Abstract of the thesis

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

Abstract submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

March 2022

Md Rafikul Islam
Cardiovascular Research Institute
Yokohama City University Graduate School of Medicine

(Doctoral Supervisor: Yoshihiro Ishikawa, Professor)

Abstract

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

Link: https://doi.org/10.1111/cas.13851

Introduction

Primary malignant bone tumors and metastatic bone tumors are mainly located in the proximal femur (Schneiderbauer MM *et al.* 2004). Surgical resection and amputation have generally been performed to treat patients with malignant tumors of the proximal femur. Although amputation may arrest the 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 the patients' limbs.

We recently identified an iron-salen, *i.e.*, μ-oxo-*N*,*N*′- bis(salicylidene)ethylenediamine iron [Fe(Salen)] as a new antitumor organic compound with intrinsic magnetic property (Eguchi H *et al.* 2015, Ohtake M *et al.* 2017, Sato I *et al.* 2016, Umemura M *et al.* 2017, Kim JH *et al.* 2017). The magnetic property of Fe(Salen) enables controlled drug delivery using a permanent or electric magnet. Fe(Salen) exhibits potent cytotoxicity, presumably via production of reactive oxygen species (ROS) that nicks DNA and then causes DNA damage (Eguchi H *et al.* 2015, Sato I *et al.* 2016, Umemura M *et al.* 2017). However, the mechanism of Fe(Salen) needs further investigation.

In addition, we have recently examined the use of this intrinsic magnetic Fe(Salen) for hyperthermic therapy, demonstrating successful targeting of a tongue cancer in rabbit (Sato I *et al.* 2016). Intravenous administration of Fe(Salen) *per se* suppressed the tumor growth before magnetically guided delivery and AMF-inducing heating were applied. Addition of these two magneto-responsive modalities further suppressed the tongue tumor. Furthermore, we have also reported the antitumor and hyperthermia-inducing effects of Fe(Salen) in human glioblastoma (GB), both *in vitro* and *in vivo* (Ohtake M *et al.* 2017). 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.

For clinical usage, we need to evaluate the cytotoxicity of Fe(Salen). Therefore, we performed 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.

Materials and Methods

We first evaluated the *in vitro* cell proliferation to confirm the effective concentration of Fe(Salen) by using xCELLigence (ACEA Biosciences, CA, USA) Real-Time Cellular Analysis system. Cell suspension containing 5×10^3 VX2 was seeded into the wells. In the mechanistic study, we performed western blotting and immunocytochemical analysis to investigate the effect of Fe(Salen) on the cell signaling pathways as we previously described (Oda K *et al.* 2017, Umemura M *et al.* 2014, Akimoto T *et al.* 2018). VX2 cells (3×10^5 cells/40 mm petri dish) were treated with 15 μM Fe(Salen) and incubated for 0.25, 1, 3 and 6 hours at 37℃ with 5% CO2 in the humidified air. We also evaluated mitochondrial potential after stimulating VX2 cells by 15 μM Fe(Salen) for 0 and 3 hours and then staining with MitoTracker Res CMXRos (ThermoFisher Scientific) (Chazotte B 2011). The mitochondrial staining was quickly observed under a microscope. Staining intensity of the mitochondria was analyzed by NIS Element software (Nikon, Tokyo, Japan).

We partly elucidated the antitumor mechanism of Fe(Salen) and performed an intravenous repeated dose toxicity study to decide the therapeutic amount. Furthermore, we evaluated the antitumor effect of selective intra-arterial injection or intravenous injection of Fe(Salen) via a catheter and the hyperthermia effect of Fe(Salen) when exposed to AMF *in vivo*. All experimental protocols were approved by the Animal Care and Use Committee at Yokohama City University, School of Medicine. The approval number is F-A-14-111. We used a rabbit model grafted with VX2 cells (rabbit squamous cell carcinoma) on the left legs (Sato I *et al*. 2016, Yoneda T *et al*. 1985). 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. The rabbits were then separated into 6 groups (4 in each group). *Group 1* rabbits received no treatment (control); *group 2* rabbits received intravenous 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. The tumor volume was calculated as previously described by the following formula: $0.5 \times (\text{length}\times\text{width}^2)$ every day. Similarly, the tumor area of rabbit was detected by magnetic resonance imaging (MRI, TOSHIBA 1.3 T) before the treatment (Yallapu MM *et al*. 2011). The tumor tissue was then excised and subjected to immunohistological analysis for hematoxylin and eosin (HE), Ki67, HSP70, and TUNEL (Ohtake M *et al.* 2017, Sato I *et al.* 2016).

Results

Fe(Salen) significantly inhibited cell proliferation of VX2 cells in a dose-dependent manner. We next evaluated the antioxidant effect of vitamin C and N-acetyl-cysteine (NAC) against Fe(Salen)-induced VX2 cells

death (Eguchi H *et al.* 2015). Both pretreatment vitamin C and NAC significantly negated the Fe(Salen)-induced antitumor effect in a dose-dependent manner. To examine the cellular signaling pathway, we performed western blotting analysis. Fe(Salen) phosphorylated the MEK and ERK, and dephosphorylated STAT $^{(tyr705)}$ in a time-dependent manner. Moreover, vitamin C and MEK inhibitor (U0126) negated the Fe(Salen)-induce effect on the phosphorylation of ERK and STAT3 in VX2 cells. In addition, immunocytochemistry showed that Fe(Salen) decreased the protein expression of STAT3 at 3 and 6 hours after the stimulation compared to 0 hour in VX2 cells. Although, there was no significant difference in STAT3 protein expression between 3 and 6 hours. Taken together, Fe(Salen) might increase phosphorylation of MEK/ERK and then suppress the phosphorylation of STAT3, resulting in cell apoptosis via down regulation of the transcription of anti-apoptotic proteins. Moreover, Fe(Salen) decreased the mitochondrial membrane potential in VX2 compared with the control.

To decide an optimal dose of Fe(Salen) in the animal study, we performed a toxicity study in rat, which was designed to study the dose level of intravenous repeated injection once per day for a week. These results showed that no deaths attributable to Fe(Salen) administration were observed in any group. No abnormal clinical signs were noted during the administration period in the 5, 25 mg/kg and 50 mg/kg groups. In a histopathological examination, aggregation of macrophage around brown pigment, assumed to be Fe(Salen), was seen in alveolar walls and liver in all groups. These results also showed that the estimated toxic dose for rat was above 50 mg/kg.

To examine the effect of an alternating current magnetic field (AMF) in the presence of Fe(Salen) in rabbits, we established the rabbit model bearing squamous cell carcinoma in the right femur (Ohtake M *et al.* 2017). Intra-arterial injection of Fe(Salen) exhibited a more significant antitumor effect than did the intravenous injection. Furthermore, the combination of Fe(Salen) intra-arterial injection and AMF exposure showed a more significant 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) via arterial injection by catheter. HE staining analysis demonstrated the significant differences in the size of the necrotic area among the 3 groups. 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%). The 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. The number of HSP70 positive cells was similar between the control and the intra-arterial Fe(Salen) group, while it was significantly increased in the intra-arterial Fe(Salen) with AMF group. AMF decreased the cell viability and increased the heat shock protein expression. Similarly, the TUNEL staining study showed the similar results to those in the HE staining study, and these results were positively correlated with each other.

In this study, we first time evaluated the effectiveness of Fe(Salen) in the point of administration route, *i.e.*, selective intra-arterial injection by catheter. Therefore, these results indicate a new administration route, *i.e.*, selective arterial injection of Fe(Salen) by catheter, and the development of a new strategy of simultaneous hyperthermia-chemotherapy in the future.

Discussion

Our results indicate that chemotherapy with Fe(Salen) nanoparticles (NPs) using the selective catheter and AMF-induced hyperthermia exhibited strong anti-tumor effects in a rabbit model of femur tumors. This strategy using the selective catheter and AMF showed a greater decrease of tumor size compared to the Fe(Salen) intravenous injection group, the MTX intra-arterial injection group or the Fe(Salen) intra-arterial injection group. Conventional chemotherapy drugs, including MTX, have anti-tumor effects, but not magnetism. In contrast, Fe(Salen) NPs could be used for both anti-tumor and hyperthermia therapies at the focal site. This magnetism is the major advantage of Fe(Salen) compared with the other conventional drugs. Furthermore, our study showed that the selective arterial injection of Fe(Salen) by catheter showed further antitumor effects of Fe(Salen) compared with the intravenous systematic injection that we previously reported (Eguchi H *et al.* 2015, Ohtake M *et al.* 2017, Sato I *et al.* 2016). Although we have reported the effect of Fe(Salen) using only intravenous injection or local injection into animal models, this is the first report that the effectiveness of the selective catheter is useful to treat them.

We have previously reported that Fe(Salen) promotes ROS, resulting in DNA nicking (Eguchi H *et al.* 2015, Ohtake M *et al.* 2017, Sato I *et al.* 2016). Fe(Salen) also increased cytochrome c in the mitochondria-enriched fraction, as shown by immunoblotting, resulting in cell apoptosis (Eguchi H *et al.* 2015).[4] However, little is known about the mechanism of the anti-tumor effect in Fe(Salen). In this study, we elucidated the mechanism of Fe(Slaen)-induced anti-tumor effect. 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 (Siddiquee KAZ *et al.* 2008). The inhibition of STAT3 induced the G1 arrest of the cell cycle and suppressed cell proliferation in human pancreatic adenocarcinoma cell lines (Venkatasubbarao K *et al.* 2005). Taken together, Fe(Salen) exhibited the antitumor effect via MEK/ERK/STAT3 signaling.

In the present study, we investigated the toxic effect of Fe(Salen) and determined the dose level of these toxic effects by repeated intravenous administration in male Sprague-Dawley rats. These 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 of mice and rabbit (Eguchi H *et al*. 2015, Ohtake M *et al*. 2017, Sato I *et al*. 2016). 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 intravenous injection (Ohtake M *et al*. 2017). 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 the embolism to the organ, or we should develop more suitable drug compounds, *e.g.*, micelles coated with Fe(Salen).

In the current study, we have demonstrated a new approach to inject Fe(Salen) via the 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.

References

Akimoto T, Nagasako A, Ohtake M et al. Alternating Magnetic Field (AMF) Enhances Cytotoxicity of Compound C. *Cancer Sci*. 2018; 109(11): 3483-3493.

Chazotte B. Labeling Mitochondria with MitoTracker Dyes. *Cold Spring Harb Protoc*. 2011;2011(8):990-992.

Eguchi H, Umemura M, Kurotani R, et al. A magnetic anti-cancer compound for magnet-guided delivery and magnetic resonance imaging. *Sci Rep*. 2015;5:9194.

Kim JH, Eguchi H, Umemura M, et al. Magnetic metal-complex-conducting copolymer core-shell nanoassemblies for a single-drug anticancer platform. *NPG Asia Mater*. 2017;9:e367.

Oda K, Umemura M, Nakakaji R, et al. Transient receptor potential cation 3 channel regulates melanoma proliferation and migration. *J Physiol Sci*. 2017; 67(4): 597-505.

Ohtake M, UM, Sato I, Akimoto T et al. Hyperthermia and chemotherapy using Fe(Salen) nanoparticles might impact glioblastoma treatment. *Sci Rep*. 2017;7:42783.

Sato I, Umemura M, Mitsudo K et al. Simultaneous hyperthermia-chemotherapy with controlled drug delivery using single-drug nanoparticles. *Sc Rep*. 2016;6:24629.

Schneiderbauer MM, von Knoch M, Schleck CD, et al. Patient Survival After Hip Arthroplasty for Metastatic Disease of the Hip. *J Bone Joint Surg.* 2004;86A(8):1684-1689.

Siddiquee KAZ, Turkson J. STAT3 as a target for inducing apoptosis in solid and hematological tumors. *Cell Res*. 2008;18(2): 254-267.

Umemura M, Baljinnyam E, Feske S, et al. Store-Operated Ca^{2+} Entry (SOCE) Regulates Melanoma Proliferation and Cell Migration. *PLoS One*. 2014;9(2):e89292.

Umemura M, Kim J-H, Aoyama H, et al. The iron chelating agent, deferoxamine detoxifies Fe(Salen)-induced cytotoxicity. *J Pharmacol Sci*. 2017;134:203-210.

Venkatasubbarao K, Choudary A, Freeman JW. Farnesyl Transferase Inhibitor (R115777)–Induced Inhibition of $STAT^{3}(Tyr^{705})$ Phosphorylation in Human Pancreatic Cancer Cell Lines Require Extracellular Signal-Regulated Kinases. *Cancer Re*s. 2005;65(7): 2861-2871.

Yallapu MM, Othman SF, Curtis ET et al. Multi-functional magnetic nanoparticles for magnetic resonance imaging and cancer therapy. *Biomaterials*. 2011;32(7):1890-1905.

Yoneda T, Kitamura M, Ogawa T, Aya S-i, Sakuda M. Control of VX_2 Carcinoma Cell Growth in Culture by Calcium, Calmodulin, and Prostaglandins. *Cancer Res*. 1985; 45: 398-405.

Publication list

Main article for the Ph.D

*Masanari Umemura and **Md. Rafikul Islam**++, Hidenobu Fukumura, Itaru Sato, Yusuke Kawabata, Kousuke Matsuo, Rina Nakakaji, Akane Nagasako, Makoto Ohtake, Fujita Takayuki, Utako Yokoyama, Tomohiro Nakayama, Haruki Eguchi, Yoshihiro Ishikawa. Simultaneous hyperthermia-chemotherapy effect by arterial injection of Fe(Salen) for femur tumor. *Cancer Science*. 110(1); 356-365: 2018. (*Masanari Umemura and **Md. Rafikul Islam**++ contributed equally to this work.).

Link: https://doi.org/10.1111/cas.13851

Publication as a coauthor

Kohei Osawa, Masanari Umemura, Rina Nakakaji, Ryo Tanaka, **Rafikul Md Islam**, Akane Nagasako, Takayuki Fujita, Utako Yokoyama, Toshiyuki Koizumi, Kenji Mitsudo, Yoshihiro Ishikawa. Prostaglandin E2 receptor EP4 regulates cell migration through Orai1. *Cancer Science*. 111; 160-174: 2019.