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Original article

Biodistribution and predictive hepatic gene expression of intravenous iron sucrose

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ABSTRACT

Introduction: We have examined iron biodistribution and hepatic gene expression in rats following administration of the generic Iron Sucrose Azad (ISA) or the reference iron sucrose drug Venofer®. **Methods:** ISA and Venofer® were administered intravenously to normal, non-anemic, male rats at 15 mg/kg (a supra-therapeutic dose-level). To evaluate biodistribution, tissue iron levels were determined over 28 days for plasma, liver, spleen, bone marrow, heart, kidney, lung and stomach using a validated ICP-MS method. Hepatic gene expression was evaluated by microarray analysis of mRNA from samples taken 24 h after drug administration. **Results:** Iron concentration/time profiles for plasma and tissues were quantitatively similar for ISA and Venofer. Following administration, circulating iron levels briefly exceeded transferrin binding capacity and there was a transient increase in hepatic iron. Bone marrow iron levels remained elevated throughout the study. No increases in tissue iron levels were observed in the heart, stomach or lungs. Spleen iron levels increased over the course of the study in treated and control rats. Small, transient increases were recorded in the kidneys of treated rats. The effects of ISA and Venofer® on hepatic gene transcription were similar. Principal components analysis showed that there was no systematic effect of either treatment on transcriptional profiles. Only a small number of genes showed significant modulation of expression. No transcriptional pattern matches with toxicity pathways were found in the ToxFX database for either treatment. No modulation of key genes in apoptosis, inflammation or oxidative stress pathways was detected. **Discussion:** These findings demonstrated that the biodistribution of administered iron is essentially similar for Iron Sucrose Azad and Venofer®, that iron sucrose partitions predominantly into the liver, spleen and bone marrow, and that hepatic gene expression studies did not provide any evidence of toxicity in animals treated at a supra-therapeutic dose-level.

1. Introduction

Optimal treatment of anemia in patients with ailments such as chronic kidney disease requires both erythropoietin to stimulate erythropoiesis and intravenous iron to provide sufficient substrate for the synthesis of hemoglobin (Macdougall et al., 1996; Schiesser et al., 2006). Iron preparations are colloids, consisting of iron-carbohydrate nanoparticles with an iron-oxyhydroxide core (Kudasheva, Lai, Ulman, & Cowman, 2004). This formulation prevents uncontrolled release of iron into the plasma, thus facilitating uptake of the particles by the mononuclear phagocyte system for short-term storage and subsequent delivery of iron via transferrin to erythroid precursors (Geisser & Burckhardt, 2011). Although preparations such as iron sucrose have been marketed for decades and their use is well established, discussions continue as to the potential for side-effects, since free iron is bioactive and can catalyze the formation of reactive oxygen species or enhance bacterial growth (Espósito, Breuer, Slotki, & Cabantchik, 2002; Parkkinen, von Bonsdorff, Peltonen, Grönhagen-Riska, & Rosenlöf, 2000; Van Wyck, Anderson, & Johnson, 2004).

The rate of iron release from iron preparations and the subsequent handling by the mononuclear phagocyte system is dependent upon structural factors, such as the size of the nanoparticles that constitute the carrier (Danielson, 2004). Therefore, iron preparations need to be well characterized and subjected to robust manufacturing procedures to ensure product uniformity with consistent pharmacokinetics and biodistribution *in vivo*.

Although Venofer® has been well-characterized, concerns have recently been raised by non-clinical data suggesting that some generic iron sucrose preparations may exhibit different properties, leading to altered iron release rates and toxic effects (Toblli, Cao, Oliveri, & Angerosa, 2012; Toblli, Cao, Oliveri, & Angerosa, 2009a, 2009b).

Abbreviations: ISA, Iron Sucrose Azad; ICP-MS, Induced Coupled Plasma Tandem Mass Spectrometry; EMA, European Medicines Agency; CS, Transferrin Saturation; TIBC, Total Iron Binding Capacity; NaCl, Sterile isotonic saline solution; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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These reports, together with the lack of a drug-receptor interaction that would allow conventional pharmacokinetic comparison to demonstrate bioequivalence (Geisser & Burckhardt, 2011), have led the European Medicines Agency (EMA) to publish a Reflection Paper. This document states the EMA's position regarding the approach to be taken for evaluation of a generic iron preparation (European Medicines Agency, 2011). The Reflection Paper states that physicochemical characterization and pharmacokinetic comparison in humans may not be sufficient to ensure comparable safety and efficacy between a reference product and a generic, and that comparative measurement of time-dependent total iron content in a "target organ" would be necessary to demonstrate essential similarity. Various compartments are proposed, such as plasma, mononuclear phagocyte system and target tissues, which can be divided into either pharmacological or toxicological targets.

Recently, a new iron sucrose generic, Iron Sucrose Azad (ISA), has been developed and its physicochemical properties have been shown to resemble those of the reference product (Venofer®) (Meier et al., 2011). ISA has also been studied using the experimental protocol used in Toblli et al. (2012); Toblli et al. (2009a, 2009b), where it was found to lack the toxic effects attributed to other generic iron sucrose preparations and to have a profile indistinguishable from that of Venofer® (Meier et al., 2011).

Following the guidance of the Reflection Paper, we report in this article the studies that we have performed to further evaluate the properties of ISA: to assess and compare tissue distribution as required by the Reflection Paper and in addition to examine the effect on hepatic gene expression, following intravenous administration to rats.

For this work, ICP-MS, an analytical technique used for elemental determinations, was chosen as an analytical method that permits quantitation of total iron in biological matrices. An ICP-MS based bioanalytical method was formally validated for the detection of total iron in rat plasma and target tissues. This method offers the advantages of detection limit sensitivity, handling of simple or complex matrices and very little sample preparation.

The dose-level of 15 mg iron/kg body weight was chosen for the biodistribution study as it allowed clearly quantifiable differences of total iron from endogenous levels to be measured and it exceeded the therapeutic dose-level. The iron biodistribution profiles of ISA and Venofer® in plasma and target tissues were determined over a range of time-points using the validated ICP-MS analytical method. This permitted direct comparison of the two treatments for iron partitioning and time-dependent biodistribution to support the claim of essential similarity.

The Sprague-Dawley rat was chosen because it is an appropriate model for the study of iron biodistribution and is accepted by regulatory authorities for this type of study. Normal (non-anemic) rats were used, as the purpose of this work was specifically to address the Reflection Paper's issues of biodistribution, and not product efficacy.

Some recent publications have suggested that generic iron compounds could modulate the expression of genes involved in oxidative stress, inflammation and apoptosis pathways, phenomena that are known to be involved in tissue injury during chronic iron therapy (Toblli et al., 2012; Toblli et al., 2009a, 2009b). In this study, we therefore also evaluated and compared the effects of the two iron sucrose products on the hepatic gene expression profiles of these biological processes.

2. Methods

2.1. Iron sucrose preparations

Iron Sucrose Azad (Batch A1122) and the reference product, Venofer® (Batch 035001) were supplied by Azad Pharma AG, Toffen, Switzerland as injectable pharmaceutical preparations. Prior to administration, ISA and Venofer® were diluted in saline solution to obtain concentrations of 7.5 mg iron/mL. Samples of the diluted dose formulations (as administered to the animals) were sent to an independent laboratory (Swiss Technology Partners, Neuhausen, Switzerland) for determination of total iron using a validated ICP-OES (Induced Coupled Plasma - Optical Emission spectrometry) procedure. Actual concentrations were determined as 7.8 mg iron/mL for ISA and 7.25 mg iron/mL for Venofer®. The BP & USP specifications for total iron in iron sucrose preparations are 95%-105% of the labeled amount of iron, therefore the actual dose-levels administered were acceptable as they were within the specified range of variation.

2.2. Animals

Male Sprague Dawley rats, Crl CD® (SD) IGS BR, *Caesarian Obtained, Barrier Sustained-Virus Antibody Free* (COBS-VAF®), were obtained from Charles River Laboratories France, l'Arbresle, France and were acclimated to the study conditions for a period of 7 or 9 days before treatment. Rats were housed in threes or fours (according to group), in polycarbonate cages with stainless steel lids, containing autoclaved sawdust (SICSA, Alfortville, France), in a barriered rodent unit (temperature 22 ± 2 °C, relative humidity 50 ± 20%, with 12 h light/dark cycles). All rats had free access to SSNIFF R/M-H pelleted maintenance diet (SSNIFF Spezialdiäten GmbH, Soest, Germany) and to bottles containing filtered tap water. For urine collection, the animals were put into individual metabolism cages for an overnight period of at least 14 h. Rats were allocated to the groups by a computerized randomization procedure, so that the average body weight of each group was similar. At the time of administration, the animals were 7 to 8 weeks old with a mean body weight of 259 g (range: 237 to 289 g). This work was performed in an AAALAC-accredited facility.

2.3. Biodistribution and gene expression study

Three groups of male Sprague-Dawley rats were treated with ISA (48 rats), Venofer® (48 rats) or the vehicle (saline solution; 36 rats). ISA and Venofer® were administered at the dose-level of 15 mg iron/kg by a single intravenous (1-min bolus) injection in the tail vein, under a dosage volume of 2 mL/kg. The control group received the vehicle [0.9% NaCl, batch No. 1 F190, supplied by Lavoisier (Paris, France)] under the same conditions.

A clinical examination was performed on each animal once daily, at approximately the same time, throughout the study. The body weight of each animal was recorded once before allocation to a group, on the day of treatment and once a week thereafter.

Routine hematology, blood biochemistry and urinalysis samples were taken before sacrifice on days 2, 4, 7, 14 or 28.

Blood and tissues were collected from 6 control and 8 iron sucrose treated animals per time-point as follows: on day 1 (day of administration), 2, 4, 7, 14 or 28 (corresponding to approximately 6, 24, 72, 144, 312 and 648 h after administration). Rats were fasted overnight prior to sampling, with the exception of the 6-h sample. Venous blood (approximately 2 × 1 mL aliquots) was collected from the orbital sinus, under light isoflurane anesthesia. A 1 mL aliquot of blood was centrifuged and used for determination of plasma iron concentration by ICP-MS. The other 1 mL aliquot was used for determination of total serum iron level and total iron binding capacity (by colorimetric analysis using the ADVIA 1650 Biochemistry analyzer). This permitted calculation of the iron saturation coefficient of transferrin using the following formula:

$$\text{Saturation coefficient} = (\text{total serum iron}/\text{TIBC})$$

Following blood sample collection, rats were weighed, then deeply anesthetized by an intraperitoneal injection of sodium pentobarbital and perfused with 0.9% NaCl for 10 min to remove blood from the

tissues. Liver, bone marrow, heart, spleen, kidney, stomach and lung were collected for analysis by ICP-MS analysis.

2.4. Determination of plasma and tissue iron levels

The organs (bone marrow, liver, heart, spleen, kidney, lung and stomach) were solubilized by immersion in pure nitric acid (≥69%) for at least 16 h at room temperature, then the homogenates were sonicated and kept at −20 °C until analysis. The final preparations were analyzed by ICP-MS using the hydrogen mode (Agilent Technologies 7700 series) equipped with an autosampler (Agilent G3160B). The analysis (signals, ratios, concentrations, calibration curve parameters) was generated by Masshunter A.01.01 software.

The ICP-MS analytical method for the determination of total iron was validated according to the EMA guideline (Bouchard et al., 2013; European Medicines Agency, 2011). Since endogenous levels of iron are present in untreated rats, phosphate buffered saline was used as a surrogate matrix during method validation for the preparation of calibration standards. Samples of plasma, heart, liver, spleen, stomach, kidney, lung and bone marrow from untreated Sprague Dawley rats were used to prepare quality control samples.

2.5. Iron biodistribution

Iron biodistribution was characterized by the mean (tissue) concentrations obtained at each time-point. The Standard Deviation (SD) and Coefficient of Variation (CV) were calculated to assess inter-individual variability. For samples with a concentration level below the Limit of Quantification (LLOQ), the values were considered to be equal to LLOQ/2.

2.6. Evaluation of gene expression

On day 2 (time-point 24 h post administration), liver tissue samples from 3 rats in each group were processed for hepatic gene expression investigations. As maximum exposure was observed 24 h after treatment, this time-point was chosen to assess and compare the effects of ISA and Venofer® on gene transcription.

The left lateral lobe of the liver was collected after sacrifice, but before NaCl 0.9% perfusion, in an RNase-free environment (sequentially collected using RNase-free instruments). It was then cut into three fragments (5 × 5 × 5 mm), rinsed with cold RNase-free PBS, snap frozen in liquid nitrogen (less than 12 min after sacrifice) and stored at −80 °C pending RNA extraction for microarray analysis.

Total RNA was extracted, using a combined Trizol/RNeasy® method, and quantified by spectrophotometry. RNA quality control was performed by micro-capillary electrophoresis on an Agilent 2100 bioanalyzer, and an RNA Integrity Number (RIN) was determined for each sample (a RIN higher than 6.0 defined a good RNA quality sample). Total RNA was labelled using the 3′IVT protocol (Affymetrix) and hybridized on a Genechip® Rat whole genome 230 2.0 array. The array was washed and stained, then scanned using a solid-state laser scanner (GeneArray Scanner 3000). The array image scans were processed with Affymetrix Genechip® Command Console software.

The data were analyzed and expression values were pre-processed using the Robust Multichip Average method, which consists of three steps: background adjustment, quantile normalization and summarization (Log 2 transformed data). Signal distribution was investigated using box plots, and relative log expression metric box plots were used to detect possible outliers. Two analyses (principal component analysis and hierarchical clustering) were performed to assess intra- and inter-group gene expression profile variability. Transcriptional pathway modulation was analyzed using the ToxFX database and DrugMatrix® (NIEHS, North Carolina, USA). The ISA and Venofer® gene expression profiles were compared to the DrugMatrix® database generated from 638 different compounds.

Furthermore, a total of 179, 94 and 103 genes involved in oxidative stress, inflammation and apoptosis, respectively, were compiled into listings using the keywords from three sources: the Kyoto Encyclopedia of Genes and Genomes (KEGG) and NetAffx™ databases, and the Qiagen website for RT^2 qPCR products. Individual expression values, the mean expression value per group and the fold-change when compared to the control group were tabulated for each biological process (a modulation of at least 2-fold is usually used to define a significant gene modulation).

3. Results

The Reflection Paper stipulates that comparative data on the time-dependent iron content in major target organs may be used to support the claim of essential similarity for generic and reference nanoparticle iron medicinal products (European Medicines Agency, 2011). However, the Reflection Paper provides no guidance on a suitable analytical method to detect total iron in the various target tissues, or on what dose or time-points should be used, so these aspects had to be addressed prior to comparison between the distribution patterns of ISA and the reference product.

3.1. ICP-MS determination of plasma and tissue iron levels

Prior to the *in vivo* study, the suitability of the ICP-MS method for quantification of total iron levels in rat tissues was confirmed by a formal validation (Bouchard et al., 2013) (see Table 1), with determination of dynamic range, accuracy, precision, matrix effect, processed sample stability (stability of the final preparation at room temperature), short term stability (stability of iron after storage in rat liver, stomach, heart, plasma, lung, kidney, spleen and bone marrow at room temperature), freeze/thaw stability (stability of the analyte in rat liver, stomach, heart, plasma, lung, kidney, spleen and bone marrow after three or four freeze/thaw cycles at a nominal temperature of −20 °C) and long term stability (stability of the analyte in rat liver, stomach, heart, plasma, lung, kidney, spleen and bone marrow after storage at a nominal temperature of −20 °C). The method was found to be precise and accurate, allowing reliable measurement of study samples. No matrix effect was observed as the calibration lines were linear from 1000 (LLOQ) to 100000 ng/mL. Method reproducibility was shown for each matrix (liver, stomach, heart, plasma, lung, kidney, spleen and bone marrow) over three analytical sequences. The coefficient of variation was low and accuracy was good. Stability tests showed no adsorption of iron on container walls, either in glass, polypropylene or polyethylene. The absence of adsorption was demonstrated after storage in matrix at room temperature and at a nominal temperature of −20 °C, after three freeze/thaw cycles and after storage of the extracted matrix at a nominal temperature of 4 °C.

3.2. In vivo study

The dose-level of 15 mg iron/kg body weight for the *in vivo* study was selected on the basis of preliminary (range-finding) experiments that showed a clear difference in measured levels of total iron between ISA-treated and vehicle control (endogenous iron levels) rats. In the preliminary study, it was demonstrated that this dose-level was sufficient to permit the detection of induced modifications of iron concentration over the planned experimental period. The range of blood and organ collection time-points used for the *in vivo* study encompassed time-points from 6 h up to 28 days, based on literature data (Danielson, Salmonson, Derendorf, & Geisser, 1996; Food, Drug Administration-Center for Drug Evaluation & Research, 2000).

After administration of ISA or Venofer®, no mortality or clinical signs related to treatment were observed. No relevant changes in hematology, blood biochemistry or urinalysis parameters were noted for samples taken on days 2, 4, 7, 14 or 28, when comparing the

Table 1
Summary of the validation results for the distribution matrices.

Validation parameters	Liver	Heart	Plasma	Lung	Kidney	Spleen	Stomach	Bone marrow
Dynamic range	1000 to 100000 ng/mL							
Regression model	Linear $1/x^2$							
Accuracy (%RE, absolute value)	<10.8%	<10.6%	<3.9%	<3.5%	<6.0%	<12.2%	<6.7%	<1.5%
Precision (%CV)	<5.3%	<4.3%	<7.1%	<6.2%	<6.1%	<6.0%	<7.9%	<4.2%
Matrix effect (%RE,absolute value)	<7.0%	<2.8%	<7.3%	<4.5%	<2.8%	<14.0%	<8.3%	<4.7%
Processed sample stability	94 h	412 h	145 h	198 h	242 h	174 h	195 h	222 h
Short-term stability	47 h	196 h	24 h	37 h	72 h	51 h	23 h	51 h
Freeze/thaw cycles	3 cycles							
Long-term stability	218 days	143 days	217 days	118 days	126 days	172 days	157 days	105 days

% CV: coefficient of variation %, %RE: relative error, 1000 ng/mL: lower limit of quantification.

Table 2
Total iron, total iron binding capacity and transferrin saturation in serum.
(Mean values, n = 8 ISA or Venofer® treated or 6 vehicle control animals).

Parameters	Formulation	6 h (day 1)	24 h (day 2)	72 h (day 4)	144 h (day 7)	312 h (day 14)	648 h (day 28)
IRON	Vehicle	57.7	25.9	28.9	12.8	20.6	30.6
(μmol/L)	Reference	116.9**	25.8	16.0*	12.9	25.1	20.5*
	ISA	126.0**	27.4	14.7**	16.8	21.7	25.2
TIBC	Vehicle	73.3	71.3	68.7	61.6	73.3	75.5
(μmol/L)	Reference	75.9	66.5	68.2	62.2	71.5	72.5
	ISA	77.2	67.2	64.6	64.0	73.7	70.3*
SC	Vehicle	0.79	0.36	0.44	0.21	0.28	0.41
	Reference	1.54**	0.39	0.24*	0.21	0.36	0.29
	ISA	1.63**	0.41	0.23*	0.26	0.30	0.36

* $p < 0.05$.
** $p < 0.01$.

iron sucrose preparations with the vehicle or with each other (data not shown). Total serum iron concentration (as measured by colorimetric analysis using the Advia 1650 biochemistry analyzer) showed a transient 5-fold increase at 6 h after iron sucrose administration, but values returned to endogenous levels on day 2. Total iron binding capacity was similar in all groups. During the transient increase in serum iron levels, the saturation coefficient of transferrin was exceeded in both iron sucrose treated groups (as shown by values greater than one); this can be expected to result in systemic exposure of the rats to free (non-transferrin-bound) iron (See Table 2).

3.3. Biodistribution

Plasma iron concentrations measured by ICP-MS confirmed the pattern seen with serum iron measurements. Total iron values were around 5-fold above the endogenous (vehicle control) level at 6 h after iron sucrose administration. This increase was transient and levels returned to baseline at 24 h post administration and remained at baseline until the end of the study (See Fig. 1). Rapid clearance from plasma was expected, given the reported plasma half-life of 5.3 h for iron sucrose (Danielson et al., 1996).

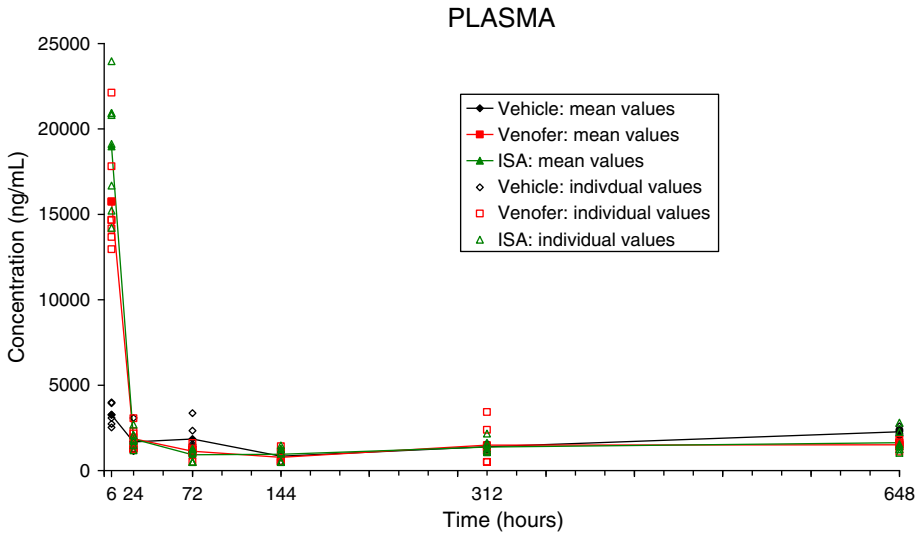

Fig. 1. Plasma iron concentrations over time. (n = 8 ISA or Venofer® treated or 6 vehicle control animals determined by ICP-MS).

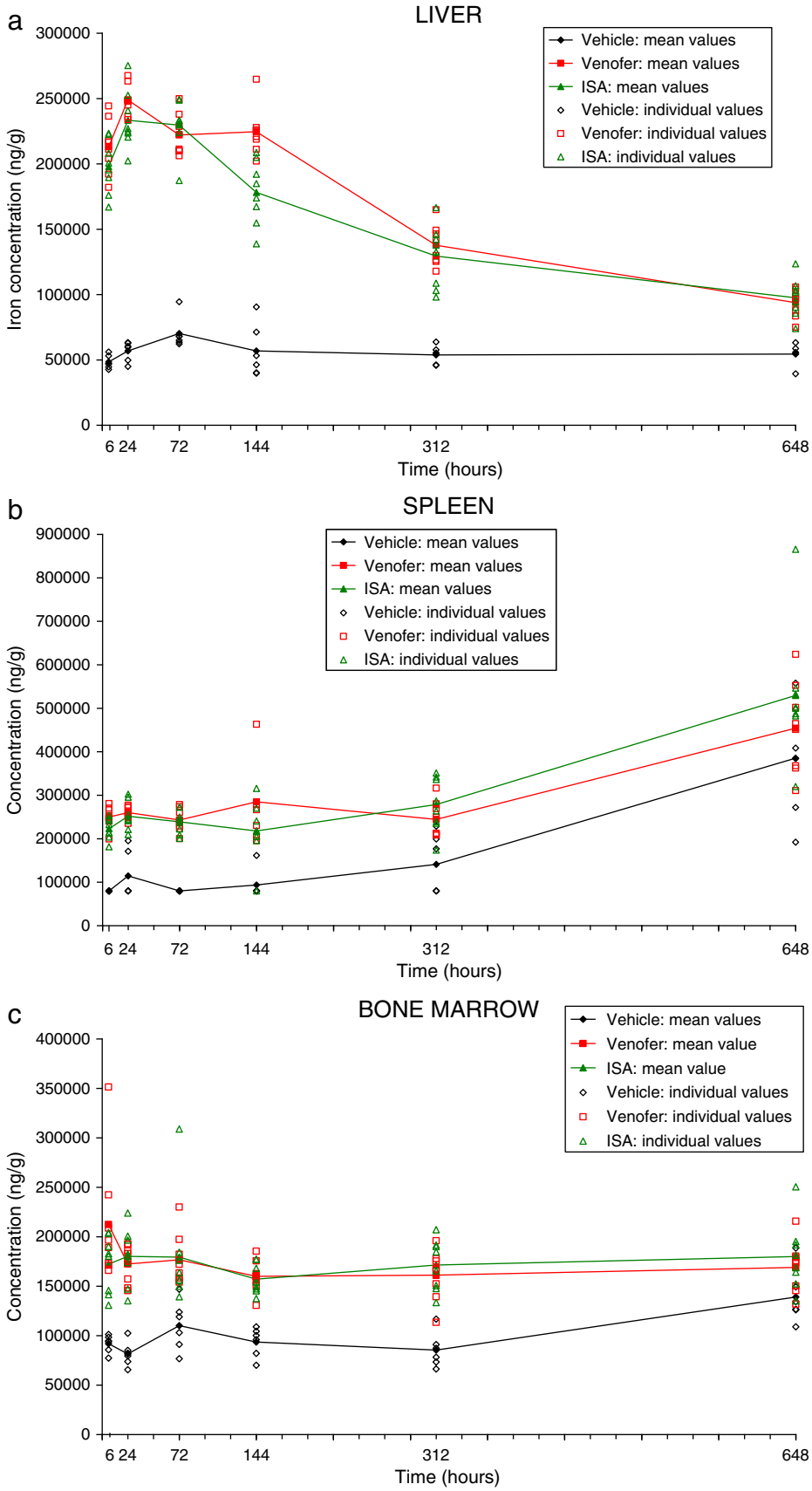

Fig. 2. a. Liver iron concentrations over time. (n = 8 ISA or Venofer® treated or 6 vehicle control animals; determined by ICP-MS). b. Spleen iron concentration over time. (n = 8 ISA or Venofer® treated or 6 vehicle control animals; determined by ICP-MS). c. Bone marrow iron concentrations over time. (n = 8 ISA or Venofer® treated or 6 vehicle control animals; determined by ICP-MS).

Analysis of the tissue samples showed that iron partitioned predominantly into the pharmacological targets, namely spleen, bone marrow and liver (See Fig. 2a, b and c).

- Spleen iron levels increased over the course of the study in all three groups including the vehicle control group. This increase was attributed to the normal physiological changes due to ageing of the rats over the course of the study. The quantitative impact of this process was much greater than the changes observed in the liver and bone marrow. Tissue levels in the spleen reached 350 to 500000 ng iron/gram, compared with levels of 250000 ng iron/gram (in liver) and 200000 ng iron/gram (in bone marrow). These figures are consistent with published results describing iron storage in the spleen in rats (Forster et al., 1994; Hegde et al., 2010)
- In both iron sucrose treated groups, bone marrow iron levels were approximately 2-fold greater than endogenous values and remained elevated at this level throughout the 28 day period of the study.
- There was a transient increase (*circa* 5-fold endogenous values) in liver iron levels in iron sucrose treated rats, over the period from administration to day 7, after which iron levels decreased gradually toward the control values.

Tissue iron analyses were also performed on four tissues that are potential targets of iron-mediated toxicity: heart, kidney, stomach and lung (see Fig. 3). No increases in tissue iron concentration and no clear differences between vehicle and treated groups were observed in the heart, stomach and lung. Small increases in tissue iron concentration were measured in the kidneys, but to a much lesser extent than for pharmacological targets.

Iron concentration/time profiles for plasma and tissues were quantitatively similar in ISA and Venofer® treated groups, thus indicating that the biodistribution of administered iron was essentially similar for these two iron sucrose preparations (see Fig. 4a and b).

3.4. Gene expression

Gene expression data from liver samples taken 24 h after iron sucrose administration were analyzed by principal component analysis (data not shown) and correlation heat map (see Fig. 5). The transcriptional profiles did not cluster according to (control or drug) treatment group in either analysis, thus showing that there was no systematic effect of ISA or Venofer® treatment on transcriptional profiles.

The number of genes for which gene expression was modulated (at $p < 0.05$) by either ISA or Venofer® was low (531 for ISA; 217 for Venofer®). This can be classified as a weak (biological) response according to the criteria of DrugMatrix® (where a weak response is defined as a maximum of 10% of genes modulated out of the 31402 genes present on the microarrays), suggesting that the two drug treatments did not induce any significant changes in biological processes. Only 33 and 38 well-annotated probes, corresponding to 30 and 33 well-annotated genes, were modulated at least 2-fold ($p < 0.05$) by Venofer® and ISA, respectively (see Table 3; figures modulated by more than 2-fold in bold type). No genes involved in iron regulation or disposition were significantly modulated by either ISA or Venofer®. Most of these genes were up-regulated and the fold inductions ranged from −2.26 to 9.31 (for Venofer®) and -3.5 to 16.71 (for ISA).

The expression pattern changes induced by ISA or Venofer® were compared to a set of gene expression signatures in the ToxFX® database which indicate effects on a range of cellular pathways involved in hepatotoxicity. Only one pathway was affected by Venofer® and two by ISA.

- The “cell-cycle G2/M transition pathway” affected by the Venofer® treatment was related to cell cycle control.
- “TGF-beta signaling” and “NF-kappaB signaling” pathways were affected by ISA treatment; in both cases this action was mediated by increased expression of IkB. This protein is responsible for binding and inactivating NF-kappaB, a key transcription factor in cellular responses to stress and inflammation. A similar effect on IkB expression was seen after treatment with Venofer®, but statistical significance was not reached.

Neither iron sucrose treatment caused a strong transcriptional response in any of the biological pathways induced by reference compounds known to cause inflammation, apoptosis or oxidative stress (such as LPS, Aflatoxin B1 or allyl alcohol). For example, caspase, interleukin and gluthathione transferase genes, described as biomarkers of these pathways, were not modulated after either Venofer® or ISA treatment.

We reviewed our data specifically for potential effects on apoptosis, inflammation and oxidative stress, using a list of relevant genes compiled from different sources (as described in the Methods section). The expression values for the listed genes are presented in Supplementary data Table S1. Only two genes involved in apoptosis (Jun and Myc) and one gene involved in oxidative stress (Fos) were affected after ISA treatment; none of these genes can be considered

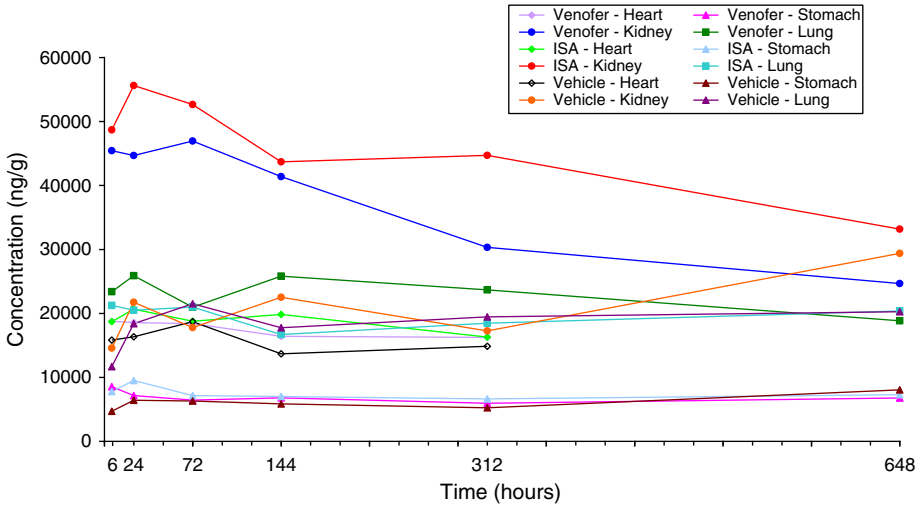

Fig. 3. Heart, kidney, stomach and lung iron concentrations over time. (n = 8 ISA or Venofer® treated or 6 vehicle control animals; determined by ICP-MS). For heart values, no data available for 648 h due to an analytical problem.

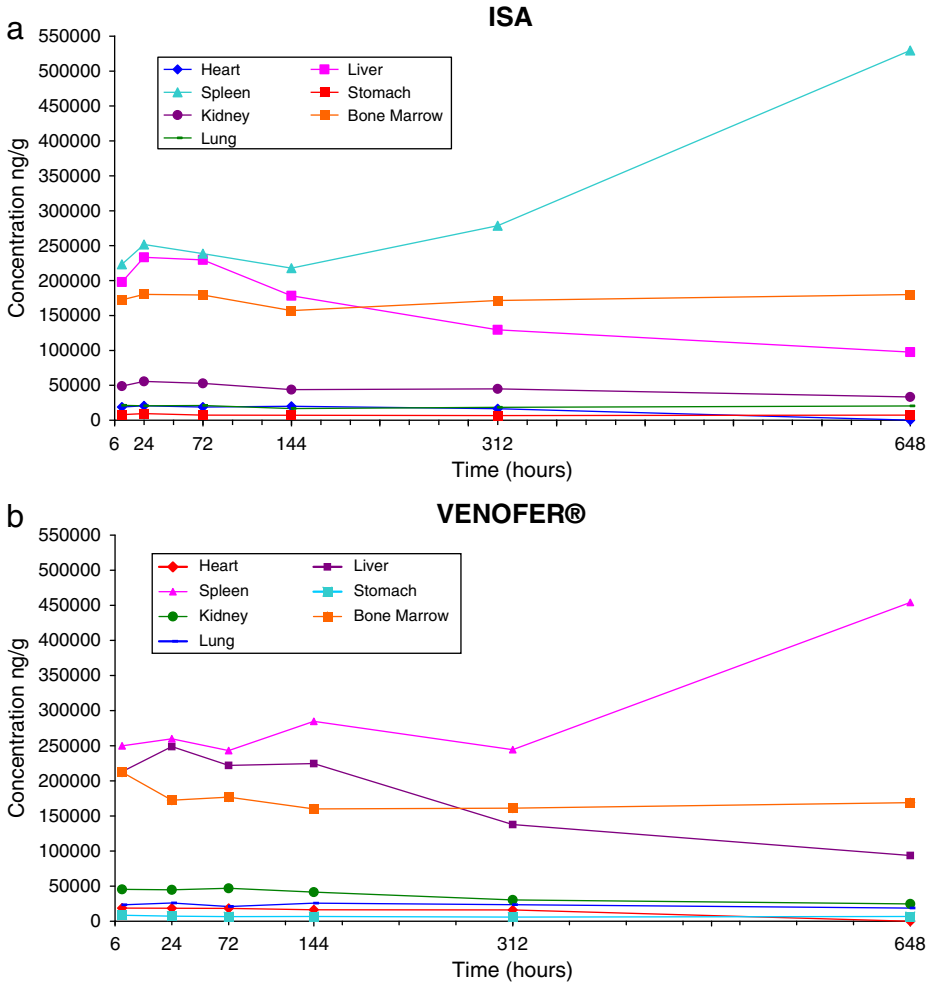

Fig. 4. a. Mean iron concentrations over time in ISA treated animals. (n = 8; determined by ICP-MS). b. Mean iron concentrations over time in Venofer® treated animals. (n = 8; determined by ICP-MS).

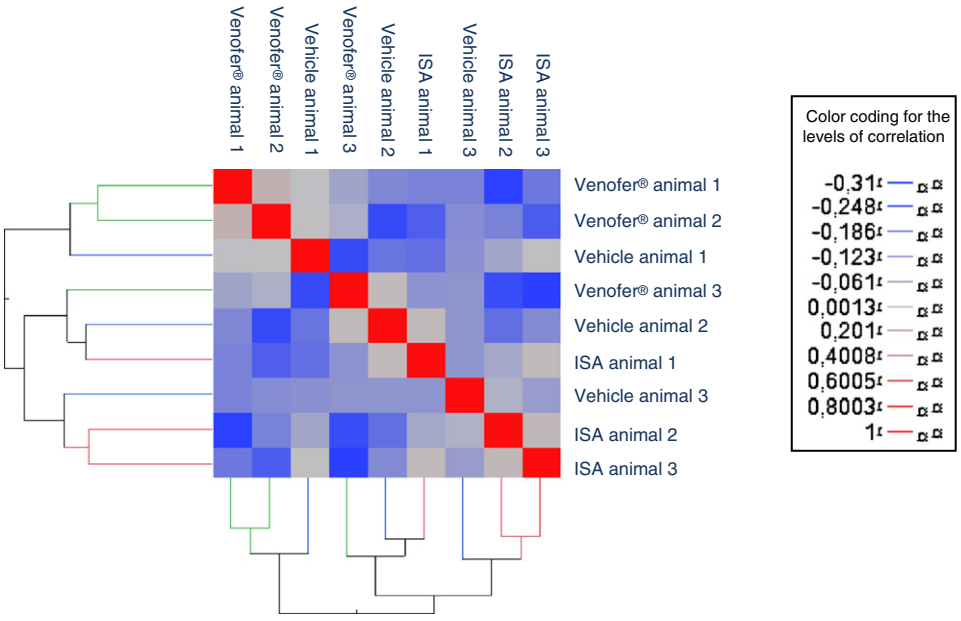

Fig. 5. Correlation heat map. This figure presents the correlation between the gene expression profiles of individual animals. The correlation coefficient is represented by the color at the intersection of two individuals. Color coding goes from red (highest) to dark blue (lowest) correlation. The correlation matrix indicates that there is no systematic effect of ISA or Venofer® on transcription profiles. The hierarchical analysis shown on the left hand and lower margins confirms this conclusion.

Table 3
Genes modulated by Venofer® or ISA at least 2 fold with p-values < 0.05 (ANOVA).

Probe Set ID	Gene symbol	Gene title	Venofer® treatment		ISA treatment	
			FC	p-value	FC	p-value
1368294_at	Dnase1l3	Deoxyribonuclease 1 like-3	−1.60	0.017	**−3.50**	0.015
1390821_at	Pcdha	Protocadherin alpha	−1.54	0.170	**−3.09**	0.18
1373233_at	Lhfpl2	Lipoma HMGICfusion partner-like 2	−1.46	0.102	**−2.06**	0.041
1389871_at	Got2	Glutamic-oxaloacetic transaminase 2, mitochondrial	−1.41	0.212	**−2.09**	0.038
1385160_at	Stab2	Stabilin 2	−1.39	0.019	**−2.94**	0.007
1393149_at	Pcdha	Protocadherin alpha	−1.34	0.289	**−2.22**	0.032
1374247_at	LOC100363145	Stabilin 1	−1.25	0.017	**−2.04**	0.008
1372868_at	Tor3a	Torsin family 3, member A	−1.11	0.602	**−2.11**	0.009
1371527_at	Emp1	Epithelial membrane protein 1	−1.04	0.777	**2.53**	0.009
1367973_at	Ccl2	Chemokine (C-C motif) ligand 2	1.07	0.801	**3.08**	0.011
1375951_at	Thbd	Thrombomodulin	1.08	0.552	**2.09**	0.037
1376632_at	Lmcd	LIM and cysteine-rich domains 1	1.08	0.589	**2.22**	0.040
1384509_s_at	Pcdh17	Protocadherin 17	1.17	0.523	**9.18**	0.000
1375138_at	Timp3	TIMP metallopeptidase inhibitor 3	1.17	0.081	**2.69**	0.003
1368505_at	Rgs4	Regulator of G-protein signaling 4	1.19	0.163	**2.44**	0.009
1370286_at	Slc38a2	Solute carrier family 38, member 2	1.23	0.189	**2.04**	0.030
1370153_at	Gdf15	Growth differentiation factor 15	1.24	0.219	**2.86**	0.001
1390687_at	Plek	Pleckstrin	1.31	0.076	**2.56**	0.002
1376100_at	Tubb6	Tubulin, beta 6	1.42	0.110	**2.21**	0.005
1371754_at	Slc25a25	Solute carrier family 25, member 25	1.45	0.070	**2.10**	0.027
1393454_at	Pcdh17	Protocadherin 17	1.46	0.058	**7.73**	0.000
1386879_at	Lgals3	Lectin, galactoside-binding, soluble, 3	1.48	0.018	**2.57**	0.000
1386969_at	Nrn1	Neuritin 1	1.59	0.054	**3.47**	0.001
1385635_at	Cd5l	Cd5 molecule-like	1.73	0.003	**2.01**	0.001
1368223_at	Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.76	0.020	**3.04**	0.001
1377869_at	Ccrn41	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	1.77	0.367	**9.51**	0.10
1395944_at	RGD1310778	Similar to putative protein C21orf45	1.89	0.064	**2.17**	0.032
1371643_at	Ccnd1	Cyclin D1	1.94	0.009	**2.38**	0.003
1384280_at	Nusap1	NUCLEOLAR and spindle associated protein 1	**2.01**	0.014	1.29	0.178
1374449_at	CDca3	Cell division cycle associated 3	**2.01**	0.001	1.41	0.090
1373722_at	kig20a	Kinesin family member 20A	**2.01**	0.015	1.31	0.227
1393581_at	Aspm	Asp (abnormal spindle) homolog, microcephaly associated (*Drosophila*)	**2.03**	0.010	1.37	0.207
1389566_at	Ccnb2	Cyclin B2	**2.03**	0.005	1.48	0.075
1370346_at	Ccnb1	Cyclin B1	**2.03**	0.013	1.39	0.236
1382419_at	Cenpk	Centromere protein K	**2.04**	0.015	1.60	0.053
1390591_at	Slc17a3	Solute carrier family 17 (sodium phosphate), member 3	**2.04**	0.038	1.88	0.028
1393848_at	Rrm2	Ribonucleotide reductase M2	**2.10**	0.020	1.47	0.069
1388484_at	Ubec2c	Ubiquitin-conjugating enzyme E2C	**2.12**	0.023	1.66	0.070
1373658_at	Racgap1	Rac GTPase-activating protein 1	**2.12**	0.020	1.35	0.195
1370345_at	Ccnb1	Cyclin B1	**2.17**	0.007	1.62	0.052
1394419_at	Arhgap11a	Rho GTpase-activating protein 11A	**2.23**	0.009	1.62	0.060
1385086_at	Bub1	Budding uninhibited by benzimidazoles 1 homolog (*S. cerevisiae*)	**2.30**	0.024	1.65	0.104
1388650_at	Top2a	Topoisomerase (DNA) II alpha	**2.32**	0.003	1.55	0.048
1368260_at	Aurkb	Aurora kinase B	**2.37**	0.018	1.47	0.107
1384068_at	Ckap2	Cytoskeleton associated protein 2	**2.45**	0.011	1.67	0.096
1389668_at	Spc25	SPC25, NDC80 kinetochore complex componentl, homolog (S. cerevisiae)	**2.61**	0.011	1.93	0.082
1388340_at	Ns5atp9	NS5A (hepatitis C virus) transactivated protein 9	**2.66**	0.005	1.86	0.025
1374775_at	Mki67	Antigen identified by monoclonal antibody Ki-67	**2.72**	0.006	1.62	0.133
1367776_at	Cdk1	Cyclin-dependent kinase 1	**2.74**	0.006	1.82	0.090
1383747_at	Ect2	Epithelial cell transforming sequence 2 oncogene	**2.88**	0.020	1.45	0.212
1372685_at	Cdkn3	Cyclin-dependent kinase inhibitor 3	**2.92**	0.016	1.71	0.091
1379582_a_at	Ccna2	Cyclin A2	**2.99**	0.011	1.76	0.105
1368826_at	Comt	Catechol-O-methyltransferase	**3.55**	0.023	1.08	0.539
1389408_at	Rrm2	Ribonucleotide reductase M2	**3.57**	0.005	**2.14**	0.075
1370902_at	Akr1b8	Aldo-keto reductase family 1, member B8	**9.31**	0.002	**16.71**	0.001
1371074_a_at	Mcm6	Minichromosome maintenance complex component 6	**2.21**	0.022	**2.42**	0.016
1388122_at	Gstp1	Glutathione S-transferase pi 1	**3.23**	0.000	**2.13**	0.005
1390112_at	Efemp1	EGF-containing fibulin-like extracellular matrix protein 1	**−2.26**	0.042	**−2.41**	0.039
1386843_at	Cd5l	Cd5 molecule-like	**2.29**	0.007	**2.22**	0.002
1371150_at	Ccnd1	Cyclin D1	**2.31**	0.015	**2.69**	0.005
1383075_at	Ccnd1	Cyclin D1	**2.42**	0.012	**2.79**	0.004
1375043_at	Fos	FBJ osteosarcoma oncogene	**2.47**	0.349	**6.00**	0.030
1387006_at	Sult2al1	Sulfotranferase family 2A, dehydroepiandrosterone (DHEA)-preferring-like 1	**2.29**	0.151	**4.77**	0.002
1368168_at	Scl34a2	Solute carrier family 34 (sodium phosphate), member 2	**2.23**	0.063	**2.72**	0.019

The numbers in bold type correspond to values where the fold count is greater than 2.

as pathognomic for apoptotic or oxidative stress changes. The increased expression of Myc and Fos genes was also induced by the Venofer® treatment. Regarding the inflammatory pathway, only two genes (Ccl2 and Cxcl10) were up-regulated after ISA treatment. Modulation of the expression of these few isolated genes is not indicative of apoptotic, inflammatory or oxidative stress processes, where extensive coordinated expression changes are observed, involving numerous genes.

Overall, the responses observed after ISA and Venofer® treatment were similar as regards the lack of a systematic effect on gene transcription, the weak response in terms of numbers of modulated genes, the lack of action on cellular pathways (as indicated by

transcriptional "signatures") and the absence of evidence of apoptotic, inflammatory or oxidative stress processes.

4. Discussion

The suggestion that conventional regulatory appraisal of generic products may not be suitable for parenteral nanoparticle products, particularly with respect to iron where *in vivo* handling is relatively complex, has led the EMA to publish a Reflection Paper on non-clinical studies for generic nanoparticle iron medicinal product applications (European Medicines Agency, 2011). As well as providing a template for pre-clinical studies to evaluate the essential similarity of such products, the scope of the Reflection Paper is also an affirmation that these products do not require the extensive in vitro and *in vivo* studies necessary to confirm the safety and efficacy of generic biologics ("biosimilars"). The Reflection Paper details which pharmacological and toxicological compartments should be assessed for total iron, but it is sparse with respect to other parameters, such as suitable analytical method, experimental period and dose-level of the iron preparation.

Although many analytical methods, *e.g.* spectrophotometry, fluorimetry and atomic absorption, have been developed for the determination of iron in biological matrices, the high sensitivity and ease of sample handling for inductively-coupled plasma mass spectrometry (ICP-MS) made it the method of choice; in this study we undertook a comprehensive validation of the ICP-MS iron quantification method for all assayed tissues, to the standards currently required by the EMA guideline (European Medicines Agency, 2011).

In the biodistribution study between ISA and Venofer®, amounts of total iron were substantially higher in the pharmacological compartments than the toxicological compartments. This pattern of iron distribution suggests that delivery of iron using intravenous iron preparations does result in delivery to pharmacological targets (bone marrow as a site for erythropoiesis, liver and spleen as iron storage sites), whilst by-passing other (toxicological) sites, except for the kidneys, where only a moderate, transient increase in iron was seen.

The behavior of the two iron sucrose preparations was very similar with respect to both the kinetic profile of iron distribution and the quantity reaching each compartment. It was interesting to note that the extent of transferrin saturation was very similar for ISA and Venofer®, since the presence of non-transferrin bound iron may be linked to the appearance of adverse events (Chandler, Harchowal, & Macdougall, 2001).

The results obtained in this comparative study, where the behavior of the two iron preparations was highly similar with respect to both the kinetic profile of iron distribution and the quantity reaching each compartment, demonstrate that according to the stipulations in the Reflection Paper, ISA and Venofer® should be viewed as essentially similar, and the findings support a previous study comparing these two preparations where no physicochemical or toxicological differences were observed (Meier et al., 2011).

In addition to reports that certain generic iron sucrose formulations induce markers of both oxidative stress and inflammation in the rat (Toblli et al., 2012; Toblli et al., 2009a, 2009b), a recent rodent immunohistochemistry study claimed that generic iron sucrose products, but not the reference product Venofer®, also induce caspase 3, a marker for apoptosis (Toblli, Cao, Giani, Dominici, & Angerosa, 2011). Therefore, in order to directly evaluate and compare the action of ISA and Venofer® on parameters of oxidative stress, inflammation and apoptosis, the induction of gene expression by iron sucrose administration was examined *ex vivo*, by determination of the hepatic gene transcription profiles in response to vehicle, ISA or Venofer® treatment. Most of the genes modulated in this study were similarly up-regulated by both treatments. As an example, Cyclin D1, involved in cellular proliferation, was induced approximately 2-fold by both Venofer and ISA; an increase in Cyclin D1 gene expression and protein expression synthesis was previously described in an iron overloaded mouse model (Moon et al., 2012; Troadec et al., 2006). No modulations of key genes in apoptosis, inflammation or oxidative stress were detected, thus indicating that the iron sucrose preparations have no effect on these pathways in normal (non-anemic) male rats treated at the dose-level of 15 mg/kg.

Many pre-clinical and clinical studies have addressed the question as to whether, and to what extent, iron-containing products may contribute toward tissue injury through induction of oxidative stress or inflammation. To our knowledge this study is the first to examine the direct effect of iron sucrose on gene transcription for proteins involved in these phenomena. In this context, no impact of iron sucrose therapy has been reported on the appearance of reactive oxygen species or the induction of leukocyte surface molecules in hemodialysis patients (Guz et al., 2006; Pai et al., 2007).

The studies in this report represent an approach to fulfill the requirements of the EMA's Reflection Paper, which stipulate that comparative data from non-clinical studies on time-dependent iron content in the major target organs (for iron) may be used to support the claim of essential similarity between a generic and reference product. This information is useful, since no attempt was made in the Reflection Paper to define a system (*e.g.* analytical method, dose/time, number of animals, *etc.*) that should be used to reliably establish and quantitate the fate of iron administered through such products. The method used in this study enabled the time-related biodistribution pattern of the generic iron sucrose preparation, ISA, to be shown as essentially similar to that of the reference product, Venofer®.

5. Conclusion

We have developed an ICP-MS bioanalytical method for sample analysis of rat plasma and tissue samples. This method can reproducibly quantitate total iron in plasma and target tissues. Using this method, together with a suitable supra-therapeutic dose-level and relevant sampling schedule, we performed a biodistribution study with the iron sucrose treatments ISA and Venofer®. Following intravenous bolus administration of ISA or Venofer®, similar partitioning profiles were obtained, predominantly into the mononuclear phagocyte system (spleen and liver) and pharmacological target tissue (bone marrow). These data support the conclusion that the time-related biodistribution profiles of ISA and Venofer® showed generic similarity. This work addressed the EMA Reflection Paper requirements for comparative data from non-clinical studies on time-dependent iron content in major target organs and supports the claim of essential similarity.

The effects of ISA and Venofer® on hepatic gene transcription were similar. Principal components analysis showed that there was no systematic effect of ISA or Venofer® treatment on transcriptional profiles. The expression of only a small number of genes was significantly affected by the drug treatments. No relevant transcriptional pattern matches with toxicity pathways were found in the ToxFX database for either the ISA or Venofer® treatment. There were no significant differences between ISA and Venofer® on the expression of genes involved in the apoptosis, inflammation or oxidative stress pathways.

Overall, the findings demonstrated that the biodistribution of administered iron is essentially similar for these two iron sucrose preparations, that iron partitions predominantly into pharmacological target tissues, and that animals treated at a supra-therapeutic dose-level show no evidence of hepatic toxicity as indicated by their gene expression profiles.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.vascn.2013.04.005.

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