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ORIGINAL ARTICLE

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# Flavone acetic acid increases the cytotoxicity of mitomycin C when combined with hyperthermia

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**Abstract** Flavone acetic acid (FAA, NSC 347512) is known to selectively reduce tumor blood flow. Taking advantage of this pharmacodynamic effect, we have previously shown that FAA in combination with hyperthermia (HT) can produce a marked improvement in antitumor response in mice. In the present study, we investigated whether FAA could increase the cytotoxicity of mitomycin C (MMC), a bioreductive drug with selective cytotoxicity against hypoxic cells, under either normothermic or hyperthermic conditions. *In vitro*, the cytotoxicity of MMC against B16 melanoma cells was not enhanced with exposure to FAA at concentrations less than 100 μg/ml, even when combined with HT (43°C, 60 min). The cytotoxicity of MMC (1 μg/ml) at pH 6.5, however, was enhanced by exposure of cells to hypoxia in combination with HT. *In vivo*, the tumor growth time, calculated as the time required to double the initial tumor volume, was 5.2, 6.8, 8.5, and 15.0 days with FAA (150 mg/kg) alone, MMC (4 mg/kg) alone, FAA + MMC, or FAA + MMC + HT (43°C, 15 min) treatment groups, respectively. Antitumor response obtained in animals treated with FAA plus MMC with HT was clearly better than that obtained in any of the other groups. Scheduling of FAA, MMC, and HT was found to be important in producing optimal antitumor response. Administration of MMC (4 mg/kg) prior to FAA (150 mg/kg) and subsequent HT treatment was superior to administration of FAA before MMC. In an attempt to explain these findings, the influence of FAA on blood flow in skeletal muscle and in tumor was examined using a laser blood flowmeter. FAA administration to mice produced a 75% reduction in blood flow to the tumor for up to 2 h but had no detectable effect on normal skeletal blood flow. Our current explanation of the increased antitumor response achieved with the combination of MMC, FAA, and HT is as follows. The FAA-mediated decrease in blood flow to the tumor, when combined with HT, may produce sufficiently hypoxic conditions to significantly increase the antitumor efficacy of the bioreductive drug, MMC. We believe that clinical testing of this combined drug treatment with hyperthermia is warranted.

**Key words** Flavone acetic acid · Mitomycin C · Hyperthermia · Tumor blood flow · Hypoxia

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## Introduction

Flavone acetic acid (FAA) was initially prepared as a potential anti-inflammatory agent by Lipha (France) in 1984. Antitumor activity against several murine solid tumors and human xenografts has been reported [10, 33, 38]. Synergistic cytotoxicity with the cytokine, interleukin 2 [44], has renewed interest in FAA and has prompted new clinical trials [18]. Recently, one partial response was reported in a patient with renal cell carcinoma [30]. Although the precise mechanism of the broad spectrum of antitumor activity of FAA against murine solid tumors is unknown, a number of unique pharmacological effects of FAA on solid tumors has been demonstrated. For example, it has been shown to: (1) increase hypoxia within solid tumors by reducing tumor blood flow, resulting in hemorrhagic necrosis [45]; (2) decrease ATP concentration and inhibit plasma membrane $Na^+/K^+$-ATPase activity by interfering with the generation of adenosine diphosphate and with inorganic phosphate utilization [9]; (3) mediate alterations in natural killer cells, tumor necrosis

factor, and other components of the immune system [3, 10]; and (4) result in extensive single-strand DNA breaks [2]. Among these mechanisms, reduction of tumor blood flow has been thought to be most responsible for the cytotoxic action towards solid tumors [27].

Mitomycin C (MMC), a naturally occurring prototype bioreductive alkylating agent with a wide range of antitumor activity against human tumors, has been shown to be selectively cytotoxic toward hypoxic tumor cells in vitro [16, 21, 22, 41] and in vivo [35] by producing DNA cross-links. Hyperthermia (HT) has now been widely used in clinical situations [14, 40], and would appear to have therapeutic potential in the treatment of solid tumors, especially when used in combination with other treatments such as radiation [13] and chemotherapy including MMC [4] and FAA [37]. The antitumor effect of HT is greatly enhanced by hypoxia [34] or acidic conditions [12]. The development of thermotolerance within cultured cells has also been shown to be inhibited by acidic pH [29].

In the present study, we investigated whether the cytotoxicity and antitumor efficacy of MMC could be enhanced in vitro and in vivo by the administration of FAA in combination with HT. We proposed that FAA would produce a marked reduction in tumor blood flow at a time when MMC would reach its maximum intra-tumor concentration. The bioreductive drug would then be trapped and kept at a high concentration in the tumor, where it would be more effective due to FAA-mediated hypoxia. Moreover, a FAA-induced hypoxic condition would also enhance the cytotoxicity of HT. Thus, the combination of FAA plus MMC with HT might be uniquely synergistic against solid tumor growth. Here, we describe the combined effect of FAA plus MMC in combination with HT against B16 melanoma cells, and discuss potential mechanisms of the enhanced cytotoxicity of the combined treatment.

## Materials and methods

### Drugs

FAA was obtained from Professor R.A. Newman (Department of Clinical Investigation, M.D. Anderson Cancer Center, Houston, Tex.). FAA was dissolved in physiological saline just prior to use. MMC was obtained from Kyowa Hakko (Tokyo, Japan) and was dissolved in Hank's solution immediately prior to use.

### B16 cell culture

For in vitro experiments, B16 melanoma cells (obtained from Dr. S. Taniguchi, Medical Institute of Bioregulaiton of Kyushu University, Fukuoka, Japan) were cultured in monolayers on 60-mm plastic dishes (Corning 25010, Iwaki Glass, Japan) using Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, N.Y.). Cells were maintained at 37°C in a humidified 5% $CO_2$ atmosphere.

### Colony formation assay

Three hundred exponentially growing B16 melanoma cells were placed in 60-mm dishes without any drugs and were incubated at 37°C for 24 h. Cells were then exposed to various concentrations of both MMC (0.1–5 μg/ml) and FAA (0.1–100 μg/ml) at 37°C for 60 min. The concentration of FAA examined in this study, below 100 μg/ml, is a clinically achievable level. In groups treated with HT, cells were incubated at 43°C for 60 min simultaneously with drugs. After treatment, cells were washed 3 times with phosphate-buffered saline and incubated in fresh medium. After 1 week, colonies were stained with Giemsa solution and those which contained more than 50 cells were counted. Effects of treatment were evaluated by determining the extent of inhibition of colony formation. Each experiment was done in triplicate.

### Hypoxic conditions

Cells were exposed to hypoxic conditions induced by incubation for 4 h in a humidified mixure of 95% $N_2$ and 5% $CO_2$, using a Bellco glass incubator (Bellco Glass, USA) [20]. Cells were preincubated for 3 h in a humidified atmosphere followed by exposure to the drugs for 1 h.

### Regulation of pH

The pH was regulated by using different concentrations of $NaHCO_3$ in the medium while maintaining the $CO_2$ concentration constant at 5% [1]. Cells were exposed to a given pH for the same duration as the hypoxic treatment, 3 h preincubation and 1 h incubation in the presence of MMC (1 μg/ml). The maximum pH deviation during hypoxic treatment was less than 0.05 throughout each experiment. Exposure of B16 melanoma cells to either hypoxia or acidic pH (in the range 6.5–7.4) or the combination of both conditions for 4 h had no adverse effect on cell survival.

### Animals

C57BL/6NCrj male mice (5 weeks old; 18–23 g) were obtained from Charles River Japan (Tokyo, Japan), and were housed under constant temperature and humidity conditions. Mice were fed a diet of standard laboratory chow, allowed free access to water, and housed six per cage in a controlled environment with a 12-h light/dark cycle. A 6-day environmental adaptation period was allowed prior to use of these animals for experiments.

### Tumors

For in vivo experiments, B16 melanoma cells were transplanted by subcutaneous injection of $5 \times 10^5$ tumor cells in a volume of 0.05 ml of Hank's solution into the lower part of thigh, where hyperthermia-related damage to intra-abdominal organs could be avoided. Tumors grew to diameters of 6–7 mm within 7–9 days after injection of the cell suspension. All experiments were performed with this size of tumor.

### Treatment of B16 melanoma solid tumor in vivo

Ninety-six tumor-bearing mice were divided into 12 groups for different treatments as shown in Table 1. Each group included eight tumor-bearing mice. Body weight was measured daily, and evidence

**Table 1.** Treatment groups. Each mouse was given saline or mitomycin C (*MMC*) and/or flavone acetic acid (*FAA*), with or without hyperthermia

| Group | Temperature (°C) | Treatment |
|---|---|---|
| 1 | 37.0 | NaCl |
| 2 | 37.0 | MMC (2 mg/kg) |
| 3 | 37.0 | MMC (4 mg/kg) |
| 4 | 37.0 | FAA (150 mg/kg) |
| 5 | 37.0 | MMC (2 mg/kg) + FAA (150 mg/kg) |
| 6 | 37.0 | MMC (4 mg/kg) + FAA (150 mg/kg) |
| 7 | 43.0 | NaCl |
| 8 | 43.0 | MMC (2 mg/kg) |
| 9 | 43.0 | MMC (4 mg/kg) |
| 10 | 43.0 | FAA (150 mg/kg) |
| 11 | 43.0 | MMC (2 mg/kg) + FAA (150 mg/kg) |
| 12 | 43.0 | MMC (4 mg/kg) + FAA (150 mg/kg) |

of gastrointestinal toxicity, as determined by the presence of bloody diarrhea, was recorded at this time. In combination experiments, drugs were administered just prior to HT, which was induced by immersing the tumor-bearing lower part of the mouse thigh into a circulating water bath (Model T-10, Thermonics, Tokyo, Japan) at 43°C for 15 min. Control mice were exposed to normothermic (37°C) treatment for 15 min.

The mean tumor volume of each group at the time of treatment on day 0 was similar. Control groups of mice received saline instead of drugs. Tumor size was measured with a digital caliper (DP-1 HS, Mitutoyo, Tokyo, Japan) every day after treatment. Body weight and macroscopic evidence of gross gastrointestinal toxicity (such as diarrhea or nasal and urogenital bleeding) were also recorded daily. Tumor volume was determined from measurement of two perpendicular diameters by use of the following formula [5]:

$$\text{Tumor volume} = 1/2 \times \text{length} \times (\text{width})^2$$

Relative tumor volume, tumor growth time (TGT), and tumor growth delay (TGD) were used to evaluate the antitumor effect. Relative tumor volume was determined from the ratio of tumor volume on each day of treatment to the initial tumor volume on day 0. TGT was calculated as the time required to reach twice the initial tumor volume. TGD was calculated by subtracting the TGT of the control tumor from that of treated tumors.

### Timing and sequence of drugs and heat

The relative antitumor efficacy of FAA followed by MMC compared to MMC followed by FAA was explored without exposure of mice to hyperthermia. These studies consisted of FAA (150 mg/kg) being administered at $-4$, $-2$, $-1$, 0, $+1$, $+2$, and $+4$ h relative to administration of MMC (4 mg/kg; hour 0). Once the optimal sequence of compounds was found, the relative antitumor efficacy produced by the sequence of drugs followed by HT (43°C, 15 min) was examined.

### Measurement of tumor blood flow

Tumor and muscle blood flow of mice, treated with FAA (150 mg/kg) alone, were measured with a laser blood flowmeter and flow probe (Biomedical Science, Kanazawa, Japan) [28]. After immobilization of the animal, a small incision was made in the skin and tumor surface to introduce the probe, where it was then fixed at a central location over the tumor or muscle tissue. The blood flow of muscle, as control tissue, and of the tumor were measured from 30 min before to 4 h after administration of FAA in four tumor-bearing mice.

### Statistical analysis

Statistical differences in data were analyzed by Student's *t*-test, where *P* values of less than 0.05 were considered to be significant.

## Results

### Effect on colony formation

The cytotoxic effects of FAA and/or MMC ( + / − HT) in B16 melanoma cells are shown in Fig. 1. At the normal body temperature of 37°C, MMC (1 μg/ml) cytotoxicity was not enhanced by coincubation with FAA up to concentrations of 100 μg/ml (Fig. 1A). Exposure of cells to HT (43°C, 60 min) produced a heat-mediated increase in cytotoxicity of MMC (3.8-fold) and FAA (1.8-fold) relative to drug responses obtained at 37°C. The enhancement of MMC cytotoxicity by heat, however, was independent of the relative FAA concentration to which cells were exposed (Fig. 1B).

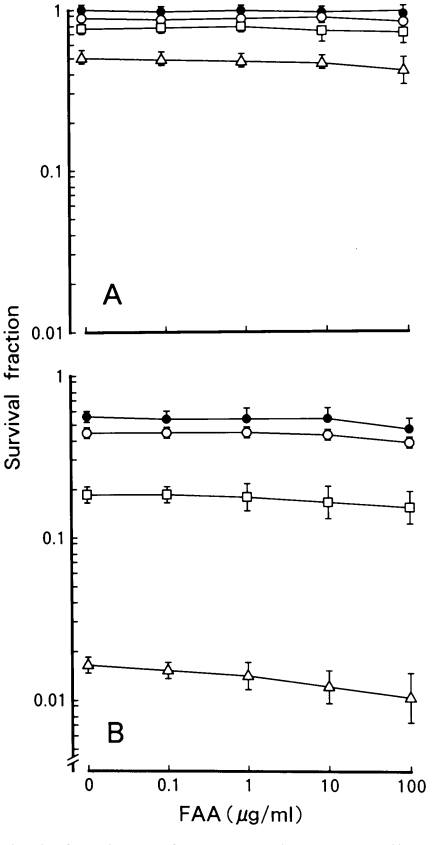


**Fig. 1** Survival fraction of B16 melanoma cells exposed to mitomycin C (*MMC*) and flavone acetic acid (*FAA*) at 37°C (**A**) or 43°C (**B**) for 60 min. The concentrations of MMC were: 0 μg/ml (●), 0.1 μg/ml (○), 1 μg/ml (□), 5 μg/ml (△). Data are presented as mean ± SD from three experiments

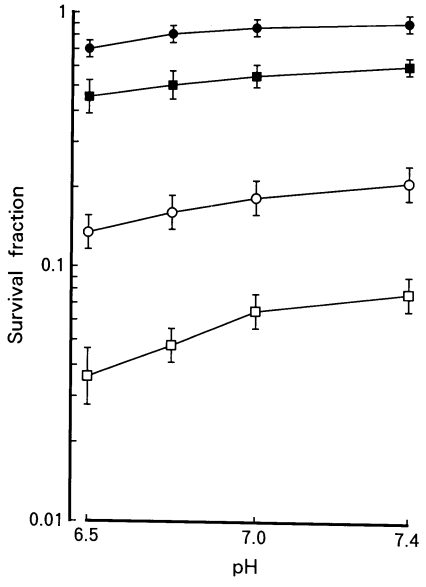


**Fig. 2** Survival fraction of hypoxic B16 melanoma cells exposed to MMC (1 μg/ml) at various pHs with or without hypoxia and hyperthermia. Hypoxic cells were preincubated for 3 h and exposed to MMC for 1 h under hypoxic conditions. *Symbols*: MMC under aerobic conditions at 37°C (●), MMC under aerobic conditions at 43°C (○), MMC under hypoxic conditions at 37°C (■), MMC under hypoxic conditions at 43°C (□). Data are presented as mean ± SD from three experiments

The survival of B16 melanoma cells exposed to MMC (1 μg/ml) at various pHs with or without HT under aerobic and hypoxic condition is presented in Fig. 2. Under aerobic conditions, the survival fraction of cells after exposure to MMC was only slightly decreased at a pH less than 7.0, with a survival fraction of $7.7 \times 10^{-1}$ at pH 7.4 and $6.9 \times 10^{-1}$ at pH 6.5. Under hypoxic conditions, the cytotoxicity of MMC in combination with HT was significantly enhanced, especially at an acidic pH. The survival fraction of cells was $7.5 \times 10^{-2}$ at pH 7.4 and $3.8 \times 10^{-2}$ at pH 6.5. The cytotoxicity of MMC was most enhanced in combination with HT at an acidic pH under hypoxic conditions.

## Effect on B16 melanoma solid tumors

Tumor growth curves after treatment with FAA (150 mg/kg) alone or in combination with MMC (2 mg/kg or 4 mg/kg) with or without HT (43°C, 15 min) are shown in Fig. 3. FAA or HT alone showed minimal effects on tumor growth. Treatment with FAA (150 mg/kg) plus MMC (4 mg/kg) in combination with HT, however, resulted in a significant reduction in tumor volume. TGT data for FAA and/or MMC with or without HT are summarized in Table 2. TGT in the control group was 4.3 ± 1.0 days. TGT for MMC (2 mg/kg) alone, FAA (150 mg/kg) alone, and MMC plus FAA was 5.8 ± 1.3, 5.2 ± 1.2, and 6.2 ± 1.1 days, respectively. There were no significant differences in TGT compared to that of control groups. In combination with HT, however, the antitumor effects of FAA, MMC (4 mg/kg), FAA plus MMC (2 mg/kg), and FAA plus MMC (4 mg/kg) were all significantly enhanced ($P < 0.05$) compared to respective groups without HT.

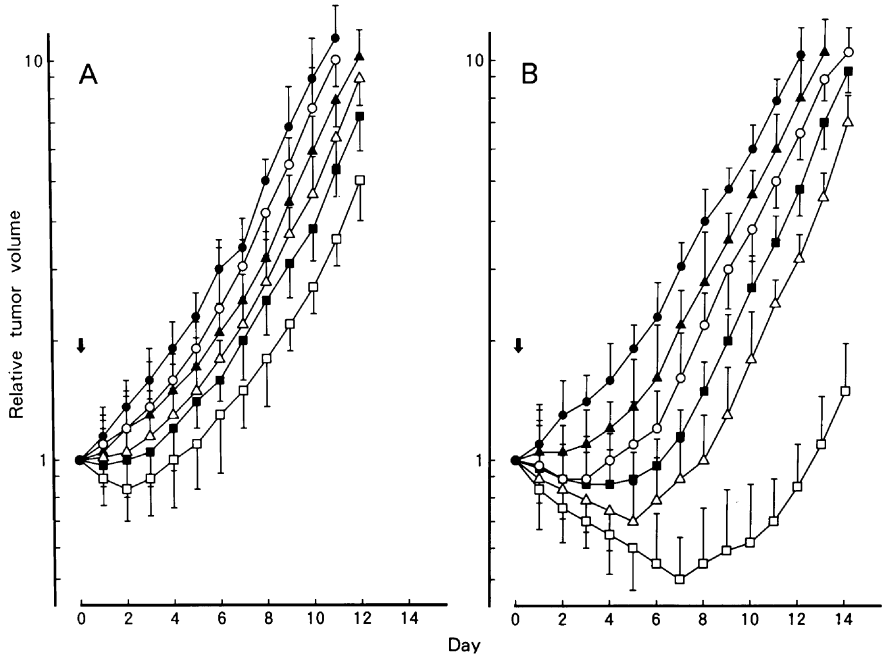


**Fig. 3** Growth curves of B16 melanoma tumors in C57BL/6NCrj male mice treated with various combinations of MMC and/or FAA under normothermic (**A**) or hyperthermic (**B**) conditions. Relative tumor volume was expressed as the ratio of tumor volume on each day to initial tumor volume at time of treatment. *Symbols*: Control (●), 2 mg/kg MMC (▲), 4 mg/kg MMC (■), 150 mg/kg FAA (○), 2 mg/kg MMC + 150 mg/kg FAA (△), 4 mg/kg MMC + 150 mg/kg FAA (□). Data are presented as mean ± SD of tumor measurements from eight mice. *Arrow* indicates first day of drug treatment

**Table 2.** Tumor growth time (*TGT*), tumor growth delay (*TGD*), and body weight changes after treatment with FAA, MMC and hyperthermia, either alone or in combination. Data are presented as mean ± SD

| Group[a] | TGT (days)[b] | TGD (days)[c] | Body weight change (%)[d] |
|---|---|---|---|
| 1 | 4.3 ± 1.0 | 0.0 | 99.8 |
| 2 | 5.8 ± 1.3 | 1.5 | 100.9 |
| 3 | 6.8 ± 0.9 | 2.5 | 98.9 |
| 4 | 5.2 ± 1.2 | 0.9 | 98.8 |
| 5 | 6.2 ± 1.1 | 1.9 | 97.9 |
| 6 | 8.5 ± 0.7 | 4.2 | 99.1 |
| 7 | 5.2 ± 1.2 | 0.9 | 99.4 |
| 8 | 6.6 ± 1.1 | 2.3 | 97.8 |
| 9 | 9.0 ± 1.1[e] | 4.7 | 98.4 |
| 10 | 7.6 ± 0.5[e] | 3.3 | 97.1 |
| 11 | 10.3 ± 1.1[e] | 6.0 | 98.8 |
| 12 | 15.0 ± 1.8[e] | 10.7[f] | 97.9 |

[a] See Table 1 for explanations of the treatment groups
[b] TGT was calculated as time required to reach a tumor volume twice that of the initial tumor volume
[c] TGD was calculated by subtracting the tumor growth time of the control group from that of treated group
[d] The mean body weights on day 7, expressed as percentage of that on day 0 in each group
[e] Significantly different from the same dose without hyperthermia ($P < 0.05$)
[f] Significantly different from all other groups ($P < 0.01$)

The greatest enhancement of antitumor effect of MMC (4 mg/kg) was observed when it was combined with FAA and HT. This response was larger than the sum of the two treatments given separately. Mean body weight 7 days after treatment in all treated groups revealed no significant difference from the control group. The mice tolerated these doses of FAA plus MMC with HT well and there was no obvious toxicity or deaths.

As shown in Fig. 4A, the antitumor effect of MMC was substantially increased when FAA was administered after rather than before administration of MMC. When combined with HT, the maximum antitumor effect was observed when FAA was administered 1 h after MMC (Fig. 4B).

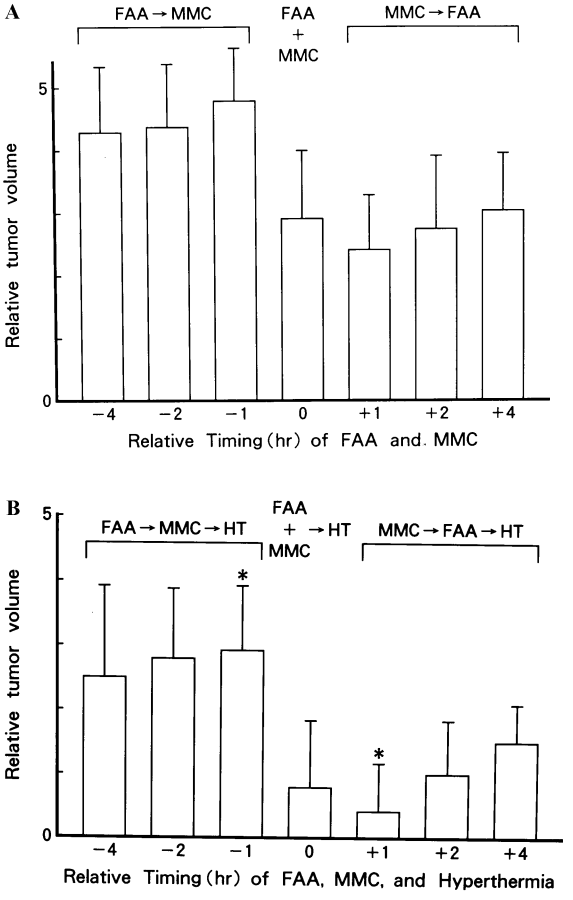


**Fig. 4A** Effect of scheduling of MMC and FAA without hyperthermia (*HT*) on mouse tumor volume. Data are presented as mean ± SD of relative tumor volumes in mice ($n = 6$) treated with MMC (4 mg/kg) and/or FAA (150 mg/kg) at various times. Measurements of relative tumor volumes were made on day 10 in each group. FAA was administered at − 4, − 2, − 1, 0, + 1, + 2, and + 4 h relative to administration of MMC at time 0. **B** Effect of scheduling of MMC (4 mg/kg), FAA (150 mg/kg), and HT (43°C, 15 min) on mouse tumor volume. Data are presented as the relative tumor volumes on day 10 in each group. HT was added 2 h after administration of the final drug (see above). *Asterisk* indicates, significant difference ($P < 0.05$) between administration of FAA 1 h after compared to 1 h before MMC treatment

## Blood flow

Figure 5 shows the effect of FAA (150 mg/kg) on both intra-tumoral and intra-muscular blood flow. The mean initial blood flow in the tumor was 88% of that in the muscle. Although the blood flow in the muscle remained unchanged after administration of FAA, tumor blood flow was significantly reduced to approximately 25% of initial muscle blood flow within 10 min after injection of FAA, and remained at this low flow rate for about 2 h before gradually returning to normal.

## Discussion

We have shown that the cytotoxicity of MMC is enhanced *in vitro* when cells are exposed to hypoxic conditions. The most marked enhancement was observed at an acidic pH in combination with HT. This is in agreement with other *in vitro* studies of oxygen dependence of cell killing by MMC [20]. MMC requires reductive transformation of its quinone group by either DT-diaphorase [8, 25] or NADPH cytochrome *c* reductase [15, 19] in order to exhibit alkylating activity. Under hypoxic conditions, certain enzyme systems are capable of transforming MMC to active metabolites [16, 17] through addition of electrons.

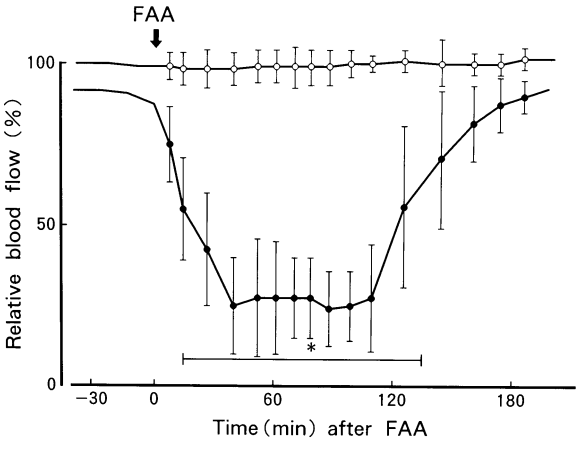


**Fig. 5** Changes in blood flow of s.c. B16 melanoma tumor and muscle in mice treated with FAA (150 mg/kg). Blood flow was measured with a laser blood flowmeter. *Symbols*: muscle blood flow (○), tumor blood flow (●). Data are presented as mean ± SD from four experiments. *Asterisk* indicates significant difference ($P < 0.05$) for four treatment groups whose blood flow was altered by administration of FAA relative to their respective control values. *Arrow* indicates time of FAA administration

This phenomenon results in enhanced cytotoxicity of MMC toward hypoxic subpopulations within solid tumors [16] by producing interstrand DNA cross-links [23]. Under acidic conditions, the cell-killing effect of heat is also enhanced [42] because of the combined effect of acidosis-induced lysosomal activity and heat-induced cell membrane damage [31, 32].

Our results clearly demonstrate that the cytotoxicity of MMC was enhanced by FAA in combination with HT in *vivo*, but not *in vitro*. *In vitro*, this result is explained by the fact that the mechanism of FAA is mainly based on a host-mediated action, such as reduction of tumor blood flow [45] and enhancement of the immune system [3, 10]. *In vivo*, significant differences were noted between the treatment of FAA given 1 h before as opposed to 1 h following MMC administration. As MMC has reached its maximum plasma concentration within almost 1 h in tumor-bearing male DBA/2J mice after i.p. administration of MMC [6], it seems possible that the maximal plasma concentration of MMC, given i.p. in C57/BL mice, also occurs within this time. Reduction of tumor blood flow occurs within 10 min after FAA administration. Thus, with the administration of MMC 1 h preceding FAA, peak plasma levels of MMC in mice would occur before the FAA-mediated reduction in tumor blood flow. This may result in MMC being trapped and maintained within the tumor at a high concentration. The reduced tumor blood flow with resulting hypoxia would tend to optimize the metabolism of MMC to active cytotoxic species and enhance cell killing effectiveness. When combined with HT, the increase in antitumor efficacy of the combined treatment may be explained by the fact that the FAA-mediated hypoxic condition has been shown to increase antitumor activity of HT alone as well as that of MMC alone [16, 37]. The combined effect of MMC plus HT was also obserevd in the *in vitro* hypoxic experimental model. When FAA was administered before MMC, FAA-induced lower tumor blood flow may have restricted the uptake of MMC into the tumors. Even when combined with HT, enhancement of the combined antitumor effect was limited. Thus, the increase in antitumor effect of MMC on B16 melanoma seems to be critically dependent on the time of its administration relative to that of FAA.

Duke et al. [7] reported the i.p. administration of FAA (200 mg/kg) produced a 60% reduction in MAC 26 tumor blood volume using an Evan's blue perfusion technique, which was first demonstrated 4 h after treatment and persisted for 24 h. Zwi et al. [45] reported that perfusion of colon 38 tumors, using the fluorescent stain, H33342, was reduced to 50% of controls within 3 h of i.p. administration of FAA (1.2 mmol/kg) and was completely inhibited by 24 h. Several factors such as tumor type, animal species, route of administration of FAA, and method of measurement of blood flow may influence the actual extent of reduction in tumor blood flow. There are now several proposed mechanisms of FAA-induced reduction of tumor blood flow. These include a change in endothelial barrier function leading to increased vascular permeability [43], an alteration of platelet function through inhibition of platelet adhesion [36], an increase in intravascular coagulopathy [26], and vascular failure mediated by induction of tumor necrosis factor [24].

There have been several reports that vasoactive drugs, which can selectively reduce the blood flow of tumors but not that of normal tissues, enhance the antitumor effect of hypoxic-target antitumor drugs and bioreductive drugs. Reduction of tumor blood flow could be potentially useful for enhancing the antitumor effect of such antitumor drugs and HT [11, 39]. In the present study, we have shown that, in a solid tumor, cytotoxicity of MMC was significantly enhanced by administration of FAA in combination with HT, and that marked enhancement was achieved when FAA was given 1 h after MMC, followed by HT. Although FAA alone has failed to show antitumor effects in clinical trials at present, it is currently being investigated for a potential synergistic effect with interleukin 2. FAA may be effective in inducing hypoxic conditions in human solid tumors, which may lead to enhancement of the amtitumor effect of hypoxic-target agents such as MMC. The enhanced antitumor efficacy of the combined treatment may represent a novel approach to selective therapy of human solid tumors, which includes hypoxic subpopulations. However, before clinical application of our proposed chemo-hyperthermia treatment, a detailed evaluation of the desired effect of FAA on human tumors, followed by combination with a single and then a multiple treatment therapy must be carried out in preclinical experiments. Pharmacokinetic

determination of MMC and FAA within tumors and the determination of these concentrations within the peripheral circulation may help in an understanding of the efficacy of this novel combination of treatments.

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