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Comparison of *in vivo* Efficacy of Hypoxic Cytotoxin Tirapazamine and Hypoxic Cell Radiosensitizer KU-2285 in Combination with Single and Fractionated Irradiation

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Development of strategies to eradicate radioresistant hypoxic cells would be of great benefit for clinical radiotherapy. In the present study, the *in vivo* effects of a promising hypoxic cytotoxin, tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-N-oxide), were examined in comparison with those of KU-2285, one of the best hypoxic cell radiosensitizers, in combination with both single and fractionated irradiation. The tumor response was assessed by the standard *in vivo-in vitro* clonogenic assay using SCCVII tumors in C3H mice and EMT-6/KU tumors in Balb/c mice with different characteristics of tumor hypoxia. With single-dose irradiation (18 Gy), both tirapazamine and KU-2285 showed significant enhancement of cell killing in a dose-dependent manner, but tirapazamine was more effective for SCCVII tumors with acutely hypoxic cells, while KU-2285 was more effective for EMT-6/KU tumors predominantly with chronically hypoxic cells. In fractionated irradiation regimens (4 fractions of 5 Gy at 12 h intervals), tirapazamine showed more marked combined effects at 10 and 20 mg/kg than KU2285 at 100–200 mg/kg in both SCCVII and EMT-6/KU tumors. We concluded that the effectiveness of KU-2285 and tirapazamine was correlated with the nature of tumor hypoxia with single-dose irradiation, whereas tirapazamine appeared more potent than KU-2285 with fractionated irradiation. These findings suggest the potential usefulness of tirapazamine in clinical fractionated radiotherapy.

Key words: Hypoxia — Radiation — Tirapazamine — KU-2285 — Sensitizer

Over the years, the existence of hypoxic cells in solid tumors has been regarded as a cause of treatment failure in radiation therapy.[1–3] Many investigators in the field of radiation biology[4–7] have detected hypoxic cells in various experimental tumors. Recent technical developments such as direct measurements of oxygen tension by microelectrodes[8] and noninvasive techniques[9,10] including labeled nitroimidazole-binding have provided compelling evidence that hypoxic cells also exist in certain types of malignancies in humans.

Possible strategies to eradicate such radioresistant cells may involve two approaches. One is the use of hypoxic cell radiosensitizers having oxygen-mimicking actions, as intensively examined over the past two decades, and the other is the use of hypoxic cytotoxins which are specifically toxic toward hypoxic cells. Unfortunately only marginal, if any, benefit has been achieved to date in most of the clinical trials with hypoxic cell radiosensitizers, partly due to dose-limiting peripheral neurotoxicity.[11] However, some positive results with hypoxic cell radiosensitizers[2,3,12] have encouraged us to search for more potent agents with lower toxicity and higher sensitizing effects.[13–17]

Recently, a new class of bioreductive antitumor agents has been developed. Tirapazamine, a benzotriazine di-N-oxide, is a lead compound among them and has the feature of preferential killing of hypoxic cells, both *in vitro* and *in vivo*.[18] The hypoxic cytotoxicity of tirapazamine is considered to result from DNA double-strand breakage by oxidizing radical anions produced through bioreductive metabolism under hypoxic conditions.[19] Brown *et al.* have shown in experimental studies[20–23] that the actual cell killing caused by the addition of tirapazamine to a fractionated radiation course is much greater than that expected from additive toxicity of irradiation and drug, and that tirapazamine produces greater tumor radiosensitization in fractionated regimens than their standard hypoxic radiosensitizer, etanidazole.[21] These positive preclinical results have supported this approach with bioreductive agents in radiation oncology and cancer research.[23–26] Tirapazamine is currently being clinically tested.[27]

KU-2285, a fluorinated 2-nitroimidazole derivative, was developed as a less toxic and more potent hypoxic

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cell radiosensitizer.[14, 15, 28] We have reported that KU-2285 has higher radiosensitizing effects than those of etanidazole in both *in vitro* and *in vivo* experiments, even at low drug dose levels and in a clinically relevant low radiation dose range.[17, 29] This compound was also expected to have lower peripheral neurotoxicity, based on pharmacokinetic studies.[30] Thus, KU-2285 is one of the best hypoxic cell radiosensitizers developed so far, and is now undergoing clinical trials. Therefore, it is worthwhile to examine whether KU-2285 or tirapazamine can play a more significant role in the control of hypoxic cells in the same preclinical experimental setting in combination with irradiation.

In this study, we have examined the *in vivo* efficacy of tirapazamine at various drug doses in combination with both single and fractionated irradiation in comparison with that of KU-2285, at doses applicable to humans, using two murine tumors having a different nature of hypoxia.

MATERIALS AND METHODS

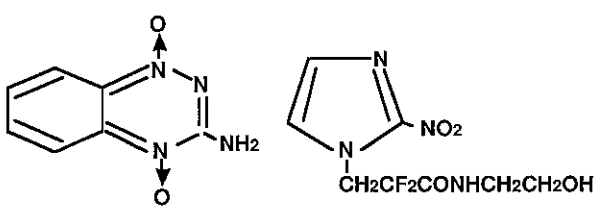
Drugs Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-N-oxide) was kindly provided by Sanofi Winthrop Pharmaceuticals Research Division (Collegeville, PA). KU-2285 was supplied by Daikin Co. Ltd. (Osaka). Both drugs were dissolved in saline immediately before use at various concentrations and administered i.p. in a volume of 0.02 ml/g body weight of mice 30 min before irradiation. These intervals have been shown to be optimal for each drug in previous studies.[14, 20, 21] Fig. 1 shows the chemical structural formulae of the two drugs. Drug doses were chosen according to the LD_{50} value: tirapazamine (89 mg/kg, i.p.)[18] and KU-2285 (2100 mg/kg, i.p.).[14]

Mice and tumors SCCVII and EMT-6/KU tumors[31, 32] were used in 8- or 10-week-old female C3H/He and Balb/c mice, respectively. Exponentially growing SCCVII or EMT-6/KU cells obtained from *in vitro* cultures were transplanted into the hind legs of the host mice by subcutaneous inoculation of 2×10^5 cells in 0.02 ml, as in the previous studies.[14, 17, 28] Experiments were started when tumors reached 10 mm in diameter after about 12 days.

Table I shows the differences in oxygenation status of the two murine tumors. SCCVII tumors have the hypoxic fraction of about 5.4–10% and contain a necrotic area of only 1.2% on histological sections. Since SCCVII tumors show rapid reoxygenation after irradiation,[32] the nature of hypoxia is supposed to be so-called "acute hypoxia" due to transient fluctuation of tumor blood flow. On the other hand, EMT-6/KU tumors have a relatively larger hypoxic fraction and also contain larger necrotic areas than SCCVII tumors.[31] Reoxygenation in EMT-6/KU tumors occurs relatively slowly[33] and the hypoxic cells in EMT-6/KU tumors are suspected to have been chronically rather than acutely produced by limited diffusion of oxygen as described in the tumor cord model. We were interested in whether tirapazamine would exhibit different effects in these two tumors having different characteristics of hypoxia.

Irradiations Whole-body irradiation of the tumor-bearing mice was performed without any physical immobilization or anesthesia because these procedures significantly influenced tumor oxygenation status, as previously reported.[31, 32, 34] Irradiation was delivered to the mice moving freely in an acrylic box in room air at a dose rate of 3.2 Gy/min using 15 MV X-rays generated by a linear accelerator. A single dose of 18 Gy or 4 fractions of 5 Gy at 12 h intervals were given.

Cell survival assay Tumor response was assessed by our standard *in vivo-in vitro* clonogenic assay. Details of this assay have been published elsewhere.[15, 17, 28] Briefly, after



Tirapazamine KU-2285

Fig. 1. The structural fomulae of the two compounds. Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-N-oxide) is thought to act as a hypoxic cytotoxin.[18] KU-2285 (a fluorinated 2-nitroimidazole derivative) is a hypoxic cell radiosensitizer having oxygen-mimicking activity.[14]

Table I. Characteristics of the Oxygenation Status of the Two Experimental Tumors

	SCCVII	EMT-6/KU
Host mice	C3H/He	Balb/c
Site of inoculation	s.c.	s.c.
Tumor diameter (mm)[a]	10	10
Hypoxic fraction (%)[b]	5.4–10	14–15
Necrotic area (%)[c]	1.2	24.5
Reoxygenation	rapid[d]	relatively slow[e]
Nature of tumor hypoxia	acute hypoxia	chronic > acute hypoxia

a) The size of each tumor reached 10 mm in diameter about 12 days after s.c. inoculation of 2×10^5 cells.
b) Hypoxic fraction determined by the paired survival curve method.[31, 32, 34]
c) Proportion of necrotic area was estimated microscopically on histological sections stained with hematoxylin and eosin.[31]
d, e) These findings were suggested in previous publications from our institute.[32, 33]

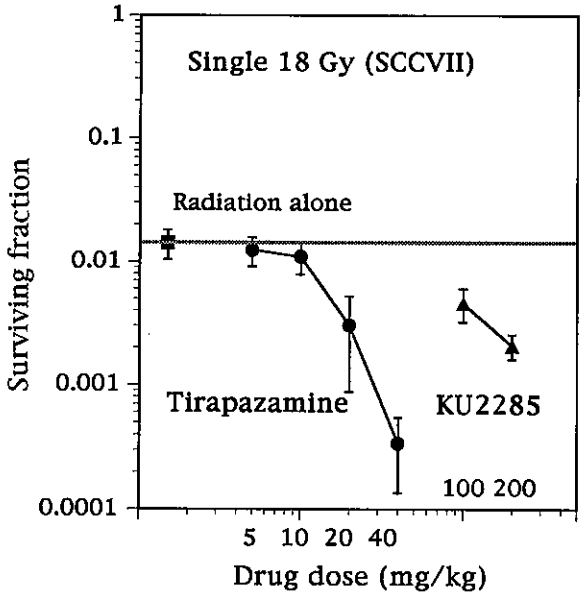


Fig. 2. Combined effects of tirapazamine (●) and KU-2285 (▲) with a single 18 Gy irradiation in SCCVII tumors. Drug doses were chosen according to the LD_{50} value: tirapazamine (89 mg/kg) and KU-2285 (2100 mg/kg). The tumor-bearing mice received i.p. injection of the compounds 30 min prior to the irradiation. The tumors were excised 12 h after the irradiation and the surviving fractions were determined by the colony formation method. Each point with error bar represents the average and SD.

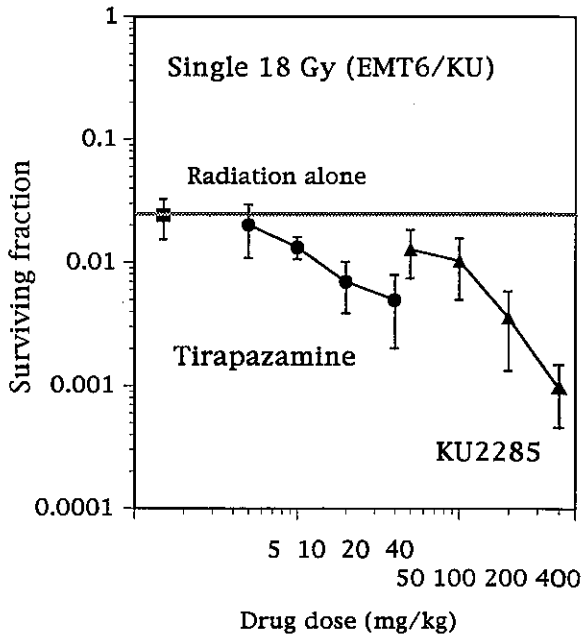


Fig. 3. Combined effects of tirapazamine (●) and KU-2285 (▲) with a single 18 Gy irradiation in EMT-6/KU tumors. Experimental procedures were similar to those described in the legend to Fig. 2. Each point with error bar represents the average and SD.

the mice were killed, the tumors were excised, minced, and dispersed into single cells with 0.1% neutral protease (Sigma Chemie, Deisenhofen, Germany). Eagle's minimum essential medium supplemented with 12.5% fetal bovine serum was used for both SCCVII and EMT-6/KU tumors throughout the experiments. Appropriate numbers of viable tumor cells were seeded into 60 mm plastic culture dishes and the tumor cell clonogenicity was determined by the standard colony formation method after a 10-day incubation. The data in each treatment group were obtained from 8–10 experimental animals and the geometrical average with the standard deviation for each treatment group was calculated and plotted by StatView4.0 (Abacus Concepts, Inc., Berkeley, CA) and DeltaGraph2.0 (DeltaPoints, Inc., Monterey, CA). The significance of differences was determined by a 2-tailed Student's *t* test.

RESULTS

Combined effect for single-dose irradiation Fig. 2 shows the results of a single 18 Gy irradiation of SCCVII tumors in the *in vivo-in vitro* assay. The hypoxic cytotoxin tirapazamine alone without irradiation reduced the surviving fraction to 0.64 ± 0.07 or 0.56 ± 0.03 (the mean $\pm$SD) for a single dose of 10 or 40 mg/kg, respectively, while the hypoxic radiosensitizer KU-2285 alone without irradiation had no significant effect on tumor cell survival. Tumor cell killing was significantly potentiated by tirapazamine at doses of $\geq$10 mg/kg and also by KU-2285 in a dose-dependent fashion. The effect of 20 mg/kg of tirapazamine was comparable to that of 100–200 mg/kg of KU-2285. A marked combined effect at 40 mg/kg of tirapazamine could be demonstrated, which may imply that larger numbers of acutely hypoxic cells produced by fluctuation of blood flow would be exposed to effective concentrations of the drug for longer durations.

Fig. 3 shows the data from the experiments of single 18 Gy irradiation with EMT-6/KU tumors. Tumor cell killing was significantly potentiated by both drugs and the effect of 20 and 40 mg/kg of tirapazamine was comparable to that of 100 mg/kg of KU-2285. At drug doses $\geq$200 mg/kg, KU-2285 showed marked radiosensitization.

Combined effect for fractionated irradiation Figs. 4 and 5 show the results with fractionated irradiation (4 fractions of 5 Gy, at 12 h intervals) with SCCVII and EMT-6/KU tumors, respectively. Tirapazamine at 10 and 20 mg/kg showed highly significant combined effects in both tumors. On the other hand, KU-2285 showed weaker,

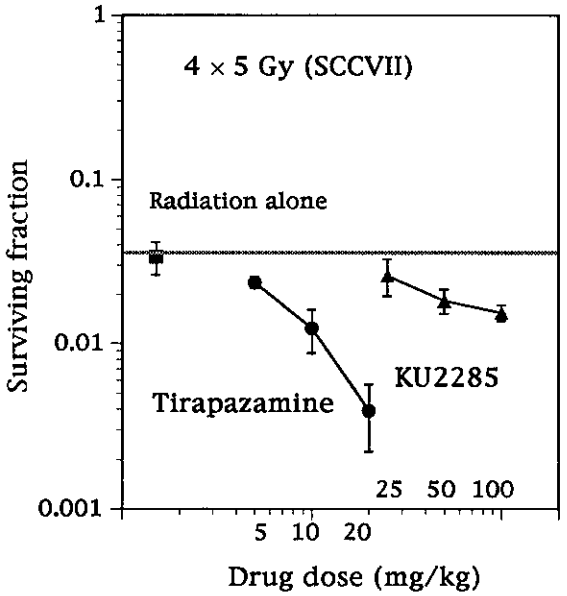


Fig. 4. Combined effects of tirapazamine (●) and KU-2285 (▲) with fractionated irradiation (4×5 Gy at 12 h intervals) in SCCVII tumors. The tumor-bearing mice received i.p. injection of the compounds 30 min prior to each irradiation. The whole-body irradiation was performed on mice moving freely in an acrylic box. The tumors were excised immediately after the last irradiation (at 36 h after the first irradiation) and the surviving fractions were determined by the colony formation method. Each point with error bar represents the average and SD.

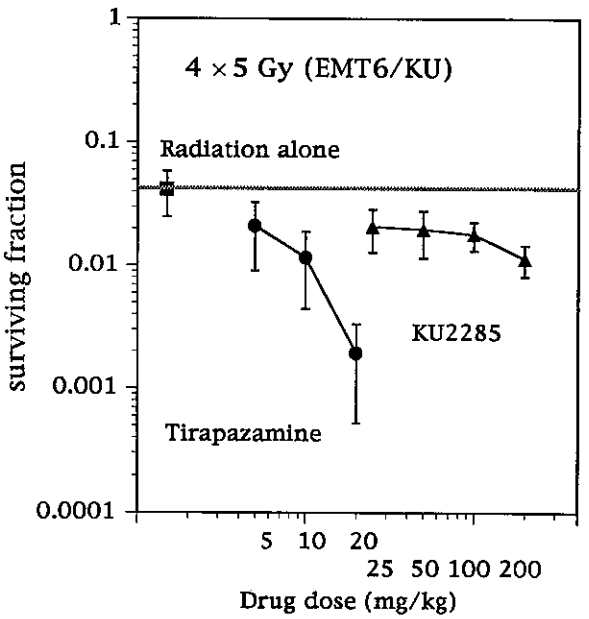


Fig. 5. Combined effects of tirapazamine (●) and KU-2285 (▲) with fractionated irradiation (4×5 Gy at 12 h intervals) in EMT-6/KU tumors. Experimental procedures were similar to those described in the legend to Fig. 4.

though still significant, enhancement of cell killing than did tirapazamine. These findings seemed different from the above findings with single-dose irradiation. Fractionated administration of tirapazamine was effective for EMT-6/KU tumors with chronically hypoxic cells. Therefore, the nature of hypoxia appears to be less important with fractionated rather than single-dose irradiation.

DISCUSSION

The presence of hypoxic cells in solid tumors is an important problem in radiotherapy. To assess the usefulness of chemical modifiers of the radiation response of the hypoxic cells, it is important to take into consideration the fact that hypoxia in tumors can result from two quite different mechanisms. Firstly, chronically hypoxic cells can be produced by limited diffusion of oxygen from capillaries, as described in the classical tumor cord theory by Thomlinson and Gray,[1] and secondly, acutely hypoxic cells can result from transient fluctuation of tumor blood flow, as postulated by Brown[4] and later demonstrated unequivocally in murine tumors by Chaplin and his colleagues.[7] In this study, we have evaluated the enhancement of tumor responses by a hypoxic cell radiosensitizer, KU-2285, and a hypoxic cytotoxin, tirapazamine, when combined with both single and fractionated irradiation. We have characterized the different effects of the two drugs and also of the two radiation regimens in two murine tumors having different characteristics of hypoxia. The hypoxic fraction determined by the paired-survival curve method was about 5.4–10% and 14–15% in SCCVII and EMT-6/KU tumors, respectively. These values appeared to be smaller than those found by Brown and Lemmon.[21] Shibamoto *et al.*[34] have reported that the use of anesthesia and the immobilization of the mice in jigs could produce significant increases (>20%) in the hypoxic fraction in SCCVII tumors. Therefore, we performed whole-body irradiation of tumor-bearing mice moving freely in an acrylic box to prevent the above-mentioned conditions from affecting the hypoxic fractions, since these procedures can artificially cause not only radioprotection but also augmentation of cytotoxicity by tirapazamine.

As Figs. 2 and 3 show, although both tirapazamine and KU-2285 could exhibit significant combined effects with single-dose irradiation, tirapazamine at higher doses seemed more effective than KU-2285 in SCCVII tumor with acutely hypoxic cells, while tirapazamine seemed less potent at high doses than KU-2285 in EMT-6/KU

tumors predominantly with chronically hypoxic cells. Zeman *et al.*[18] have already reported that the rapid loss of patent tirapazamine due to extremely rapid metabolism occurred with a half life of the order of minutes in tumors rendered 100% hypoxic by clamping. Durand and Olive[35] have demonstrated using the V79 multicell spheroid system that rapid bioreduction of tirapazamine occurred at rates that exceeded drug delivery, resulting in considerably less efficacy when a large hypoxic fraction was present. These previous findings could account for the lesser effectiveness of tirapazamine in chronically hypoxic cells, because of lower ability to reach the entire hypoxic cell subpopulation. This hypothesis can also explain the previous results by Brown *et al.*[36,37] that the addition of hydralazine to tirapazamine did not affect the amount of cell killing in combination with 8×2.5 Gy, although hydralazine could produce essentially 100% hypoxia in the tumors. Because KU-2285 is thought to be chemically stable and not subject to rapid metabolic breakdown, and moreover to have adequate lipophilicity,[14,15,28,30] a sufficient amount of KU-2285 may penetrate into the entire hypoxic subpopulation and larger radiosensitization may occur with a large single-dose irradiation. It is not difficult to find situations similar to the delivery of a single high-dose irradiation in the clinic. For example, in cases of intraoperative radiation therapy performed at Kyoto University Hospital,[38,39] patients with advanced pancreatic cancers are usually given single-dose irradiation of more than 25 Gy and those with osteosarcomas receive more than 50 Gy; they represent possible candidates for the use of KU-2285. One important conclusion is that the *in vivo* effect of tirapazamine appears to be considerably influenced by the nature of tumor hypoxia, especially with single-dose irradiation. When a large hypoxic fraction is present in tumors, KU-2285 may exhibit greater radiosensitization than tirapazamine with single-dose irradiation. We generally agree with the previous observation by Brown and Lemmon[21] that there appeared to be an inverse correlation between the extent of radiosensitization of etanidazole and the radiation potentiation by tirapazamine.

As Figs. 4 and 5 show, KU-2285 had significant combined effects in both tumors at low doses clinically achievable in humans with oral administration.[17] These findings confirm the previous findings by Sasai *et al.*[28] with other regimens in combination with radiation dose fractionation that KU-2285 was able to sensitize SCCVII and transplantable mammary tumors *in vivo* even at such low drug doses. However, as clearly shown in these figures, the effects of KU-2285 were considerably smaller than those of tirapazamine. These results may confirm the findings[11,25,40] that the radiosensitizing activity of hypoxic cell radiosensitizers is less efficient at a smaller radiation dose. On the other hand, the hypoxic cytotoxicity of tirapazamine seems to be independent of the size of the radiation dose per fraction, and it appears more important with a fractionated regimen that tumors have multiple chances to be exposed to tirapazamine.

Interestingly, tirapazamine with this fractionated protocol could demonstrate great potentiation of cell killing in a similar fashion for both tumors in spite of the differences of hypoxia. The behavior of the hypoxic fraction between the fractions must play an important role in these effects observed with fractionated irradiation. Reoxygenation may be one of the major reasons why fractionated irradiation gives better results than single-dose irradiation. Reoxygenation between the fractions may also diminish the role of hypoxic cell radiosensitizers in clinical practice.[2,6,11,40] According to Kitakabu *et al.*,[32] who used the same irradiation conditions as in the present study, the hypoxic fraction of SCCVII tumors after single and fractionated irradiation showed a transient increase, followed by rapid decreasing trends to the pretreatment level. The recent report by Shibamoto *et al.*[41] on the hypoxic fraction after KU-2285 sensitization plus irradiation has also shown that reoxygenation occurred quite efficiently in SCCVII tumors, mainly due to the mechanism of acute hypoxia. More recently, Brown *et al.*[23,25,42] have introduced the term "rehypoxiation" based on the findings that re-establishment of the hypoxic fraction in SCCVII tumor will occur after a single dose of tirapazamine with the same kinetics as for reoxygenation. According to these experimental findings and hypotheses concerning the existence of acute hypoxia,[4,6,7,25] hypoxic cells may exist essentially at every fractionation, and any treatment strategy to target the hypoxic cells would theoretically be able to demonstrate significant therapeutic gains. We have shown that KU-2285 is one of the most efficient hypoxic cell radiosensitizers even at clinically relevant low radiation doses (0–4.5 Gy), by using the micronucleus assay.[16,17,29] However, the sensitization of hypoxic cells by KU-2285 cannot exceed the radiosensitivity of the fully oxygenated cells. In contrast, the cytotoxicity toward hypoxic cells by tirapazamine, depending on both the drug concentration and exposure duration, can easily go beyond the radiation effects on the oxygenated cells. Although the reoxygenation in EMT-6/KU tumors was found to occur more slowly than in the SCCVII tumors,[33] the result did not show directly whether acutely hypoxic cells might be involved in EMT-6/KU tumors. Zwi *et al.*[43] have clearly demonstrated that a spontaneous loss of perfusion occurred in EMT-6 tumors by using a double-label fluorescence technique, providing evidence for the existence of acute hypoxia in addition to chronic hypoxia in EMT-6 tumors. Although tirapazamine seemed less effective for chronically hypoxic cells in the single-dose regimen, fractionated administration may enable tirapazamine not

only to reach the residual hypoxic cells repeatedly in the chronically hypoxic areas, but also to kill the acutely hypoxic cells produced because of spontaneous perfusion loss.

In conclusion, tirapazamine appeared more effective when combined with fractionated rather than single-dose irradiation in two murine tumors, possibly due to the effect on acutely hypoxic cells. These findings suggest the potential usefulness of tirapazamine in clinical radiation therapy. However, it seems premature to apply the results obtained on the fractionated regimens directly to clinical radiotherapy, because it is still not clear whether reoxygenation and rehypoxiation occur so efficiently in human tumors[6, 25] and so constantly throughout the overall treatment period as in the preclinical studies using murine tumors. It is still unclear to what extent acute hypoxia may occur in human tumors. Further investigations on hypoxia in human tumors are under way.

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