Preclinical study on hypoxic radiosensitizing effects of glycididazole in comparison with those of doranidazole *in vitro* and *in vivo*

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Abstract. To overcome the radioresistance of hypoxic cells in solid tumor, numerous types of radiosensitizers specifically against them have been developed. Glycididazole has a chemical structure in which two metronidazole forms are combined, and is widely used as a hypoxic radiosensitizer in China. However, a detailed investigation of its radiosensitizing properties has not been performed. The present study reported a comparative assessment of glycididazole and doranidazole, another hypoxic radiosensitizer. All experiments were performed using the murine squamous cell carcinoma cell line SCCVII. Prior to X-irradiation, the cells were treated with the test drugs at concentrations of 10 mM and 200 mg/kg *in vitro* and *in vivo*, respectively. Uptake and their intratumor chemical forms were analyzed by high performance liquid chromatography (HPLC). Both drugs enhanced the reproductive cell death induced by X-irradiation under hypoxia. However, the growth delay assay of the transplanted tumor revealed the combination of X-irradiation and glycididazole showed a similar antitumor effect to that of X-irradiation alone, whereas doranidazole significantly sensitized the cells to X-irradiation. HPLC analysis revealed that incorporated glycididazole was decomposed to metronidazole and was therefore present at a lower concentration compared with that of doranidazole. The decomposition of glycididazole to metronidazole reduced its radiosensitizing efficiency *in vivo*. Elucidation of the kinetics of drugs containing metabolizable chemical forms is necessary for the optimization of clinical treatment.

Introduction

Tumor hypoxia is recognized as a well-known obstacle to radiotherapy due to the limitation of oxygen effect, i.e., oxygen-dependent sensitization of radiation efficiency (1). To reduce the radioresistance of hypoxic tumors, several strategies, such as fractionated radiotherapy, increased oxygen delivery, hypoxia inducible factor (HIF)-1 inhibitors, and radiosensitizers, have been developed (2). Since the introduction of misonidazole in the 1970s, numerous hypoxic cell radiosensitizers have been screened; several have undergone clinical evaluation (3). However, clinical trials with nitroimidazole derivatives have demonstrated their limited therapeutic benefit and remain inconclusive. Furthermore, misonidazole and etanidazole, the well-known nitroimidazole derivatives, induced undesirable side effects such as neurotoxicity (4). Additionally, the low tolerable dose produced an inconclusive outcome and restricted the clinical use of these drugs. In contrast to these results, a Danish trial of nimorazole, another radiosensitizer, significantly improved the radiotherapy of squamous head and neck cancers without major side effects (5,6). In these trials, clinically relevant hypoxia markers allowed the identification of patients that benefit from the combination of nimorazole and radiotherapy.

Furthermore, doranidazole [1-(1',3',4'-trihydroxy-2'-butoxy)-methyl-2-nitroimidazole, PR-350; Fig. 1] was established in Japan; its structure is intended to reduce neurotoxicity owing to its impermeability across the blood-brain barrier (BBB) (7). The radiosensitizing effect of this compound under hypoxia has been clarified *in vitro* (8-10) and *in vivo* (8,9,11,12). Recently, our group demonstrated the radiosensitizing effect of doranidazole on C6 rat intracranial glioma, which shows wide range of hypoxia and disruption of the BBB (13). Based on these studies, a phase I/II trial against locally advanced non-small-cell lung cancer (14) and phase III trials of doranidazole against advanced pancreatic cancer (15,16) were performed. Some studies demonstrated

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that doranidazole treatment after irradiation significantly improved the local tumor control and overall survival. These recent clinical reports have encouraged the further development and improvement of nitroimidazoles.

In China, sodium glycididazole, a nitroimidazole derivative with a chemical structure containing a dimer of metronidazole (Fig. 1), was designed, screened, and studied by the Second Military Medical University (17). There are several literature reports in Chinese that indicate the radiation-enhancing potential of glycididazole. In addition, glycididazole was reported to have radiosensitizing and chemosensitizing effects in clinical trials (17-19) and is marketed and used clinically. However, there are few preclinical studies and no investigations have been performed to evaluate the radiosensitizing efficacy of glycididazole in comparison with that of other nitroimidazole derivatives. Therefore, in this study, we aimed to evaluate the radiosensitizing effects of glycididazole and doranidazole on tumor cells with different oxygenation conditions *in vitro* and on the tumor growth of subcutaneous xenografts *in vivo*.

Materials and methods

Reagents. Glycididazole was purchased from Luye Pharma Group Co., Ltd, (Beijing, China). Doranidazole was supplied by Pola Pharma Inc. (Tokyo, Japan). Ultrapure N_2 gas (99.999%) was obtained from Air Water Technical Supply (Ishikari, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), unless otherwise stated.

Cell culture. Murine squamous cell carcinoma SCCVII cells were grown in α-MEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Filtron Pty. Ltd., Brooklyn, Australia). Cells were maintained at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Drug treatment, hypoxic incubation, and X-irradiation in vitro. Tumor cells were allowed to adhere to a 6-cm plastic dish and treated with 10 mM glycididazole or doranidazole before hypoxic incubation. To establish the hypoxic conditions (oxygen concentration ≤10 mmHg [1.3%]; unpublished data) for tumor cells, the dish was placed in a gas-exchangeable chamber and ultrapure N_2 gas was continuously passed over the dish, which was placed on ice, for 25 min. The cells were then exposed to the indicated doses of X-rays while maintaining the gas flow. X-irradiation was performed with a Shimadzu PANTAK HF-350 X-ray generator (1.0 mm Al filter; 200 kVp; 20 mA; Shimadzu, Kyoto, Japan).

Clonogenic survival assay. At 5 h before hypoxic induction, 100-20,000 of SCCVII cells were seeded on a 6-cm plastic dish. After drug treatment, hypoxic induction, and X-irradiation as described above, the cells were incubated with fresh medium supplemented with 10% FBS under normoxia at 37˚C for 8 days. The cells were subsequently fixed with methanol, stained with Giemsa solution, and scored under a microscope. Only colonies that contained more than 50 cells were scored as surviving cells. After the surviving fraction at each dose was calculated with respect to the plating efficiency of the non-irradiated control, the mean ± standard error of the surviving fractions obtained from three experiments was plotted. The survival curves were

fitted to a linear-quadratic (LQ) model: SF=exp (-αD-βD^2), where SF is the surviving fraction and D is the physical dose, by using the data analysis software Origin Pro 7 (OriginLab Co., Northampton, MA, USA).

Animal model. All animal experiments in this study were conducted according to the guidelines of the Law for The Care and Welfare of Animals in Japan and approved by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Male C3H/HeN mice were purchased from Japan SLC (Hamamatsu, Japan). SCCVII solid tumors were established via s.c. injection of $2x10^5$ cells into the limbs of micle.

Drug treatment and X-irradiation in vivo. The experiment was initiated when the tumor volume (V=0.5 x length x width^2) reached 250-450 mm^3. The body weights measured before the experiments were in the range from 20-25 g. The animals were randomized into six groups of 9-10 animals per group: i) saline only; ii) saline and X-irradiation (30 Gy); iii) glycididazole only; iv) glycididazole and X-irradiation; v) doranidazole only; and vi) doranidazole and X-irradiation. Glycididazole and doranidazole were intravenously injected into mice at a dose of 200 mg/kg, 20 min before X-irradiation. For the irradiation of transplanted tumors, the mice were shielded with lead panels, except for the tumor-bearing limbs. X-irradiation was performed with an X-Rad iR-225 (1.0 mm Al + 0.5 mm Cu filters; 250 kVp; 16.7 mA; Precision X-Ray; North Branford, CT, USA) at a dose rate of 1.7 Gy/min. The tumor volume was measured three times per week and the tumor growth was monitored for a maximum of 47 days.

high performance liquid chromatography (HPLC) analysis. At 20 min after i.v. injection of the compounds, the mice were sacrificed and blood and tumor tissues were sampled. Serum was obtained via centrifugation of the blood sample at 5,000 x g for 15 min and passed through an Ultrafree-MC filtration device (EMD Millipore, Billerica, MA, USA). Tumor samples were homogenized with Polytron (Kinematica, Luzern, Switzerland) and centrifuged at 3,000 x g for 5 min. The supernatant was diluted with two volumes of methanol and centrifuged at 2,500 x g for 10 min. The obtained supernatant was mixed with two volumes of ethanol, which was subsequently evaporated completely. Aqueous serum and resolved tumor samples were analyzed using an HPLC system comprising a gradient pump (LC-10ADvp), an autosampler (SIL-10ADvp), an in-line degasser (DGU-20A3), a column oven (CTO-10ACvp), and a UV detector (SPD-10Avp) (all from Shimadzu). System control and data analysis were performed by using LCsolution (Shimadzu). Separation was performed by using a reversed phase column (4.6x250 mm) of TSK gel ODS-80Tm (TOSOH).

Statistical analysis. The Kaplan-Meier method was used to analyze the doubling times. Log-rank test was used to compare the doubling times between any 2 treatment groups.

Results

Glycididazole radiosensitized cancer cells under hypoxic conditions to a similar extent as doranidazole did in vitro.

Figure 1. Chemical structures of glycididazole and doranidazole.
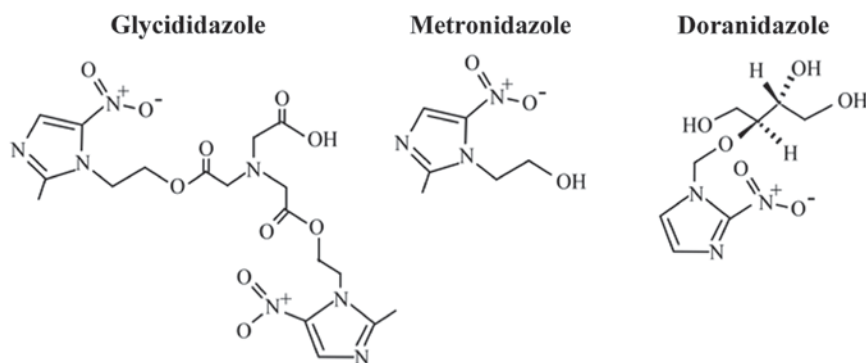
To investigate the effects of glycididazole and doranidazole on the radiosensitivity of cancer cells, colony formation assay was performed. The clonogenic survival curves of SCCVII cells irradiated *in vitro* under normoxic and hypoxic conditions, with or without these drugs, are shown in Fig. 2A. In the absence of glycididazole, X-irradiation under hypoxia reduced the radiosensitivity of SCCVII cells. Under both normoxia and hypoxia without irradiation, 10 mM glycididazole was not toxic to SCCVII cells. The survival curves of SCCVII cells irradiated under normoxia show that glycididazole decreased the clonogenic ability. The dose that reduced cell survival to 10% (D_{10}) obtained from the normoxic cell survival curve was 6.55 Gy, which decreased to 6.08 Gy when the cells were irradiated in the presence of glycididazole (Table I). The enhancement of radiation-inducing cell death was more pronounced in hypoxic condition; the D_{10} values were 9.18 and 7.09 in the absence and presence of glycididazole, respectively. The sensitizing enhancement ratio (SER) for glycididazole was 1.08 and 1.29 for normoxic and hypoxic conditions, respectively, which suggested that glycididazole sensitized both hypoxic and normoxic cells to radiation *in vitro*.

Alternatively, although 10 mM doranidazole exerted no sensitizing effect when combined with irradiation in aerobic conditions, a significant sensitizing activity was observed in combination with irradiation under hypoxic conditions (Fig. 2B). The SER for doranidazole, which was calculated from the D_{10} values in each condition, was 1.02 and 1.24 for normoxic and hypoxic conditions, respectively (Table I). These results suggested that glycididazole exerted a radiosensitizing effect on hypoxic cells that was comparable to that of doranidazole.

Glycididazole showed weak radiosensitizing effect on tumor growth compared with that of doranidazole in vivo. To evaluate the radiosensitizing effects of glycididazole and doranidazole on squamous carcinoma *in vivo*, SCCVII tumor-bearing mice were treated with 30 Gy X-irradiation 20 min after the i.v. administration of 200 mg/kg glycididazole or doranidazole. As shown in Fig. 3 and Table II, a single administration of either glycididazole or doranidazole exerted little effect on tumor growth. X-irradiated tumors exhibited delayed growth and the doubling time of tumor volume after X-irradiation was 18 days. The treatment with 200 mg/kg doranidazole significantly enhanced the inhibition of radiation-induced growth and extended the doubling time to 42 days (P<0.001). Although treatment with glycididazole at the same dose extended the

Figure 2. The radiosensitizing effect of the tested compounds in SCCVII cells. The clonogenic survival of SCCVII cells after treatment with glycididazole (A) or doranidazole (B) and X-irradiation was assessed by colony formation assay. Each compound was treated at a concentration of 10 mM. Hypoxic treatment and X-irradiation were performed as described in Materials and Methods. Figure symbols are as follows: X-irradiation only under normoxia (closed circles); tested compound + X-irradiation under normoxia (open circles); X-irradiation alone under hypoxia (closed squares); and tested compound + X-irradiation under hypoxia (open squares). Data are expressed as the mean ± standard error for three experiments.

doubling time to 20 days significantly (P<0.05), the enhancement rate was clearly smaller than that of doranidazole.

Glycididazole was decomposed to metronidazole and its concentration was relatively low compared with that of

Table I. Survival parameters and sensitizer enhancement ratios for SCCVII cells treated with X-irradiation combining with glycididazole or doranidazole.

Treatment	Oxygen condition	Drug	α (Gy^{-1})	β (Gy^{-2})	D_{10}	SER$_{D10}$
Glycididazole	Normoxia	-	0.183	0.026	6.55	1.08
		+	0.186	0.032	6.08	
	Hypoxia	-	0.088	0.018	9.18	1.29
		+	0.214	0.016	7.09	
Doranidazole	Normoxia	-	0.173	0.031	6.29	1.02
		+	0.191	0.030	6.16	
	Hypoxia	-	0.052	0.021	9.38	1.24
		+	0.189	0.015	7.53	

The biological parameters and sensitizer enhancement ratios (SER) were calculated based on survival curves. Calculation formula is shown in Materials and methods.

Table II. Tumor doubling time.

Treatment	Days (lower, upper limits)
Saline	4 (4, 8)
Glycididazole	4 (2, 6)
Doranidazole	4 (4, 6)
Saline + X-rays	18 (8, 20)
Glycididazole + X-rays	20 (18, 33)[a]
Doranidazole + X-rays	42 (27, 47)[b,c]

The median time required for tumor volume doubling is illustrated with lower and upper limits in parentheses. Statistical significance is indicated as [a]P<0.05, [b]P<0.001, vs. Saline + X-rays, [c]P<0.01, vs. Glycididazole + X-rays.
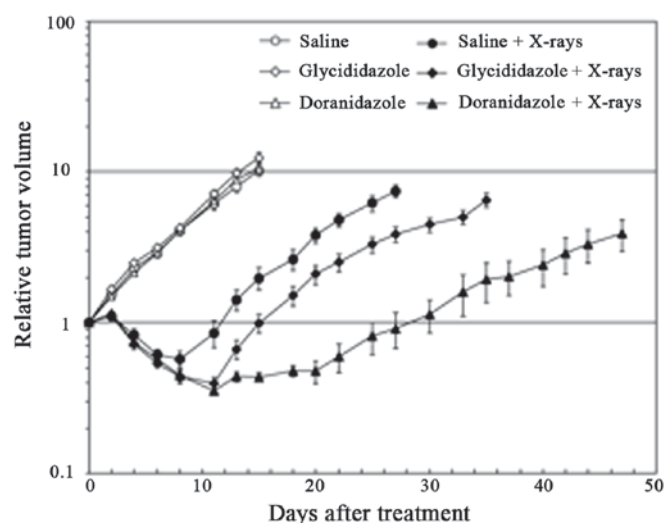

Figure 3. Tumor growth curves in mice treated with 30 Gy of X-irradiation and/or 200 mg/kg drugs. Drug treatment and X-irradiation were performed as described in Materials and methods. Tumor volume on the y-axis is presented as the relative size to the initial volume when the treatment was performed. Figure symbols are as follows: Saline only (open circles); saline and X-irradiation (closed circles); glycididazole only (open diamonds); glycididazole and X-irradiation (closed diamonds); doranidazole alone (open triangles); and doranidazole and X-irradiation (closed triangles). Data are expressed as the mean ± standard error for 9-10 animals per group.

doranidazole. To explain why glycididazole did not enhance radiation-induced tumor suppression, we assessed the chemical forms and tissue concentrations after the administration of glycididazole and doranidazole *in vivo*. The results of the HPLC analysis showed that the incorporated glycididazole was barely detected in its original form within the tumor and that the only detectable metabolite was metronidazole, as shown in Fig. 4A. In contrast, the administered doranidazole was detected as the undecomposed form (Fig. 4B). The concentrations of these drugs in the tumor and blood serum were also examined. As shown in Fig. 4C, the amount of glycididazole incorporated into tumor in the form of metronidazole was significantly lower (26.07±8.25 μg/g of tissue) than that of doranidazole (57.16±11.79 μg/g of tissue). This trend was also observed in blood serum.

Discussion

The purpose of this study was to re-evaluate the radiosensitizing effect of glycididazole, which is widely used in China for clinical treatment, *in vitro* and *in vivo*. As shown in Fig. 2, treatment with 10 mM glycididazole improved the radiosensitivity of SCCVII cells under hypoxia, similar to doranidazole. Interestingly, the enhancement of radiation-induced cell death by glycididazole

was observed even in normoxia. A recent report by Zeng *et al* demonstrated the similar effect of glycididazole on normoxic laryngeal cancer cells and revealed significant downregulation of ataxia-telangiectasia mutated (ATM), p-ATM, CHK2, and p53 and the upregulation of MDM2 and Cdk2 after the combined treatment with glycididazole and X-irradiation, not only in hypoxic conditions, but also in normoxic conditions, and concluded that glycididazole has the potential to inhibit the ATM signaling response after X-irradiation (20). Although glycididazole is composed of a metronidazole dimer, to the best of our knowledge, there are no reports to demonstrate that metronidazole exerted radiation modifying effects, except for its oxygen-mimetic properties owing to the electron affinity. Therefore, glycididazole may have unknown physiological activity and further investigation is necessary.
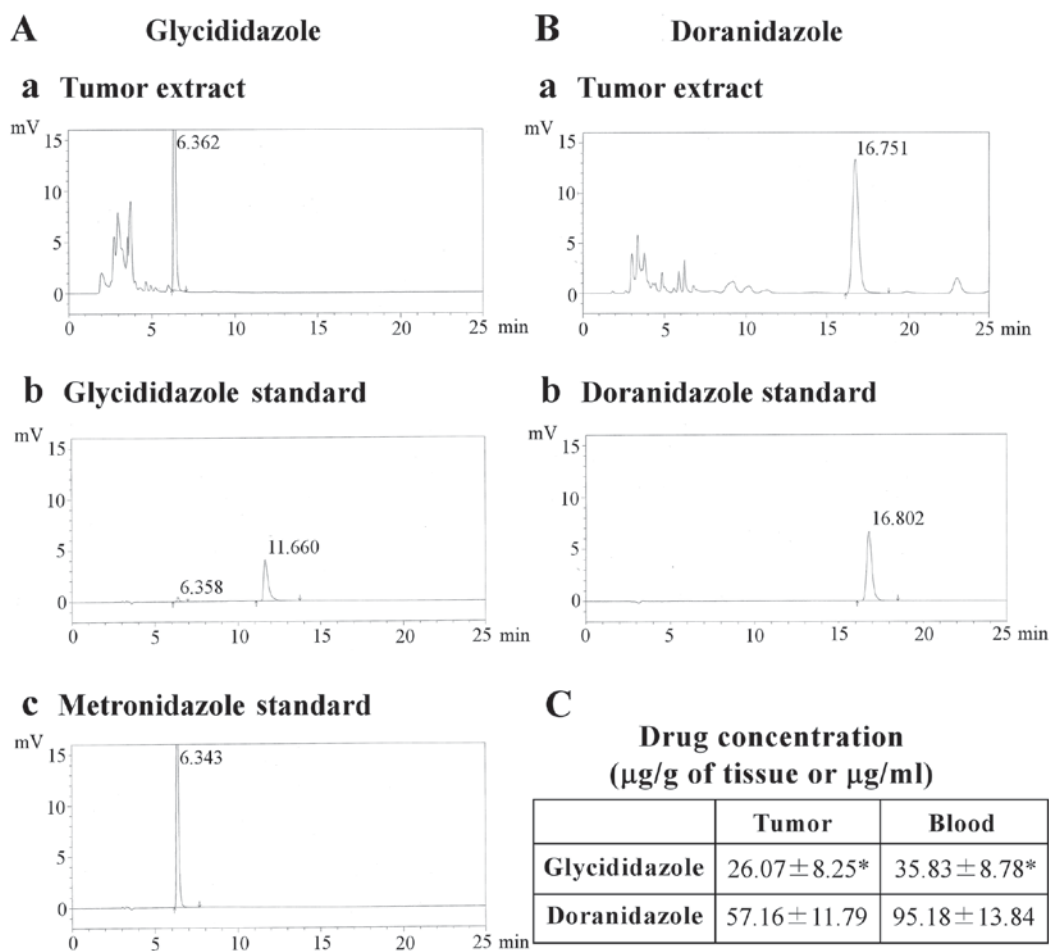
Figure 4. HPLC profiles of tumor homogenates from SCCVII-bearing mice treated with 200 mg/kg glycididazole (Aa) and doranidazole (Ba). Lower panels represent the spectrum of the standards of glycididazole (Ab), metronidazole (Ac), or doranidazole standard (Bb). (C) Drug concentrations in the tumor and blood of mice treated with glycididazole and doranidazole. Note that the concentration of glycididazole was shown as that of metronidazole, which was calculated from standard metronidazole, because we could not detect glycididazole in the tumor or blood.

In contrast to the *in vitro* study that showed that the radiosensitizing effect of glycididazole (SER=1.29) was similar to that of doranidazole (SER=1.24) under hypoxic conditions, 200 mg/kg glycididazole exhibited a relatively weak sensitizing effect compared with that of doranidazole in SCCVII-transplanted tumor cells (Fig. 3). Only one study has examined the antitumor effect of glycididazole using the growth delay assay in a transplanted tumor model (20). The authors demonstrated the significant radiosensitizing potential of glycididazole in a xenografted Hep-2 tumor mouse model, but they employed a high dose of glycididazole (3×700 mg/m^2/day). Combined with our results, glycididazole did not show as strong a radiosensitizing effect as expected from the *in vitro* study. The inconsistencies between the *in vitro* and *in vivo* studies may be explained by the results of the HPLC analysis (Fig. 4). In tumor lysate obtained 20 min after glycididazole administration, glycididazole was not detected in the HPLC spectra, but metronidazole was detected. These results suggested that the glycididazole incorporated into the tumor was rapidly decomposed and bound to biomolecules as metronidazole adducts. This hydrolysis is thought to occur in the presence of esterase or ester hydrolase. Mahfouz and Hassan examined the hydrolysis kinetics of a series of metronidazole amino acid ester prodrugs (21). In an aqueous phosphate solution of pH 7.4,

the prodrugs exhibited adequate chemical stability (half-life, $t_{1/2}$, 4-16 h). However, in 80% human plasma, the drugs were hydrolyzed to metronidazole within a few min. Metronidazole benzoate, an internal remedy, also underwent rapid hydrolysis; however, no significant hydrolysis of the ester in gastric fluid and intestinal fluid was observed, but only metronidazole was reported in the sera and urine of patients administered metronidazole benzoate (22,23). In addition, it was reported that a twin ester prodrug, which consisted of a dimer of metronidazole and twin esters, was chemically stable at the physiological pH buffer solution ($t_{1/2}$, 13-40 h) but the release of metronidazole from the prodrug ensued rapidly in 80% human plasma ($t_{1/2}$, 10-150 min) (24). These reports suggest that glycididazole, in which two metronidazoles are linked by the ester bond, can be easily decomposed to the parental form *in vivo*. Unfortunately, a high-dose amount of metronidazole is required to achieve radiosensitization; at this level, metronidazole causes severe nausea and vomiting (25). Therefore, to increase the bioavailability of glycididazole and to obtain a sufficient response in the tumor, it was necessary to increase its stability in blood through modification of the linker bond that connected the two metronidazoles. For example, the twin ester prodrug derived from phthalic acid is not a substrate for the enzyme esterase and shows a slow release of metronidazole ($t_{1/2}$, 12.4 h) (24).

We think two advantages will be obtained if the stability of such a twin drug is improved as follows. First is that the availability of 2-nitroimidazole moiety for radiosensitization against hypoxic cells can be increased. Because 2-fold of 2-nitroimidazole moieties per mole are provided, the chance of oxidation of radiation-induced free radicals is thought to be increased, leading to the induction of much more cell killing. This effect may be more pronounced in a condensed microenvironment *in vivo* than a monolayer culture *in vitro*. Second, to establish the method to stabilize the linker bond that connected the two metronidazoles is important as a toehold to develop the stable polymeric nitroimidazole compounds. Thus far, few attempts to develop and evaluate the polymeric compounds have been achieved. The promising compounds with multiple 2-nitoroimidazole moieties have a potential not only to exhibit stronger radiation-enhancing effect as a hypoxic radiosensitizer, but also to accumulate in hypoxic region more efficiently as a hypoxic imaging probe.

In conclusion, glycididazole efficiently sensitized cancer cells to radiation under both hypoxia and normoxia *in vitro*. However, the expected extent of tumor regression could not be achieved by the combination of X-irradiation and glycididazole. The modification of the chemical structure to reduce the decomposition of glycididazole to metronidazole is necessary to improve the cure rate of the combined therapy with X-irradiation in clinic.

Acknowledgements

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