Sequential Impact of Tiazofurin and Ribavirin on the Enzymic Program of the Bone Marrow

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ABSTRACT

Tiazofurin and ribavirin are clinically used inhibitors of IMP dehydrogenase (DH), binding to the NAD and IMP sites, respectively, of the target enzyme. In patients with chronic granulocytic leukemia in blast crisis, daily tiazofurin infusions decreased the high IMP DH activity in blast cells and resulted in 77% response (G. Weber. In: R. A. Harkness et al., Purine and Pyrimidine Metabolism in Man, Vol. VII, Part B, pp. 287–292, 1991). However, patients relapsed in a few weeks with emergence of high IMP DH activity (G. Tricot et al., Int. J. Cell Cloning, 8: 161–170, 1990). The present study showed that the tiazofurin-induced depression of IMP DH activity in rat bone marrow can be maintained by ribavirin injection. Tiazofurin (150 mg/kg, i.p., once a day for 2 days) decreased IMP DH activity to 10% and ribavirin (250 mg/kg, i.p., once a day for the subsequent 3 days) maintained the enzymic activity at 20 to 30% of control values. In control rats where no ribavirin was given, IMP DH activity of the tiazofurin-treated rats rapidly returned to the range of untreated animals. The decrease of IMP DH activity (t1/2 = 2.6 h) sharply preceded that of the bone marrow cellularity (t1/2 = 17.4 h). In addition to the target enzyme, IMP DH, tiazofurin also decreased activities of the guanylate metabolic enzymes, guanine phosphoribosyltransferase and GMP reductase, and the pyrimidine salvage enzymes, deoxycytidine and thymidine kinases with t1/2 of 2.6, 4.7, 6.0, 3.4, and 6.5 h, respectively. In cycloheximide-treated rats, where much of protein biosynthesis was blocked, the t1/25 of these five enzymes in bone marrow were shorter, 1.6, 4.3, 3.0, 0.6, and 0.8 h, respectively.

Thus, the impact of tiazofurin in the bone marrow entails a decrease in the activity of the target enzyme, IMP DH, and also of other enzymes in purine and pyrimidine biosynthesis as a result of the enzyme half-lives shortened by this drug. These novel observations should assist in achieving better protection and recovery of bone marrow during and after chemotherapy.

INTRODUCTION

It is well known that various anticancer agents damage the bone marrow which, in many cases, is able to recover. However, little is understood of the metabolic impact of anticancer drugs on the bone marrow and the biochemical basis of its ability to recover. In the first systematic study of the enzymic strategy of the bone marrow in the rat, Prajda et al. (1) reported that, as compared with normal liver, there were markedly elevated activities of the key enzymes of de novo purine and pyrimidine biosynthesis. It was also striking that, in the bone marrow, the activities of the salvage enzymes were considerably higher than those of the rate-limiting enzymes of purine and pyrimidine biosynthesis (1).

The purpose of the present investigation was to gain insight into the mechanism of action of tiazofurin and ribavirin on the enzymic program of the bone marrow. Tiazofurin is a produg that in sensitive cells after its 2-step enzymatic conversion to the active metabolite, TAD, powerfully inhibits IMP DH, the rate-limiting enzyme of de novo GTP biosynthesis and results in a decrease in the concentration of guanylates and in oncolytic action (2–5). Tiazofurin treatment is effective in chronic granulocytic leukemia in blast crisis with a 77% response (6, 7). Tiazofurin has a three-pronged impact involving (a) chemotherapy, (b) induced differentiation, and (c) down-regulation of oncogenes in the human leukemic blast cells (8) and in K562 cells (9). Ribavirin also is an inhibitor of IMP DH, blocking the enzyme at the IMP attachment site, whereas TAD blocks at the NAD/NADH ligand site (10) (Fig. 1). A further purpose was to determine whether tiazofurin is effective in decreasing the bone marrow IMP DH activity and whether subsequent ribavirin administration could keep the enzyme activity at low levels. To obtain more complete information on the impact of these drugs we tested the responses of other enzymes involved in IMP and GMP metabolism and clarified the behavior of the activities of salvage enzymes.

The results indicate that tiazofurin administration and subsequent treatment with ribavirin were successful in keeping IMP DH activity low in the bone marrow. Enzymes with short half-lives, such as dCyd and dThd kinases, also rapidly decreased in activity, whereas other enzymic activities changed less extensively. A brief preliminary report was published (11).

MATERIALS AND METHODS

Drugs. Tiazofurin and ribavirin were obtained from the Viratek Company, Costa Mesa, CA. Cycloheximide and various reagents were purchased from Sigma, St. Louis, MO. The drugs were dissolved in 0.9% NaCl solution immediately before treatment of the animals.

Animals. Male ACI/N inbred strain rats of 180 to 220 g of weight were obtained from Harlan Sprague Dawley, Indianapolis, IN, and were fed Purina Laboratory Chow and water ad libitum.

Treatment. For dose-response studies normal rats were given injections i.p. of various doses of drugs. Rats were killed 6 h after treatment, a time point selected on the basis of time sequence results. Rats were given injections i.p. of tiazofurin (150 mg/kg) once a day for 2 days. Subsequently, for an additional 3 days, control rats received daily 0.9% NaCl solution injections, and experimental rats received daily ribavirin treatment (250 mg/kg, i.p.).

Tissue Preparation, Biochemical Studies, and Cell Counts. Rats were stunned, decapitated, and bled 6 h after the final injection. Bone marrow was obtained from both femurs by flushing with isotonic KCl (12). This cell suspension was used for preparation of homogenates, 100,000 × g cytosolic extract, and for cell counts. For cell counts the nucleated cells of the bone marrow were counted in a hemocytometer. IMP DH activity was determined in the cytosol fractions of tissue homogenates by an isotope technique (13). dThd and dCyd kinase, GPRT (14, 15), and GMP R (16) activities were assayed as reported. Protein concentrations were measured by a routine method (17) using crystalline bovine serum albumin as a standard.

5 The abbreviations used are: TAD, thiazole-4-carboxamide adenine dinucleotide; IMP DH, inosine 5′-monophosphate dehydrogenase (EC 1.1.1.205); GPRT, guanine phosphoribosyltransferase (EC 2.4.2.8); dCyd, deoxycytidine; dThd, thymidine; t1/2, the time required for a 50% decrease in activity; IC50, dose required for a 50% decrease in activity; GMP R, GMP reductase (EC 1.6.6.8).

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Pro-drug | Active Metabolite | Target
---|---|---
![Tiazofurin](image1) | ![TAD](image2) | ![NAD](image3) | IMP DH
![Ribavirin](image4) | ![RMP](image5) | IMP

Fig. 1. Attacking points of active metabolites of tiazofurin and ribavirin on IMP dehydrogenase, i.e., targets of tiazofurin and ribavirin on IMP dehydrogenase.

Expression and Evaluation of Results. Enzymic activities were calculated in specific activity as nmol of substrate metabolized per h per mg of protein; data were also expressed as percentages. Cell counts were calculated in millions of nucleated cells counted per rat femur. Differences between means were subjected to statistical evaluation by the t test; those yielding a probability of less than 5% were considered statistically significant.

RESULTS AND DISCUSSION

Dose-Response Studies: Effect of Tiazofurin or Ribavirin on IMP DH Activity of Rat Bone Marrow. Animals were treated with tiazofurin or ribavirin and killed 6 h later as described in “Materials and Methods.” Fig. 2 shows that 50 mg/kg of tiazofurin sharply decreased IMP DH specific activity in the bone marrow to 17% of controls, and dose increments up to 200 mg/kg failed to further lower the activity. By contrast, ribavirin caused a step-wise decrease in IMP DH activity; 150 or 200 mg per kg depressed the activity to 15% of the controls. The results show that at low concentrations tiazofurin was more effective than ribavirin in inhibiting IMP DH activity; however, the two drugs were equally potent at 150 and 200 mg/kg. The effectiveness of tiazofurin and ribavirin treatment on IMP DH activity in bone marrow is compared by the IC₅₀ values of these drugs. Tiazofurin (IC₅₀ = 29 mg/kg) was approximately twice as effective as ribavirin (IC₅₀ = 65 mg/kg).

Effect of Tiazofurin and Subsequent Ribavirin Treatment on Cellularity and IMP DH Activity in Bone Marrow. The normal cellularity of the adult rat bone marrow is 70 million cells per femur. Daily tiazofurin injections decreased the cellularity (with a t½ = 17.4 h) to 42% in 48 h (Fig. 3). The cellularity remained at this level for 78 h, then returned to normal range by 102 h. In animals where 2 days of tiazofurin were followed by 3 days of ribavirin administration, cellularity leveled off at 30% of the control values from 54 through 126 h.

Tiazofurin produced a rapid decline in IMP DH activity (t½ = 2.6 h) of the bone marrow which preceded the decrease in cellularity (Fig. 4). At 6 h after the first tiazofurin injection, IMP DH activity was 18% of the controls; with the second tiazofurin injection, the activity decreased to 4% at 30 h. In the 0.9% NaCl solution-treated controls, activity remained at this level for 54 h when it slowly rose, reaching normal range at 102 h. When ribavirin treatment followed the two injections of tiazofurin, IMP DH activity rose only slightly and remained at about 20% of the control for 126 h (Fig. 4). These results provide evidence that ribavirin was able to keep IMP DH activity at a low level in the bone marrow following tiazofurin treatment.

Effect of Tiazofurin and Subsequent Ribavirin Treatment on Activities of GMP Reductase and GPRT. GPRT activity contributes to the overall impact of tiazofurin because through this enzyme activity guanine can be salvaged in one step to form GMP and, thus, the...
guanylate pool can be replenished. The activity of GMP reductase directly opposes that of IMP DH by converting GMP back to its substrate, IMP (16). Tiazofurin administration decreased GMP reductase activity with a $t_{1/2}$ of about 6 h, and the activity remained at 30 to 40% of control for 76 h. In ribavirin-treated animals the activity decreased and was maintained at 50% to 60% of that of the controls for 126 h (not shown).

Tiazofurin administration decreased GPRT activity with a $t_{1/2} = 4.7$ h, reaching 20% of control at 48 h. Ribavirin maintained GPRT activity at 20% for 126 h. In saline-treated animals GPRT activity returned only to about 60% of controls and did not completely recover during the experiment (126 h) (Fig. 5).

**Effect of Tiazofurin and Ribavirin Treatment on Activities of dThd and dCyd Kinases.** The activities of the pyrimidine salvage enzymes influence the overall impact of tiazofurin. As tiazofurin down-regulates the production of guanylates and dGTP, the activity of thymidine kinase provides thymidylates and eventually dTTP, and dCyd kinase leads eventually to formation of dCTP, required with dGTP and dATP for the formation of DNA. Tiazofurin caused a decrease in dThd kinase activity with a $t_{1/2} = 6.5$ h. Ribavirin administration kept the enzyme activity at 14 to 17% of the control. By contrast, in the 0.9% NaCl solution-treated animals the activity returned to normal range by 78 h (Fig. 6).

Tiazofurin administration sharply depressed dCyd kinase activity with a $t_{1/2} = 3.4$ h. Enzymic activity decreased to about 5% of control by 6 h and remained at this level for 2 days. With ribavirin administration the low activity was maintained for 126 h. In saline-treated animals, activity returned to normal range by 78 h (Fig. 7).

**Effect of Cycloheximide on Enzymic Activities in Rat Bone Marrow.** To throw light on the mechanism of changes in enzymic activities that occurs during tiazofurin and ribavirin treatment, the rates of decrease in enzymic activities were measured in cycloheximide-injected rats. Cycloheximide is useful in such an evaluation because it markedly curtails the biosynthesis of proteins so that, by following the sequence of events after injection of this drug, the decay rates of the enzymes, largely unopposed by protein biosynthesis, can be measured (11, 18, 19, 23). Comparison of the half-lives of bone marrow enzymes after tiazofurin and cycloheximide is given in Table 1. Cycloheximide treatment decreased the activities with $t_{1/2}$
Fig. 7. Effect of tiazofurin and ribavirin on dCyd (Cdr) kinase activity of rat bone marrow. The activity of dCyd kinase in the bone marrow was 19.2 ± 0.3 nmol/h/mg of protein (mean ± SE). There were 3 or more rats in each group. Asterisks indicate statistically significant differences from the control value ($P < 0.05$).

Table 1 Effect of tiazofurin and cycloheximide on enzyme activities and cellularity in rat bone marrow

<table>
<thead>
<tr>
<th>Enzymes and activities</th>
<th>Tiazofurin</th>
<th>Cycloheximide</th>
<th>TR/CX ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellularity/femur</td>
<td>17.4</td>
<td>8.8</td>
<td>1.9</td>
</tr>
<tr>
<td>dThd kinase</td>
<td>6.5</td>
<td>0.8</td>
<td>8.1</td>
</tr>
<tr>
<td>GMP reductase</td>
<td>6.0</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>GPRT</td>
<td>4.7</td>
<td>4.3</td>
<td>1.1</td>
</tr>
<tr>
<td>dCyd kinase</td>
<td>3.4</td>
<td>0.6</td>
<td>5.7</td>
</tr>
<tr>
<td>IMP dehydrogenase</td>
<td>2.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

TR, tiazofurin (2-ß-D-ribofuranosylthiazole-4-carboxamide, NSC 286193); CX, cycloheximide.

...for GMP reductase, 3.0, GPRT, 4.3, IMP DH, 1.6, dThd kinase, 0.8, and dCyd kinase, 0.6 h.

...With tiazofurin, the activity of the target enzyme of this drug, IMP DH, rapidly declined with a $t_{1/2}$ of 2.6 h. The half-lives were for the other enzymes, dThd kinase, 6.5, GMP reductase, 6.0, GPRT, 4.7, and dCyd kinase, 3.4 h. These $t_{1/2}$s are assumed to be due to their degradation rates in the presence of active protein biosynthetic processes because tiazofurin inhibits RNA and DNA, but not protein, biosynthesis.

...Thus, the enzymic activities which are not immediate targets of tiazofurin are assumed to have been reduced because the tiazofurin action caused RNA and DNA biosynthesis. Therefore, in the tiazofurin-treated animals enzymes that turn over rapidly with a short $t_{1/2}$ decreased sharply, whereas enzymes with slower turnover rates such as GMP reductase decreased and were slower in returning to normal. It is assumed that IMP DH activity decreased because of inhibition by tiazofurin and ribavirin, but the other enzyme activities decreased because of the partially unopposed decay rates. Table 1 shows that the $t_{1/2}$s in tiazofurin-treated rats were 1.1- to 8.1-fold longer than those in cycloheximide-treated ones where there is little protein biosynthesis.

Effect of Chemotherapy on Targeted and Nontargeted Enzymatic Activities. Earlier work in this laboratory showed that the tiazofurin-induced decrease in IMP DH activity was due to inhibition of the type 2 isozyme (20, 21). The existence of IMP DH isozymes and their increased amount in cancer cells were recently confirmed (20, 22). This was followed by a marked rise in the production of mRNA for this enzyme (20, 21). The return of IMP DH activity to the control level in the present study is thought to be due to detachment of tiazofurin and TAD from the NAD ligand site and an increase in mRNA-directed new biosynthesis of this enzyme (20, 21).

Our observations indicate that the overall impact of tiazofurin not only affects the activities of the targeted enzyme, IMP DH, but also of other enzymes (GPRT, GMP reductase) that are involved in guanylate metabolism. Moreover, other enzymic activities (dCyd and dThd kinases) are also reduced presumably because of their high turnover rates (11, 18, 19). These results agree with our report on methotrexate which is known to reduce macromolecular biosynthesis through inhibiting the targeted enzyme, dihydrofolate reductase, leading to a reduction in the conversion of dUMP to dTMP (catalyzed by dTMP synthase). We showed that with methotrexate injection the activities of dThd kinase and dTMP synthase also decreased sharply because of their rapid decay rates (11, 18, 19). These new data agree with our report on the action of the antiguanine agent, acivicin (NSC 163501), which reduces macromolecular biosynthesis and, in addition, to decreasing the target enzyme activities in the bone marrow (glutamine-utilizing enzymes, amidophosphoribosyltransferase, GMP synthase, carbamoyl-phosphate synthase II, CTP synthase), it also decreases the activity of dThd kinase (1). This hypothesis is also supported by the fact that injection of the anticancer drug, taxol, which does not act through curtailing purine or pyrimidine biosynthesis does not cause a decrease in the activities of the enzymes which have short $t_{1/2}$s (not shown). Another possible interpretation is based on our observation that tiazofurin down-regulates the expression of the ras oncogene (8, 9). Thus, the enzymic decreases may be due to the phenotypic change that is linked with the down-regulated ras oncogene expression. Taken together, these observations should be helpful in understanding the bone marrow damage in treatment with anticancer drugs and should provide new strategies for the protection and improved recovery of the bone marrow.

Novel Aspects of This Report Include the Following. (a) Tiazofurin was twice as effective as the other IMP DH inhibitor, ribavirin, in decreasing the activity of bone marrow IMP DH. (b) Ribavirin was able to keep IMP DH activity at low levels after tiazofurin reduced the enzyme activity. (c) In addition to decreasing the activity of the target enzyme, IMP DH, tiazofurin also decreased the activities of the purine metabolic enzymes, GPRT and GMP reductases, and the pyrimidine salvage biosynthetic enzymes, dCyd and dThd kinases. (d) Cycloheximide studies indicated that the shortest half-lives are those for dCyd and dThd kinases. These enzymic activities also decline rapidly after tiazofurin treatment as an indirect, nontargeted result of this drug. (e) The impact of tiazofurin on targeted and nontargeted enzymes of the bone marrow is in line with the effect of other inhibitory drugs of macromolecular synthesis, including methotrexate and acivicin. (f) The decrease in enzymic activities in the bone marrow indicates that, if protection is to be achieved with thymidine or deoxy- cytidine, these precursors would have to be administered within 4 h after tiazofurin injection when the activities of dThd and dCyd kinases are still high enough to utilize these substrates.


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