Usefulness of combined treatment with continuous administration of tirapazamine and mild temperature hyperthermia in γ-ray irradiation in terms of local tumor response and lung metastatic potential

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Continuously administered TPZ and MTH with γ-rays

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Abstract

Purpose: To evaluate the usefulness of combined treatment with continuous administration of a hypoxic cytotoxin, tirapazamine (TPZ), and mild temperature hyperthermia (MTH) in γ-ray irradiation in terms of local tumor response and lung metastatic potential, referring to the response of intratumor quiescent (Q) cells.

Materials and Methods: B16-BL6 melanoma tumor-bearing C57BL/6 mice were continuously given 5-bromo-2'-deoxyuridine (BrdU) to label all proliferating (P) cells. The tumor-bearing mice then received γ-ray irradiation after a single intraperitoneal injection or 24 h continuous subcutaneous infusion of TPZ, either with or without MTH. Immediately after the irradiation, cells from some tumors were isolated and incubated with a cytokinesis blocker. The responses of the Q and total (= P + Q) cell populations were assessed based on the frequency of micronuclei using immunofluorescence staining for BrdU. In other tumor-bearing mice, 17 days after irradiation, macroscopic lung metastases were enumerated.

Results: Continuous administration elevated the sensitivity of both the total and Q cells, especially the total cells. MTH raised the sensitivity of Q cells more remarkably in both single and continuous administrations, probably because of more exposure to TPZ in intermediately hypoxic areas derived mainly from chronic hypoxia through MTH. With or without irradiation, TPZ, especially administered continuously and combined with MTH, decreased the number of lung metastases.

Conclusion: The combination of continuous long-term administration

of TPZ and MTH in γ-ray irradiation was thought to be promising because of its potential to enhance local tumor response and repress lung metastatic potential.

Key words:
tirapazamine; mild temperature hyperthermia; quiescent cell; acute hypoxia, chronic hypoxia

The authors have no conflict of interest to declare.

Introduction

Many of the cancer cells in solid tumors are non-proliferating (quiescent), and many of the features of quiescent (Q) cells are still unknown [1]. To improve cancer treatment, the response of Q cells to anticancer treatment should be determined, since often tumor cells that are quiescent *in situ* are still clonogenic [1].

The development of bioreductive agents that are particularly toxic to hypoxic cells is considered a promising approach to solving the problem of radio-resistant tumor hypoxia in cancer radiotherapy [2]. Tirapazamine (TPZ), a lead compound in the development of bioreductive hypoxic cytotoxins, in combination with radiation has been shown to be very useful for controlling solid tumors as a whole, especially for controlling Q tumor cell populations that are rich in hypoxic region [2,3]. Tumor hypoxia results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia, transient hypoxia or ischemic hypoxia). Chronically hypoxic tumor cells existing at the rim of the oxygen diffusion distance can be killed by just a single administration of TPZ [3]. Acutely hypoxic tumor cells occurring sporadically throughout solid tumors can be killed by TPZ during long-term continuous administration. Namely, the long-term continuous administration of TPZ can kill both chronically and acutely hypoxic tumor cells [4].

Mild temperature hyperthermia (MTH) was reported to increase the response of tumors to radiation by improving oxygenation through an increase in tumour blood flow [5]. Further, MTH was also shown

to enhance the tumor response, especially of the intratumor Q cell population, to TPZ [6].

Metastasis is a leading cause of cancer deaths and involves a complex, multistep process by which tumor cells disseminate to distant sites to establish discontinuous secondary colonies [7,8]. It was reported that acute and cyclic, but not chronic, hypoxia significantly increased the number of spontaneous lung metastases in mice by a factor of about 2, and that this effect was due to the influence of the acute hypoxia treatment on the primary tumor and not to other potential effects of the treatment such as damage to the lung epithelium [9,10]. Based on this report, we recently reported the significance of injections of an acute hypoxia-releasing agent, nicotinamide, into tumor-bearing mice as a combined treatment with high dose rate γ-ray irradiation in terms of reducing the number of lung metastatic nodules [11].

Here, using a readily metastasizing murine melanoma cell line, we tried to analyze the usefulness of combined treatment with continuous long-term administration of TPZ and MTH in radiotherapy with γ-rays in terms of local tumor response and lung metastatic potential. Concerning the local tumor response, the effect not only on the total (= proliferating (P) + Q) tumor cell population but also on the Q cell population was evaluated using our original method for selectively detecting the response of Q cells in solid tumors [3].

Materials and Methods

Mice and tumors

B16-BL6 murine melanoma cells (Institute of Development, Aging and Cancer, Tohoku University) derived from C57BL/6 mice were maintained *in vitro* in RPMI-1640 medium supplemented with 10 % fetal bovine serum. Tumor cells (1.25×10^5) were inoculated subcutaneously into the left hind leg of 8-week-old syngeneic female C57BL/6 mice (Japan Animal Co., Ltd., Osaka, Japan). Eighteen days later, the tumors, approximately 7 mm in diameter, were employed for γ-ray irradiation in this study, and the body weight of the tumor-bearing mice was 20.1 ± 2.3 (mean ± standard error) g. Mice were handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety Handling Regulations for Laboratory Animal Experiments at our university. The p53 of B16-BL6 tumor cells is the wild type [12].

Labeling with 5-bromo-2'-deoxyuridine (BrdU)

Twelve days after the inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously into the animals' backs for 6 days to label all P cells. The percentage of labeled cells after continuous labeling with BrdU was 54.3 ± 6.1 %, and reached a plateau at this stage. Therefore, tumor cells not incorporating BrdU after continuous labeling were regarded as Q cells.

Treatment

After the labeling with BrdU, TPZ dissolved in physiological saline was administered at a dose of 224 micromoles/kg (40 mg/kg) singly by intra-peritoneal injection or continuously for 24 h by subcutaneously implanting mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing TPZ dissolved in physiological saline in the backs of mice. Right after the intra-peritoneal injection or during the last hour of the 24 h of continuous subcutaneous infusion, the tumors grown in the left hind legs of mice were heated at 40°C for 60 min by immersing the tumor-bearing foot in a water bath. Right after the heating, solid tumors grown in the left hind legs of mice were irradiated with a cobalt-60 γ-ray irradiator.

Concerning MTH, the tumors grown in the left hind legs of mice were heated at 40°C for 60 min by immersing the tumor-bearing foot in a water bath. The mouse was held in a specially constructed device with the tail and right leg firmly fixed with an adhesive tape. The left tumor-bearing leg was pulled down by a special sinker (approximately 45 g) which was affixed to the skin of the toe with Superglue (Arone-arufa, Konishi Co., Osaka, Japan). The mice were then placed on a circulating water bath maintained at the desired temperature. The mice were air-cooled during the heat treatment [13]. Temperatures at the tumor center equilibrated within 3 to 4 min after immersion in the water bath and remained 0.2-0.3°C below the bath's temperature. The water bath's temperature was maintained at 0.3°C above the desired tumor temperature.

γ-Ray irradiation was performed with a cobalt-60 γ-ray irradiator at a dose rate of 2.75 Gy/min as conventionally employed

high dose-rate irradiation with tumor-bearing mice held in a specially constructed device with the tail firmly fixed with an adhesive tape. Lead blocks were used to avoid irradiating other body parts than the tumor-bearing left hind leg.

Each irradiation group also included mice that were not pretreated with BrdU.

Immunofluorescence staining of BrdU-labeled cells and micronucleus (MN) assay

Immediately after irradiation, the tumors excised from the mice given BrdU were minced and trypsinized (0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline [PBS], 37°C, 15 min). Tumor cell suspensions were incubated for 72 hours in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division. The cultures were trypsinized, and cell suspensions were fixed and resuspended with cold Carnoy's fixative (ethanol:acetic acid = 3:1 in volume). Each suspension was placed on a glass microscope slide, dried at room temperature and treated with 2 M hydrochloric acid for 60 min at room temperature to dissociate the histones and partially denature the DNA. The slides were immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected by indirect immunofluorescence staining using a monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, CA) and a fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma, St. Louis, MO). To distinguish the tumor cells stained with green-emitting FITC

and observe them separately, cells on the slides were treated with red-emitting propidium iodide (PI, 2 µg/ml in PBS) as a background staining and monitored under a fluorescence microscope.

When cell division is disrupted, or the chromosomes are broken or damaged by chemicals or radiation, the distribution of genetic material between the two daughter nuclei during cell division is affected and pieces or entire chromosomes fail to be included in either of the daughter nuclei. The genetic material that is not incorporated into a new nucleus forms a "micronucleus".Thus, the frequency with which micronuclei form (MN frequency) reflects the genotoxicity of a chemical compound and radiation very well. The MN frequency in cells not labeled with BrdU could be examined by counting the micronuclei in the binuclear cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed [3].

The ratios obtained in tumors not pretreated with BrdU indicated the MN frequency at all phases in the total tumor cell population. More than 300 binuclear cells were counted to determine the MN frequency.

Clonogenic cell survival assay

The clonogenic cell survival assay was also performed for tumors implanted in mice given no BrdU using an *in vivo-in vitro* assay method immediately after irradiation. The tumors not labeled with BrdU were excised, weighed, minced, and disaggregated by stirring for 20 min at 37 °C in PBS containing 0.05 % trypsin and 0.02% EDTA.

The cell yield was 1.2 ± 0.4 x 10^7/g tumor weight. Appropriate numbers of viable tumor cells from the single cell suspension were plated on 60 or 100-mm tissue culture dishes, and, 12 days later, colonies were fixed with ethanol, stained with Giemsa, and counted. The cut-off cell number to be considered a colony was 50. For the tumors that received no irradiation, the surviving fractions (SFs) for the total tumor cell populations and the MN frequencies for the total and Q cell populations are shown in **Table 1**. The fraction of cells surviving a given radiation dose is determined by counting the number of macroscopic colonies as a fraction of the number of cells seeded, followed by allowance, that is, dividing by the fraction of cells surviving no radiation dose.

As stated above, the MN frequencies for Q cells were obtained from BrdU-unlabeled cells in tumors after continuous BrdU labeling *in vivo*. The MN frequencies and surviving fractions (SFs) for total tumor cell populations were obtained from cells in tumors not pretreated with BrdU. Thus, we could not detect any interaction between BrdU and irradiation in our data for the MN frequency and SF.

Growth of B16-BL6 tumors

After γ-ray irradiation at an absorbed dose of 0 or 16 Gy with or without TPZ in combination with MTH on the 18th day after inoculation, the size of the tumors implanted in the left hind legs of some tumor-bearing mice was checked 2-3 times a week for about 20 days. Tumor volume was calculated using the formula: $V = \pi/6 \times a \times b^2$, where a and b are respectively the longest and shortest

diameters of the tumor measured with calipers.

Metastasis assessment

Seventeen days after irradiation (= 35 days after the inoculation of B16-BL6 melanoma cells), the tumor-bearing mice were sacrified by cervical dislocation, and their lungs were removed, briefly washed with distilled water, cleaned of extraneous tissue, fixed in Bouin's solution overnight (Sigma), and stored in buffered formalin 10 % (Sigma) until metastases were counted. Macroscopically visible metastases were counted under a dissection microscope [14]. Eighteen days after the inoculation and immediately before exposure to γ-rays, macroscopic lung metastases were also counted as background data. The number was 5.2 ± 1.6.

Data analysis and statistics

Three mice with a tumor in the left hind leg were used to assess each set of conditions and each experiment was repeated three times. Namely, nine mice were used for each set of conditions. To examine the differences between pairs of values, Student's *t*-test was used when variances of the two groups could be assumed to be equal with Shapiro-Wilk normality test; otherwise the Welch *t*-test was used. *p*-Values are from two-sided tests. The data on cell survival and MN frequencies were fitted to the linear quadratic dose relationship [15].

Results

Table I shows the SFs for the total tumor cell population and the MN frequencies without irradiation for the total and Q cell populations. TPZ treatment resulted in significantly lower SFs and higher MN frequencies in both the total and Q cell populations under each set of conditions. In addition, the continuous administration of TPZ produced significantly lower SFs and higher MN frequencies than a single intraperitoneal administration in the two cell populations under all conditions. Further, addition of MTH to the TPZ treatment led to lower SFs and higher MN frequencies in both cell populations under all conditions. On the other hand, the Q cell population showed significantly higher MN frequencies than the total cell population under each set of conditions.

Figure 1 shows cell survival curves for the total cell population as a function of the absorbed dose of γ-rays with or without TPZ in combination with MTH. **Figure 2** shows net MN frequencies as a function of irradiated absorbed dose with or without TPZ in combination with MTH in the total and Q tumor cell populations. The net MN frequency was the MN frequency in tumors that received irradiation minus that in tumors that did not. Overall, the net MN frequencies were significantly smaller in Q cells than the total cell population.

To estimate the radio-enhancing effect of TPZ, irradiation with TPZ in both the total and Q cell populations was compared with irradiation only, using the data obtained without MTH shown in **Figures 1** and **2** (**Table II**). The radio-enhancing effect of TPZ was

significantly larger than 1.0, and TPZ enhanced the sensitivity of the Q cell population significantly more than that of the total cell population. Further, continuous administration increased the sensitivity of both cell populations significantly more than single intraperitoneal administration.

To estimate the radio-enhancing effect of combined treatment with MTH in both the total and Q cell populations, the data shown in **Figures 1** and **2** were used (**Table III**). Whether TPZ was administered singly or continuously, the sensitivity of the Q cell population seemed to be slightly more enhanced with MTH than that of the total cell population, although there were no significant differences. There were also no significant differences in the radio-enhancing effects between single and continuous administration of TPZ.

To examine the difference in radio-sensitivity between the total and Q cell populations, dose-modifying factors were calculated using the data in **Figures 1** and **2** (**Table IV**). Overall, all the values were significantly larger than 1.0. In combination with TPZ, the difference was significantly decreased. In further combination with MTH, the difference in radio-sensitivity seemed to be smaller, although there were no significant differences. There were also no significant differences in the values between single and continuous administration of TPZ.

Figure 3 shows tumor growth curves after γ-ray irradiation with or without TPZ administration in combination with MTH 18 days after the tumor cell inoculation. To evaluate tumor growth, the period

required for each tumor to become twice as large as on day 18 was obtained using the data shown in **Figure 3** (**Table V**). Without irradiation, the period required for TPZ combination was significantly prolonged with as compared to without TPZ. Continuous administration further lengthened the period with significant differences, with or without MTH. Whether TPZ was administered singly or continuously, MTH seemed to prolong the period without any significant differences. With irradiation at an absorbed dose of 16 Gy, overall, the period required was significantly prolonged compared with no irradiation, and the treatments ranked in the following order; without TPZ < with singly administered TPZ < with continuously administered TPZ, with or without MTH. Again, whether TPZ was administered singly or continuously, MTH seemed to prolong the period without any significant differences.

Figure 4 shows the numbers of lung metastases on day 35 after inoculation as a function of the absorbed dose of γ-ray irradiation with or without TPZ administration in combination with MTH. Without irradiation, TPZ, especially when administered continuously, seemed to decrease the numbers of macroscopic metastases. Whether TPZ was administered singly or continuously, MTH seemed to further decrease the number. With irradiation, as the absorbed dose increased, the numbers decreased. Further, the numbers decreased more remarkably with TPZ than without. Again, especially when TPZ was administered continuously, the numbers decreased further, and MTH seemed to cause a greater decrease. With or without MTH, the curves for the tumors treated by radiation with TPZ were slightly

steeper than those for the tumors treated by radiation only. However, there were no significant differences between the slopes of the curves for tumors treated by radiation with TPZ and those for tumors treated by radiation only. Consequently, this means there was no apparent radio-sensitizing effect of TPZ on repressing lung metastases from treated tumors. Meanwhile, there was an almost parallel shift in the curves and no apparent changes in the slopes of the curves for the tumors treated with TPZ with or without MTH. This means there was no apparent difference in the radio-sensitizing or -protecting effect between single intraperitoneal and continuous subcutaneous administration with or without MTH in terms of the numbers of lung metastases. Anyway, with or without irradiation, continuous administration of TPZ combined with MTH, which was most cytotoxic as an initial effect, seemed to reduce the numbers of lung metastases from the local tumors most efficiently.

The numbers of lung metastases from local tumors that received irradiation under each set of conditions, which produced an identical SF of 0.03 as an initial effect (**Figure 1**), were estimated using the data shown in **Figure 4** (**Table VI**). Overall, the combination with TPZ tended to decrease the numbers more than γ-ray irradiation only. Especially when TPZ was administered continuously, the number was significantly smaller than that for γ-ray irradiation only. Further combination of MTH with TPZ resulted in a slightly smaller number than without MTH, although not significantly. Thus, also when TPZ was administered continuously combined with MTH, the number was significantly smaller than that for γ-ray irradiation only.

Discussion

Tumor microvasculatures frequently have structural and functional abnormalities, such as a disorganized vascular network, dilations, an elongated and tortuous shape, an incomplete endothelial lining, a lack of physiological/pharmacological receptors, an absence of flow regulation, and intermittent stasis [1,16]. Perfusion-related O_2 delivery leads to ischemic hypoxia, which is often transient. Thus, perfusion-limited acute hypoxic areas are distributed throughout the tumor depending on these causative factors [1,16,17]. Diffusion-related chronic hypoxia is caused by an increase in diffusion distances with tumor expansion. This results in an inadequate O_2 supply for cells distant (>70 μ m) from the nutritive blood vessels. Diffusion-limited hypoxia may also be caused by a deterioration of diffusion "geometry," for example, concurrent versus countercurrent blood flow within the tumor microvessel network [1,16,17]. MTH before irradiation decreased the hypoxic fraction (HF), even combined with nicotinamide treatment that prevents transient fluctuations in tumor blood flow that lead to the development of acute hypoxia [18,19]. In contrast, MTH did not decrease the HF when tumor-bearing mice were placed in a circulating carbogen (95% O2 / 5% CO2) chamber during irradiation [20]. Thus, MTH was shown to increase the tumor response to radiation by improving tumor oxygenation through an increase in tumor blood flow, thereby preferentially overcoming chronic hypoxia rather than acute hypoxia [20].

In this study, the enhancing effect of the hypoxic cytotoxin

TPZ with or without MTH was more remarkable in Q cells than in the total cell population (**Table II**) mainly because of the much larger size of the hypoxic fraction (HF) in the Q cell population [3,11]. When administered continuously, TPZ can produce DNA breaks in larger areas of solid tumors where not only chronic hypoxia exists but also acute hypoxia has occurred, whereas a single administration mainly kills chronically hypoxic tumor cells [4]. In the previous study using theoretical predictions [21], it was also shown that fluctuating pO2 increases the efficacy of TPZ, and that the longer time frame of TPZ delivery causes slow fluctuations in pO2, resulting in increasing the efficacy of TPZ.

According to our previous report [11], in the B16-BL6 tumor, the HF of the total cell population includes a large acutely HF and small chronically HF. In contrast, the HF of Q cells is made up of a large chronically HF and small acutely HF. Consequently, when MTH was combined with TPZ before irradiation, more TPZ could be exposed to intermediately hypoxic areas derived mainly from chronic hypoxia in solid tumors through the increase in tumor blood flow induced by MTH [4], resulting in greater enhancing effects preferentially on chronic hypoxia-rich Q cell fractions. Anyway, the use of TPZ, especially in combination with MTH, is very useful for enhancing the sensitivity of tumor cells, especially of radio-resistant Q tumor cells.

Meanwhile, tumor growth as a whole showed a close correlation with the initial response of the total cell population in a tumor. Thus, it is reasonable that the changes in tumor growth as a whole

(**Fig. 3, Table V**) were consistent with and well supported the changes in the radio-sensitivity of total tumor cell populations in cell survival curves (**Fig. 1**) and dose-response curves of the net MN frequency (**Fig. 2**).

The presence of Q cells is probably due, at least in part, to hypoxia and the depletion of nutrition, a consequence of poor vascular supply [1,16]. As a result, Q cells are viable and clonogenic, but have ceased dividing. This might promote the formation of micronuclei at 0 Gy in Q tumor cells (**Table 1**). Q cells were shown to have significantly less radiosensitivity than the total cell population [1,3,16], that is, more Q cells survive radiotherapy than P cells (**Fig. 2**, **Table IV**). Thus, the control of chronic hypoxia-rich Q cells has a great impact on the outcome of conventional radiotherapy for controlling local tumors, resulting in the superiority of the combination of TPZ and MTH in radiotherapy. As a result, the combined use of TPZ and MTH led to a decrease in the difference in radiosensitivity (**Table IV**). After all, the use of TPZ as a hypoxic cytotoxin in combination with MTH is thought to be very promising in terms of local tumor response in conventional radiotherapy.

Hypoxia is suggested to enhance metastasis by increasing genetic instability [10]. Acute but not chronic hypoxia increased the number of macroscopic metastases in mouse lungs [9,10]. We recently reported the significance of the administration of an acute hypoxia-releasing agent, nicotinamide, into tumor-bearing mice as a combined treatment with γ-ray irradiation in terms of repressing

lung metastasis [11]. Also in this study, continuous administration of TPZ, by which acutely hypoxic tumor cells are effectively killed, decreased the number of lung metastases, especially when combined with MTH that increased the dose of TPZ delivered. Meanwhile, as the delivered γ-ray dose increased with irradiation, the number of macroscopic lung metastases decreased reflecting the decrease in the number of clonogenically viable tumor cells in the primary tumor (**Fig. 4**). Consequently, continuous administration of TPZ in combination with MTH in γ-ray irradiation showed a little more potential to reduce the number of metastases.

It was elucidated that control of the chronic hypoxia-rich Q cell population in primary solid tumors has the potential to impact the control of local tumors as a whole, while control of the acute hypoxia-rich total tumor cell population has the potential to impact the control of lung metastases. Namely, in conventional radiotherapy, continuous TPZ administration combined with MTH is thought to have a great potential to control both local solid tumors and lung metastases from the local tumors.

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Table I.

Surviving fractions and micronucleus frequencies at 0 Gy

Treatment	Total cells	Quiescent cells
<Plating efficiency (%)>		
Without TPZ* or MTH**	**84.4 ± 8.2[a]**	**----**
With TPZ(*i.p.*[+])	**65.7 ± 6.6[b]**	**----**
With TPZ(*cont.*[++])	**37.0 ± 3.5[c]**	**----**
With TPZ(*i.p.*) and MTH	**57.6 ± 5.8[d]**	**----**
With TPZ(*cont.*) and MTH	**31.4 ± 2.1[e]**	**----**
<Micronucleus frequency>		
Without TPZ or MTH	**0.050 ± 0.007[f]**	**0.077 ± 0.008[k]**
With TPZ(*i.p.*)	**0.104 ± 0.010[g]**	**0.129 ± 0.013[l]**
With TPZ(*cont.*)	**0.147 ± 0.015[h]**	**0.181 ± 0.017[m]**
With TPZ(*i.p.*) and MTH	**0.128 ± 0.012[i]**	**0.163 ± 0.016[n]**
With TPZ(*cont.*) and MTH	**0.170 ± 0.019[j]**	**0.212 ± 0.020[o]**

*; Tirapazamine

**; Mild temperature hyperthermia

+; Single intraperitoneal administration

++; Continuous subcutaneous administration

Values are presented as mean ± standard error (n = 9).

Micronucleus frequencies for quiescent cells were significantly larger than those for total cells under all treatment conditions ($p < 0.05$). In addition, in each following combination of two values, there was the significant difference between the two values ($p < 0.05$); a&(b,c,d or e), f&(g,h,i or j), k&(l,m,n or o), b&c, d&e, g&h, i&j, l&m, n&o.

Table II.

The effect* of tirapazamine on each end-point

Type of administration	Total cells	Quiescent cells
<Surviving fraction = 0.03>		
Single intraperitoneal	**1.2 ± 0.1[a]**	**----**
Continuous subcutaneous	**1.5 ± 0.15[a]**	**----**
<Net micronucleus frequency = 0.6>		
Single intraperitoneal	**1.1 ± 0.05[b,d]**	**1.3 ± 0.1[c,d]**
Continuous subcutaneous	**1.3 ± 0.1[b,e]**	**1.55 ± 0.15[c,e]**

***;** The dose of radiation required to obtain each end-point without each treatment in relation to that required to obtain each endpoint with each treatment.

Values are presented as mean ± standard error (n = 9).

Letters[a-e] represent the significant differences between two values ($p < 0.05$).

Table III.

The effect* of mild temperature hyperthermia on each end-point

Type of administered tirapazamine	Total cells	Quiescent cells
<Surviving fraction = 0.03>		
Single intraperitoneal	**1.1 ± 0.05**	**----**
Continuous subcutaneous	**1.05 ± 0.05**	**----**
<Net micronucleus frequency = 0.6>		
Single intraperitoneal	**1.1 ± 0.05**	**1.15 ± 0.05**
Continuous subcutaneous	**1.05 ± 0.05**	**1.1 ± 0.05**

*; The ratio of the dose of radiation necessary to obtain each end-point without mild temperature hyperthermia to that needed to obtain each end-point with mild temperature hyperthermia.

Values are presented as mean ± standard error (n = 9).

Table IV.

Dose-modifying factors for quiescent cells relative to the total cell population*

Type of tirapazamine administered	Without MTH**	With MTH
<Net micronucleus frequency = 0.6>		
Without TPZ	1.65 ± 0.15[a,b]	----
Single intraperitoneal	1.4 ± 0.1[a]	1.35 ± 0.1
Continuous subcutaneous	1.45 ± 0.1[b]	1.4 ± 0.1

*; The ratio of the dose of radiation necessary to obtain each end-point in the quiescent cell population to that needed to obtain each end-point in the total tumor cell population.

**; Mild temperature hyperthermia

Values are presented as mean ± standard error (n = 9).

Letters[a,b] represent the significant differences between two values ($p < 0.05$).

Table V.

The period (days) required for each tumor to become twice as large as on day 18 after tumor cell inoculation

Type of tirapazamine administered	Without MTH*	With MTH
<Without irradiation>		
Without tirapazamine	2.2 ± 0.3[a,b]	----
Single intraperitoneal	3.8 ± 0.4[a,c]	4.2 ± 0.4[d]
Continuous subcutaneous	4.7 ± 0.5[b,c]	5.4 ± 0.5[d]
<With irradiation at a dose of 16 Gy>		
Without tirapazamine	11.0 ± 1.2[e,f]	----
Single intraperitoneal	16.5 ± 1.7[e,g]	19.4 ± 2.0[h]
Continuous subcutaneous	22.0 ± 2.4[f,g]	24.2 ± 2.5[h]

*; Mild temperature hyperthermia

Values are presented as mean ± standard error (n = 9).

Values for irradiation at the dose of 16 Gy were significantly larger than those for no irradiation under all treatment conditions ($p < 0.05$). In addition, letters[a–h] represent the significant differences between two values ($p < 0.05$).

Table VI.

The numbers of metastases from the irradiated tumors that received cytotoxic treatment producing a similar initial local effect*

Type of tirapazamine administered	Without MTH**	With MTH
<Surviving fraction = 0.03>		
Without tirapazamine	14.2 ± 1.4[a,b]	----
Single intraperitoneal	13.0 ± 1.3	12.2 ± 1.2
Continuous subcutaneous	11.4 ± 1.1[a]	10.8 ± 1.1[b]

*; Based on the data shown in Figure 4, the estimated numbers of lung metastatic nodules from local tumors that received γ-ray irradiation with or without tirapazamine in combination with mild temperature hyperthermia, which produced an identical surviving fraction of 0.03 as an initial effect in Figure 1.

**; Mild temperature hyperthermia

Values are presented as mean ± standard error (n = 9).

Letters[a,b] represent the significant differences between two values ($p < 0.05$).

Figure legends

Fig. 1 Cell survival curves for the total cell population from B16-BL6 tumors irradiated with γ-rays following the single intraperitoneal (*i.p.*) or continuous subcutaneous (*cont.*) administration of tirapazamine (TPZ) in combination with mild temperature hyperthermia (MTH) on day 18 after tumor cell inoculation. ○ γ-ray irradiation only; △ γ-ray irradiation after single intraperitoneal administration of TPZ; ▲ γ-ray irradiation after single intraperitoneal administration of TPZ with MTH; □ γ-ray irradiation after continuous subcutaneous administration of TPZ; ■ γ-ray irradiation after continuous subcutaneous administration of TPZ with MTH. Bars represent standard errors (n = 9).

Fig. 2 Dose response curves of the net micronucleus frequency for total (left panel) and quiescent (right panel) cell populations from B16-BL6 tumors irradiated with γ-rays following the single intraperitoneal (*i.p.*) or continuous subcutaneous (*cont.*) administration of tirapazamine (TPZ) in combination with mild temperature hyperthermia (MTH) on day 18 after tumor cell inoculation. ○ γ-ray irradiation only; △ γ-ray irradiation after single intraperitoneal administration of TPZ; ▲ γ-ray irradiation after single intraperitoneal administration of TPZ with MTH; □ γ-ray irradiation after continuous subcutaneous administration of TPZ; ■ γ-ray irradiation after continuous subcutaneous administration of TPZ with MTH. Bars represent standard errors (n = 9).

Fig. 3 Tumor growth curves for B16-BL6 tumors with (solid lines)

or without (dotted lines) γ-ray irradiation at a dose of 16 Gy following the single intraperitoneal (*i.p.*) or continuous subcutaneous (*cont.*) administration of tirapazamine (TPZ) in combination with mild temperature hyperthermia (MTH) on day 18 after tumor cell inoculation. ○ γ-ray irradiation only; △ γ-ray irradiation after single intraperitoneal administration of TPZ; ▲ γ-ray irradiation after single intraperitoneal administration of TPZ with MTH; □ γ-ray irradiation after continuous subcutaneous administration of TPZ; ■ γ-ray irradiation after continuous subcutaneous administration of TPZ with MTH. Bars represent standard errors (n = 9).

Fig. 4 Counted numbers of macroscopic metastases in the lung on day 35 after tumor cell inoculation as a function of the dose of γ-ray irradiation following the single intraperitoneal (*i.p.*) or continuous subcutaneous (*cont.*) administration of tirapazamine (TPZ) in combination mild temperature hyperthermia (MTH) on day 18 after tumor cell inoculation. ○ γ-ray irradiation only; △ γ-ray irradiation after single intraperitoneal administration of TPZ; ▲ γ-ray irradiation after single intraperitoneal administration of TPZ with MTH; □ γ-ray irradiation after continuous subcutaneous administration of TPZ; ■ γ-ray irradiation after continuous subcutaneous administration of TPZ with MTH. Bars represent standard errors (n = 9).

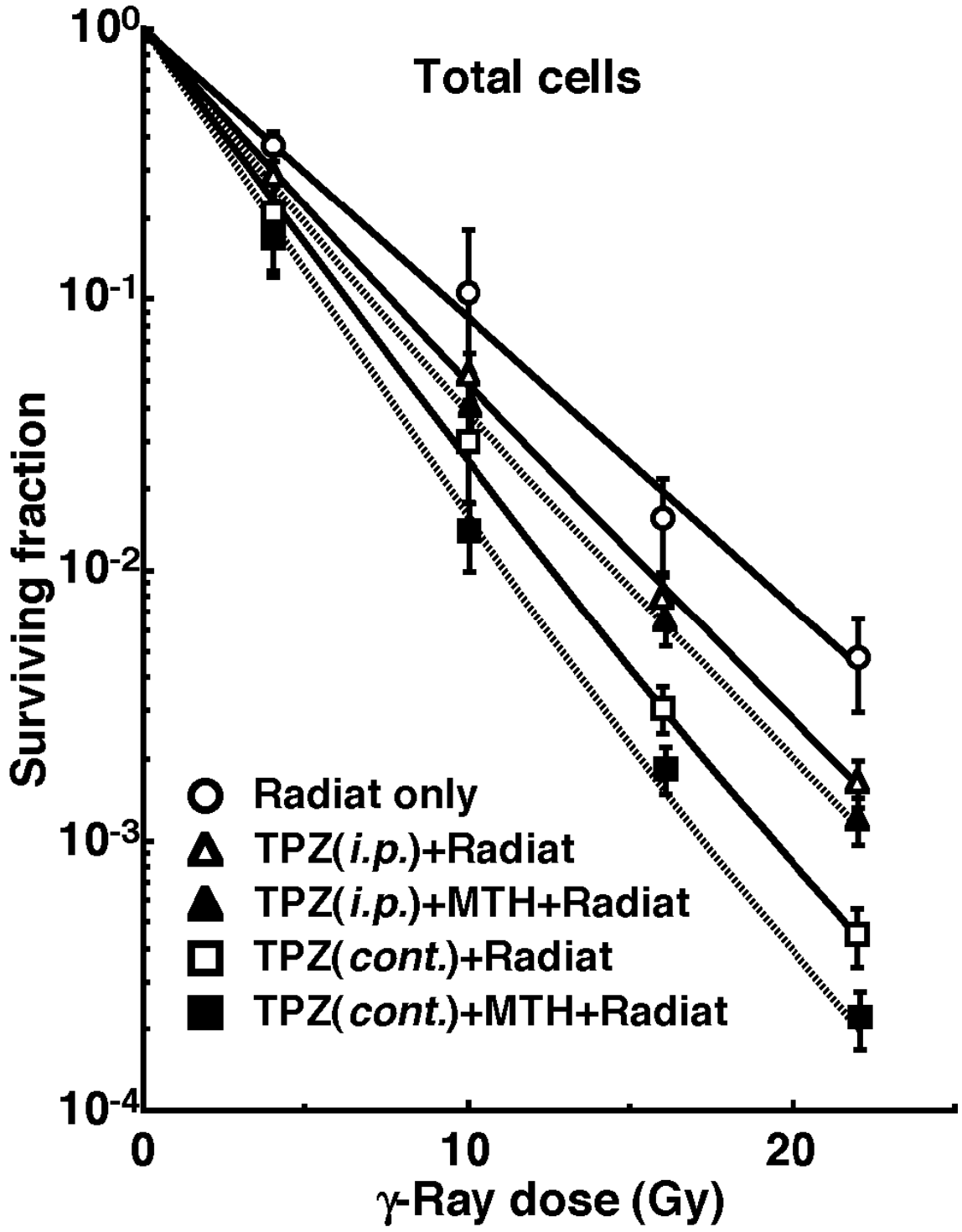
Total cells
Surviving fraction
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
10
20
γ-Ray dose (Gy)
Radiat only
TPZ(*i.p.*)+Radiat
TPZ(*i.p.*)+MTH+Radiat
TPZ(*cont.*)+Radiat
TPZ(*cont.*)+MTH+Radiat

Figure 1

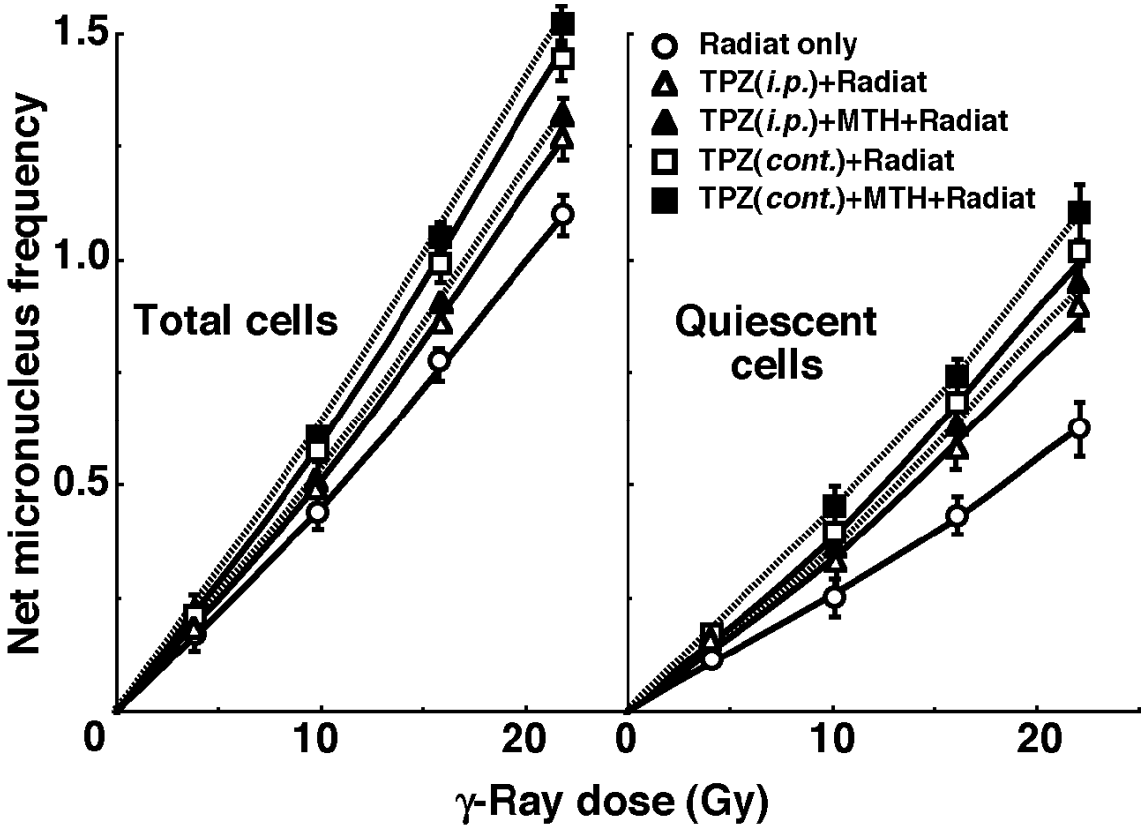

Figure 2

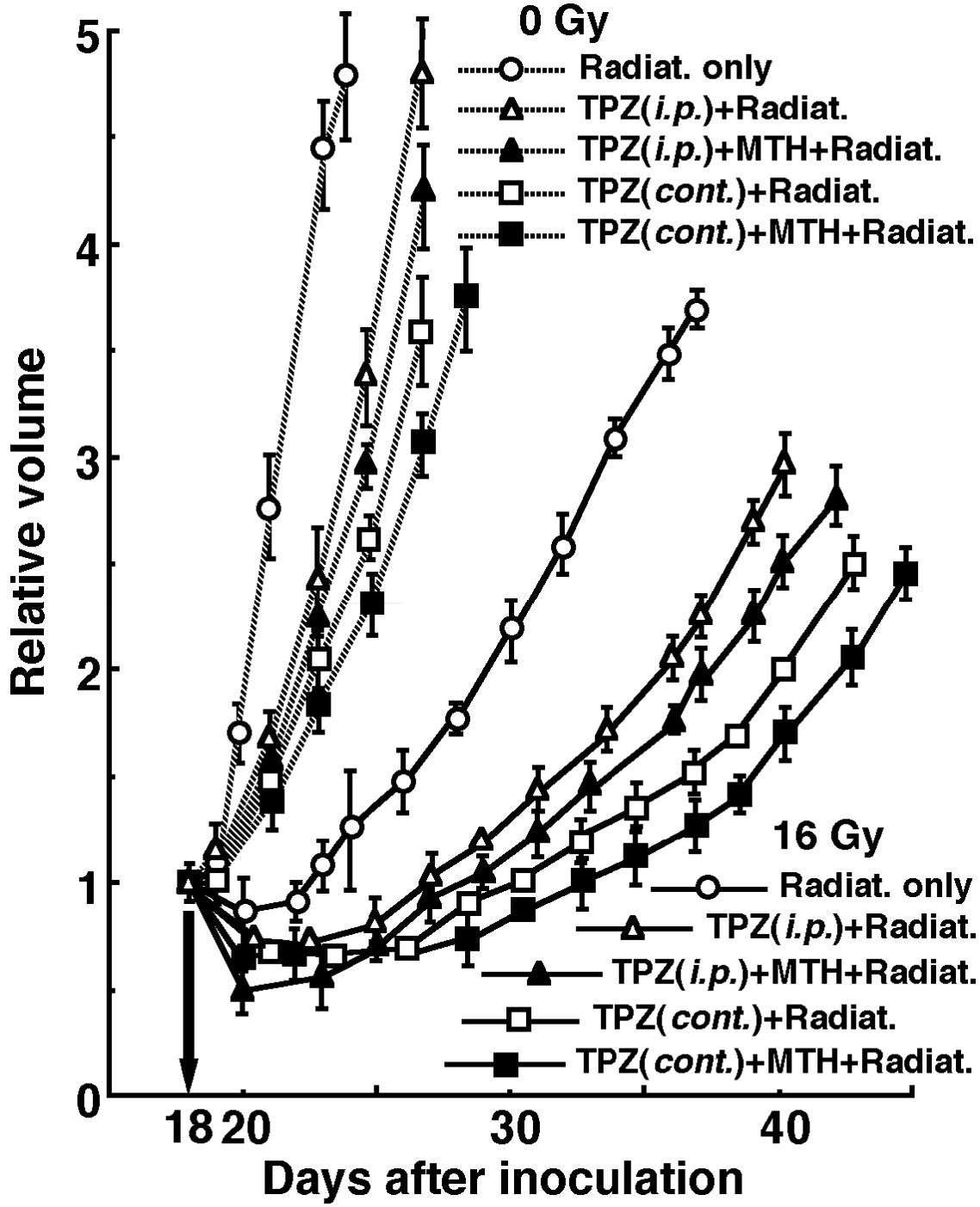
0 Gy
Radiat. only
TPZ(i.p.)+Radiat.
TPZ(i.p.)+MTH+Radiat.
TPZ(cont.)+Radiat.
TPZ(cont.)+MTH+Radiat.
16 Gy
Radiat. only
TPZ(i.p.)+Radiat.
TPZ(i.p.)+MTH+Radiat.
TPZ(cont.)+Radiat.
TPZ(cont.)+MTH+Radiat.
Relative volume
0
1
2
3
4
5
18 20
30
40
Days after inoculation

Figure 3

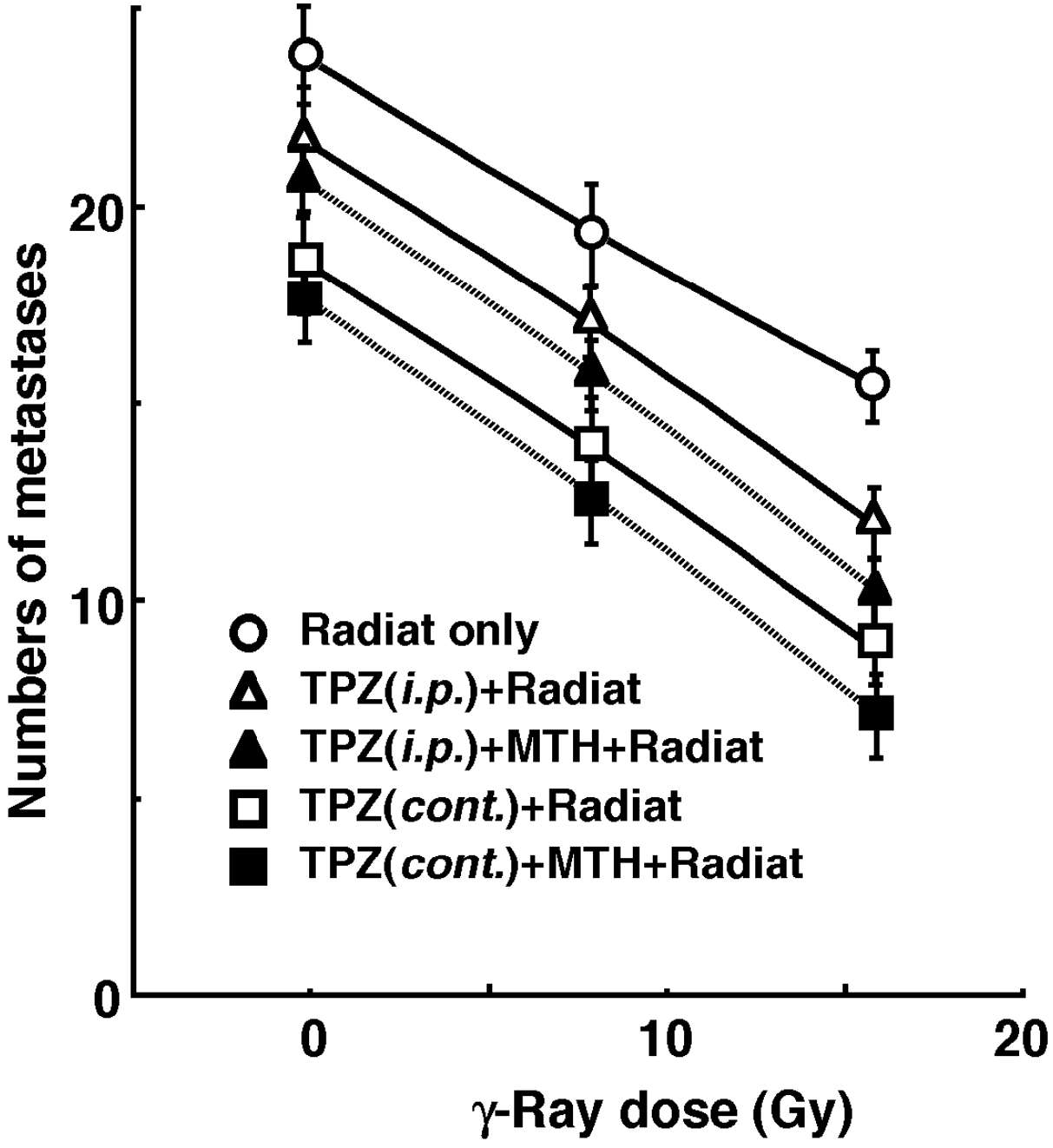
Numbers of metastases
20
10
0
0
10
20
γ-Ray dose (Gy)
Radiat only
TPZ(i.p.)+Radiat
TPZ(i.p.)+MTH+Radiat
TPZ(cont.)+Radiat
TPZ(cont.)+MTH+Radiat

Figure 4