


# 5-fluorouracil combined with cisplatin and mitomycin C as an optimized regimen for hyperthermic intraperitoneal chemotherapy in gastric cancer

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**Background and Objectives:** Optimized drug regimens for hyperthermic intraperitoneal chemotherapy (HIPEC) have not been standardized completely in patients with advanced gastric cancer (GC). We evaluated an optimized anti-tumor protocol comprising 5-fluorouracil (5-FU) combined with cisplatin (CDDP) and mitomycin C (MMC) *in vitro* for clinical use of HIPEC.

**Methods:** The sensitivities of 5-FU, CDDP, or MMC, alone or in combination, using different drug concentrations, exposure times, and hyperthermic conditions (42°C) were determined *in vitro* by the CD-DST method using 3 different differentiated GC cell lines.

**Results:** The tumor cell growth-inhibitory effect of 5-FU was concentration-dependent for all cell lines. In addition, 5-FU showed a hyperthermic sensitization effect at all drug concentrations for all cell lines. The appropriate concentration of each drug was 5-FU, 200 µg/mL; CDDP, 10 µg/mL; MMC, 2 µg/mL. Under hyperthermic conditions, most growth-inhibitory effects for each drug at 30 min was equivalent to 60 min of exposure; use of three drugs combined significantly inhibited growth compared with any of the drugs alone.

**Conclusion:** An appropriate *in vitro* intraperitoneal chemotherapy regimen for GC was combined use of 5-FU, CDDP, and MMC at 42°C for 30 min.

## KEYWORDS

gastrectomy, gastrointestinal carcinoma, HIPEC, hyperthermia, intraperitoneal chemotherapy, peritoneal metastasis

## 1 | INTRODUCTION

Gastric cancer (GC) with peritoneal metastasis continues to exhibit an extremely poor prognosis, and early postoperative peritoneal metas-

tasis is a major problem for the treatment of advanced GC even with curative intent.<sup>1</sup> Hyperthermic intraperitoneal chemotherapy (HIPEC) has emerged as a promising procedure for the prevention and treatment of peritoneal metastasis during surgery for pseudomyxoma

peritonei, malignant mesothelioma, ovarian cancer, colorectal cancer, and GC.<sup>2-10</sup> However, some adverse effects have been reported regarding cytoreductive surgery and HIPEC. Thus, more effective and less adverse effective treatment regimens regarding the optimal drug regimens, treatment durations, and HIPEC temperatures should be developed.

The drugs selected for HIPEC, which have tumoricidal activity following brief exposure, are generally cell cycle phase-nonspecific agents. They are characterized by direct cytotoxic effects and synergistic anti-tumor activity when used in combination with hyperthermia. The drugs most widely used for HIPEC for GC are mitomycin C (MMC) and cisplatin (CDDP), used either alone or in combination. These drugs are independent of the cell cycle and are thermosensitive.<sup>11,12</sup>

5-Fluorouracil (5-FU) has been the mainstay of adjuvant or palliative treatment for patients with gastrointestinal malignancies, and it is usually administered intravenously. 5-FU is considered a cell cycle- and time-dependent drug, because 5-FU exhibits cytotoxic effects due to the inhibition of deoxyribonucleic acid (DNA) replication. However, 5-FU metabolites, namely, 5-fluorouridine diphosphate and 5-fluorouridine triphosphate, have direct cytotoxic effects because they are incorporated into ribonucleic acid (RNA).<sup>13,14</sup> Moreover, 5-FU has been shown to be thermosensitive.<sup>15,16</sup> In addition, 5-FU also induces reactive oxygen species that are directly involved in cell killing.<sup>17,18,19,20</sup> These different lines of evidence provide a rationale for administering HIPEC that includes 5-FU. Hence, we sought to add 5-FU to the HIPEC protocol that included MMC and CDDP to improve its cytotoxic effects and enhance its protective and therapeutic effects against peritoneal metastasis following surgery for advanced GC.

This *in vitro* study aimed to define the appropriate conditions for HIPEC by evaluating the antitumor effects under several drug concentrations and different durations of drug exposure, to determine the efficacy of 5-FU administered in combination with MMC and CDDP under hyperthermic conditions.

## 2 | MATERIALS AND METHODS

### 2.1 | Assessment of anticancer drug sensitivity

The collagen gel droplet-embedded culture drug sensitivity test (CD-DST) (Kurabo Industry Ltd, Osaka, Japan) was used to examine the sensitivity of MMC (Kyowa Hakko Kogyo), CDDP (Kaken, Tokyo, Japan), and 5-FU (Kyowa Hakko Kogyo)—either alone or in combination—against human GC cell lines (Riken BioResource Centre Tsukuba, Ibaraki, Japan) MKN7,<sup>21</sup> MKN45,<sup>21,22</sup> and GCIY,<sup>23</sup> derived from well-differentiated tubular, poorly differentiated tubular, and mucinous adenocarcinomas, respectively.

The sensitivity of each anticancer drug was tested using different concentrations of the drug on each cell line under normothermic (37°C) or hyperthermic (42°C) conditions. Considering clinical relevance, we used the following concentrations for the administered drugs: 5-FU: 100-600 µg/mL, CDDP: 5-30 µg/mL, and MMC:

1-16 µg/mL. Next, we examined tumor growth inhibition based on the amount of time the cells were exposed to each drug. Then, we evaluated the efficacy of the 3 drugs in combination. Finally, we determined the concentration of each drug that was considered optimal when incorporated into HIPEC.

The tumor cell growth inhibition assay was conducted using the CD-DST with a modified version of the manufacturer's instructions.<sup>24</sup> Briefly, the GC cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (10% FBS-RPMI) at 37°C in 5% CO<sub>2</sub>. Using a collagen gel culture kit (Nitta Gelatin, Inc., Osaka, Japan), each of the cell lines was mixed with molten collagen to achieve a final concentration of  $1 \times 10^5$  cells/mL. After solidification, the collagen gel-embedded cancer cells were overlaid with 10% FBS-RPMI that contained either a single drug or a mixture of 5-FU, CDDP, and MMC at the designated final concentrations. The drug solutions were kept incubated at 37°C or 42°C to attain the desired temperature. The cancer cells were incubated with the drug solutions for 30 or 60 min at 37°C or 42°C. Then, the solution containing the drug(s) was removed and the culture maintained in a motionless state at 37°C in 5% CO<sub>2</sub> for an additional 7 days. At the end of the incubation period, neutral red was added to each well at a final concentration of 50 µg/mL, and the colonies of cancer cells in the collagen gel droplets were stained for 2 h. The cells were then fixed with 10% neutral-buffered formalin. Images of the stained gels were acquired using a video microscope (VH-5910; Keyence, Osaka, Japan), and cell proliferation rates were obtained by measuring the optical densities. The sensitivity was expressed as a percentage of the T/C ratio, where T was the optical density of the images from the treated group and C was the optical density of the images from the control group.

### 2.2 | Statistical analysis

Student's *t*-test was used to compare the groups in relation to the continuous variables. *P* values <0.05 with two-tailed tests were considered statistically significant.

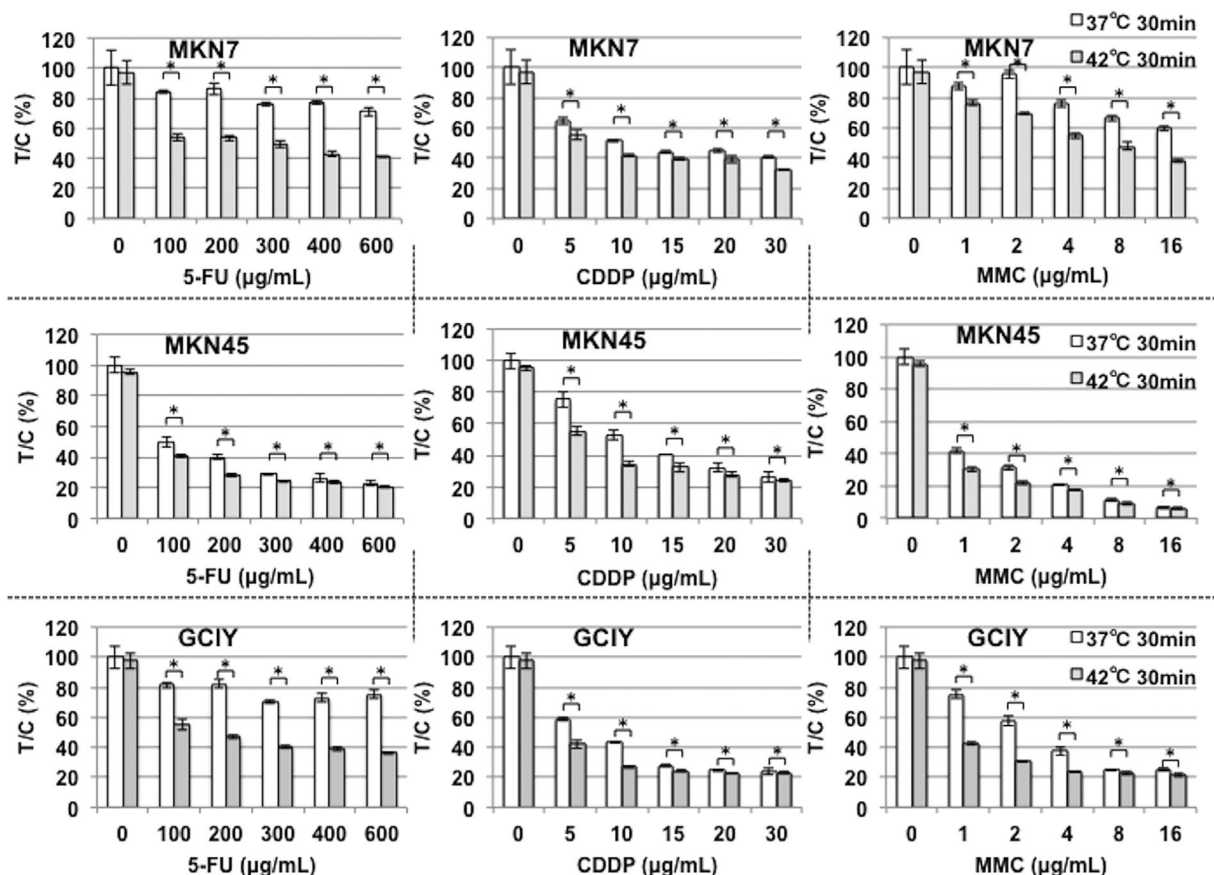
## 3 | RESULTS

### 3.1 | Drug concentrations and hyperthermic sensitization

We performed tumor growth inhibition assays using the CD-DST and measured the hyperthermic sensitization effects of the anti-tumor drugs against the 3 GC cell lines (Figure 1).

Under normothermic conditions, the tumor cell growth-inhibitory effect of 5-FU was dependent on the drug's concentration for the MKN45 cell line, but was independent of the drug's concentration for the MKN7 and GCIY cell lines. Under hyperthermic conditions, the tumor cell growth-inhibitory effect of 5-FU was dependent on the drug's concentration for all cell lines. In addition, 5-FU showed a hyperthermic sensitization effect at all drug concentrations examined and on all cancer cell lines tested.

The tumor cell growth-inhibitory effects of CDDP and MMC against the 3 GC cell lines were dependent on the concentrations of



**FIGURE 1** The tumor cell proliferation assay was conducted using the collagen gel droplet-embedded culture drug sensitivity test. The sensitivities of 5-fluorouracil (5-FU), cisplatin (CDDP), and mitomycin C (MMC) against each cell line (MKN7, MKN45, and GCIY) were compared at different concentrations of each drug (5-FU: 100–600  $\mu\text{g/mL}$ ; CDDP: 5–30  $\mu\text{g/mL}$ ; and MMC: 1–16  $\mu\text{g/mL}$ ) under normothermic (37°C) or hyperthermic (42°C) conditions. T/C (%) denotes the sensitivity. At 42°C, the chemosensitivity significantly increased compared with that of 37°C at all drug concentrations in all cell lines ( $P < 0.05$ ). \* $P < 0.05$ ; 5-FU, 5-fluorouracil; CDDP, cisplatin; MMC, mitomycin C; T/C (%), sensitivity, where T was the image optical density of the treated group and C was the image optical density of the control group

the drugs, and they were significantly enhanced at 42°C compared with 37°C at all of the drug concentrations examined.

### 3.2 | Drug concentrations for HIPEC

Regarding the clinical dosages of the anticancer drugs for HIPEC, our view was that the drug dosages should be as low as possible to reduce the incidence of adverse events caused by HIPEC, while securing an anti-tumor effect.

Under hyperthermic conditions, a greater tumor growth-inhibitory effect was observed when the concentration of each drug was doubled, trebled, or quadrupled (Table 1). The tumor growth-inhibitory effect of 5-FU at 400  $\mu\text{g/mL}$  (2000 mg/5 L perfusate) and 600  $\mu\text{g/mL}$  (3000 mg/5 L perfusate) at 42°C showed increases of  $7.5 \pm 3.3\%$  and  $10.1 \pm 2.4\%$ , respectively, in relation to the T/C compared with 5-FU at 200  $\mu\text{g/mL}$  (1000 mg/5 L perfusate). The tumor growth-inhibitory effect of CDDP at 20  $\mu\text{g/mL}$  (100 mg/5 L perfusate) and 30  $\mu\text{g/mL}$  (150 mg/5 L perfusate) at 42°C increased by  $4.5 \pm 2.3\%$  and  $7.7 \pm 3.3\%$ , respectively, in relation to the T/C compared with CDDP at 10  $\mu\text{g/mL}$  (50 mg/5 L perfusate). The tumor growth-inhibitory effect of MMC at 4  $\mu\text{g/mL}$

(20 mg/5 L perfusate) and 8  $\mu\text{g/mL}$  (40 mg/5 L perfusate) at 42°C increased by  $8.7 \pm 5.1\%$  and  $13.9 \pm 7.0\%$ , respectively, in relation to the T/C compared with MMC at 2  $\mu\text{g/mL}$  (10 mg/5 L perfusate).

Given the small differences between the low and high concentrations of each anticancer drug in relation to their anti-tumor effects under hyperthermic conditions, we determined that the appropriate concentration of each anticancer drug in this clinical study for HIPEC was as follows: 5-FU: 200  $\mu\text{g/mL}$  (1000 mg/5 L saline perfusate); CDDP: 10  $\mu\text{g/mL}$  (50 mg/5 L saline perfusate); and MMC: 2  $\mu\text{g/mL}$  (10 mg/5 L saline perfusate).

### 3.3 | Duration of HIPEC

Next, we tried to determine the optimal duration of HIPEC. Each GC cell line was exposed to each anticancer drug for 30 or 60 min under normothermic (37°C) or hyperthermic (42°C) conditions in vitro (Figure 2). After exposure for 30 or 60 min, each anticancer drug significantly inhibited the growth of all of the cancer cell lines under hyperthermic conditions, compared with that of any cancer cell line under normothermic conditions.

**TABLE 1** Drug concentrations and anti-tumor effects under hyperthermic conditions

5-FU				
Cell lines				
*Sensitivity at 42°C for 30 min (%)				
Concentration (µg/mL)	MKN7	MKN45	GCIY	
200	53.5 ± 2.0	28.0 ± 0.7	46.5 ± 1.1	
400	42.8 ± 1.9	23.9 ± 0.9	38.7 ± 1.3	
600	41.2 ± 2.4	20.5 ± 0.7	36.0 ± 0.6	
	Difference in *sensitivity (%)			Mean ± standard deviation (%)
400 µg/mL vs 200 µg/mL	10.7	4.1	7.8	7.5 ± 3.3
600 µg/mL vs 200 µg/mL	12.3	7.5	10.5	10.1 ± 2.4
CDDP				
Cell lines				
*Sensitivity at 42°C for 30 min (%)				
Concentration (µg/mL)	MKN7	MKN45	GCIY	
10	41.8 ± 0.9	34.4 ± 1.3	26.8 ± 0.7	
20	39.3 ± 2.4	27.4 ± 1.6	22.9 ± 0.1	
30	32.4 ± 0.4	24.5 ± 1.1	23.0 ± 0.6	
	Difference in *sensitivity (%)			Mean ± standard deviation (%)
20 µg/mL vs 10 µg/mL	2.5	7.0	3.9	4.5 ± 2.3
30 µg/mL vs 10 µg/mL	9.4	9.9	3.8	7.7 ± 3.3
MMC				
Cell lines				
*Sensitivity at 42°C for 30 min (%)				
Concentration (µg/mL)	MKN7	MKN45	GCIY	
2	69.3 ± 0.6	22.0 ± 1.1	30.4 ± 0.4	
4	54.9 ± 1.9	17.3 ± 0.2	23.3 ± 0.2	
8	47.7 ± 2.4	9.4 ± 1.0	22.7 ± 1.1	
	Difference in *sensitivity (%)			Mean ± standard deviation (%)
4 µg/mL vs 2 µg/mL	14.4	4.7	7.1	8.7 ± 5.1
8 µg/mL vs 2 µg/mL	21.5	12.6	7.7	13.9 ± 7.0

\*Sensitivity was expressed as the percentage of the T/C ratio, where T was the optical density of the images from the treated group and C was the optical density of the images from the control group.

5-FU, 5-fluorouracil; CDDP, cisplatin; MMC, mitomycin C.

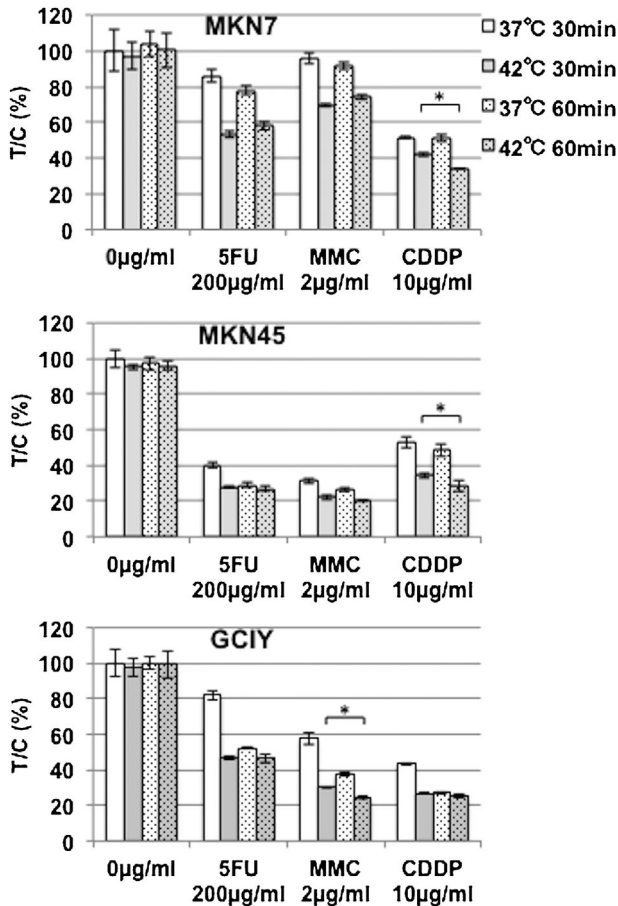
Under hyperthermic conditions, the exposure of all 3 cell lines to 5-FU for 30 min showed a tumor growth-inhibitory effect that was equivalent to 60 min of exposure to 5-FU. Under hyperthermic conditions, exposure of the cell lines MKN7 and MKN45 to MMC for 30 min led to a tumor growth-inhibitory effect that was equivalent to 60 min of exposure to MMC, and exposure of the cell line GCIY to CDDP for 30 min was equivalent to 60 min of exposure to CDDP. Compared with 30 min of exposure, 60 min of exposure to MMC significantly inhibited the growth of the GCIY cells and 60 min of exposure to CDDP significantly inhibited the growth of the MKN7 and MKN45 cell lines. After 60 min of exposure, MMC induced a 13.1% increase in the T/C in the GCIY cell line, while CDDP induced a 7.5% increase in the T/C in the MKN7 cell line and a 5.9% increase in the T/C in the MKN45 cell line. Hence, we decided that the appropriate duration for HIPEC was 30 min, because exposure to the drugs for 30 min under hyperthermic conditions had sufficient anti-tumor effects, and 30 min of exposure might reduce the risks associated

with prolonged hyperthermia and minimize the movement of the drugs from the peritoneum into the bloodstream.

### 3.4 | Combined effects of the anticancer drugs

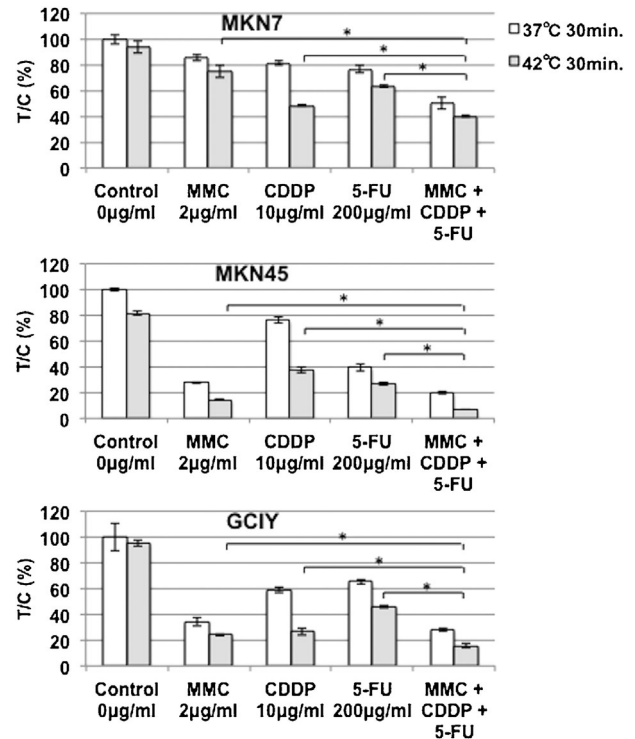
The use of 5-FU (200 µg/mL), MMC (2 µg/mL), and CDDP (10 µg/mL) in combination for 30 min significantly inhibited the growth of the 3 GC cell lines compared with the use of any of the anticancer drugs alone under both hyperthermic and normothermic conditions (Figure 3). Using the three drugs in combination under hyperthermic conditions at 42°C significantly enhanced the drugs' anti-tumor effects on all 3 GC cell lines compared with the anti-tumor effects achieved by the three drugs used in combination under normothermic conditions at 37°C.

The combined use of the three drugs for 30 min significantly inhibited the growth of the 3 GC cell lines, as compared with the use of any combination of two drugs analyzed in this study under hyperthermic conditions (data not shown).



**FIGURE 2** The sensitivities of each drug (5-fluorouracil: 200 μg/mL, mitomycin C: 2 μg/mL, and cisplatin: 10 μg/mL) against the cell lines (MKN7, MKN45, and GCIY) that were exposed to the drugs for 30 or 60 min under normothermic (37°C) or hyperthermic (42°C) conditions. \* $P < 0.05$ ; 5-FU, 5-fluorouracil; CDDP, cisplatin; MMC, mitomycin C; T/C (%), sensitivity, where T was the image optical density of the treated group and C was the image optical density of the control group

Next, we examined the antitumor effects of HIPEC with the three drugs combined in the 3 GC cancer cell lines, for a duration of 30–120 min. Each GC cell line was exposed to the three drugs combined for 30, 60, 90, and 120 min under hyperthermic (42°C) conditions in vitro (Figure 4). Compared with 30 min of exposure, 90 and 120 min of exposure to the combination of the three drugs under hyperthermic conditions significantly inhibited the growth of the MKN7 cells with a small T/C increase of 7.7% or 10.1%, respectively. Sixty, 90, and 120 min of exposure to the 3-drug combination significantly inhibited the growth of the MKN45 cells, as compared with 30 min of exposure with a small T/C increase of 2.0%, 4.5%, or 5.4%, respectively. Thirty minutes of exposure to the 3-drug combination showed a tumor growth-inhibitory effect on GCIY cells equivalent to 60, 90, and 120 min of exposure. Again, we found limited antitumor benefits for long time exposures to anticancer drugs under hyperthermic conditions, and propose that the appropriate duration for HIPEC using the 3-drug combination should be 30 min.



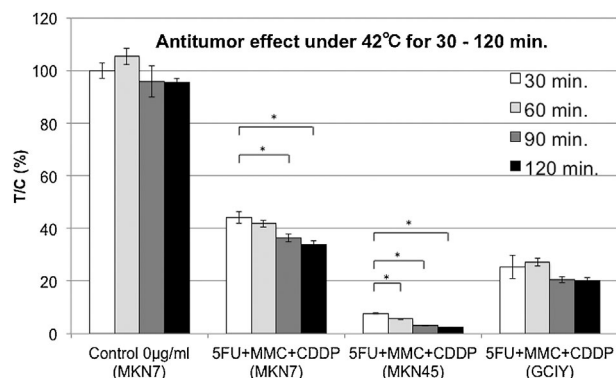
**FIGURE 3** The sensitivity of each drug used alone (mitomycin C [MMC]: 2 μg/mL, cisplatin [CDDP]: 10 μg/mL, or 5-fluorouracil [5-FU]: 200 μg/mL) or in combination (MMC: 2 μg/mL, CDDP: 10 μg/mL, and 5-FU: 200 μg/mL) against the cell lines (MKN7, MKN45, and GCIY) exposed to the drugs for 30 min under normothermic (37°C) or hyperthermic (42°C) conditions. \* $P < 0.05$ ; 5-FU, 5-fluorouracil; CDDP, cisplatin; MMC, mitomycin C; T/C (%), sensitivity, where T was the image optical density of the treated group and C was the image optical density of the control group

## 4 | DISCUSSION

In this experimental study, we found that 5-FU, CDDP, and MMC used in combination under hyperthermic conditions had a greater antitumor effect compared with their use in combination under normothermic conditions or compared with their use as single agents, suggesting that it would be worthwhile to clinically investigate the feasibility and safety of HIPEC for advanced GC.

Many reports describe the use of surgery plus HIPEC for the treatment of advanced gastrointestinal cancers; however, HIPEC protocols have not yet been established. The CDDP and MMC dosages, which are mostly used for HIPEC in gastrointestinal cancers, range from 50 to 300 mg and 10 to 50 mg, respectively, and the HIPEC duration varies from 30 to 150 min.<sup>4,5,9,10,20,25,26</sup>

The greatest systemic toxicities resulting from the high doses of CDDP and/or MMC administered with HIPEC are acute renal failure and myeloid suppression.<sup>26</sup> Our in vitro study that used CD-DST to assess the anti-tumor effects of the drugs determined that the increases in tumor growth inhibition caused by higher doses of CDDP or MMC were limited compared with those induced by lower doses of each drug, and that hyperthermia or using the anticancer drugs (5-FU,



**FIGURE 4** The sensitivity of the GC cell lines (MKN7, MKN45, and GCIY) to the 3-drug combination (mitomycin C [MMC]: 2 µg/mL, cisplatin [CDDP]: 10 µg/mL, and 5-fluorouracil [5-FU]: 200 µg/mL) when exposed to the drugs for 30, 60, 90, and 120 min under hyperthermic (42°C) conditions. \* $P < 0.05$ ; 5-FU, 5-fluorouracil; CDDP, cisplatin; MMC, mitomycin C; T/C (%), sensitivity, where T is the image optical density of the treated group, and C is the image optical density of the control group

CDDP, and MMC) in combination enhanced the anti-tumor effect. Hence, our approach that involved reducing the doses and using the anti-tumor drugs in combination under hyperthermic conditions was reasonable and warranted examination in a clinical study of HIPEC.

5-FU has been used extensively in peri-operative chemotherapy for GC. Chemotherapeutic agents that act in a non-cell cycle-dependent manner are preferable for HIPEC from a pharmacological perspective, because it is administered within a short timeframe. 5-FU, folic acid, and nucleic acid analogs exhibit cytotoxic and anti-tumor effects that result from the inhibition of nucleotide metabolism and/or DNA replication, thereby indicating the specificity of these agents for cells in the S-phase. Therefore, 5-FU is considered a cell cycle- and time-dependent drug, and it is currently used in repeated early postoperative intraperitoneal chemotherapy following HIPEC.<sup>9,27</sup> However, 5-fluorouridine diphosphate and 5-fluorouridine triphosphate, which are 5-FU metabolites, exert their cytotoxic effects by becoming incorporated into RNA.<sup>13,14</sup> In addition, 5-FU acts not only as an anti-metabolite in pyrimidine metabolism, but also as an inducer of reactive oxygen species, which are involved in the mechanisms that lead to cell death and have anti-tumor effects.<sup>17,18</sup> Furthermore, cancer chemotherapy increases the production of reactive oxygen species via an interaction with hyperthermia.<sup>19,20</sup> In the present study, a tumor-suppressive effect was found after the GC cells were briefly exposed (30 min) to a high concentration of 5-FU, which may reflect a direct cytotoxic effect independent of the cell cycle. Moreover, the anti-tumor effect of 5-FU combined with hyperthermia was dose-dependent rather than time-dependent. These findings suggest that 5-FU is a promising drug for HIPEC in GC.

We acknowledge that our study had some limitations. Although we examined the antitumor effects of different concentrations of each drug in detail, we did not investigate all possible combinations of different concentrations for each drug, a various hyperthermic conditions, or a range of drug exposure times to determine whether

improved antitumor effects could be achieved. It is possible that there exists an optimal combination of drug concentration and exposure time in a strict temperature of hyperthermic conditions in vitro. However, optimum conditions in vitro are not always equivalent to conditions in vivo. In addition, the optimum combinations of HIPEC determined from the antitumor effects in vitro would be extremely limited in patients who have experienced extensive surgical stress associated with GC surgery. Thus, it is acceptable to determine a HIPEC protocol in vitro for clinical application that minimizes adverse effects by reducing the dose of drugs and HIPEC duration, while achieving an adequate antitumor effect.

On the other hand, as shown in Figures 1-3, each cell line displays a different sensitivity to hyperthermia. Therefore, as a next step, it is important to analyze the effect of hyperthermia on each cancer cell line in detail by comprehensively combining the drugs and drug exposure periods, because it might be possible to reduce the dose of anticancer drugs or to use a single agent or two agents in combination by adjusting the thermal period in hyperthermia-sensitive tumor cells. These findings will provide an opportunity to identify biomarkers associated with thermally sensitive or insensitive tumors. Thereafter, we can choose optimal HIPEC methods that maintain sufficient antitumor effects with minimal toxicity to each cancer patient based on the hyperthermia-sensitivity of the tumor.

## 5 | CONCLUSIONS

In conclusion, we have shown the new regimens of 5-FU combined with MMC and CDDP with a relatively small amount of each drug, short exposure time against cancer cells, and 42°C of mild hyperthermia for HIPEC after gastrectomy for GC. Clinical studies should be conducted to confirm the safety and efficacy of this promising treatment regimen for advanced GC.

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