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COMPARISON OF BIOCHEMICAL PARAMETERS OF BENZAMIDE RIBOSIDE, A NEW INHIBITOR OF IMP DEHYDROGENASE, WITH TIAZOFURIN AND SELENAZOFURIN

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Abstract—The biochemical and cytotoxic activities of the IMP dehydrogenase (IMPDH) inhibitors benzamide riboside, tiazofurin, and selenazofurin were compared. These three C-nucleosides exert their cytotoxicity by forming an analogue of NAD, wherein nicotinamide is replaced by the C-nucleoside base. The antiproliferative activities of these three agents were compared in a panel of 60 human cancer cell lines. To examine the relationship of benzamide riboside and selenazofurin to tiazofurin, COMPARE computer analysis was performed, and correlation coefficients of 0.761 and 0.815 were obtained for benzamide riboside and selenazofurin, respectively. The biochemical activities of these agents were examined in human myelogenous leukemia K562 cells. Incubation of K562 cells for 4 hr with 10 μM each of benzamide riboside, selenazofurin and tiazofurin resulted in a 49, 71, and 26% decrease in IMPDH activity with a concurrent increase in intracellular IMP pools. As a consequence of IMPDH inhibition, GTP and dGTP concentrations were curtailed. These studies demonstrated that selenazofurin was the most potent of the three agents. To compare the cellular synthesis of NAD analogues of these agents, K562 cells were incubated with 10 μM each of benzamide riboside, tiazofurin and selenazofurin after prelabeling the cells with [2, 8-^{3}H]adenosine. The results demonstrated that benzamide riboside produced 2- and 3-fold more of NAD analogue (BAD) than tiazofurin and selenazofurin did. To elucidate the effects of the three compounds on other NAD-utilizing enzymes, the inhibitory activities of purified benzamide adenine dinucleotide (BAD), thiazole-4-carboxamide adenine dinucleotide (TAD) and selenazole-4-carboxamide adenine dinucleotide (SAD) were studied in commercially available purified preparations of lactate dehydrogenase, glutamate dehydrogenase and malate dehydrogenase. TAD and SAD did not inhibit these three dehydrogenases. Although BAD did not influence lactate and glutamate dehydrogenases, it selectively inhibited 50% of malate dehydrogenase activity at a 3.2 μM concentration. These studies demonstrate similarities and differences in the biochemical actions of the three C-nucleosides, even though they share similar mechanisms of action.

Key words: benzamide riboside; IMP dehydrogenase inhibitor; selenazofurin; tiazofurin; malate dehydrogenase inhibitor; NAD analogues

The C-nucleosides benzamide riboside, tiazofurin, and selenazofurin have generated interest because of their antitumor activities against murine and mammalian tumor cells [1–6]. A considerable effort has been dedicated to the search for more active derivatives of tiazofurin; however, the structural requirements for the antitumor activity appear to be rather stringent [1]. Benzamide riboside, 3-(1-deoxy-β-D-ribofuranosyl)benzamide, was synthesized recently [7] and shown to be a potent inhibitor of IMPDH¶ activity [5]. These three C-nucleoside analogues exert their cytotoxic effects on the cells by inhibiting IMPDH activity leading to reduced synthesis of guanylates, since the effects of these agents are largely preventable by the exogenous addition of guanine or guanosine, both of which replenish the supply of guanylates. Tiazofurin is now in phase II clinical trials for treating patients with end-stage leukemia and produces good responses in patients with chronic myelogenous leukemia [8, 9]. Selenazofurin, an analogue of tiazofurin in which the sulfur moiety is replaced by selenium, displays antiviral and antitumor properties [2, 10], but has yet to reach the clinic.

As part of the screening program at the National

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¶ Abbreviations: BAD, benzamide adenine dinucleotide; GDH, L-glutamate dehydrogenase; IC_{50}, a concentration of an agent that produces a 50% reduction in cell proliferation; IMPDH, inosine 5′-monophosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; SAD, selenazole-4-carboxamide adenine dinucleotide; and TAD, thiazole-4-carboxamide adenine dinucleotide.

Cancer Institute, the three compounds were examined for their cytotoxicity against 60 different human tumor cell lines [11]. The data generated from the cytotoxicity studies were evaluated by a computer COMPARE analysis [12]. This program constructs pair-wise correlates of the cell line data for a new compound being examined with the corresponding information for other agents, thus providing clues to the *in vitro* biochemical mechanisms of action of a given new compound. In this report we present a comparative evaluation of correlation coefficients obtained with COMPARE analysis of the three aforementioned nucleosides.

The present study was initiated to elucidate similarities and differences between the three C-nucleoside derivatives in order to understand the reasons for their differential cytotoxicity.

MATERIALS AND METHODS

Materials. Benzamide riboside, tiazofurin and selenazofurin were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. [2, 8-^{3}H]IMP (specific radioactivity 20 mCi/mmol) was synthesized as reported [13]. BAD, TAD and SAD were synthesized as described previously [6, 14]. [2, 8-^{3}H]Adenosine (specific radioactivity 54 mCi/mmol) was procured from Moravek Biochemicals Inc., Brea, CA. Partisil 10-SAX columns and an HPLC apparatus consisting of an NEC computer (APC IV, Power Mate 2 mode), a 991 photodiode array detector, a 600E multisolvent delivery system, and a refrigerated 712-WISP autoinjector were purchased from the Millipore Corp., Bedford, MA. The Foxy fraction collector was from ISCO Inc., Lincoln, NE. Partisil PXS 10/25 was purchased from Fisher Scientific, Pittsburgh, PA. Purified LDH from hog muscle (EC 1.1.1.27; specific activity 450 U/mg protein), GDH from beef liver (EC 1.4.1.3; specific activity 120 U/mg protein) and MDH from pig heart mitochondria (EC 1.1.1.37; specific activity 1200 U/mg protein) were products of the Boehringer Mannheim Corp., Indianapolis, IN. All other chemicals of the highest purity were obtained from the Sigma Chemical Co., St. Louis, MO.

Cytotoxicity studies. K562 cells in the logarithmic phase of growth were used for all experiments. K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37° in an atmosphere of 95% air and 5% CO_2 [15]. The cytotoxicity of benzamide riboside, tiazofurin and selenazofurin in human cell lines was determined as described [11].

IMPDH activity. K562 cells were incubated with saline or with the agents (10 μM) for 2 hr; then cells were harvested by centrifugation, washed once with cold PBS and lysed in buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% NP-40 and 2 μg/mL aprotinin. The lysate was kept for 20 min on ice and centrifuged at 13,000 g for 20 min, and the supernatant was used as a source of enzyme. The enzyme activity was assayed according to the method cited in Ref. 13; briefly, 5-μL aliquots of 0.5 M KCl with 20 μM allopurinol were dispensed into the apex of Eppendorf tubes and dried at room temperature (25°). For the assay, in a total volume of 10 μL, Eppendorf tubes contained: 5 μL of the substrate mixture containing 286 μM [2, 8-^{3}H]IMP (200 μCi/mL) and 1 mM NAD, and the reaction was initiated by the addition of a 5-μL aliquot of enzyme extract. IMPDH activity was expressed as nanomoles of XMP formed per milligram protein per hour.

Analysis of ribonucleotides. Cells in culture were treated with saline or 10 μM each of benzamide riboside, tiazofurin or selenazofurin for 4 hr at 37°; cells were harvested by centrifugation and washed once with cold saline. Cells were extracted with 10% trichloroacetic acid and centrifuged for 0.5 min; the supernatant was immediately neutralized with 0.5 M tri-*n*-octylamine in freon. An aliquot of the neutralized extract was analyzed on HPLC using a Partisil 10-SAX column [16].

Determination of the concentration of intracellular deoxyribonucleoside triphosphates. To examine the effect of the agents on 2′-deoxyribonucleoside triphosphate levels, K562 cells were incubated with 10 μM each of the C-nucleosides for 4 hr, and the intracellular levels of the 2′-deoxyribonucleotides were determined by the cited methodology with minor modification [17]. Briefly, deoxyribonucleotides were separated on a Whatman Partisil PXS 10/25 column equilibrated with 5 mM ammonium phosphate buffer, pH 2.8, and developed with a linear gradient to 0.75 M ammonium phosphate buffer, pH 3.7, over 38 min at a flow rate of 2 mL/min and then maintained at 0.75 M ammonium phosphate for an additional 8 min. Under the conditions of assay, >95% of dCTP, dTTP, dATP and >90% of dGTP were recovered; the deoxyribonucleotides dCTP, dTTP, dATP and dGTP eluted at 29.9, 31.1, 34.4 and 40.3 min, respectively.

Cellular synthesis of the NAD analogues BAD, TAD and SAD. K562 cells (1×10^7 cells/10 mL) were incubated with [2, 8-^{3}H]adenosine (185 nmol, specific radioactivity 200 mCi/mmol) for 1 hr at 37°. Saline or 10 μM each of benzamide riboside, tiazofurin or selenazofurin was then added, incubated for a further 2 hr, and centrifuged at 400 g for 5 min. The cell pellet was washed once with PBS, homogenized in cold 10% trichloroacetic acid, neutralized with 0.5 M tri-*n*-octylamine in freon, and analyzed on HPLC as described above.

Analysis of malate, L-glutamate and lactate dehydrogenase activities. Rates of the three dehydrogenases were determined by measuring the decrease in absorbance at 340 nm in 10 mM Tris–HCl buffer, pH 9.5 [18]. Rates were calculated using a millimolar extinction coefficient of 6.22/cm for NADH. The MDH assay was performed in 1-mL cuvettes containing a 1-mM solution of oxaloacetic acid, various concentrations of NADH (15–125 μM), and 1 μg purified MDH. Reaction cuvettes for the assay of LDH contained 1 mM pyruvate, NADH (15–225 μM), and 50 ng/mL purified LDH. Reaction cuvettes for the assay of GDH contained 1 mM α-ketoglutaric acid, 1 mM ammonium chloride, NADH (25–225 μM), and 1 μg/mL purified GDH. Kinetic assays were conducted in triplicate, and results were plotted by the method of Lineweaver–Burk.

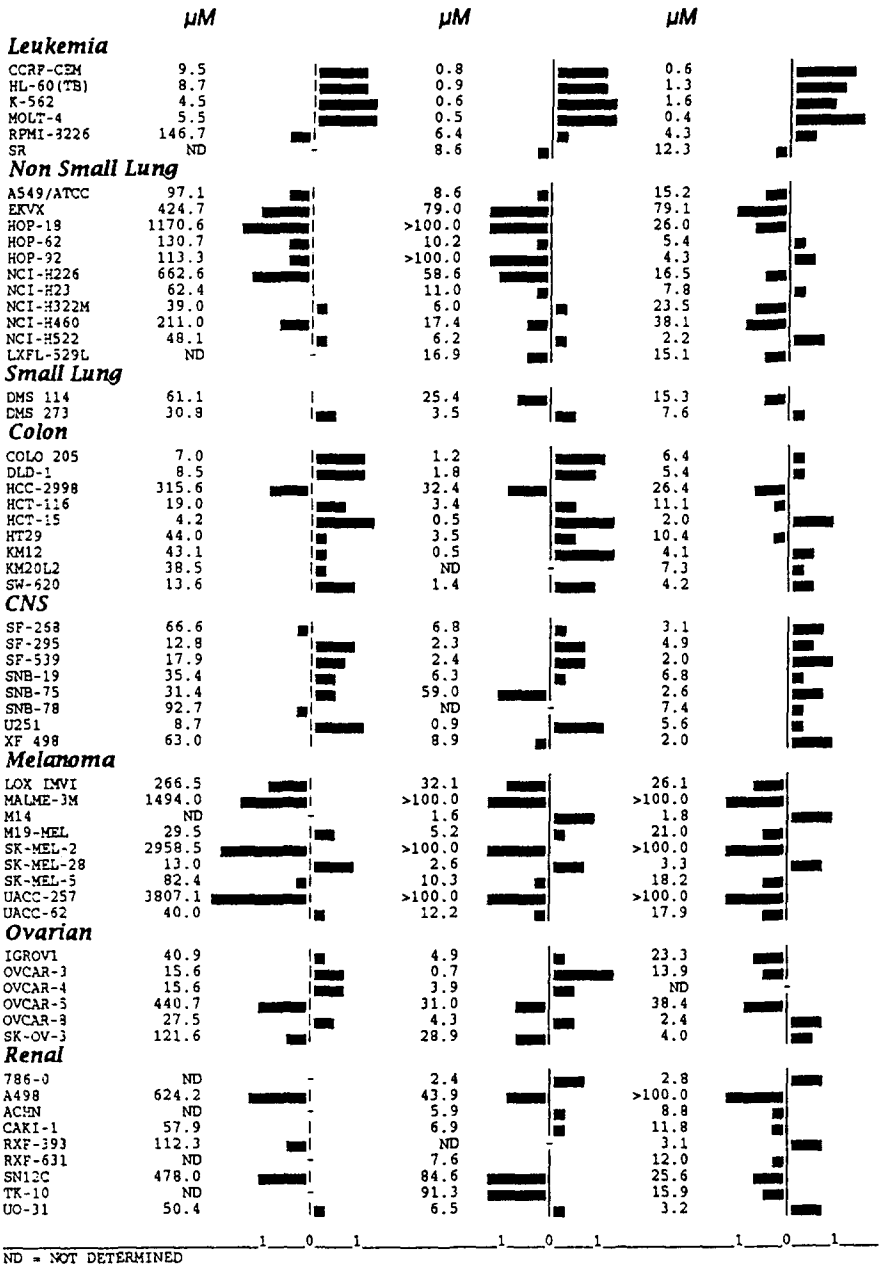

Fig. 1. Differential cytotoxicity of tiazofurin, selenazofurin and benzamide riboside against 60 different human cancer cell lines. These are depicted by a comparative mean graph as described [12]. Data in the form of $\log_{10}GI_{50}$ values for each cell line are averaged. The GI_{50} is the National Cancer Institute's notation for the concentration of an agent that causes a 50% inhibition of cell growth where the mean optical densities are used to estimate the number of cells in the test wells and the number of cells in the control wells and both are corrected for the mean optical densities at the time immediately preceding addition of the test agent. In other words, a GI_{50} is an IC_{50} corrected to time zero [11]. The $\log_{10}GI_{50}$ values for each cell line are subtracted from this mean value to compute the bar lengths for the mean graph. The sign convention adopted is such that cells having $\log_{10}GI_{50}$ values that are less than the mean (and therefore are more sensitive to the test drug than the average cell line) are graphed pointed toward the right from the mean line with a length proportional to the difference from the mean. Thus, in Fig. 1 the three agents are more potent to the leukemic cell lines CCRF-CEM, HL-60 (TB), K562, and Molt-4 than, for instance, the non-small lung cancer cell lines EKVX or HOP-18. The scales at the bottom of the graphs (_1_0_1_) are in units of $\log_{10}$ differences from the mean which is centered at _0_.

Table 1. Effect of benzamide riboside, selenazofurin and tiazofurin on IMPDH activity

Agents	IMPDH activity (nmol XMP formed/ hr/mg protein)	Inhibition (%)
Control	19.86 ± 1.03	0
Benzamide riboside	10.06 ± 0.6	49*
Selenazofurin	5.85 ± 0.58	71*
Tiazofurin	14.86 ± 0.23	26*

Logarithmically growing cells (1×10^7 cells/flask) were incubated with saline or 10 μM each of tiazofurin, benzamide riboside or selenazofurin for 2 hr, and enzyme activity was assayed as described in Materials and Methods. Data are means ± SD of triplicate samples.

* Significantly different from the control ($P < 0.05$).

RESULTS

Cytotoxicity of benzamide riboside, tiazofurin and selenazofurin to human tumor cells. Figure 1 is a comparative mean graph depicting the patterns of cytotoxicity of the three agents tiazofurin, selenazofurin, and benzamide riboside. These mean graphs were generated as described [12] from cytotoxicity data obtained by the drug screening program of the National Cancer Institute [11]. These cell lines were representative of leukemia, non-small cell lung cancer, small cell lung cancer, colon cancer, melanoma, ovarian cancer and renal cancer. Selenazofurin and benzamide riboside exhibited 3- to 10-fold greater cytotoxicity than tiazofurin to human leukemic cell lines. Although there was a difference in the effectiveness of the three agents on non-small cell lung cancer cell lines, no selectivity was discernible. Benzamide riboside and selenazofurin were more cytotoxic than tiazofurin to small cell lung cancer cell lines. In general, human colon, CNS and melanoma cells showed greater susceptibility to the action of selenazofurin or benzamide riboside than to that of tiazofurin, although CNS tumor cell lines were selectively sensitive to benzamide riboside ($IC_{50} < 10$ μM). Among the three compounds, selenazofurin was more cytotoxic to ovarian cancer cells. Overall, selenazofurin and benzamide riboside exhibited potent cytotoxicity compared to tiazofurin in these cell lines.

Comparison of the correlation coefficients of benzamide riboside, tiazofurin and selenazofurin in the COMPARE program. The three compounds were examined for their cytotoxicity against 60 different human tumor cell lines. The data generated from the cytotoxicity studies were evaluated by a computer COMPARE algorithm [12]. This program devises pair-wise correlates of the cell line data for a "seed" compound being examined with the corresponding information for other compounds, allowing the prediction of the *in vitro* biochemical mechanisms of action of the seed compound. When tiazofurin was used as the seed compound for the COMPARE analysis, the correlation coefficients for benzamide riboside and selenazofurin were 0.761 and 0.815. When tiazofurin and selenazofurin were used as seeds and the COMPARE analysis repeated, the correlation coefficient with benzamide riboside was 0.761 and 0.619, respectively. Thus, the overall patterns of relative potencies compared by algorithm suggests that all three agents might share similar biochemical mechanisms of action.

Effects of benzamide riboside, tiazofurin and selenazofurin on IMPDH activity. K562 cells were incubated with saline, or a 10 μM concentration of the three agents for 2 hr at 37°, prepared and assayed for IMPDH activity as detailed in Materials and Methods. Among the three compounds, selenazofurin was the most potent IMPDH inhibitor with 71% inhibition, compared with benzamide riboside and tiazofurin, which showed 49 and 26% inhibition (Table 1).

Effects of benzamide riboside, tiazofurin and selenazofurin on the concentration of ribonucleotides. K562 cells in culture were incubated with saline or with 10 μM each of the agents for 4 hr, and the pools of ribonucleotides were measured by HPLC (Table 2). The results showed that there was a decrease in GMP and GTP levels with a concurrent increase in IMP pools in treated cells. ATP concentrations were not perturbed.

Effects of benzamide riboside, tiazofurin and selenazofurin on the pools of 2'-deoxyribonucleoside triphosphates. K562 cells in culture were incubated

Table 2. Biochemical effects of benzamide riboside, selenazofurin and tiazofurin on the ribonucleotide levels in K562 cells

Nucleotides	Control (pmol/10^6cells)	Benzamide riboside	Selenazofurin	Tiazofurin
		(% of control)		
ATP	3966 ± 16	100	96	102
IMP	19.9 ± 0.6	691*	934*	805*
GMP	15.6 ± 2.8	27*	15*	22*
GDP	142 ± 2.3	90	85	81*
GTP	836 ± 16	54*	44*	63*

K562 cells (1×10^7 cells/flask) were incubated with 10 μM each of benzamide riboside, selenazofurin or tiazofurin for 2 hr at 37° and prepared for the assay of ribonucleotides as described in Materials and Methods. Results are expressed as means ± SD of three samples.

* Significantly different from the control values ($P < 0.05$).

Table 3. Effects of benzamide riboside, selenazofurin and tiazofurin on the concentration of 2'-deoxyribonucleoside triphosphates in K562 cells

dNTP	Control (pmol/10^6 cells)	Benzamide riboside	Selenazofurin	Tiazofurin
		(% of control)		
dCTP	60 ± 4	183	130	153
dTTP	13 ± 1	161	153	146
dATP	30 ± 2	100	100	83
dGTP	39 ± 1	43*	41*	38*

K562 cells (5×10^7 cells/flask) in culture were incubated with saline or 10 μM each of benzamide riboside, selenazofurin or tiazofurin for 4 hr at 37°. Cells were harvested by centrifugation, washed once with cold PBS, extracted with 500 μL of 60% methanol, and heated for 1 min in boiling water. The samples were clarified by centrifuging at 18,000 *g* for 2 min. The concentration of deoxyribonucleoside triphosphate was analyzed as described in Materials and Methods.

* Significantly different from the control values ($P < 0.05$).

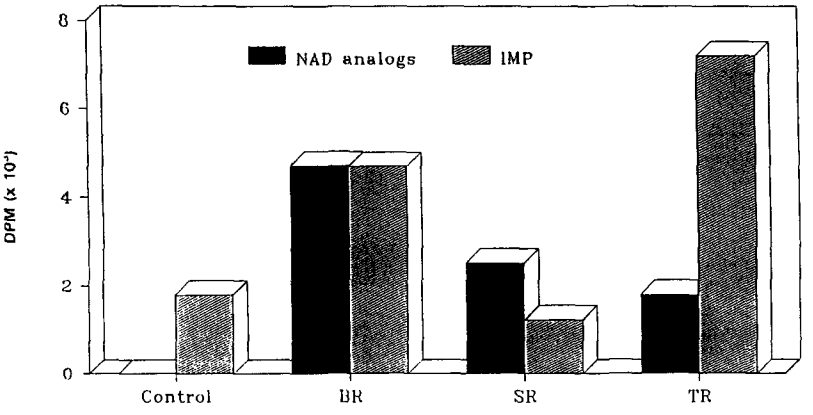

Fig. 2. Synthesis of NAD analogues of benzamide riboside (BR), selenazofurin (SR) and tiazofurin (TR). K562 cells (1×10^7) in culture were prelabeled with [2,8-^{3}H]adenosine (10 μCi/flask, specific radioactivity 200 mCi/mmol) for 2 hr at 37° and then 10 μM each of BR, SR or TR was added and the cells were further incubated for 2 hr. The cells were prepared as described in Materials and Methods for HPLC analysis.

with saline or with 10 μM each of benzamide riboside, tiazofurin or selenazofurin for 4 hr at 37°. This treatment resulted in a decrease in dGTP concentration to 43, 38 and 41%, respectively, with a concomitant increase in dTTP and dCTP pools. There was no change in the dATP level (Table 3).

Biological synthesis of NAD analogues of benzamide riboside (BAD), tiazofurin (TAD) and selenazofurin (SAD). To determine whether there was a difference in the cellular biosynthesis of NAD analogues of the three agents responsible for cytotoxic action in cancer cells, K562 cells were labeled with [2,8-^{3}H]adenosine for 2 hr at 37°. Then benzamide riboside, tiazofurin or selenazofurin (10 μM each), or saline was added and the cells were further incubated for 2 hr at 37°. The cells were processed as described. Under these conditions, the K562 cells formed 2- and 3-fold more BAD than SAD and TAD, respectively (Fig. 2).

Effects of BAD, TAD and SAD on the activity of malate, L-glutamate and lactate dehydrogenases. NAD analogues of the three agents did not perturb the activities of LDH and GDH even up to a 50 μM concentration. In contrast, among the three NAD analogues, only BAD inhibited the activity of MDH a key enzyme in the citric acid cycle. The K_m of MDH for NADH at a fixed concentration of oxaloacetate was 24.3 ± 3.9 μM. A Lineweaver–Burk plot for MDH with various NADH concentrations in the presence of several concentrations of BAD is shown in Fig. 3. The K_i for BAD was determined to be 3.18 ± 0.79 μM. The Lineweaver–Burk plot indicates that BAD acts as a competitive inhibitor with respect to NADH utilization by MDH.

DISCUSSION

The C-nucleoside antimetabolites benzamide riboside, tiazofurin and selenazofurin were selected for comparison to determine the relationship of biochemical parameters with cytotoxicity. These three compounds showed differential cytotoxicity

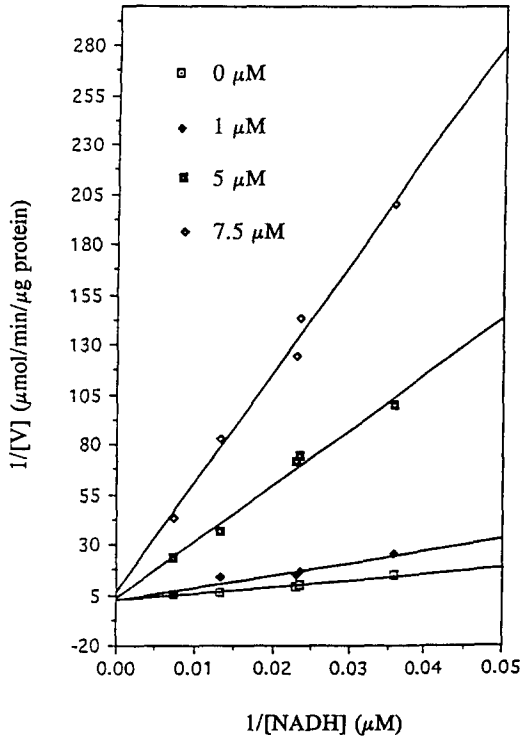

Fig. 3. Inhibition of malate dehydrogenase activity by BAD. NADH and BAD concentrations were varied in the presence of a fixed 1 mM oxaloacetate concentration and assayed as described in Materials and Methods.

toward some of the 60 human cancer cell lines of the National Cancer Institute's current drug screen. The COMPARE algorithm being developed at the National Cancer Institute permits the rapid selection of compounds with similar patterns of cytotoxicity toward the tumor panel. When the cytotoxicity data on the three compounds were evaluated in the COMPARE program, the correlation coefficients obtained suggested that the three compounds might share similar biochemical mechanisms of action. Our earlier studies have demonstrated that tiazofurin and selenazofurin exert their action through the formation of NAD analogues, TAD and SAD, which inhibit IMPDH activity, leading to a reduction in cellular guanylate levels [2, 19]. We have demonstrated recently, that benzamide riboside also shares the same biochemical mechanisms of action [6]. The present studies provide additional evidence for the relevance of the COMPARE algorithm in selecting compounds with similar mechanisms of action.

For the design of new antimetabolites, IMPDH is a good target, because it catalyzes a key step in the *de novo* synthesis of guanylates and its activity is increased in cancer cells [20]. This suggests that tumor cells depend more on the *de novo* guanylate synthesis than do normal cells and, hence, IMPDH inhibitors should be cytotoxic to these cells. Tiazofurin exhibits potent antitumor activity against murine [1] and human tumor cells including human myelogenous leukemia K562 cells in culture [3, 15]. Tiazofurin is being evaluated in phase I/II studies to assess its efficacy against end-stage leukemia in adult patients. At present, a 47% response rate has been obtained [8, 9].

Although the structural requirements for cytotoxic activity in the thiazole-4-carboxamide molecule of tiazofurin are stringent [1], selenium can replace sulfur in the heterocyclic ring, with a 5-fold increase in cytotoxicity [2]. The greater cytotoxicity of selenazofurin compared with tiazofurin in susceptible tumor cells appears to result from more extensive formation of SAD than TAD and a 4-fold greater affinity of SAD than TAD for its enzymatic target, IMPDH [14].

The most recent C-nucleoside inhibitor of IMPDH is benzamide riboside [5]. We have shown that it exerts its biochemical action through its NAD analogue, BAD [6]. Among the three C-nucleoside inhibitors of IMPDH, 2- to 3-fold more of BAD than of SAD or TAD was synthesized by K562 cells (Fig. 2). Benzamide riboside exerts selective cytotoxicity against CNS tumors in the panel of human tumor cells.

Nicotinamide and its analogues are inhibitors of poly(ADP-ribose)transferase, and thus a combination with other cytotoxic agents should be useful in the treatment of neoplasms [21]. Benzamide is a potent inhibitor of poly(ADP-ribosyl)ation [7]. However, it does not affect significantly other reactions that utilize NAD as the coenzyme at concentrations that completely inhibit poly (ADP-ribosyl)ation. Present studies on the inhibition of NAD, utilizing reactions other than IMPDH, by the three compounds showed that only benzamide riboside selectively inhibited malate dehydrogenase activity with a K_i of 3.2 μM (Fig. 3). The K_m for oxaloacetate utilization by MDH was 24 μM and a similar order of K_m values has been reported for this enzyme from *Bacillus subtilis* (61 μM; Ref. 22) and from bovine heart (40 μM; Ref. 23).

In summary, the three C-nucleoside analogues benzamide riboside, tiazofurin and selenazofurin have shown oncolytic activity. These agents exert their cytotoxic actions by forming NAD analogues that inhibit IMPDH activity. Benzamide riboside seems to synthesize the maximum amount of NAD analogue among the three compounds in K562 cells. Because of the current interest in anticancer antimetabolites that inhibit IMPDH and since more of BAD than of SAD or TAD is formed by human myelogenous leukemia K562 cells, future studies are being directed at developing benzamide riboside by determining its *in vivo* antitumor activity, therapeutic index, toxicity and bioavailability.

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