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KIH-802, an acetohydroxamic acid derivative of 2-nitroimidazole, as a new potent hypoxic cell radiosensitizer: radiosensitizing activity, acute toxicity, and pharmacokinetics*

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Summary. The radiosensitizing activity, acute toxicity, and pharmacokinetics of a new hypoxic cell radiosensitizer, potassium 2-nitroimidazole-1-acetohydroxamate (KIH-802), were compared with those of misonidazole (MISO) and etanidazole (SR-2508). The radiosensitizing activity of KIH-802 was slightly higher than that of MISO and SR-2508 in vitro and was similar to or slightly higher than that of MISO or SR-2508 in vivo. The acute toxicity of KIH-802 was slightly higher than that of MISO. The concentrations of KIH-802 in the brains and peripheral nerves of mice were as low as those of SR-2508 and lower than those of MISO.

Introduction

To overcome the resistance of hypoxic cells to radiation, hypoxic cell radiosensitizers are being tested. Misonidazole (MISO) was the first to be extensively investigated clinically. Unfortunately, its clinical value was found to be limited by its severe side effects, which include irreversible peripheral neuropathy [4, 18].

Development of new hypoxic cell radiosensitizers began in Japan in the late 1970s. Since then, many new compounds that have potent sensitizing activities against single cells in vitro have been synthesized. Some of them have been found to have a sufficient sensitizing effect in vivo [6, 10, 13] but were not suitable for clinical use because of their relatively strong acute toxicity. To develop a new hypoxic cell radiosensitizer, we incorporated an acetohydroxamic acid moiety into the side chain of 2-nitroimidazoles [5]. Among these compounds, potassium 2-nitroimidazole-1-acetohydroxamate (KIH-802) was evaluated as being potentially the most useful as a hypoxic cell radiosensitizer [5]. In this report, the further examination of this compound is described in comparison with that of MISO and etanidazole (SR-2508).

Materials and methods

Compounds. Figure 1 shows the structural formula of KIH-802, which was developed by Hori and Inayama. MISO and SR-2508 were obtained from Nippon Roche (Tokyo) and the 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.

The reduction potential [$E_{1/2}^{RED}$ (S/S^+)/V vs Ag/Ag^+] and partition coefficient (P) of each compound, measured in buffer at pH 7.4 by methods described previously [10, 12, 15], are shown in Table 1. The acidity constant (pK_a) of KIH-802 was evaluated from the pH change in aqueous solutions by titration with 0.01–0.1 mol dm^{-3} NaOH or HCl.

Radiosensitizer testing systems. The effects of the radiosensitizers were evaluated using one in vitro system and two in vivo systems. In the in vitro system, EMT6/KU cells were used as previously described [9, 10, 12, 13]. The EMT6/KU cells were maintained in alternate passages in BALB/c mice and in cell culture in Eagle's minimum essential medium (MEM) containing 12.5% fetal bovine serum (FBS). This complete medium was used for all experiments. To test sensitizing activity under hypoxic conditions, exponentially growing cells were suspended in glass test tubes (4×10^5 cells/0.25 ml MEM). Immediately after the radiosensitizers had been added to the suspension, the tubes were made hypoxic by purging with gas comprising 95% N_2/5% CO_2 for 40 min. Tubes were then sealed for irradiation. Immediately after irradiation, the num-

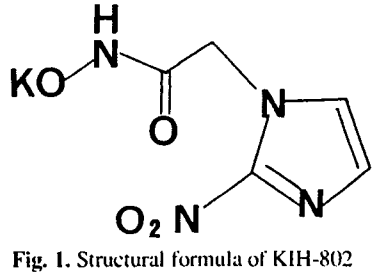

Fig. 1. Structural formula of KIH-802

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Table 1. Chemistry, sensitization, toxicity, and pharmacokinetic data for MISO, SR-2508, and KIH-802

	MISO	SR-2508	KIH-802
p[a]	0.422[f]	0.048[f]	0.044
$E_{1/2}^{RED}$(V)[b]	−1.04[g]	−1.05[g]	−0.98[i]
pK_a[c]			8.05
SER (in vitro)[d]:			
1.0 mmol dm^{-3}	1.65[g]	1.65[g]	1.70
0.5 mmol dm^{-3}	1.45[g]	1.45[g]	1.50
SER (in vivo-in vitro):			
50 mg/kg			1.30
100 mg/kg	1.35[g]	1.30[g]	1.40
200 mg/kg	1.50[g]	1.45[g]	1.75
300 mg/kg			1.95
SER (growth delay):			
100 mg/kg	1.45[g]	1.50[g]	1.40
200 mg/kg	1.60[g]	1.65[g]	1.65
$LD_{50/7}$ (g/kg)	1.3[g]	4.1[g]	0.9
AUC (mg/kg × min)[e]:			
Brain	12,400[h]	280	<100[j]
Sciatic nerve	13,300[h]	6,200	7,000[j]

[a] Partition coefficient in octanol/water
[b] $E_{1/2}^{RED}$ (S/S$^+$)/V vs Ag/Ag$^+$
[c] Acidity constant
[d] Sensitizer enhancement ratio
[e] Area under the curve from 0 to 240 min
[f] 0.43 for MISO and 0.046 for SR-2508 [1]
[g] Data from [9]
[h] MISO plus its metabolite, desmethylmisonidazole
[i] This value is described for KIH-801, the free acid of KIH-802
[j] KIH-802 plus its metabolite, 2-nitroimidazole-1-acetic acid as an minor component

ber of cells in the suspension was counted and an assay for cell survival was carried out using the colony formation method. The control plating efficiency was 94% ± 3% (mean ± SD).

The in vivo tumor response was measured by an in vivo-in vitro assay and a growth delay assay using SCC VII tumors of C3H/He mice as previously described [8–11]. In the in vivo-in vitro assay, the cell yield was 3.2 ± 1.4 × 10^7 cells/g (mean ± SD). The control plating efficiency was 42% ± 9% (mean ± SD). In the growth delay assay, the volume of the developing tumors was estimated by caliper measurement of the three perpendicular diameters on alternate days, assuming an ellipsoid shape as described previously [8, 9]. Each treatment group consisted of eight or nine mice.

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 irradiation conditions for the in vivo-in vitro assay and that of 28% for the growth delay assay [14].

Irradiation. Irradiation was carried out using 10 MV X-rays generated by a medical linear accelerator at a dose rate of 5.6 Gy/min, as previously described [9, 12, 14, 15]. Single cells in test tubes were irradiated in a water bath at 37° C. For the in vivo-in vitro assay, the mice received whole-body irradiation, and for the determination of the growth curves of tumors, only the tumor-bearing leg was irradiated. For whole-body irradiation, mice were not restrained, but for local irradiation they were fixed with adhesive tape, with their limbs extended and without anesthesia.

Calculation of sensitizer enhancement ratios. Sensitizer enhancement ratios (SERs) were calculated from the ratio of the two radiation doses

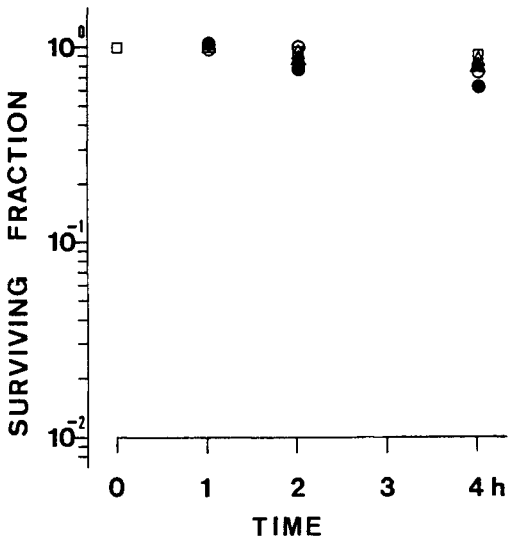

Fig. 2. Survival of EMT6/KU cells exposed to various concentrations of KIH-802 under hypoxic conditions: *open circle*, 0.5 mmol dm^{-3}; *closed circle*, 1.0 mmol dm^{-3}. For comparison, data are also included for exposure to 1.0 mmol dm^{-3} MISO under hypoxic conditions (*closed triangle*) and 1.0 mmol dm^{-3} KIH-802 under oxic conditions (*open triangle*), as well as for untreated cells under hypoxic conditions (*open square*). Points represent the mean of four experiments. Error bars are smaller than the plotted symbols in all cases

required to reduce the surviving fraction to 1% in single-cell experiments, whereas in solid SCC VII tumor experiments they were calculated for a surviving fraction of 0.1% in the in vivo-in vitro assay [9, 12, 13]. In the growth delay assay, SERs were calculated from the radiation doses necessary to obtain the same volume doubling time with or without drug treatment [9].

Toxicity to mice. The $LD_{50/7}$ (the drug dose necessary to kill 50% of the mice within 7 days) was determined for MISO, SR-2508, and KIH-802 in 5-week-old female ICR mice using 30 mice for each compound. KIH-802 and MISO were injected i.p. and SR-2508 was given i.v., according to the results of the radiosensitization experiments.

Pharmacokinetic studies. The pharmacokinetic studies were carried out in C3H/He mice bearing SCC VII tumors inoculated s.c. into the right hind leg as previously described [9]. Mice were used 2 weeks after tumor transplantation, when the tumors were approximately 500 mm^3 in size. KIH-802 and MISO were injected i.p. and SR-2508 was given i.v.; all compounds were given at a dose of 200 mg/kg.

Serum and tissue homogenates were extracted with methanol and analyzed with a reversed-phase high-performance liquid chromatograph (HPLC) equipped with a Hitachi 655-2525 column (4 × 150 mm; C_{18}; particle size, 5 μm). The flow rate was 0.6 ml/min. The eluents were as follows: MeOH: H_2O (20:80), 0.02 mol dm^{-3} NaH_2PO_4, H_3PO_4 (to pH 3.0) for MISO; MeOH: H_2O (10:90), 0.02 mol dm^{-3} NaH_2PO_4, H_3PO_4 (to pH 3.0) for KIH-802 and SR-2508. The drug absorbance peak was detected by a Hitachi variable wavelength UV monitor 638-0410 at 320 nm. The retention times of MISO, KIH-802, and SR 2508 were 5.5, 3.9, and 4.0 min, respectively.

Results

Cytotoxicity

Figure 2 shows the data on the cytotoxicity of KIH-802 to oxic and hypoxic EMT6/KU cells. The data are presented as the surviving fraction of EMT 6/KU cells exposed to

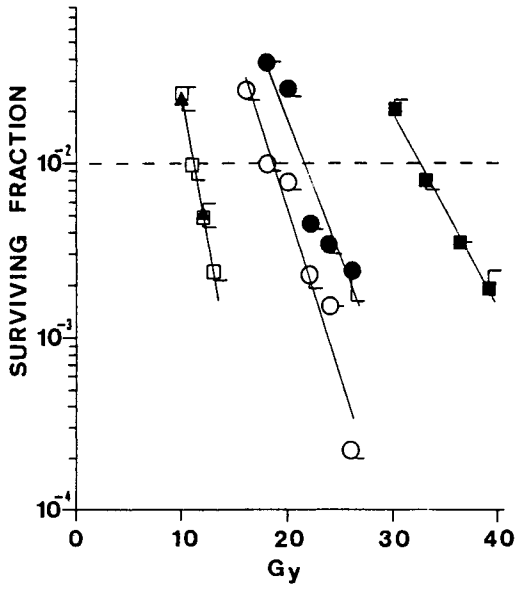

Fig. 3. Survival data for hypoxic or oxic EMT6/KU cells X-irradiated in the presence or absence of KIH-802. Error bars represent 1 SD from four experiments. *Open square*, oxic cells in the absence of KIH-802; *closed triangle*, oxic cells with 1.0 mmol dm^{-3} KIH-802; *closed square*, hypoxic cells in the absence of KIH-802; *closed circle*, hypoxic cells with 0.5 mmol dm^{-3} KIH-802; *open circle*, hypoxic cells with 1.0 mmol dm^{-3} KIH-802

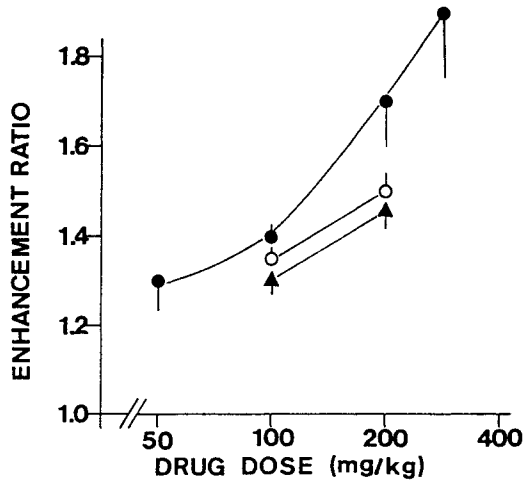

Fig. 5. Sensitizer enhancement ratios as a function of the delivered dose of MISO (*open circle*), SR-2508 (*closed triangle*), or KIH-802 (*closed circle*), calculated from the results of the in vivo-in vitro assay using SCC VII tumors. Error bars represent 1 SE from more than four experiments

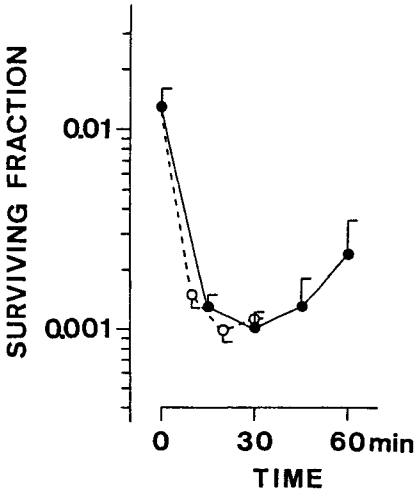

Fig. 4. The surviving fraction of the cells in SCC VII tumors after a dose of 18 Gy had been given to the tumors at different times after an i.p. (*closed circle*) or i.v. (*open circle*) injection of 100 mg/kg KIH-802. Time 0 represents no drug treatment before irradiation. Error bars represent 1 SE from four experiments

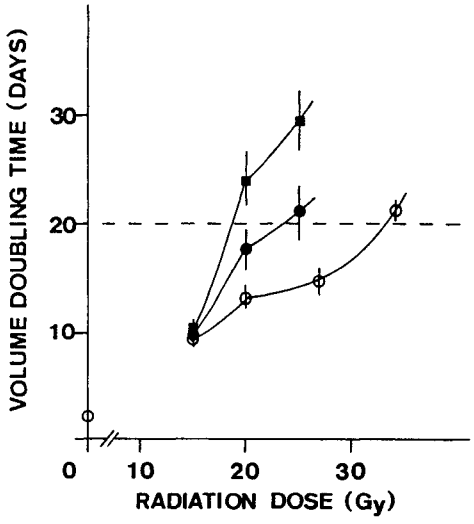

Fig. 6. Dose-response curves of SCC VII tumors, i.e., volume doubling time as a function of the irradiation dose. Error bars represent 1 SE from 8 or 9 mice. *Closed square*, 200 mg/kg KIH-802; *closed circle*, 100 mg/kg KIH-802; *open circle*, no drug

various concentrations of KIH-802 under oxic or hypoxic conditions for intervals of 1, 2, and 4 h. Incubation of oxic EMT6/KU cells with 1 mmol dm^{-3} KIH-802 for 4 h did not significantly inhibit colony formation, but under hypoxic conditions this compound was slightly cytotoxic.

Radiosensitization in vitro

Figure 3 shows the radiation survival curves for EMT6/KU cells exposed to 0.5 and 1.0 mmol dm^{-3} KIH-802 under hypoxic conditions. KIH-802 showed radiosensitizing activity in hypoxic cells; the SERs for concentrations of 0.5 and 1.0 mmol dm^{-3} were 1.50 and 1.70, respectively. Under oxic conditions, 1.0 mmol dm^{-3} KIH-802 did not sensitize EMT 6/KU cells to ionizing radiation. Table 1 shows the SERs for KIH-802, MISO, and SR-2508 at concentrations of 0.5 and 1.0 mmol dm^{-3}.

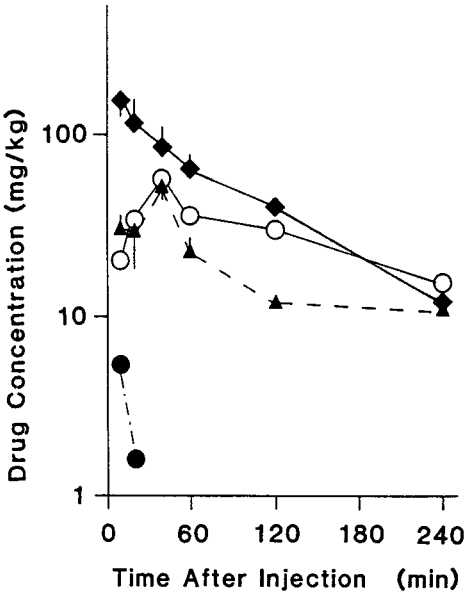

Fig. 7. Concentrations of KIH-802 in the serum (*closed diamond*), tumors (*closed triangle*), brains (*closed circle*), and sciatic nerves (*open circle*) of SCC VII tumor-bearing C3H/He mice as a function of time after i.p. injection of 200 mg/kg of the drug. Error bars represent 1 SD from more than four experiments

Radiosensitization in vivo

In vivo-in vitro assay. For the time-course experiment, we made no attempt to produce full survival curves; rather a single dose of 18 Gy was given at various times after the i.p. or i.v. injection of 100 mg/kg KIH-802 (Fig. 4). Radiosensitization appeared to be maximal 20–30 min after i.v. injection and 15–45 min after i.p. treatment; therefore, KIH-802 was given i.p. 30 min before irradiation in subsequent experiments to determine the SERs. Based on the results of previous experiments [12, 13], MISO was injected i.p. 40 min before irradiation and SR-2508 was given i.v. 20 min prior to irradiation.

From the radiation survival data (data not shown), the SER was determined for each compound at various doses. Figure 5 shows the SER of each compound as a function of the dose delivered. KIH-802 appeared to have a slightly higher radiosensitizing activity than either MISO or SR-2508.

Growth delay assay. On the basis of the findings obtained in the in vivo-in vitro assay, KIH-802 was given i.p. 30 min before irradiation. MISO was injected i.p. 40 min prior to irradiation and SR-2508 was given i.v. 20 min before irradiation. Figure 6 shows the dose-response curves for tumors treated with X-rays in the absence of drug as well as after various doses of KIH-802. The SER for each dose delivered was calculated at the volume doubling time equal to 20 days. Table 1 shows the SER values for KIH-802 in comparison with those for MISO and SR-2508; there was no significant difference in radiosensitizing activity among these three compounds.

Pharmacokinetic studies

Figure 7 shows drug concentrations in the serum, tumor, brain, and sciatic nerve after i.p. administration of KIH-802. Table 1 shows the exposure to KIH-802, MISO, and SR-2508 in the brains and peripheral nerves and the biologic half-lives of these compounds in each tissue. The concentration of KIH-802 in the brains and sciatic nerves was similar to that of SR-2508, whereas that of MISO was much higher. The tumor concentration of KIH-802 was slightly lower than that of either MISO or SR-2508.

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 SR-2508 (Table 1); the value for KIH-802 was 0.9 g/kg.

Discussion

KIH-802 belongs to a group of 2-nitroimidazoles containing a hydroxamic acid unit in their side chain and has a structure [-CO-NH-$(CH_2)n$-OH, $n = 0$] similar to that of SR-2508 [-CO-NH-$(CH_2)n$-OH, $n = 2$]. Some hydroxamic acid derivatives act as antitumor agents [17] and protease inhibitors [7] due to their metal chelating ability. In these molecules, the -CO-NH-OH unit is more sensitive to ionizing irradiation [16]. It has been reported that the existence of a hydroxamic acid moiety in the side chain of 2-nitroimidazole enhanced its radiosensitizing ability both in vitro and in vivo [5]. Therefore, KIH-802 was expected to show good radiosensitizing activity. As reported above, this compound sensitized well the response of SCC VII tumor to irradiation, as expected.

The acute toxicity of KIH-802 was slightly higher than that of MISO and 4 times higher than that of SR-2508. However, the dose-limiting factor for MISO and SR-2508 in clinical use was not their acute toxicity but their chronic neurotoxicity, especially the irreversible peripheral neuropathy [2–4, 18]. The pharmacokinetic study showed that the concentrations of KIH-802 in neural tissue were lower than those of MISO and as low as those of SR-2508. The octanol/water partition coefficient of KIH-802 is very low, and this low lipophilicity was responsible for the drug's low concentration in the brains [1, 19]. A physiologically similar barrier is assumed to exist around the peripheral nervous system [1, 19]; therefore, this compound is expected to have low central and peripheral neurotoxicity, as does SR-2508.

In conclusion, KIH-802 showed good radiosensitizing activity in vivo. Its concentration in neural tissue was very low; therefore, this compound would be expected to show low neurotoxicity. These results have encouraged us to proceed with the further examination of this compound and to develop more useful hydroxamic derivatives of 2-nitroimidazoles as hypoxic cell radiosensitizers.

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