Effects of Hypoxic Cell Radiosensitizer Doranidazole (PR-350) on the Radioresponse of Murine and Human Tumor Cells

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Doranidazole/Hypoxic cell/Sensitizer/Pancreatic tumor/Oxygenation status.

We have investigated the radiosensitizing effect of doranidazole, a hypoxic cells radiosensitizer, using SCCVII tumor cells of C3H mice and CFPAC-1 and MIA PaCa-2 human pancreatic tumor cells. The radiosensitivity of hypoxic SCCVII cells in vitro increased with 1 mM doranidazole by a factor of 1.34 and 1.68, when determined by clonogenic survival and micronucleus (MN) formation, respectively. The radiation-induced growth delay of SCCVII tumors was significantly enhanced and the TCD_{50/120} was reduced by a factor of 1.33 when 200 mg/kg doranidazole was injected, i.v., 20 min prior to tumor irradiation. The in vivo-in vitro excision assay showed that radiosensitivity of SCCVII cells in vivo increased by a factor of 1.47 with 200 mg/kg doranidazole. The radiation-induced growth delay of CFPAC-1 xenografts in nude mice was significantly enhanced and the TCD_{50/90} was reduced by a factor of 1.30 by 200 mg/kg doranidazole. On the other hand, 200 mg/kg of doranidazole exerted no influence on the radiation-induced growth delay in MIA PaCa-2 xenografts. The tumor oxygenation status, as determined with an oxygen sensitive needle probe and the immunohistological study using pimonidazole, indicated that MIA PaCa-2 tumors are better oxygenated than CFPAC-1 tumors. The relatively well-oxygenated status in MIA PaCa-2 tumor may account for the lack of radiosensitization by doranidazole. It is concluded that the magnitude of radiosensitization of tumors by doranidazole is dependent on the oxygenation status of the tumors and that doranidazole may be useful in increasing the response of hypoxic human pancreatic tumor to IORT.

INTRODUCTION

The presence of radioresistant hypoxic cells in human malignant tumors is believed to be one of limiting factors for complete eradication of tumors by conventional radiotherapy. Therefore, various means to overcome the hypoxic resistant to radiotherapy have been developed and underwent clinical trials in the past. Radiosensitization of hypoxic cells in human tumors using electron affinitive compounds such as nitroimidazole derivatives received considerable attention after they were demonstrated to be effective to radiosensitize hypoxic cells in vitro as well as in experimental animal tumor models. Unfortunately, clinical application of the chemical hypoxic cell radiosensitizer was not materialized mainly due to undesirable side effects of the drugs such as unacceptable neurotoxicity.

It has been shown that varying fractions of hypoxic cells in tumors undergo reoxygenation during the course of fractionated radiotherapy implying that the potential therapeutic gain by hypoxic cells radiosensitizer may be substantially compromised for fractionated radiotherapy as compared with that for a single radiation exposure. Recently, however, certain types of human tumor are treated with Intra-Operative Radiation Therapy (IORT), or hypofractionated conformal radiotherapy, which may limit reoxygenation of hypoxic cells. Therefore, it would be reasonable to expect that hypoxic cell radiosensitizers may be useful to increase the response of tumors to such a single or hypofractionated radiotherapy in which considerable fractions of target cell population are hypoxia. Unresectable pancreatic tumors are frequently treated with IORT in Japan. Pancreatic tumors are reported to be radioresistant due, at least in part, to poor oxygenation probably caused by poor vascularization. Therefore, we hypothesized that hypoxic cell radiosensitizers may be able to improve the response of pancreatic tumors to
IORT.

Doranidazole (1-(1′,3′,4′-trihydroxy-2′-butoxy)methyl-2-nitroimidazole, PR-350) is a hypoxic cell radiosensitizer. Because of the presence of low lipophilic side chain to the 2-nitroimidazole structure, this drug is rapidly eliminated from body. Therefore, this compound is less toxic than other hitherto known chemical hypoxic cell radiosensitizers to animals or human. Currently phase I and II clinical trials to evaluate the toxicity and effectiveness of doranidazole to enhance the response of human pancreatic tumors to IORT are in progress in Japan. In this study, as a part of our investigation to further reveal various aspects of radiosensitization by doranidazole, we have studied the implication of tumor oxygenation status in the response to a single dose of radiation exposure in vitro and in vivo using human pancreas xenografts and a mouse tumor model.

MATERIALS AND METHODS

Hypoxic cell radiosensitizer doranidazole

Doranidazole (1-(1′,3′,4′-trihydroxy-2′-butoxy)methyl-2-nitroimidazole, PR-350) is synthesized by POLA Pharmaceutical R and D Laboratory (Yokohama, Japan). Doranidazole was dissolved in PBS (Phosphate Buffered Saline) for in vitro study and in physiological saline for in vivo study. 2-14C-labeled doranidazole (14C-doranidazole) was synthesized at Daiichi Pure Chemicals, Co. Ltd (Tokyo, Japan) and used for the pharmacodynamic study of doranidazole in mice.

Reagents

Eagle’s minimum essential medium (MEM) was purchased from Nissui (Tokyo, JAPAN). Dulbecco’s modified minimum essential medium (DMEM) and Iscove’s modified Dulbecco’s medium (IMDM) were purchased from GIBCO BRL (Rockville, MD). Fetal bovine serum was purchased from SERO (St. Louis, MO, U.S.A.). Pimonidazole hydrochloride and anti-pimonidazole adducts monoclonal antibody (HypoxyprobeTM-1 kit) were obtained from Natural Pharmacia International, Inc. (Research triangle Park, NC). Serum-free protein block, streptavidin-conjugated peroxidase and 3,3′-diaminobenzidine peroxidase substrate (DAB) were obtained from DAKO JAPAN (Kyoto, JAPAN). Biotin conjugated affinity-purified antibody from a goat anti-mouse IgG was obtained from TAKARA (Osaka, Japan). Pronase and Crystal/Mount were obtained from Biomeda Corp. (Foster city, CA). The Soluenen350 tissue solubilizer and Hionic-Fluor scintillation cocktail were purchased from Packard Japan Company (Tokyo, Japan).

Cells

SCCVII mouse tumor cells were provided by Dr Murayama, Tokai University. Human pancreatic tumor cell line CFPAC-1 and MIA PaCa-2 were obtained from American Type Culture Collection (Manassas, VA). The SCCVII cells were cultured in MEM with 10% heat-inactivated fetal bovine serum (FBS). The CFPAC-1 cells were cultured in IMDM with 10% FBS and the MIA PaCa-2 cells were cultured in DMEM supplemented with 2.5% heat-inactivated horse serum and 10% FBS. All the cells were cultured in humidified mixture of 95% air / 5% CO2 in a 37°C incubator.

Irradiation

Cells in vitro or tumors grown in hind leg of mice were irradiated with a 250 kVp X-ray machine at an average dose rate of 1.6–1.8 Gy/min. For the in vitro MN assay, cells were irradiated with 60Co-iradiator at a dose rate of 1.0 Gy/min.

Pharmacokinetics of doranidazole

SCCVII tumor cells in exponential growth phase in culture were harvested by trypsinization and washed, about 5×106 cells in 0.05ml PBS were inoculated s.c. into the right hind legs of female C3H/HeN mice. The mice (10 weeks old, 19–22g, Nippon CLEA JAPAN, INC) bearing 8–9 mm diameter SCCVII tumors in hind legs were injected with 14C-doranidazole at 200 mg/5mL/kg (1.85 MBq/kg) through tail vein. At 5, 15, 30 minutes and 1, 2, 4 and 24 hours after the 14C-doranidazole injection, the concentration of the labeled drug in serum and tumor were determined. The blood drawn from tail vein was centrifuged, serum was collected and dissolved in Soluene-350. The excised tumors were weighed, minced and dissolved in Soluene-350. The volume of Soluene-350 containing the serum or tumor tissues was adjusted with toluene, mixed with scintillator Hionic-Fluor and the radioactivities of the samples were counted with a liquid scintillation counter.

Radiosensitization of hypoxic SCCVII cells in vitro with doranidazole

SCCVII cells in exponential growth phase in culture were harvested by trypsinization, washed and suspended in serum free medium at a concentration of 0.5–1×106 cells/mL in glass tubes. The tubes were plugged with stoppers, and two hypodermic needles were pierced though the stopper into tubes. A gas mixture of 95% N2 and 5% CO2 was flushed through one of the needles of each tube for 20 min at room temperature while another needle served as an outlet of the gas. Doranidazole solution in serum free medium in glass tubes was also flushed with 95% N2/5% CO2 for 20 min at room temperature. The hypoxic doranidazole solution was transferred using a glass syringe into the tube containing hypoxic cell suspension mixture, and the cells were irradiated immediately. The cells were then washed twice with serum-free medium to remove doranidazole and the closo-
genic cell survival or the frequency of micronucleus in the cells were determined.

Clonogenic cell survival Assay. Appropriate numbers of irradiated and unirradiated control SCCVII cells were seeded in 60 mm diameter plastic culture dishes, incubated for 7 days under 5% CO\textsubscript{2}/95% air atmosphere at 37°C, fixed with methanol and stained with Giemsa solution. The number of colonies containing more than 50 cells were counted and the surviving fraction was calculated.

Micronucleus (MN) Assay. The irradiated and unirradiated control SCCVII cells were seeded in 60 mm diameter plastic culture dishes containing cytochalasin B (2 μg/mL) to inhibit cell division. After a 24 hr incubation, the cells were fixed with Carnoy solution (ethanol : acetic acid) and stained with propidium iodide. The micronuclei in the binucleated cells were counted with fluorescence microscope. At least 400 binucleated cells were assessed per dish, and the mean number of micronuclei per single binucleated cell was defined as the micronucleus frequency.\textsuperscript{39}

Radiosensitization of SCCVII tumor in vivo

Effect of doranidazole on the radiosensitivity of SCCVII tumors was evaluated by determining the survival of clonogenic tumor cells using in vivo-in vitro excision assay method and also by determining the tumor growth delay and TCD\textsubscript{50/120}. About 2 × 10\textsuperscript{5} SCCVII tumor cells in 0.05 ml PBS were inoculated s.c. into the right hind legs of female C3H/HeN mice. When the tumors grew to 8–10 mm in diameters, the host mice were injected with 200 mg/kg of doranidazole dissolved in saline through tail vein. The tumor volume was measured 2–3 times a week.

TCD\textsubscript{50/120} assay. The radiation dose required to cure 50% of tumors 120 days after irradiation (TCD\textsubscript{50/120}) was calculated.

Radiosensitization of human pancreatic tumor in vivo

Tumor growth delay. Human pancreatic tumor cells (CFPAC-1 and MIA Paca-2) in exponential growth phase in culture were harvested by trypsinization and washed, and about 5 × 10\textsuperscript{6} single cells in 0.1 ml PBS were inoculated s.c. to the flank of BALB/c-\(\nu\)/nu mice (NIPPON CLEA JAPAN, INC.). When the xenografts grew to about 1 cm in diameters, they were excised, cut into small pieces and transplanted s.c. into the right hind leg of BALB/c-\(\nu\)/nu mice. Attempts were made to inoculate a similar size of tumor piece to each mouse. When the tumors grew to about 10 mm in diameters, the host mice were injected with 200 mg/kg of doranidazole dissolved in saline through tail vein. The volume of doranidazole solution was kept at 0.02 mL/g body weight. The tumors were irradiated locally as described above and the tumor volume was measured 2–3 times a week.

TCD\textsubscript{50/90} assay. The radiation dose required to cure 50% of tumors 90 days after irradiation (TCD\textsubscript{50/90}) was calculated by the method using SCCII tumors.

Determination of pO\textsubscript{2} in tumors

The tumor pO\textsubscript{2} was determined with Eppendorf pO\textsubscript{2} Histograph (Eppendorf, Hamburg, Germany).\textsuperscript{30–32} The mice bearing tumors in hind legs were anesthetized and laid on a Plexiglas board, which was placed on a heating pad. The body temperature (rectal) of mice was maintained at 36–37°C by warming the Plexiglas board with the underlying heating pad. Self-adhering reference electrodes (SynCor Neonatal ECG Electrode, Lectec Corp., Minnetonka, MN) were attached to the shaved dorsal surface of the animals. A 0.3 mm diameter pO\textsubscript{2} electrode was inserted about 2 mm deep into tumors by hand through a small incisions made in the skin over the distal side of the tumors. The electrode was then inserted into the tumors by a computer-controlled system.\textsuperscript{30–32} The pO\textsubscript{2} was measured along 3 horizontal and parallel tracks in the lower layer of the tumors and 2 parallel tracks in the upper layer of the tumors. The mean and median pO\textsubscript{2} as well as the % frequency of pO\textsubscript{2} readings lower than 5.0 mm Hg in each tumor were determined.

Immunohistological visualization of hypoxic loci with Pimonidazole

The degree of hypoxia in tumors was examined also by visualizing hypoxic loci in tumors using 10 mg/mL pimonidazole hydrochloride (Hypoxyprobe-1) as previously described.\textsuperscript{33} The tumor bearing mice were injected with 60 mg/kg pimonidazole hydrochloride through tail vein, sacrificed 90 min later, the tumors were excised and immediately fixed with 20% formalin. After paraffin embedding and sectioning of the fixed tumor tissue, monoclonal antibody
against the protein adducts of pimonidazole was added. To reveal the location of these adducts, a chromogenic secondary antibody was added and the sections were counterstained with hematoxylin. The stained sections were examined with stereoscopic microscope for the pimonidazole binding, and the captured digital images (NIKON, Tokyo, JAPAN) were analyzed with computer software. The extent of immunostaining for pimonidazole was expressed as the percentage of stained region’s pixels.

Statistical Analysis

Statistical analyses of results were performed with the SAS software, Version 6.12. The difference in tumor doubling times among different groups were assessed by a tarone test, a test using log-rank type score with MULTTEST procedure in tumor growth delay. The TCD50/120 value and their 95% confidence limit were calculated from logistic regression analysis using PROBIT procedure of SAS. Differences with \( p \) value < 0.05 were considered to be statistically significant.

RESULTS

Radiosensitization of hypoxic SCCVII cells in vitro

Clonogenic survival assay. The radiation survival curves of clonogenic SCCVII cells irradiated in vitro under oxic and hypoxic condition with or without presence of 1mM doranidazole are shown in Fig. 1. The \( D_0 \) of hypoxic cell survival curve was 3.27 Gy and it decreased to 2.48 Gy when cells were irradiated in the presence of 1mM doranidazole. The sensitizing enhancement ratio (SER), i.e. ratio of these \( D_0 \) values was 1.32. The ratio of doses required to reduce the cell survival to 1% under hypoxic and oxic conditions was 1.34. Under hypoxic conditions without irradiation, survival fraction with or without doranidazole was 0.834 ± 0.083 (mean ± S.D.) and 0.886 ± 0.212, respectively. The survival curve of oxic cells irradiated with 1mM doranidazole was identical to that irradiated without doranidazole (data not shown).

Micronucleus (MN) formation. The results of radiosensitizing effect of doranidazole on SCCVII cells as determined with MN method are shown in Fig. 2. The frequency of micronucleus in the cells irradiated in the presence of various dose of doranidazole under oxic or hypoxic conditions is plotted as a function of radiation dose to the cells. Under oxic condition, the radiation-induced MN was not affected...
by doranidazole (Fig. 2A). However, under hypoxic condition, the radiation-induced MN increased as a function of doranidazole dose (Fig. 2B). The SERs, as calculated from the slope of the linear regression lines of the frequency of MN, for 0.3 mM, 1 mM and 3.0 mM doranidazole were 1.42, 1.68 and 1.95, respectively. Under both hypoxic andoxic condition with doranidazole, no apparent induction of MN was observed in nonirradiated condition.

**Pharmacokinetics of doranidazole in mice bearing SCCVII tumor**

The concentrations of $^{14}$C-doranidazole in SCCVII tumors grown in the hind leg of C3H mice and that in the serum in the same mice were determined at various times after an i.v. administration of 200 mg/kg of $^{14}$C-doranidazole. The concentration of $^{14}$C-doranidazole in tumor peaked reading to 134 μg eq/g at 15 min after injection then gradually declined to 9 μg eq/g at 24 h (Fig. 3). The concentration of $^{14}$C-doranidazole in serum increased to a maximum of 362 μg eq/ml at 5 min after the drug injection and then declined at a half-life of 2.2 h during the initial 4 h and then at a slower rate thereafter. The serum concentration of $^{14}$C-doranidazole was almost undetectable at 24 hours after the drug administration.

**Radiation-induced clonogenic cell death in SCCVII tumors**

Figure 4 shows the effect of an i.v. injection of 200 mg/kg doranidazole on the radiation survival curves of clonogenic cells in SCCVII tumor as determined with *in vivo-in vitro* excision assay. Under nonirradiated condition, survival fraction with or without doranidazole was $0.445 \pm 0.131$ (mean ± S.D.) and $0.388 \pm 0.134$, respectively. From the slopes of similar survival curves for different doses of doranidazole, the SERs at various times after doranidazole injection was calculated (Table 1). The SERs were 1.09–

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![Graph](https://via.placeholder.com/150)

**Fig. 3.** Pharmacokinetics of doranidazole in the tumor and plasma in C3H mice bearing SCCVII tumor after a single i.v. injection. Each value represents the mean ± S.D. (n = 5). (○) plasma; (□) tumor

![Graph](https://via.placeholder.com/150)

**Fig. 4.** Radiation survival curves of SCCVII cells *in vivo* irradiated 20 min after an i.v. injection of 200 mg/kg doranidazole. Each value represents the mean ± S.E. (n = 5). (○) Saline; (□) doranidazole 200mg/kg.

**Table 1.** The sensitizer enhancement ratios (SERs) for SCCVII cells in tumors irradiated at various time after an i.v. administration of different doses of doranidazole

| Dose       | Sensitizer enhancement ratio (SER) |
|------------|-----------------------------------|
|            | 10 min   | 20 min   | 30 min   | 40 min   | 60 min   |
| 50mg/kg    | 1.09     | 1.22     | 1.03     | 1.08     | 1.06     |
| 100mg/kg   | 1.20     | 1.26     | 1.07     | 1.13     | 1.01     |
| 200mg/kg   | 1.36     | 1.47     | 1.20     | 1.27     | 1.10     |

The SERs were calculated using survival curves, and each point of survival curves was consisted of 4 tumors.

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1.36 at 10 min after 50–200 mg/kg doranidazole injection indicating doranidazole caused significant radiosensitization of tumors within 10 min after the drug administration. The maximum radiosensitization of tumor with a SER of 1.47 occurred 20 min after doranidazole administration at 200 mg/kg. Thereafter, the effect of doranidazole gradually declined although considerable radiosensitization was still evident 40–60 min after drug administration.

**Radiation-induced growth delay of SCCVII tumors**

Figure 5 shows the effect of doranidazole on the radiation-induced growth delay of SCCVII tumors. Tumors were irradiated with 30 Gy of X-ray 20 min after an i.v. administration of various doses of doranidazole. An administration of 200 mg/kg doranidazole alone exerted no effect on the growth of SCCVII tumors. The growth rate of tumors irradiated with 30 Gy of X-rays after an administration of 50 mg/kg doranidazole was similar to that after irradiation alone (data not shown) and that of 100 mg/kg doranidazole was slightly slower than that of the tumors irradiated after saline injection. The radiation-induced growth delay was further enhanced with 200 mg/kg doranidazole. The tumor volume doubling time after irradiation following saline injection and 200 mg/kg doranidazole injection was about 20 days and 36 days, respectively. The difference in the suppression of tumor growth in the saline injected group and doranidazole (200 mg/kg) injected group was statistically significant ($p = 0.00156$).

**TCD$_{50/120}$ of SCCVII tumors**

The percent of tumors considered to be cured at 120 days after irradiation with various dose of X-rays following an i.v. injection of saline or different doses of doranidazole are shown in Fig. 6. The TCD$_{50/120}$ for each groups are shown in Table 2, the TCD$_{50/120}$ for saline group was 55.4 Gy and it decreased to 50.4 Gy, 41.9 Gy and 41.7 Gy when tumors were irradiated after an i.v. injection of 50, 100 or 200 mg/kg of doranidazole, respectively. The corresponding SERs were 1.10, 1.32 and 1.33.

**Fig. 6.** Tumor control rate for SCCVII tumor after irradiation. Each plot of tumor control curves was consisted of 14–15 mice. (●) Saline ; (△) 50 mg/kg; (□) 100 mg/kg; (○) 200 mg/kg.

**Table 2.** TCD$_{50/120}$ values and SERs for SCCVII tumor irradiated after administration

| Treatment         | TCD$_{50/120}$ (Gy) | SER  |
|-------------------|----------------------|------|
| Saline            | 55.4 (51.2–59.9)*    | 1.00 |
| Doranidazole 50mg/kg | 50.4 (46.7–54.3)     | 1.10 |
| Doranidazole 100mg/kg | 41.9 (21.0–46.4)     | 1.32 |
| Doranidazole 200mg/kg | 41.7 (38.1–45.1)     | 1.33 |

Each control rate was calculated using 14–15 mice in each radiation dose.

*95% confidence interval

**Radiation-induced growth delay of human pancreatic tumor xenografts**

The effect of doranidazole on the radiation-induced growth delay of CFPAC-1 human pancreatic tumor xenografts in nude-mice is shown in Fig. 7A. The CFPAC-1 tumors irradiated with 15 Gy of X-ray 20 min after an i.v. saline injection initially regressed but regrew to the original volume in about 54 days. When CFPAC-1 tumors were irradiated with 15 Gy 20 min after an i.v. injection of 200 mg/kg doranidazole, the tumors regrew to the original volume after temporal regression in 91 days, which was significantly longer than that caused by saline and radiation ($p = 0.0463$).

The effect of 200 mg/kg doranidazole on the growth delay of MIA PACa-2 tumors irradiated with 4 Gy X-rays is shown.
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in Fig. 7B. The growth rate of tumors treated with 200 mg/kg doranidazole and irradiation was not significantly different from that of tumors treated with saline and irradiation.

**TCD$_{50/90}$ of CFPAC-1 tumors**

The percent of CFPAC-1 tumors considered to be cured at 90 days after irradiation with various dose of X-rays following an i.v. injection of 200 mg/kg doranidazole is shown to Fig. 8. As shown in Table 3, the TCD$_{50/90}$ of CFPAC-1 tumor for the radiation alone group was 44.2 Gy and it decreased to 34.1 Gy when tumors were irradiated after an i.v. injection of 200 mg/kg of doranidazole. The corresponding SERs was 1.30.

**Oxygenation status of tumors**

Table 4. shows the median $pO_2$ values and the % frequency of $pO_2$ readings lower than 5.0 mmHg in SCCVII, CFPAC-1 and MIA PaCa-2 tumors. These $pO_2$ were obtained from 1031-1287 measured values in 26–29 tumors for each tumor group. In SCCVII tumors, the median $pO_2$ value was 2.1 ± 0.6 mmHg and 88.6 ± 3.0% of $pO_2$ readings were lower than 5.0 mmHg. In CFPAC-1 human tumor xenografts, the median $pO_2$ value was 4.5 ± 0.7 mmHg and 60.5 ± 4.8% of $pO_2$ readings were lower than 5.0 mmHg. In MIA PaCa-2 tumors the median $pO_2$ value was 8.0 ± 1.1 mmHg and 47.2 ± 3.7% of $pO_2$ readings were lower than 5.0 mmHg.

Figure 9 shows the immunostaining for pimonidazole binding in a SCCVII mouse tumor and CFPAC-1 and MIA PaCa-2 human pancreatic tumor xenografts. The immunostaining was most strong in SCCVII tumors and was rather weak in MIA PaCa-2 tumor. The percentage of Pimonidazole positive regions, i.e. hypoxic region, were obtained and averaged in 5 tumors for each tumor line and averaged. As shown in Table 4, the three different tumors showed considerable differences in the size of hypoxic region. In SCCVII
DISCUSSION

The results of present study conformed our previous observations that doranidazole is an effective hypoxic cell radiosensitizer both in vitro and in vivo. Further, the results obtained in the present study indicated that the radiosensitizing efficacy of doranidazole is related to the oxygenation status of tumors.

In the past clinical studies on the chemical radiosensitizer for hypoxic cells such as misonidazole and similar electron affinic compounds, the increase in tumor response to radiotherapy was disappointing. Such disappointing clinical results may be attributed to two major reasons. First, the past clinical trials for hypoxic cell radiosensitizers were done with conventional fractionated radiotherapy. Second, the doses of the sensitizers administered with each fractionated radiation exposure were limited due to side effects particularly in the nervous systems. Recently, increasing number of certain tumors are treated with Intra-Operative Radiation Therapy (IORT). It may be expected that the effect of IORT would be greatly enhanced by hypoxic cell radiosensitizers if the tumors contain hypoxic cells. On the such assumption, doranidazole, a hypoxic cell radiosensitizer, is currently undergoing clinical trials in combination with IORT for pancreatic cancer in Japan.

Our in vitro study using SCCVII tumor cells clearly demonstrated that doranidazole is able to radiosensitize hypoxic, as determined with clonogenic cell survival assay (Fig. 1) and micronucleus assay (Fig. 2). These radiosensitizing effects are in good agreement with previous reports. Doranidazole is highly soluble in water, and have a characteristic required for effective diffusion in tumors and for rapid excretion. The partition coefficient of doranidazole in octanol/water (p) is 0.035 and half-wave reduction electric potential is –0.297 mV. As shown in Fig. 3, the concentration of doranidazole in serum in mice injected with the drug through tail vein decreased rapidly while the concentration of the drug in tumors remained at considerable levels during 15–30 min after the drug administration. Therefore, we irradiated tumors 20 min after doranidazole administration in the subsequent in vivo experiments to study the radiosensitizing effect of doranidazole on tumors in mice (Table 1). The in vivo-in vitro excision assay for clonogenic cell survival (Fig. 4), tumor growth delay study (Fig. 5) and TCD50/120 assay (Fig. 6) all indicated that an i.v. administration of doranidazole at 100–200 mg/kg significantly enhances the response of SCCVII tumors to a single dose of radiation with an enhancement would significantly minimize the effect of the hypoxic cell radiosensitizers. Second, the doses of the sensitizers administered with each fractionated radiation exposure were limited due to side effects particularly in the nervous systems.

Table 4. Comparison of \( pO_2 \) distribution with pimonidazole binding in tumors

| Cell line   | \( pO_2 < 5.0 \text{ mmHg} \) (%) | Median \( pO_2 \) (mmHg) | Pimonidazole immunostaining |
|-------------|---------------------------------|--------------------------|-----------------------------|
| SCCVII      | 88.6 ± 3.0                      | 2.1 ± 0.6                | 21.8 ± 4.2                  |
| CFPAC-1     | 60.5 ± 4.8                      | 4.5 ± 0.7                | 10.3 ± 1.4                  |
| MIA PaCa-2  | 47.2 ± 3.7                      | 8.0 ± 1.1                | 4.3 ± 0.8                   |

Each value represents the mean ± S.E. The total number of values of \( pO_2 \) distribution used to construct each set of data was 1031~1287 from 26~29 tumors. Immunostaining data were calculated from 5 sections in 5 tumors.

Doranidazole is currently undergoing clinical trials in combination with IORT for pancreatic cancer in Japan.
ratio of about 1.3. On the other hand, doranidazole is
considered to be nontoxic for normal or tumor cell because it had
no effect for SCCVII cells in vitro surviving fractions under
hypoxic condition and in vitro micronucleus frequencies in
hypoxic and oxic condition for 0 Gy, and normal cells are
usuallyoxic.

CFPAC-1 human pancreatic tumors grown in the hind legs of BALB/c-nu/nu mice were also radiosensitized by 200 mg/
kg doranidazole administered i.v. 20 min before the tumor
radiation as judged from the increase in radiation-induced
tumor growth delay (Fig. 7). However, in MIA PaCa-2
tumors, the growth delay caused by a 4 Gy irradiation was
not enhanced by 200 mg/kg doranidazole (Fig. 7). Probably we
fail to observe radiosensitization in MIA PaCa-2 tumors with
doranidazole in the present study partly because the
tumors were irradiated with a low radiation dose, i.e.4 Gy.
Doranidazole had no effect for SCCVII cells in vivo sur-
viving fractions without irradiation. Tumor growth in the
mice given doranidazole without irradiation was not differ-
ent from that in mice treated with saline without irradiation
in SCCVII, CFPAC-1 and MIA PaCa-2 tumors. These sug-
gest that doranidazole should be easily tolerated in clinical
use.

Nordsmark et al.\textsuperscript{36} reported that there was no correlation
between the pimonidazole staining and the degree of
hypoxia determined with Eppendorf oxygen probe in prima-
ry cervix carcinomas. On the other hand, Raleigh et al.\textsuperscript{33}
observed that the oxygenation status determined with
pimonidazole staining was correlated with \(p_O_2\) determined
with oxygen probe or radiobiologically determined hypoxic
fraction in C3H mammary tumors. In the present study we
observed that the oxygenation status determined with pimo-
 nidazole staining (Fig. 9) and that with \(p_O_2\) probe (Table 4)
were correlated. More specifically, both pimonidazole stain-
ing and \(p_O_2\) determined with oxygen probe indicated that
SCCVII tumor is most hypoxic and MIA PaCa-2 tumors is
least hypoxic among the three tumors we studied (Table 4
and Fig. 9). However, the degree of hypoxia determined with
the two different methods varied considerably. For example,
in SCCVII tumors, 88.6% of \(p_O_2\) readings were lower than
5.0 mm Hg, i.e. hypoxic, whereas the hypoxic areas stained
with pimonidazole was 21.8%. Similarly, the \% of \(p_O_2\) read-
ings lower than 5 mmHg were considerably larger than the
\% area stained with pimonidazole in both CFPAC-1 tumors
and MIA PaCa-2 tumors. As mentioned above, the enhance-
ment of radiation-induced growth delay by doranidazole in
CFPAC-1 tumors was greater than that in MIA PaCa-2
tumors (Fig. 7). It may be concluded that doranidazole was
ineffective to radiosensitize MIA PaCa-2 tumor because this
tumor is relatively well oxygenated (Table 4 and Fig. 9).
Further experiments are in progress in our laboratory to
reveal the possible relationship between the oxygenation sta-
tus and the radioreponse in human pancreatic tumor
xenografts. Nevertheless, we may conclude based on the
results obtained in the present study that doranidazole is a
hypoxic cell radiosensitizer potentially useful to enhance the
response of human pancreatic tumors to IORT.

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