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A New, Potent 2-Nitroimidazole Nucleoside Hypoxic Cell Radiosensitizer, RP170

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The radiosensitizing activity, acute toxicity and pharmacokinetics of RP170, a new hypoxic cell radiosensitizer, were compared with those of misonidazole (MISO) and SR2508. RP170 belongs to the group of 2-nitroimidazole nucleosides, which are designed to be selectively excluded from the neural tissue. The reduction potential of RP170 was similar to that of MISO and SR2508. The partition coefficients in octanol/water of RP170, MISO, and SR2508 were 0.094, 0.35, and 0.021, respectively. The radiosensitizing activity of RP170 was similar to that of MISO and SR2508 *in vitro* and *in vivo*. There was no significant difference in the radiosensitizing activity of RP170 *in vivo* between intravenous and intraperitoneal administration. The acute toxicity of RP170 was the same as that of SR2508. Pharmacokinetic evaluation showed that the concentration of RP170 in the brain was as low as that of SR2508. RP170 is expected to have the same radiosensitizing effects as MISO and SR2508, and to be less neurotoxic than MISO.

Key words: Radiosensitization — Hypoxic cell radiosensitizer — 2-Nitroimidazole — 2-Nitroimidazole nucleoside — RP170

The existence of hypoxic, and hence radiation-resistant cells in malignant tumors is considered one possible cause of the failure of radiation to produce local control. Several methods to overcome this resistance of hypoxic cells are being tested. Hypoxic cell radiosensitizers are one of these. Misonidazole (MISO) was the first hypoxic cell radiosensitizer to be extensively investigated clinically. Unfortunately, the initial optimism has been tempered by the realization that the use of the drug may be associated with the development of severe side effects. These include irreversible peripheral neuropathy, which has seriously limited its potential.[1,2] Two 2-nitroimidazoles, SR2508 and Ro 03-8799 are now being tested clinically. SR2508 is a compound with reduced lipophilicity and shows reduced uptake in neural tissue. Although the clinical studies have confirmed that it is 3 times less toxic than MISO, its dose-limiting factor is still peripheral neuropathy.[3,4] In addition, because SR2508 has impaired oral bioavailability owing to its decreased lipophilicity, it must be given intravenously to obtain a sufficient concentration in plasma or tumor.[5,6] This is time-consuming and troublesome compared to oral administration.

Ro 03-8799 has high lipophilicity and a basic side chain.[7] It shows temporary central neurotoxicity rather than the peripheral neurotoxicity.[8,9] Because of this, the maximum dose tolerated with a single injection is limited to 0.75 g/m^2.

Another approach to reduce the neurotoxicity of 2-nitroimidazole sensitizers is incorporation of nucleoside analogues into the 2-nitroimidazole ring. RA263, one of this group of compounds, has been shown to be less toxic in mice than MISO, but it is less effective *in vivo* when compared at an equivalent drug dose.[10,11]

More recently developed compounds, RK28 and RK29, have high *in vitro* activity and are almost as efficient *in vivo* as MISO.[12,13] However, the acute toxicity of these compounds in mice is higher than that of MISO. As part of the search for a better nucleoside analogue sensitizer, RP170 (Fig. 1) has recently been developed, and has proved to be better than any other currently known 2-nitroimidazole nucleoside sensitizer. In this report, the sensitizing activity *in vitro* and *in vivo*, the acute toxicity and pharmacokinetics of RP170 are presented in detail.

MATERIALS AND METHODS

Compounds RP170 (Fig. 1) has a sugar analogue at the N1-position of the 2-nitroimidazole ring and is thus a nucleoside analogue. It was developed and kindly provided by M. Sakaguchi (Pola Corporation, Yokohama). MISO and SR2508 were obtained from Nippon Roche (Tokyo) and National Cancer Institute (USA), respectively. For *in vitro* experiments, the compounds were dissolved in phosphate-buffered saline and diluted with the medium. For *in vivo* experiments, they were dissolved in physiological saline.

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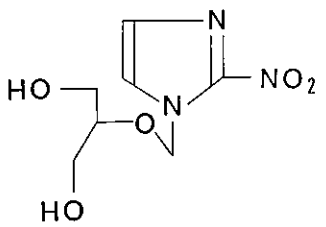

RP170

Fig. 1. Structural formula of RP170.

The reduction potential ($E_{1/2}^{RED}$) of each compound was measured by a method described previously[12, 14, 15] and was evaluated relative to the Ag/AgCl(saturated)/3.5 *M* KCl electrode by means of cyclic voltammetry with Ar-purged N, N-dimethylformamide solution (0.01 *M*) containing 0.1 *M* tetra-N-butylammonium perchlorate as a supporting electrode. The partition coefficient (*P*) in octanol/water of each compound was measured in buffer at pH 7.4 according to the method of Fujita *et al.*[16]

Sensitizer testing system EMT6/KU cells cultured in Eagle's minimum essential medium (MEM) containing 12.5% fetal bovine serum (FBS) were used for *in vitro* study as described in detail previously.[12, 14, 17] To evaluate cytotoxicity, 2×10^5 EMT 6/KU cells in 0.25 ml MEM were exposed to various concentrations of RP170 under oxic or hypoxic conditions for 1, 2, and 4 h. To evaluate the sensitizing activity under hypoxic conditions, exponentially growing cells were suspended in glass test tubes (2×10^5/0.25 ml MEM). Immediately after the sensitizers were added to the suspension, the tubes were made hypoxic by purging with 95%N_2/5%CO_2 gas for 40 min. The tubes were then sealed for irradiation. Immediately after the irradiation, the drug was removed and cells were assayed for survival. The control plating efficiency was 91±4% (mean±SD).

In vivo tumor response was measured by an *in vivo-in vitro* assay and a growth delay assay using SCC VII tumor of C3H/He mice as described previously.[14, 18, 19] SCC VII tumor cells growing in MEM supplemented with 12.5% FBS were inoculated subcutaneously in both hind legs for *in vivo-in vitro* assay or the right hind leg for growth delay assay of syngeneic 8- to 9-week-old female C3H/He mice.

In the *in vivo-in vitro* assay, the cell yield was $3.2\pm1.8\times10^7$ cells/g (mean±SD), and the control plating efficiency was 43±8% (mean±SD).

In the growth delay assay, the volume of the developing tumors was estimated every other day by caliper measurements of 3 perpendicular diameters, assuming an ellipsoid shape. In both assays, the tumor was irradiated when it reached a volume of about 500 mm^3. The tumor had a hypoxic fraction of 5.4% under the conditions of the irradiation for the *in vivo-in vitro* assay, and 28% in the case of the growth assay.[20]

Irradiation Irradiation was carried out using 10 MV X-rays generated by a linear accelerator at a dose rate of 5.6 Gy/min as previously described.[18–20] Single cells were irradiated in a water bath at 37°C. For the *in vivo-in vitro* assay, the mice received whole-body irradiation and to obtain the growth delay curves of tumors, only the tumor-bearing leg was irradiated. For whole-body irradiation, mice were not restrained, but for local irradiation, mice were fixed with adhesive tape with their limbs extended without anesthesia.

Calculation of sensitizer enhancement ratio (SER) SERs were calculated from the ratio of two radiation doses required to reduce the surviving fraction to 1% in single-cell experiments, while in solid SCC VII tumor experiments they were calculated for the surviving fraction of 0.1% in the *in vivo-in vitro* assay.[12, 17] In the growth delay assay, SERs were calculated from the radiation doses necessary to get the time for tumors to regrow to 2× treatment volume.

Toxicity to mice The $LD_{50/7}$ (drug dose necessary to kill 50% of the mice within seven days) in 5-week-old female ICR mice was determined for MISO, SR2508 and RP170 using 30 mice for each compound.

Pharmacokinetic studies The pharmacokinetic studies were carried out in C3H/He mice bearing the SCC VII tumor. Mice were used two weeks after subcutaneous tumor transplantation when the tumors were approximately 500 mm^3 in size. MISO was administered intraperitoneally, and RP170 and SR2508 intravenously on the basis of the results of the sensitization experiments. All compounds were given at a dose of 200 mg/kg.

Serum and tissue homogenates were extracted with methanol and analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a Hitachi 655-2525 column (4×150 mm, C_{18}, particle size=5 μm). The flow rate was 0.6 ml/min. The eluants were as follows: MeOH:H_2O (20:80) 0.02 *M* NaH_2PO_4, H_3PO_4 (to pH 3.0) for MISO, and MeOH:H_2O (10:90) 0.02 *M* NaH_2PO_4, H_3PO_4 (to pH 3.0) for RP170 and SR2508. The drug absorbance peak was detected at 320 nm. The retention times of MISO, RP170 and SR2508 were 5.5 min, 4.9 min, and 4.0 min, respectively.

RESULTS

Cytotoxicity Figure 2 shows the data on the cytotoxicity of RP170 to oxic and hypoxic EMT 6/KU cells. Incubation of oxic EMT6/KU cells with 1 m*M* RP170 for 4 h

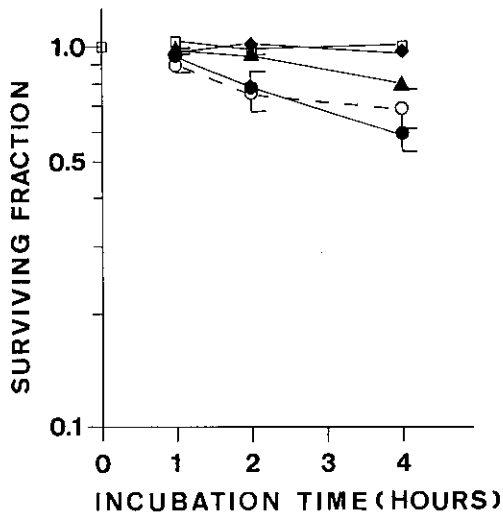

Fig. 2. Survival of EMT 6/KU cells exposed to various concentrations of RP170 under hypoxic conditions: open circles, 0.5 m*M*, closed circles 1.0 m*M*. For comparative purposes, data are also included for 1.0 m*M* MISO under hypoxic conditions (closed triangles), 1.0 m*M* RP170 under oxic conditions (closed diamonds), and under hypoxic conditions without drugs (open squares). Error bars represent 1 SE.

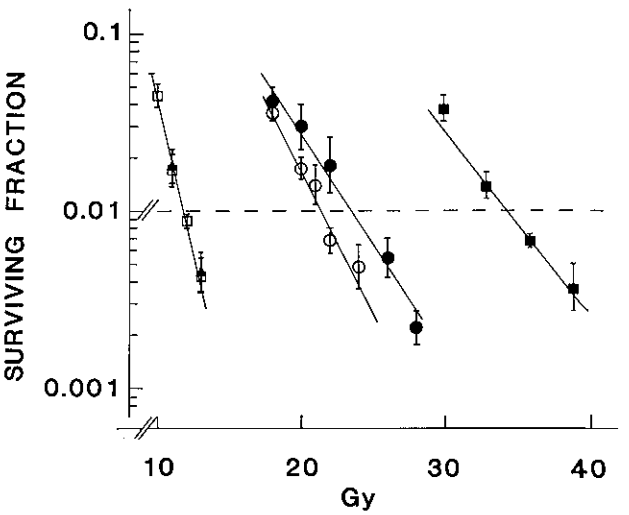

Fig. 3. Survival data for hypoxic or oxic EMT 6/KU cells X-irradiated in the presence or absence of RP170. Error bars indicate 1 SD. Open squares, oxic in the absence of RP170; closed triangles, oxic with 1.0 m*M* RP170; closed squares, hypoxic in the absence of RP170; closed circle, hypoxic with 0.5 m*M* RP170; open circles, hypoxic with 1.0 m*M* RP170.

did not significantly inhibit colony formation. However, under hypoxic conditions RP170 was slightly cytotoxic.

Radiosensitization *in vitro* Figure 3 shows the radiation survival curves for EMT 6/KU cells exposed to 0.5 m*M* and 1.0 m*M* RP170 under hypoxic conditions. RP170 showed the sensitizing activity to hypoxic cells. The SERs for 0.5 m*M* and 1.0 m*M* RP170 are 1.45, and 1.65, respectively. Under oxic conditions, 1.0 m*M* RP170 did not sensitize EMT 6/KU cells to ionizing radiation.

Table I shows the SERs for RP170, MISO and SR 2508 at the concentrations of 0.5 m*M* and 1.0 m*M*. There was no difference in sensitizing activity among these three compounds at the same concentration.

Radiosensitization *in vivo*

In vivo-in vitro assay: For the time-course experiment, a single dose of 18 Gy was administered at various times after injection of 100 mg/kg of RP170, or 100 mg/kg of SR2508 (Fig. 4). The radiosensitizing activity of SR2508 was impaired when it was given intraperitoneally. There was no significant difference in the radiosensitizing activity of RP170 between the intravenous and intraperitoneal administrations. However there was a trend that RP170 had a slightly higher radiosensitizing activity when given intravenously rather than intraperitoneally. Based on these results, RP170 was administered intravenously 20 min before irradiation in the following experiments to determine SERs. According to the results of previous experiments,[17] MISO was injected intraperitoneally 40 min before irradiation, and SR2508 was administered intravenously 20 min before irradiation.

Table I. Chemistry, Sensitization, and Toxicity Data for MISO, SR2508, and RP170

	MISO	SR2508	RP170
$P^{a)}$	0.35	0.021	0.094
$E_{1/2}^{RED}$ (V)$^{b)}$	−1.04	−1.05	−1.01
SER (*in vitro*)$^{c)}$			
1.0 m*M*	1.65	1.65	1.65
0.5 m*M*	1.45	1.45	1.45
SER (*in vivo-in vitro*)			
50 mg/kg			1.10
100 mg/kg	1.35	1.30	1.30
200 mg/kg	1.50	1.45	1.45
300 mg/kg			1.55
SER (regrowth delay)			
50 mg/kg			1.30
100 mg/kg	1.45	1.50	1.45
200 mg/kg	1.60	1.65	1.65
$LD_{50/7}$ (g/kg)	1.3	4.1	4.1

a) *P*: Partition coefficient in octanol/water.
b) $E_{1/2}^{RED}$: Reduction potential.
c) SER: Sensitizer enhancement ratio.

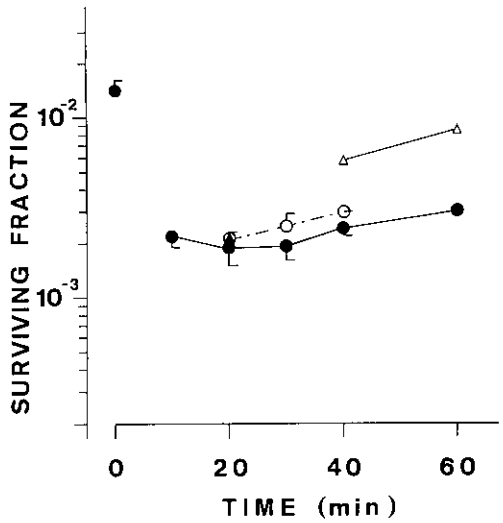

Fig. 4. The surviving fraction of the cells in SCC VII tumors after a dose of 18 Gy given to the tumors at different times after an iv (closed circles) or ip (open circles) injection of 100 mg/kg of RP170. Zero (0) min means no treatment before irradiation. For comparative purposes, data are also included for iv (closed triangle) or ip (open triangles) injection of 100 mg/kg of SR2508. Error bars represent 1 SE.

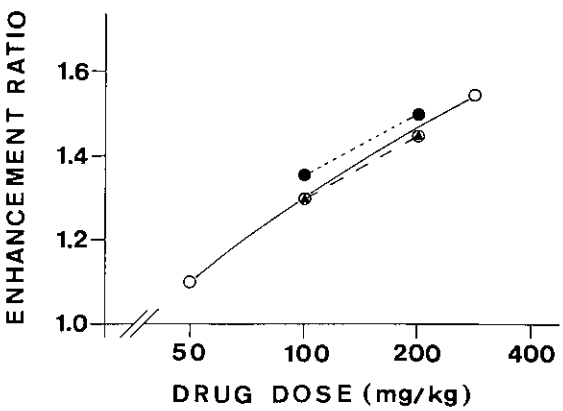

Fig. 5. Sensitizer enhancement ratios as a function of administration doses of MISO (closed circles), SR2508 (closed triangles), and RP170 (open circles) from the results of *in vivo-in vitro* assay using SCC VII tumors.

From the radiation survival curves (data not shown), SERs were determined for each compound at various administration doses. Figure 5 shows the SERs of each compound as a function of the administration dose. RP170 appeared to sensitize the tumor to the X-irradiation at all doses as effectively as did SR2508.

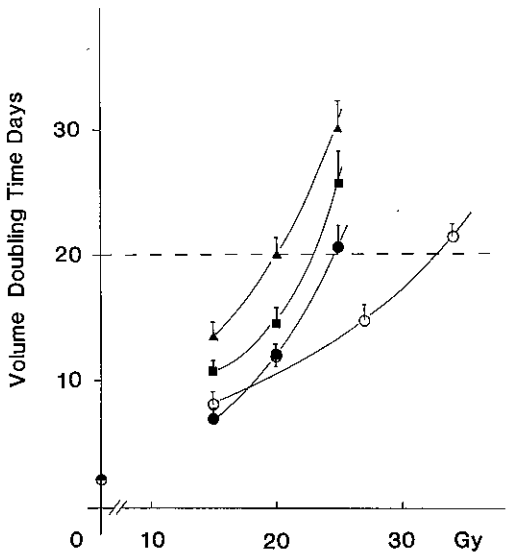

Fig. 6. Dose-response curves of SCC VII tumors, i.e. volume doubling time as a function of radiation dose. Error bars represent 1 SE. Closed triangles, 200 mg/kg RP170; closed aquares, 100 mg/kg RP170; closed circles, 50 mg/kg RP170; open circles, no treatment before irradiation.

Growth delay assay: According to the findings obtained in the *in vivo-in vitro* assay, RP170 and SR2508 were administered intravenously 20 min before irradiation. MISO was injected intraperitoneally 40 min before irradiation. In Fig. 6, dose-response curves are shown for the tumor treated with X-rays in the absence of a drug, or in the presence of various doses of RP170. The SER for each administration dose was calculated at the time for tumors to regrow to 2× treatment volume equal to 20 days. Table I shows the SERs for each administration dose of RP170 in comparison with those for MISO and SR2508. There was no significant difference in the radiosensitizing activities of these three compounds at the same administered dose.

Pharmacokinetic studies Figure 7A shows the serum, tumor and brain concentrations after intraperitoneal administration of MISO. The concentrations of this drug in brain were similar to those in tumor. RP170 and SR2508 showed quite different pharmacokinetics from those of MISO (Fig. 7B, C). The concentrations of these two drugs in brain were very low throughout the observation period. There was no significant difference in the drug concentrations in the SCC VII tumor between SR2508 and RP170.

Acute toxicity The acute toxicity measured as the $LD_{50/7}$ was found to be 1.3 g/kg for MISO, and 4.1 g/kg for SR2508 and RP170 (Table I). Therefore RP170 and SR2508 are approximately 3 times less toxic than MISO.

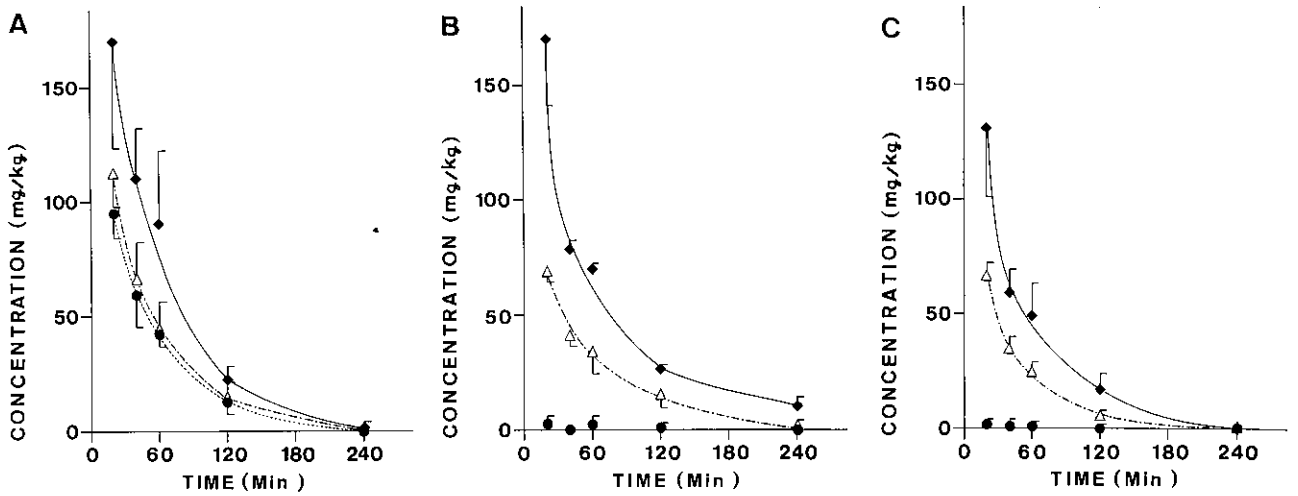

Fig. 7. Concentrations of MISO (A), SR2508 (B), and RP170 (C) in the serum (closed diamonds), tumor (open triangles), and brain (closed circles) of SCC VII bearing C3H/He mice as a function of time after ip (MISO) or iv (SR2508, RP170) injection of 200 mg/kg of the drug. Error bars represent 1 SD.

DISCUSSION

RP170 belongs to a group of 2-nitroimidazole nucleosides designed to be selectively excluded from the neural tissue, of which other members are RA263[10, 11] and RK28.[12, 13] It was hypothesized that nucleosides in general may not cross the blood-brain barrier effectively.[21] The pharmacokinetic study on RA263 revealed a low concentration of the compound in neural tissue.[10, 11] This is similar to the results for RP170 in this study.

Among the group of 2-nitroimidazole nucleosides, RK28 and RK29 are similar to MISO in their ability to sensitize the hypoxic cells to irradiation,[12, 13] whereas RA263 was reported to require a larger dose to get the same effect as MISO *in vitro*.[10, 11] RP170 showed the same activity as MISO in the animal experiments at an equal administration dose. In this study, the $LD_{50/7}$ for RP170 was 4.1 g/kg whereas $LD_{50/7}$ for MISO was 1.3 g/kg. It was reported that the $LD_{50/24h}$ for RA263 was 3.3 g/kg when the $LD_{50/24h}$ for MISO was 1.4 g/kg,[10] and the acute toxicity of RK28 or RK29 was higher than that of MISO.[12, 13] Therefore, we concluded that RP170 has the best properties among 2-nitroimidazole nucleoside hypoxic cell sensitizers as regards its radiosensitizing activity *in vivo* and acute toxicity.

The pharmacokinetic study showed that the concentration of RP170 in the brain is lower than that of MISO and as low as that of SR2508. The partition coefficient of RP170 is 0.094 versus 0.35 for MISO and 0.021 for SR2508. The low lipophilicity and the 2-nitroimidazole nucleoside structure of RP170 should account for this low concentration in the brain. These special properties of RP170 limit its ability to penetrate the blood brain barrier. A physiologically similar barrier is assumed to exist around the peripheral nervous system.[5, 6] Therefore it is expected that the concentration of RP170 in the peripheral nervous tissue is as low as that in the central nervous tissue.

RP170 showed the same characteristics in the radiosensitizing activity *in vitro* and *in vivo*, acute toxicity, and pharmacokinetics as SR2508. However there are some differences between these two radiosensitizers. The first is that RP170 had a good radiosensitizing activity when injected intraperitoneally, whereas the activity of SR2508 was impaired when it was administered intraperitoneally. The second difference is in the structure of these two drugs: SR2508 is an amide,[22] whereas RP170 is a nucleoside. Some biological characteristics not tested in this study, such as the mutagenicity and chronic toxicity, of these two drugs may be different. Some 2-nitroimidazole nucleosides were reported to have very low mutagenicity.[10, 11] The present results encourage further studies of RP170, including toxicity studies in large animals.

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