Anti-Cancer Drugs 1995, **6**, pp. 158–162

Short report

In vitro and *in vivo* antitumor activity of the interferon inducer bropirimine

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***In vivo* and *in vitro* antitumor effects of an interferon (IFN)-α inducer, bropirimine (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone), were examined in the murine tumor system. The antitumor effects were studied in Meth A cells, which were the most sensitive to bropirimine in the murine cell lines tested. The direct inhibitory activity of the drug was not reduced when Meth A cells were incubated with bropirimine and anti-IFNs, indicating that the inhibition is not due to autocrine IFN induction from tumor cells. The drug partially inhibited the uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine. Cell cycle analysis with flow cytometry showed that the drug decreased G_0/G_1 phase and increased G_2/M phase Meth A cells. The drug administered i.p. exhibited a remarkable antitumor effect against Meth A cells which were implanted i.p. These results suggested that the drug induced the *in vivo* antitumor effect by its direct antitumor activity.**

Key words: Antitumor effect, bropirimine, interferon inducer.

Introduction

Bropirimine (molecular weight 266), a derivative of phenylpyrimidinone (Figure 1), is reported to be a biological response modifier (BRM) through the induction of endogeneous interferon (IFN)-α in several animal species[1] and in man.[2–5] Bropirimine showed antiviral activity,[6] and activated natural killer cells,[7] macrophages[8] and antibody response.[9] The compound caused antitumor action such as prolongation of survival time and reduction of metastasis in the mouse.[10] A carcinogen-induced autochthonous bladder carcinoma was also inhibited in growth by the compound.[11] In the rat, it reduced the nodule size of a low antigeneic 7,12-dimethylbenzo[*a*]anthracene-induced mammary carcinoma.[12] Synergistic effects were observed when bropirimine was combined with cyclophosphamide 5or other chemotherapeutic agents.[13] However, little is known about the direct antitumor action of bropirimine. The aim of the present study is to determine whether or not broirimine has antitumor action in *in vitro* cultivated and *in vivo* passaged tumor cells and to clarify the inhibition mechanism.

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Materials and methods

Tumors and mice

Methylcholanthrene-induced fibrosarcoma Meth A cells[14] were suspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (culture medium) and incubated at 37°C in a 5% CO_2 incubator. For the culture of 3LL, 40 μM of 2-mercaptoethanol was added to the culture medium. Meth A cells have been maintained by i.p. passages in BALB/c mice (Charles River Japan, Atsugi, Japan) and were used for *in vivo* experiments.

In vitro tumor growth inhibition by bropirimine

The tumor cells (2×10^3) were suspended in 0.5 ml of culture medium. Since bropirimine (Upjohn Pharma, Tsukuba, Japan) is not sufficiently soluble in water (< 2 mg/ml), the compound was first dissolved in dimethylsulfoxide (DMSO) (133 mg/ml) and then this solution was diluted to culture medium. The final concentration of DMSO was 0.08%. DMSO did not affect the tumor growth, DNA, RNA and protein syntheses or the cell cycle. After

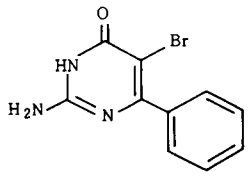

Figure 1. Chemical structure of bropirimine (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone).

culture, the cell number was determined with a Coulter Counter (Model Z_{BI}; Coulter Electronics, FL, USA).

Treatment with anti-IFNs in *in vitro* growth inhibition

Meth A cells (5×10^3 cells/ml) were incubated in a 96-well tissue culture plate with anti-murine IFN-α monoclonal antibody (mAb) (rat IgG1, specific for the most of mouse IFN-α produced by mouse L cells and recombinant mouse IFN-α; no cross-reaction was seen with mouse IFN-β, IFN-γ and human IFNs; at a dilution of 500-fold, this antibody neutralizes 10 U/ml of mouse IFN- α in the system of L cells challenged with VSV) and anti-murine IFN-β (rat IgG1, specific for mouse IFN-β produced by mouse L cells and recombinant mouse IFN-β; no cross-reaction with mouse IFN-α, IFN-γ and human IFNs; at a dilution of 400 000 times, this antibody neutralizes 10 U/ml of mouse IFN-β in the system of L cells challenged with VSV) (Yamasa Shoyu, Chiba-Ken, Japan) (500-fold dilution) and murine recombinant IFN-α (10 000 U/ml) (Sumitomo, Pharma, Osaka, Japan) and IFN-β (20 000 U/ml) (Toray, Tokyo, Japan). After 4 days, the cell number was determined with the Coulter Counter. Tumor growth (%) was calculated as follows: (number of cells with drug)/(number of cells without drug) × 100 (%).

DNA, RNA and protein synthesis

Meth A cells (2×10^4) were incubated in 0.5 ml of culture medium for 24 h. After addition of 1 μCi of [^{3}H]thymidine (specific activity 247.9 GBq/mmol), [^{3}H]uridine (specific activity 1.6 TBq/mmol) or [^{3}H]leucine (specific activity 185.0 GBq/mmol) (NEN, Boston, MA), the cells were further incubated for 2.5 h. The cells were filtered and then washed with 5% trichloroacetic acid and 0.9% NaCl. The radioactivity incorporated into cells was analyzed by a liquid scintillation counter. At the same time, the cell number was determined by a Coulter Counter. The percentage of radioactivity per cells was calculated as follows: [(c.p.m. with drug/no. of cells with drug)/(c.p.m. without drug/no. of cells without drug)] × 100 (%).

Cell cycle

The cell cycle analysis was determined according to the method of Krishnan.[15] The cells were washed and fixed with 70% ethanol for more than 3 h at 4°C. After washing the cells with PBS, the cells were incubated with PBS containing 1 mg/ml of heat-inactivated (80°C for 10 min) RNase (Sigma, St Louis, MO) at 37°C for 40 min. After centrifugation, the cells were resuspended in 50 μg/ml propidium iodide (PI) (Sigma) dissolved in 0.1% sodium citrate solution. The DNA content of 10^4 cells was determined with a flow cytometer (Profile; Coulter Electronics).

Antitumor activity of bropirimine

Meth A (2×10^5) cells were injected i.p. into BALB/c mice on day 0. Bropirimine was suspended in PBS and administered i.p. at a dose of 200 or 100 mg/kg daily on five consecutive days from day 0. Control mice were injected i.p. with the same volume of PBS (0.2 ml).

Results

In vitro growth inhibition

In vitro growth inhibition against murine cell lines, colon 26, Meth 1, L1210, Meth A and 3LL was examined for sensitivity to bropirimine (data not shown). In cell lines tested, the drug is effective to Meth A and 3LL. Therefore, the 50% inhibitory concentration (IC_{50}) of these cells was determined after day 4 of culture (Table 1). The

Table 1. IC_{50} of bropirimine against Meth A and 3LL cells

Tumor cell	IC_{50} (μg/ml)[a]
Meth A	24.4 ± 5.1
3LL	50.0 ± 5.8

[a]Mean ± SD of three experiments.

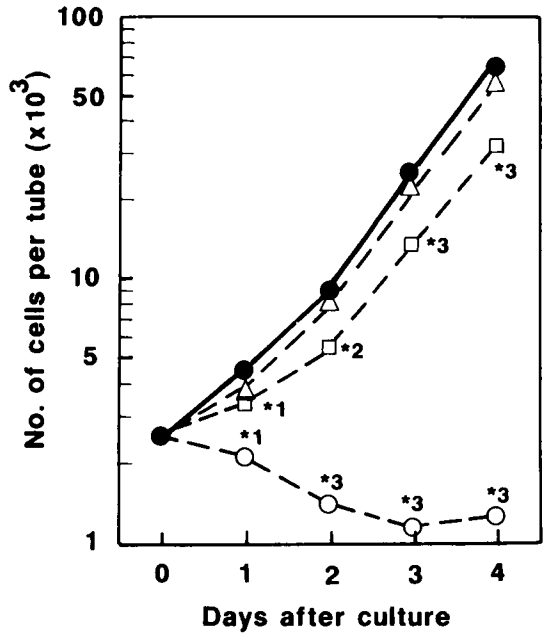

Figure 2. Growth-inhibition curve of Meth A cells with bropirimine. Since the coefficient of variation of cell number was less than 0.1, the standard deviation was omitted. One representative result of three experiments is shown. Bropirimine: 100 μg/ml (○), 33 μg/ml (□), 11 μg/ml (△); control (●). Statistically significant versus control by Student's *t*-test, *1, $0.02 < p$ 0.05; *2, $0.01 < p < 0.02$; *3, $p < 0.001$.

drug dose-dependently inhibited the growth of Meth A (Figure 2) and 3LL cells (data not shown). These results indicated that bropirimine possessed a direct growth-inhibitory action against Meth A and 3LL.

Effect of anti-IFNs on bropirimine-induced growth inhibition

We studied in detail the effect of the drug on Meth A cells, which were the most sensitive tumor tested. Since bropirimine is an IFN inducer, there is a possibility that IFN released from tumor cells by the drug inhibited tumor growth in an autocrine fashion. Therefore, we examined this hypothesis using IFNs and anti-IFN antibodies (Table 2). The growth of Meth A cells was inhibited with IFN-α and/or IFN-β to a similar degree as bropirimine. Anti-IFN-α and/or anti-IFN-β mAb concentrations were high enough to reverse the growth inhibition with IFN(s). The growth-inhibitory activity with bropirimine was not affected by treatment with anti-IFN antibodies. These results indicated that the inhibition of Meth A cells with bropirimine was not dependent on the IFN(s).

Effect on DNA, RNA and protein synthesis

After incubation with bropirimine for 24 h, Meth A cells were pulse-labeled with [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine. The incorporation of these precursors into Meth A cells was partially and almost equally inhibited by the drug (Table 3), indicating that DNA, RNA and protein syntheses were inhibited with the drug to the same degree.

Effect on cell cycle

After incubation with the drug for 1, 2 and 3 days, the DNA content of cells was determined by flow cytometry using PI (Figure 3). The G_0/G_1 phase of the cell cycle was decreased while the G_2/M phase was increased, indicating that the drug arrested the cells at the G_2/M phase.

Antitumor effect in mice

The antitumor effect was determined in an i.p. (tumor)–i.p. (drug) system to examine the direct activity not only *in vitro* but also *in vivo*. As shown

Table 2. Effect of anti-IFN-α and anti-IFN-β antibodies on growth inhibitory activity of Meth A cells by bropirimine

	Growth inhibition (%)				
	bropirimine (μg/ml)		IFN		
Anti-IFN antibody	30	15	α + β	α	β
–	35 ± 1	84 ± 7	24 ± 1	19 ± 2	37 ± 3
+	40 ± 3	80 ± 1	58 ± 2[a]	66 ± 3[a]	71 ± 3[a]

The anti-murine IFN mABs (α and β) were diluted 500-fold and titers of murine recombinant IFN -α and -β were 10 000 U/ml and 20 000 U/ml.
[a]Statistically significant versus each anti-IFN from sample by Student's *t*-test ($p < 0.001$).

Table 3. Effect of bropirimine on incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into Meth A cells[a]

Bropirimine (μg/ml)	Incorporation (%)		
	[^{3}H]thymidine	[^{3}H]uridine	[^{3}H]leucine
100	68.0 ± 3.5[b]	72.7 ± 8.1[c]	64.8 ± 14.3[d]
33	97.0 ± 10.6	97 ± 13.2	98.3 ± 7.1
0	100	100	100
0[e]	109.0 ± 8.5	97.3 ± 6.4	94.5 ± 13

[a]Mean ± SD of three experiments for thymidine and uridine incorporation, and of four experiments for leucine incorporation.
Statistically significant versus control by Student's *t*-test: [b]$0.002 < p < 0.005$, [c]$0.02 < p < 0.05$, [d]$0.01 < p < 0.002$.
[e]Without DMSO.

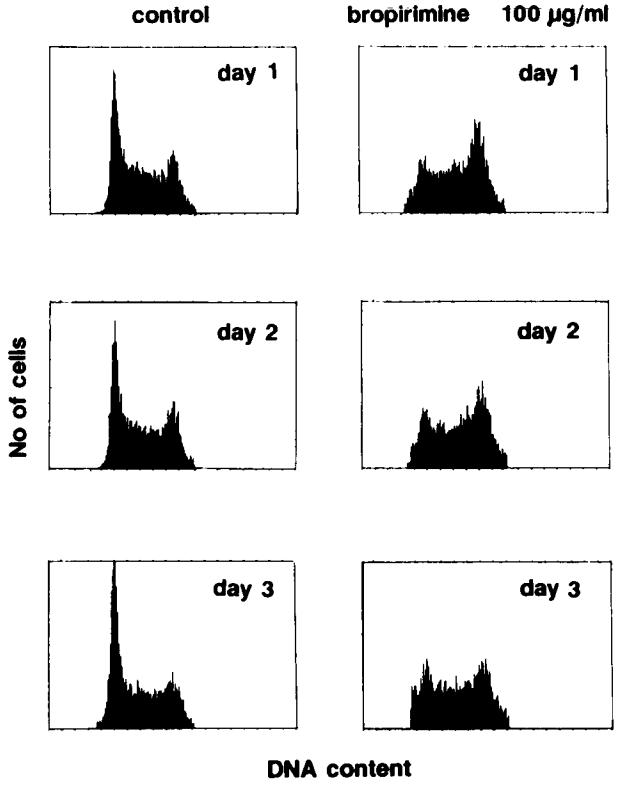

Figure 3. Cell cycle analysis of bropirimine-treated Meth A cells. Meth A cells was cultured with or without bropirimine and the cell cycle was analyzed by flow cytometry using PI. One representative result of three experiments is shown.

in Table 4, the drug exhibited the antitumor effect dose-dependently. These results suggested that the antitumor effect of the drug was dependent on the direct activity as well as the BRM activity as an IFN inducer.

Table 4. Antitumor activity of bropirimine against Meth A cells

Bropirimine	Survival days [mean ± SD(n − 1)]	T/C[a] (%)
200	40.3 ± 14.8[b]	206
100	> 30.4 ± 14.4[c]	155
0	19.6 ± 3.7	100

Eight BALB/c mice were implanted i.p. with Meth A (2×10^5) on day 0. Bropirimine (mg/kg) was injected i.p. on five consective days from day 0.
[a]T/C: survival days of bropirimine-treated mice/survival days of control mice.
[b]Statistically significant with Student's *t*-test versus control: $0.001 < p < 0.002$.
[c]One mouse survived more than 59 days. The duration of survival days of the surviving mouse was counted as 59, and mean survival days and standard deviation were calculated.

Discussion

Bropirimine is currently under clinical trial for its antitumor potential in phase I trials,[2–5] and its effectiveness to renal and bladder cancer in phase II trials. The drug was excreted without chemical modification or as the glucuronide-conjugated form. The drug concentration (C_{max}) was 170–300 μg/ml in urine and 50–90 μg/ml in serum.[2–5] These results suggested a direct antitumor effect of the drug. Therefore, we studied *in vitro* and *in vivo* antitumor effects. IC_{50}s for Meth A and 3LL were 24.4, and 50.0 μg/ml, respectively (Table 1), which were lower concentrations than in urine and serum. Growth inhibition by the drug is not dependent on IFN of tumor cells (Table 2). The drug partially inhibited DNA, RNA and protein syntheses (Table 3), and arrested cells at the G_2/M phase (Figure 3).

An antitumor effect was observed in the i.p.–i.p. system of BALB/c mice (Table 4). After *in vitro* treatment with bropirimine, Meth A cells with var-

ious sizes of cytoplasm and nucleus in comparison with control were observed with Wright–Giemsa staining (data not shown). These results indicated that bropirimine possesses direct antitumor activity.

The drug showed antitumor effects against human SK MEL-3 melanoma and Caki-1 cell carcinoma, which were implanted in nude mice.[16] Murine IFN, which was induced by the drug in nude mice, is not effective in human tumors because of species restriction. This report also supported the direct antitumor effect of the drug.

Antibiotics such as actinomycin cause a block in the S and G_2/M phase.[17] Thus, the cell cycle block by the drug is similar to that by antibiotics. The drug blocked the cells at the S and G_2/M phase (Figure 3). Since DNA, RNA and protein syntheses decreased at the G_2/M phase,[18] the partial reduction of these syntheses by the drug (Table 3) may be due to the block in the G_2/M phase.

We have previously reported that the IC_{50} of Meth A cells for mitomycin C (MMC) as an authentic drug was 0.23 μg/ml.[19] The IC_{50} values of bropirimine for Meth A and 3LL were much higher than that of MMC. On the other hand, IFN was induced in human peripheral blood mononuclear cells at bropirimine concentrations above 12 μg/ml *in vitro*[20] and was predicted to be induced at above 50 μg/ml *in vivo* (human plasma).[21] Thus, the drug showed IFN-inducing and direct activities under almost the same drug concentration. These results should be taken into consideration for the further analysis of the antitumor activity of the drug, because the sensitivity to IFN and direct activity of the drug are dependent on tumors.

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(Received 6 June 1994; received in revised form September 1994; accepted 22 September 1994)