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Metabolism and action of benzamide riboside in Chinese hamster ovary cells

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Benzamide riboside (3-(1-deoxy-β-D-ribofuranosyl)benzamide, BR) a new analog of nicotinamide riboside, is toxic to Chinese hamster ovary cells and inhibits guanine nucleotide synthesis in a manner comparable to that of tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide). Adenosine kinase deficient cells demonstrate slight resistance but retain the ability to form the NAD analog, benzamide adenine dinucleotide (BAD). HPLC analysis of BAD containing cells is described. A BR resistant cell line was isolated that demonstrates cross-resistance to both tiazofurin and 6-aminonicotinamide, suggesting a common metabolic step; enzymatic analysis indicates reduced levels of NAD pyrophosphorylase in these cells. BR toxicity was only partially reversed or prevented by the presence of guanosine, suggesting either that BR inhibits guanine salvage to some extent or, more probably, that BR can, at high concentration, inhibit cell growth by another mechanism in addition to inhibition of guanine nucleotide synthesis. Cells incubated with BR for several hours retain the ability to salvage exogenously provided guanosine. The demonstration that BAD can be phosphorylated by NAD kinase, presumably to form BADP, suggests that this metabolite may be formed in cells and may have inhibitory activity at high concentrations of BR.

Key words: Benzamide riboside, guanine nucleotides, NAD kinase, nicotinamide riboside, tiazofurin.

Introduction

In recent years there has been renewed interest in metabolic pathways associated with NAD stemming from observations linking NAD derivatives with DNA repair[1–3] and, more recently, the identification of cyclic ADP-ribose as a second messenger.[4–6] The recent synthesis[7] of benzamide riboside (BR), a C-glycosidic nicotinamide riboside analog, has provided another potential tool for probing the metabolic intricacies of NAD, its precursors and its derivatives.

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Originally prepared in a series as a potential inhibitor of poly(ADP-ribosy)lation, BR demonstrated potent toxicity toward S49.1 lymphoma cells but appeared not to be selectively inhibitory to poly (ADP-ribose) synthetase. It has more recently been suggested[8] to have toxic properties similar to tiazofurin; the latter is a thiazole C-nucleoside that is metabolized to an analog of NAD and, as such, is a potent inhibitor of IMP dehydrogenase.[9] The recent work of Gharebaghi *et al.*[10,11] confirmed the previous indications that BR inhibits guanylate synthesis and that it does so by formation of an adenine dinucleotide derivative that inhibits IMP dehydrogenase. This communication addresses the metabolism and inhibitory activity of BR in Chinese hamster ovary (CHO) cells and various mutant cell lines derived from them, and describes the formation of an additional metabolite that appears to be the phosphorylated form of the adenine dinucleotide derivative.

Methods

Chemicals

BR was synthesized as described elsewhere.[7] Tiazofurin was provided by the Drug Synthesis and Evaluation Branch of the National Cancer Institute. 6-Aminonicotinamide was purchased from Sigma (St Louis, MO). [2-^{3}H]Adenine (27.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), [carbonyl-^{14}C]nicotinamide (56 mCi/mmol) from ICN Radiochemicals (Irvine, CA) and [8-^{14}C]adenine (50 mCi/mmol) from Amersham (Arlington Heights, IL).

Cell lines and growth conditions

The CHO cell line was as described previously.[12] Cell lines Rb^R-1 (deficient in adenosine kinase) and

RAT (deficient in adenosine kinase, adenine phosphoribosyltransferase and hypoxanthine, guanine phosphoribosyltransferase) were derived from the parent CHO line and have been described elsewhere.[12] All cell lines were carried in McCoy's 5a medium supplemented with 10% fetal bovine serum as well as penicillin and streptomycin. Medium supplemented with exhaustively dialyzed serum was used for all experiments.

Determination of minimum inhibitory drug concentration (MIC)

The MIC was determined as described previously[12] by allowing cells to form clones in the presence of a series of drug concentrations for 7 days. The MIC is the lowest tested concentration of drug in which clones contain fewer than 50 cells. In experiments to determine the abilities of various agents to reverse or prevent growth inhibition by BR, the agents were added at the same time as drug. Observed effects could therefore reflect either reversal of toxicity or its prevention.

Induction and selection of BR resistant cell lines

CHO cells were treated with MNNG by conventional procedures, after which the mutagen was removed, the cells washed and allowed to grow with splitting when necessary, for 7 days. The cultures were then detached with trypsin and dispensed into T-75 flasks, having one peel-back side, in medium containing dialyzed serum and 100 μM BR. Growth was allowed for 10 days after which the resistant clones were selectively trypsinized with the aid of glass cylinders and transferred to clean flasks and allowed to grow in the absence of drug in medium containing non-dialyzed serum. The resistant phenotype was subsequently verified by challenge with varying concentrations of BR.

Metabolic experiments

These were carried out as previously described.[12] Briefly, cells were dispensed into T-25 flasks, 3×10^6 per flask, and incubated overnight. The media were then aspirated and replaced with fresh medium supplemented with dialyzed serum and the desired additions. After 5–6 h incubation the media were removed and the cell monolayers were washed. Cells were extracted *in situ* with cold 0.4 N perchloric acid. The extracts were neutralized with KOH and, after removal of precipitate by centrifugation, analyzed by HPLC. The cell number was determined in duplicate culture as previously described.[12]

Fractionation of metabolites by HPLC

Nucleotides were fractionated on a Partisil-10 SAX column as previously described.[12] NAD, BAD and related compounds were separated on a 300 × 3.9 mm I.D. Resolve C_{18} reversed-phase column using a concave gradient from 100% 0.1 M $(NH_4)_3PO_4$, pH 5.1, to 30% of 25% methanol.[13] Metabolites were detected by their absorbance at 254 nm.

Enzymatic phosphorylation of BAD

BAD was purified by reversed-phase HPLC (described above) from perchloric acid extracts of CHO cells that had been treated with BR for 22 h. The peak at 33 min was collected from several runs, pooled and the BAD removed from the high salt solvent by adsorption onto activated charcoal with subsequent removal with ammoniacal ethanol (47.5% ethanol, 5% concentrated NH_4OH). After lyophilization the residue was dissolved in water and used directly. For preparation of [^{3}H]BAD, cells were incubated for 4–8 h with 150 μM BR and 25 μCi/ml [2-^{3}H]adenine followed by purification of the metabolite as described above.

Phosphorylation was effected with commercially purified NAD^+ kinase. Incubation mixtures contained 10 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 4 mM ATP, 50 μM NAD or BAD and 1 unit of NAD^+ kinase in a final volume of 100 μl. After 30 min incubation the reaction was terminated by the addition of 100 μl of perchloric acid. After neutralization with KOH and removal of the precipitate by centrifugation, aliquots of the supernatant were analyzed by HPLC as described above.

Determination of NAD pyrophosphorylase activity

Extracts of CHO and BR-1 cells were prepared and assayed for NAD pyrophosphorylase activity according to the procedures described by Paulik *et al.*[14]

Results

BR demonstrates toxicity to CHO cells that is essentially comparable to that of tiazofurin and 6-aminonicotinamide (Table 1). Since the activity of nucleoside analogs is dependent upon phosphorylation to the monophosphate derivative, the activity of BR was tested toward the adenosine kinase deficient cell line, Rb^R-1, and compared with the activity of tiazofurin which has been shown[12] to reflect phosphorylation by adenosine kinase as well as by two other cellular enzymes. Line Rb^R-1 is highly resistant to the adenosine analog ribaverin[15] but demonstrates no resistance to tiazofurin and only slight (5-fold) resistance to BR. The RAT cell line, which is deficient in not only adenosine kinase but HGPRT and APRT as well, shows increased sensitivity to both tiazofurin and BR, which most likely reflects the inability of these cells to salvage guanine, adenine and adenosine. Drug resistant cell lines can be very useful in clarifying metabolic routes and mechanisms of action. With this in mind a BR resistant clone of CHO cells was selected after mutagenesis with MNNG. This cell line, BR-1, was selected for resistance to 100 μM BR and demonstrates substantial resistance compared to the parent CHO cells (Table 1). In addition it demonstrated cross-resistance to both tiazofurin and 6-aminonicotinamide, suggesting a common metabolic pathway. Cell lines selected for resistance to tiazofurin that have reduced levels of NAD pyrophosphorylase demonstrate cross-resistance to both BR and 6-aminonicotinamide.[11,16] A comparison of the NAD pyrophosphorylase activities of the CHO and BR-1 cell lines was consistent with this; the specific activity of this enzyme in CHO cells was 16 nmol/h/mg protein while that of the BR-1 cells was 0.42 nmol/h/mg.

Attempts to reverse or prevent BR induced toxicity with natural cell components (Table 2) showed a partial reversal (or prevention) by nicotinamide riboside, suggesting competition at some level; however, it did not appear to be dose dependent. This may reflect activation (phosphorylation) of the drug by more than one enzyme, which is probably the case since the adenosine kinase deficient cell line (Table 1) demonstrated partial resistance to BR. Nicotinamide, when added in addition to that already in the medium (4.1 μM, McCoy's 5a medium), had no demonstrable growth inhibition reversal or prevention properties. A partial reversal (or prevention) was accomplished with guanosine.

If BR is metabolized by these cells to form an analog of NAD it should be possible to detect such a metabolite by the same HPLC methodology used for the analysis of the tiazofurin metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD).[13] Figure 1 shows the HPLC profile of perchloric acid extracts of CHO cells treated (top) and untreated (bottom) with 25 μM BR. There is clearly an additional UV-absorbing peak in the drug treated cells eluting at a time that would be consistent with BAD. The amount of metabolite formed is both concentration and time dependent, and the UV absorbance spectrum is characteristic of an adenine dinucleotide derivative. Further evidence for the identity of

Table 2. Reversal of BR toxicity to CHO cells

Additions	Concentration (μM)	MIC (μM) of BR
None	—	10
Nicotinamide riboside	30	50
	300	50
Nicotinamide	30	10
	300	10
Guanine	50	10
	100	10

MICs were determined as described for Table 1 with indicated additions.

Table 1. Cross-resistance properties of mutant cell lines

		MIC (μM)			
Cell line	Deficiency	ribavirin	BR	TR	6-AN
CHO (parent)	none	50	10	5	10
Rb^R-1	AK	>500	50	5	ND
RAT	AK, HGPRT APRT	>500	5	1	ND
BR^R-1	ND	ND	250	250	100

The MICs were determined by plating cells in the presence of drug as described in Methods. AK, adenosine kinase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; ND, not determined.

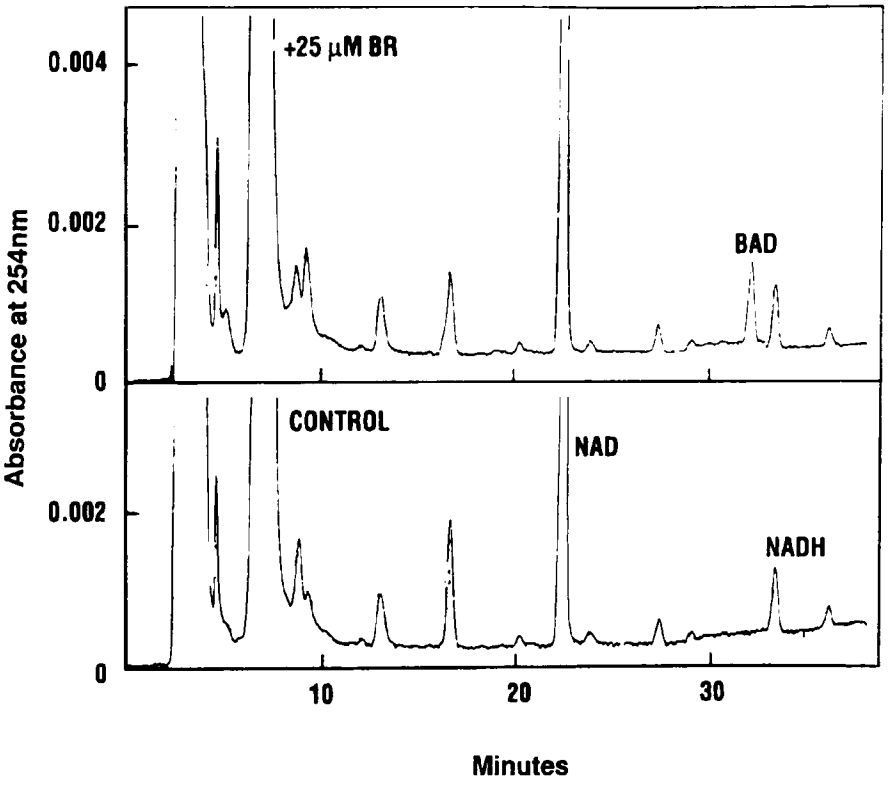

Figure 1. Formation of a putative metabolite of BR in CHO cells. CHO cells, 4×10^6 per T-75 flask, were incubated 5 h in the presence (top) or absence (bottom) of 25 μM BR. The cells were then washed and extracted *in situ* with 0.4 N perchloric acid. The extract was neutralized with KOH as described in Methods followed by HPLC analysis as described. The profiles represent 5×10^5 cell equivalents.

Table 3. Incorporation of labeled precursors into cellular adenine dinucleotide derivatives

Additions	d.p.m./10^6 cells	
	NAD	BAD
[^{14}C]Adenine	17459	242
[^{14}C]Adenine + 100 μM BR	9745	3452
[^{14}C]Nicotinamide	18678	98
[^{14}C]Nicotinamide + 100 μM BR	12591	148
	12591	148

Cultures were incubated with the indicated precursors and extracted as described in Methods for metabolic experiments. Guanosine was added to the cultures either at the beginning of the incubation or after 4 h. Total incubation time was 5 h. Cell extracts were analyzed by HPLC as described; fractions in which the NAD and BAD peaks elute were collected and counted.

Table 4. Inhibition of BAD formation by nicotinamide ribonucleoside (NR)

Additions	pmol BAD formed/10^6 cells
50 μM BR	158
50 μM BR + 50 μM nicotinamide	149
50 μM BR + 50 μM NR	82

CHO cells were incubated 5 h with the indicated additions followed by extraction with perchloric acid as described in Methods. Analysis was by HPLC as described. The data are representative of three experiments that were carried out.

this compound as an adenine dinucleotide was obtained by following the incorporation of label from [^{14}C]adenine and [^{14}C]nicotinamide into metabolites in the presence and absence of BR (Table 3). The putative BAD peak was efficiently labeled by the adenine but not by the nicotinamide. The presence of NR in the medium during exposure of cells to BR substantially reduced formation of the metabolite (Table 4). This could result either from competition for phosphorylation of the derivative or at the level of NAD pyrophosphorylase.

The formation of an NAD analog from BR could result in inhibition by a variety of mechanisms, one being inhibition of IMP dehydrogenase as earlier shown for the tiazofurin metabolite, TAD. If IMP dehydrogenase is, in fact, inhibited by BAD there should be a dramatic decrease in the cellular GTP concentration after treatment of cells with BR. The data of Table 5 indicate that this was indeed the

Table 5. Effect of BR on GTP pools in CHO and Rb^R-1 (adenosine kinase deficient) cells

Cell line	Additions (100 μM)	pmol/10^6 cells			
		NAD	TAD	BAD[a]	GTP
CHO	none	1654	—	—	819
	tiazofurin	1442	186	—	159
	BR	1094	—	536	137
Rb^R-1	none	1264	—	—	976
	tiazofurin	680	282	—	132
	BR	648	—	254	144

Cells were incubated with the indicated additions for 5 h and extractd with 0.4 N perchloric acid as described in Methods. NAD, TAD and BAD were analyzed by reverse-phase HPLC; GTP concentrations were determined by anion exchange HPLC as described.
[a]BAD concentrations were calculated assuming the same extinction coefficient as NAD.

result of treatment of both CHO and Rb^R-1 cells with BR. Although the amount of accumulated BAD was significantly lower in the adenosine kinase deficient cells, it was still sufficient to interfere with GTP synthesis to essentially the same extent as in the CHO cells. Both tiazofurin and BR appeared to have some effect on cellular NAD levels; in both cell lines the NAD concentrations were significantly depressed after exposure to drug compared with the control levels.

The inability of guanosine to completely reverse the inhibition produced by BR suggests either that salvage of guanosine is inhibited by BR or that there is another toxic mechanism that occurs at the higher concentrations of drug. Analysis of GTP pools after incubation with BR in the presence and absence of 50 μM guanosine, added either at the beginning of incubation with drug or after 4 h (Table 6), showed dramatic expansion of the cellular GTP pool. This

Table 6. Effect of BR on the GTP pool in CHO cells in the presence of 50 μM guanosine

Initial Additions (50 μM)	Additions after 4 h (50 μM)	pmol/10^6 cells	
		BAD[a]	GTP (%)
—	—	—	784 (100)
BR	—	416	166 (21)
BR, Guo	—	422	2072 (264)
BR	Guo	644	1692 (216)
Guo	—	—	2614 (333)
—	Guo	—	1750 (223)

Cells were incubated with the indicated additions for 5 h and extracted with 0.4 N perchloric acid as described in Methods. BAD was analyzed by reverse phase HPLC; GTP was determined by anion exchange HPLC as described.
[a]BAD concentrations were calculated assuming the same extinction coefficient as NAD.

allows the conclusion that BR does not substantially affect the guanosine salvage pathway.

It is quite possible that BAD affects cellular oxidative reactions other than IMP dehydrogenase; the formation of the phosphorylated derivative of BAD, BADP, could be another source of cytotoxic activity. If BADP were formed in the cell it would most likely be at a concentration so low that it might be difficult to detect and analyze by HPLC techniques. As an alternative to this the ability of commercially purified NAD^+ kinase to phosphorylate BAD was determined (Figure 2). HPLC analysis of these reaction mixtures clearly showed the formation of an additional UV absorbing peak (Figure 2E) in the region in which a phoshorylated derivative would be expected, based on the behavior of NADP (Figure 2A–C). This observation was subsequently confirmed using BAD (isolated from a cell extract as described in Methods) containing a tritium label in the adenine moiety (not shown).

Discussion

The metabolism and toxic mechanisms of BR appear, in many ways, to be similar to those of tiazofurin, as predicted earlier by Jayaram *et al.*[8] Our early observation that the RAT cell line which is deficient in adenosine kinase, HGPRT and APRT is significantly more sensitive to both tiazofurin and BR is suggestive that these agents have the common property of interfering with guanylate synthesis. Both agents thus appear to be converted, presumably via phosphorylation (by more than one enzyme) to the monophosphate derivative followed by condensation with ATP catalyzed by NAD pyrophosphorylase. The resulting compounds, TAD and BAD, are highly effective inhibitors of IMP dehydrogenase resulting in depletion of the cellular GTP pool. The inhibition of CHO cells by tiazofurin can be reversed (or prevented) by the presence of guanine or guanosine.[17] Inhibition by BR can also be reversed to some extent by guanine and guanosine, however, relatively high concentrations are required. If the reversal or prevention reflected satisfaction of a requirement created by inhibition of guanylate biosynthesis relatively low concentrations should suffice. This observation suggests that BR is either able to interfere with salvage of guanosine or that it has another mechanism by which it inhibits cell growth. Since we have demonstrated that the presence of BR in the medium does not interfere with utilization of guanosine for guanine nucleotide synthesis it seems most probable that

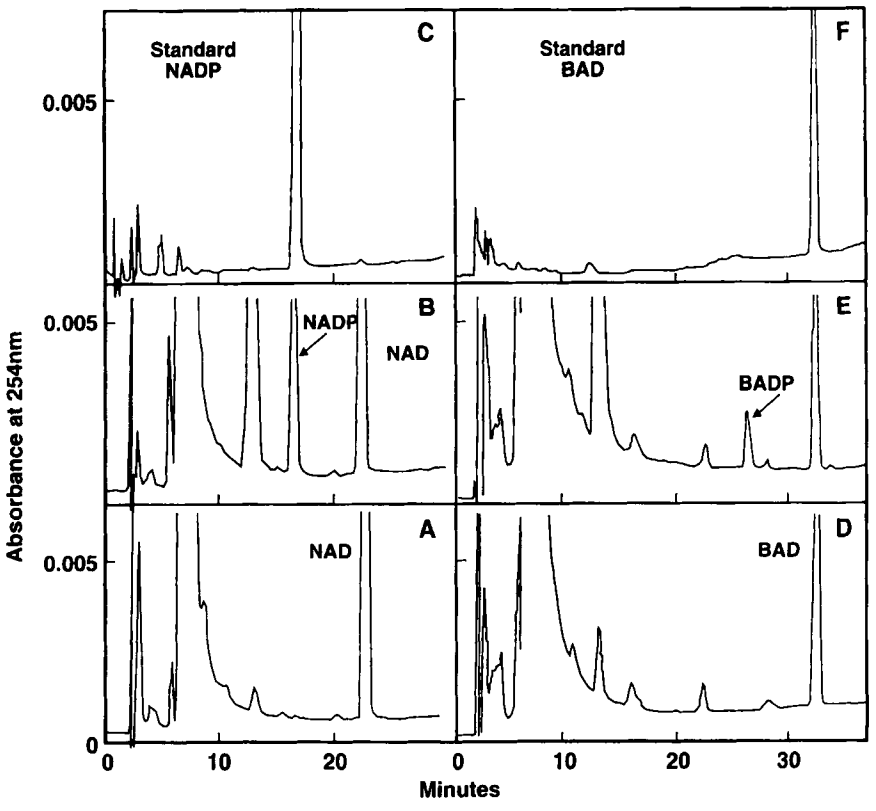

Figure 2. Phosphorylation of BAD by NAD^+ kinase; HPLC profiles of incubation mixtures. Isolation of BAD and incubation mixtures for the phosphorylation reaction are described in Methods. Graphs A, B and C show control reactions with NAD as substrate; graph A (reaction mixture without NAD^+ kinase), B (complete reaction mixture) and C (standard NADP). Graphs D, E and F show reactions relating to phosphorylation of BAD; graph D (reaction mixture without enzyme), E (complete reaction mixture) and F (standard BAD). In each case 100 μl of the perchloric acid treated reaction mixture was analyzed by the described HPLC methodology.

BAD may be interfering with some other cellular reaction. The demonstration that BAD can be phosphorylated by NAD^+ kinase provides a basis for considering potential interactions of BADP in the inhibitory action of BR. It is not clear, however, whether BAD is phosphorylated to BADP in the cell. NADP is present in CHO cells at low levels that make HPLC analysis difficult; however, it may be possible to label BAD by incubation of cells with high specific activity adenine in the presence of BR and thus to possibly see a phosphorylated form of BAD. 6-Aminonicotinamide, for example, is metabolized to the adenine nucleotide derivative which is further phosphorylated to 6-ANADP.[18] The latter is a highly effective inhibitor of 6-phosphogluconate dehydrogenase. We have isolated several 6-AN resistant cell lines which we have tested for sensitivity to BR. None of them have demonstrated cross resistance.

In summary, observations in this communication not previously reported include the demonstration that a BR resistant cell line has reduced levels of NAD pyrophosphorylase rendering it unable to form significant quantities of BAD and the observation that BAD can be converted, by NAD kinase, to the phosphorylated derivative, BADP. Since NAD and its derivatives are important in many aspects of cell growth and metabolism additional studies of the mechanism(s) of BR action and metabolism could provide useful leads and perhaps tools to aid in our understanding of some of these phenomena.

References

1. Sato MS, Lindahl T. Role of poly(ADP-ribose) formation in DNA repair. *Nature* 1992; **336**: 356–8.
2. Shall S. ADP-Ribose in DNA repair: a new component of DNA excision repair. *Adv Radiat Biol* 1984; **11**: 1–69.
3. Althaus FR. Poly-ADP-ribosylation and chromatin organization, In: Althaus R, Richter C, eds. *ADP-ribosylation of proteins: molecular biology biochemistry and biophysics.* Berlin: Springer-Verlag 1987: 59–65.
4. Koshiyama H, Lee LC, Tashjian AH Jr. Novel mechanisms of intracellular calcium release in pituitary cells. *J Biol Chem* 1991; **266**: 16985–8.

5. Lee HC, Aarhus R. ADP-Ribosyl cyclase: an enzyme that cyclizes NAD^+ into a calcium mobilizing metabolite. *Cell Reg* 1991; **2**: 203–9.
6. Clapper DL, Walseth TF, Dargie PJ, *et al.* Pyridine nucleotide metabolites stimulate calcium release from sea urchin eggs microsomes desensitized to inositol trisphosphate. *J Biol Chem* 1987; **262**: 9561–8.
7. Krohn K, Heins H, Wielckens K. Synthesis and cytotoxicity of C-glycosidic nicotinamide riboside analogues. *J Med Chem* 1992; **35**: 511–7.
8. Jayaram HN, Gharehbaghi K, Jayaram NH, *et al.* Cytotoxicity of a new IMP dehydrogenase inhibitor, benzamide riboside, to human myelogenous leukemia K562 cells. *Biophys Res Commun* 1992; **186**: 1600–6.
9. Plunkett W, Saunders PP. Metabolism and action of purine nucleoside analogs. *Pharmacol Ther* 1991; **49**: 239–68.
10. Gharehbaghi K, Jayaram NJ, Reiser J, *et al.* Oncolytic activity of a new IMP dehydrogenase inhibitor, benzamide riboside, in human myelogenous leukemia K562 and in human colon carcinoma HT-29 cells. *Proc Am Ass Cancer Res* 1993; **34**: 370.
11. Gharehbaghi K, Paull KD, Kelley JA, *et al.* Cytotoxicity and characterization of an active metabolite of benzamide riboside, a novel inhibitor of IMP dehydrogenase. *Int J Cancer* 1994; **56**: 892–9.
12. Saunders PP, Spindler CD, Tan MT, *et al.* Tiazofurin is phosphorylated by three enzymes from Chinese hamster ovary cells. *Cancer Res* 1990; **50**: 5269–74.
13. Saunders PP, Alvarez E, Kantarjian HM. Determination of nicotinamide-adenine dinucleotide and thiazole-4-carboxamide-adenine dinucleotide in human leukocytes by reversed-phase high-performance chromatography. *J Chromatogr* 1992; **577**: 37–41.
14. Paulik E, Jayaram HN, Weber G. Determination of NAD pyrophosphorylase activity in biological samples. *Anal Biochem* 1991; **197**: 143–8.
15. Saunders PP, Lai MM. Nucleoside kinase activities of Chinese hamster ovary cells. *Biochim Biophys Acta* 1983; **761**: 135–41.
16. Arimilli S, Saunders PP. Characterization of CHO cell lines resistant to tiazofurin (TR) and 6-aminonicotinamide (6-AN). *Proc Am Ass Cancer Res* 1992; **33**: 465.
17. Saunders PP, Kuttan R, Lai MM, *et al.* Action of 2-β-D-ribofuranosylthiazole-4-carboxamide (tiazofurin) in Chinese hamster ovary and variant cell lines. *Mol Pharmacol* 1983; **23**: 534–9.
18. Herken H, Lange K, Kolbe H. Brain disorders induced by pharmacological blockade of the pentose phosphate pathway. *Biochem Biophys Res Commun* 1969; **36**: 93–100.

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