The iron chelating agent, deferoxamine detoxifies Fe(Salen)-induced cytotoxicity

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Abstract

Iron-salen, i.e., μ-oxo-NN’-bis(salicylidene)ethylenediamine iron (Fe(Salen)) was a recently identified as a new anti-cancer compound with intrinsic magnetic properties. Chelation therapy has been widely used in management of metallic poisoning, because an administration of agents that bind metals can prevent potential lethal effects of particular metal. In this study, we confirmed the therapeutic effect of deferoxamine mesylate (DFO) chelation against Fe(Salen) as part of the chelator antidote efficacy. DFO administration resulted in reduced cytotoxicity and ROS generation by Fe(Salen) in cancer cells. DFO (25 mg/kg) reduced the onset of Fe(Salen) (25 mg/kg)-induced acute liver and renal dysfunction. DFO (300 mg/kg) improves survival rate after systematic injection of a fatal dose of Fe(Salen) (200 mg/kg) in mice. DFO enables the use of higher Fe(Salen) doses to treat progressive states of cancer, and it also appears to decrease the acute side effects of Fe(Salen). This makes DFO a potential antidote candidate for Fe(Salen)-based cancer treatments, and this novel strategy could be widely used in minimally-invasive clinical settings.

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1. Introduction

Chemotherapy is a key treatment for patients with cancer, together with surgery and radiotherapy. However, excess administration of anti-cancer drugs is a major problem causes adverse effects during treatment.

We previously reported that an iron-salen, i.e., \( \mu \)-oxo-N,N'-bis(salicylidene)ethylenediamine iron \((\text{Fe(Salen)})\) is a magnetic compound, that has several roles in anti-cancer activity, magnetic resonance imaging (MRI), and a drug delivery system (DDS).\(^1\) Fe(Salen) was classified as a magnetic substance that can be made to accumulate locally using a permanent magnet, and it can be heated up inside the tumor tissue under an alternating magnetic field (AMF), thereby locating and targeting a tumor in situ. In our previous study, Fe(Salen) was mostly metabolized throughout liver-gallbladder.\(^3\) Fe(Salen) injection (50 mg/kg) did not change serum hepatic enzyme (AST) and ALT in mice for 7 days. The excess Fe(Salen) induced liver dysfunction and renal function.

We have recently examined the use of intrinsic magnetic Fe(Salen) in hyper-thermic therapy, demonstrating successful targeting of tongue cancer in a rabbit model.\(^7\) In the study, we examined the combination of systemic intravenous Fe(Salen) injection, controlled drug delivery using a magnet, followed by hyper-thermic therapy using AMF. We used a rabbit model of tongue cancer because the application of magnet and AMF are relatively easy in this model. We have also reported the anti-tumor and hyperthermia-inducing effects of Fe(Salen) in human glioblastoma (GB), both in vitro and in vivo.\(^3\) Therefore, Fe(Salen) could be a potent single-drug anti-cancer agent for clinical applications.

Conversely, excess administration of iron often induces toxicities or side effect such as chronic iron overload, resulting in increased morbidity and mortality. For example, cyclophosphamide and ifosfamide are widely used antineoplastic agents, but their side-effect of hemorrhagic cystitis is still encountered and it is an important problem. Acrolein is the main molecule responsible for this side-effect and 2-mercaptoethane sulfonate (mesna) is the commonly used preventive agent. Mesna binds acrolein and prevents it from direct contact with uropoietic.\(^4\)

Suitable antidotes for Fe(Salen) are also required in clinical use. Because Fe(Salen) is an organic compound that includes iron (Fe), we focused on deferoxamine mesylate (DFO). DFO is derived from Streptomyces pilus,\(^5\) and it is a hexadentate siderophore molecule that has high affinity for Fe, resulting in formation of a stable 1:1 complex.\(^5,6\) Over the past 30 years, DFO has been a gold standard for controlling body iron which can prolong survival and prevent potential organ dysfunction, and it is generally considered safe and efficacious.\(^7\) Currently, DFO is widely used to treat Fe overload-associated disease such as thalassemia\(^5,6\) and tumors,\(^3,10\) and allergic or anaphylactic reaction to DFO are rare.\(^3,11\) DFO-Fe chelation using intra-lysosomal ferritin and ferrioxamine, allows the chelated complex to be excreted by the kidney and in the feces via the bile.\(^3\)

In this study, our aim is to develop a DFO-assisted antidote method in Fe(Salen) administration, where the iron-chelating agent binds free iron in Fe(Salen), thereby boosting the efficacy of Fe(Salen) in cancer treatments and also reducing possible side effects.

2. Materials and methods

2.1. Reagents and cell culture

DFO (\(>92.5\%\) salt was purchased from Sigma (St. Louis, MO, USA). Fe(Salen) (\(>95.0\%\)) was purchased from Tokyo Chemical Industry (Japan). Fe(Salen) was sonicated for 6 h at 4 °C, and was used in normal saline suspension. Sample preparation was conducted under ambient conditions (23 °C). Rabbit squamous cell carcinoma (VX2) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). YKG (YKG-1, JCRB0746), which are human glioblastoma malignant glioma cells, were purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank.\(^4\) OVK18, human ovarian carcinoma cells were purchased from Riken Bioresource Center, cell bank. Early passage cultures of each group of cells were stored and used for these experiments. VX2 cell lines were cultured in RPMI-1640 with l-glutamine and phenol red medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. YKG were cultured in D-MEM, containing 10% FBS and 1% penicillin–streptomycin. OVK18 were cultured in MEM, containing 10% FBS and 1% penicillin–streptomycin.

2.2. Optical properties of Fe(Salen)–DFO mixture

The Ultraviolet Visible Absorption Spectroscopy (UV–Vis) absorbance and Fourier Transform Infrared Spectroscopy (FT-IR) characteristics of Fe(Salen)–DFO mixture samples were determined using a UV–Vis spectrophotometer (Nanodrop, Fisher-Scientific) and a FT-IR spectrophotometer (JASCO FT/IR-4100) in an ATR mode, respectively.

2.3. Sodium 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT) assay

A cell proliferation assay was performed using a commercially available kit, XTT Cell Proliferation Assay Kit (ATCC) (Manassas, VA, USA) according to the manufacturer’s protocol.\(^1,3\) VX2, OVK18 and YKG cells (\(5 \times 10^3\) cells/ml, \(1 \times 10^4\) cells/ml and \(8 \times 10^3\) cells/ml, respectively) were seeded on 96-well plates. The inoculated plate was incubated at 37 °C for 2 h in a 5% CO2 humidified atmosphere. Blank control wells contained medium without Fe(Salen). Cells were incubated for 24 h, at 37 °C, and 5% CO2 and Fe(Salen) was added with or without DFO. After incubation, 50 μ of the activated–XTT solution was added to each well. The plate was returned to the incubator for 3 h. The wells containing the cells and blank controls were measured using a micro plate reader.

2.4. Measurement of reactive oxygen species

Measurement of reactive oxygen species (ROS) was performed as previously reported.\(^1,2\) The cells were plated in 96-well culture plates (\(1 \times 10^5\) cells/ml) overnight. The cells were then treated with 7.5 or 15 μM Fe(Salen) in the presence or absence of DFO at 37°C for 24 h. The intracellular ROS level was then measured using the fluorescent dye 2’,7’-dichlorofluorescin diacetate (DCFH-DA) (Sigma–Aldrich, Tokyo) as previously described. In the presence of oxidant, DCFH is converted into the highly fluorescent 2’,7’-dichlorofluorescin. The cells were first washed with PBS, and serum-free MEM containing 10 μM DCFH-DA was added to each well, and then incubated at 37°C for 40 min. ROS production was measured using a microplate reader equipped with a spectrofluorometer (PerkinElmer ARVO MX, Yokohama) at an emission wavelength of 538 nm and excitation wavelength of 485 nm.

2.5. Acute side effect of Fe(Salen) and survival experiment in mice

Animal experiments were performed according to the Yokohama City University guidelines for experimental animals. Male ICR mice (n = 50) weighting 30–38.5 g were purchased from Nihon SLC. Fe(Salen) and DFO were mixed, incubated at 37°C for 10 min, and sonicated before injection. Avertin (12.5 g/L 2,2,2-tribromoethanol, 25 ml/L, 2-methyl-2-butanol) was injected intraperitoneally for general anesthesia.

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The mice (n = 12) were then divided into three groups: intravenous saline injection (control group) and intravenous 25 mg/kg Fe(Salen) injection; and intravenous 25 mg/kg DFO injections (Fe(Salen) + DFO group). DFO and/or Fe(Salen) was injected slowly via a tail vein in all groups. Change in parameters of serum lactate dehydrogenase (LD), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine were examined before (pre), 4 and 8 h after injection in the three groups.

The mice (n = 50) were then divided into three groups: intravenous 200 mg/kg Fe(Salen) injection (Fe(Salen) group); intravenous 200 mg/kg Fe(Salen) injection and 300 mg/kg DFO injections (Fe(Salen) + DFO group); and intravenous 300 mg/kg DFO injection (DFO group). DFO and/or Fe(Salen) was injected slowly via a tail vein in all groups. The mice were immediately returned to their cages and observed for mortality over 24 h.

2.6. Statistical analysis

Data are expressed as the means ± Standard Error of the Mean (SEM). Data were analyzed using a one-way ANOVA followed by Tukey's post hoc test, except for the survival experiment in mice where the using \( \chi^2 \)-test was used. Statistical significance was set at \( p < 0.05 \). Graph-pad Prism software was used for the analysis. Statistical significance was set at \( p < 0.05 \).

2.7. Ethics approval and consent to participate

Animal experiments were performed according to the Yokohama City University guidelines for experimental animals. All experimental protocols were approved by the Animal Care and Use Committee at Yokohama City University, School of Medicine.

3. Results

3.1. Chemical chelating reaction between Fe(Salen) and DFO

To address the chemical chelating reaction between Fe(Salen) and DFO, we performed an optical analysis using UV–Vis spectroscopy (Fig. 1a). After reacting with DFO, the absorbance curve of aqueous Fe(Salen) at 490 nm that corresponded with the charge transfer characteristic between the Fe and the Salen ligand was substantially blue-shifted to 430 nm, which was proportional to the

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**Fig. 1. Chemical effect of Fe(Salen) in response to DFO.** (a) UV–Vis absorption spectral changes in the reaction of Fe(Salen) with DFO as a function of DFO concentration. The inset is a photo of Fe(Salen) solutions before and after adding DFO, showing an immediate color change from orange to light red. (b) Schematic chelating mechanism of Fe(Salen) over DFO. Detoxification of Fe(Salen) by chelation of DFO. (i) Fe(Salen), (ii) DFO, and (iii) Fe-DFO and Salen

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DFO concentration (10–40 μM), This is consistent with a previous report using the Fe complex in the presence of DFO.15 This optical shift is also correlated with the sample color change from orange to red, as shown in the photo (inset), which indicates that the DFO chelating agent formed water–soluble DFO–Fe complexes by the chelating to the Fe via the hydroxamate ligands in DFO, but not the Salen ligands (Fig. 1b).

3.2. DFO was successfully conjugated to the Fe from Fe(Salen)

To further characterize the optical properties of Fe(Salen) over DFO, FT-IR analysis was conducted (Fig. 2). For DFO alone, the bands at 2858–2931 cm⁻¹ and 2995–3004 cm⁻¹ are attributed to the asymmetric and symmetric stretching vibrations of CH₂ and N–H stretching vibrations, correspondingly. In the low wave-number region, the bands at 1053, 1200, 1460, 1566 and 1628 cm⁻¹ are result from the stretching vibration of N–O of the hydroxamate groups, the stretching vibration of C–C the bending vibration of CH₂, and the bending vibration of N–H (amine II), and stretching vibration of C=O (amine I) respectively. When comparing these spectra with those of DFO and Fe(Salen), the FTIR spectrum of the Fe(Salen)–DFO mixture is distinctive. The bending vibration of the CH₂ at approximately 1460 cm⁻¹ in DFO was also observed in the conjugates. These results confirmed that DFO was successfully conjugated to the Fe ions from Fe(Salen).

3.3. DFO decreases the magnetism of Fe(Salen)

To evaluate the DFO chelating effect on Fe(Salen) magnetism, we measured of Fe(Salen) magnetism using electron spin resonance (ESR). Fe(Salen) sample magnetism was decreased in the presence of DFO in a dose-dependent manner (Fig. 3). Additionally, Fe(Salen) magnetism with DFO was substantially weakened after reaction for 1 day, compared with that of Fe(Salen) measured immediately after adding DFO. Acid condition (pH 3.8) or alkaline condition (pH 8.9) did not affect the magnetism of the Fe(Salen) DFO mixture (Supplemental Fig. 1).

3.4. DFO negates the anti-cancer effect of Fe(Salen) in cancer

We previously reported that Fe(Salen) inhibited cell proliferation of various kinds of cancer cells including VX2 and YGK which were used in this study.1–3 To estimate the inhibitive effect of DFO over Fe(Salen) on cancer cells proliferation, XTT assay was performed using human ovarian carcinoma cells (OVK18) in addition to rabbit squamous cell carcinoma (VX2) cells and human glioblastoma cells (YKG) because OVK18 cell line is sensitive for Fe(Salen).2,3 Our previous data showed that IC₅₀ of VX2 and YKG are approximately 7.5 μM and 30 μM. IC₅₀ of OVK is also about 7.5 μM (Fig. S2). Our previous studies have showed that the sensitivity of Fe(Salen) cytotoxicity was different among diverse cancer cell lines for little understood reasons.

DFO decreased anti-cancer effect of Fe(Salen) in VX2, OVK18 and YKG cells in a dose-dependent manner (Fig. 4). DFO per se did not exhibited the anti-cancer effect (Supplemental Fig. 2). These data suggested that DFO inhibits the anti-cancer effect of Fe(Salen) in various cancer cells, by altering the anti-cancer Fe motif via chelating. Thus, DFO may be a promising candidate as a Fe(Salen) detoxicant.

![Fig. 2. FT-IR spectra of DFO, DFO–Fe(Salen), and Fe(Salen) solution. The curve signature of DFO–Fe(Salen) confirms the successful chelating-induced conjugation between DFO and Fe(Salen) drug molecules, distinct from those of the free DFO and Fe(Salen) molecules.](image)

![Fig. 3. DFO decreases the magnetism of Fe(Salen). ESR analysis of Fe(Salen) magnetism. Magnetism was evaluated using an EMX-8/2.7 ESR spectrometer (Bruker Biospin, Billerica, MA, USA) with 250 mM sonicated Fe(Salen) and with various concentrations of DFO immediately after mixing and after 1 day (n = 4, N.S., not significant, **p < 0.01, ***p < 0.001 vs. 0 mM DFO).](image)

Fig. 4. DFO negates the anti-cancer effect of Fe(Salen) in various human cancer cells. XTT assay was performed in the presence of 15 μM Fe(Salen) and 0, 5, 10, and 20 μM DFO in VX2 (rabbit squamous cell carcinoma), OVK18 (human ovarian cancer cells) and YKG (human glioma cancer cells) (n = 4, N.S., not significant, **p < 0.01, ***p < 0.001 vs. control).

Fig. 5. DFO negates ROS production in the presence of Fe(Salen) in cancer cells. DFO negated ROS production of Fe(Salen) in the presence of 7.5 or 15 μM Fe(Salen) and 0, 5, 10, and 20 μM DFO in VX2, OVK18 and YKG cells. DFO negated ROS production of Fe(Salen) in VX2, OVK18 and YKG cells (n = 4, N.S., not significant, *p < 0.05, ***p < 0.001 vs. control).
3.5. DFO negates ROS production of Fe(Salen) in cancer cells

We then examined whether DFO negates ROS production of Fe(Salen) in VX2, OVK18 and YKG. We previously reported that Fe(Salen) showed anticancer effect via ROS production.1,2 It is widely known that Fe^{2+} can react with H_{2}O_{2} to produce ROS via the Fenton reaction (Fe^{2+} + H_{2}O_{2} \rightarrow Fe^{3+} + OH^- + OH^+),16–18 which is most likely associated with the mechanism of ROS production and cytotoxicity of Fe(Salen). DFO inhibited ROS production of Fe(Salen) in various cancer cells in a dose-dependent manner (Fig. 5).

Fig. 6. DFO decreases acute side effects of Fe(Salen) in mice and improves mouse survival rates after injection of a fatal dose of Fe(Salen). (a) Examination of systematic side-effects of Fe(Salen) with/without DFO 0, 4 and 8 h after injection into mouse (n = 4, N.S., not significant, **p < 0.01, ***p < 0.001 vs. control). (b) Acute toxicity of Fe(Salen) (left, n = 20), Fe(Salen) + DFO (middle, n = 20) and DFO (right, n = 10) administered intravenously in mice.
3.6. The side effect of Fe(Salen) and DFO

We first examined a 4 week intravenous repeated dose toxicity of Fe(Salen) (10 mg/kg and 50 mg/kg) in male Sprague-Dawley rats to investigate in detail the toxic effect of the nanoparticles.

Our results showed that no deaths attributable to Fe(Salen) administration were observed in any group and no abnormal clinical signs were noted during the administration period in the 10 mg/kg and 50 mg/kg groups. During the administration period, significant suppression of body weight gain and food consumption were not observed in any groups (Table S1).

We performed hematologic, blood chemical, and histopathological examinations (Tables S2–4). In the histopathological examination, an aggregation of macrophage around brown pigment, which is assumed to be Fe(Salen), was seen in alveolar walls in the 50 mg/kg groups and was slightly seen in the 10 mg/kg (Table S4).

Fe(Salen) did not change obviously other vital organ (cerebellum, heart, kidney, liver). Under the condition of this study, these results showed that estimated toxic dose for rats was above 50 mg/kg.

We next performed the examination of systematic side-effect of DFO in mice (Fig. S4). Change in parameters of renal function (creatinine) and liver function (ALT, AST and ALP) were examined 4 h and 8 h after DFO injection (25 mg/kg). There were no differences between the control and experimental groups. Our results showed that no abnormal clinical signs attributable to DFO were noted during the administration period in the 25 mg/kg groups.

3.7. DFO decreases acute side effects of Fe(Salen) and improves mouse survival rates after administrating a fatal dose of Fe(Salen)

The ability of immobilized DFO to chelate Fe(Salen) in vivo and reduced its toxicity was assessed using a rodent model. We first examined whether DFO decreased acute side effects of Fe(Salen) in mice. We evaluated the systematic side-effect of Fe(Salen) (25 mg/kg) with/without DFO (25 mg/kg) before (pre), 4 and 8 h after injection into mice (Fig. 6a). Creatinine was significantly greater in the Fe(Salen) treated group than in the control (saline) and Fe(Salen) + DFO group 8 h after injection. Serum lactate dehydrogenase (LD), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also significantly greater in the Fe(Salen) treated group than in the control (saline) and Fe(Salen) + DFO group 4 and 8 h after injection. These results indicated that DFO decreased acute side effects of Fe(Salen).

We next evaluated the survival rate, i.e., 50% Lethal Dose (LD50), in mice 24 h after injection. The rate in the Fe(Salen) group was about 50%. Conversely, the rate was 80% in Fe(Salen) + DFO group, resulting in an increase the survival rate of 30% compared with the Fe(Salen) group (Fig. 6b). DFO did not show acute toxicity. These results suggest that DFO decreased the Fe(Salen) toxicity in vivo.

4. Discussion

Fe(Salen) is a versatile anti-cancer agent that can be delivered using a permanent magnet and visualized using MRI, and it can also produce heat upon AMF stimulation, which is beneficial for treating various cancers. However, direct use of Fe(Salen) in vivo might cause possible side effects because of its intrinsic cytotoxicity and poor solubility. As an alternative pathway to reduce possible side effects of Fe(Salen) while boosting its targeting efficacy in cancer treatment, a drug delivery system involving nanocarriers, e.g., micelles, was recently investigated. To the best of our knowledge, this is the first report regarding the use of antidotes for direct Fe(Salen) administration.

We previously reported that Fe(Salen) has a similar mechanism for its anti-cancer effect that of cisplatin. The major side effect of cisplatin is nephrotoxicity, which is a dose-limiting factor in cisplatin therapy. It has been reported that sodium thiosulfate (STS) binds to cisplatin in vitro and in vivo, resulting in a reduction of cisplatin nephrotoxicity. Similarly, Fe(Salen) also requires antidote such as DFO to be used in minimally-invasive clinical applications.

DFO was introduced in the 1960s and was used as the first iron chelator for treatment of chronic iron overload. Because DFO has long been used in clinics, much long-term data is available to support its use as a chelation agent in chronic iron overload. Additionally, deferasirox, another common iron chelator, is the most current oral chelator to be used for the management of chronic Fe overload because of its good oral bioavailability. Although there is less long-term outcome data available for deferasirox, many studies on its efficacy and safety have been reported. We also examined the effect of deferasirox, for chelating of Fe(Salen). Unfortunately, the chelating effect of deferasirox was not stronger than that of DFO at the initial examination (data not shown), and DFO was a more promising chelating agent candidate for Fe(Salen). This result is consistent with the trivalent metals’ affinity for the ligand, which generally decrease in the following sequence: hexadentate > tridentate > bidentate, because their coordination requirements are best satisfied by six donor atoms ligating to the metal center in an octahedral fashion. Moreover, the cost of deferasirox is considerably higher than that of DFO. Thus, future studies should focus on the development of more cost-effective antidotes for Fe(Salen). Smith et al. reported that an increasing dose of DFO administration caused higher iron excretion, especially in those patients who had severe iron overload. In our animal study, the survival rate was dramatically increased in the Fe(Salen) + DFO group in contrast to the control group when a higher concentration of DFO was used, suggesting that systemic adjustment of the DFO dose to reduce Fe(Salen) cytotoxicity are required in future clinical studies.

Drug repositioning is when existing drugs are used for different purpose. This is a cost-effective strategy to identify new treatments for condition, and it has been used to identify new uses for several drugs. Our study also identified DFO as a potential candidate for drug positioning, which should lead to novel clinical uses in the near future.

Cohen et al. demonstrated that intensive chelation significantly reduced liver iron stores in children within 52–83 months after DFO initiation. We reported that Fe(Salen) is a hepatic metabolism drug, and DFO can be used as a suitable antidote for Fe(Salen). Moreover, our previous report demonstrated that Fe(Salen) can induce apoptosis that occurs in response to ROS production, and in this study, DFO inhibited Fe(Salen)-induced ROS production. Further research should focus on the functional molecular mechanism of how DFO inactivates the anti-cancer effect of Fe(Salen).

5. Conclusion

Our in vitro and in vivo studies suggested that DFO is not only an efficient iron chelator compared with a cytotoxic iron-based drug, e.g., Fe(Salen), and it can also reduce potential side effects by constraining the anti-cancer effect in a modifiable fashion, enabling minimally-invasive cancer therapy.

Conflict of interest

The authors declare that they have no competing interests.

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Authors’ contributions
MU, TF, UY, SO, MS, HE and YI conceived and designed the experiments; HA, YH, HF, MU, RN, IS, MO, TA and RT performed the experiments; MU and MT analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2017.07.002

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