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Tomoaki Fujioka · Koich Ishikura
Michihiko Hasegawa · Kazunori Ogyu
Yasushi Matsushita · Masatsugu Sato
Fumio Sato · Hikaru Aoki · Takashi Kubo

Antitumor effects of oral administration of an interferon-inducing pyrimidinone, Bropirimine, on murine renal-cell carcinoma

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Abstract Bropirimine [2-amino-5-bromo-6-phenyl-4-(3H)-pyrimidinone] is a low-molecular-weight compound that acts as an inducer of interferon in several animal species. Experiments were designed to explore the possibility of using this drug for the treatment of renal-cell carcinoma (RCC). Euthymic BALB/c mice were inoculated with murine RCC (Renca) cells and given graded doses of Bropirimine p.o. for 5 consecutive days beginning on day 1 following tumor inoculation. These mice were killed and tumors were excised on day 21. Bropirimine significantly ($P < 0.01$) inhibited the tumor growth at a daily dose of 1,000 or 2,000 mg/kg. No adverse effect or toxicity was noted at 1,000 mg/kg, and at 2,000 mg/kg there was only a marginal body-weight reduction without any other appreciable side effect. In addition to the inhibition of tumor growth, there was a small yet significant ($P < 0.05$) increase in the duration of survival (in days) in the Bropirimine-treated animals. When the treatment was delayed to begin on day 6 following tumor inoculation, Bropirimine did not suppress tumor growth in euthymic mice, pointing to the importance of the timing of the treatment. In athymic nude BALB/c mice lacking T-cells or T-cell function, Bropirimine also inhibited tumor growth ($P < 0.01$). The antitumor effect of this drug was abolished by pretreatment with anti-asialo GM1 serum, which eliminated natural killer (NK) activity in euthymic mice. In vivo treatment with Bropirimine augmented the cytotoxicity of lymphocytes isolated from the spleens or lungs of the tumor-bearing mice, which were active against Renca and YAC-1 cells in vitro. This activity was NK-cell-dependent as judged on the basis of the results of the in vitro complement-dependent cytotoxicity assay. Since Bropirimine induced interferon (IFN)-α/β production, significantly ($P < 0.05$) elevating its serum concentration, and since this drug mimics the effects of IFN-α/β, it seemed likely that the Bropirimine-induced NK cell augmentation we found was mediated by IFN-α/β. These results suggest that Bropirimine, a booster of NK activity, may have potential as an adjunct to other therapeutic modalities in the treatment of human RCC.

Key words Bropirimine · Immunotherapy · Murine renal-cell carcinoma

T. Fujioka (✉) · K. Ishikura · M. Hasegawa · K. Ogyu · Y. Matsushita · M. Sato · F. Sato · H. Aoki · T. Kubo
Department of Urology, Iwate Medical University School of Medicine, 19–1, Uchimaru, Morioka 020, Japan

Introduction

Renal-cell carcinoma (RCC) patients have metastatic disease at the time of diagnosis in 25%–57% of cases and develop metastases within 10 years in approximately 60% of cases after a curative nephrectomy [1, 2]. Aside from surgical resection, metastatic RCC is difficult to treat because it is resistant to both chemotherapy and readiotherapy [3]. Recently, attempts have been made to use biological response modifiers (BRMs) such as interferons (IFNs) or interleukin 2 as well as the adoptive transfer of lymphokine-activated killer (LAK) cells or tumor-infiltrating lymphocytes (TILs) for the treatment of advanced RCC, with the rate of efficacy reported being 30% [4–8]. However, these results have emphasized the need for more powerful therapeutic approaches for metastatic RCC.

Bropirimine [2-amino-5-bromo-6-phenyl-4 (3H) pyrimidinone], one such BRM, has been shown to have several immunomodulatory activities, including interferon induction, and antiviral as well as antitumoral activities, offering attractive potential for a variety of immunomodulatory applications [9–14].

In the present studies, we examined the effects of Bropirimine on the in vivo growth of murine RCC as well as on the in vitro cytotoxic activity of lymphocytes

against this type of tumor cell, for which no information is available at present. The killer cells responsible for the Bropirimine-induced antitumor cytotoxicity were also characterized.

Materials and methods

Animals

Female, euthymic hairy or athymic nude BALB/c mice were housed in a special pathogen-free animal facility and were used for experiments at an age of 8–10 weeks.

Tumors

The Renca cell line, renal adenocarcinoma syngeneic in BALB/c mice, kindly provided by Dr. R. H. Wiltrout (NCI, Frederick, Md. USA), was maintained in vivo by serial intrarenal passage, YAC-1, a moloney leukemia line commonly used as a reference target for natural killer (NK) activity, was maintained in vitro. Tumor lines were shown to be mycoplasma-free as tested with a commercially available kit (Flow Laboratories, McLean, Va. USA).

Drugs and antibodies

Bropirimine was manufactured and provided by Upjohn Co. (U-54461S, Kalamazoo, Mich. USA). This compound was given p.o. in fine suspension form in a vehicle composed of 5 mg/100 ml carboxymethylcellulose, 4 mg/100 ml polysorbate 80, 9 mg/100 ml sodium chloride, and 9% benzyl alcohol (0.5% CMC NaCl solution). Lyophilized anti-asialo GM1 serum (014–09801, Wako Pure Chemical Industries, Tokyo, Japan) and monoclonal anti-Thy-1, 2. antibody (CL8959, Cedarlane Laboratories, Hornby, Ontario, Canada) were reconstituted with balanced salt solution. Anti-asialo GM1 (1:50 dilution) and anti-Thy-1, 2 (1:100 dilution) antibodies, respectively can completely eliminate NK activity and T-cell activity in vitro in the presence of low toxic rabbit complement (1:10 dilution) (CL3051, Cederlane) [15]. For the in vivo experiments, 0.3 ml of 1:20 diluted anti-asialo GM1 serum was injected i.v. into the tail vein of euthymic BALB/c mice on the same day the tumor cells were inoculated.

Experimental tumor model

The Renca tumor was aseptically excised from a tumor-bearing mouse, and single-cell suspensions were prepared by mechanical dissociation and filtration of the resulting tumor-cell suspension through a 45-mesh stainless-steel sieve. Injection of 5×10^5 tumor cells under the capsule of the left kidney in euthymic BALB/c mice was performed through a flank incision. In athymic nude BALB/c mice, 1×10^6 Renca cells were inoculated s.c. Then, graded doses of Bropirimine were given p.o. in a volume of 0.1 ml through a 24-gauge curved animal-feeding needle for 5 consecutive days beginning on either day 1 or day 6 following tumor inoculation. Control mice were given 0.1 ml of 0.5% CMC–NaCl solution orally. All mice were killed and tumors, excised at 21 days after tumor inoculation. Each experimental group consisted of 10 animals in all in vivo experiments unless otherwise indicated. All experiments were performed at least twice with similar findings.

Preparation of lymphoid cells

Lymphocytes were isolated from the spleens and lungs of untreated and Bropirimine-treated mice. The spleens were cut into small pieces with scissors and squeezed through a steel sieve under sterile conditions. After centrifugation the pellet was treated with ammonium chloride TRIS-hydrochloric acid buffer (0.75% ammonium chloride in 17 m*M* TRIS-hydrochloric acid, pH 7.65) to remove red blood cells. Lymphocytes were then collected, washed, and suspended in RPMI medium (13200, Gibco, Grand Island, N.Y., USA) containing 10% heat-inactivated fetal calf serum (FCS; 16000, Gibco). Lungs of Renca-bearing mice were excised and a single-lymphoid-cell suspension was obtained by enzymatic and mechanical dispersion according to the methods described by Belldegrun and associates [16]. The chopped tissue was dissociated by mechanical stirring for 4 h at 37°C in a flask containing RPMI medium (without FCS) with 0.01% hyaluronidase type V (1,000–2,000 units/mg; H6254, Sigma Chemical Co., St. Louis, Mo., USA), 0.1% collagenase type IV (163–230 units/mg; C5138, Sigma), and 0.002% deoxyribonuclease I (100 units/mg; D4263, Sigma). The resulting mixture was then filtered through the steel mesh, washed and separated on a Ficoll-Hypaque gradient.

Cell-mediated cytotoxicity in vitro

The cytotoxicity assay was performed by a 4-h chromium-release method [14]. Target cells were labeled with 100 μCi of Na_2-[^{51}Cr]-O_4 for 60 min at 37°C. After being washed three times, target-cell suspensions were mixed with the effector cells and then incubated for 4 h at 37°C in a U-bottomed 96-well microtiter plate. Samples of the supernatant were harvested and counted in a gamma counter (Aloca, Auto Well Gamma System, ARC-500, Tokyo, Japan). The percentage of specific cytotoxicity was calculated according to the formula:

$$\%\text{Specificity} = \frac{\text{experimental counts/min-spontaneous counts/min}}{\text{total incorporated counts/min-spontaneous counts/min}} \times 100.$$

IFN assay

Serum IFN-α/β levels were determined at 3 or 6 h after oral administration of 1,000 mg/kg Bropirimine in the Renca-bearing euthymic BALB/c mice. Each group consisted of six mice. IFN-α/β activity was determined by a cytopathic-effect assay involving vesicular stomatitis virus cultured on murine LY cells (ATCC CRL 1722) and a dye-uptake method [17]. The murine IFN standard (IFN-α/β; 0820–11, Lee Biomolecules, San Diego, Calif, USA) had a concentration of 6.400 IRU/ml.

Statistical analysis

Data were expressed as mean values ± standard deviation (SD). For individual tumor weights and serum IFN levels, the Dunnett test or generalized Wilcoxon test was performed using the Fisher statistical software to determine the significance of the effects of Bropirimine. The analysis of survival was performed by plotting the survival curves and calculating the differences utilizing the Kaplan-Meier test or generalized Wilcoxon test. Differences were considered statistically significant when the *P* value was 5% or less.

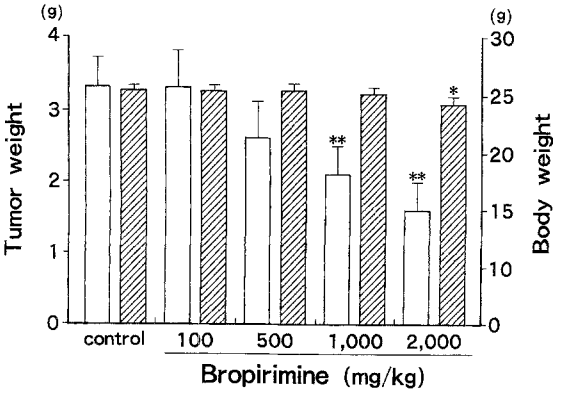

Fig. 1 Effects of graded doses of oral Bropirimine on tumor weight (*open bars*) and body weight (*hatched bars*) at 21 days after renal inoculation of Renca tumor cells (5×10^5) in female euthymic BALB/c mice. Bropirimine was given for 5 consecutive days following inoculation of the tumor cells. Each *bar* and *vertical line* indicates the mean value and SD for 10 animals, respectively. $^*P < 0.05$; $^{**}P < 0.01$ as compared with the respective controls

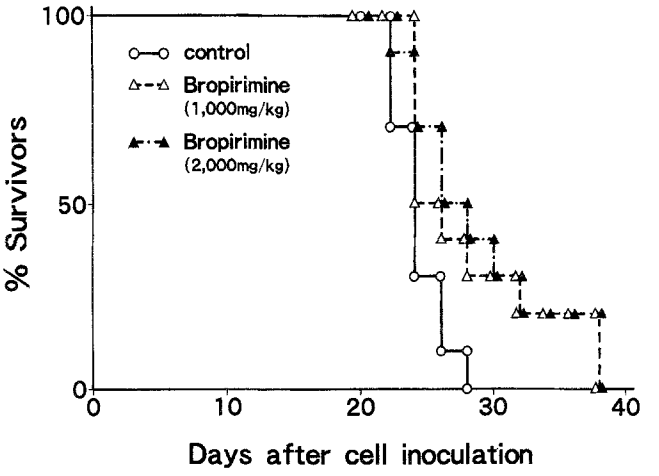

Fig. 2 Temporal course of the percentage of survival of Bropirimine-treated female euthymic BALB/c mice bearing Renca tumor cells under the renal capsules. Both of the Bropirimine-treated groups (1,000 and 2,000 mg/kg) survived significantly ($P < 0.05$) longer than the control group

Results

In vivo antitumor effect of Bropirimine treatment against Renca tumor

When Bropirimine was given orally for 5 days beginning on day 1 following tumor inoculation in euthymic hairy BALB/c mice and the tumor weight was measured on day 21, there was a significant ($P < 0.01$) inhibition of Renca growth at a daily Bropirimine dose of 2,000 or 1,000 mg/kg (Fig. 1). No significant antitumor effect was observed at 500 or 100 mg/kg. The mean weights of tumors in the control, 2,000-mg/kg-treated, 1,000-mg/kg-treated, 500-mg/kg-treated, and 100-mg/kg-treated mice were 3.3 ± 0.4, 1.6 ± 0.3, 2.1 ± 0.4, 2.6 ± 0.5, and 3.3 ± 0.5 g, respectively. There was no significant difference in tumor weight between the 2,000-mg/kg- and 1,000-mg/kg-treated groups. At the daily dose level of 1,000 mg/kg, no toxicity attributable to the drug was observed. At the dose level of 2,000 mg/kg there was only marginal body-weight reduction without any other appreciable side effect (Fig. 1). When the administration of Bropirimine (2,000 or 1,000 mg/kg daily) began on day 6 and continued for a total of 5 days following Renca inoculation, there was no difference in tumor weight as measured on day 21 between the control group and the experimental groups.

In another set of experiments with euthymic hairy BALB/c mice, Bropirimine treatment at a daily dose of 1,000 or 2,000 mg/kg beginning on day 1 following the Renca tumor inoculation resulted in a significant ($P < 0.05$) prolongation of the survival of treated mice as compared with controls (Fig. 2). There was no significant difference in survival between the 2,000-mg/kg- and 1,000-mg/kg-treated groups. All the control mice died by day 28, whereas 30% of the mice in the treated groups were alive on day 30. On the basis of these results, we used a dose of 1,000 mg/kg in subsequent experiments.

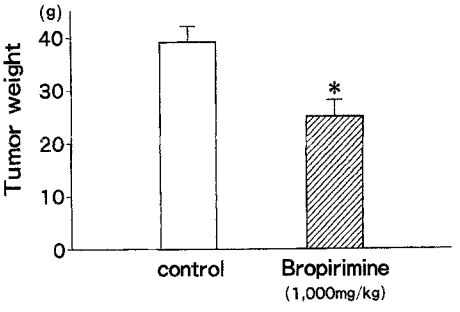

Fig. 3 Effects of 1,000 mg/kg p.o. Bropirimine on tumor weight at 21 days after s.c. inoculation of Renca tumor cells (1×10^6) in female athymic BALB/c nude mice. Bropirimine was given for 5 consecutive days following inoculation of the tumor cells. Each *bar* and *vertical line* indicates the mean value and SD for 10 animals, respectively. $^*P < 0.01$ as compared with the Bropirimine-untreated, tumor-bearing control mice

Growth-inhibitory effects of Bropirimine in athymic nude mice and euthymic mice with depressed NK activity

As shown in Fig. 3, Bropirimine (1,000 mg/kg) inhibited tumor growth significantly ($P < 0.01$) in athymic nude mice on day 21, these results being similar to those obtained in euthymic hairy mice. The mean tumor weight was 2.5 ± 0.3 g in the treated mice and 3.7 ± 0.5 g in the controls. However, Bropirimine was

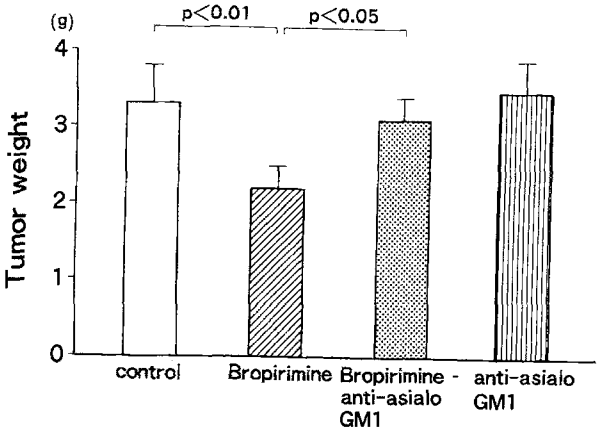

Fig. 4 Effects of p.o. administration of 1,000 mg/kg Bropirimine for 5 days on tumor weight at 21 days after renal inoculation of Renca tumor cells (5×10^5) in female euthymic BALB/c mice that also received i.v. injection of anti-asialo GM1 serum on the same day the tumor cells were inoculated. Appropriate control groups were also studied as shown. Each *bar* and *vertical line* indicates the mean value and SD for 10 animals, respectively

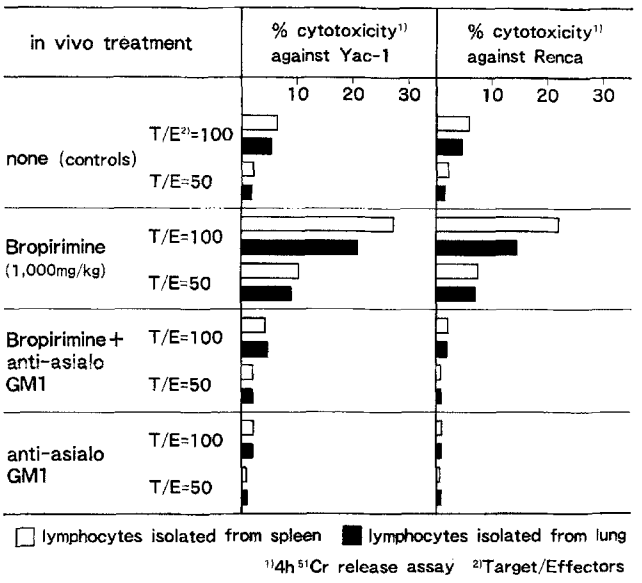

Fig. 5 Effects of in vivo treatment of Renca cell-inoculated female euthymic BALB/c mice with 1,000 mg/kg oral Bropirimine for 5 days with or without anti-asialo GM1 serum on the in vitro cytotoxicity of the lymphocytes isolated from their spleens and lungs as tested against Renca and YAC-1 cells in the 4-h ^{51}Cr-release assay. Lymphocytes were derived from spleens and lungs 7 days after renal inoculation of Renca tumor cells (5×10^5)

ineffective ($P > 0.1$) in reducing the tumor weight in euthymic mice pretreated with anti-asialo GM1 serum, in whom NK activity was specifically eliminated (Fig. 4).

Modulation of host immunity

The Bropirimine-treated and untreated control euthymic hairy BALB/c mice were killed on day 7 following Renca inoculation and lymphocytes were isolated from their spleens and lungs. As shown in Fig. 5, lymphocytes from both organs of the treated mice markedly lysed the Renca cells as well as the murine NK-sensitive YAC-1 cells. In contrast, the lymphocytes from the control mice failed to show such activity. When pretreatment with anti-asialo GM1 serum was given on day 0, these in vitro cytotoxic activities of lymphocytes from the Bropirimine-treated animals were abolished with both types of target cells.

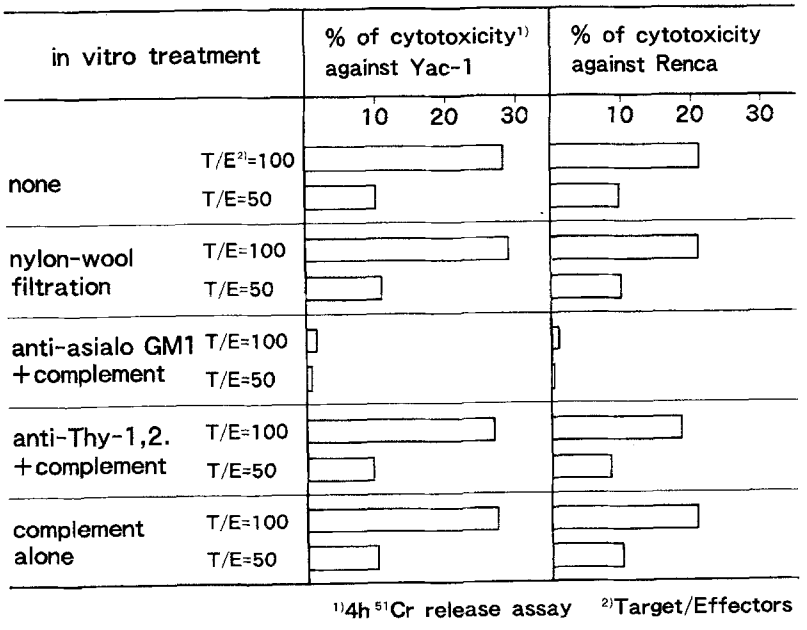

Fig. 6 Characterization of Bropirimine-activated lymphocytes derived from spleens at 7 days after renal inoculation of female euthymic BALB/c mice with Renca tumor cells (5×10^5). Bropirimine (1,000 mg/kg) was given for 5 days. The activated lymphocytes were characterized as NK cells on the basis of nylon-wool filtration and attenuation of cytotoxicity by anti-asialo GM1 + complement. The 4-h ^{51}Cr-release assay was used to assess the cytotoxic activity against YAC-1 and Renca cells. Appropriate control groups were also studied as shown

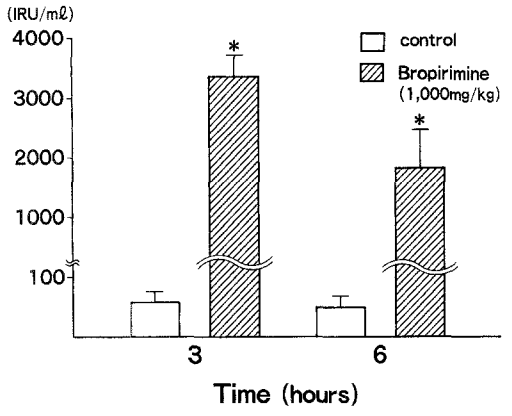

Fig. 7 Serum IFN levels determined 3 or 6 h after administration of a single oral dose of 1,000 mg/kg Bropirimine or of the control vehicle. IFN levels were determined by a cytopathic-effect assay involving vesicular stomatitis virus cultured on murine LY cells and a dye-uptake method. Each *bar* and *vertical line* indicates the mean value and SD for 6 animals, respectively. $*P < 0.05$ as compared with the respective vehicle-treated controls

Characterization of syngeneic killer cells

Lymphocytes were isolated from the spleens of Bropirimine-treated euthymic hairy BALB/c mice on day 7 following tumor inoculation and the lymphocyte type that was active against Renca cells was characterized. When lymphocytes were separated by nylon-wool filtration, the active cells were present in the filtered fraction, but not in the retained macrophage or B-cell fraction (Fig. 6). Bropirimine-induced antitumor activity against Renca and YAC-1 cells was not affected by anti-Thy 1, 2 treatment but was abolished almost completely by anti-asialo GM1 treatment in the in vitro complement-dependent cytotoxicity assay (Fig. 6). These findings indicated that the Bropirimine-activated effector cells responsible for the lysis of the Renca cells were NK cells.

IFN level of murine serum

Serum IFN levels were determined in the tumor-inoculated euthymic hairy BALB/c mice at 3 or 6 h after oral administration of a single dose of 1,000 mg/kg Bropirimine or of the vehicle. Serum levels of IFN-α/β in the treated mice were significantly ($P < 0.05$) elevated at both 3 and 6 h as compared with the control values (Fig. 7). Thus, it is likely that Bropirimine-induced enhancement of the NK-cell activity was IFN-mediated.

Discussion

A murine renal cortical adenocarcinoma of the dark cell type, Renca, which is of spontaneous origin in BALB/c mice, has been well characterized and is sensitive to lysis by NK cells [18, 19]. It has been proposed that this tumor provides an excellent experimental model in which tumor growth and progression can be quantitated accurately, and it has therefore been used to screen drugs for potential activity against RCC [19–22]. In the present investigation, we examined the antitumor effects of a unique oral drug, Bropirimine, using this murine RCC model.

Bropirimine exhibited antitumor activity against Renca cells in both euthymic and athymic BALB/c mice. Since a body-weight reduction due to Bropirimine treatment, albeit marginal, was observed at the highest dose tested, i.e., 2,000 mg/kg, the optimally effective dose of this agent seemed to be 1,000 mg/kg in these murine experiments. When the treatment was delayed to begin on day 6 following Renca inoculation, Bropirimine did not inhibit tumor growth, pointing to the importance of the timing of drug administration in relation to the disease stage. From our experiments, it appears that greater effects could be expected if intervention with this drug were to take place earlier.

Bropirimine treatment suppressed Renca tumor growth in athymic nude mice, which lack T-cells and T-cell function congenitally, as it did in hairy euthymic mice. However, when the NK activity was depleted by anti-asialo GM1 serum in the euthymic mice, the antitumor efficacy of the drug was abolished. These results suggest that the involvement of NK cells, but not T-cells, was essential to produce the antitumor effect of Bropirimine.

In our in vitro experiments, the lymphocytes isolated from the spleens and lungs of the Bropirimine-treated tumor-bearing euthymic BALB/c mice exhibited augmented cytotoxic activity against Renca cells. This in vitro antitumor activity was abolished by in vivo administration of anti-asialo GM1 serum. Thus, there was a good agreement between the results of our in vivo and in vitro experiments, both pointing to the importance of NK cells as mediators of the antitumor effects of Bropirimine. The NK identity of the lymphocytes activated by Bropirimine was further confirmed by in vitro testing with nylon-wool filtration and with a complement-dependent cytotoxicity using anti-asialo GM1 and anti-Thy 1, 2 antibodies.

The reduction of rat mammary tumor size in Bropirimine-treated Sprague-Dawley rats has been reported to correlate significantly with the titers of the induced IFN [12]. It was also reported that the induction of IFN led to an augmentation of NK cytotoxic potential [23, 24]. We previously reported on the in vivo antitumor activity of IFN-α/β against Renca tumors transplanted under the renal capsules. When 1×10^4 U of natural IFN-α/β was injected i.p. into these tumor-bearing mice, the tumor weight (1.42 ± 0.32 g) was significantly ($P < 0.05$) suppressed as compared with the control values (3.9 ± 0.11 g). This

was associated with significantly ($P < 0.05$) extended survival [25]. We also demonstrated IFN induction by Bropirimine in the present investigation and, thus, the NK-cell augmentation produced by Bropirimine in our present experiments is likely to have been mediated by IFN-α/β. As roughly estimated, oral administration of 1,000 mg/kg Bropirimine for 5 days seems to induce NK antitumor activity equivalent to that produced by a single i.p. injection of 1×10^4 U IFN-α/β in the murine RCC model.

The present observation suggests that Bropirimine is potentially effective in murine RCC as a booster of NK activity. In patients who undergo curative operation for RCC, recurrence or metastasis frequently develops even after complete nephrectomy, and long-term adjuvant therapy is needed in most RCC patients. It is in this context that Bropirimine, which can be given orally, might have a place as an adjunct to other therapeutic modalities in the treatment of RCC.

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