Cancer Chemother Pharmacol (1993) 31: 455–458

Cancer Chemotherapy and Pharmacology

Effect of hyperthermia on the activity of 1-[(4′-hydroxy-2′-butenoxy)methyl]-2-nitroimidazole, which is cytotoxic to hypoxic cells

Tetsuya Kusumoto[1], Yoshihiko Maehara[2], Yasunori Emi[3], Yoshihisa Sakaguchi[3], Hideo Baba[3], Masakazu Sakaguchi[4], and Keizo Sugimachi[2, 3]

Clinical Research Division, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 815
[2] Department of Surgery II, [3] Cancer Center of Kyushu University Hospital, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812
[4] POLA Pharmaceutical R&D Laboratory, 560, Kashiwao-cho, Totsuka-ku, Yokohama 244, Japan

Received 10 April 1992/Accepted 26 October 1992

Summary. The effect on EMT6/KU cells of a newly synthesized hypoxic cell sensitizer, 1-[(4′-hydroxy-2′-butenoxy)methyl]-2-nitroimidazole (RK28), combined with heat was determined in vitro under conditions of hypoxia. As compared with aerobic conditions, hypoxia produced a 1.30-fold increase in the cytotoxicity of the drug for mouse mammary EMT6/KU cells induced by 1 h heat treatment at 43° C in medium with a normal pH. Hypoxia also reduced the surviving fraction of cells treated with both RK28 alone for 2 h and the same concentrations of RK28 and heat (43° C) in combination. Those enhancement ratios corresponded to a 20.3- and >345-fold increase, respectively. Moreover, concomitant treatment with RK28 and heat greatly inhibited the clonogenic activity of the EMT6/KU cells under conditions of in vitro hypoxia and in all experimental groups; there was a statistically significant difference in the time-response curves ($P < 0.05$). As hypoxic cells in a solid tumor are resistant to various anticancer drugs, RK28 combined with hyperthermia deserves further study for possible clinical applications.

Introduction

Radiation-sensitizing agents, including misonidazole (MISO) [1], and chemotherapeutic agents [12] in conjunction with hyperthermia [2, 22, 28] are prescribed for the treatment of subjects with a malignant tumor. This combined therapy is more advantageous in disseminated disease such as leukemia than in solid tumors [9]. Therapeutic strategies that can effectively attack radio- or chemotherapy resistant subpopulations of a tumor, such as hypoxic cells, need to be developed.

Hypoxic cell radiosensitizers that are superior to MISO in cytotoxicity and have less neurotoxic properties have been developed in Japan [18]. MISO, a hypoxic-cell cytotoxic agent studied in a clinical setting, produces side effects of peripheral neurotoxicity [1]. A 2-nitroimidazole derivative sensitizer, 1-[(4′-hydroxy-2′-butenoxy)methyl]-2-nitroimidazole (RK28), was recently synthesized in an attempt to reduce the neurotoxicity seen for MISO [18].

Murayama et al. [18] reported that RK28 produced a marked cytotoxicity for both HeLa S3 cells and Chinese hamster V79 cells under conditions of hypoxia and that RK28 could function as a hypoxic-cell cytotoxin.

There are now reliable data indicating that the addition of local or whole-body hyperthermia can substantially improve tumor control or result in cell lethality [20, 21]. Thermal damage to tissues is greatly affected by changes in tissue blood flow during heat treatment [26, 27]. The intratissue microenvironment controlled by blood flow affects the thermosensitivity of the tissues, and an acidic condition potentiates the thermal injury to mammalian cells [8, 26, 27]. Although both hypoxia and low pH enhance heat-induced cellular damage in tumors, as we have noted using various lines of cultured cells [23], the effects of hypoxia alone and the change in heat sensitivity need to be investigated.

Hyperthermia potentiates the cytotoxicity of chemotherapeutic agents such as doxorubicin [10], cisplatin [11, 17], and mitomycin C [29, 31] as well as that of some radiation sensitizers, including MISO [1]. We directed our attention to radiosensitizers for the targeting of hypoxic subpopulations in a solid tumor. We determined and compared the effects of heat alone, RK28 alone, and the combination of RK28 and heat under both aerobic and hypoxic conditions. The utility of RK28 combined with hyperthermia as an effective therapy for patients with a malignancy is discussed.

Correspondence to: Yoshihiko Maehara, Department of Surgery II, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

Materials and methods

Chemicals. RK28, obtained from POLA Pharmaceutical R&D Laboratory, Yokohama, Japan, was dissolved in Eagle's minimal essential medium (MEM)-1 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) just before its use. The medium contained 292 mg *L*-glutamine/ml, 100 units penicillin/ml, 0.1 mg streptomycin/ml, and 0.04 mg gentamycin/ml supplemented with 15.0% fetal calf serum (FCS; Gibco Laboratories, N.Y., USA).

Cells. The EMT6 (subline EMT6/KU) murine mammary-carcinoma cells used in this study were kindly provided by Prof. M. Abe, Department of Radiology, Faculty of Medicine, Kyoto University, Kyoto, Japan. The cells were maintained by serial subcutaneous implantation of tumor fragments into the leg of BALB/c female mice aged 7–8 weeks (Japan Charles-River Co. Ltd., Kanagawa, Japan). When required for experiments, the cells were taken directly from subcutaneous-passage tumors in mice. Single-cell suspensions were prepared from tumors using a combined mechanical and enzymatic dissociation procedure. The tumors were finely minced with scissors, transferred to enzyme preparations (0.04% trypsin: 30 ml enzyme solution), and suspended in MEM-1 according to the experimental design.

In vitro hypoxic conditions. The exponentially growing EMT6/KU cells were suspended in a glass test tube (approximately 5×10^5 cells/0.3–0.4 ml MEM-1) fitted with needles for the inflow and outflow of gases through sterile rubber stoppers. To attain hypoxic conditions in the presence or absence of the addition of designated concentrations of RK28 to the suspension, the tubes were made hypoxic by purging with gas comprising 95.0% N_2: 5.0% CO_2 for a 1-h preincubation period followed by hyperthermic treatment under hypoxic conditions [18]. Aerobic conditions were produced by purging with gas comprising 95.0% air: 5.0% CO_2 in the same manner. Prior to the start of the experiments, we confirmed that the pH of the medium did not change in case of a 2-h treatment under hypoxic conditions and that the oxygen concentration of the medium reached 0 after a 1-h gas purging.

Heating. Exposure to heat was done by immersing the glass tube with the sterile rubber stopper and keeping the oxygen environment intact in a water bath heated precisely at various temperatures. The temperature of the water was automatically controlled at the required temperature within an accuracy of ±0.1°C. The cells were simultaneously treated by heat combined with RK28 exposure under either aerobic or hypoxic conditions immediately following hypoxic pretreatment.

Cell-survival assay. The cell-survival assay was carried out using the colony-formation method. The exponentially growing EMT6/KU cells were exposed to anoxic gas in a glass tube as described above for 1 h together with various designated concentrations of RK28 for preincubation and were then treated with heat, whereby the conditions of hypoxia were maintained using continuous gas purging through the sterile rubber stoppers. The temperatures used for heat treatment were 37.0°C for controls and 41.0°C–44.0°C for treated cells. After RK28 or heat treatment under either aerobic or hypoxic conditions, the cells were washed with Hanks' balanced salt solution and enumerated. The EMT6/KU cells were seeded in sterile plastic culture dishes in triplicate according to the experimental design and then incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day 10, the colonies in each group were fixed with ethanol, stained with Giemsa solution, and counted. More than 50 cells were considered to be one colony. All experiments were independently performed at least three times.

Statistical analysis. The Welch-Aspin *t*-test [16, 25] was used to determine the significance of differences in the slopes of time-dependent survival curves obtained under aerobic versus hypoxic conditions. When the *P* value was less than 0.05, the difference was considered to be statistically significant.

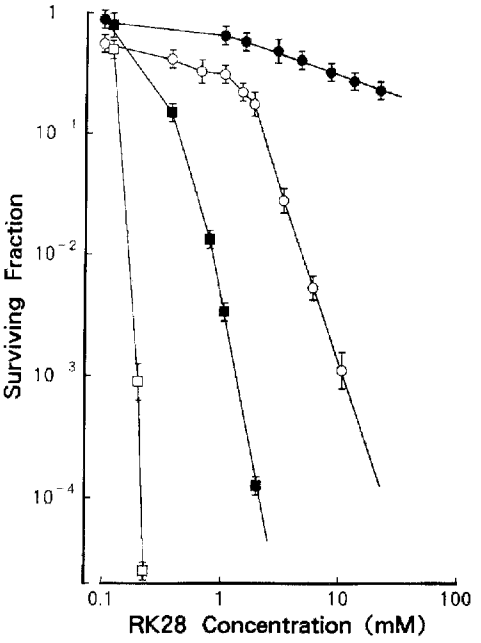

Fig. 1. Survival of EMT6/KU cells following 1 h exposure to RK28 alone (●, ○) or to RK28 plus heat (■, □). The temperature was 43.0°C. *Closed and open symbols*, findings obtained under aerobic and hypoxic conditions, respectively; *points*, means of 3 independent experiments; *bars*, SD

Table 1. Effect of 1 h heat treatment on the survival of EMT6/KU cells under aerobic or hypoxic conditions

Temperature (°C)	Plating efficiency	
	Aerobic	Hypoxic
37.0	95.9±8.1[a]	82.7±15.0
41.0	50.3±0.5*	36.8± 2.1*
43.0	9.5±0.8**	7.3± 0.6**
44.0	3.9±0.2*	2.6± 0.1*

[a] Percentage of surviving fraction following 1 h exposure to heat
* $P < 0.01$, ** $P < 0.05$

Results

Survival of hypoxic EMT6/KU cells treated with heat or with a combination of RK28 and heat

The plating efficiency for the controls was 95.9% ± 8.1%. The EMT6/KU cells were preincubated under hypoxic conditions for 1 h followed by a 1-h period of heat exposure under the same conditions. Each response curve had a shoulder at 41°C, and there was a log-linear response at temperatures exceeding 41°C. When heat was applied to the EMT6/KU cells kept under hypoxic conditions, the response curve shifted to levels lower than those observed under aerobic conditions (Table 1). The effects on the heat-induced decrease in cell survival by hypoxia were thus evident in the EMT6/KU cells.

We then examined the surviving fraction of EMT6/KU cells treated with various concentrations of RK28 for 2 h (1 h preincubation with RK28 and 1 h drug-specific treatment time) under hypoxic conditions (Fig.1). RK28 was

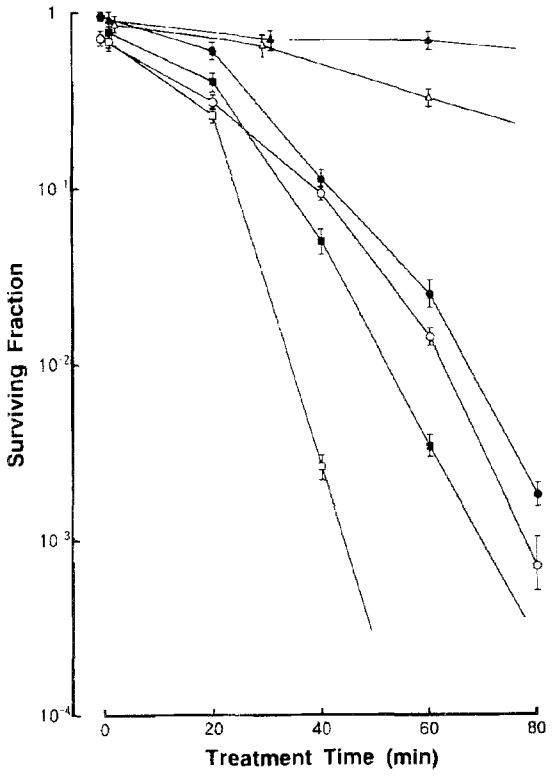

Fig. 2. Survival of EMT6/KU cells treated with RK28 alone (▲, △), with heat alone (●, ○), or with a combination of RK28 and heat (■, □). The cells were exposed to a temperature of 43.0°C and to 1.0 mM RK28 for 0–80 min. *Closed and open symbols*, findings obtained under aerobic and hypoxic conditions, respectively; *points*, means of 3 independent experiments; *bars*, SD

more cytotoxic to EMT6/KU cells under hypoxic conditions than under aerobic conditions, with hypoxia producing a 20.3-fold increase in cytotoxicity. At doses exceeding 1.5 mM RK28, the dose-dependent survival curve showed a log-linear response. When heat exposure at 43°C was added for the last 1 h in each group, there was more than a 99% cell kill under both aerobic and hypoxic conditions, as shown in Fig. 1.

Time-dependent survival of hypoxic EMT6/KU cells treated with RK28 and heat

After preincubation with 1 mM RK28 under hypoxic conditions, the combination of RK28 treatment plus heat exposure at 43°C was applied to the EMT6/KU cells for 0–80 min, whereby the same environment was maintained except for the temperature. As shown in Fig. 2, in cells exposed to 30–40 min of heat or RK28, the surviving fraction in each group showed a log-linear response. Time-dependent survival was inhibited to the greatest degree in all groups when the EMT6/KU cells were treated with RK28 plus heat under conditions of hypoxia. When the data were analyzed using the Welch-Aspin *t*-test, significant differences were found in the regression coefficients as revealed by the slopes of the time-response curves generated for the group treated with RK28 plus heat under hypoxic conditions and those plotted for the other groups ($p < 0.05$).

Discussion

It is generally accepted that the effects of hyperthermia in cancer treatment depend on tumor environmental factors [19]. A reduction in tumor blood flow creates a region of localized chronic hypoxia and a low pH. Other investigators report that incubation and heating of cells at elevated temperatures in low-pH medium greatly increases the cell kill as compared with heating at a normal pH [6, 7, 13]. Killing the radioresistant subpopulations of cells in tumors should improve local tumor control, as such cells have been found to be hypoxic within an acidic environment [14, 30]. Chaplin and co-workers [4] propose that hypoxia in cells is presumably induced by chronic, diffusion-limited hypoxia, as mentioned above, or by a state of acute, transient hypoxia. The temporary intermittent cessation of blood flow within the tumor vasculature results in the latter type of hypoxia in in vivo tumors. The cells within this area are thought to be resistant to radiation and chemotherapy but relatively sensitive to hyperthermia at a low pH [23].

We found that hypoxia significantly inhibited the survival of EMT6/KU cells. As the pH of the medium was confirmed to be constant, the change in thermosensitivity significantly correlated with oxygen conditions and with the depletion of the intracellular pH caused by hypoxic treatment.

As is the case for various nitro compounds, the cytotoxicity of RK28 toward EMT6/KU cells is increased in the presence of hypoxia, which implies an increase in the cytotoxicity of the drug to hypoxic tumor-cell subpopulations (unpublished data). Under hypoxic or acidic (low-pH) conditions, the nitro ($-NO_2$) group in RK28 is presumably reduced to the amine ($-NH_2$), including the intermediates by both electron-transfer reactions and hydrogen abstraction from cell-target-producing intermediates, as is the case for MISO [3]. The preferential action of 2-nitroimidazoles is associated with an appropriate reducing system in neoplastic cells, as has been noted for alkylating agents [15, 24]. As reviewed by Coleman [5], products in the reducing process of 2-nitroimidazoles contribute to their cytotoxicity under hypoxic conditions. The activity of RK28 against a solid tumor depended on diffusion of the agent to the hypoxic cells. As RK28 has a sugar component and a high electron affinity, it is relatively water-soluble and can readily be delivered to tissues.

We found that hyperthermia increased the cytotoxicity of RK28 to both well-oxygenated and hypoxic cells in culture. At normal pH and 43.0°C, the cytotoxicity of RK28 depended on oxygenation of the cell cultures, and heat at normal pH significantly sensitized the hypoxic EMT6/KU cells to RK28 cytotoxicity. The most likely explanation for our results would be the activation of RK28 under hypoxic conditions at elevated temperatures. The function of RK28 in the hypoxic cells might be explained by electron-transfer reactions and hydrogen abstraction, which is probably the case for MISO as described by Coleman [5]. Hyperthermia may increase the cell-target-

producing intermediates that contribute to the cytotoxicity of RK28. It was postulated that the chemical activation of RK28 might be increased by the addition of thermal energy and that cellular repair systems would thereby be inhibited. As another explanation, we propose that selective intracellular glutathione depletion by RK28 exposure may be involved under conditions of hypoxia.

RK28 was prescribed by a radiation oncologist for Japanese patients with a malignancy, and good effects were observed [18]. RK28 seems to improve local solid-tumor control and to increase the therapeutic gain. Moreover, the additive conditions of low pH, which are close to the conditions of an in vivo solid tumor, increase the grwoth-inhibitory activity of the drug in solid tumors.

If the toxicities of RK28 are tolerable, this agent should increase the potential of hyperthermia to provide local control of a malignant tumor. Our findings of the in vitro hyperthermic potentiation of RK28 under hypoxic conditions pave the way for in vivo studies and potential clinical applications.

Acknowledgements. This work was supported by a Grant-in-Aid for Cancer Research from Japanese Cancer Research of the Ministry of Health and Welfare (2-18). We thank Dr. S. A. Holden, Dana-Farber Cancer Institute, Boston, for her advice on the manuscript.

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