as cyproterone acetate, flutamide, nilutamide, bicalutamide, abiraterone acetate, seviteronel, enzalutamide, apalutamide, darolutamide, and a combination thereof.

The chemotherapeutic agent as disclosed herein can be cyclophosphamide,
20 mechlorethamine, chlorambucil, melphalan, dacarbazine, nitrosoureas, temozolomide, daunorubicin, doxorubicin, epirubicin, aclarubicin, amrubicin, pirarubicin, valrubicin, zorubicin, idarubicin, mitoxantrone, cabazitaxel, larotaxel, ortataxel, tesetaxel, paclitaxel, docetaxel, abraxane, taxotere, epothilone A, epothilone B, epothilone C, epothilone D, epothilone E, epothilone F, vorinostat, romidepsin, irinotecan, topotecan,
25 etoposide, teniposide, tafluposide, bortezomib, erlotinib, gefitinib, imatinib, vemurafenib, vismodegib, azacitidine, azathioprine, capecitabine, cytarabine, doxifluridine, fluorouracil, gemcitabine, hydroxyurea, mercaptopurine, methotrexate, tioguanine, bleomycin, actinomycin, carboplatin, cisplatin, oxaliplatin, nedaplatin, triplatin tetranitrate, phenanthriplatin, picoplatin, satraplatin, tretinoin, alitretinoin,
30 bexarotene, vinblastine, vincristine, vindesine, vinflunine, vinorelbine, aminopterin, pemetrexed, pralatrexate, raltitrexed, pentostatin, cladribine, clofarabine, fludarabine, nelarabine, carmofur, floxuridine, tegafur, cytarabine, gemcitabine, decitabine, hydroxycarbamide, belotecan, camptothecin, cositecan, etirinotecan pegol, exatecan, gimatecan, lurtotecan, rubitecan, silatecan, mitoxantrone, losoxantrone, pixantrone,

- bendamustine, chlormethine, ifosfamide, trofosfamide, prednimustine, uramustine, carmustine, fotemustine, lomustine semustine, nimustine, ranimustine, streptozocin, mannosulfan, treosulfan, carboquone, thiotepa, triaziquone, triethylenemelamine, altretamine, procarbazine, mitobronitol, pipobroman, dacarbazine, temozolomide,
- 5 dactinomycin, mitomycin, plicamycin, aminolevulinic acid, efaproxiral, methyl aminolevulinate, padeliporfin, porfimer sodium, talaporfin, temoporfin, verteporfin, tipifarnib, abemaciclib, alvociclib, palbociclib, ribociclib, seliciclib, bortezomib, carfilzomib, oprozomib, ixazomib, anagrelide, tiazofurin, masoprocol, niraparib, olaparib, rucaparib, belinostat, entinostat, panobinostat, romidepsin, vorinostat, pi3k,
- 10 alpelisib, copanlisib, duvelisib, idelalisib, umbralisib, atrasentan, bexarotene, testolactone, amsacrine, arsenic trioxide, asparaginase, pegaspargase, belzutifan, celecoxib, demecolcine, elesclomol, elsamitucin, eribulin, estramustine phosphate, etoglucid, lonidamine, lucanthone, mitoguazone, mitotane, oblimersen, omacetaxine mepesuccinate, trabectedin, alitretinoin, bexarotene, tretinoin, veliparib, venetoclax,
- 15 salinomycin, gemifloxacin, adriamycin, ciprofloxacin, dactinomycin, mitomycin, actinomycin, anastrozole, letrozole, exemestane, tamoxifen, toremifene, fulvestrant, leuprolide, goserelin, triptorelin, histrelin, buserelin, degarelix, cyproterone acetate, flutamide, nilutamide, bicalutamide, abiraterone acetate, seviteronel, enzalutamide, apalutamide, darolutamide, or any combination thereof.
- 20 Combinations of chemotherapeutic agents may be used for the treatment of cancer. Said combinations include, but are not limited to, an alkylating agent such as cyclophosphamide and an antimetabolite such as methotrexate and/or 5-fluorouracil; an anthracycline and a taxane, optionally in combination with a hormone receptor inhibitor such as the monoclonal antibody trastuzumab; capecitabine and oxaliplatin;
- 25 leucovorin and 5-fluorouracil; leucovorin, 5-fluorouracil and irinotecan, optionally in combination with oxaliplatin, or with an angiogenesis inhibitor such as bevacizumab, or with an epidermal growth factor receptor (EGFR) inhibitor such as cetuximab; leucovorin, 5-fluorouracil and oxaliplatin; a vinca alkaloid such as vincristine, in combination with an antibiotic such as dactinomycin, optionally further combined with
- 30 an alkylating agent such as cyclophosphamide, a vinca alkaloid such as vincristine, in combination with a topoisomerase inhibitor such as irinotecan, an alkylating agent such as carboplatin, in combination with a taxane such as paclitaxel, and an antimetabolite such as gemcitabine, in combination with an alkylating agent such as cisplatin.

One or more chemotherapeutic agents may be combined with other forms of therapy such as hyperthermia, immunotherapy, photodynamic therapy, radiation therapy, targeted therapy, and/or surgery.

The term “chelating agent”, interchangeably used with the term “chelator”, as
 5 used herein, includes reference to an agent that reacts with metal ions to form a metal-chelating agent complex, or chelate. This type of complexing generally involves the formation or presence of two or more separate coordinate bonds between a polydentate ligand and a single central atom. Such ligands are also referred to as chelants, chelators, chelating agents, or sequestering agents. The skilled person is well
 10 aware of the vast array of chelating agents available, including but not limited to Acetylacetone, Alizarin, Alizarin Red S, Amidoxime, Amidoxime group, Aminoethylethanolamine, Aminomethylphosphonic acid, Aminopolycarboxylic acid, ATMP, BAPTA, Bathocuproine, BDTH2, Benzotriazole, Bipyridine, 2,2'-Bipyridine, 2,2'-Bipyrimidine, Bis(dicyclohexylphosphino)ethane, 1,2-Bis(dimethylarsino)benzene,
 15 1,2-Bis(dimethylphosphino)ethane, 1,4-Bis(diphenylphosphino)butane, 1,2-Bis(diphenylphosphino)ethane, Calixarene, Carcerand, Catechol, Cavitand, Citrate, Citric acid, Clathrochelate, Corrole, 2.2.2-Cryptand, Cyclam, Cyclen, Cyclodextrin, Deferasirox, Deferiprone, Deferoxamine (DFO) such as Desferal, Denticity, Dexrazoxane, Diacetyl monoxime, Trans-1,2-Diaminocyclohexane, 1,2-
 20 Diaminopropane, 1,5-Diaza-3,7-diphosphacyclooctanes, 1,4-Diazacycloheptane, 1,5-Diazacyclooctane, Dibenzoylmethane, Diethylenetriamine, Diglyme, 2,3-Dihydroxybenzoic acid, Dimercaprol, 2,3-Dimercapto-1-propanesulfonic acid, Dimercaptosuccinic acid, 1,1-Dimethylethylenediamine, 1,2-Dimethylethylenediamine, Dimethylglyoxime, DIOP, Diphenylethylenediamine, 1,5-Dithiacyclooctane, Domoic
 25 acid, DOTA, DOTA-TATE, DTPMP, EDDHA, EDDS, EDTA, EDTMP, EGTA, 1,2-Ethanedithiol, Ethylenediamine, Ethylenediaminediacetic acid, Ethylenediaminetetraacetic acid, Etidronic acid, Fluo-4, Fura-2, Gallic acid, Gluconic acid, Glutamic acid, Glyoxal-bis(mesitylimine), Glyphosate, Hexafluoroacetylacetone, Homocitric acid, Iminodiacetic acid, Indo-1, Isosaccharinic acid, Kainic acid, Malic
 30 acid, Metal acetylacetonates, Metal dithiolene complex, Metallacrown, Nickel bis(stilbenedithiolate), Nitrilotriacetic acid, Oxalic acid, Oxime, Pendetide, Penicillamine, Pentetic acid, Phanephos, Phenanthroline, O-Phenylenediamine, Phosphonate, Phthalocyanine, Phytochelatin, Picolinic acid, Polyaspartic acid, Porphine, Porphyrin, 3-Pyridylnicotinamide, 4-Pyridylnicotinamide, Pyrogallol,

Salicylic acid, Sarcophagine, Sodium citrate, Sodium diethyldithiocarbamate, Sodium polyaspartate, Terpyridine, Tetramethylethylenediamine, Tetraphenylporphyrin, Tetrasodium EDTA, Thenoyltrifluoroacetone, Thioglycolic acid, TPEN, 1,4,7-Triazacyclononane, Tributyl phosphate, Tridentate, Triethylenetetramine, 1,1,1-Trifluoroacetylacetone, 1,4,7-Trimethyl-1,4,7-triazacyclononane, Triphos, Trisodium citrate, 1,4,7-Trithiacyclononane and TTFA. Chelating agents may be lipophilic, such as dimercaprol (BAL), deferasirox (marketed as Exjade™, Desirox™, Defrijet™, Desifer™, Rasiroxpine™ and Jadenu™), N,N'-bis(2-mercaptoethyl)isophthalamide (also referred to as BDTH2, BDET and BDETH2; trade names B9™, MetX™ and OSR#1™), prussian blue (Radiogardase™), α-lipoic acid, mono-alkylated DMSA (e.g. monoisoamylDMSA), oximes (e.g. dimethylglyoxime, salicylaloxime), diethyldithiocarbamate (DDC) and derivatives thereof (e.g. N(methoxybenzyl)-D-glucamine dithiocarbamate) and dexrazoxane; or hydrophilic, such as dimercaptosuccinic acid (DMSA), dimercaptopropanoic acid (DMPA) or derivatives thereof, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), N-acetylcysteine (NAC), malic acid, succinic acid, citric acid, tartaric acid, deferoxamine, penicillamine, nitrilotriacetate (NTA), histidine, 2,3-dimercapto-1-propanesulfonic acid (DMPS). When reference is made to a specific chelator, such as DMSA, included within said definition are derivatives thereof. As an example, monoisoamylDMSA (miaDMSA) is a derivative of DMSA. The term "chelator" includes reference to the metal complexes of chelators such as, for EDTA, Ca-EDTA, sodium calcium EDTA and Zn-EDTA, and for DTPA for instance Zn-DTPA, Ca-DTPA, etc.

The term "broad-spectrum chelating agent", as used herein, includes reference to a chelating agent that is capable of chelating different metals such as at least 2, 3, 4, 5 or at least 6 different metals. Said broad-spectrum chelating agent preferably binds with similar strength (spanning only a few orders of magnitude) to the different metals such as at least 2, 3, 4, 5 or at least 6 different metals. As is known to a person skilled in the art, the strength with which a chelating agent binds to the different metals may differ. For example, deferoxamine (desferal) is known to strongly bind iron, but weakly to zinc and other metals, rendering deferoxamine (desferal) not a broad-spectrum chelating agent as defined herein above. Preferably, the chelating agent as disclosed herein is a broad-spectrum chelating agent. Examples of broad-spectrum chelating agents are EDTA, DMSA, DMPS, DTPA, BAL, etc. Preferably, the chelating agent as disclosed herein is miaDMSA.

The term “tumor grafting”, as used herein, includes reference to a method to translocate tumor cells, tissue and/or cell conglomerates such as spheroids and organoids from one site to another, e.g., from one organism and/or cell culture to another organism and/or cell culture. Alternatively, tumor grafting for example also includes to translocate tumor cells, tissue and/or cell conglomerates of the same origin. For example, tumor cells, tissue and/or cell conglomerate is grafted onto a (test) model of the invention. In embodiments, said tumor cells, tissue and/or cell conglomerates may comprise solid tumors or liquid tumors. In case of translocating solid tumors, the transplant may be termed a xenograft.

For example, a tumor tissue is grafted onto a (test) model of the invention prior to loading of the heavy metal mixture or after equilibrating is performed. For example, the tumor cell, tissue and/or cell conglomerate which is grafted onto a (test) model is derived from another (test) model, such as an *in vitro* (test) model. For example, the tumor cell, tissue and/or cell conglomerate to be grafted is loaded, i.e. derived from a loaded test model, such as an *in vitro* (test) model. For example, the tumor cell, tissue and/or cell conglomerate to be grafted is unloaded, i.e. derived from a naïve model. For example, an unloaded tumor cell, tissue and/or cell conglomerate is grafted onto an unloaded model prior to loading of the heavy metal mixture. For example, a loaded tumor cell, tissue and/or cell conglomerate, i.e. a tumor cell, tissue and/or cell conglomerate or organoid having a sub-toxic heavy metal accumulation, is grafted onto a loaded test model, i.e. a test model having a sub-toxic heavy metal accumulation. For example, the loaded tumor cell, tissue and/or cell conglomerate is grafted onto the loaded test model after equilibration is performed. For example, a loaded tumor cell, tissue and/or cell conglomerate is grafted onto a unloaded model. For example, an unloaded tumor cell, tissue and/or cell conglomerate is grafted onto a loaded test model.

The term “combination”, or “in combination with”, as used herein, includes reference to a combination of compounds, including active compounds or compounds that become active when provided along with certain molecules, and molecules that may be provided in one preparation, or as two or more distinct preparations. When provided as two or more distinct preparations, they may be provided to a model or test model at the same time or consecutively. When provided as two or more distinct preparations, they may be provided to a model or test model using the same or different routes of delivery.

Heavy metal mixture

The invention comprises at least one heavy metal mixture. The at least one heavy metal mixture of the invention is for example a mixture of at least two, more preferably a mixture comprising 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, at least 23, or all heavy metals selected from antimony (Sb), cerium (Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), gallium (Ga), germanium (Ge), holmium (Ho), indium (In), lanthanum (La), lutetium (Lu), neodymium (Nd), niobium (Nb), praseodymium (Pr), samarium (Sm), tantalum (Ta), terbium (Tb), thulium (Tm), tungsten (W), uranium (U), ytterbium (Yb), iridium (Ir), osmium (Os), palladium (Pd), platinum (Pt), rhodium (Rh), ruthenium (Ru), gold (Au), silver (Ag), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), molybdenum (Mo), nickel (Ni), tin (Sn), zinc (Zn), arsenic (As), bismuth (Bi), cadmium (Cd), hafnium (Hf), manganese (Mn), mercury (Hg), protactinium (Pa), rhenium (Re), selenium (Se), tellurium (Te), thallium (Tl), thorium (Th), vanadium (V), zirconium (Zr), actinium (Ac), polonium (Po), promethium (Pm), radium (Ra), technetium (Tc), astatine (At), and rubidium (Rb).

For example, the heavy metal mixture of the invention comprises 2 – 23, 5 – 20, 7 – 14, 10 – 13 or 6 – 8 heavy metals. For example, the heavy metal mixture comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, or at least 30 heavy metals. For example, cadmium, lead and mercury; manganese, cadmium and mercury; manganese, cadmium, lead and mercury; manganese, cadmium, lead, arsenic and mercury; manganese, cadmium, lead, arsenic, mercury, chromium, iron, nickel, zinc, cobalt, and copper; manganese, cadmium, lead, arsenic, mercury, vanadium, chromium, iron, zinc, cobalt, copper, and arsenic; and including manganese, cadmium, lead, arsenic, mercury, vanadium, chromium, iron, nickel, zinc, cobalt, copper, nickel, and arsenic. Said mixture for example comprises cadmium, such as cadmium, lead and mercury; or for example comprises mercury, such as mercury, lead and manganese. For example, said heavy metals are applied as metal salts. For example, said heavy metals are applied as metal salts selected from MnCl_2 , CdCl_2 , $(\text{CH}_3\text{COO})_2\text{Pb}$, NaAsO_2 , HgCl_2 , Na_3VO_4 , $\text{K}_2\text{Cr}_2\text{O}_7$, $(\text{NH}_4)_y\text{Fe}_x(\text{C}_6\text{H}_5\text{O}_7)$, NiCl_2 , ZnCl_2 , ZnSO_4 , CoCl_2 , CuSO_4 , or a combination thereof. The heavy metal mixture is flexible

and is for example adapted to the model's loading sensitivity or a specific loading profile, observing rule that the model heavy metal mixture must be non-toxic, i.e., that the loaded heavy metals are present in the model in sub-toxic amounts, meaning that the model has elevated levels of the heavy metals, but does not show any signs of toxicity.

For example, the heavy metal mixture is a liquid, wherein the heavy metals are dissolved. For example, the heavy metals are dissolved in water, an alcohol such as ethanol, a polar aprotic solvent such as acetone, acetonitrile, dichloromethane, and dimethyl sulfoxide (DMSO), an acid, preferably a mild acid such as citric acid, acetic acid, nitric acid and phosphoric acid, or a mixture thereof.

As is known to a person skilled in the art, iron salts may be dissolved under mild acidic conditions, ideally providing a pH of about 2 to prevent their precipitation. For example, ferrous or ferric salts may be dissolved in mild acid, such as 20 mM HNO₃. Hydrophobic metal compounds, such as organic derivatives of Pb and Hg (e.g. methyl mercury) may be dissolved in an apolar solvent, such as diethyl ether, or in a polar solvent, e.g., ethanol or DMSO.

For example, the heavy metals are applied in concentrations of 0.0001 – 100 mg/kg, 0.001 – 80 mg/kg, 0.01 – 50 mg/kg, 0.025 – 40 mg/kg, 0.05 – 25 mg/kg, 0.1 – 20 mg/kg, 0.2 – 15 mg/kg, 0.5 – 12 mg/kg, 0.7 – 10 mg/kg, 0.8 – 9 mg/kg, 0.9 – 8 mg/kg, 1 – 7 mg/kg of (test) model. For example at least 0.001 mg/kg, at least 0.01 mg/kg, at least 0.05 mg/kg, at least 0.1 mg/kg, at least 0.3 mg/kg, at least 0.5 mg/kg, at least 0.6 mg/kg, at least 0.8 mg/kg, at least 1 mg/kg, at least 3 mg/kg, at least 5 mg/kg, at least 10 mg/kg of (test) model weight.

For example, the heavy metal mixture according to the invention further comprise a buffering compound such as 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES), 3-(morpholin-4-yl)propane-1-sulfonic acid (MOPS), phosphate buffer, phosphate buffered saline (PBS) and/or any other buffer or combinations of buffers, one or more salts, one or more sugars, and the like. The concentration of the mixture is for example adjusted to the delivery volume, depending on, for example, the model that is used. Mixtures are preferably made fresh before use, e.g. within 12 hours to 1 minute before use. Mixtures are preferably vortexed vigorously before each administration.

For example, the invention comprises at least two heavy metal mixtures of different heavy metal composition. For example, the invention comprises at least two heavy metal mixtures having different heavy metal concentrations.

5 *Methods of the invention*

The model used in the method of the invention is an *in vitro* model and/or an *in vivo* model. In methods of the invention, both an *in vitro* model and an *in vivo* model are used. In embodiments, an *in vitro* model is used prior to an *in vivo* model. In methods of the invention, the results obtained with the *in vitro* model may determine
10 whether or not the *in vivo* model will be used. In methods of the invention, both the *in vitro* and the *in vivo* model are used in the presence and absence of a chelator. In methods of the invention, both the *in vitro* model and the *in vivo* model are used in parallel to a control. In methods of the invention, an *in vitro* model and an *in vivo* model may be combined for testing the efficacy of the active compound for cancer
15 treatment after providing an *in vivo* non-human test model with an *in vitro* model such as a tumor graft.

For example, the model is an *in vitro* cell culture. For example, the *in vitro* model is a cell culture of cells, preferably mammalian cells such as primary cells or cell lines, including fibroblast cells such as MRC-5, NIH3T3, L929, CHO, and BHK-21 cell lines,
20 epithelial cells such as HeLa, Vero, and HEK293 cells, liver cells such as HepG2 cells, endothelial cells such as BAE-1 cells, kidney cells such as MDCK cells; pancreatic cells such as DSL-6A/C1 and DSL-6B/C2; pancreatic island cells such as TGP52 and TGP61 cells; neuroblastoma cells such as SH-SY5Y cells; lymphoblasts such as NS0, HL60, Jurkat and U937 cells. For example, the *in vitro* model is a cell culture of cells selected
25 from the group consisting of 5637, 769-P, 786-O, A-172, A-204, A-375, A-388, A-427, A-498, A-549, A-704, ACHN, AN3-CA, AsPC-1, AU565, Beas-2B, BT-20, BT-549, BxPC-3, C-33 A, CAL27, CCF-STTG1, CCRF-CEM, COLO 205, COLO 829, Daoy, DB, DLD-1, DoTe2 4510, DU145, DU4475, ES-2, FaDu, G-361, H2170, HCT-116, HCT-15, Hec-1-A, Hep-3B, HL-60, Hs 578T, Hs 746.T, Hs 766T, HT, HT-1080, HuTu 80, J82, JAR,
30 Jurkat E6-1, K-562, KATO III, KG-1, KLE, KY-821, Hu812, LNCaP clone FGC, LoVo, LS174T, LS411N, MCF-7, MeWo, MG-63, MIA PaCa-2, MOLM-13 (AML), MOLT-4, NCC-IT, NCI-H460, NCI-H661, NCI-H82, OCI-AML3 (AML), OVCAR-3, PA-1, PC-3, PFSK-1, RD, Rh30, RKO, RL, RL-95-2, RPMI-7951, RS4;11, RT-4, SHP-77, SK-N-AS, SK-N-FI, SNU-5, SNU-C2B, SR, SU-DHL-1, SU-DHL-6, SW48, SW480, SW620,

SW626, SW837, SW872, SW900, SW948, SW982, T24, T98G, TCCSUP, THP-1, TT, U-118MG, U20S, U-87MG ATCC, U-937, VA-ES-BJ, and a combination thereof. For example, the *in vitro* model is a cell culture of cells selected from the group consisting of A-549; Hec-1A; MCF7; OVCAR3; Beas-2B; H-2170; BxPC3; SW1990; Hep-3B; 5 MOLM-13; MOLM-14; OCI-AML3; THP-1; U-937 (large B-cell lymphoma); HL-60; KY821, and a combination thereof. For example, the *in vitro* model is a 2-D, or 3-D cell culture like spheroids and organoids.

For example, the *in vitro* model is an 3-D organoid selected from a ductal adenocarcinoma such as HCM-CSHL-0094-C25 (ATCC reference number PDM41) 10 and/or an pancreatic adenocarcinoma such as HCM-BROD-0008-C25 (PDM106). Suitable *in vitro* models are available from the Human Cancer Models Initiative (HCMI).

For example, said *in vitro* model may be cultured under conditions that allow maintenance or proliferation of the cells or organoid. For example, the cells or 15 organoid are kept at a temperature between 20 °C and 40 °C, such as between 30 °C and 37 °C, in the presence of a gas mixture comprising O₂. Said gas mixture may further comprise CO₂, which may be included to control the pH of the growth medium. For example, buffering of the growth medium may be achieved by including an organic (e.g., HEPES) or CO₂-bicarbonate based buffer in the growth medium. In general, 4 - 20 10% CO₂, such as 5 - 7% CO₂, is routinely used for most cell and organoid culture experiments. Said growth medium may be any growth medium suitable for culturing animal or human cells such as Dulbecco's Modified Eagle Medium (DMEM), minimum essential medium (MEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Ham's tissue medium, etc..

25 For example, said growth medium may be supplemented with growth factors, which are often derived from serum of animal blood. For example, the growth medium may comprise 20% (v/v), or less than 20 %, less than 10%, less than 7 %, less than 5%, less than 3 %, less than 2 %, less than 1 %, or less than 0.5 % serum. For example, the growth medium comprises less than 1 % (v/v) serum. For example, a growth medium 30 contains 20% of serum, or less than 20 %, less than 10 %, less than 8 %, less than 5 %, less than 3 %, or about 1 % (v/v) serum.

Said serum may be any serum that can be used as a growth supplement for culturing of animal and/or human cells, tissues and organs, including horse serum,

rabbit serum, goat serum, porcine serum, and bovine serum such as fetal bovine serum (FBS).

For example, for loading of an *in vitro* model, the at least one heavy metal mixture according to the present invention is added to a growth medium, for example
5 DMEM, which contains 10% of serum, or less than 10 %, less than 8 %, less than 5 %, less than 3 %, or 1 % fetal bovine serum.

For example, the model is an *in vivo* model such as a non-human living organism. For example, the *in vivo* model is a rodent such as a rat and/or a mouse, a rabbit, a guinea pig, zebrafish, a cat, a dog, a chicken, or a non-human primate such as
10 rhesus macaque or chimpanzee.

For example, the model is an *in vivo* model that resembles a human disease and/or disorder such as Parkinson's disease, a neuropsychiatric disorder such as depression, epilepsy, cardiovascular disease, rheumatoid arthritis, osteoarthritis, atherosclerosis, and the like. Non-limiting examples of such *in vivo* models have been
15 described in, e.g., Dovonou et al., 2023. Translational Neurodegeneration 12: 36; Fuchs and Flügge, 2022. Dialogues Clin Neuroscience 8: 323-333; Wang et al., 2022, Aging Dis 13: 215–231; van der Velden et al., 2022. Cardiovasc Res 118: 3016–3051; Wang et al., 2023. Europ J Immunol 53: 2249962; Szponder et al., 2023. J Clin Med 12: 5; Gistera et al., 2022. Circulation Res 130: 1869–1887.

The loading of at least one heavy metal mixture into the model is for example performed by pipetting and/or mixing the heavy metal mixture to the culture medium of the model, cultivating the model in the heavy metal mixture, injecting the at least one heavy metal mixture into the model, oral gavage of the heavy metal mixture in the model, for example using a flexible or curved 16G-20G gavage needle, or a combination
25 thereof. For example, for an *in vitro* model, such as a cell culture, used as test model the loading is performed by pipetting and/or mixing the at least one heavy metal mixture to the growth medium of the model and/or cultivating the model in the heavy metal mixture.

For example, for an *in vitro* model such as organoids, the loading of heavy
30 metals is performed before, or during formation and/or differentiation of the *in vitro* model. For example, for an *in vitro* model the loading of the heavy metals by the heavy metal mixture is performed after formation and/or differentiation of the *in vitro* model. For example, for an *in vitro* model, such as organoids, the loading is performed by culturing the *in vitro* cell culture in the presence of the heavy metal mixture during

proliferation and differentiation into 3D organoid structures. For example, for an *in vivo* model, such as a non-human organism, the loading is performed by injecting the at least one heavy metal mixture in the model and/or by oral gavage of the at least one heavy metal mixture in the model. The loading is for example performed once or
5 multiple consecutive times. For example, the loading is performed once a day, twice a day, three times a day, four times a day, once per week, every second day, or once every three days. For example, the loading is performed consecutively for 2 – 60 days, including for at least 1 hour, for at least 8 hours, for at least 12 hours, for at least 24 hours, for at least 32 hours, for at least 2 days, for at least 3 days, for at least 5 days,
10 for at least 7 days, for at least 10 days, for at least 14 days, for at least 21 days, or for at least one month. For example, the loading is performed > 24 hrs. For example, the loading is performed for at least 2 days. For example, the loading is performed daily for 10 consecutive days. For example, the loading is performed in a repetitive series with a pause in between. For example, the loading is performed for at least five days
15 following a loading pause of at least two days, following at least another five days of loading.

Optionally, loading is performed with at least two different heavy metal mixtures. For example, the at least two different heavy metal mixtures may differ in the presence of one or more of the metals. For example, one of the heavy metal
20 mixtures may comprise a number of heavy metals without iron, for example between 2-22, such as 4, 10, 15 or 20 heavy metals, and the second heavy metal mixtures may comprise the same composition of heavy metals with the addition of iron. For example, the at least two different heavy metal mixtures are loaded at the same time or alternately during the loading period or in consecutive periods. For example, first mix
25 1 for a period of time and then mix 2 for a period of time. Mix 1 and mix 2 may be loaded successively, or with x time in between such as 1 day, 2 days, 3 days, or 1 week.

For example, loading is performed with a volume of 0.1 – 50 ml/kg, 0.5 – 25 ml/kg, 1 – 20 ml/kg, 1.5 – 15 ml/kg, 2 – 10 ml/kg of heavy metal mixture. For example, loading is performed with a volume of at least 0.1, at least 0.5, at least 1.0, at least 1.5,
30 at least 2.0, at least 2.5, at least 3, at least 3.5, at least 4, at least 4.5, at least 5, at least 7, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50 ml/kg of heavy metal mixture.

For example, loading is performed with at least one heavy metal mixture comprising 10.0 mg/kg Na₃VO₄, 10.0 mg/kg K₂Cr₂O₇, 6.0 mg/kg CoCl₂, 5.0 mg/kg NiCl₂,

- 10.0 mg/kg NaAsO₂, 1.0 mg/kg CdCl₂, 1.5 mg/kg HgCl₂, 30.0 mg/kg (CH₃COO)₂Pb. For example, loading is performed with at least one heavy metal mixture comprising 4.5 mg/kg Na₃VO₄, 4.5 mg/kg K₂Cr₂O₇, 8.0 mg/kg (NH₄)_yFe_x(C₆H₈O₇), 2.7 mg/kg CoCl₂, 2.25 mg/kg NiCl₂, 8.0 mg/kg CuSO₄, 16.0 mg/kg ZnCl₂, 4.5 mg/kg NaAsO₂, 1.0 mg/kg CdCl₂,
5 0.68 mg/kg HgCl₂, 13.50 mg/kg (CH₃COO)₂Pb. For example, loading is performed with at least one heavy metal mixture comprising 2.25 mg/kg Na₃VO₄, 2.25 mg/kg K₂Cr₂O₇, 5 mg/kg MnCl₂, 4.0 mg/kg (NH₄)_yFe_x(C₆H₈O₇), 1.35 mg/kg CoCl₂, 1.13 mg/kg NiCl₂, 4.0 mg/kg CuSO₄, 8.0 mg/kg ZnCl₂, 2.25 mg/kg NaAsO₂, 0.5 mg/kg CdCl₂, 0.34 mg/kg HgCl₂, 6.75 mg/kg (CH₃COO)₂Pb.
- 10 The method of the invention for example comprises a step of equilibrating the model after the loading of the heavy metal mixture is performed. The equilibrating step of the method, also termed clearing step, of the invention is for example performed in order to equilibrate and stabilize the heavy metal levels throughout the model, e.g., *in vitro* cell culture and/or body of the *in vivo* model. For example,
15 equilibration facilitates transition from acute to chronic sub-toxicity of the heavy metal accumulation in the test model. For example, equilibration is performed in an *in vivo* model in order to produce an *in vivo* test model having a chronic sub-toxic heavy metal accumulation. The equilibrating step is, for example, performed in absence of a heavy metal mixture or in the presence of a heavy metal mixture. For example, the
20 equilibrating step is performed in the presence of a heavy metal mixture having a reduced concentration of heavy metals, when compared to the heavy metal mixture used for loading. The equilibrating step, may also be termed incubating step, stabilizing step, clearing step, and/or washout step. For example, the equilibrating step is performed for 1 hour – 1 year, including at least 1 hour, at least 2 hours, at
25 least 1 day, at least 2 days, at least 3 days, for at least 5 days, for at least 7 days, for at least 10 days, for at least 2 weeks, for at least 3 weeks, for at least 4 weeks, for at least 5 weeks, for at least 6 weeks, for at least 7 weeks, for at least 8 weeks, for at least 9 weeks, or for at least ten weeks. For example, the equilibrating is performed for 3 to 8 weeks, for 3 to 5 weeks, for 4 to 6 weeks or for 5 to 7 weeks. For example, for *in vivo*
30 test models the equilibrating step is performed for a period of at least 2 days, at least 3 days, for at least 5 days, for at least 7 days, for at least 10 days, for at least 2 weeks, for at least 3 weeks, for at least 4 weeks, for at least 5 weeks, for at least 6 weeks, for at least 7 weeks, for at least 8 weeks, for at least 9 weeks, for at least ten weeks, for at least 4 month, or for at least 6 months. For example, for *in vitro* models the

equilibrating step is not performed, or performed for a period of at least 30 seconds, one minute, 5 minute, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, least 6 hours, 8 hours, 10 hours, 12 hours, 18 hours, or at least 24 hours. For example, for an *in vitro* model, such as a cell culture, the equilibration comprises replacing the cultivation
5 medium of the test model at least once, at least twice, at least three times, at least four times, at least five times, at least six times, or at least 10 times during the equilibrating (clearing) period. In case no equilibration step is performed for an *in vitro* model, an active compound or mixture of compounds, in the presence or absence of a chelating agent, may be added directly to the loading mixture in the absence of an
10 equilibrating step.

Optionally the method of the invention of producing a test model comprises a step of confirming the sub-toxicity of the heavy metal accumulation in the test model, meaning the absence of overt toxic symptoms. For example, this step comprises determining general parameters selected from mortality, viability, growth rate,
15 morphology, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof. As is indicated herein above, confirming the sub-toxicity of the heavy metal accumulation is preferably performed after loading of an *in vitro* test model, and after loading and equilibrating for an *in vivo* test model.

20 For example, sub-toxicity is confirmed if at least 90 %, preferably all, of the individuals of a population of the test models which were loaded with the heavy metal mixture do not show a difference of more than more than 20%, more than 15%, more than 10 %, more than 5 %, more than 2 %, more than 1 %, or show no significant difference, preferably no difference, in one or more of said parameters tested,
25 preferably in all parameters tested, after loading and/or after equilibration, when compared to the model's performance before loading of the at least one heavy metal mixture and/or compared to said parameters of an unloaded control.

For example, sub-toxicity of the heavy metal mixture is confirmed if a test model, or a group of test models, shows at most 20 %, at most 15 %, at most 10 %, at most 5
30 %, at most 2 %, at most 1 % difference, or show no difference, preferably no significant difference, in one or more of the above mentioned parameters tested.

For example, sub-toxicity of the heavy metal mixture is confirmed if a test model, or a group of test models, shows at most at most 20 %, at most 15 %, at most 10 %, at

most 5 %, at most 2 %, at most 1 % difference, or show no difference, preferably no significant difference, in all of the above mentioned parameters.

For an *in vivo* test model, no significant difference preferably is no clinically significant difference.

5 For example, for *in vitro* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the individual populations of the test model which were loaded with the heavy metal mixture do not show more than more than 20 %, more than 15 %, more than 10 %, more than 5 %, more than 2 %, more than 1 % difference, or show no difference, preferably no significant difference, in one
10 or more, preferably all of the parameters selected from viability, growth rate, morphology including shape, when compared to the model's performance in the parameters before loading of the at least one heavy metal mixture and/or compared to the performance in the parameters of a unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90 % of a
15 population of the test model which were loaded with the heavy metal mixture, or a population of said test model as a whole, do not show more than 10 % difference in one or more, preferably all, parameters tested selected from viability, growth rate, morphology including shape compared to the model's performance in any of such parameters before loading of the at least one heavy metal mixture and/or compared to
20 the performance in any of such parameters of a unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal mixture or the population of test models, do not show a difference of more than more than 20 %, more than 15 %, more than 10 %, more than 5
25 %, more than 2 %, more than 1 %, or do not show a difference in viability after loading compared to the viability of an unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90% of the test models which were loaded with the heavy metal mixture or the population of test models, preferably all test models, do not show a difference of more than 10 % in
30 viability after loading compared to the viability an unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90% of the test models which were loaded with the heavy metal mixture, preferably all test models, do not show a difference in viability after loading compared to the viability before loading and/or of an unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if a population of the test model, such as a cell culture, which was loaded with the heavy metal mixture, does not show a difference of more than 20 %, more than 15 %, more than 10 %, more than 5 %, more than 2 %, more than 1 %, or does not show a difference, in growth rate after loading compared to the growth rate before loading of the heavy metal mixture and/or compared to the growth rate of an unloaded control population.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal mixture do not show a difference, preferably no significant difference, in morphology, after loading compared to the morphology before loading of the heavy metal mixture and/or compared to the morphology of an unloaded control.

For example, for *in vitro* test models sub-toxicity is confirmed if at least 90% of the test models which were loaded with the heavy metal mixture do not show a significant difference in morphology after loading compared to the morphology before loading and/or compared to the morphology of an unloaded control.

For example, for determining sub-toxicity of *in vitro* test models, combinations of parameters including viability and morphology; viability and growth rate; morphology and growth rate; viability, morphology and growth rate; may be analyzed. Sub-toxicity may be confirmed if the test models which were loaded with the heavy metal mixture do not show a significant difference, preferably no difference, in said combination of parameters, when compared to said parameters before loading of the heavy metal mixture and/or compared to the growth rate of an unloaded control population, or at least show no biologically significant difference.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal mixture do not show more than 10 %, more than 5 %, more than 2 %, more than 1 % difference, or show no clinically significant difference, preferably no difference, after equilibration in one or more, preferably all, of the parameters tested selected from mortality, weight, weight gain, behavior, appearance and a standard clinical blood biochemistry or hematology panel after loading and equilibrating, when compared to the parameters of the model before loading of the at least one heavy metal mixture and/or compared to the parameter of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 %, at least 99% or 100 % of the individuals of a population of the test model which were loaded with the heavy metal mixture do not show a difference of more than 10 %, more than 5 %, more than 2 %, more than 1 %, or show no
5 significant difference, preferably no difference, in one or more of the parameters tested, preferably all parameters tested, after loading and equilibrating, when compared to the model's performance before loading of the at least one heavy metal mixture and/or compared to the performance in the parameters of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at
10 least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metals, meaning the group of test models, do not show a difference in percentage of mortality after loading and equilibrating compared to the mortality of the models before loading and/or of an unloaded controls, meaning the group of control non-human animals.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90% of
15 the test models, preferably all test models, which were loaded with the heavy metal mixture do not show a difference in the percentage of mortality after loading and equilibrating, compared to the mortality of the model before loading or of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at
20 least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal mixture, do not show a difference of more than 10 %, more than 5 %, more than 2 %, more than 1 %, or do not show a difference, in weight and/or in weight gain after loading and equilibrating, compared to the weight and/or weight
25 gain of before loading and/or of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90% of
the test models, preferably all test models, which were loaded with the heavy metal mixture do not show a difference of more than 20 % in weight and/or in weight gain after loading and equilibrating compared to the weight and/or weight gain before
30 loading and/or of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal, do not show a significant difference, preferably not a difference,

in behavior after loading and equilibrating compared to the behavior of the model before loading and/or an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90% of the test models, preferably all test models, which were loaded with the heavy metal mixture do not show a significant difference in behavior, preferably not a difference, after loading and equilibrating compared to the behavior before loading and/or of an unloaded control.

Behavior is for example determined by (loss of) balance, drinking behavior, eating behavior, walking behavior and/or social behavior of the test model.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded with the heavy metal, do not show a significant difference, preferably not a difference, in appearance after loading and equilibrating, compared to the appearance before loading and/or of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90% of the test models, preferably all test models, which were loaded with the heavy metal mixture do not show a significant difference, preferably not a difference, in appearance after loading and equilibrating compared to the appearance before loading and/or of an unloaded control.

For example, appearance is determined by the appearance of the skin, fur, nose, eyes, ears, tail, etc.. For example, appearance is determined by the appearance of any feature of the test model which is discernible by the human eye.

A standard clinical blood hematology or biochemistry panel is for example performed to detect biochemical or hematological differences between the loaded test model and an unloaded model. Said biochemical or hematological differences may be clinically significant biochemical or hematological differences. A standard clinical blood biochemistry or hematology panel for example reports on the health status of a (test) model. For example, a standard clinical blood biochemistry or hematology panel includes parameters selected from hemoglobin (Hb) level, bilirubin level, hematocrit (Hct) level, red blood cell (RBC) level, white blood cell (WBC) level, platelet count, and hematological indices such as mean cellular volume (MCV), mean cellular hemoglobin (MCH), and/or mean cellular hemoglobin concentration (MCHC). Optionally, a clotting test may be included. Said standard clinical blood biochemistry or hematology panel for example includes determining enzyme levels such as aspartate phosphatase,

alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK; also termed creatine kinase (CK)), or blood levels of lipids, metabolites or nutrients such as cholesterol, triglycerides, creatinine, glucose, and electrolytes such as Na⁺, Cl⁻, K⁺ and Ca⁺⁺. For example, sub-toxicity is confirmed if
5 no clinically significant differences in the parameters of a standard clinical blood biochemistry or hematology panel are present between a test model and an unloaded model.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 %, at least 95 %, at least 98 % at least 99% or 100% of the test models which were loaded
10 with the heavy metal, do not show a clinically significant difference, preferably do not show a difference, in parameters of a standard clinical blood biochemistry or hematology panel after loading and equilibrating, compared to parameters of a standard clinical blood biochemistry or hematology panel before loading and/or of an unloaded control.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90% of the test models, preferably all test models, which were loaded with the heavy metal mixture do not show a clinically significant difference in parameters of a standard
15 clinical blood biochemistry or hematology panel after loading and equilibrating, compared to the parameters of a standard clinical blood biochemistry or hematology panel before loading and/or compared to the parameters of a standard clinical blood
20 biochemistry or hematology panel of an unloaded control.

For example, a standard clinical blood biochemistry or hematology panel as may be used in the invention is described in Washington IM, Van Hoosier G. Clinical Biochemistry and Hematology. The Laboratory Rabbit, Guinea Pig, Hamster, and
25 Other Rodents. 2012:57–116. Such standard clinical blood biochemistry or hematology panel may be used for determining sub-toxicity of heavy metal accumulation in a test model. In embodiments, the values obtained in a test model may be compared to the reference values provided in Washington IM, Van Hoosier G. Clinical Biochemistry and Hematology. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents.
30 2012:57–116, and values close to the reference values may be considered indicative for the presence of sub-toxic metal accumulation without toxic symptoms. As an alternative, or in addition, clinically significant deviations from the reference values may be considered indicative for the presence of toxic metal accumulation.

For example, a test to determine standard biochemical or hematological parameters is a standard blood test which reports on the health status of multiple organs. For example, if the outcome of such standard clinical test is within reference ranges (reference intervals), or not clinically significant outside of the reference
5 ranges, for the model may be considered indicative for the presence of sub-toxic metal accumulation without toxic symptoms. For example, physical activity can raise CPK levels up to 10 times the upper reference value for a period of days or weeks without indicating a disease or toxicity and, therefore, is not considered clinically significant.

For example, for *in vivo* test models sub-toxicity is confirmed if at least 90 % of
10 the test models which were loaded with the heavy metal mixture do not show a significant difference, preferably not a clinically significant difference, preferably not a difference, in one or more, preferably all, of the parameters selected from behavior, appearance and a standard clinical blood biochemistry or hematology panel after loading and equilibrating, when compared to the parameter before loading of the at
15 least one heavy metal mixture and/or compared to the parameter of an unloaded control.

For example, sub-toxicity of *in vivo* test models, preferably a group of *in vivo* test models, may be determined by analyzing combinations of parameters selected from mortality and weight; mortality and weight gain, mortality and behavior; mortality
20 and appearance; mortality and parameters of a standard clinical blood biochemistry or hematology panel; weight and behavior; weight and appearance; weight and parameters of a standard clinical blood biochemistry or hematology panel; weight gain and behavior; weight gain and appearance; weight gain and parameters of a standard clinical blood biochemistry or hematology panel; behavior and appearance; behavior
25 and parameters of a standard clinical blood biochemistry or hematology panel; appearance and parameters of a standard clinical blood biochemistry or hematology panel; mortality, weight and appearance; mortality, weight gain and appearance; mortality, weight and behavior, mortality, weight gain and behavior; mortality, weight and parameters of a standard clinical blood biochemistry or hematology panel;
30 mortality, weight gain and parameters of a standard clinical blood biochemistry or hematology panel; mortality, behavior and appearance; mortality, behavior and parameters of a standard clinical blood biochemistry or hematology panel; weight, behavior and appearance; weight, behavior and parameters of a standard clinical blood biochemistry or hematology panel; mortality, weight, behavior, and appearance

and parameters of a standard clinical blood biochemistry or hematology panel; weight gain, behavior and appearance; weight gain, behavior and parameters of a standard clinical blood biochemistry or hematology panel; behavior, appearance and parameters of a standard clinical blood biochemistry or hematology panel; mortality, weight gain, behavior, and appearance and parameters of a standard clinical blood biochemistry or hematology panel; mortality, weight, weight gain, behavior, and appearance and parameters of a standard clinical blood biochemistry or hematology panel. Sub-toxicity may be confirmed if the test models which were loaded with the heavy metal mixture do not show a significant difference, preferably not a difference, in said combination of parameters after loading and equilibrating, when compared to said parameters before loading of the heavy metal mixture and/or compared to an unloaded control population, or at least show no clinically significant difference.

For example, a test model may still harbor subtle changes and effects after loading of the heavy metal mixture and/or after loading and equilibrating is performed which are not captured by the above mentioned parameters, or are not considered to confer clinically significant toxicity.

For an *in vitro* model, the equilibration step or clearing step may be performed by applying a clearing medium to the test model. For example, the clearing step is a washing step. For example, the clearing step is a washing step comprising the application of a washing medium, such as phosphate buffered saline, saline, growth medium, growth medium with supplements, growth medium with serum, buffers such as Tris buffer, HEPES buffer, MOPS buffer and/or any other buffer or combinations of buffers or combinations of buffers with additional solutions (e.g. saline or sucrose) or any other solution that does not cause gross damage to cells or cell function during or following the wash. For example, for *in vitro* test models the clearing step is a washing step comprising applying a washing medium to the *in vitro* test model. For example, for *in vitro* test models the washing step is performed in repetitive washing cycles comprising removal of used washing medium and application of new washing medium. For example, for *in vitro* test models the washing step is performed by at least two, at least four, at least five, at least six, or at least ten washing cycles. When two or more washing steps are performed, the washing medium may be the same, or different.

For example, for an *in vitro* test model, such as a cell culture, a clearing medium is replaced at least once, at least twice, at least three times, at least four times or at least five times during the clearing.

Optionally, a method of the invention comprises determining the heavy metal accumulation within the test model after the loading step and/or after the equilibrating step is performed. Determining the heavy metal accumulation within the test model comprises determining that the heavy metals loaded via the heavy metal mixture in the model, e.g., *in vitro* cell culture and/or body of the *in vivo* model, are increased after the loading and/or after the, equilibrating step is performed.

For example, at least 10 %, at least 15 %, at least 20 %, at least 25 %, at least 30 %, at least 40 %, at least 50%, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 %, or 100 % of the individual heavy metals which were loaded are increased in the test model after the loading and/or after the equilibrating step is performed. For the avoidance of any doubt, the percentage of individual heavy metals does not refer to any amount and/or level of heavy metals, but to the different heavy metals (sort of heavy metals) included in the at least one heavy metal mixture used for loading. For example, at least 1, at least 2, at least 3, at least for 4, at least 5, at least 6, at least 8, at least 9, at least 10, at least 11, at least 12, or all individual heavy metals which were loaded are increased in the test model after the loading and/or after the equilibrating/step is performed. As is known to a person skilled in the art, each cell type may have its own specific metal uptake profile, for example depending on the organ it comes from. In addition, subtle differences may be present between cells or cell lines from the same organ.

For example, the heavy metal levels are at least 20 %, at least 30%, at least 40%, at least 50%, at least 60%, at least 70% at least 80% at least 90%, at least 100%, at least 120%, at least 130%, at least 140%, at least 150%, at least 180%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400% or at least 500% increased, when compared to the heavy metal levels of the model prior to loading and/or compared to an unloaded control. As is indicated herein above, some cells/cell lines may not take up certain metals at all or may do so very poorly.

For example, at least 20 % of the individual heavy metals which were loaded are increased by at least 50 % in the test model when compared to the heavy metal levels of the model prior to loading and/or compared to an unloaded control.

For example, the method of the present invention comprises determining the chronic state of the heavy metal accumulation within the test model after the equilibrating step is performed. Determining the chronic state of the heavy metal accumulation within the test model comprises determining that the heavy metals

loaded via the heavy metal mixture in the model, e.g., *in vitro* cell culture and/or body of the *in vivo* model, remain at constant or near-constant increased levels after the equilibrating step is performed. For example, chronic heavy metal accumulation is defined by levels of loaded heavy metals remaining constantly increased for at least 3 days, for at least 5 days, for at least 7 days, for at least 14 days, for at least 21 days, for at least 1 month or for the remaining lifetime of the test model compared to the heavy metal levels of the model prior loading and/or compared to an unloaded control. For example, the loaded heavy metal levels remain increased for at least 3 days, for at least 5 days, for at least 7 days, for at least 14 days, for at least 21 days, for at least 1 month, or for the remaining lifetime of the test model after equilibrating is performed. For example, the constantly increased heavy metal levels are at least 10%, at least 20% at least, 30%, at least 40%, at least 50%, at least 60%, at least 70% at least 80% at least 90%, at least 100%, at least 120%, at least 130%, at least 140%, at least 150%, at least 180%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400% or at least 500% increased, when compared to the heavy metal levels of the model prior to loading and/or compared to an unloaded control. For example, the increased and/or constantly increased, i.e. chronic, heavy metal levels are at least 1.2, at least 1.5, at least 1.8 at least 2, at least 3, at least 4, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 100, at least 150, or at least 200 times increased compared to the heavy metal levels of the model prior loading and/or compared to an unloaded control. As is known to a person skilled in the art, said increase of heavy metal levels may differ between organs, and may differ between individual metals.

As will be clear to a skilled person, the actual heavy metal levels in a test model may slightly decrease after equilibrating is performed, for example due to leaking out through the kidneys. However, the heavy metal levels in a test model will remain increased for at least 3 days, for at least 5 days, for at least 7 days, for at least 14 days, for at least 21 days, for at least 1 month or for the remaining lifetime of the test model compared to the heavy metal levels of the model prior loading and/or compared to an unloaded control.

For example, the heavy metal accumulation and/or distribution is confirmed by measuring the heavy metal levels via mass spectrometry (MS), such as inductively coupled plasma (ICP) MS, total reflection X-rays fluorescence (TXRF), such as TXRF using Bruker S2 PICOFA, optical emission spectroscopy (OES), such as ICP-OES,

atomic emission spectroscopy (AES), such as ICP-AES, atomic absorption spectroscopy (AAS), HPLC, or any other method suitable to the skilled person or a combination thereof. For example, determining the heavy metal accumulation and/or its chronic state within the test model comprises at least one measurement of the heavy metal levels in the test model. For example, the at least one measurement is performed at a time point at which the heavy metal accumulation and/or its chronic state is assumed. For example, determining the heavy metal accumulation and/or its chronic state within the test model comprises at least two, at least three, at least four or at least five measurements of the heavy metal levels on different days. For example, the heavy metal levels are measured once before the loading and at least two times on different days after equilibrating is performed. For example, the heavy metal levels are measured in cell samples for *in vitro* test models. For example, the heavy metal levels are measured in the urine, feces, serum, tissue samples, or biopsy samples, or a combination thereof for *in vivo* test models.

Methods of the invention may further comprise a tumor grafting step onto the *in vivo* (test) model. For example, tumor grafting is performed at any time in the method, i.e. before loading, during loading, after loading, before equilibration, during equilibration, or after equilibration. For example, the tumor grafting onto the (test) model is performed prior to a loading step of the heavy metal mixture and/or after equilibrating of the test model is performed. For example, tumor grafting is performed after loading is performed. For example, the tumor grafting is performed after equilibrating the *in vivo* test model, wherein the tumor graft, such as a tumor cell, tissue and/or cell conglomerate, has a sub-toxic heavy metal accumulation (i.e., *in vitro* test model) before the step of tumor grafting is performed. For example, the tumor graft being grafted onto the test model after equilibrating is performed has a sub-toxic heavy metal accumulation which is similar to the sub-toxic heavy metal accumulation of the test model. For example, the tumor graft is a test model according to the invention. For example, the sub-toxic heavy metal accumulation of tumor graft is obtained by the method of the invention. For example, the tumor graft, such as a tumor cell, tissue and/or cell conglomerate, is unloaded, i.e., does not have a sub-toxic heavy metal accumulation. For example, the unloaded tumor graft is grafted onto a (naïve) model.

Test model

The test model according to the invention has a sub-toxic accumulation of heavy metals. This means the test model according to the invention has increased levels of heavy metals compared to an unloaded control and/or compared to the (test) model prior to loading of at least one heavy metal mixture according to the invention was performed but does not have any overt signs or symptoms of toxicity. For example, the test model according to the invention has a chronic sub-toxic accumulation of heavy metals. This means that the test model according to the invention for example has chronically/constant increased levels of heavy metals and/or constant distribution of increased heavy metal levels compared to an unloaded control and/or compared to the (test) model prior to loading of at least one heavy metal mixture according to the invention. For example, the test model of the invention is obtainable by the method of the invention. For example, the model is an *in vivo* model such as a non-human living organism. For example, the *in vivo* model is a rodent such as a rat and/or a mouse, a rabbit, a guinea pig, zebrafish, a cat, a dog, a chicken, or a non-human primate such as rhesus macaque or chimpanzee.

For example, the test model is used for studying of emergence and/or treatment of diseases and/or disorders. For example, studying of emergence and/or treatment of diseases and/or disorders comprises applying an active compound to the test model. In particular, the test model of the invention is used for determining the toxicity and/or efficacy of active compounds, drugs and/or drug candidates in treating disorders and/or diseases. For example, for determining the toxicity and/or efficacy of active compounds, drugs and/or drug candidates in treating disorders and/or diseases in a human-like pre-clinical setting. Such testing will predict the likelihood of success or failure in a clinical trial which is not revealed in a classical heavy metal free preclinical animal model. For example, such testing comprises applying an active compound, drug and/or drug candidate, optionally in combination with a chelating agent, to the test model and comparing the toxicity and/or efficacy of the active compound, drug and/or drug candidate to an unloaded control. For example, the active compound, drug and/or drug candidate is novel. For example, the active compound, drug and/or drug candidate is known. For example, the test model is used for studying of emergence and/or treatment of diseases and/or disorders, wherein a novel active compound is applied to the test model. For example, the test model is used for studying of emergence and/or treatment of a disease and/or disorder, wherein a known active compound having a novel medical use is applied to the test model.

As will be known to a skilled person, emergence of a disease and/or disorder may be studied by prophylactic treatment with an active compound, with the aim of preventing induction or onset of the disease or disorder. Said active compound may be known, or novel.

- 5 For example, the test model is used for studying the emergence and/or treatment of diseases and disorders selected from tumors, cancer, inflammatory diseases, infectious diseases and/or neurodegenerative disorders. For example, the test model is used for studying the emergence and/or treatment of tumors and/or cancers selected from the group consisting of solid tumors, blood born tumors, leukemias, tumor
- 10 metastasis, hemangiomas, acoustic neuromas, neurofibromas, trachomas, pyogenic granulomas, psoriasis, astrocytoma, acoustic neuroma, blastoma, Ewing's tumor, craniopharyngioma, ependymoma, medulloblastoma, glioma, hemangioblastoma, Hodgkin's lymphoma, medullablastoma, leukaemia, mesothelioma, neuroblastoma, neurofibroma, non-Hodgkin's lymphoma, pinealoma, retinoblastoma, sarcoma,
- 15 seminoma, trachomas, and Wilms' tumor, bile duct carcinoma, bladder carcinoma, brain tumor, breast cancer, bronchogenic carcinoma, carcinoma of the kidney, cervical cancer, choriocarcinoma, choroid carcinoma, cystadenocarcinoma, embryonal carcinoma, epithelial carcinoma, esophageal cancer, cervical carcinoma, colon carcinoma, colorectal carcinoma, endometrial cancer, gallbladder cancer, gastric
- 20 cancer, head cancer, liver carcinoma, lung carcinoma, medullary carcinoma, neck cancer, non-small-cell bronchogenic/lung carcinoma, ovarian cancer, pancreas carcinoma, papillary carcinoma, papillary adenocarcinoma, prostate cancer, small intestine carcinoma, prostate carcinoma, rectal cancer, renal cell carcinoma, retinoblastoma, skin cancer, small-cell bronchogenic/lung carcinoma, squamous cell
- 25 carcinoma, sebaceous gland carcinoma, testicular carcinoma, uterine cancer, or a combination thereof.

- For example, the test model is used for studying the emergence and/or treatment of inflammatory diseases selected from the group consisting of psoriasis, rheumatoid arthritis (RA), multiple sclerosis, systemic lupus erythematosus (SLE), diverse
- 30 phenotypes of inflammatory bowel diseases (IBD), asthma, liver fibrosis, liver cirrhosis, allergy, or a combination thereof.

For example, the test model is used for studying the emergence and/or treatment of infectious disease selected from the group consisting of hepatitis B and/or C virus, human immune deficiency virus, cytomegalovirus, Herpes Simplex virus, Measles

virus, respiratory syncytial virus, Helicobacter pylori infection, SARS-CoV-2, Influenza or a combination thereof.

For example, the test model is used for studying the emergence and/or treatment of neurodegenerative disorders selected from the group consisting of Alzheimer's
5 disease, Huntington's disease, Parkinson's disease, multiple sclerosis, Amyotrophic lateral sclerosis (ALS), or a combination thereof.

For example, the active compound, drug and/or drug candidate which is applied to the test model is used or intended for use against any of the above mentioned diseases and/or disorders. For example, the active compound, drug and/or drug
10 candidate is an anti-cancer drug, anti-inflammatory drug, anti-infection drug, a drug for treating neurodegenerative disorders, or a combination thereof. For example, the active compound, drug and/or drug candidate is selected from a chemotherapeutic agent and/or immunotherapeutic agent.

For example, for an *in vitro* test model the active compound is applied by
15 pipetting and/or mixing the active compound to the culture medium and/or injecting the active compound into the *in vitro* test model, such as a cell culture. For example, for an *in vivo* test model the active compound is applied enterally or parenterally.

Said parenteral administration is for example epidural, intracerebral, intracerebroventricular, epicutaneous, sublingual, extra-amniotic, nasal, intra-
20 arterial, intra-articular, intracardiac, intracavernous, intradermal, intralesional, intramuscular, intraocular, intraosseous, intraperitoneal (IP), intrathecal, intrauterine, intravaginal, intravenous (IV), intravesical, intravitreal, subcutaneous, transdermal, perivascular, transmucosal or intratumoral administration; more preferably the administration is intravenous or intratumoral administration. For
25 example, for an *in vivo* test model the active compound is applied by injection to the test model.

Said enteral administration is, for example, oral, sublingual, gastric and rectal administration. For example, for an *in vivo* test model the active compound is applied by a feeding tube such as an oral gavage to the test model.

30 Optionally, the test compound is applied in combination with at least one chelating agent. The at least one chelating agent is for example applied together with active compound, separately of each other at the same time or separately of each other staggered in time. Preferably, at least one chelating agent is selected from the group comprising DMPS, EDTA, DTPA, dimercaprol, DMSA, and derivatives thereof, such as

miaDMSA, or a combination thereof. More preferably, said at least one chelating agent is a combination of DMPS and EDTA; of DMPS and DTPA; of DMPS and dimercaprol; of DMPS and DMSA, preferably miaDMSA; of EDTA and DTPA; of EDTA and dimercaprol; of EDTA and DMSA, preferably miaDMSA; of DTPA and dimercaprol; of DTPA and DMSA, preferably miaDMSA; of dimercaprol and DMSA, preferably miaDMSA. Even more preferably, one or more chelating agent is selected from the group comprising DMSA, DMPS, EDTA, and derivatives thereof, such as miaDMSA. In a preferred embodiment, the chelating agent is DMPS, a combination of at least DMPS and EDTA, or a combination of at least DMSA, preferably miaDMSA, and EDTA.

For example, for an *in vitro* test model the at least one chelating agent is applied by pipetting and/or mixing the at least one chelating agent to the culture medium and/or injecting the at least one chelating agent into the *in vitro* test model, such as a cell culture. For example, for an *in vivo* test model the at least one chelating agent is applied enterally or parenterally. Said enteral administration is, for example, oral, sublingual, gastric and rectal administration. Said parenteral administration can be epidural, intracerebral, intracerebroventricular, epicutaneous, sublingual, extra-amniotic, nasal, intra-arterial, intra-articular, intracardiac, intracavernous, intradermal, intralesional, intramuscular, intraocular, intraosseous, intraperitoneal, intrathecal, intrauterine, intravaginal, intravenous, intravesical, intravitreal, subcutaneous, transdermal, perivascular, transmucosal or intratumoral administration; more preferably the administration is intravenous or intratumoral administration. For example, for an *in vivo* test model the at least one chelating agent is applied by injection (IV or IP) and/or oral gavage to the test model.

A chelating agent as disclosed herein can be applied in any acceptable pharmaceutical dosage form. For example a chelating agent is applied as an aqueous medium such as a solution, suspension, emulsification and/or as nanoparticle, such as liposome, lipid vessel or coated solid nanoparticle etc.. A chelating agent can also be applied orally as a pill, tablet, capsule etc..

For example, a chelating agent is applied parenterally. Said parenteral administration is for example epidural, intracerebral, intracerebroventricular, epicutaneous, sublingual, extra-amniotic, nasal, intra-arterial, intra-articular, intracardiac, intracavernous, intradermal, intralesional, intramuscular, intraocular, intraosseous, intraperitoneal, intrathecal, intrauterine, intravaginal, intravenous, intravesical,

intravitreal, subcutaneous, transdermal, perivascular, transmucosal or intratumoral administration. For example, for an in vivo test model the chelating agent is applied by injecting and/or oral gavage to the test model.

Optionally, an active compound and/or chelating agent as disclosed herein is in
5 composition that further comprises a pharmaceutically acceptable excipient (or carrier). As used herein, pharmaceutically acceptable excipient or carrier includes reference to any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels,
10 binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and a combination thereof, as would be known to one of ordinary skill in the art. Except insofar as any conventional carrier is incompatible with the active ingredient and/or chelating agent, its use in the pharmaceutical compositions is contemplated. Acceptable excipients, carriers or diluents for
15 therapeutic use are well known in the pharmaceutical art, and are described, for example, in standard textbook references such as Remington's Pharmaceutical Sciences (e.g. Mack Publishing Co., A.R. Gennaro edit., 1985).

EXAMPLES

20

General Materials and Methods

In vitro models

The *in vitro models*, both 2-D and 3-D experimental systems, were loaded with a solution of a number of metals for up to several days. These metal loaded systems
25 were either used directly for experiments, or, alternatively, washed to eliminate excess metals and/or incubated with metal free or low-level metal solutions for a period of time varying from a few minutes to a few days to allow equilibration (washout) of metals. Within this period, metals not tightly bound to cell components wash out and reach a stable distribution as in chronic metal overload. Subsequently the *in vitro*
30 models were ready for experimental work.

The composition of the metal mix, duration of loading and duration of equilibration period were experimentally optimized. For each parameter multiple options exist to mimic both general and specific human conditions as may be the object of the research. This is presented in the following.

For cells, including 3-D cultures like spheroids and organoids, metal mixtures were prepared as described below and provided in growth medium to a cell culture for a specified time period. The present experimental work points to an optimal period of 24 hours but shorter or longer periods may be used. After loading, cells may be washed
5 and used for induction of tumors in an animal or used for independent *in vitro* experiments. In the latter case, test compounds such as chemotherapeutics and/or chelators may be added directly to the medium without prior washing, or, alternatively, cells may be washed and either incubated directly with the test compounds or incubated in medium for a period ranging from a few minutes to several
10 days for leaching and stabilizing metals before test compounds are added.

In vivo models

Rodents, including rats and mice, were loaded with a solution of a number of metals by oral gavage for several days, followed by an equilibration period of several
15 weeks without treatment to allow the metals to equilibrate across the body and reach a stable distribution as in chronic metal overload. Subsequently the animals were ready for experimental work.

The composition of the metal mix, duration of loading and duration of equilibration period were experimentally optimized as described below. For each
20 parameter multiple options exist to mimic both general and specific human conditions as may be the object of the research. This is presented in the following.

Metal solutions and profiles

Metal solutions used for metal loading are preferably prepared from metal salts.
25 For *in vitro* use, metal salts are typically dissolved in mild acid, such as 1-50 mM HNO₃, preferably 10 mM, to prevent their precipitation. Hydrophobic metal compounds, such as organic derivatives of Hg (e.g. methyl mercury) and Pb are dissolved in appropriate solvent (e.g. ethanol, DMSO). Individual metal solutions are combined according to the desired composition. Mixtures may preferably be made
30 fresh before use.

For use with growth media or other solutions used with *in vitro* cultures, an equivalent amount of base, e.g. NaOH, may be added to the medium/solution to prevent pH fluctuations. The base is typically added to the growth medium and mixed before the metal mix is added.

Metal mixes used are described for each example below. A sample metal solution is provided in Table 1.

Metal solutions used for *in vivo* model metal loading are preferably prepared from metal salts (examples in Tables 2-3). Most metal salts were dissolved in water. Fe salts were dissolved in mild acid, such as 20 mM HNO₃ to prevent their precipitation. Hydrophobic metal compounds, such as organic derivatives of Pb and Hg (e.g. methyl mercury) were dissolved in an appropriate aprotic solvent (e.g. ethanol, DMSO).

Composition of sample metal mix for cell loading				
Metal	Preferred salt	Name	Metal Mix 100% Target conc. (μM)	Metal Mix 75% Target conc. (μM)
V	Na ₃ VO ₄	Sodium orthovanadate	0.34	0.26
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.43	0.32
Mn	MnCl ₂	Manganese(II) chloride tetrahydrate	4.00	3.00
Fe	(NH ₄) _y Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	3.40	2.55
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	3.00	2.25
Ni	NiCl ₂	Nickel(II) chloride	8.50	6.38
Cu	CuSO ₄	Cupric sulfate pentahydrate	4.25	3.19
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	4.25	3.19
As	NaAsO ₂	Sodium (meta)arsenite	1.70	1.28
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.51	0.38
Hg	HgCl ₂	Mercury(II) chloride	2.55	1.91
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	1.70	1.28

Table 1. Sample metal mixtures used for metal loading in cells.

Individual salt solutions were combined according to the desired composition. Fe is typically given on an intermittent schedule. On days when Fe is not included in the mix, the acid used for dissolving the Fe is added instead.

Mixtures are preferably made fresh before use and vortexed vigorously before each administration.

Composition of sample metal mix for loading of rats					
Metal	Preferred salt	Name	MW	Group I	
				Metal (mg/kg bw)	Salt (mg/kg bw)
V	Na ₃ VO ₄	Sodium orthovanadate	183.91	4.50	16.23
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	294.18	4.50	12.73
Fe	(NH ₄) _y Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	320.00	8.0	45.71
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	237.93	2.70	10.89
Ni	NiCl ₂	Nickel chloride hexahydrate	237.69	2.25	8.91
Cu	CuSO ₄	Cupric sulfate pentahydrate	249.69	8.0	31.71
Zn	ZnCl ₂	Zinc chloride	136.30	16.0	33.04
As	NaAsO ₂	Sodium (meta)arsenite	129.91	4.50	7.79
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	228.36	1.0	2.06
Hg	HgCl ₂	Mercury(II) chloride	271.5	0.68	0.91
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	379.33	13.50	24.62

Table 2. A sample metal mixture used for metal loading in rats.

5 The concentration of the mixtures was adjusted to the delivery volume (see below).

Composition of sample metal mix for loading of mice					
Metal	Preferred salt	Name	MW	Metal (mg/kg bw)	Salt (mg/kg bw)
V	Na ₃ VO ₄	Sodium orthovanadate	183.91	2.25	8.11
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	294.18	2.25	6.36
Mn	MnCl ₂	Manganese(II) chloride tetrahydrate	197.90	5.00	17.99
Fe	(NH ₄) _y Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	320.00	4.0	22.86
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	237.93	1.35	5.44
Ni	NiCl ₂	Nickel chloride hexahydrate	237.69	1.13	4.46
Cu	CuSO ₄	Cupric sulfate pentahydrate	249.69	4.0	15.85
Zn	ZnCl ₂	Zinc chloride	136.30	8.0	16.52
As	NaAsO ₂	Sodium (meta)arsenite	129.91	2.25	3.90
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	228.36	0.5	1.03
Hg	HgCl ₂	Mercury(II) chloride	271.5	0.34	0.45
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	379.33	6.75	12.31

Table 3. A sample metal mixture used for metal loading in mice.

Metal loading

10 Metal loading for *in vitro* model was performed by incubating cells or 3-D systems, such as organoids and spheroids, with a metal-containing growth medium or

alternative solution for a period ranging from a few minutes to several days.

Alternatively, 3-D systems were grown in the presence of the metal mixture.

Upper limits for duration and concentration are limited by potential acute toxicity.

- 5 Metal loading was either performed in the native growth medium for a particular cell line, preferably containing up to 10% serum (Full), or in serum reduced (SR) medium with less than 10% serum, for example 1%. As serum concentration in the growth medium is known to affect metal uptake, metal levels that are well tolerated in medium with 10% serum may be toxic at lower serum concentrations. Conversely,
- 10 metal levels that result in effective cellular uptake in SR medium, for example with 1% serum, may be less effective in medium with 10% serum.

Metal loading for *in vivo* models was performed by oral gavage, using a 20G gavage needle. For rats, solutions were delivered at 2ml/kg and for mice at 5ml/kg.

- 15 Typically, metals were applied daily for 7 or 10 consecutive days to optimize speed and efficiency but slower (e.g. intermittent), longer or shorter regimens may be engaged if required. Upper limits for duration and concentration are limited by potential acute toxicity.

20 *Washing and equilibration of in vitro models*

- Washing was used to remove residual metals from the solution, where indicated. For adherent cultures, the medium was aspirated and replaced by a washing solution, such as PBS or growth medium, whereas non-adherent cultures were centrifuged at non-damaging conditions prior to aspiration of the medium. According to the
- 25 performed experimental work, full removal of the metal mixtures typically required three cycles of washing (data not shown). Less than three washing cycles left a small residual level of the metal mix, which, however, may be compatible with experimental designs.

- Equilibration was optionally performed after washing by incubating the culture
- 30 in growth medium without added metals, or with reduced metals, for a period of a few minutes to several days. The equilibration medium may be either be serum free (SF), SR or Full serum levels. The performed experimental work (not shown) indicated that equilibration was complete (i.e. metal levels were stable) after 24 hours. Shorter or longer periods may be used.

Equilibration of in vivo models

Animals were allowed at least three weeks for equilibration with no further treatment during that period. There is no upper limit to the equilibration period.

5

Tumor induction in mice

Xenografts were produced by subcutaneous injection of cancer cells. Typically, a predetermined amount of cells was prepared and mixed with an equal volume of a matrix (e.g. MatriGel) and injected subcutaneously. Tumor size was monitored by width and length measurements and size was calculated using the equation:

10

$$(\text{Length} * \text{width}^2)/2.$$

Treatment was typically started when tumors were 120-250 mm³ in size. Animals were sacrificed according to ethical directives at 1500 mm³, to prevent undue suffering of the animals.

15

Leukemia and *in-situ* tumors were induced by injection of a predetermined number of cells into the tail vein.

*Treatment**In vitro models*

20

Cultures were treated as described in each experiment. For chelation and treatment studies, chelators and other test substances, such as anti-cancer treatment, were added to the growth medium after loading. This step may be performed by direct addition to the loading medium without washing or equilibrating, or by including them in, or adding them to, the growth medium after washing or after equilibration.

25

The duration of the treatment was determined by the activity of the compounds. Cells were collected for cell count and metal assessment, using EDTA-free trypsin for cell release.

In vivo models

30

Animals were treated as prescribed in each experiment. For chelation studies, chelators and other test substances were delivered by iv, ip, im or subcutaneous injection or by oral gavage or in the food or the drinking water.

For tumor studies, anti-cancer treatment was delivered as prescribed by the drugs used and chelators and other test compounds were delivered as above.

Monitoring

In vitro models

Key parameters monitored were cell number, viability and metal content.

5

In vivo models

Animals were routinely monitored for weight, appearance and behavior.

Blood was collected for standard clinical hematology and biochemistry panels.

Serum was obtained for metal detection.

10

Urine and feces were collected for 24 hour periods in metabolic cages for metal detection.

At sacrifice, key organs (including tumors) were collected. Samples of organs were examined by histology where required and otherwise organs were homogenized for metal detection.

15

Blood collection and organ harvesting were done 72 hours after the last treatment as metal levels were markedly perturbed by chelator treatments but stabilized after 72 hours.

Viability assay for in vitro models

20

Resazurin cell survival assays were used to determine cell viability. Typically, 2×10^3 cells were seeded in 100 μ l SR medium in 96 well plates. Loading, washing and equilibration was performed as described above. A stock solution of resazurin was made at 880 μ M in PBS adjusted to pH 7.8, filtered 0.2 μ m and kept at 4°C. Medium was replaced with 75 μ l of resazurin solution (final concentration 44 μ M). Cells were

25 incubated at 37°C for 3-4 hours and fluorescence emission was measured at 583nm with excitation at 555nm using a spectrophotometer. Incubation time may vary to optimize the development of the color reaction for quantitation.

Metal detection

30

All biological samples were digested in concentrated HNO_3 and H_2O_2 in preparation for metal analysis and digested samples were assayed by ICP-MS for multiple metals.

Example 1. *In vivo* model (Rat).

In this example rats were loaded with a mixture of 11 metals (Table 2) by oral gavage for either 10 consecutive days followed by equilibration for at least 5 weeks or loaded for 7 days followed by equilibration for at least 4 weeks. At intervals, urine and feces were collected and blood samples were obtained. At the end of the experiment
5 animals were sacrificed and organs were collected for metal measurements. Weight was monitored throughout. The experimental setup is presented in Figure 1.

Results

Characterization of the model

10 The most readily available samples in a medical setting are urine, feces and blood samples. Thus, these are closely monitored in this example. Results are presented in Figures 2-15.

Urinary excretion of metals was a very sensitive parameter of metal loading, particularly for the acute phase and the way metals settle into a chronic pattern.
15 Results show that excretion of supplemented metals peak right after completion of loading and then, for most metals, decreases sharply over a few days to 2 weeks whereafter levels are stable or decrease very slowly over time. A few notable exceptions include Hg and As that present a slower return towards long term steady state levels and Fe that shows an inverse excretion patterns, reflecting its strict
20 regulation and trafficking in the body rather than a relatively uncontrolled flux of metals as seen with most other metals. Non-supplemented metals (Mg, Ca, Mn, Se and Rb) are unaffected or mildly affected by the supplementation of other metals.

Fecal excretion primarily reflects dietary intake with a contribution from biliary excretion when present. Results show that fecal metal excretion is high following
25 metal administration, in line with expectation from an oral gavage delivery route. Excretion returns to baseline levels immediately after the administration is discontinued and remains low throughout. Fecal excretion of non-supplemented metals is mildly affected during the acute phase of metal loading.

Serum levels reflects the labile pool of metals in transit or, at steady state, the
30 equilibrium of circulating metals with tissue bound metals. Metals “in transit” include both incoming metals from the GI tract moving to the body tissues and metals from the GI tract and tissues moving to the kidneys for excretion. It is at all times a dynamic pool that depends on influx, tissue uptake, tissue release and efflux by excretion. As seen in the results, the serum pool of metals presents a more complex

pattern than metal excretion. Thus, highly regulated essential metals (Fe, Cu and Zn) deviate only slightly from their normal levels, whereas other supplemented metals increase sharply after administration and then decrease to a steady state level.

Notably, serum levels of As remain markedly elevated even 6 weeks after completion
5 of administration. Non-supplemented metals largely remain stable in serum.

Organ metal levels were obtained after equilibration and each organ exhibits a distinct pattern of metal uptake, dictated more by tissue characteristics than by the flooding by metals, Figures 16-31. Results are the mean of 3-5 experiments stabilized for at least 5 weeks. For better visualization, some graphs are presented at two
10 resolutions, using the same data.

V is taken up in all organs with particularly high levels reached in spleen, kidney, liver and bone marrow;

Cr is primarily elevated in spleen and kidney;

Fe, Ni and Zn are largely unaffected by the supplementation;

15 Co is slightly (40-130%) elevated across organs;

Cu is primarily elevated in the kidney;

As is strongly elevated – up to 50 times the background – in all organs;

Cd is strongly elevated across organs, with particularly high levels achieved in liver and kidney;

20 Hg (provided as an inorganic salt) is mildly elevated across organs with the exception of spleen where no elevation was observed. Particularly high levels are found in the kidneys;

Pb is also elevated in all organs, in particular bone marrow, spleen and brain, with very high concentrations in the kidneys.

25 The patterns of metal uptake in specific organs is largely similar to that observed and reported in literature for single metals even though in the current model multiple metals were available at the same time.

Body weight monitoring revealed a mild transient decrease in growth rate during loading (acute phase), amounting to only a few percent of the body weight,
30 which is not unusual during an active treatment by oral gavage. It was followed by catch up growth during the equilibration phase, Figure 32. Catch up growth was complete after four weeks (chronic phase). Subsequently, the minimum period for equilibration in rats was set at five weeks to include an extra week for variability.

Hematology and blood chemistry panels revealed no clinically meaningful effect of metal loading. Thus, no clinically significant findings were observed in a 4 week monitoring period that followed a 7 days loading period, using two different concentrations of the metal mix (data not shown).

5 Equilibration period was demonstrated by the migration of metals in organs over a period of 4 weeks following a 7 day loading period, Figure 33 (as examples of the entire panel of metals). At the end of 4 weeks, equilibration was largely complete. Thus, a minimum of 5 weeks was selected as the standard equilibration interval.

10 In summary, the rat model has been thoroughly characterized and found suitable for use as a model system for human metal overload, so common in modern man. Notably, the serum levels of metals obtained in our model system follow a similar pattern to that observed in AML patients [Ohanian, M. et al., A heavy metal baseline score predicts outcome in acute myeloid leukemia. Am J Hematol. 2020:422–434], Table 4.

Metal (units)		Ohanian et. al.		Rat experiments	
		AML Median	Control Median	Loaded Mean	Control Mean
Na	(mmol/L)	134.737	129.759	Not measured	Not measured
Mg	(mmol/L)	0.97	1.04	0.923	0.954
Ca	(mmol/L)	2.437	2.463	2.229	2.295
V	(µmol/L)	Not measured	Not measured	0.199	0.113
Cr	(µmol/L)	0.062	0.02	0.033	0.039
Mn	(mmol/L)	Not measured	Not measured	0.923	0.954
Fe	(µmol/L)	35.111	28.945	51.934	48.113
Co	(µmol/L)	0.011	0.011	0.032	0.039
Ni	(µmol/L)	Not measured	Not measured	0.024	0.083
Cu	(µmol/L)	19.494	16.406	21.462	17.496
Zn	(µmol/L)	17.461	14.907	18.837	15.331
As	(µmol/L)	0.005	0.02	3.738	0.062
Se	(µmol/L)	1.196	2.558	12.574	11.989
Rb	(µmol/L)	2.993	3.166	3.102	2.997
Cd	(nmol/L)	0	0.089	1.245	0.626
Hg	(nmol/L)	Not measured	Not measured	2.679	2.378
Pb	(nmol/L)	25.965	2.172	3.845	2.138

15 **Table 4.** Serum metal levels in patients and in the rodent model.

20 The model reveals only a low grade adverse effects in the acute phase as seen in the slight transient depression of weight gain during and immediately following metal loading but otherwise shows no other signs of toxicity, including biochemistry and hematology blood panels, appearance, behavior and survival.

Example 2. Using the *in vivo* model to study metal flux in an organism.*Methods*

5 In this example rats were loaded with a mixture of 11 metals (Table 2) by oral gavage for 10 consecutive days followed by equilibration for at least 5 weeks. Subsequently, animals were treated with one of three chelators, DMPS, DMSA or miaDMSA, at a daily dose of 0.3-0.4 mmol/kg (75-100 mg/kg) for 9 consecutive days. Urine and feces were collected on days 1, 5, 9 and 20 and serum was collected 72 hours
10 after the respective chelator treatment. Animals were sacrificed and organs were collected for metal analysis 72 hours after the respective chelator treatment and immediately after a holding period of 11-20 days (denoted as +16 days in Figures) following the ninth treatment. Naïve and metal-loaded control groups, untreated by chelators, were followed for comparison. Each results is an average of at least 2
15 experiments. The experimental setup is presented in Figure 1.

Description

 In the context of cancer, neurodegenerative disorders and additional diseases where a link has been made to heavy metal accumulation, some of the key heavy
20 metals implicated are As, Cd, Hg and Pb, which are focal in this analysis. Furthermore, a focus is placed on three essential metals, Fe, Cu and Zn, as representative of those metals that should be conserved.

 The results for excretion and serum levels are shown for the three chelators in Figures 34-49. It is seen that the model provides a clear picture of the way each
25 chelator provokes metal excretion and affects serum metal levels during its action. Moreover, the efficacy of each chelator is measured in a human-like environment, where multiple contaminating metals are present together. This already highlights the importance of the model as a tool for studying metal toxicity and its treatment in a realistic setup. Hitherto, such studies have been limited to single or dual (or in rare
30 cases triple) metal studies in animals, whereas real-life broad metal spectrum studies have been performed only in humans. It is emphasized that due to competitive and reinforcing interactions among the metals, many of the observations cannot be fully predicted from single (or dual or triple) metal studies. A notable example of such surprising interactions are the decreased excretion of Ni, Co and Mn in the urine in

the presence of chelators. This is ascribed to a lower affinity of these metals for the chelators relative to other metals, resulting in more efficient excretion of other metals in the kidney and outcompeting of the weaker metals.

5 Results

Overall, chelators increase urinary excretion of the heavy metals, with the notable exception of DMSA that showed no or little effect on As and Cd excretion, a moderate effect on Hg and a strong effect on Pb.

For As chelator-provoked urinary excretion is ranked

10 miaDMSA > DMPS > DMSA;

For Cd the ranking is miaDMSA >>> DMPS > DMSA;

For Hg: miaDMSA > DMPS > DMSA; and

For Pb: DMSA > miaDMSA = DMPS.

It is generally considered desirable to spare essential metals that are typically
15 tightly regulated in the body and involved in a large number of biochemical functions. For that reason, bulk removal of essential metals has generally failed to achieve a therapeutic benefit, except in clinical overload such as hemochromatosis and Wilson's disease, since it is impossible to control whether the metals are removed from their beneficial or detrimental functions. Thus, in this context, removal of essential metals
20 is considered undesirable.

In line with the heavy metals, DMSA was overall the weakest chelator, whereas miaDMSA removed some Fe and DMPS removed large amounts of Cu and Zn.

Ranking by efficiency of provoked urine excretion:

Fe is not excreted by DMPS and DMSA: DMSA = DMPS < miaDMSA;

25 Cu: DMSA = miaDMSA < DMPS; and

Zn : DMSA < miaDMSA << DMPS.

The relative efficacy of DMSA and DMPS is in line with human data.

Chelators had, in general, little effect on fecal excretion of metals. Fecal excretion primarily reflects the nutrition of the animal with a potential contribution
30 from metal chelates that may be excreted in the bile.

Thus, only Fe excretion in feces was reduced by all chelators whereas As excretion was mildly increased by all.

Notably, however, fecal excretion of Cd was strongly increased by miaDMSA but not by DMSA and DMPS; Hg excretion was strongly increased by miaDMSA but only

mildly by DMSA and DMPS; and Pb excretion was increased slightly by DMSA and miaDMSA. These findings indicate that fecal excretion of metal is more likely to occur with miaDMSA, in line with its more hydrophobic character.

Serum levels of metals are perturbed by chelators that mobilize the metals from tissues. While the majority of mobilized metals were excreted through the kidneys and, to a lesser degree, in the feces, serum levels often increased transiently after chelator treatment. It was found that serum metal levels generally stabilize around 72 hours after chelator treatment (data not shown), which was subsequently used as the standard time-point for serum sampling.

It was observed that non-supplemented metals were minimally affected by chelation treatment, with the exception of Mn that had decreased serum values after treatment with all chelators.

Among the supplemented metals, Fe, Co, Cu, Zn and V were minimally affected by the chelators. The serum levels of other metals had distinct patterns after chelator treatment. Thus, Ni levels were increased after treatment with DMSA and miaDMSA, peaking after 5 treatments, whereas levels were decreased by DMPS; As levels were decreased by chelators but miaDMSA caused a complex pattern that peaked on day 5; Cr levels were decreased by DMPS but not by DMSA, and once again miaDMSA caused a complex pattern that peaked on day 5; Cd levels were increased by DMSA, decreased by DMPS after peaking on day 1, and decreased consistently by miaDMSA; Hg levels peaked after one treatment with all chelators and then dropped backed to control levels with DMPS and DMSA, whereas levels decreased substantially below control level with miaDMSA; Pb levels dropped with all chelators with a relative efficiency ranking miaDMSA > DMSA > DMPS.

Metal levels in organs are presented in Figures 50-54. The levels of metals across non-supplemented metal is only minimally affected (Mn, slightly increased) or not at all (Mg, Ca, Se and Rb). Mg is shown as an example. The key toxic metals are affected differentially by organ and by metal with different chelating agents. As, Cd and Hg are shown as examples. Tissue levels of essential metals, that are typically highly regulated, are largely unaffected. They are represented by Cu.

Thus, Cd is reduced by miaDMSA in liver and kidney and, notably, remains low after cessation of treatment, whereas, in contrast, DMSA and DMPS increase liver Cd levels slightly, and DMSA but not DMPS slightly decreases kidney Cd levels.

Notably, DMPS had little to no effect on Cd levels in lung, brain and bone marrow (BM), whereas DMSA slightly increased lung levels but was neutral for brain and BM and miaDMSA was neutral for Cd in lung and BM but slightly increased brain levels.

Hg levels were consistently decreased by miaDMSA in all organs. DMSA and
5 DMPS also reduced Hg in liver and kidney but reduced brain Hg slightly and were ineffective in BM. In lungs, DMSA increased Hg levels while DMPS had no effect.

Notably, tissue levels of As were only mildly affected by chelators across the board. This finding is surprising since multiple studies report significant removal of As by chelators, in particular miaDMSA. A likely explanation is that a finite amount of
10 chelator is saturated by the metals with the highest affinity and As is outcompeted by these metals in a multi-metal heavy metal accumulation. This finding emphasized the unique value of the novel animal model for commonplace human metal contamination.

Cu, an essential metal whose bulk removal is deemed undesirable because of its involvement in multiple biochemical processes, was almost unaffected by all chelators.
15 Cu differs from other essential metals by its accumulation in kidneys after loading. This increase is normalized by all three chelators.

Tissue levels of Mg, an essential element that was not supplemented, are largely unaffected by the chelators.

20 *Discussion*

The reporting of the results above has been primarily descriptive with little or no attempt to analyze the biology of metals behind the results, which is not the subject of interest in this report. The results, however, clearly show the power and sensitivity of the model in the investigation of metal chelation in a multi-metal contaminated rodent
25 model that mimics the commonplace contamination inherently present in the modern human. The strength of the model is evident in its ability to show how chelators affect all aspects of metal metabolism, including excretion, serum levels and residual tissue levels over time and through multiple treatments with metal-specific resolution. It is further shown that the model is highly sensitive to the differences between chelators,
30 clearly revealing both major and minor differences, and is thus a competent tool to studying therapy-related use of chelators, whether for metal toxicity and sub-toxicity or for the treatment of cancer and other diseases for which drugs may have a metal related aspect. Notably, our results often differ from those of single- or dual metal studies where major effects of chelators on the supplemented metal(s) may be

observed, while in our studies such effects may be substantially altered by competitive binding of other metals.

Example 3. Using the *in vivo* model for studying cancer treatment.

5

Methods

A model system was established in mice, based on the rat model and with appropriate adjustments. The same metal mix was adopted for convenience and only the strength of the mix was adjusted to accommodate for the metal tolerance of the mice. It was found that mice tolerated the metal mix well at 30-50% of the strength used in rats. This was tolerated well in both NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) and SCID-NOD mice. Some variability in tolerance was noted between batches of mice.

In the present example, NSG mice were loaded with the metal mix at 50% the strength used in rats (Table 5) for 10 consecutive days and then allowed 4-6 weeks to equilibrate. Control mice (naïve), started out at the same time, were not treated during the loading and equilibration period.

Metals for Mice in Xenograft Study ("50%" solution)					
Metal	Preferred salt	Name	MW	Metal (mg/kg bw)	Salt (mg/kg bw)
V	Na ₃ VO ₄	Sodium orthovanadate	183.91	2.25	8.11
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	294.18	2.25	6.36
Fe	(NH ₄) _y Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	320.00	4.0	22.86
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	237.93	1.35	5.44
Ni	NiCl ₂	Nickel chloride hexahydrate	237.69	1.13	4.46
Cu	CuSO ₄	Cupric sulfate pentahydrate	249.69	4.0	15.85
Zn	ZnCl ₂	Zinc chloride	136.30	8.0	16.52
As	NaAsO ₂	Sodium (meta)arsenite	129.91	2.25	3.90
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	228.36	0.5	1.03
Hg	HgCl ₂	Mercury(II) chloride	271.5	0.34	0.45
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	379.33	6.75	12.31

Table 5. Metal mixture used for loading of mice in Example 3.

20 Tumors were induced by subcutaneous injection of 2 million A549 cells in MatriGel. Cells were loaded with metals by incubation for 24 hours with a metal mix for cells

detailed in Table 6. For naïve mice, cells were used without metal loading and for metal loaded mice metal loaded cells were used.

The target size for commencing treatment was 150-180 mm³ with an actual range of 120-250 mm³. Treatments constituted of doxorubicin (dox), 2 mg/kg iv twice weekly, or
5 miaDMSA, 0.3 mmol/kg iv + dox 2 mg/kg iv twice weekly. A control group receiving DMSA, 0.3 mmol/kg iv twice weekly, was also included. When dox and chelators were given to the same animal, the chelator was applied first, then the chemotherapy.

Metal mix for cells in Example 3			
Metal	Preferred salt	Name	Metal Mix 100% Target conc. (μM)
V	Na ₃ VO ₄	Sodium orthovanadate	0.34
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.43
Fe	(NH ₄) ₃ Fe ₂ (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	3.40
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	3.00
Ni	NiCl ₂	Nickel(II) chloride	8.50
Cu	CuSO ₄	Cupric sulfate pentahydrate	4.25
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	4.25
As	NaAsO ₂	Sodium (meta)arsenite	1.70
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.51
Hg	HgCl ₂	Mercury(II) chloride	2.55
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	1.70

Table 6. Metal mixture used for cell loading in Example 3.

10

Tumors were monitored three times per week by caliper measurements and volume was calculated using the formula:

$$(\text{length} \times \text{width}^2)/2.$$

15 *Results*

Doxorubicin caused a minor decrease in tumor size in naïve mice, as expected from chemotherapy, whereas it has no effect in the metal loaded mice (Figure 55 A-B). This indicates that the metal contamination induces chemoresistance as already observed in cell studies (data not shown).

20

In metal loaded mice, a chelator alone had no effect on tumor size. However, the combination of miaDMSA and dox resulted in a 30-40% reduction in tumor size as compared to control and to single treatments (Figure 55 C).

These results show that our model provides surprising insight into resistance to cancer therapy and its connection to chronic heavy metal accumulation. This is evidenced by the difference in sensitivity to doxorubicin in naïve and metal loaded animals. It was further shown that the model is instrumental in the demonstration of the role of metals in cancer therapy, and how treatment of the metal accumulation by broad-range chelators can potentiate cancer therapy. In other words, the model allows us to demonstrate how the presence of metals at elevated but sub-toxic levels in the organism can cause resistance to treatment, while the removal of such metals by suitable chelators can restore sensitivity to treatment.

10

Example 4. *In vitro* model

Materials and methods

Cell lines

MCF7, Hec-1a and Beas-2B cells were obtained from ATCC (https://www.atcc.org). Cells were typically maintained in DMEM high Glucose and 10% FCS at 37°C and 5% CO₂.

15

Metals/chelators

Metals were dissolved individually in 10mM HNO₃ and combined in the desired ratio in a metal mixture which was sterilized by filtration. Small volumes of the metal mixture were added to the incubation media to provide the desired amounts of the

20

Metals for MCF7 and Hec-1a cells			50%	100%
Metal	Preferred salt	Name	Concentration (μM)	Concentration (μM)
V	Na ₃ VO ₄	Sodium orthovanadate	0.40	0.80
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.16	0.32
Mn	MnCl ₂	Manganese(II) chloride tetrahydrate	4.00	8.00
Fe	(NH ₄) ₂ Fe ₂ (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	4.00	8.00
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	3.25	6.50
Cu	CuSO ₄	Cupric sulfate pentahydrate	2.00	4.00
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	5.00	10.00
As	NaAsO ₂	Sodium (meta)arsenite	1.25	2.50
Se	Na ₂ SeO ₃	Sodium selenite	0.80	1.60
Rb	RbCl	Rubidium chloride	30.00	60.00
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.33	0.65
Hg	HgCl ₂	Mercury(II) chloride	1.60	3.20
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	0.33	0.65

metals to the cells. An equivalent amount of NaOH was added to prevent pH fluctuations. The metal concentrations are presented in Tables 7 and 8.

Table 7. Composition of metal mix used for MCF7 and Hec-1a cells.

Metals for Beas-2b cells				
Metal	Preferred salt	Name	Metal Mix 50%	Metal Mix 100%
			Target conc. (µM)	Target conc. (µM)
V	Na ₃ VO ₄	Sodium orthovanadate	0.17	0.34
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.21	0.43
Fe	(NH ₄) _y Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	1.70	3.40
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	1.50	3.00
Ni	NiCl ₂	Nickel(II) chloride	4.25	8.50
Cu	CuSO ₄	Cupric sulfate pentahydrate	2.13	4.25
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	2.13	4.25
As	NaAsO ₂	Sodium (meta)arsenite	0.85	1.70
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.26	0.51
Hg	HgCl ₂	Mercury(II) chloride	1.28	2.55
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	0.85	1.70

Table 8. Composition of metal mix used for Beas-2b cells.

5

For investigation of the effect of serum on metal uptake in cells, MCF7 cells were exposed to the same metal mix (MMI) in either SR medium with 1% serum or Full medium with 10% serum. Additional metal mixes (MMII and MMIII) were investigated in Full medium to demonstrate the ability to compensate for the lower fractional uptake of metals. The metal mixes are presented in Table 9.

10

Metals for MCF7 cells in serum experiment (Example 4)					
Metal	Preferred salt	Name	Metal Mix I Target conc. (μM)	Metal Mix II Target conc. (μM)	Metal Mix III Target conc. (μM)
V	Na ₃ VO ₄	Sodium orthovanadate	0.34	0.51	0.68
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.43	0.64	0.85
Fe	(NH ₄) _x Fe _x (C ₆ H ₈ O ₇)	Ammonium iron(III) citrate	3.40	5.10	5.10
Co	CoCl ₂	Cobalt(II) chloride hexahydrate	3.00	3.00	3.00
Ni	NiCl ₂	Nickel(II) chloride	8.50	12.75	12.75
Cu	CuSO ₄	Cupric sulfate pentahydrate	4.25	7.44	10.63
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	4.25	6.38	6.38
As	NaAsO ₂	Sodium (meta)arsenite	1.70	2.55	2.55
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.51	1.02	1.53
Hg	HgCl ₂	Mercury(II) chloride	2.55	5.10	7.65
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	1.70	3.40	5.10

Table 9. Composition of metal mixes used for MCF7 cells in the studies of the effect of serum content in growth media on metal uptake in cells.

5 Treatments

Cells were seeded and grown overnight in SR medium. 24 hours later the medium was replaced with metal mixtures in SR medium or control SR medium (Vehicle) for another 24 hours. Subsequently, cells were washed and, where applicable, were incubated in SF medium for another 24 hours for equilibration. At each stage, cells were collected for cell count and metal assessment, using EDTA-free trypsin for cell release.

For the studies of the effect of serum levels on metal uptake, one set of cells was prepared as above whereas another was prepared the same way except that SR medium was replaced with Full medium.

15

Results - CHARACTERIZATION OF THE MODEL

1) Cell count and viability

Cell count and viability determination were performed to ensure that no overt toxicity occurred (sub-toxicity). Thus, an initial panel of metal combinations and concentrations were screened and eventually optimized to a composition that had low impact on cell count and viability. This optimization was repeated for each cell line or specific condition (e.g. duration of metal loading) employed. The data are not shown but the results are reflected in the metal mix used for each experimental set-up.

20

Notably, cell count and viability were also used for calculation of metal uptake, which is typically expressed as ng metal/ 10^5 cells.

2) Metal uptake

5 In order to determine the uptake of metals into the cells and the distinct effects of different metal mix concentrations added to the medium, cells were incubated with different concentrations of an extended metal mix for 24 hours in SR medium, washed and collected for ICP-MS assessment. In three different cell lines, metals were taken up in a dose-dependent fashion, yet each cell line had its own specific pattern of metal
10 uptake (Figure 56, representative metals shown).

It is seen that levels of toxic metals (cadmium and lead are shown as examples) are substantially increased in all cell lines and the effect is dose dependent. The uptake of essential metals (copper and zinc are shown as examples) is less consistent but still exhibits a general pattern of uptake and dose response.

15

3) Equilibration (recovery)

After loading and washing, cells were incubated in SF medium without added metals for 24 hours, washed and collected for ICP-MS assessment. The results are presented in Figure 57 and show that cells first take up key metals during the loading
20 phase and then lose some during equilibration, as loosely bound metals are washed out and equilibrated with the medium. Cell types differ in the way they take up metals but the principle of metal release is the same for different cell types.

Toxic metals are represented by arsenic, cadmium, mercury and lead and essential metals are represented by copper.

25

4) Serum content in medium

It has been shown in the past that the serum content in the incubation medium may affect the metal uptake of cells, with low serum content being associated with higher metal uptake. However, low serum content also starves cells and reduces
30 growth rates (not shown). In order to map the effects of serum content and explore the ability to compensate for lower metal uptake in high serum medium, metal content was studied in cells following incubation in medium with either 1% (SR medium) or 10% serum (Full medium). In addition, metal uptake in medium with 10% serum at different metal levels was determined.

The effect of serum on the uptake of representative metals is presented in Figure 58. It is seen that uptake of metals is lower in the Full medium than in SR medium. Different metal concentrations, as listed in Table 9, are generally well reflected in uptake which is mostly dose responsive even in 10% serum. Notably, uptake of vanadium, cadmium, arsenic (for arsenic MMII = MMIII in metal mix) and lead is dose responsive, but even the highest levels of cadmium and lead provided in Full medium do not reach the uptake levels observed in SR medium. Copper and zinc are taken up poorly in Full medium, with a weak dose response in copper (for copper MMIII > MMII > MMI in metal mix) but no dose response in zinc (for zinc MMIII = MMII > MMI in metal mix). Uptake of mercury is truncated at the highest level added (for mercury MMIII > MMII > MMI in metal mix).

Discussion

The results indicate that multi-metal loading and equilibration occurs in cells under a variety of conditions. Uptake may be affected by multiple parameters, such as the cell line used, the composition of the metal mix, its concentration, the composition of the incubation medium with special emphasis on serum content and duration of exposure. The versatility of parameters allows a broad approach that must be optimized for each set of conditions and fit the aim of the experiment. With this being said, whichever specific conditions are used, the common aim is to create a cell system that exhibits a metal overload suitable for studying aspects of the almost universal human chronic, sub-toxic metal accumulation.

Further, the results demonstrate that post-loading handling may influence the amount of metal retained in a stable fashion. Washing may be necessary for metal determination but otherwise may not be required. Similarly, equilibration has been shown to cause leeching of metals from the cells, which may in some instances weaken the model whereas in others it may facilitate a specific research aim. Thus, this *in vitro* model may be employed with multiple variations for diverse studies of the chemistry, biochemistry and biology of the multi-metal accumulation in modern humans.

Example 5. Using the *in vitro* model to study metal removal from cells

Materials and methods

Cell lines

MCF7 cells were obtained from ATCC (<https://www.atcc.org>). Cells were maintained in DMEM high Glucose and 10% FCS at 37°C and 5% CO₂.

Metals/chelators

5 Metals mixtures used are detailed in Table 10.

Metals for MCF7 cells for chelation studies			
Metal	Preferred salt	Name	Target conc. (μM)
Cr	K ₂ Cr ₂ O ₇	Potassium dichromate	0.50
Mn	MnCl ₂	Manganese(II) chloride tetrahydrate	4.00
Cu	CuSO ₄	Cupric sulfate pentahydrate	5.00
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	5.00
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.60
Hg	HgCl ₂	Mercury(II) chloride	3.00
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	2.00

Table 10. Composition of metal mix used for MCF7 cells in chelation studies.

Chelators were prepared shortly before use to avoid their degradation in solution. DMPS was dissolved in double distilled water or SR medium, British Anti Lewisite (BAL) in SR medium, and miaDMSA in 5% sodium bicarbonate, or at low concentrations, in SR medium. Chelators were added to SF medium to provide the indicated final concentrations.

15 *Treatments*

Following loading and equilibration, cells were exposed to 100 μM chelators in SF medium for 1, 4 and 24 hours. Subsequently, cells were harvested for cell count, viability and metal assessment.

20 *Results*

For demonstration of metal removal by chelators, loaded and equilibrated cells were incubated with three different chelators and cellular metal content was measured at different time points.

The results indicated that all three chelators, DMPS, BAL and miaDMSA, were capable of removing metals from the cells. Figure 59 presents results after 1 hour of

incubation with the chelators and shows toxic metals (cadmium, mercury and lead are shown as examples) (A), and essential metals, represented by copper and zinc (B). MiaDMSA was the most efficient remover of cadmium and mercury, whereas BAL was most efficient for removal of lead, closely followed by miaDMSA. The chelators also
5 removed some of the essential metals. It is seen that miaDMSA was the chelator that removed the least of the essential metals.

Discussion

The cell system, characterized in Example 4, for mimicking a metal
10 contaminated organism, such as modern humans, is shown to be a capable reporter of metal removal by chelators. Chelation, as reflected in the residual metal content of cells, is evident for both toxic and essential metals. Moreover, the model system shows differential action of various chelators, emphasizing thereby its capacity as reporting system. As is shown in the international application WO2023/229462A, cells such as a
15 tumor or tumor cell culture may become resistant towards treatment with a chemotherapeutic agent due to heavy metal accumulation. A chelating agent can be successfully employed to reverse said metal-induced resistance of a resistant cancer. See, for example, WO2023/229462A, in which it is shown that EDTA, DMPS, miaDMSA, and DMSA can reverse metal-induced resistance to cancer treatment in
20 doxorubicin treated cells.

Example 6. Using the *in vitro* model to study cancer treatment

Materials and methods

Organoids PDM41 HCM-CSHL-0094-C25; and PDM106, HCM-BROD-0008-C25
25 were obtained from ATCC (<https://www.atcc.org>). The 3D organoids were maintained in Complete Human Feeding Media (CHFM), 50% v/v L-WRN conditioned media, 500 nM A83-01 (Sigma-Aldrich, SML0788, St. Louis, MO, USA), 100 ng/mL hFGF10 (Biolegend, 559308, San Diego, CA, USA), 50 ng/mL EGF (ThermoFisher, PHG0311, Waltham, MA, USA), 0.01 μ M Gastrin 1 (Tocris, 3006, Bristol, UK), 1.25 mM N-acetylcysteine (R&D, 5619, Minneapolis, MN, USA), 10 mM nicotinamide (Sigma-Aldrich, N0636), 1 $\times$ B-27 supplement (Life Technologies, 17504-044, Carlsbad, CA, USA), 10.5 μ M Y-27632 (Sigma-Aldrich, Y0503) in DMEM-F12 (Merck, D642, Darmstadt, Germany) supplemented with 1 mM HEPES buffer (Merck, H3375) and 1 $\times$
30 antibiotic-antimycotic (ThermoFisher, 15240062).

Metals/chelators and chemotherapy

Metals were used in solution or dissolved individually in 10 mM HNO₃ as described above, and then combined in the desired ratio in a heavy metal mixture which was sterilized by filtration. Small volumes of the heavy metal mixture were added to the incubation media to provide the prescribed respective concentrations, as detailed in Table 11. An equivalent amount of NaOH was added to balance the HNO₃ and prevent pH fluctuations.

DMSA was dissolved in water; miaDMSA in water and 5% NaOH, to provide a master stock solution of 1 mM in water, and sterilized by filtration.

Metals for organoids			
Metal	Preferred salt	Name	Target conc. (μM)
Mn	MnCl ₂	Manganese(II) chloride tetrahydrate	4.00
Cu	CuSO ₄	Cupric sulfate pentahydrate	5.00
Zn	ZnCl ₂ /ZnSO ₄	Zinc chloride	5.00
Cd	CdCl ₂	Cadmium chloride hemipentahydrate	0.60
Hg	HgCl ₂	Mercury(II) chloride	3.00
Pb	(CH ₃ COO) ₂ Pb	Lead(II) acetate trihydrate	2.00

Table 11. Composition of metal mix used for pancreatic organoid studies.

Notably, organoids were grown in a metal-containing growth medium to ensure that the entire organoid was metal loaded, rather than using post-formation loading which may not reach all parts equally, due to the 3-D structure. This approach is also a more accurate model of the development of pancreatic cancer as it occurs in modern man who is almost universally contaminated with heavy metals.

3D organoid viability assays

Organoids were seeded at 5 × 10³ in 10 μL of BME (Bio-Techne) and incubated with metal solution or control medium. After three days, cells were treated with chelators at 150 μM and increasing concentrations of drugs. After five days' incubation, proliferation was measured using Cell Titre Glo (Promega, G9682), as per manufacturer's instructions. The luminescence was then read with an integration of

1.0 s and gain of 100 on a plate reader (Biotek) using GenU software. Percentage viability was calculated relative to each control condition.

Results

5 In order to determine whether organoids could serve as test models for metal-induced resistance to cancer treatment and its reversal by chelators, two distinct patient-derived organoids, PDM41 which was isolated from a primary pancreatic tumor and PDM106 which was isolated from a hepatic metastasis of pancreatic cancer, were tested with different chemotherapeutics. Results are shown for gemcitabine and
10 nab-paclitaxel.

Gemcitabine: Figure 60 demonstrates that PDM41 organoids had increased resistance to Gemcitabine in the presence of metals. Addition of chelators miaDMSA and DMSA strongly decreased the resistance to gemcitabine, particularly at lower drug concentrations (< 20 ng/ml).

15 Similarly, it may be seen in Figure 61 that the metals increased the resistance of PDM106 organoids to gemcitabine. Both chelators were able to restore sensitivity to control levels.

Nab-paclitaxel: The loading of PDM41 organoids with metals induced resistance to nab-paclitaxel, as may be seen in Figure 62. MiaDMSA resensitized the organoids to
20 the drug whereas DMSA had no effect.

 PDM106 organoids, on the other hand, displayed intrinsic resistance to nab-paclitaxel. Addition of metals did not increase the resistance to the drug and chelators were not effective at sensitizing the organoids to nab-paclitaxel as presented in Figure 63.

25

Conclusion

 Metal induced resistance to therapy, as represented by gemcitabine and nab-paclitaxel, was seen in both the PDM41 and PDM106 organoids, but in the latter it was selective in the sense that the PDM106 organoids were intrinsically resistant to
30 nab-paclitaxel and that resistance was not exacerbated by addition of metals, nor alleviated by chelators.

 It was shown that metal-induced resistance could be reversed by treatment with chelators. However, a differentiation was observed: Both DMSA and miaDMSA

reversed resistance to gemcitabine in both organoid types, whereas only miaDMSA reversed metal-induced resistance to nab-paclitaxel.

The implication of the results, as obtained from two independent populations of patient derived pancreatic cancer cells grown into 3-D organoids, is that the effects of metals and chelation may be clearly established and differentiated in the organoid model. Moreover, the clarity of results was such that it could be demonstrated that these populations had different sensitivities and resistance patterns related to certain drugs. Furthermore, the *in vitro* model allowed to differentiate between metal-associated and metal-dissociated cases of drug resistance, and between the efficacy of different chelators.

It is thus concluded that the *in vitro* 3-D model system is a sensitive research tool for investigating metal-related aspects of cancer research and, in particular, metal-associated resistance to cancer therapy and the reversal of such resistance by chelators.

General Discussion

It was observed that the metal loaded models provide a surprising way to study the involvement of background metal contamination that is common in modern man, living in an industrial society and constantly exposed to pollution in air, water and food. This background contamination is emerging as a suspected factor in the etiology of many diseases including neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, ALS and more, in some infectious diseases, in the emergence and propagation of cancer and, in particular, in cancer treatment.

The surprising findings place a spotlight on the hitherto unappreciated role of multi-metal accumulation in resistance to treatment, in particular in cancer treatment. The importance of the models described herein is that they provide a novel and unexpected means to fill the gap between preclinical and clinical studies of medicines and thereby provide both a predictive and a corrective tool for bringing new medicines to clinical studies and to the market.

In current practice, new medicines undergo extensive preclinical studies, typically in rodent models, and, if proven successful, will progress to clinical studies. However, roughly 9 of 10 drugs eventually fail in clinical trials due to excessive toxicity or poor efficacy. The cases of poor efficacy are particularly vexing as these drugs all exhibited satisfactory efficacy in preclinical models. The differences are

typically ascribed to the differences between species as a general label for reasons that are unknown. The presently described test model shows that such differences may reflect the difference between a “clean” background in models, such as mice, vs. the metal elevated state in modern man.

5 This brings into focus the current methodology where the environment and nutrition of the laboratory animals is kept meticulously clean of heavy or toxic metals such as V, Cr, As, Cd, Hg, Pb and others. This, of course, sets laboratory animals apart from real world animals or humans that accumulate contaminating metals over a lifetime.

10 Similar considerations apply to *in vitro* models that are normally kept clean of heavy and toxic metals and therefore fail to mimic the actual human counterpart it seeks to study. The methods and systems proposed herein and described in the examples provides a much more realistic model of human tumors than the “clean” *in vitro* models commonly engaged in cancer research. In particular, the demonstration
15 that the 3-D models reliably reflect metal-associated effects in cancer is an important point since they combine the simplicity of cell culture with the 3-D nature of real life tumors.

Consequently, the unexpected implication of these models is that they may be used as a preclinical discriminator between treatments that are metal sensitive and
20 those that are not. In other words, a drug candidate successful in traditional preclinical studies may be tested in the metal loaded model as well. If the efficacy of the drug candidate is lower or absent in the metal loaded model a choice may be made not to bring it into clinical trials or, alternatively, to retest it in combination with a broad-spectrum chelator. If the combination with the chelator restores efficacy fully or
25 in part, a choice may be made to enter the drug candidate into clinical trials in combination with a chelator. The studies reported above have shown that miaDMSA is a particularly efficient candidate for this purpose. This method may also be used to revisit treatments that have already failed in the clinic and give them a second chance to become an approved and beneficial medicine when combined with a chelator.

30

CLAIMS

1. A method for testing the effects of elevated heavy metal levels on the efficacy of an active compound for cancer treatment, comprising
 - 5 (i) providing an in *vivo* non-human test model having elevated heavy metal levels,
 - (ii) providing the model with a tumor graft, and
 - (iii) testing the efficacy of the active compound for cancer treatment by providing the active compound to the model, optionally wherein at least one chelating agent is
 - 10 applied in combination with the active compound.
2. The method of claim 1, whereby the in *vivo* non-human test model may be obtained by a method comprising
 - (a) providing at least one heavy metal mixture,
 - 15 (b) loading a model with the at least one heavy metal mixture,
 - (c) equilibrating the model and, optionally,
 - (d) confirming the elevated heavy metal levels and/or distribution thereof in the model.
- 20 3. The method according to claim 2, further comprising confirming that the heavy metal accumulation is sub-toxic, meaning that the model does not show symptoms of toxicity.
4. The method according to claim 3, wherein confirming the sub-toxicity of the
- 25 heavy metal accumulation comprises determining parameters selected from mortality, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof.
5. The method according claim 3 or 4, wherein sub-toxicity of the heavy metal
- 30 accumulation in the test model is confirmed when the test model does not show a clinically significant difference in parameters tested after loading and equilibrating, when compared to the parameters of the model before loading and/or compared to an unloaded control.

6. The method according to any one of claims 1-5, whereby tumor graft has elevated heavy metal levels prior to the step of tumor grafting.
- 5 7. The method according to any one of claims 2-6, wherein the loading step is performed over a certain period of time, such as once a day, several times a day, or once a week.
8. The method according to any one of claims 2-7, wherein the equilibrating step (c)
10 is performed for at least one week, preferably wherein the equilibrating step is performed for 3 to 6 weeks.
9. The method according to any one of claims 1-8, wherein the testing of the efficacy of the active compound further provides knowledge whether the active compound is
15 safe in a metal-contaminated model.
10. A method of producing a test model having elevated heavy metal levels, comprising the steps of
- (i) providing at least one heavy metal mixture,
20 (ii) loading a model with the at least one heavy metal mixture,
(iii) equilibrating the model and, optionally,
(iv) confirming the elevated heavy metal levels and/or distribution thereof in the model.
- 25 11. The method according to claim 10, wherein the test model has increased levels of heavy metals loaded by the at least one heavy metal mixture after the equilibrating step, when compared to an unloaded control and/or compared to the levels of the model prior to the loading step.
- 30 12. The method according to claim 10 or 11, further comprising confirming that the heavy metal accumulation is sub-toxic, meaning that the model does not show symptoms of toxicity.

13. The method according to claim 12, wherein the sub-toxicity of the heavy metal accumulation is confirmed when the test model does not show a biologically or clinically significant difference in general parameters selected from mortality, viability, growth rate, morphology, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof.
14. The method according to any one claims 10-13, wherein the test model is an *in vitro* or *in vivo* test model, wherein the *in vivo* test model is non-human.
15. The method according to claim 14, wherein the *in vitro* model is a cell culturing system, a tissue culture system, a coculturing system, a spheroid culture, or an organoid culture, or wherein the *in vivo* model is selected from rat, mouse, rabbit, guinea pig, zebrafish, cat, dog, chicken, non-human primate such as rhesus macaque or chimpanzee, or a combination thereof.
16. The method according to any one of claims 12-15, wherein the sub-toxicity of the heavy metal accumulation for an *in vitro* test model is confirmed when the test model does not show biologically significant differences in parameters selected from viability, growth rate, morphology, or a combination thereof, and the sub-toxicity of the heavy metal accumulation for an *in vivo* test model is confirmed when the test model does not show biologically or clinically significant differences in parameters selected from mortality, weight, weight gain, behavior, appearance, parameters of a standard clinical blood biochemistry or hematology panel, or a combination thereof.
17. The method according to any one of claims 2-16, wherein the heavy metals of the at least one heavy metal mixture are selected from manganese, nickel, cadmium, lead, mercury, arsenic, chromium, iron, zinc, cobalt, copper, vanadium, platinum, titanium, antimony, tin or a combination thereof, preferably wherein the heavy metals of the at least one heavy metal mixture are applied as heavy metal salts.

18. The method according to any one of claims 2-17, wherein the loading step is performed over a certain period of time,
for *in vivo* models, preferably wherein the loading step is performed once a day, several times a day, or once a week,
5 for *in vitro* models, preferably wherein the loading step is performed in a time span of between 30 minutes and four days, such as 4 hours, 1 day, 2 days or 3 days.
19. The method according to any one of claims 2-18, wherein the equilibrating step is performed
10 for *in vivo* models, for at least one week, preferably wherein the equilibrating step is performed for at least 3 to 6 weeks,
for *in vitro* models, for a time span of between 5 minutes and 4 days, preferably wherein the equilibrating step (iii)) is performed for 1 minute, 5 minutes, 30 minutes, 1 hour, 2 hours, 6 hours 12 hours or 24 hours.
15
20. The method according to any one of claims 10-19, wherein the method further comprises a tumor grafting step onto an *in vivo* model.
21. The method according to claim 20, wherein the step of tumor grafting is
20 performed prior to the loading step or after the equilibrating step.
22. The method according to claim 20 or 21, wherein the tumor grafting is performed after the equilibrating step of the model, wherein the tumor graft has a heavy metal accumulation prior to the step of tumor grafting.
25
23. The method according to any one of claims 2-22, wherein elevated heavy metal levels and/or distribution is confirmed by measuring the heavy metal levels via mass spectrometry (MS), such as inductively coupled plasma (ICP) MS, total reflection X-rays fluorescence (TXRF), such as TXRF using Bruker S2 PICOFAF, optical emission
30 spectroscopy (OES), such as ICP-OES, atomic emission spectroscopy (AES), such as ICP-AES, atomic absorption spectroscopy (AAS), HPLC, or any other method suitable to the skilled person or a combination thereof.

24. The method according to claim 16, wherein sub-toxicity of the heavy metal accumulation in the *in vitro* test model is confirmed when the test model does not show a difference of more than 20%, more than 15%, more than 10 % in one or more of said parameters tested, when compared to the parameters of the model before loading
5 and/or compared to an unloaded control.

25. The method according to claim 16, wherein for *in vivo* test models sub-toxicity of the heavy metal accumulation in the *in vivo* test model is confirmed when the test model does not show a clinically significant difference in all parameters tested
10 after loading and equilibrating, when compared to the parameters of the model before loading and/or compared to an unloaded control.

26. A heavy metal mixture for producing a test model having a sub-toxic heavy metal accumulation comprising heavy metals selected from manganese, cadmium,
15 lead, mercury, arsenic, chromium, iron, nickel, zinc, cobalt, copper, vanadium, platinum, titanium, antimony, tin or a combination thereof,
preferably wherein the heavy metals of the heavy metal mixture are applied as heavy metal salts.

20 27. Use of the heavy metal mixture according to claim 26 for producing a test model having a sub-toxic heavy metal accumulation.

28. A test model obtainable by the method according to any one of claims 10-25.

25 29. The test model according to claim 28, wherein the test model is an *in vitro* or *in vivo* test model, wherein the *in vivo* test model is non-human.

30. The test model according to claim 28 or 29, wherein the test model is an *in vivo* test model having a chronic sub-toxic heavy metal accumulation.

30

31. Use of the test model according to any one of claims 28-30, for studying of emergence and/or treatment of diseases and/or disorders,
preferably for studying of emergence and/or treatment of cancer, tumors, infectious diseases, inflammatory diseases and/or neurodegenerative disorders.

32. Use of the test model according to claim 31, wherein the disease or disorder is a cancer.
- 5 33. Use of the test model according to claim 31 or 32, whereby an active compound is applied to the test model,
optionally wherein at least one chelating agent is applied in combination with the active compound.
- 10 34. Use of the test model according to claim 33, wherein the active compound is an anti-cancer drug.
35. Method for determining the influence of elevated heavy metal levels on toxicity and/or efficacy of an active compound comprising applying an active compound
15 to a test model according to any one of claims 28-30, and comparing the toxicity and/or efficacy of the active compound obtained in the test model with the toxicity and/or efficacy obtained in a naïve model.
36. The method of claim 35, further comprising applying a chelating agent in
20 combination with the active compound to the test model and, optionally, also to the naïve model.
37. Use of an *in vitro* test model according to any one of claims 28-30, that is grafted on an *in vivo* test model according to any one of claims 28-30, for studying of
25 emergence and/or treatment of diseases and/or disorders,
preferably for studying of emergence and/or treatment of cancer, tumors, infectious diseases, inflammatory diseases and/or neurodegenerative disorders.
38. Use of the test model according to claim 37, wherein the disease or disorder is a
30 cancer.