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Simultaneous hyperthermia-chemotherapy effect by arterial injection of Fe(Salen) for femur tumor

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We previously identified a novel nanomagnetic particle, *N*,*N*′-bis(salicylidene)ethylenediamine iron [Fe(Salen)]. Fe(Salen) not only shows antitumor effects but also magnetic properties. We found that Fe(Salen) can be used for magnet-guided drug delivery and visualization of accumulated drug by magnetic resonance imaging (MRI) because of its magnetism. In addition, Fe(Salen) can generate heat by itself when exposed to an alternating current magnetic field (AMF), resulting in a hyperthermia effect. Herein, we partly elucidated the antitumor mechanism of Fe(Salen) and carried out an i.v. repeated dose toxicity study to decide the therapeutic amount. Furthermore, we evaluated the antitumor effect of selective intra-arterial injection or i.v. injection of Fe(Salen) by catheter and the hyperthermia effect of Fe(Salen) when exposed to AMF in vivo. We used a rabbit model grafted with VX2 cells (rabbit squamous cell carcinoma) on the right leg. Intra-arterial injection of Fe(Salen) showed a greater antitumor effect than did i.v. injection. The combination of Fe(Salen) intra-arterial injection and AMF exposure showed a greater antitumor effect than did either Fe(Salen) or methotrexate (MTX) without AMF exposure, suggesting that AMF exposure greatly enhanced the antitumor effect of Fe(Salen) by arterial injection by catheter. This is the first report that the effectiveness of Fe(Salen) was evaluated in the point of administration route; that is, selective intra-arterial injection by catheter.

Umemura and Islam contributed equally to this study.

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Taken together, these results indicate a new administration route; that is, selective arterial injection of Fe(Salen) by catheter, and the development of a new strategy of simultaneous hyperthermia-chemotherapy in the future.

KEYWORDS
alternating current magnetic field, arterial injection, Fe(Salen), femur tumor, hyperthermia

1 | INTRODUCTION

Primary malignant bone tumors and metastatic bone tumors are mainly located in the proximal femur.[1] Approximately 16% of Ewing's sarcomas, 13% of chondrosarcomas and 10% of osteosarcomas form at this location.[2] Surgical resection and amputation have generally been carried out to treat patients with malignant tumors of the proximal femur. Although amputation may arrest tumor progression, it causes severe physical disability for patients. Therefore, more effective treatment instead of surgery needs to be developed for femur tumors to save patients' limbs.

Hyperthermia therapy is a type of cancer treatment in which body tissue is exposed to high temperature (up to 113°F). Hyperthermia in the range of 41°C to 45°C induces apoptosis in many cell lines.[3] Despite its effectiveness, it has not been widely used in the clinic. One reason is that it is technically difficult to increase the desirable temperature at the target tumor site only without damaging the surrounding normal tissue.

By computer analysis, we previously searched a magnetic compound in a library with more than 3 million compounds. We recently identified an iron-salen; that is, μ-oxo-*N*,*N*′- bis(salicylidene) ethylenediamine iron [Fe(Salen)] as a new antitumor organic compound with intrinsic magnetic property.[4-8] The magnetic property of Fe(Salen) enables controlled drug delivery using a permanent or electric magnet. The antitumor effect is similar to that of cisplatin. Fe(Salen) shows potent cytotoxicity, presumably through production of reactive oxygen species (ROS) that nicks DNA and then causes DNA damage.[4,6,7] However, the mechanism of Fe(Salen) needs further investigation.

We have recently examined the use of this intrinsic magnetic Fe(Salen) for hyperthermic therapy, demonstrating successful targeting of a tongue cancer in a rabbit.[6] In that study, we examined the combination of systemic i.v. Fe(Salen) injection, controlled drug delivery using magnet, and hyperthermic therapy by an alternating current magnetic field (AMF) in a rabbit model of tongue cancer because the application of magnet and AMF is relatively easy.[6] Giving i.v. Fe(Salen) per se suppressed tumor growth before magnetically guided delivery, and AMF-inducing heating was applied. Addition of these two magneto-responsive modalities further suppressed the tongue tumor.

In addition, we have also reported the antitumor and hyperthermia-inducing effects of Fe(Salen) in human glioblastoma (GB), both in vitro and in vivo.[5] The combination of local Fe(Salen) injection and AMF exposure (combined hyperthermia-chemotherapy) showed greater antitumor effects in a mouse model of GB than did either Fe(Salen) alone or carmustine (BCNU) alone. Based on these results, Fe(Salen) would be a potent, single-drug anticancer agent in future clinical applications. Moreover, we have recently reported the therapeutic effect of deferoxamine mesylate (DFO) chelation against Fe(Salen) as part of chelator antidote efficacy.[7] Giving DFO reduced cytotoxicity and ROS generation by Fe(Salen) in cancer cells and improved survival rate after systematic injection of a fatal dose of Fe(Salen) in mice.

For clinical use, we need to evaluate the cytotoxicity of Fe(Salen). Therefore, we carried out a preclinical toxicity study of Fe(Salen) nanoparticles using rat and then analyzed hematological examinations and blood chemistry tests. Furthermore, we examined the feasibility and effectiveness of combined hyperthermia-chemotherapy with catheter-guided Fe(Salen) injection into the feeding artery to treat femur tumor in a rabbit model. Because catheters can readily select feeding arteries of tumors, it is easy to expose an AMF. Our results indicate that this method is indeed promising.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell culture

μ-Oxo *N*,*N*′-bis(salicylidene)ethylenediamine iron, Fe(Salen) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Fe(Salen) was sonicated for 30 minutes and was used in suspension.[4-6] Methotrexate was purchased from Sigma-Aldrich (St Louis, MO, USA). Primary antibodies used on western blots were as follows: anti-phosphorylated-MEK1/2 (Ser 217/221) (#9121s; Cell Signaling, Danvers, MA, USA), anti-MEK1/2 (47E6) (#9126s; Cell Signaling), anti-phosphorylated-ERK1/2 (#4370s; Cell Signaling), anti-ERK1/2 (sc94; Santa Cruz Biotechnology, Dallas, TX, USA), anti-phosphorylated-STAT3 (Tyr705) (#9131S; Cell Signaling) and anti-STAT3 (#9139; Cell Signaling). Monoclonal antibody for Ki67 (Santa Cruz Biotechnology) and heat shock protein (HSP)70 (Thermo Fisher Scientific, Waltham, MA, USA) was used for immunohistochemical study using the avidin-biotin-peroxidase kit (Vector Laboratories, Burlingame, CA, USA).[4-6] Rabbit squamous cell carcinoma (VX2) cells were purchased from ATCC (Manassas, VA, USA).

2.2 | Real-time cell growth assay

In vitro cell proliferation was measured using xCELLigence (ACEA Biosciences, San Diego, CA, USA) Real-Time Cellular Analysis

system.[1] Briefly, background impedance was measured following the addition of 100 μL growth medium to 16-well E-plates. Cell suspension containing 5×10^3 VX2 was seeded into the wells. Attachment and proliferation were monitored using the xCELLigence Real-Time Cellular Analysis system. Upon reaching logarithmic phase, cells were further treated with Fe(Salen) and continuously monitored for ~100 hours. Impendence changes expressed as the cell index (CI) were automatically calculated as live cells interacting with electrodes in the E-plates, correlating with viability or cytotoxicity of cells over time. Growth curves were normalized to CI at 48 hours just before the antitumor effect of Fe(Salen).

2.3 | Western blot analysis

Western blot analysis was carried out as previously described.[9-11] VX2 cells (3×10^5 cells/60 mm petri dish) were treated with 15 μmol/L Fe(Salen) and incubated for 0.25, 1, 3 and 6 hours at 37°C with 5% CO_2 in humidified air.

2.4 | Mitochondrial test

We assumed that Fe(Salen) causes cell apoptosis through the damage of mitochondrial potential. VX2 cells were seeded in a 60-mm petri dish and then incubated overnight. Cells were then stimulated by 15 μM Fe(Salen) for 0 and 3 hours. After three PBS washes, MitoTracker Red CMXRos (Thermo Fisher Scientific) was added to each group, incubated for 45 minutes at 37°C and washed again with PBS.[12] Warm RPMI-1640$^{+/+}$ medium was added to dishes, and mitochondrial staining was quickly observed under a microscope. Staining intensity of the mitochondria was analyzed by NIS Element software (Nikon, Tokyo, Japan).

2.5 | Animals

Twenty-two male Sprague-Dawley rats (Crj:CD), 6 weeks of age, were obtained from Charles River Japan (Yokohama, Japan). The animals were acclimated to the laboratory conditions for 7 days, and 20 healthy animals were selected for use in the study at 7 weeks of age.

2.6 | Experimental design and dose level

Ten rats each were randomly allocated to the following four groups: Fe(Salen) 5 mg/kg; 25 mg/kg; 50 mg/kg; and 0.9% saline (0 mg/kg, control). Fe(Salen) or 0.9% saline was given through the coccygeal vein at an injection rate of 1 mL/kg per minute using an injection pump once daily for 7 days under unanesthetized conditions. The dosing volume was 20 mL/kg in all groups, including the control group.

2.7 | Observation

Clinical signs were assessed and recorded twice daily, before and after dosing, during the administration period. Body weight was measured twice weekly, and daily food consumption was calculated by measuring the amount of unconsumed food every 3 or 4 days.

2.8 | Animal tumor model

Male Japanese White rabbits (2.0-2.5 kg body weight; Oriental, Tokyo, Japan) were housed individually in a room with an artificial 12/12 hour light/dark cycle. The animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, the National Research Council (1996). We established the rabbit femur tumor model. Rabbits bearing squamous cell carcinoma were generated as we previously described.[6,13] Briefly, rabbits were purchased from SLC Co. (Shizuoka, Japan). Under general anesthesia, we implanted VX2 cells into the lateral quadriceps of the rabbits. The tumors were allowed to grow to a size of 50 mm in length (usually for 14 days) prior to treatment.

2.9 | Intra-arterial injection of Fe(Salen) nanoparticles or MTX

Isoflurane-oxygen gas was used for anesthesia, which was maintained for the entire surgical procedure. The incision was made at the tumor site of the right inner femur, and the right saphenous artery was exposed (Figure S1A). A 1.2-Fr funnel polyurethane catheter (Primetech, Tokyo, Japan) was inserted from the aorta at 2 cm above the bifurcation, and the distal end was advanced to the feeding artery of the tumor while checking the angiography with injection of contrast medium (Figure S1B). The proximal side of the catheter was processed underneath the skin to the lateral back and secured at the site. We also confirmed blood flow at the distal end of catheter by Doppler echo (Figure S2A). When Fe(Salen) or MTX was given through the catheter, the proximal end of the catheter was exposed. After dosage, the distal end was embedded and fixed underneath the skin (Figure S2B).

2.10 | Evaluation of antitumor and hyperthermia effects in rabbit tumor model

The rabbits were then separated into six groups (four in each group). Group 1 rabbits received no treatment (control); group 2 rabbits received i.v. Fe(Salen) injections (5 mg/kg); group 3 rabbits received intra-arterial Fe(Salen) injection (5 mg/kg); group 4 rabbits received intra-arterial MTX injections (5 mg/kg); group 5 rabbits received intra-arterial Fe(Salen) injections, followed by exposure to AMF; and group 6 rabbits were exposed to AMF without Fe(Salen) injection. MTX or Fe(Salen) was injected once daily for a week. Tumor size was measured manually for 7 days. Tumor volume was calculated as previously described by the following formula: $0.5 \times (\text{length} \times \text{width}^2)$ every day. Similarly, tumor area of the rabbit was detected by magnetic resonance imaging (MRI; TOSHIBA 1.3 T; Canon Medical Systems Corporation, Otawara, Japan) before the treatment (Figure S3).[14] The tumor tissue was then excised and histological analysis was carried out.

2.11 | Hyperthermia treatment

An alternating magnetic field (AMF) was driven by a transistor inverter (Hot Shot; Ameritherm Inc., Rochester, NY, USA) and generated by a solenoid copper coil (resistivity: 1.673×10^{-8} Ωm) with an inner diameter of 4 cm and an outer diameter of 5 cm. Experiments were carried out at a frequency of 280 kHz and a current of 335 amps.[5,6,15] The rabbit hind limb was placed into the coil of the device. AMF (30 mT) was applied for 60 minutes and repeated at 48-hour intervals for the hyperthermia group (groups 5 and 6) twice a week.

2.12 | Immunohistochemical and TUNEL staining

Immunohistochemical staining for Ki67 was done as previously described.[5,6] Ten non-overlapping fields were examined with WinROOF imaging software (Mitani, Tokyo, Japan) by a pathologist in a blinded method. Determination of apoptotic cells was made by the TUNEL technique using the Dead End Fluorometric TUNEL system (Promega, Madison, WI, USA) as previously described.[4] The nuclei of these tissues were also stained with DAPI as an indication of DNA fragmentation.

2.13 | Ethics statement

Animal experiments were carried out according to the Yokohama City University guidelines for experimental animals. The Animal Care and Use Committee at Yokohama City University, School of Medicine, approved all animal studies. All experimental protocols were approved by the Animal Care and Use Committee at Yokohama City University, School of Medicine.

2.14 | Statistical analysis

Statistical significance of the differences noted in the biochemical parameters was evaluated using the one-way analysis of variance (ANOVA) method or unpaired test, followed by Prism as a post-hoc test. For the histopathological analysis, significant differences were evaluated by the Kruskal-Wallis one-way method of variance analysis.

3 | RESULTS

3.1 | Fe(Salen) showed potent antitumor effect in rabbit squamous cancer cells

We previously reported that Fe(Salen) nanoparticles (NP) showed potent, dose-dependent antitumor effects for 24 hours in various cancer cell lines[4] and increased generation of ROS in VX2, rabbit squamous cell carcinoma.[6,7] To evaluate the antitumor effect of Fe(Salen) in the rabbit model, we first evaluated the continuous antitumor effect of Fe(Salen) in VX2 cell lines for 2 days. The result showed that Fe(Salen) significantly inhibited cell proliferation of VX2 cells in a dose-dependent method for 2 days (Figure 1A). We next evaluated the antioxidant effect of vitamin C and *N*-acetyl-cysteine (NAC), which are widely used for their antioxidant activities, against Fe(Salen)-induced ROS production because Fe(Salen) contains iron and promotes ROS production by the Fenton reaction.[4] This

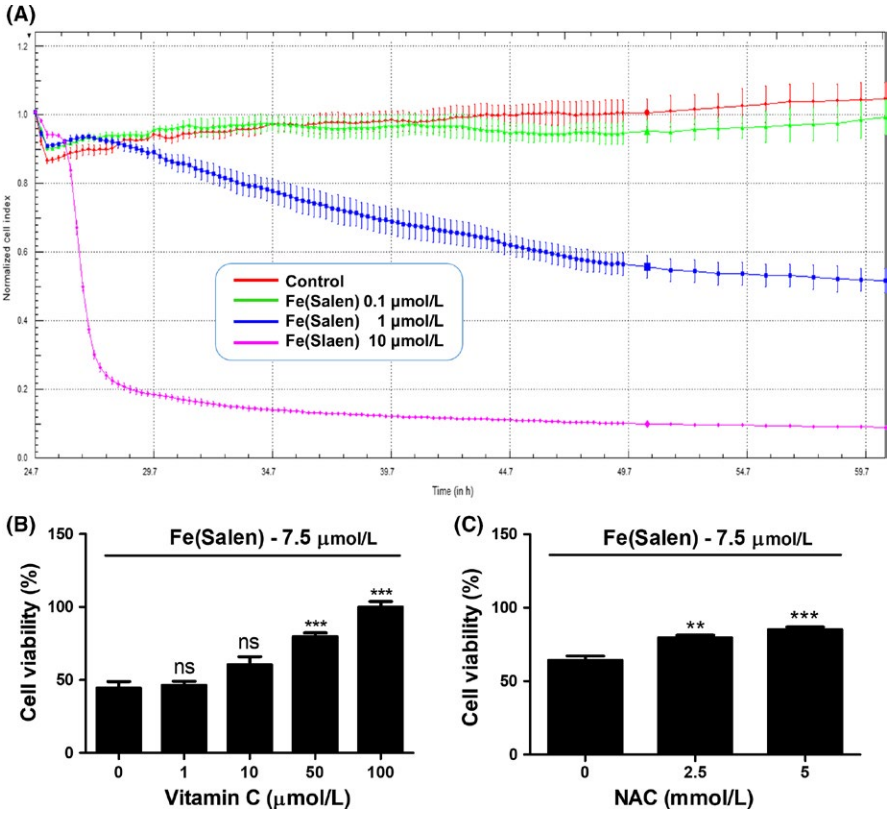

FIGURE 1 Continuous monitoring of cell growth in the presence of Fe(Salen). A, In vitro cell proliferation was measured using xCELLigence Real-Time Cellular Analysis system (ACEA Biosciences, San Diego, CA, USA). Fe(Salen) inhibited antitumor effects in VX2 cell lines in a time-dependent method. B, Effect of vitamin C on Fe(Salen)-induced cytotoxicity in VX2. Vitamin C negated Fe(Salen)-induced antitumor effects in VX2 in a dose-dependent method (n = 4; ns, not significant; ***P < .001). C, Effect of *N*-acetyl-cysteine (NAC) on Fe(Salen)-induced antitumor effects in VX2. NAC also negated Fe(Salen)-induced antitumor effect in VX2 in dose-dependent method (n = 4; **P < .01, ***P < .001)

mechanism of Fe(Salen)-induced ROS production was supported by our previous finding in cyclic voltammetry.[4] Both pretreatment vitamin C and NAC significantly negated the Fe(Salen)-induced antitumor effect in a dose-dependent way (Figure 1B,C).

3.2 | Fe(Salen) increased phosphorylation of MEK and ERK and dephosphorylation of STAT3 in VX2 cells

To examine the cellular signaling pathway, we carried out western blotting analysis. The MEK/ERK signaling pathway is known to play a major role in mammalian cell proliferation, differentiation, development, transformation and apoptosis.[16,17] Fe(Salen) phosphorylated MEK and ERK in a time-dependent method (Figure 2A,B). A previous report claimed that ROS increased the phosphorylation of ERK1/2, which ultimately caused cell apoptosis through decreasing mitochondrial membrane potential.[18] Expression of constitutively active MEK-1 increased ERK activity and inhibited STAT3$^{(Tyr705)}$ phosphorylation in a human pancreatic adenocarcinoma cell line.[19] STAT3 is an important molecule downstream of ERK1/2.[20] Therefore, we hypothesized that the phosphorylation of ERK1/2 downregulated the phosphorylation of STAT3 in the presence of Fe(Salen) in VX2 cells. STAT3 is a common signal transduction and transcription factor for anti-apoptotic proteins such as Bcl-2 and Mcl-1.[21] In the current study, Fe(Salen) dephosphorylated STAT$^{(tyr705)}$ (Figure 2A,B). Immunocytochemistry showed that Fe(Salen) decreased the protein expression of STAT3 3 and 6 hours after the stimulation compared to 0 hour in VX2 cells (Figure 2C,D). There was no significant difference in STAT3 protein expression between 3 and 6 hours.

3.3 | Vitamin C and MEK inhibitor U0126 negated Fe(Salen)-induced phosphorylation of ERK and Fe(Salen)-induced dephosphorylation of STAT3 in VX2 cells

Vitamin C negated the phosphorylation of ERK in the presence of 15 μmol/L Fe(Salen) in VX2 cells (Figure 3A,B). Vitamin C also recovered the dephosphorylation of STAT3 in the presence of 15 μmol/L

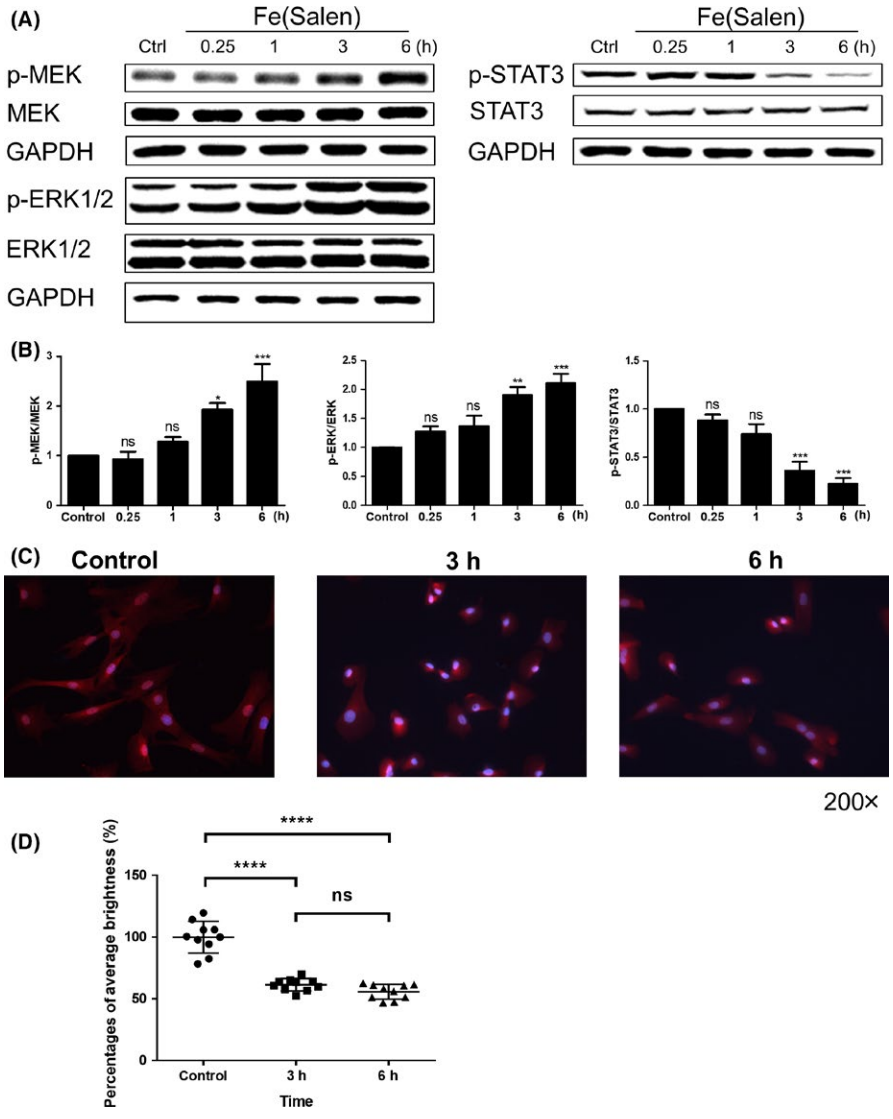

FIGURE 2 Fe(Salen) increased phosphorylation of MEK and ERK and dephosphorylation of STAT3. A, Western blot analysis of phosphorylation of MEK, ERK and STAT3 in VX2 cells stimulated by Fe(Salen) (15 μmol/L) for 6 h. B, Densitometric analysis (bar graph) of the western blot shows that Fe(Salen) increased phosphorylation of MEK and ERK and dephosphorylation of STAT3 (n = 4; ns, not significant; *P < .05, **P < .01, ***P < .001). C, Representative images of STAT3 (red) and nucleus (blue). D, Percentage of average brightness of STAT3 (n = 4; ns, not significant; ****P < 0.0001)

Fe(Salen) in VX2 cells. Furthermore, U0126, a MEK inhibitor, negated the phosphorylation of ERK in the presence of 15 μmol/L Fe(Salen) in VX2 cells (Figure 3C,D). U0126 recovered the dephosphorylation of STAT3 in the presence of 15 μmol/L Fe(Salen) in VX2 cells. Taken together, Fe(Salen) might increase phosphorylation of MEK/ERK and then suppress the phosphorylation of STAT3, resulting in cell apoptosis by downregulation of the transcription of anti-apoptotic proteins.

3.4 | Fe(Salen) decreased mitochondrial membrane potential, resulting in mitochondrial damage

As previously mentioned, ROS may play an important role in inducing an antitumor effect of Fe(Salen).[4] Therefore, we hypothesized that Fe(Salen) decreased the mitochondrial membrane potential and caused mitochondrial damage, resulting in the generation of ROS. Fe(Salen) decreased the mitochondrial membrane potential in VX2 compared with the control (Figure 4).

3.5 | Experimental design and dose level of i.v. repeated dose toxicity study of Fe(Salen) once per day for a week in male rats

To decide an optimal dose of Fe(Salen) in the animal study, we carried out a toxicity study that was designed to study the dose level of i.v. repeated injection once per day for a week. Results showed that no deaths attributable to Fe(Salen) dosage were observed in any group. No abnormal clinical signs were noted during the dosage period in the 5, 25 and 50 mg/kg groups.

During the dosage period, significant suppression of body weight gain and food consumption was not observed in any group (Figure S4). Results of hematology and blood chemistry are summarized. Hematological examination showed no significant changes in any group in hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHV) (Table S1). Blood chemistry tests showed no significant changes in any group (Table S2). An increase in lung weight was noted in the 25 and 50 mg/kg groups. In a histopathological examination, aggregation of macrophages around brown pigment, assumed to be Fe(Salen), was seen in alveolar walls and liver in all groups (Table S3 and Figure S5). These results also showed that the estimated toxic dose for rat was above 50 mg/kg.

3.6 | Alternating current magnetic field enhanced the antitumor effect of Fe(Salen) by arterial injection in rabbits

To examine the effect of an AMF in the presence of Fe(Salen) in rabbits, we established the rabbit model bearing squamous cell carcinoma in the right femur.[6] After tumors grew to approximately 50 mm in diameter, we started to inject Fe(Salen) or MTX into rabbit models by i.v. injection or intra-arterial injection by catheter with/without AMF (Figure S2b). Tumors were manually measured every day and were compared to the basal volume on the first treatment day (Figure 5A).

In control animals that received no therapy, tumor volume increased to 189.6 ± 9.6% of the basal volume for 7 days (Figure 5B).

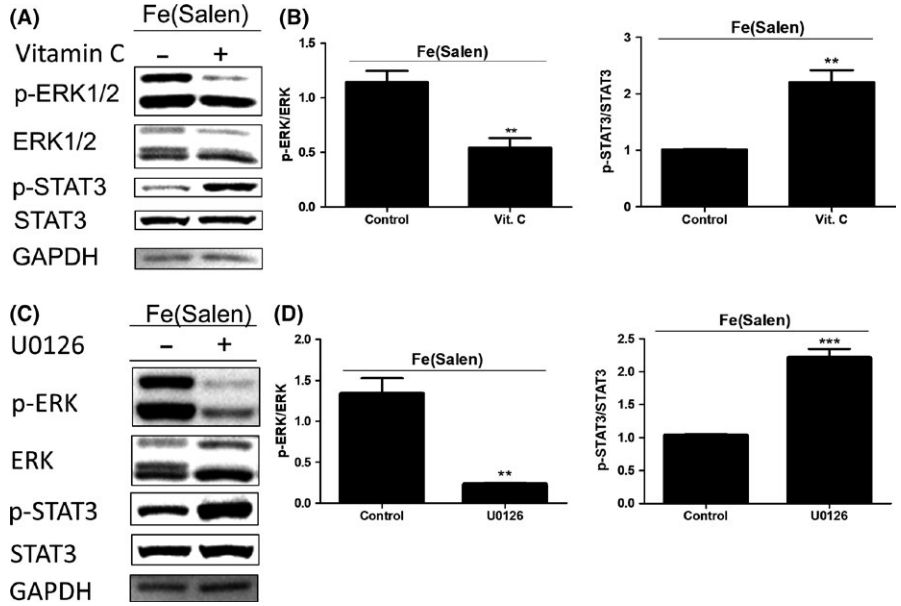

FIGURE 3 Fe(Salen) dephosphorylated STAT3 in VX2 cells. A, Western blot analysis of phosphorylation of ERK and STAT3 in VX2 cells stimulated by Fe(Salen) (15 μmol/L) and vitamin C for 6 h. B, Densitometric analysis (bar graph) of the western blot shows that vitamin C negated Fe(Salen)-induced phosphorylation of ERK and dephosphorylation of STAT3 (n = 4; $^{**}P < .01$). C, Western blot analysis of phosphorylation of ERK and STAT3 in VX2 cells stimulated by Fe(Salen) (15 μmol/L) and U0126 for 6 h. D, Densitometric analysis (bar graph) of the western blot shows that U0126 negated the Fe(Salen)-induced phosphorylation of ERK and dephosphorylation of STAT3 (n = 4; $^{**}P < .01$, $^{***}P < .001$)

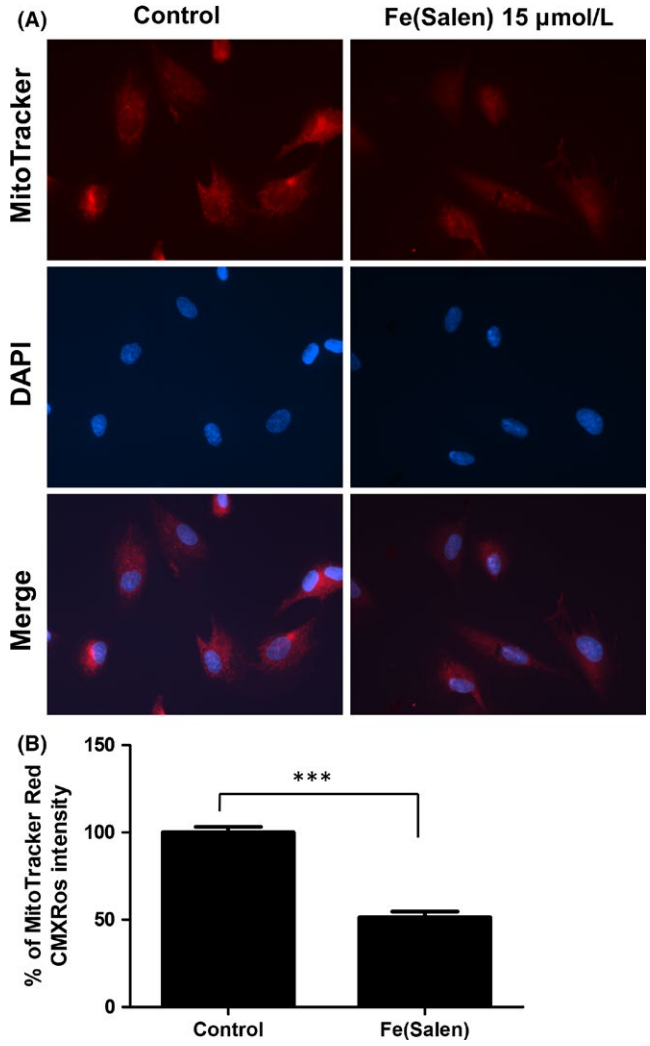

FIGURE 4 Fe(Salen) decreased mitochondrial membrane potential, resulting in mitochondrial damage. Confocal immunofluorescent analysis of VX2 cells using MitoTracker Red CMXRos (Thermo Fisher Scientific, Waltham, MA, USA). Magnification, ×20. A, Representative images of mitochondrial membrane potential. B, Densitometric quantification of the immunofluorescent analysis (n = 4; ***P < .001)

In animals that received i.v. Fe(Salen) dosage, tumor volume was smaller (135.9 ± 15.6%) than in the control. In animals that received intra-arterial MTX or Fe(Salen) injection, tumor volume was much smaller than in the control (65.9 ± 6.5% and 66.8 ± 3.3%, respectively). In animals that received intra-arterial Fe(Salen) dosage, followed by AMF, tumor volume significantly decreased to 42.9 ± 1.9%. This volume was 28% smaller than that with intra-arterial injection of Fe(Salen) only.

VX2 carcinoma is a rapidly growing tumor with an ischemic center and well-vascularized periphery, as previously reported.[22] To further explore the effect of AMF and Fe(Salen) in the rabbit model, we evaluated the pathological changes among the control group, the intra-arterial Fe(Salen) group, and the intra-arterial Fe(Salen) with AMF group. We carried out H&E staining, immunohistochemistry with Ki67 and TUNEL stain of the tumor tissue sections.

Hematoxylin and eosin staining analysis showed significant differences in the size of the necrotic area among the three groups (Figure 5C,D). In the control group, the size of the necrotic area was 56.8 ± 8.1%. In the intra-arterial Fe(Salen) group, it was 76.8 ± 3.0%. Further, in the intra-arterial Fe(Salen) with AMF group, the size of necrosis was the greatest (89.6 ± 1.6%). Median ratio of Ki67 index was 33% in the control group, 19% in the intra-arterial Fe(Salen) group, and 10% in the intra-arterial Fe(Salen) with AMF group (Figure 5C,E).

3.7 | Alternating current magnetic field increased HSP70 expression in the presence of Fe(Salen) by arterial injection in rabbits

Number of HSP70-positive cells was similar between the control and the intra-arterial Fe(Salen) group, whereas it was significantly increased in the intra-arterial Fe(Salen) with AMF group (Figure 6A,B). AMF decreased cell viability and increased heat shock protein expression. Similarly, the TUNEL staining study showed similar results to those in the HE staining study, and these results were positively correlated with each other (Figure 6).

4 | DISCUSSION

Our results indicate that chemotherapy with Fe(Salen) NP using the selective catheter and AMF-induced hyperthermia showed strong antitumor effects in a rabbit model of femur tumors. This strategy using the selective catheter and AMF showed a greater decrease in tumor size compared to the Fe(Salen) i.v. injection group, the MTX intra-arterial injection group or the Fe(Salen) intra-arterial injection group. Conventional chemotherapy drugs, including MTX, have antitumor effects, but not magnetism. In contrast, Fe(Salen) NP could be used for both antitumor and hyperthermia therapies at the focal site. This magnetism is the major advantage of Fe(Salen) compared with other conventional drugs. Furthermore, our study showed that selective arterial injection of Fe(Salen) by catheter had further antitumor effects of Fe(Salen) compared with the i.v. systematic injection that we previously reported.[4,6] Although we have reported the effect of Fe(Salen) using i.v. injection or local injection into animal models only, this is the first report showing the effectiveness of the selective catheter in their treatment.

We have previously reported that Fe(Salen) promotes ROS, resulting in DNA nicking.[4-6] Fe(Salen) also increased cytochrome *c* in the mitochondria-enriched fraction, as shown by immunoblotting, resulting in cell apoptosis.[4] However, little is known about the mechanism of the antitumor effect in Fe(Salen). In this study, we elucidated the mechanism of the Fe(Salen)-induced antitumor effect. Phosphorylation of ERK1/2 downregulated phosphorylation of STAT3 in the presence of Fe(Salen) in VX2 cells. STAT3 is a common signal transduction and transcription factor for anti-apoptotic proteins such as Bcl-2 and Mcl-1 21. Inhibition of STAT3 induced G1 arrest of the cell cycle and suppressed cell proliferation in human

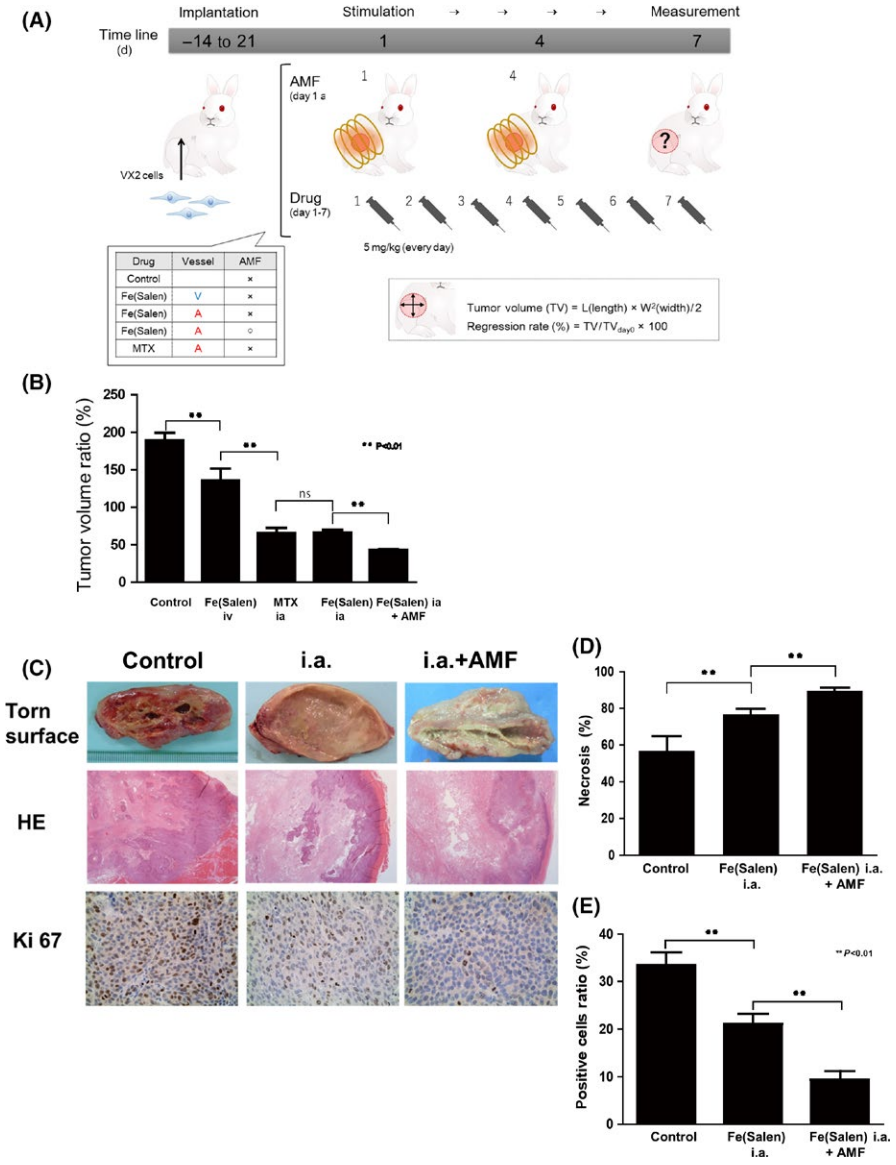

FIGURE 5 Alternating current magnetic field (AMF) enhanced the antitumor effect of Fe(Salen) by arterial injection in rabbits. A, Treatment schedule of Fe(Salen) for a rabbit model bearing squamous cell carcinoma (VX2) in the right femur. B, Change of tumor volume ratio for 7 days (n = 5, ns, not significant, $^{**}P < .01$). Each of the samples was significantly different, as shown by Kruskal-Wallis test. C, Representative pictures of torn surface in tumor (upper), HE staining (middle) and Ki67 (lower) of femur tumor. D, Quantification of necrotic area by H&E staining ($^{**}P < .01$). E, Positive ratio of Ki67 ($^{**}P < .01$). Ki67-positive cells were also counted in 3000 cells for each group, and the positive cell ratio was determined. i.a., intra-arterial; MTX, methotrexate

pancreatic adenocarcinoma cell lines.[19] Taken together, Fe(Salen) showed the antitumor effect through MEK/ERK/STAT3 signaling (Figure S6).

We have already established a reproducible protocol for synthesis of Fe(Salen) NP with consistent purity and magnetic properties in line with Good Manufacturing Practice (GMP) as recommended by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (Y. Hoshina, M. Umemura, H. Eguchi, & Y. Ishikawa, unpublished data). In future clinical use, we should use the Fe(Salen) that we synthesized with the GMP standard.

In the present study, we investigated the toxic effect of Fe(Salen) and determined the dose level of these toxic effects by repeated i.v. dose in male Sprague-Dawley rats. Results showed that the estimated toxic dose for rat was above 50 mg/kg. We previously reported that 5 mg/kg Fe(Salen) with magnet caused a robust decrease in tumor sizes in mice and rabbits.[4,6] Therefore, this result is consistent with our previous reports. Although we have previously reported the examination of systematic side-effects of Fe(Salen) and distribution of ^{14}C-Fe(Salen) in local injection into the brain, the genotoxicity study report of Fe(Salen) is not available in the study of systematic i.v. injection.[5] Therefore, this study may provide the first evidence that supports the application of future clinical studies using Fe(Salen).

In our pathological examination of the toxicity study, brown pigmentations were seen in lung and liver. These are assumed to be due to the embolism of Fe(Salen) nanoparticles because Fe(Salen) is insoluble. These results indicated that we should sonicate this nanoparticle to prevent embolism to these organs, or we should develop more suitable drug compounds, for example, micelles coated with Fe(Salen). We have previously reported spontaneous self-assembly of the water-insoluble prodrug μ-oxo-bis(*N*,*N*′-ethylenebis(salicylideniminato)iron) [Fe(Salen)] (magnetic core) with polypyrrole (PPy)-*b*-polycaprolactone (PCL) smart diblock copolymers.[8] In this system, PCL serves as a heat-responsive core scaffold, and PPy serves as an electronic core-size controller and

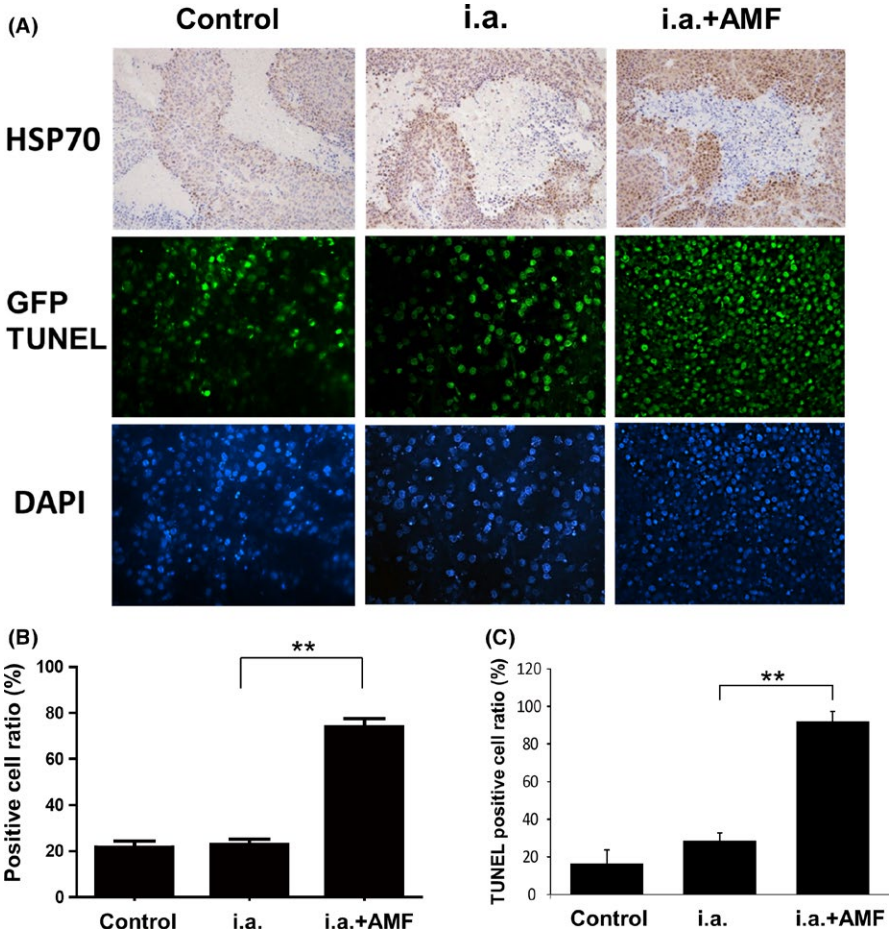

FIGURE 6 Alternating current magnetic field (AMF) increased heat shock protein (HSP)70 expression and cell apoptosis in the presence of Fe(Salen) by arterial injection in rabbits. A, Representative histological photo of HSP70 (upper), GFP TUNEL staining (middle) and DAPI (lower) of femur tumor. B, HSP70 positive cells were counted as a marker of heat stress, similarly in 3000 cells in each group (**$P < .01$). C, GFP TUNEL-positive cells were counted as a marker of apoptosis, similarly in 3000 cells in each group (**$P < .01$). i.a., intra-arterial

pH-responsive shell. We may use this micelle coated with Fe(Salen) instead of using conventional Fe(Salen) NP.

In the present study, we evaluated the effect of Fe(Salen) or MTX into rabbit models either by i.v. catheter or selective intra-arterial catheter injection. Our results showed that the effect of Fe(Salen) therapy by selective arterial injection was stronger than that by i.v. systematic injection. It was previously reported that mean concentration of anticancer agent (carboplatin) in tongue tumor was greater after selective arterial injection by the lingual artery than that by the femoral artery.[23] Therefore, we believe that more Fe(Salen) was delivered to the site of the tumor when injected intra-arterially through its feeding artery rather than when injected systemically through a vein.

We evaluated the antitumor effect of selective intra-arterial injection or i.v. injection of Fe(Salen) by catheter and the hyperthermia effect of Fe(Salen) when exposed to AMF in vivo. Combination of Fe(Salen) intra-arterial injection and AMF exposure showed a greater antitumor effect than did either Fe(Salen) or MTX without AMF exposure, suggesting that AMF exposure showed a hyperthermia effect and greatly enhanced the antitumor effect of Fe(Salen) by arterial injection by catheter because Fe(Salen) had magnetism. We should have directly measured the temperature at the site of the rabbit tumor. However, it was technically difficult to measure the temperature with our thermometer because the probe tip of our thermometer was made of metal, which, per se, generated heat upon exposure to AMF. Therefore, instead, we evaluated the protein expression of heat shock protein (HSP), which is well known to increase with temperature rise.

In the current study, we have demonstrated a new approach to inject Fe(Salen) by selective catheter to treat femur tumors. Moreover, our results indicated that hyperthermia and chemotherapy with single-drug nanoparticles could be used for femur tumor treatment.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for the present article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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