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# Changes in the Sensitivity of Intratumor Cells during Fractionated Tirapazamine Administration

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**Mice bearing solid tumors received 10 intraperitoneal administrations of 5-bromo-2′-deoxyuridine (BrdU) to label the proliferating (P) tumor cells. Then, as a priming treatment, tirapazamine (TPZ) was intraperitoneally administered. Further, 0 through 48 h later, the tumor-bearing mice received TPZ again at various doses. The tumor cells were isolated and incubated with a cytokinesis blocker. The micronucleus (MN) frequencies in cells with and without BrdU labeling, which were regarded as P and quiescent (Q) cells at the priming treatment, respectively, were determined using immunofluorescence staining for BrdU. The MN frequency in the total (P+Q) tumor cells was determined from the tumors that were not pretreated with BrdU. In addition, P cell ratios in the tumors at the second treatment were determined using immunofluorescence staining for P cell nuclear antigen. In each cell fraction, the longer the interval between the two treatments, the higher was the sensitivity to TPZ, except 1 h after the priming treatment. More than 24 h later, total and P cells, especially P cells, showed significantly higher sensitivity to TPZ than in the case of a single TPZ treatment. The longer the period between the two TPZ treatments, the lower was the P cell ratio at the second treatment. These findings were thought to indicate that the use of TPZ in the treatment of solid tumors causes a shift from the P to the Q state *in vivo*.**

Key words: Tirapazamine — Fractionated treatment — Quiescent cell — Proliferating cell — Cell kinetics

Recent studies have suggested that tumor hypoxia could be exploited by the use of bioreductive agents that preferentially kill hypoxic cells.[1,2] Tirapazamine (TPZ, SR-4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), one of the most well-known bioreductive agents, was reported to have highly selective cytotoxicity toward hypoxic cells in both *in vivo* and *in vitro* tumor systems.[1–3] Severe hypoxia was not required and the level of hypoxia found in many human tumors was sufficient for the toxicity.[2]

In general, to perform cancer therapy more efficiently, it is essential to clarify the response of nonproliferating (quiescent (Q)) cells in solid tumors to the therapy, because many tumor cells are quiescent *in situ* but still have clonogenicity.[1] Until recently, a comparatively simple assay for assessing the response of intratumor Q cells was not available. For analyzing the responses of Q cells in solid tumors, we have developed a combined method using micronucleus (MN) assay and identification of proliferating (P) cells with 5-bromo-2′-deoxyuridine (BrdU) and anti-BrdU monoclonal antibody.[4] This method has already yielded several radiobiologically important findings concerning intratumor Q cells.[4,5] Further, the question of how Q cell populations behave in solid tumors during radiotherapy in two fractions has also been clarified.[6,7] However, the behavior of intratumor Q cells following hypoxia-specific cytotoxin (TPZ) administration is still unknown. Therefore, in this study, we examined the behavior of P and Q cells in solid tumors following TPZ administration, using our method for selectively detecting the response of Q cells in solid tumors.[4]

## MATERIALS AND METHODS

**Tumors, mice and labeling with BrdU** SCC VII squamous cell carcinomas derived from C3H mice were maintained *in vitro* in Eagle's minimum essential medium. Cells were collected from monolayer cultures, and approximately $1.0\times10^5$ cells were inoculated subcutaneously into the left hind legs of 8- to 11-week-old syngeneic female C3H/He mice. Nine days after the inoculation, 100 mg/kg of BrdU dissolved in physiological saline was adminis-

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tered intraperitoneally, 10 times at 12 h intervals to label all P cells in the tumor. Administration of BrdU did not change the tumor growth rates. The tumors were 1 cm in diameter on treatment. The labeled tumor cell ratio after 10 doses of BrdU was 55.3 (50.8−59.8) % (mean (95% confidence limit)), and reached a plateau at this stage. Therefore, in this study, we regarded tumor cells not incorporating BrdU after 10 doses as Q tumor cells.[4)]

**Drug** Hori *et al*. synthesized TPZ according to the established method,[8)] which Brown and colleagues also employed,[3)] and analyzed it by chemical ionization mass spectrometry [*m*/*z*, 179 (MH+)], electron ionization mass spectrometry [*m*/*z*, 178 (M+)] and infrared spectrometry [KBr]. This synthesized TPZ was regarded as identical to commonly used TPZ.

**Treatment** After the labeling with BrdU, the tumor-bearing mice were assigned to two groups to receive the priming treatment or not. The first group of mice received TPZ at a dose of 5 to 40 mg/kg intraperitoneally.

For the second group of mice, TPZ dissolved in physiological saline was administered intraperitoneally at the dose of 20 mg/kg as a priming treatment after labeling with BrdU. The tumor-bearing mice then further received TPZ at a dose of 5 to 20 mg/kg intraperitoneally, 0 through 48 h after the priming treatment. During the interval between the two administrations of TPZ, no BrdU was administered.

The tumors of the first and second groups of mice were excised 1 h after TPZ administration and 1 h after the second TPZ administration, respectively. The concentrations and time course employed here have been shown to be appropriate for TPZ to function completely.[9, 10)]

Each treatment group included mice pretreated with and without BrdU.

**Immunofluorescence staining of BrdU-labeled cells and observation of MN formation** The procedures we used have been described in detail elsewhere.[4)] Excised tumors from mice given BrdU were minced and trypsinized. Tumor cell suspensions were incubated for 48 h in tissue culture dishes containing complete medium and 1.0 $\mu$g/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division. The cultures were trypsinized and single cell suspensions were fixed with 70% ethanol. After centrifugation, the cell pellet was resuspended with cold Carnoy's fixative. The suspension was then placed on a glass microscope slide and the sample was dried at room temperature. The slides were treated with 2 *N* hydrochloric acid for 45 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody. To observe double staining of tumor cells with FITC and propidium iodide (PI), cells on the slides were treated with PI (1−5 $\mu$g/ml in phosphate-buffered saline (PBS)) and monitored under a fluorescence microscope. The MN frequency in BrdU-unlabeled cells (=Q cells at the time of priming treatment) could be examined by counting the micronuclei in the binuclear cells that showed only red fluorescence. In the same manner, the micronuclei in binuclear cells could also be counted in BrdU-labeled cells (=P cells at the time of priming treatment), in which at least part of the nucleus or micronucleus showed green 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.[11)]

The ratio obtained in tumors not pretreated with BrdU indicated the MN frequency of all phases of the total (P+Q) tumor cell populations. More than 300 binuclear cells were counted to determine the MN frequency.

**Determination of P tumor cell ratios at the time of the priming and second TPZ treatments** Just before the priming and second TPZ treatment, tumors from other mice not given BrdU were also excised and trypsinized. Then, tumor cell suspensions were subjected to detergent extraction of soluble P cell nuclear antigen (PCNA) by incubating the cells in 500 $\mu$l ethylenediamine tetraacetate (2 $\mu$g/ml) in PBS containing Triton X-100 (2.5 $\mu$l/ml) and bovine serum albumin (5 $\mu$g/ml) for 15 min on ice, followed by fixation with 3 ml of 100% methanol for 10 min at −20°C,[12)] and resuspended in cold modified Carnoy's fluid. This suspension was placed on a microslide glass using a dropper in the manner mentioned above. Thereafter, tumor cells on the microslide glass were treated with indirect immunofluorescence staining using monoclonal anti-PCNA (PC10) and FITC-conjugated antimouse IgG antibody, and then they were treated with nuclear staining with PI as a background staining. The P tumor cell ratio for each interval group after the priming treatment was obtained from the ratio of the number of tumor cells exhibiting green fluorescence (FITC) to the total number of tumor cells observed.

Four mice were used to assess each set of conditions and each experiment was repeated 3 times. To examine the differences between pairs of values, Student's *t* test was used when the variances of the two groups could be assumed to be equal; otherwise the Welch *t* test was used. *P* values were from two-sided tests.

## RESULTS

Table I shows the MN frequencies for total, P and Q cells when no TPZ was given and when TPZ (20 mg/kg) was given only as a priming treatment. When no TPZ was administered, the MN frequencies decreased in the following order, with significant differences ($P<0.05$): Q cells >

Table I. Micronucleus Frequencies for No Treatment and after Tirapazamine Treatment

| Treatment | Total cells | Proliferating cells | Quiescent cells |
|---|---|---|---|
| No tirapazamine treatment | | | |
| | 0.054 | 0.029 | 0.086 |
| | (0.052−0.056) | (0.015−0.035) | (0.065−0.107) |
| Hours after tirapazamine (20 mg/kg) treatment | | | |
| 0 h | 0.155 | 0.135 | 0.288 |
| | (0.142−0.168) | (0.120−0.150) | (0.260−0.316) |
| 1 h | 0.164 | 0.136 | 0.316 |
| | (0.149−0.179) | (0.120−0.152) | (0.288−0.346) |
| 12 h | 0.152 | 0.135 | 0.269 |
| | (0.140−0.164) | (0.121−0.149) | (0.243−0.295) |
| 24 h | 0.151 | 0.135 | 0.255 |
| | (0.139−0.163) | (0.120−0.150) | (0.229−0.281) |
| 48 h | 0.151 | 0.135 | 0.253 |
| | (0.138−0.164) | (0.122−0.148) | (0.227−0.279) |

Numbers in parentheses are 95% confidence limits, determined from mean values, standard deviations, and the numbers of observations on which the means and standard deviations were based.

P+Q cells > P cells. Following the priming treatment, the MN frequencies decreased in the same order, but significant differences were observed only between Q cells and other cells. One hour after the priming treatment, each cell fraction showed a relative increase in MN frequency. The overall decrease in MN frequency was evaluated in relation to time after the priming treatment, with the values at the time point showing the maximum effect (=1 h later) taken to be 1.0 after normalizing with respect to the MN frequencies for no TPZ treatment. Forty-eight hours after the priming treatment, the determined values for the decrease in MN frequency were 0.88 (0.76−1.00), 0.99 (0.87−1.00), and 0.72 (0.61−0.84) for P+Q, P and Q cell populations, respectively. The difference between P and Q cells was significant ($P<0.05$).

Fig. 1 shows the dose-response relation between TPZ dose and the corrected MN frequency (MN frequency−C, where C is the MN frequency in TPZ non-treated tumors) obtained 1 h after TPZ administration to the first group of tumor-bearing mice. P and Q cells showed significantly lower and higher corrected MN frequencies than P+Q cells, respectively ($P<0.05$). All of these three dose-response curves had upward convex shapes.

Fig. 2 shows the dose-response relation between the second treatment dose of TPZ and the corrected MN frequency (MN frequency−C, where C is the MN frequency in TPZ non-treated tumors at the time of second treatment) obtained 1 h after the second TPZ administration to the second group of tumor-bearing mice. One hour after the priming treatment, each corrected MN frequency reached a low in each cell population. More than 12 h later, the values for these three cell populations were higher than immediately after the priming treatment. In addition, the longer the interval between the two TPZ administrations, the higher was the corrected MN frequency for each cell population. The corrected MN frequencies for P+Q and P

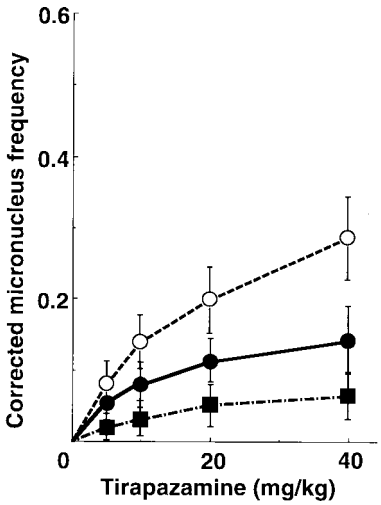


Fig. 1. The dose-response relation between tirapazamine (TPZ) dose and the corrected micronucleus (MN) frequency (MN frequency−C, where C is the MN frequency in TPZ-untreated tumors) obtained 1 h after TPZ treatment. Total tumor cells (closed circles), quiescent cells (open circles) and proliferating cells (closed squares) were treated with TPZ at doses of 5, 10, 20 and 40 mg/kg. There were significant differences in corrected MN frequency with $P<0.05$. Bars represent standard deviations. The number of observations on which mean values and standard deviations were based is 12. ● total cells, ○ quiescent cells, ■ proliferating cells.

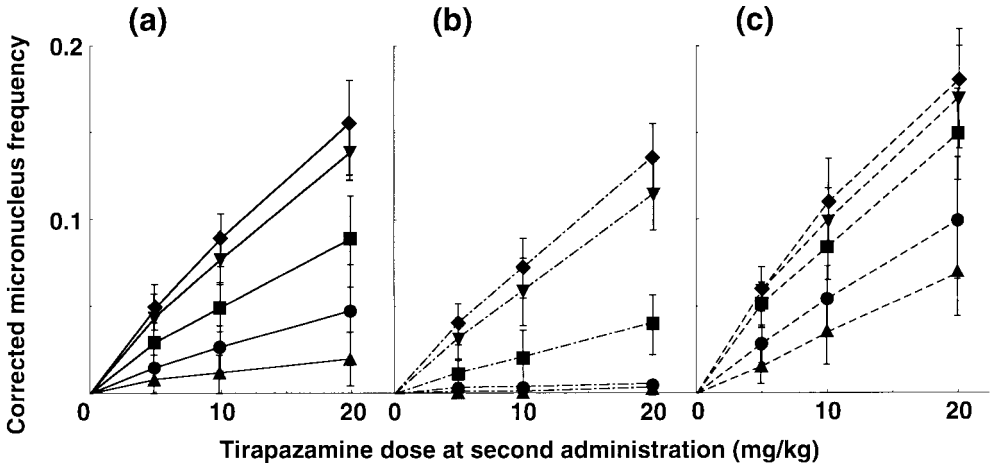


Fig. 2. The dose-response relation between the second treatment dose of tirapazamine (TPZ) and the corrected micronucleus (MN) frequency (MN frequency−C, where C is the MN frequency in TPZ-untreated tumors at the time of second treatment) obtained 1 h after the second treatment. Total tumor cells (a) proliferating cells (b), and quiescent cells (c) at the time of the priming treatment were given the second treatment with TPZ immediately after (solid circles), 1 h after (solid triangles), 12 h after (solid squares), 24 h after (reverse solid triangles) or 48 h after (solid rhombuses) the priming treatment. In the total and proliferating cells, there were significant differences in corrected MN frequency between immediately after and more than 24 h later with $P<0.05$. Bars represent standard deviations. The number of observations on which mean values and standard deviations were based is 12. ● immediately after, ▲ 1 h after, ■ 12 h after, ▼ 24 h after, ◆ 48 h after.

cells were significantly higher at more than 24 h later than immediately after the priming treatment ($P<0.05$). Further, more than 24 h later, P+Q and P cells, especially P cells, showed significantly higher corrected MN frequencies than those obtained for single TPZ treatment, as shown in Fig. 1 ($P<0.05$).

Table II shows the P tumor cell ratios at the time of the priming and second TPZ treatments. The longer the interval between the two treatments, the lower was the P cell ratio at the second treatment. The P cell ratios after 24 and 48 h were significantly lower than that at the time of the priming TPZ treatment ($P<0.05$).

## DISCUSSION

Micronucleus appears in cells treated with ionizing radiation or cytotoxic agents which damage DNA. Micronucleus is formed from acentric chromosomal fragments or whole chromosomes, which are not incorporated into daughter nuclei at mitoses.[11, 13] A relationship between cell survival and MN frequency has been reported. However, dose-response curves of MN frequency differ from report to report. This is partly because of the effect of the mitotic delay induced by DNA-damaging treatment and partly because of the different proportions of cells which pass through the mitotic phase. To solve these kinetic problems in MN frequency assay, Fenech and Morley developed a cytokinesis-blocked method using cytochalasin-B.[13] The effects of cytochalasin-B on chromosome damage in cells have not been completely elucidated. Nevertheless, a close relation has been reported between cell survival and the MN frequency obtained with cytochalasin-B treatment after irradiation with X-rays.[11, 13] Similar to the findings for X-irradiation, DNA is considered to be the primary target for cell killing by TPZ.[1, 2] Therefore, the effects of cytochalasin-B may be disregarded and MN frequency would reflect sensitivity to TPZ treatment.

TPZ is a first-generation hypoxic cytotoxin that can, by itself, exert cytotoxic activity against hypoxic mammalian tumor cells *in vitro* and rodent tumors *in vivo*.[1, 2] Nuclear magnetic resonance studies indicated that TPZ loses an electron under hypoxic conditions, thereby becoming a free radical capable of producing DNA breaks, resulting in the death of cancer cells when they attempt to undergo mitosis.[14] In our preliminary experiment, the response to TPZ of SCC VII tumor cells was not sensitized or attenuated significantly by the BrdU labeling used here. Thus, the MN frequency of BrdU-labeled cells should also reflect sensitivity to TPZ treatment.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells. The presence of these cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, which is another consequence of poor vascular supply.[1] Therefore, Q cells showed higher MN frequencies than other cells when no TPZ was administered (Table I). As shown in Table I and

Table II. Proliferating Tumor Cell Ratios at the Time of the Priming Tirapazamine Treatment and the Second Tirapazamine Treatment (%)

| At the priming tirapazamine treatment | At the second tirapazamine treatment Hours after the priming tirapazamine treatment | | | |
|---|---|---|---|---|
| | 1 h | 12 h | 24 h | 48 h |
| 59.3 (56.8−61.8)[a)] | 59.0 (54.8−63.2) | 57.2 (53.2−61.2) | 54.3 (51.8−56.8)[b)] | 53.4 (49.8−57.0)[c)] |

Numbers in parentheses are 95% confidence limits, determined from mean values, standard deviations, and the numbers of observations on which the means and standard deviations were based. *b*) and *c*); $P < 0.05$ compared with *a*).

Fig. 1, TPZ treatment produced larger MN frequencies in Q cells than in other cells. This is thought to reflect the fact that intratumor Q cell populations are fairly easy to kill with the hypoxia-specific cytotoxin TPZ because of the much larger hypoxic fraction in Q cells, compared with other cell populations.[4, 10)] Further, the findings concerning the decrease in the MN frequency with time (Table I) showed that the repair of potentially lethal damage (PLDR) following TPZ treatment was marked in Q cells. However, our previous report demonstrated that DNA breaks induced by TPZ are much less repairable than those produced by conventional DNA-damaging treatments such as X-irradiation or cisplatin administration.[15)] This is thought to be mainly because active TPZ radicals are produced at high local concentrations by activating enzymes close to DNA.[16)] That the maximum cell killing effect of TPZ occurred 1 h after administration was probably because TPZ required this period to function completely.[17)]

Fractionation is often employed in clinical chemotherapy as well as radiotherapy. However, a proper analysis of tumor cell kinetics in solid tumors during fractionated chemotherapy, especially the separate evaluation of the behavior of P and Q tumor cells, remains to be conducted. As shown in Figs. 1 and 2 and Table II, more than 24 h after the priming treatment, the sensitivity of P and P+Q cells, especially P cells, was significantly elevated at the time of the second TPZ treatment compared with the value for single TPZ treatment, and the P cell ratio was significantly reduced at the time of the second treatment compared with the priming treatment. This implies that during the interval between the two TPZ treatments, a shift from the P to the Q state occurred in the solid tumors, probably because the priming treatment caused cell loss preferentially in TPZ-sensitive Q cell fractions.[10)] Needless to say, sublethal damage (SLD) repair could occur during the interval, and rehypoxiation following the priming treatment could also influence the sensitivity of tumor cells.[17)] Concerning rehypoxiation, even long after the priming TPZ treatment, the hypoxic fraction of tumor cells returns to at most the level of that of non-treated control tumor.[17)] That is to say, from the viewpoint of rehypoxiation, the sensitivity of tumor cells to TPZ at the time of the second treatment cannot exceed that for single TPZ treatment. With regard to the repair of SLD, some degree of repair may have occurred after the priming treatment, because the dose-response curve between TPZ dose and the corrected MN frequency for single TPZ treatment had an upward convex shape (Fig. 1), and because the longer the interval between the two TPZ treatments, the higher was the corrected MN frequency for each cell population (Fig. 2). However, the MN frequency at the time of the second TPZ treatment, based on the upward convex shape of the dose-response curve for single TPZ treatment and the repair of SLD, cannot increase beyond the value obtained for single TPZ treatment. That each cell population showed the lowest corrected MN frequencies 1 h after the priming treatment (Fig. 2) was partly because TPZ required this period to function completely,[17)] partly because 1 h was too short for repair to occur,[15)] and partly because the shift from P to Q state occurred comparatively slowly, presumably more than 24 h after the priming treatment.

It has been reported that Q cells have less sensitivity to radiation or conventional chemotherapeutic agents and greater PLDR capacities than P cells in solid tumors *in vivo*.[1, 18)] This means that more Q cells can survive after radiotherapy or conventional chemotherapy, as compared with P cells. Consequently, the control of Q cells, some of which still have clonogenicity,[1)] is thought to greatly influence the outcome of anticancer therapy. Thus, the use of TPZ as an anticancer agent is promising for the treatment of solid tumors, due to its potent cytotoxicity to Q cell fractions and the small repair capacities following TPZ treatment.[16)]

As shown in our previous reports, X-ray irradiation and thermal neutron irradiation following administration of *dl*-*p*-boronophenylalanine, a well-known thermal neutron capture compound, caused recruitment from the Q to the P state.[6, 7)] The present study showed that the use of TPZ in

the treatment of solid tumors causes a shift from the P to the Q state *in vivo*. Namely, posttreatment changes in tumor cell kinetics within solid tumors depend on cancer treatment characteristics. These changes may have to be taken into account in the scheduling of treatments with combined modalities, using several different treatments that have different relative toxicities for each type of cell. Also the relative sensitivity to treatment of each type of cell after treatment may have to be taken into account.

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