

# Hyperthermia generated with ferucarbotran (Resovist®) in an alternating magnetic field enhances cisplatin-induced apoptosis of cultured human oral cancer cells

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**Abstract** Hyperthermia is a promising anti-cancer treatment in which the tissue temperature is increased to 42–45 °C, and which is often used in combination with chemotherapy or radiation therapy. Our aim in the present work was to examine the feasibility of combination therapy for oral cancer with cisplatin and hyperthermia generated with ferucarbotran (Resovist®; superparamagnetic iron oxide) in an alternating magnetic field (AMF). First, we established that administration of ferucarbotran at the approved dosage for magnetic resonance imaging provides an iron concentration sufficient to increase the temperature

to 42.5 °C upon exposure to AMF. Then, we examined the effect of cisplatin combined with ferucarbotran/AMF-induced hyperthermia on cultured human oral cancer cells (HSC-3 and OSC-19). Cisplatin alone induced apoptosis of cancer cells in a dose-dependent manner, as is well known. However, the combination of cisplatin with ferucarbotran/AMF was significantly more effective than cisplatin alone. This result suggests that it might be possible to reduce the clinically effective dosage of cisplatin by administering it in combination with ferucarbotran/AMF-induced hyperthermia, thereby potentially reducing the incidence of serious cisplatin-related side effects. Further work seems justified to evaluate simultaneous thermo-chemotherapy as a new approach to anticancer therapy.

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## Abbreviations

AMF Alternating magnetic field  
MNPs Magnetic nanoparticles  
MRI Magnetic resonance imaging  
SPIO Superparamagnetic iron oxide

## Introduction

Cancer cells are more vulnerable to increased temperature than normal cells [1]. Thus, hyperthermia is viewed as a promising approach in cancer therapy [2]. Many techniques have been reported to increase the temperature of cancer tissues, such as whole-body hyperthermia [3], radiofrequency hyperthermia [4], microwave-induced hyperthermia [5], and implantable needles [6]. However, with all

these modalities, it remains difficult to increase the temperature of only the cancer tissues in a controlled manner without damaging surrounding normal tissues.

More than 40,000 people are diagnosed with oral cancer, including cancers of the mouth, tongue, tonsils, and throat, every year in the US alone. Oral cancer can cause functional damage and disfigurement, and, in its advanced stages, it invades surrounding organs, causing disorders of speech, swallowing, and even chewing. Surgery may have serious adverse effects, so chemotherapy or radiation therapy is often favored in oral cancer patients, not withstanding potentially serious systemic side effects. Hyperthermia is often preferred, e.g., for metastatic N3 cervical lymph nodes, because it has fewer adverse side effects. However, it is difficult to induce hyperthermia in a metastatic node-specific manner. Nevertheless, selective hyperthermia has been studied as a possible approach to obtain tumor-specific cytotoxicity, e.g., by ferromagnetic embolization [7]. More recently, magnetic nanoparticles (MNPs) have been investigated for this purpose, because MNPs generate heat when they are exposed to an alternating magnetic field (AMF) as a result of hysteresis and relaxational losses [8].

Ferucarbotran (Resovist<sup>®</sup>) is an organ-specific contrast agent used in magnetic resonance imaging (MRI) of local tumors, and the permissible dose in humans has been established by at least two studies [9, 10]. Because ferucarbotran consists of superparamagnetic iron oxide (SPIO) coated with carboxydextran, it generates heat when it is exposed to an AMF [11, 12], and it has been reported to induce selective hyperthermia when used in arterial embolization [11]. However, it has not been established whether ferucarbotran is suitable for inducing hyperthermia in cancer treatment.

Cisplatin (*cis*-diaminedichloroplatinum II; CDDP) is widely used in chemotherapy in many types of cancer, including oral cancers [13]. However, it has serious side effects, including acute kidney damage and/or renal failure [14–16]. Recent studies have demonstrated that hyperthermia stimulates cellular uptake of cisplatin [17, 18] and consequently enhances the cytotoxicity of cisplatin in cancer cells, both *in vitro* and *in vivo* [19–21]. Thus, combined treatment with cisplatin plus hyperthermia may allow the effective dose of cisplatin to be decreased sufficiently to minimize serious side effects.

Accordingly, in order to examine the feasibility of using combination therapy with cisplatin and ferucarbotran/AMF-induced hyperthermia in the therapy of oral cancer, in this study we examined the effect of the combined treatment on oral cancer cells in culture. Our results confirmed that ferucarbotran/AMF-induced hyperthermia significantly enhances the effect of cisplatin. Because both cisplatin and ferucarbotran have already been approved for

clinical use, early introduction of this technique, at least for oral cancers, should be feasible.

## Materials and methods

### Reagent, drug and cell lines

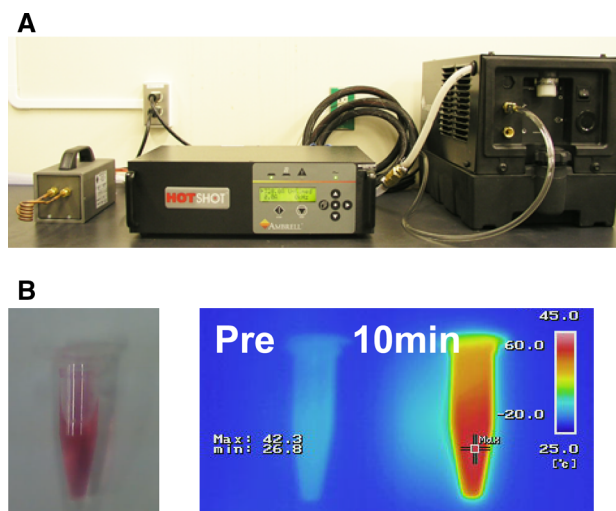
Ferucarbotran (Resovist<sup>®</sup>) was purchased from FUJIFILM Pharma (Tokyo, Japan) [11]. Cisplatin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Human oral squamous cell carcinoma cell lines OSC-19 and HSC-3 were purchased from the Japan Health Sciences Foundation, Health Science Research Resources Bank (Osaka, Japan). In all cases, cells from early passage cultures were stored and used for the experiments. OSC-19 and HSC-3 were cultured in Dulbecco's modified Eagle's medium (DMEM), 1 % penicillin–streptomycin, and 1 % L-glutamine.

### Thermography

Thermal images were taken using a thermograph (infrared thermal imaging camera InfReC R300SR; Nippon Avionics, Tokyo, Japan). Temperature was also measured using a thermograph.

### Alternating magnetic field (AMF) generator

An AMF was generated by a vertical coil with an inner diameter of 6.5 cm, driven by a transistor inverter (HOT SHOT; Ameritherm, New York, USA) operated at a



**Fig. 1** Heat generation by ferucarbotran in an alternating magnetic field (AMF). **a** The alternating magnetic field (AMF) generator, **b** a photograph of ferucarbotran in medium (*left*), and thermal images of ferucarbotran in medium before (*middle*), and 10 min after AMF (308 kHz, EC 270 A) (*right*)

frequency of 308 kHz and electric current (EC) 250 A [12, 22–26]. Temperature was measured using a hand-held thermometer, HA-200 (Anritsu Meter, Tokyo, Japan).

#### Apoptosis assay

HSC-3 cells and OSC-19 ( $6 \times 10^4$  cells/well) were seeded on 6-cm dishes and incubated for 24 h. Cisplatin was then added to a concentration of 0  $\mu$ M (control), 7.5 or 15  $\mu$ M. When hyperthermia was to be applied, 10 mM ferucarbotran was added and AMF was performed with a HOT SHOT under the conditions described above [22, 25, 26]. Incubation was continued for 12 h at 37 °C, in an atmosphere of 5 % CO<sub>2</sub> in air. Cells were washed twice with cold PBS and suspended in 1 $\times$  binding buffer at a concentration of  $1 \times 10^6$  cells/ml. Next, a 100- $\mu$ l aliquot of the solution, containing  $1 \times 10^5$  cells, was transferred to a 5-ml culture tube. Then, 5  $\mu$ l of allophycocyanin (APC) Annexin V and 5  $\mu$ l of 7-aminoactinomycin D (AAD) (BD Biosciences, CA, USA) [27] were added to the tube. Incubation was continued for 15 min at room temperature (25 °C) in the dark. Finally, 400  $\mu$ l of 1 $\times$  binding buffer were added to each tube. Cells were examined by flow cytometry (BD FACSCanto II; BD Biosciences).

#### Cell cycle analysis

Cell cycle analysis was performed using The Cycletest™ Plus DNA Reagent Kit (BD Biosciences) according to the manufacturer's protocol [28]. Briefly, HCS-3 and OSC-19 cells treated with 0  $\mu$ M (control), 7.5 or 15  $\mu$ M cisplatin, with or without hyperthermia (10 mM ferucarbotan/AMF), were washed in PBS and fixed in 90 % ethanol. Fixed cells were washed twice in PBS and stained with 50  $\mu$ M propidium iodide containing 5  $\mu$ g/ml DNase-free RNase for 1 h, then analyzed by flow cytometry using a FACScan (BD FACSCanto II).

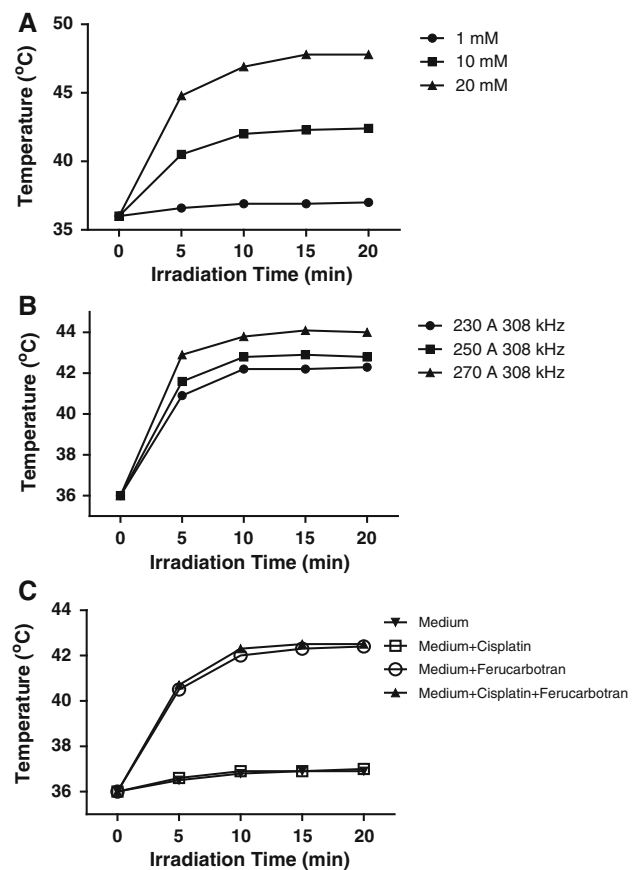
#### Statistical analysis

Data were analyzed using BD FACSDiva software (BD Biosciences). Data are expressed as mean  $\pm$  SEM. Data were analyzed by one-way ANOVA followed by the Tukey post hoc test using GraphPad Prism software (GraphPad Software, CA, USA). The criterion of statistical significance was set at  $p < 0.05$ .

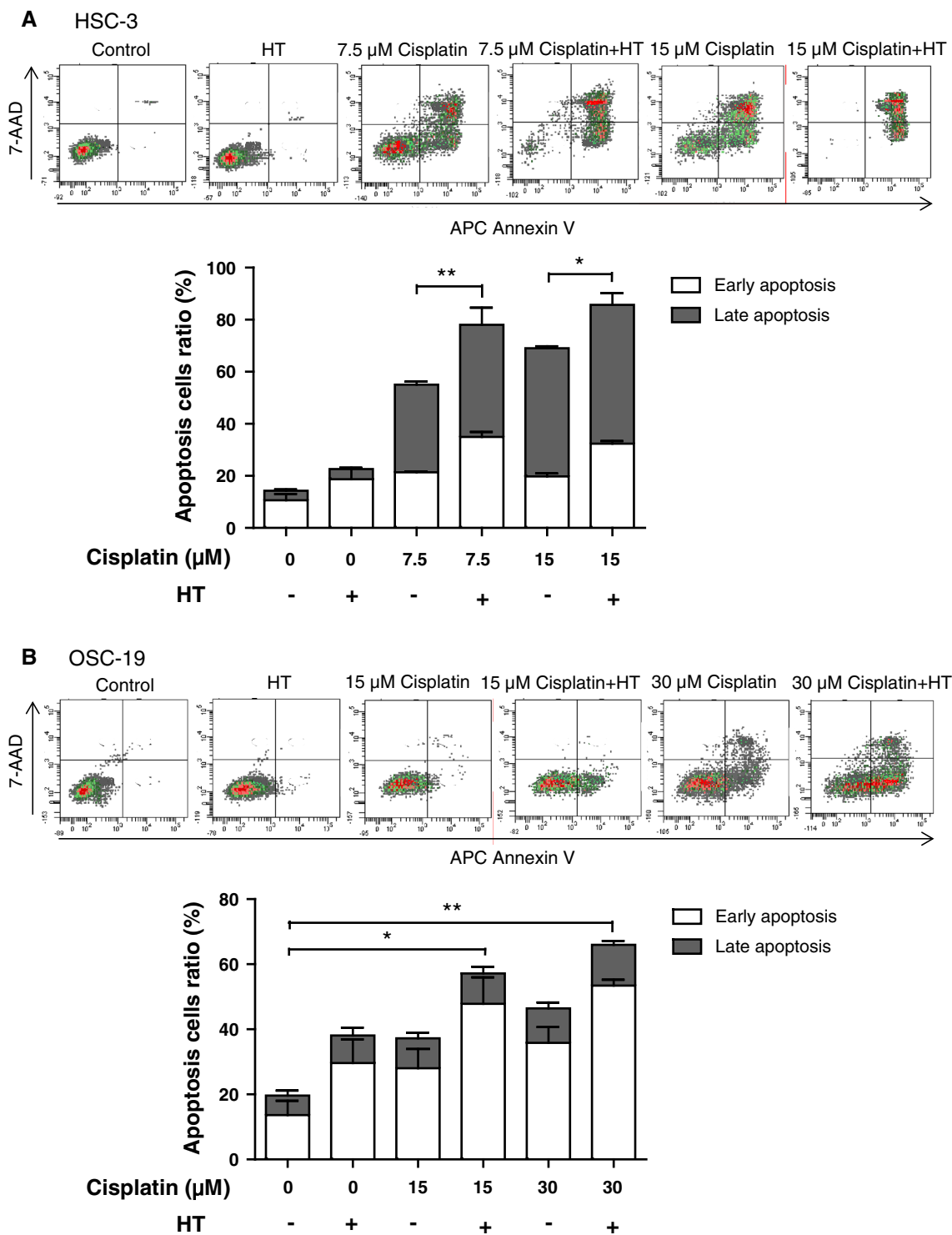
## Results

### Heat generation by ferucarbotran in an alternating magnetic field (AMF)

Heat production is determined by the magnetic properties of ferucarbotran, its concentration, and the strength of the AMF [12]. Therefore, we examined the heating effect of AMF on medium containing ferucarbotran by thermography (Fig. 1b). As shown in Fig. 2, the temperature increased time-dependently, and the extent of the increase was dependent on the concentration of ferucarbotran (Fig. 2a) and the magnitude of the EC used to generate AMF (Fig. 2b). The results showed that AMF produced at



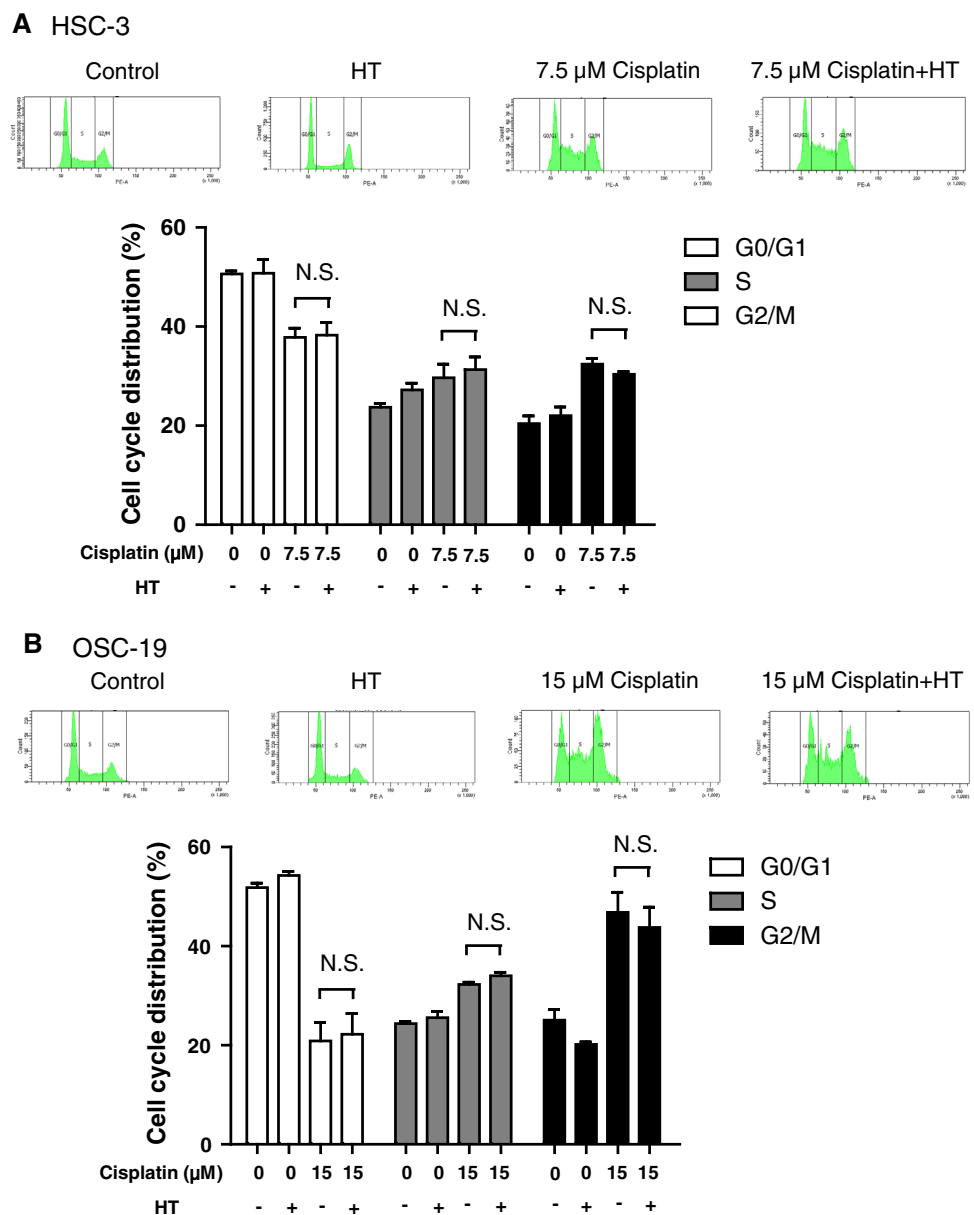
**Fig. 2** Dependence of heat generation on ferucarbotran concentration and alternating magnetic field (AMF) strength. **a** Temperature–time curves at different concentrations of ferucarbotran (1, 10, or 20 mM equivalent of iron) on AMF at 308 kHz and EC 230 A. **b** Temperature–time curves in the presence of 10 mM ferucarbotran on AMF at different levels of electric current (230–270 A) at 308 kHz. **c** Effect of cisplatin (30  $\mu$ M) on ferucarbotran (1, 10, or 20 mM equivalent of iron)/AMF (308 kHz, EC 230 A)-induced increase of temperature; medium only, medium + cisplatin, medium + ferucarbotran, and medium + cisplatin + ferucarbotran



**Fig. 3** Ferucarbotran/AMF-induced hyperthermia enhances the pro-apoptotic effect of cisplatin in human oral cancer cells. Annexin-V/PI staining of human oral cancer cells at 12-h intervals after treatment with 0, 7.5, or 15 μM cisplatin with or without hyperthermia (HT) in HSC-3 cells, and 0, 15, 30 μM cisplatin with or without HT in OSC-19 cells. **a** Representative analysis of apoptosis of HSC-3 cells and OSC-19 cells exposed to cisplatin and ferucarbotran with or without

AMF. Annexin-V/PI method with FACS scan dot plot analysis was used to divide the treated and control cells into four groups: (1) living cells (*lower left quadrant*); (2) necrotic cells (*upper left quadrant*); (3) early apoptotic cells (*lower right quadrant*); and (4) late apoptotic cells (*upper right quadrant*). **b** Representative analysis of apoptosis of OSC-19 cells exposed to cisplatin with or without AMF. \* $p < 0.05$ , \*\* $p < 0.01$ ;  $n = 4$

**Fig. 4** Combination of cisplatin and ferucarbotran/AMF-induced hyperthermia causes G2/M arrest. Cell cycle analysis of HSC-3 and OSC-19 cells at 48-h intervals after treatment with 0, 7.5, or 15  $\mu$ M of cisplatin with or without hyperthermia (HT). **a** Representative cell cycle analysis of HSC-3 cells in response to cisplatin treatment with or without AMF (*upper panel*) and of OSC-19 cells in response to cisplatin treatment with or without AMF (*lower panel*). NS not significant;  $n = 4$



generator settings of 308 kHz and EC 250 A in the presence of 10 mM (equivalent of iron) ferucarbotran was sufficient to generate a temperature of 42.5 °C, and we adopted these conditions for the subsequent assays. We confirmed that cisplatin did not alter the heating effect under these conditions (Fig. 2c).

**Ferucarbotran-enhanced cisplatin-mediated apoptosis**

It has been reported that cisplatin induces apoptosis in cancer cells [29]. We thus examined whether ferucarbotran/AMF-induced hyperthermia further increased cisplatin-induced apoptosis in oral cancer cells. FACS analysis demonstrated that cisplatin increased both early and late

apoptosis in a dose-dependent manner in HSC-3 cells (Fig. 3a) and OSC-19 cells (Fig. 3b). Ferucarbotran/AMF-induced hyperthermia for an hour significantly increased the apoptotic effect of cisplatin.

**Cisplatin-induced G2/M arrest of human oral cancer cells was unaffected by hyperthermia**

To examine whether hyperthermia modifies the mechanism of anti-cancer action of cisplatin, flow-cytometric cell-cycle analysis of treated cells was performed. Cisplatin induced potent G2/M arrest in both HSC-3 cells (Fig. 4a) and OSC-19 cells (Fig. 4b). We found that ferucarbotran/AMF-induced hyperthermia did not alter the effect of

cisplatin on the cell cycle. Thus, hyperthermia per se had no effect on the anti-cancer mechanism of cisplatin.

## Discussion

Ferucarbotran is an organ-specific superparamagnetic contrast agent used in MRI, and its safety and maximum dosage (10 mM; 0.016 mL/kg, which contains 8  $\mu$ mol (0.45 mg) Fe/kg equivalent of iron [30]) have been well established [9, 10]. Since hyperthermia has already been shown to enhance the anti-cancer effect of cisplatin [31] in the treatment of oral cancer, we anticipated that combination therapy with cisplatin and ferucarbotran/AMF-induced hyperthermia might be suitable for oral cancer treatment, making it possible to reduce the necessary dose of cisplatin and consequently reduce the risk of serious side effects.

Hyperthermia to induce apoptosis of cancer cells is best performed at about 42 °C, because temperatures above 44 °C have been reported to cause necrosis and damage to surrounding normal tissues [32]. Therefore, we first confirmed that the above concentration of ferucarbotran was sufficient to maintain a temperature of 42.5 °C under appropriate AMF conditions, and this level of hyperthermia could induce apoptosis of oral cancer cells, as evaluated by FACS analysis. It should be noted that it would still be necessary to optimize AMF conditions for clinical treatment. Similarly, it would be desirable to deliver cisplatin and ferucarbotran to oral cancer tissue in a selective manner. This may be achieved by the use of superselective intra-arterial infusion with a catheter, as we previously reported in oral cancer patients [33].

We previously reported that ROS production was higher in cancer cells than in normal cells, and was further increased when the temperature was increased [34]. Cisplatin also increases ROS production, and this is most likely the mechanism responsible for its anti-cancer effect [34, 35]. We confirmed that the combination of cisplatin and ferucarbotran/AMF-induced hyperthermia further enhanced ROS production (data not shown). This is important, because cisplatin may cause ototoxicity [36], so it is desirable to minimize the necessary cisplatin dose, as far as is consistent with therapeutic effectiveness, in the clinical context.

It is well known that cisplatin causes accumulation of cells in S phase and blocks the G0/G1 phases in xenografted human head and neck carcinoma cells [37], leading to apoptosis. [38, 39]. Our data showed that ferucarbotran/AMF-induced hyperthermia enhanced the anti-cancer effect of cisplatin without altering its characteristic effect on the cell cycle. Accordingly, ferucarbotran/AMF-induced hyperthermia did not appear to modify the mechanism of action of cisplatin in human oral cancer cells. Because both cisplatin and ferucarbotran are already in clinical use, we

believe the combination of cisplatin with ferucarbotran/AMF-induced hyperthermia has the potential for early clinical application. It should at least be possible to reduce the clinically effective dosage of cisplatin by administering it in combination with ferucarbotran/AMF, thereby reducing the risk of serious cisplatin-related side effects. Further investigation seems warranted to confirm the safety and effectiveness of this combined treatment for oral cancers in humans.

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**Conflict of interest** The authors declare no potential conflicts of interest.

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