Biochemical Pharmacology, Vol. 46, No. 11, pp. 1903–1907, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

SYNERGISTIC ACTION OF TIAZOFURIN WITH HYPOXANTHINE AND ALLOPURINOL IN HUMAN NEUROECTODERMAL TUMOR CELL LINES

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(*Received* 2 *February* 1993; *accepted* 20 *August* 1993)

Abstract—The activity of IMP dehydrogenase (EC 1.2.1.14), the key enzyme of *de novo* guanylate biosynthesis, was shown to be increased in tumor cells. Tiazofurin (TR), a potent and specific inhibitor of this enzyme, proved to be effective in the treatment of refractory granulocytic leukemia in blast crisis. We examined the effects of tiazofurin as a single agent and in combination with hypoxanthine and allopurinol in six different neuroectodermal tumor cell lines, the STA-BT-3 and 146-18 human glioblastoma cell lines, the SK-N-SH, LA-N-1 and LA-N-5 human neuroblastoma cell lines, and the STA-ET-1 Ewing tumor cell line. Tiazofurin inhibited tumor cell growth with IC_{50} values between 2.2 μM (LA-N-1 cell line) and 550 μM (LA-N-5 cells) and caused a significant decrease of intracellular GTP pools (GTP concentrations decreased to 39–79% of control). Incorporation of [8-^{14}C]guanine into GTP pools was determined as a measure of guanylate salvage activity; incubation with 100 μM hypoxanthine caused a 62–96% inhibition of the salvage pathway. Incubation with tiazofurin (100 μM) and hypoxanthine (100 μM) synergistically inhibited tumor cell growth, and the addition of allopurinol (100 μM) strengthened these effects. Therefore, this drug combination, inhibiting guanylate *de novo* and salvage pathways, may prove useful in the treatment of human neuroectodermal tumors.

Inosine monophosphate dehydrogenase (IMP DH¶; EC 1.2.1.14) catalyzes the reaction of IMP to XMP and is the rate-limiting enzyme of *de novo* guanylate biosynthesis. IMP DH was shown to be increased significantly in cancer cells and therefore considered to be a sensitive target for cancer chemotherapy [1, 2]. Out of several IMP DH inhibitors that were synthesized, tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) entered clinical studies. In sensitive cells, tiazofurin is metabolized to thiazole-4-carboxamide adenine dinucleotide (TAD), an analogue of NAD with high affinity for the NAD/NADH binding site of IMP DH [3, 4]. Tiazofurin treatment causes inhibition of IMP DH activity leading to decreased intracellular GTP and dGTP concentrations followed by inhibition of cell proliferation, tumor cell differentiation and down-regulation of c-*myc* and Ha-*ras* oncogenes *in vitro* and in patients [3, 5–11]. Tiazofurin proved to be effective in the treatment of end-stage chronic granulocytic leukemia in blast crisis through lowering the guanylate pools in the blast cells [12, 13].

Although tiazofurin is capable of inhibiting guanylate *de novo* synthesis, it has little or no effect on the salvage pathway; guanine can be metabolized by hypoxanthine guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8) to GMP and, at least in part, circumvents the action of tiazofurin. However, the guanylate salvage pathway can be inhibited by hypoxanthine and allopurinol through inhibition of HGPRT activity [14]. To demonstrate that both guanylate *de novo* and salvage pathways can be inhibited by biochemical modulation, we incubated six different human neuroectodermal tumor cell lines with tiazofurin, in combination with hypoxanthine and allopurinol, and examined the biochemical and growth inhibitory effects of this drug combination in seven different human neuroectodermal tumor cell lines.

MATERIALS AND METHODS

Chemicals and cell culture. Culture medium (RPMI 1640), fetal bovine serum and trypsin were from GIBCO, Grand Island, NY. Tiazofurin was a gift from Dr. Ven Narayanan, National Cancer Institute, Bethesda, MD, U.S.A. All other chemicals were commercially available and of the highest purity. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin at 37° in a humidified atmosphere containing 5% CO_2.

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¶ Abbreviations: IMP DH, inosine monophosphate dehydrogenase; IC_{50}, drug concentration that causes a 50% reduction in cell proliferation; TAD, thiazole-4-carboxamide adenine dinucleotide; PBS, phosphate-buffered saline; HGPRT, hypoxanthine guanine phosphoribosyltransferase; and PNET, primitive neuroectodermal tumor.

For growth inhibition assays, cells were seeded at a density of 2–4 × 10^4, except for the STA-BT-3 cells, which were seeded at a density of 1 × 10^5 cells in 24-well plates or 25-cm^2 tissue culture flasks and incubated for 24 hr. Drugs were added consecutively and cells were grown for an additional 4 days; then cells were trypsinized and counted, using a microscope.

Cell lines. The SK-N-SH human neuroblastoma cell line was purchased from the American Type Culture Collection (Rockville, MD, U.S.A). The LA-N-1 and LA-N-5 neuroblastoma cell lines were gifts from Dr. R. C. Seeger, Department of Pediatrics, University of California, Los Angeles (UCLA), CA, U.S.A. The STA-BT-3 glioblastoma cell line, from tumor cells of a 28-year-old female suffering from glioblastoma, the STA-BT-6 cell line from a primitive neuroectodermal tumor of the brain with differentiation towards ependymoblastoma (central PNET) and the STA-ET-1 peripheral primitive neuroectodermal tumor (Ewing tumor) cell line were established at the Children's Cancer Research Institute (CCRI), St. Anna Kinderspital, Vienna, Austria, by Dr. Peter F. Ambros [15]. The STA-BT-6 cell line is derived from a 12-year-old boy suffering from Li-Fraumeni syndrome and p53 tumor suppressor gene mutation [16]. The 146-18 glioblastoma cell line was provided by Dr. Ulrich Bogdahn (Neurologische Abteilung, Universitätsklinik Würzburg, Würzburg, FRG) and was established from a 64-year-old female patient.

HPLC determination of ribonucleotides. Control and tiazofurin-treated cells (100 μM tiazofurin for 4 hr) were pelleted, washed twice with cold phosphate-buffered saline (PBS), extracted with cold trichloroacetic acid (TCA) and processed for HPLC analysis of nucleotide pools as described earlier [4, 17, 18].

Measurement of IMP DH activity. Cells were washed three times with PBS and extracted with 20 mM Tris containing 5 μM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 1 mM dithiothreitol (DTT) by three cycles of freezing and thawing. Cell extracts were spun at 100,000 *g* in a Beckman TL 100 centrifuge, and supernatants were stored in liquid nitrogen until used. IMP DH activity was measured according to the method of Holmes *et al.* [19]. Briefly, the incubation mixture contained 50 mM potassium phosphate buffer (pH 7.4), 100 mM potassium chloride, 1 mM EDTA, 0.3 mM NAD^+ (Boehringer Mannheim, Mannheim, FRG), 5 mM NaF, [8-^{14}C]IMP (Amersham Int., Amersham, U.K.) at a final concentration of 11 μM, and 0.15 to 0.20 mg of enzyme protein in a total volume of 60 μL. The reaction was terminated by spotting 2-μL aliquots onto polyethylenimine cellulose plates (Merck, Darmstadt, FRG), and IMP and XMP were separated by 10% KH_2PO_4 (w/v) in water. Spots were visualized under UV light (254 nm) and cut out, radioactivity was determined using a Packard CA 2000 liquid scintillation counter. Enzyme activity is expressed as nanomoles product formed per milligram protein per hour.

Determination of tiazofurin metabolites. To examine the metabolism of tiazofurin, 4 × 10^6 cells were incubated with [2-^{14}C]tiazofurin (100 μM, sp. act. 27 mCi/mmol) for 4 hr at 37°. Cells were then centrifuged (1,000 *g* for 5 min), washed twice with cold PBS, extracted with 300 μL of cold 10% TCA, and promptly neutralized with tri-*n*-octylamine in freon and an aliquot was analyzed by HPLC on a Partisil-10-SAX column using an ammonium phosphate buffer system as described earlier [4, 20]

Determination of guanine incorporation into the GTP pool. To examine the effect of hypoxanthine on guanine salvage synthesis, 2 × 10^7 cells were incubated with 100 μM hypoxanthine for 3.5 hr, 0.5 μCi [8-^{14}C]-guanine was added at a final concentration of 0.5 μM (a concentration measured in human brain tumors *in vivo*) [21] and then the cells were incubated at 37° for another 30 min. Cells were centrifuged and washed twice with cold PBS, and the nucleotides were extracted and analyzed as described earlier [20].

Protein concentrations. Protein concentrations were measured with a protein-assay-kit (Bio-Rad) according to the method of Bradford [22].

RESULTS AND DISCUSSION

Effect of tiazofurin on cell growth. Tiazofurin inhibited the growth of neuroectodermal cell lines with IC_{50} values between 2.2 μM (LA-N-1 cells) and 550 μM (LA-N-5 cells) (Fig. 1). The IC_{50} values were 4.2 μM for the SK-N-SH neuroblastoma cell line, 51 μM in 146-18 glioblastoma cells and 71 μM in STA-ET-1 Ewing tumor cells. A negative correlation between the IC_{50} values of tiazofurin and the IMP DH activities was established. IMP DH activities ranged from 0.99 in the STA-BT-3 to 2.2 nmol/hr/mg protein (LA-N-1 cell line) (Fig. 1A). The generation times and IC_{50} values of tiazofurin in the respective cell lines showed a positive correlation (Fig. 1B). The most sensitive LA-N-1 cell line had a doubling time of 13 hr and the virtually tiazofurin resistant LA-N-5 and STA-BT-3 cell lines exhibited a generation time of 70 and 71 hr, respectively. These findings are in line with results obtained from other tumor systems demonstrating the progression-linked increase of IMP DH activity [1, 2, 23].

Formation of TAD in neuroectodermal tumor cell lines. We determined the intracellular concentrations of TAD, the active metabolite of tiazofurin, in five different neuroectodermal tumor cell lines. After incubation of the STA-BT-3 glioblastoma cell line with 100 μM [8-^{14}C]tiazofurin (for 4 hr) cells formed 71 pmol/mg TAD; the neuroblastoma cell lines SK-N-SH, LA-N-1 and LA-N-5 formed 54, 34 and 30 pmol/mg TAD, respectively, and the STA-ET-1 Ewing tumor cell line was able to form 15 pmol/mg TAD (data are means of three determinations). However, no linear correlation was established between TAD levels and the respective IC_{50} values of tiazofurin.

Serum levels above 100 μM tiazofurin can be maintained in patients over 4 hr [8, 15]; therefore this concentration was selected for all cell lines studied [24]. It is noteworthy that all cell lines examined formed TAD, which supports the notion that the relative resistance towards tiazofurin, observed in LA-N-5 and STA-BT-3 cells, is caused

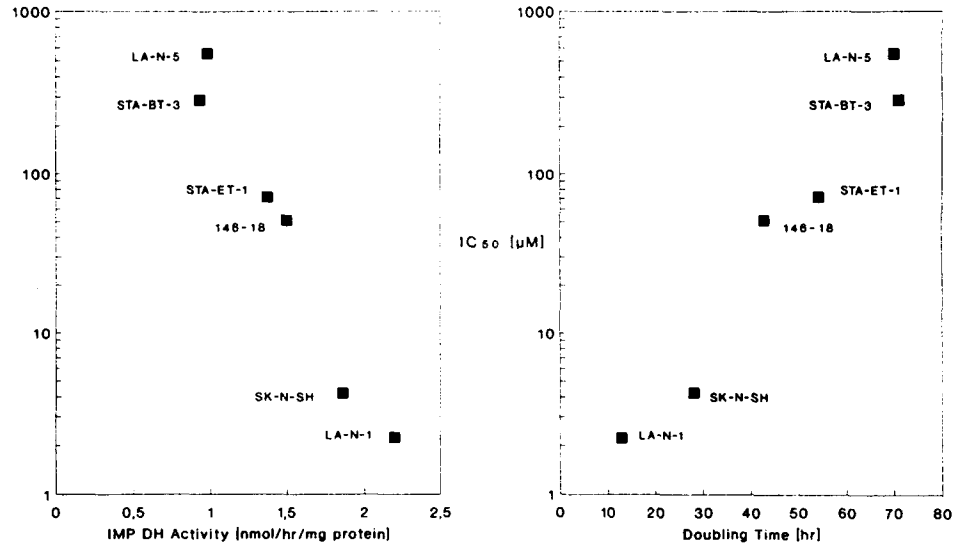

Fig. 1. Correlation between IMP dehydrogenase activity (A) and doubling times (B) and IC_{50} values of tiazofurin in various human neuroectodermal tumor cell lines.

Table 1. Effect of tiazofurin on GTP pools of human neuroectodermal tumor cell lines

Cell line	GTP (pmol/mg protein)	
	Control	+ TR (100 µM)
Glioblastoma		
146-18	1348 ± 163 (100)	1334 ± 86 (99)
STA-BT-3	848 ± 212 (100)	669 ± 38* (79)
Neuroblastoma		
SK-N-SH	3303 ± 655 (100)	1282 ± 75* (39)
LA-N-1	296 ± 130 (100)	217 ± 20* (73)
LA-N-5	1177 ± 167 (100)	630 ± 47* (54)
Ewing tumor		
STA-ET-1	2296 ± 203 (100)	1406 ± 521* (61)

Values are means ± SD of three determinations; values in parentheses are percentages of the untreated control. Cells were treated with 100 µM tiazofurin (TR) for 4 hr, and then GTP pools were determined by HPLC as described in Materials and Methods.

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

by a relative prevalence of guanylate salvage capacity versus guanylate *de novo* synthesis rather than inhibition of TAD formation [25].

Effect of tiazofurin on GTP pools in neuroectodermal tumor cell lines. When neuroectodermal tumor cells were incubated with 100 µM tiazofurin for 4 hr, a significant decrease of intracellular GTP concentrations was observed in five out of six cell lines (Table 1). GTP concentrations decreased to values between 39 and 79% of control, which is in line with results observed in other cell lines and patient cells [3, 13]. However, neither the effects of tiazofurin on GTP pools nor basal levels of GTP correlated with the IC_{50} value for tiazofurin in the respective cell line.

Effect of hypoxanthine on guanylate salvage activity. To examine the inhibition of the guanylate salvage pathway by hypoxanthine, cells were incubated with 100 µM hypoxanthine for 3.5 hr and pulse labeled with [8-^{14}C]guanine for 30 min. Guanine incorporation into GTP pools was then determined. Hypoxanthine was capable of inhibiting the guanylate salvage metabolism in all cell lines tested. Guanine incorporation decreased to values between 4 and 38% of control (Table 2). The inhibition of guanylate salvage metabolism by hypoxanthine is due to the inhibition of HPRT activity, as shown by Weber *et al.* [14] in chronic granulocytic leukemia cells. Since serum hypoxanthine levels in the range of 10–400 µM were found in the serum of females and the cord blood of their newborns during and after delivery (data not shown) and 100 µM hypoxanthine did not show any significant growth inhibitory effect in the cell lines tested (Table 3), a therapeutic administration of this physiological substance seems possible.

Synergistic growth inhibitory effect of tiazofurin, hypoxanthine and allopurinol. When cells were incubated with both tiazofurin (100 µM) and hypoxanthine (100 µM), synergistic growth inhibitory effects were observed in the LA-N-1 and LA-N-5 human neuroblastoma cell lines, the STA-BT-3 glioblastoma cells, as well as in STA-ET-1 Ewing tumor cells (Table 3). Hypoxanthine incubation alone did not inhibit tumor cell growth, though combined with tiazofurin it significantly enhanced the growth inhibitory effects of tiazofurin in all cell lines examined. Allopurinol was used as a second agent to inhibit guanylate salvage metabolism. It

Table 2. Inhibitory effect of hypoxanthine on guanine incorporation into GTP pools

Cell line	[8-^{14}C]Guanine (pCi/mg protein)	
	Control	+ HX (100 μM)
Glioblastoma		
146-18	267 ± 21 (100)	85 ± 23* (32)
STA-BT-3	555 ± 36 (100)	71 ± 2* (13)
Neuroblastoma		
SK-N-SH	1044 ± 160 (100)	313 ± 50* (30)
LA-N-1	273 ± 23 (100)	103 ± 1* (38)
LA-N-5	676 ± 30 (100)	132 ± 17* (20)
Ewing tumor		
STA-ET-1	4614 ± 1501 (100)	185 ± 61* (4)

Values are means ± SD of three determinations; values in parentheses are percentages of the untreated control. Cells were treated with 100 μM hypoxanthine (HX) for 3.5 hr, and then were pulse labeled for 30 min with [8-^{14}C]-guanine; the incorporation of guanine into GTP pools was determined as described in Materials and Methods.

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

was shown that allopurinol is capable of increasing hypoxanthine levels through inhibition of xanthine oxidase and xanthine dehydrogenase activity and thus improves the clinical effects of tiazofurin treatment in leukemia patients [12, 13]. We incubated neuroectodermal cell lines with tiazofurin and allopurinol (100 μM each) for 5 days and observed synergistic growth inhibition in SK-N-SH and LA-N-1 neuroblastoma cells, 146–18 and STA-BT-3 glioblastoma as well as STA-ET-1 Ewing tumor cells. At the concentration used, allopurinol did not inhibit cell growth; however, in combination with tiazofurin, allopurinol was capable of potentiating the growth inhibitory effects of tiazofurin. When cells were incubated with tiazofurin, hypoxanthine and allopurinol, synergistic effects were even more pronounced.

The cell number of STA-ET-1 decreased to 3.7% of untreated controls which is 7% of the predicted value for additive growth inhibition. In the other cell lines, incubated with the combination of tiazofurin, hypoxanthine and allopurinol, we observed cell numbers between 17 and 53% of the calculated values for additive effects. These results demonstrate that co-incubation of neuroectodermal cells with tiazofurin, hypoxanthine and allopurinol potentiates the growth inhibitory effects achieved by tiazofurin alone.

Clinical aspects. Despite combined modality treatment with chemotherapy, surgery and radiation, malignant neuroectodermal tumors have a poor prognosis. Except for Ewing sarcoma, no effective chemotherapeutic treatment has been established and most attempts of designing successful treatment regimens have failed. We have previously shown increased IMP DH activities in glioblastomas *in vivo* [21]. We selected tiazofurin in our studies since it selectively inhibits IMP DH, is capable of crossing the blood–brain barrier, and has only mild myelotoxic side-effects [4, 12].

New observations. Novel aspects of this study include the following new observations: (1) Tiazofurin effectively inhibited the growth of SK-N-SH

Table 3. Synergistic cytotoxicity of tiazofurin, hypoxanthine and allopurinol on tumor cell growth

Cell line	Tumor cell number (% of control)					
	TR	HX	Allop	TR +HX	TR +Allop	TR +HX+Allop
Glioblastoma						
146-18	38.0[a]	96[b]	117[c]	27.2	21.6*	20.7(43[d])*
STA-BT-3	74.5	109	89	47.9*	29.0*	23.0(72)*
Neuroblastoma						
SK-N-SH	14.8	100	90	12.1	9.3*	4.9(13.3)*
LA-N-1	8	102	92	3.5*	3.4*	1.3(7.5)*
LA-N-5	62.5	95	110	34.6*	65.4	34.6(65)*
Ependymoblastoma						
STA-BT-6	75.0	ND†	ND	54.2	45.8	20.8
Ewing tumor						
STA-ET-1	43.9	113	102	14.6*	14.0*	3.7(51)*

Values are percentages of the untreated control. Control cell numbers for the respective cell lines were: 146-18: 13.8 ± 0.9 × 10^4; STA-BT-3: 32.3 ± 4.1 × 10^4; SK-N-SH: 39 ± 3 × 10^4; LA-N-1: 24 ± 1.9 × 10^6; LA-N-5: 13 ± 1.1 × 10^4; STA-BT-6: 12.5 ± 1.3 × 10^4; STA-ET-1: 18.7 ± 1.6 × 10^4 cells/flask (means ± SD of three or more determinations). Cells were treated with 100 μM tiazofurin (TR), hypoxanthine (HX), allopurinol (Allop) or a combination for 4 days, and then the cell number was determined. Values in parentheses are predicted values for additive growth inhibitory effects. Predicted values (%) were calculated as: $d = a \times b \times c/10{,}000$.

* Synergism (values are less than 70% of the predicted value for additive effects).

† ND: not determined.

and LA-N-1 neuroblastoma, 146-18 glioblastoma, as well as STA-ET-1 Ewing tumor cells. All neuroectodermal tumor cell lines examined could metabolize tiazofurin to its active form TAD, and tiazofurin treatment significantly decreased intracellular GTP pools in five of the six cell lines. (2) Incubation of neuroectodermal cells with hypoxanthine significantly inhibited the guanylate salvage pathway without exerting any effects on the growth of these cell lines. (3) Tiazofurin and hypoxanthine, as well as tiazofurin and allopurinol, synergistically inhibited the growth of neuroectodermal tumor cells. Incubation of the cells with all three agents, tiazofurin, hypoxanthine and allopurinol, strengthened these effects.

The results presented in this paper support the approach of "enzyme pattern targeted chemotherapy" [1], yielding inhibition of guanylate *de novo* and salvage metabolic pathways, resulting in synergistic growth inhibitory effects. Considering the slow progress in the development of effective chemotherapy regimens for the treatment of neuroectodermal tumors, the combination of tiazofurin, hypoxanthine and allopurinol may offer a promising option in the treatment of these tumors.

Acknowledgements—This investigation was supported by the "Jubiläumsfonds der Österreichischen Nationalbank" Grant No. 3890, the "Fonds zur Förderung der Wissenschaftlichen Forschung", Grant No. P6634 and the "Medizinisch Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien" to K.P.

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