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GENETIC AND BIOCHEMICAL STUDIES ON THE ACTIVATION AND CYTOTOXIC MECHANISM OF BREDININ, A POTENT INHIBITOR OF PURINE BIOSYNTHESIS IN MAMMALIAN CELLS*

HIDEKI KOYAMA† and MASAE TSUJI
Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 170, Japan

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Abstract—To study the activation and cytotoxic mechanism of bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate), a novel nucleoside antibiotic with potent cytotoxic and immunosuppressive effects, we isolated in a single-step manner five mutants resistant to 10 μM bredinin from cultured mouse mammary carcinoma FM3A cells mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Such resistant (Brd^r) mutants were 15- to 19-fold less sensitive to the antibiotic than wild-type cells and maintained stably their resistant phenotypes in the absence of bredinin for more than 3 months. They were cross-resistant to tubercidin, an adenosine analog. Like wild-type cells, Brd^r mutants were capable of incorporating radioactivity from ring-labeled adenosine into the acid-insoluble macromolecular fraction. However, hypoxanthine-guanine phosphoribosyltransferase-deficient (HGPRT$^-$) mutants derived from the Brd^r cells did not incorporate the radioactivity at all or at a markedly reduced rate, indicating that blockade of the pathway via adenosine deaminase present in the Brd^r cells resulted in loss of their ability to utilize adenosine. Enzyme assays using cell-free extracts revealed that all the Brd^r mutants had less than 3% of the adenosine kinase (AK) activity found in wild-type cells. These results demonstrate that the bredinin resistance is attributed to a defective AK activity and, therefore, that bredinin is metabolized by AK, which may phosphorylate it to a toxic nucleotide, bredinin 5'-monophosphate (Brd-MP), in sensitive cells. Among exogenously added purine bases, guanine was able to reverse the cytotoxic effect of bredinin on both wild-type cells and F5 cells carrying the vector pSV2-*Escherichia coli* xanthine-guanine phosphoribosyltransferase (XGPRT) gene, while xanthine was able to do so only in F5 cells because the base was metabolized to XMP by the cells. These results support the mechanism of bredinin cytotoxicity, that Brd-MP formed in sensitive cells exposed to the antibiotic blocks the conversion of IMP to XMP by inhibiting IMP dehydrogenase.

Bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) has potent cytotoxic effects on mammalian cells in culture [1, 2] and is reported to be a promising immunosuppressive agent [1, 3–5]. In addition, 4-carbamoylimidazolium-5-olate (CIO)‡, the aglycone of bredinin, has been shown to have similar toxic effects [6]. Sakaguchi *et al.* [2, 7] studied the mechanism of such cytotoxicity and found that the growth-inhibitory effects of both bredinin and CIO on L5178Y mouse leukemia cells were reversed by guanine, guanosine, and GMP, but not by hypoxanthine, xanthine, IMP, and XMP. They failed to find either any metabolite of bredinin or its incorporation into the nucleic acids in L5178Y cells grown in medium containing radioactive bredinin [7]. They also observed bredinin in the urine and serum of rats which had been administered radioactive CIO orally [6]. Based on these and other data, they concluded that exogenous bredinin itself, or bredinin formed from exogenous CIO by the action of some enzyme such as adenine phosphoribosyltransferase (APRT), without being further metabolized, inhibited the conversion of IMP to GMP in the biosynthetic pathway of purine nucleotides [7].

Recently, Fukui *et al.* [8] demonstrated the formation of Brd-MP from CIO and 5-phosphoribosyl-1-pyrophosphate using cell-free extracts made from Ehrlich carcinoma cells and further showed that this nucleotide competitively inhibited IMP dehydrogenase (EC 1.2.1.14), an enzyme which catalyzes the conversion of IMP to XMP (Step 3 in Fig. 1). Koyama and Kodama [9] isolated eleven mutants resistant to CIO from mouse FM3A cells and found all these mutants to be completely deficient in APRT activity. Therefore, they concluded that the mechanism of CIO resistance was attributed to a defect in APRT and that CIO was

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† To whom all correspondence should be addressed.

‡ Abbreviations: AK, adenosine kinase (EC2.7.1.20); APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); APRT$^-$, APRT-deficient; Brd-MP, bredinin 5'-monophosphate; Brd^r, bredinin-resistant; CIO, 4-carbamoylimidazolium-5-olate; cio^r, CIO-resistant; *E. coli* XGPRT, *Escherichia coli* xanthine-guanine phosphoribosyltransferase (EC 2.4.2.22); *Ecogpt*, *E. coli* XGPRT gene; HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); HGPRT$^-$, HGPRT-deficient; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TCA, trichloroacetic acid; and TG, 6-thioguanine.

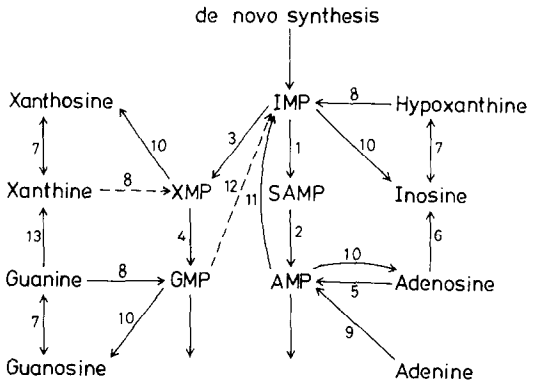

Fig. 1. Purine biosynthesis and salvage pathways in mammalian cells. Steps: 1, adenylsuccinate synthetase (EC 6.3.4.4); 2, adenylsuccinate lyase (EC 4.3.2.2); 3, IMP dehydrogenase (EC 1.2.1.14); 4, GMP synthetase (EC 6.3.4.1); 5, adenosine kinase (EC 2.7.1.20); 6, adenosine deaminase (EC 3.5.4.4); 7, purine nucleoside phosphorylase (EC 2.4.2.1); 8, HGPRT (EC 2.4.2.8); 9, APRT (EC 2.4.2.7); 10, 5′-nucleotidase (EC 3.1.3.5); 11, AMP deaminase (EC 3.5.4.6); 12, GMP reductase (EC 1.6.6.8); and 13, guanine deaminase (EC 3.5.4.3). The dotted lines show non-significant steps for growth of mammalian cells in culture.

converted by the enzyme to a toxic metabolite, Brd-MP [9]. Accordingly, these data reported by Fukui *et al.* [8] and Koyama and Kodama [9] argue against the above-mentioned view of Sakaguchi *et al.* [7] that bredinin itself was an active inhibitor of *de novo* purine synthesis.

In attempts to understand the mechanisms of the activation and cytotoxicity of bredinin, we isolated Brd^r mutants and other purine analog-resistant mutants from FM3A cells and their derivatives after mutagenesis with MNNG. Subsequent biochemical analyses revealed that the mechanism of bredinin resistance was attributable to loss of AK activity. Therefore, it is concluded that the kinase activates the antibiotic by phosphorylating it to the corresponding mononucleotide, Brd-MP.

To study the mechanism of bredinin toxicity, we took advantage of a peculiar cell line, F5, which is able to utilize xanthine for growth, because F5 cells were derived from F28-7 cells transformed by pSV2-*Ecogpt* [10]. Unlike wild-type and $APRT^-$ cells, the growth-inhibiting effects of bredinin were substantially reversed by xanthine in F5 cells. These findings support the following proposed mechanism of cytotoxicity: in sensitive cells bredinin is metabolized by AK to Brd-MP, which then blocks the conversion of IMP to XMP by inhibiting IMP dehydrogenase.

MATERIALS AND METHODS

Chemicals. Bredinin, Brd-MP, and CIO (SM-108) were provided by the Sumitomo Chemical Co. Ltd., Takarazuka, Japan. Coformycin was a gift from Meiji Seika Kaisha Ltd., Pharmaceutical Division, Tokyo, Japan. TG and tubercidin were obtained from the Sigma Chemical Co., St. Louis, MO; all the purine bases, nucleosides and nucleotides were from the Yamasa Shoyu Co. Ltd., Choshi, Japan, and MNNG was from the Aldrich Chemical Co., Milwaukee, WI. [8-^{14}C]Adenine (55.6 mCi/mmole), [8-^{14}C]adenosine (45.5 mCi/mmole), and [^{3}H]hypoxanthine monohydrochloride (3.8 Ci/mmole) were purchased from the New England Nuclear Corp., Boston, MA.

Cells and culture methods. The subclonal line, F28-7, derived from mouse mammary carcinoma FM3A cells, was used for wild-type cells [9, 11]. The cio^r3 and cio^r8 lines were $APRT^-$ derivatives selected for CIO resistance from F28-7 cells [9]. F5 cells were a transformant of F28-7 cells with a pSV2 vector carrying *Ecogpt* and the human β1 interferon gene [10] and were supplied by Dr. S. Ohno of this laboratory.

All these cell lines were maintained in suspension in synthetic medium ES (Nissui Seiyaku Co, Tokyo), supplemented with 2% fetal bovine serum (FBS; GIBCO Ltd., Grand Island, NY) as described by Koyama and Kodama [9]. Cells were cultured at 37° in fully humidified air containing 5–10% CO_2 and passaged twice a week at appropriate dilutions.

Isolation of drug-resistant mutants. Procedures for mutation induction and mutant selection were as reported previously [9, 11]. Logarithmic phase cells were treated with MNNG at 0.5 μg/ml for 2 hr, which resulted in about 50% cell survival. The cells were cultured for 5 days in normal medium to allow phenotypic expression. Five 100-mm plastic dishes (Lux Sci. Corp., Newbury Park, CA) were plated with 5×10^5 cells on agar medium consisting of 95% ES medium, 5% dialyzed fetal bovine serum (DFBS), and a selective agent such as bredinin, TG, or tubercidin. At the same time, three 60-mm plastic dishes (Lux Sci. Corp.) were plated with 100 cells on the selective agent-free, agar medium in order to determine the plating efficiency. After 7–10 days of cultivation, surviving colonies were counted, and some were picked, transferred to nonselective medium, and grown up to mass cultures. The frequency of bredinin-resistant cells was expressed as the number of Brd^r colonies on selective medium divided by the number of cells assayed (survivors), which were calculated from the number of cells plated and their plating efficiency.

Assay of drug sensitivity. Drug sensitivity of cells was tested by two assay methods. First, the degree of growth inhibition was determined by a method modified slightly from that previously reported [9]. Logarithmic phase cells were plated in duplicate at 5×10^3 cells/well in Linbro Tissue Culture plates containing 24 flat bottom wells (Flow Laboratories, Inc., McLean, VA) in 1 ml of 98% medium and 2% DFBS containing various concentrations of the drug, incubated at 37° for 72 ± 2 hr, and counted with a Coulter counter model D (Coulter Electronics Inc., Hialeah, FL). The number of cells in wells containing the drug was expressed as a percentage of the cells in control wells lacking it. To generate growth inhibition curves, these percentages were plotted against the drug concentration. The drug concentration which reduced the percentage to 50% (EC_{50}) was then determined from the curves.

Second, the colony-forming ability of each cell line was determined in drug-containing agar plates.

Three 60-mm plastic dishes (Falcon 1007; Falcon, Oxnard, CA) were plated with 10^2–10^4 cells growing logarithmically on 5 ml of agar medium containing various amounts of a drug to be assayed, incubated for 12–14 days, and the resulting colonies with more than 50 cells were counted. The relative plating efficiency of cells in dishes containing the drug was defined as a percentage of the plating efficiency observed in control dishes (no addition). The survival curve was depicted by plotting the relative plating efficiency against the drug concentration. The drug concentration which decreased the relative plating efficiency to 10% (D_{10}) was determined from the survival curves.

Incorporation of radioactive precursors. Cells in the logarithmic growth phase were plated in duplicate at 10^5 cells/well in Linbro plates containing 24 flat bottom wells in 1 ml of growth medium and cultured overnight. At zero time the cells were exposed to 0.05 μCi of [^{14}C]adenosine (diluted to 5 mCi/mmole with nonradioactive adenosine) or 0.5 μCi of [^{3}H]hypoxanthine monohydrochloride and incubated for various periods of time. Then 1 ml of 10% ice-cold TCA was added to each well, and the plates were kept on ice for over 15 min. The following procedures were carried out as described previously [9]: the radioactive samples collected on Whatman glass fiber filters (GF/C) were counted in a liquid scintillation counter. Simultaneously, at the beginning of labeling, cell counts were performed in two replicate wells to calculate the radioactivity incorporated per 10^6 cells.

Enzyme assay. Logarithmic phase cells were harvested, washed, and stored frozen at −20°. Cell-free extracts were prepared from the cell pellets and used for enzyme assays. AK activity was determined as described before [9], except that 2.5 μM coformycin was added to reaction mixtures to prevent degradation of [8-^{14}C]adenosine (substrate) by adenosine deaminase (Step 6, Fig. 1). APRT and HGPRT activities were also assayed as reported [9], using radioactive substrates [8-^{14}C]adenine and [^{3}H]hypoxanthine monohydrochloride respectively. Protein was determined by the method of Lowry *et al.* [12], using bovine serum albumin as a standard.

RESULTS

Isolation of bredinin-resistant cells. Wild-type F28-7 cells were treated with or without MNNG and assayed for appearance of bredinin-resistant cells as described in Materials and Methods. Table 1 shows the frequency of the antibiotic-resistant colonies. In untreated cells, no resistant colonies have appeared so far. In contrast, MNNG-treated cells gave rise to such colonies at a frequency of 5.8×10^{-5}. Five colonies were picked from independent plates, transferred to drug-free medium, and grown up to mass cultures. These cells were designated as Brd^{r}1 through Brd^{r}5 and used for subsequent studies. Bredinin-resistant cells also occurred in APRT$^-$ cio^{r}8 cells mutagenized in the same way as above. Ten colonies were isolated and maintained as usual in normal medium, and one of these was designated as CB7 and examined.

MNNG mutagenesis was also used to induce mutations resistant to an adenosine analog, tubercidin. The antibiotic-resistant colonies appeared only in the F28-7 cells exposed to MNNG at a frequency of 8.2×10^{-6}, the value being 7-fold less than that found for bredinin resistance. The reason for this difference is not known. We isolated four independent colonies as tubercidin-resistant (Tub^r) mutants and used them for the present study.

These resistant cells were cultured for more than 3 months in the absence of selective drugs and stably maintained their resistant phenotypes.

Sensitivity to bredinin. We tested the sensitivity of wild-type F28-7 and resistant cells to bredinin. Figure 2 shows the survival curves of such cell lines on agar plates containing various amounts of the drug. Wild-type cells were very sensitive to the antibiotic, showing a D_{10} of as low as 1 μM. In contrast, Brd^{r}3 and Brd^{r}4 were much less sensitive; that is, the D_{10} values for them ranged from 15 to 19 μM. This indicates that these mutants were 15- to 19-fold more resistant to the antibiotic than the wild-type cells. Brd^{r}1, Brd^{r}2, and Brd^{r}5 also had drug sensitivities similar to the above Brd^r mutants (unpublished data).

Figure 2 also shows that Tub^{r}5 was as resistant to bredinin as the Brd^r cells. This cross-resistance was also observed in Tub^{r}2 and Tub^{r}4. In other experiments, we found Brd^{r}3 and Brd^{r}4 cells to be clearly cross-resistant to tubercidin (data not shown). These findings suggest a common resistance mechanism operating in both Brd^r and Tub^r cell lines.

Brd^{r}3, Brd^{r}5, and Tub^{r}4 were observed to be about 2-fold more resistant to CIO, the aglycone of bredinin, than wild-type cells. However, it is unlikely that this degree of resistance is significant for the resistance mechanism of Brd^r and Tub^r mutants.

Table 1. Frequency of bredinin-resistant colonies in wild-type F28-7 cells*

Treatment	No. of cells assayed†	No. of Brd^r colonies observed	Mutant frequency‡
None	9.6×10^6	0	$<1.0 \times 10^{-7}$
MNNG	3.7×10^6	213	5.8×10^{-5}

* Wild-type F28-7 cells were treated with or without MNNG at 0.5 μg/ml for 2 hr, grown in normal medium for 5 days, plated on 10 μM bredinin-containing medium, and cultured for 9 days, as described in Materials and Methods.

† Calculated by the total number of cells plated in selective medium containing 10 μM bredinin and their plating efficiency in nonselective medium.

‡ Expressed as the number of Brd^r colonies divided by the number of cells assayed.

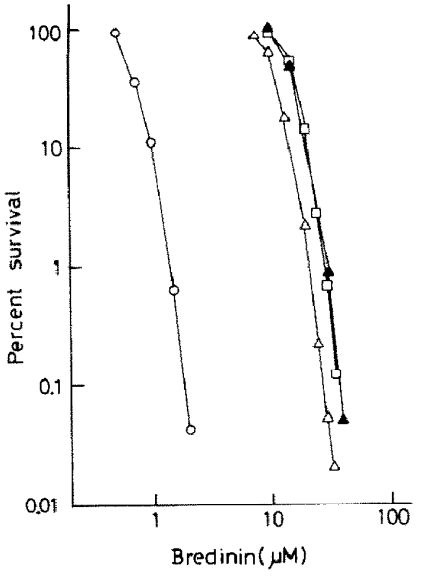

Fig. 2. Survival curves of wild-type F28-7 and drug-resistant cell lines in the presence of increasing concentrations of bredinin. Cells (10^2–10^4) were plated on agar medium containing various concentrations of bredinin and cultured for 12–14 days as described in Materials and Methods. Key: F28-7 (○—○); Brd^{r}3 (△—△); Brd^{r}4 (▲—▲); and Tub^{r}5 (□—□).

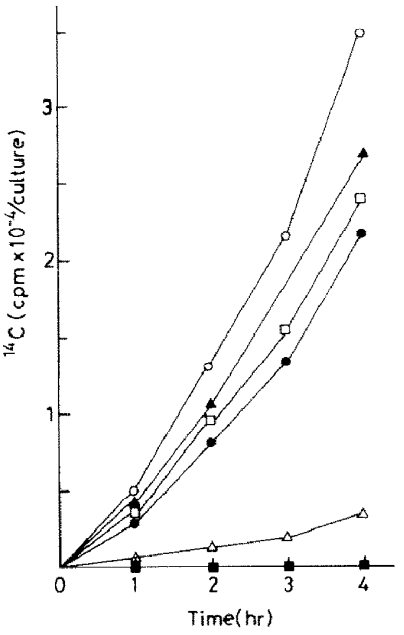

Fig. 3. Time course of radioactive adenosine incorporation into the acid-insoluble fraction of wild-type F28-7 and drug-resistant cell lines. Cells were exposed to 0.05 μCi of [8-^{14}C]adenosine at zero time, incubated for various lengths of time, and assayed for radioactivity incorporated as described in Materials and Methods. Key: F28-7 (○—○); Brd^{r}4 (●—●); BT4 (△—△); cio^{r}8 (▲—▲); CB7 (□—□); and CBT4 (■—■).

Incorporation of radioactive adenosine. We next studied whether or not Brd^r mutants were able to metabolize radioactive adenosine. As shown in Fig. 1, exogenous adenosine enters the purine nucleotide pool by being metabolized through two pathways. The first is phosphorylation by AK to AMP (Step 5). The second is deamination by adenosine deaminase to inosine (Step 6), followed by the sequential conversion to hypoxanthine and then to IMP by the action of two enzymes, purine nucleoside phosphorylase (Step 7) and HGPRT (Step 8). The formed mononucleotides are metabolized further and incorporated into the macromolecular cell fractions.

Table 2. AK, APRT, and HGPRT activities in wild-type F28-7 and drug-resistant cell lines*

Cell line	Specific activity† [nmoles·min^{-1}·(mg protein)$^{-1}$]		
	AK	APRT	HGPRT
F28-7	3.1	2.3	1.0
Brd^{r}1	0.013	2.2	ND‡
Brd^{r}2	0.037	1.8	ND
Brd^{r}3	0.096	2.0	0.97
Brd^{r}4	0.043	1.8	0.87
Brd^{r}5	0.014	1.4	ND
Tub^{r}4	0.028	1.9	ND
Tub^{r}5	0.022	1.9	ND
BT4	0.028	1.2	<0.01
cio^{r}8	2.5	<0.01	0.95
CB7	0.008	0.01	1.3
CBT4	0.016	<0.01	0
F5	2.2	1.9	1.5

* The procedures for preparation of cell extracts and enzyme assays are described in Materials and Methods.

† Average of two to three determinations, each in duplicate.

‡ Not determined.

For this experiment, we isolated HGPRT$^-$ BT4 cells from Brd^{r}4 cells by selecting for spontaneously appearing TG-resistant colonies on agar plates containing 1 μM TG (unpublished data). We also isolated a bredinin-resistant mutant CB7 from APRT$^-$ cio^{r}8 cells and then TG-resistant CBT4 cells from the CB7 mutant in the same way. Neither BT4 nor CBT4 had detectable HGPRT activity (see Table 2), nor did they incorporate [^{3}H]hypoxanthine into the acid-precipitable cell material (data not shown).

Figure 3 illustrates the time course of [8-^{14}C]adenosine uptake into the 5% TCA-insoluble fraction of cells. Wild-type F28-7 and cio^{r}8 cells incorporated it exponentially during a 4-hr incubation. Brd^{r}4 and CB7 were also able to do so at slightly reduced rates. In contrast, in BT4 cells, the amount of radioactivity taken up for 4 hr was only 10% of that observed in wild-type cells. Furthermore, CBT4 did not exhibit any incorporation activity under the same assay conditions. These results indicate that blockade of the above second pathway via adenosine deaminase, by rendering Brd^r mutants HGPRT-deficient, caused them to decrease [^{14}C]adenosine uptake, therefore suggesting that the mechanism for bredinin resistance should come from a defective AK activity.

AK deficiency in Brd^r cell lines. Table 2 summarizes the results of enzyme activity determinations in cell-free extracts prepared from wild-type F28-7,

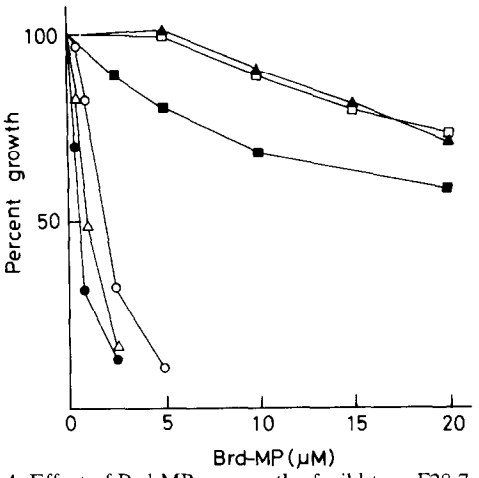

Fig. 4. Effect of Brd-MP on growth of wild-type F28-7 and drug-resistant cell lines. Cells (5×10^3) were cultured for 72 ± 2 hr in medium containing various concentrations of Brd-MP and counted as described in Materials and Methods. Key: F28-7 (○—○); cio^{r}3 (●—●); cio^{r}8 (△—△); Brd^{r}3 (▲—▲); Brd^{r}4 (□—□); and CB7 (■—■).

Brd^r, and Tub^r cell lines, and other double or triple mutants. It is quite evident that all the five Brd^r mutants had AK activities as low as 3% of the activity found in wild-type cells. This was also the case for Tub^{r}4 and Tub^{r}5 cells. Moreover, BT4, CB7, and CBT4 mutants were all devoid of AK activity. In contrast, all these lines, except for cio^{r}8 and CBT4, had APRT activities comparable to wild-type levels. Mixing experiments with wild-type and Brd^r or Tub^r cell extracts ruled out the presence of a soluble inhibitor(s) influencing AK activity.

Together with the results shown in Figs. 2 and 3, the data demonstrate that the resistance mechanism for bredinin cytotoxicity is due to AK deficiency.

Cytotoxicity of exogenous Brd-MP. Since AK transfers a phosphate moiety of ATP to the 5′-position of adenosine sugar [13], bredinin may be converted into Brd-MP in sensitive cells. Brd-MP was chemically synthesized and reported to be 2-fold as active as bredinin in suppressing the growth of L5178Y cells in culture [14]. Organic phosphate compounds are generally known to penetrate the cell membrane very poorly. Therefore, we studied whether exogenous Brd-MP affected the growth of wild-type and drug-resistant cell lines. As shown in Fig. 4, growth of wild-type and APRT$^-$ cio^{r}3 and cio^{r}8 cells was suppressed by the nucleotide at an EC_{50} of 0.6 to 2 μM. These values were a little higher than the value of bredinin observed to be toxic to wild-type cells [9], confirming the data reported by Mizuno and Miyazaki [14]. However, it should be noticed that Brd^{r}3, Brd^{r}4, and CB7, all lacking AK activity (Table 2), were much less sensitive to Brd-MP; i.e. the EC_{50} values for them were over 20 μM.

Mechanism of bredinin cytotoxicity. Now we have much evidence indicating that the toxic metabolite of bredinin is Brd-MP. This nucleotide was suggested to suppress the biosynthesis of guanine nucleotides by inhibiting IMP dehydrogenase [8]. We examined the reversal by purine bases of bredinin effects on the growth of wild-type and F5 cells. In cultured mammalian cells, hypoxanthine and guanine are easily phosphoribosylated by HGPRT to the corresponding 5′-monophosphates, IMP and GMP, while xanthine is only very poorly phosphoribosylated by the enzyme [15] (Step 8, Fig. 1). However, F5 cells are able to utilize xanthine for growth, because they are producing active *E. coli* XGPRT that is capable of converting the base to XMP efficiently [15, 16].

Table 3 indicates that only guanine at 25–50 μM was growth-inhibitory for both cell lines. The reason for this will be studied later. It is evident that 2 μM bredinin almost completely inhibited the growth of wild-type cells and that this inhibition was not

Table 3. Reversal of bredinin cytotoxicity by purine bases in wild-type F28-7 and F5 cell lines*

Addition		Cell growth† (%)	
		Cell line	
Drug	Purine base	F28-7	F5
None	None	100	100
	Adenine (50)‡	95	92
	Hypoxanthine (50)	100	95
	Guanine (25)	86	63
	Guanine (50)	65	32
	Xanthine (50)	98	101
Bredinin (2)	None	5.0	5.5
	Adenine (50)	3.7	5.2
	Hypoxanthine (50)	3.9	5.8
	Guanine (25)	68	31
	Guanine (50)	34	10
	Xanthine (50)	5.5	52

* Cells (5×10^3) were cultured for 72 ± 2 hr in medium containing bredinin, or purine bases, or both, and counted as described in Materials and Methods. Less than 10% growth means almost complete cell death accompanying vigorous degradation and lysis, as checked by phase-contrast microscopy.

† Average of two to four determinations, each in duplicate.

‡ Figures in parentheses are the concentrations of chemicals (μM).

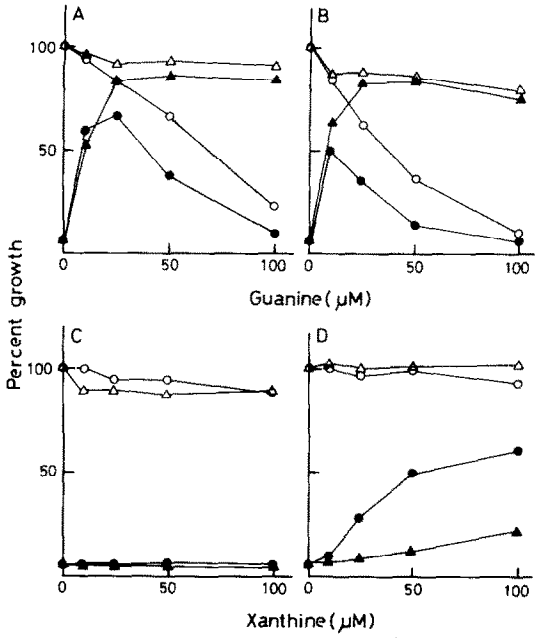

Fig. 5. Reversal of bredinin cytotoxicity by varying concentrations of guanine and xanthine in the presence or absence of adenine. The growth inhibitory effects of 2 μM bredinin on wild-type F28-7 cells (A and C) and F5 cells (B and D) were reversed by varying amounts of guanine or xanthine in combination with, or without, 50 μM adenine, as described in the legend to Table 3. Key: guanine (or xanthine) alone (○—○); guanine (or xanthine) + bredinin (●—●); guanine (or xanthine) + adenine (△—△); and guanine (or xanthine) + adenine + bredinin (▲—▲).

reversed by 25 times higher amounts (50 μM) of either adenine, hypoxanthine, or xanthine, but was greatly reversed by the same or one-half the amount of guanine. These results agree with those reported by others [2, 6, 7] and support the inhibition of conversion of IMP to GMP as a mechanism of bredinin action. On the other hand, in F5 cells, the bredinin effect was not only reversed by guanine but also by xanthine to a rather greater extent, while neither adenine nor hypoxanthine was active as reversing agents, showing that XMP produced from the exogenous xanthine by the activity of *E. coli* XGPRT in the F5 cells sufficiently prevented the growth-inhibitory action of bredinin.

In wild-type and APRT$^-$, cio^r cells, reversal was also seen with externally applied guanosine and GMP, while in HGPRT$^-$ mutants such 25 FC-1 and FG*hpt*$^-$ lines, neither guanine nor its derivatives were effective in preventing growth inhibition by the antibiotic (data not shown). This indicates that guanine itself or guanine derived from guanosine or GMP becomes active after being converted to GMP by HGPRT within the cells.

In Table 3, guanine is seen to be significantly growth-inhibitory for wild-type cells and even more markedly so for F5 cells. As a result, the reversal effect of guanine was not complete or very poorly so. In cultured mammalian cells, guanine is metabolized by HGPRT to GMP, which is thought to be one of the endogenous purine nucleotides to exert feedback inhibition on 5-phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14), the first enzyme in the pathway of purine biosynthesis [17, 18]. In addition, GMP is not efficiently converted to adenine nucleotides by the cells due to a deficiency in GMP dehydrogenase [19]. Therefore, addition of guanine may lead to a depletion of cellular adenine nucleotide pools and interfere with cell growth.

We tested the reversal effects of varying concentrations of guanine and xanthine either in the presence or absence of adenine. These data are illustrated in Fig. 5. Similar to the results shown in Table 3, guanine inhibited the growth of wild-type F28-7 cells with increasing concentration (Fig. 5A). The severe growth-inhibitory effect of 2 μM bredinin was reversed most effectively by simultaneous addition of 25 μM guanine. Inclusion of adenine at 50 μM not only suppressed the toxicity of guanine itself, but almost completely prevented the bredinin effects in combination with more than 25 μM guanine. Since adenine itself was inactive as a reversing agent (Table 3), it is likely that the base compensated for a deficiency of the adenine nucleotide pool caused by external guanine. Figure 5C shows xanthine to be totally ineffective under the same assay conditions.

These data are seen more clearly in F5 cells. Like wild-type cells, guanine alone exhibited a still larger inhibitory effect on F5 cells, but this effect was reversed by 50 μM adenine (Fig. 5B). Furthermore, adenine, added to the medium along with over 25 μM guanine, fully reversed the bredinin toxicity. Xanthine itself had no effect on F5 cells in combination with, or without, adenine (Fig. 5D). It is clear that xanthine was able to reverse the bredinin effect in a dose-dependent manner. Unexpectedly, however, this reversal was greatly suppressed by addition of adenine. The reason is not known, but *E. coli* XGPRT in F5 cells might be inhibited by adenine or its metabolite(s), so that the rate of conversion from xanthine to XMP might be reduced.

These lines of evidence obtained by the reversal studies with F5 cells strongly suggest that the site of action of bredinin is IMP dehydrogenase.

DISCUSSION

Bredinin-resistant mutants were isolated in a single-step procedure from mouse mammary carcinoma FM3A cells mutagenized with MNNG. Such mutants were 15- to 19-fold more resistant to the antibiotic than wild-type cells (Fig. 2) and displayed this resistance under prolonged cultivation. The mutants were found to be cross-resistant to tubercidin; conversely, Tub^r cells were cross-resistant to bredinin (Fig. 2). Tubercidin-resistant CHO cells have been shown to be defective in AK activity [20, 21]. Comparison of the ability to incorporate [^{14}C]adenosine between wild-type, Brd^r, or other double and triple mutant cell lines showed that the bredinin resistance was associated with loss or reduction of the precursor uptake into the acid-precipitable cell fraction (Fig. 3). Subsequent enzyme assays clearly demonstrated all the five Brd^r mutants isolated independently to be defective in AK activity (Table 2). It is therefore evident that the mechanism for bredinin resistance is attributed to AK deficiency.

This means that bredinin is activated by being phosphorylated by the action of AK to the toxic metabolite, Brd-MP, within bredinin-sensitive cells. Bredinin has a structure similar to 5-amino-1-β-ribofuranosyl-imidazole-4-carboxamide (AICAR), the precursor of purine biosynthesis [2], but the antibiotic seems to be recognized by the enzyme as an analog of adenosine.

MNNG treatment markedly induced mutations to bredinin resistance in our wild-type mouse cells, while no resistant colonies occurred in cells not treated with the mutagen (Table 1). This may indicate an extremely low probability of spontaneous events which lead to loss of AK activity, probably because two copies of the enzyme gene residing on autosome 10 [22] may normally be functioning in our mouse cells.

As described by Mizuno and Miyazaki [14] in mouse L5178Y cells, exogenous Brd-MP was almost as toxic to our wild-type and APRT$^-$ cells as was bredinin (Fig. 4). These observations may be rather unexpected, since organic phosphate compounds pass very poorly through the cell membrane. However, our findings that AK-deficient, Brd^r mutants were much more resistant to Brd-MP than both wild-type and APRT$^-$ cells strongly suggest the following mechanism for Brd-MP toxicity: (1) exogenous Brd-MP is hydrolyzed to bredinin and inorganic phosphate nonenzymatically or by some enzyme such as 5′-nucleotidase or alkaline phosphatase which is known to be located on the plasma membrane [23, 24]; and (2) the resulting bredinin enters the cells, is rephosphorylated by AK, and exerts its toxic effects on the cells. This unstability could be the reason that Brd-MP has not yet been identified in L5178Y cells cultured with [^{14}C]bredinin [7] or in serum and urine of rats given orally radioactive CIO [6]. These findings with Brd-MP effects may also support the involvement of AK in activation of bredinin.

With respect to the molecular mechanism of bredinin cytotoxicity, Sakaguchi *et al.* [2] suggested, from reversal studies of the bredinin effect with natural purine bases, that bredinin itself blocked the purine biosynthetic step from IMP to GMP, in which IMP dehydrogenase and GMP synthetase are involved (Fig. 1). However, our previous and present studies and enzymatic studies done by Fukui *et al.* [8] provide evidence that Brd-MP, but not bredinin, is a toxic metabolite to suppress the above step. They [8] found this nucleotide to inhibit competitively IMP dehydrogenase with a very low K_i value using cell-free extracts prepared from Ehrlich carcinoma and suggested that this inhibition was the mechanism for bredinin action. This indicates that Brd-MP has a chemical structure analogous to IMP, the substrate for IMP dehydrogenase. Since Brd-MP also resembles GMP, it might also inhibit GMP synthetase. However, our present data on the successful reversal of bredinin effects by xanthine seen in F5 cells producing *E. coli* XGPRT (Table 3 and Fig. 5) show that IMP dehydrogenase is, in fact, a target for Brd-MP in bredinin-sensitive cells.

Quite recently, Kusumi *et al.* [25] reported that Brd-MP inhibited competitively both IMP dehydrogenase and GMP synthetase from rat liver, but that the K_i value for the dehydrogenase was three orders of magnitude less than that for the synthetase. Clearly, our data and data reported by Fukui *et al.* [8] and Kusumi *et al.* [25] are complementary. It is therefore concluded that Brd-MP formed following exposure of cells to bredinin and to its aglycone CIO by the action of AK and APRT, respectively, exhibits its cytotoxic effects by inhibiting IMP dehydrogenase.

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