

# Use of Tiazofurin to Enhance the Metabolism and Cytotoxic Activities of Analogues of Guanine, Guanosine, and Deoxyguanosine<sup>1</sup>

Priscilla P. Saunders,<sup>2</sup> Mei-Tao Tan, Christy D. Spindler, and Roland K. Robins

Department of Chemotherapy Research, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030 [P. P. S., M-T. T., C. D. S.] and Nucleic Acid Research Institute, 3300 Hyland Ave., Costa Mesa, California 92626 [R. K. R.]

## ABSTRACT

An effective modulator of cellular guanine nucleotide pools, 2- $\beta$ -D-ribofuranosylthiazole-4-carboxamide (tiazofurin) was tested for its ability to affect utilization of certain guanine, guanosine, and deoxyguanosine analogues by Chinese hamster ovary cells and hypoxanthine guanine phosphoribosyltransferase (HGPRTase)-deficient variants. The nucleoside analogues investigated were chosen for their potential to be metabolized to the nucleotide level by pathways other than those requiring the action of HGPRTase. Exposure of tiazofurin-treated (500  $\mu$ M for 3 h) cells to 3-deazaguanosine (200  $\mu$ M for 3 h) resulted in enhanced 3-deazaGTP formation and an increase (5-10-fold) in the ratio 3-deazaGTP/GTP. Tiazofurin treatment also stimulated [<sup>3</sup>H]deoxyguanosine utilization (8-fold) by HGPRTase-deficient cells, and accordingly, greatly increased the cytotoxicity of 2'-deoxy-3-deazaguanosine and arabinosylguanine. This study emphasizes the potential usefulness of tiazofurin in sequential combination with appropriate analogues of guanosine and deoxyguanosine in a clinical setting and as a tool in studying the metabolism of these agents.

## INTRODUCTION

Inosine monophosphate dehydrogenase has been recognized (1) as a promising target for cancer chemotherapy and, accordingly, several compounds such as tiazofurin<sup>3</sup> (2) have been synthesized having inhibitory activity for this enzyme (3). One of the most striking biochemical effects of tiazofurin action is a dramatic depletion of cellular GTP pools. This property, which is presumably responsible for the cytotoxic activity of the drug, reflects formation of an NAD analogue, referred to as TAD, in which the thiazole portion of tiazofurin has replaced the nicotinamide moiety. TAD is a highly efficient inhibitor of IMP dehydrogenase, doing so in a fashion which is not competitive with respect to NAD (2, 4). Among the effects resulting from this inhibition other than depletion of the GTP pool, is an accumulation of IMP which, in turn, inhibits HGPRTase (5), the primary guanine salvage mechanism for the cell. These phenomena, taken together, render the cell essentially devoid of guanine nucleotides.

Of the numerous guanosine analogues that have been synthesized, most have been active only after degradation by purine nucleoside phosphorylase making the base that was then available for utilization via the action of HGPRTase. 3-Deazaguanosine is unique in this class of compounds in that it appears to be phosphorylated directly in CHO cells (6) and thus may be one of the few guanosine analogues whose activity is not totally dependent on the action of HGPRTase.

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<sup>2</sup>To whom requests for reprints should be addressed, at Chemotherapy Research Department, P. O. Box 52, M. D. Anderson Hospital, 6723 Bertner Avenue, Houston, TX 77030.

<sup>3</sup>The abbreviations and trivial names are: tiazofurin, 2- $\beta$ -D-ribofuranosylthiazole-4-carboxamide; TAD, tiazofurin adenine-5'-dinucleotide; HGPRTase, hypoxanthine, guanine phosphoribosyltransferase; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography.

This paper addresses the potential usefulness of tiazofurin in combination with appropriate analogues of guanosine. Experiments are described in which cultured cells were treated with sequential combinations of tiazofurin with 3-deazaguanosine and certain deoxyguanosine analogues with resultant stimulation of metabolic and cytotoxic activity.

## MATERIALS AND METHODS

**Chemicals.** 3-Deazaguanine, 3-deazaguanosine, deoxy-3-deazaguanosine, tiazofurin, and TAD were prepared as described elsewhere (3, 7, 8). [<sup>5-3</sup>H]Deoxycytidine (24 Ci/mmol) and [<sup>8-3</sup>H]deoxyguanosine (5 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA) and diluted with unlabeled material such that the specific activities were 4 Ci/mmol each.

**Cells and Media.** CHO cells, as described previously (9), were carried in monolayer culture in McCoy's 5a medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in humidified 5% CO<sub>2</sub>. All experiments were carried out in medium containing dialyzed serum to avoid competition by natural serum components. Detachment of cells was with 0.02% trypsin in solution A (0.14 M NaCl, 5.3 mM KCl, 5.6 mM glucose, 4.2 mM NaHCO<sub>3</sub>). The mutant cell line, designated TG<sup>R</sup>-3, was derived from the parent CHO line by conventional methods of mutagenesis utilizing treatment with ethyl methanesulfonate (10), and was selected with 6-thioguanine. TG<sup>R</sup>-3 is deficient in HGPRTase and resistant to 3-deazaguanine but sensitive to 3-deazaguanosine (6) as described in Table 1. These cells are maintained in the absence of drug and tested periodically for resistance to 6-thioguanine and the ability to grow in selective media. The mutation is stable.

**Survival Experiments.** Cells were dispensed into sterile 20-ml glass vials, 2.5  $\times$  10<sup>4</sup> per vial in 2-4 ml of medium containing undialyzed serum. After 6-h incubation to allow attachment, the medium was removed and replaced with fresh medium containing the indicated additions (in duplicate) and dialyzed serum. The vials were returned to the incubator at 37°C as indicated, after which the media were removed and the cell monolayers were washed twice with 5 ml of solution A. The cells were detached with 1-2 ml of trypsin, disaggregated by repeated pipetting, and diluted with medium to 10 ml for plating into four 35-mm plastic petri dishes, approximately 200 cells per dish. Growth of clones at 37°C in humidified CO<sub>2</sub> was allowed for 8 days after which the clones were stained as previously described (9) and counted to give the viable cell count. The total number of cells plated was determined with the aid of an electronic particle counter at the time of plating. The plating efficiency was calculated by dividing the number of viable cells per plate by the total number of cells plated.

**Metabolic Experiments.** Cells were dispensed into 125-ml tissue culture flasks, 3  $\times$  10<sup>6</sup> per flask, in 5-ml medium containing undialyzed serum, and incubated for 18 h. The media were then aspirated and replaced with 5 ml of fresh medium containing dialyzed serum and the desired additions (tiazofurin) for preincubation. The flasks were incubated (see Figs. 1-6, legends) in humidified 5% CO<sub>2</sub> at 37°C for the desired time. The flasks were then removed, the medium aspirated, and the cells washed once with 5 ml of solution A. Medium containing dialyzed serum and the desired analogues was then added and the flasks returned to the incubator for the indicated time. The media were removed, the monolayers washed with solution A, and extracted with 2 ml of cold 0.4 N perchloric acid which was neutralized and analyzed as described below. Cell numbers were determined in duplicate flasks.

**Analysis of Cellular GTP, 3-DeazaGTP, and TAD.** Nucleotide pools

were extracted *in situ*, with 2 ml of cold 0.4 N perchloric acid and the extracts neutralized with KOH and centrifuged to remove the precipitate. Nucleotides were separated by a modification of the HPLC method of Nelson *et al.* (11) using an ALC 204 high-pressure liquid chromatograph (Waters Associates, Milford, MA) equipped with two model 6000A pumps, a system controller, and a column of Partisil-10 SAX anion exchange resin (25 x 4.6 mm; Whatman, Inc., Bedford, MA). Cell extracts ( $1-2 \times 10^6$  cell equivalents) were injected by the U6K injection system and eluted with a concave gradient (curve 7 on the system controller) of from 40 to 100% buffer B in 40 min at a flow rate of 2 ml/min. Buffer A was composed of 0.006 M  $\text{NH}_4\text{H}_2\text{PO}_4$ , pH 2.8, and buffer B contained 0.75 M  $\text{NH}_4\text{H}_2\text{PO}_4$ , pH 3.2. The eluted compounds were detected at 280 nm by a Waters model 440 detector and quantitated using a model 730 data module. Nucleotides were identified by comparing their retention times with those of known standards.

Determination of cellular TAD concentration was carried out using the system described above with a concave gradient (curve 7 on the system controller) from 100% buffer A to 40% buffer B (pH 3.5) in 30 min at a flow rate of 2 ml/min. Absorbance was monitored at 254 nm with the model 440 detector and quantified as described previously.

Measurement of Precursor Incorporation into Perchloric Acid-soluble and -insoluble Material. The incorporation of [ $^3\text{H}$ ]deoxycytidine and [ $^3\text{H}$ ]deoxyguanosine was measured essentially as described previously (9). Briefly, TG<sup>R</sup>-3 cells were dispensed into sterile 20-ml glass vials,  $2 \times 10^5$  cells per vial in 4 ml of medium, and incubated 18 h. The medium in each vial was removed and replaced with 1 ml of fresh medium containing dialyzed serum and the indicated concentrations of tiazofurin. After 3 h of preincubation, the media were removed, the monolayers were washed twice with solution A, and 1 ml fresh medium was added containing 3  $\mu\text{Ci}$  of the indicated radioactive precursors, [ $^3\text{H}$ ]deoxycytidine or [ $^3\text{H}$ ]deoxyguanosine. After 3 h incubation, the media were removed, the monolayers washed twice with cold 5 ml solution A and 1 ml of cold 0.4 N perchloric acid was added to each vial. After 15 min the perchloric acid (perchloric acid-soluble fraction) was removed and 0.2-ml aliquots were counted with Aquasol (New England Nuclear Corp., Boston, MA). The residue in the vials was washed with additional perchloric acid and counted directly after the addition of Aquasol.

## RESULTS

Depletion of the GTP pool in cells treated with tiazofurin and its recovery after removal of drug are illustrated in Fig. 1. Two or three hours of incubation with drug were required, regardless of concentration, for substantial depletion of the cellular GTP pool. The rate of recovery, however, differed considerably depending on the dose, being much slower in those cells treated with the higher concentration of tiazofurin. This observation indicated that treatment of cells with 500  $\mu\text{M}$  tiazofurin for 3 h would render cells deficient in guanine nucleotides for several hours and would be the appropriate treatment for the ensuing experiments. Another important consideration is that these conditions produce minimal toxicity as discussed later in this paper. Since cellular TAD can be determined by HPLC (Fig. 2) its stability in the cell was determined. Cellular

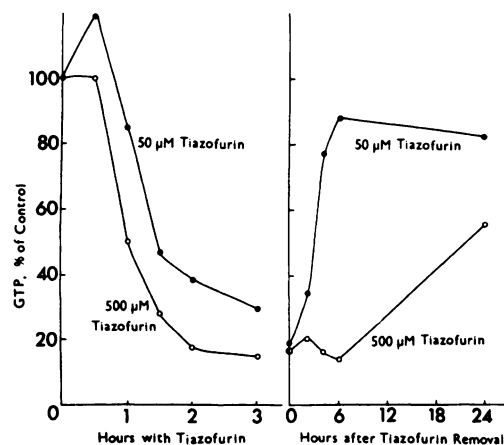


Fig. 1. Cellular GTP levels during (*left*) and after (*right*) treatment with tiazofurin. Cultures of CHO cells were treated as described in "Materials and Methods" with 50  $\mu\text{M}$  (●) or 500  $\mu\text{M}$  (○) tiazofurin for 3 h. Each determination was made using perchloric acid extract equivalent to  $1 \times 10^6$  cells. Values given are the average of determinations from duplicate flasks. Control values were 814 pmol GTP/ $10^6$  cells (*left*) and 841 pmol GTP/ $10^6$  cells (*right*).

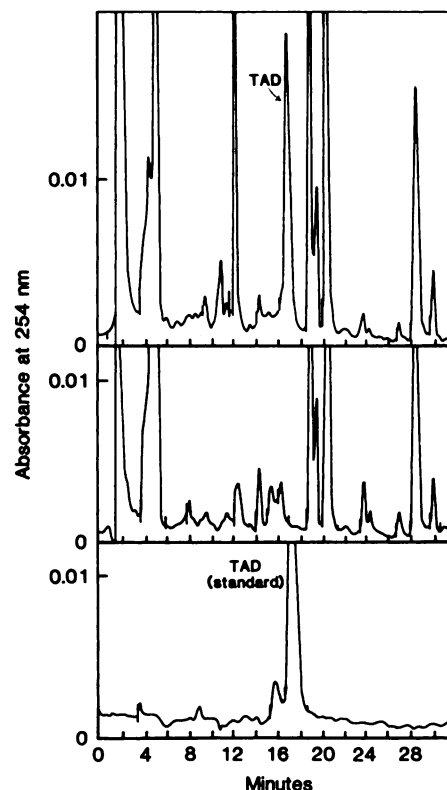


Fig. 2. HPLC analysis of TAD in cell extracts. CHO cells were incubated 3 h with (*top*) and without (*middle*) 500  $\mu\text{M}$  tiazofurin, extracted with perchloric acid, and analyzed by HPLC as described in "Materials and Methods." Profiles reflect  $1 \times 10^6$  cell equivalents. *Bottom graph*, HPLC profile of an authentic TAD standard.

degradation of TAD under these conditions (Fig. 3) was fairly rapid with a half-life of less than 1 h.

The data of Table 2 compare the effect of tiazofurin preincubation on the metabolism of 3-deazaguanine and 3-deazaguanosine to the common triphosphate derivative in CHO cells. The metabolism of the base was clearly inhibited by pretreatment with tiazofurin. This presumably reflects inhibition of HGPRTase activity by accumulated IMP or perhaps competition by its degradation product, hypoxanthine. In spite of this apparent inhibition of 3-deazaguanine metabolism, pretreatment with tiazofurin resulted in a greater ratio 3-deazaGTP/GTP owing to the rapid depletion of the cellular GTP pool.

Table 1 Properties of cell lines\*

Cell extracts were prepared and assayed as described previously (6). The minimum inhibitory drug concentrations were determined by allowing the cells to clone for 7 days in the presence of drug as described elsewhere (6). The minimum inhibitory concentration is the lowest tested concentration of drug that results in formation of clones containing fewer than 50 cells.

Cell line	HGPRTase specific activity (nmol/min/mg protein)	Minimum inhibitory concentration ( $\mu\text{M}$ )	
		3-Deazaguanine	3-Deazaguanosine
CHO	3.27	10	2.5
TG <sup>R</sup> -3	<0.01	>1000	10.0

\* Published elsewhere (6).

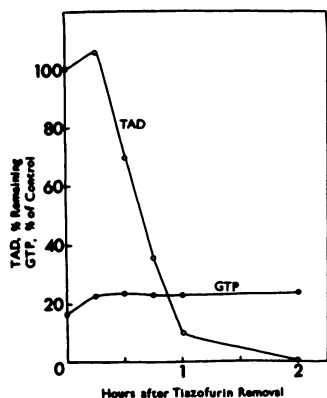


Fig. 3. Stability of cellular TAD after removal of tiazofurin from culture medium. Treatment of CHO cultures and HPLC analysis of TAD (○) and GTP (●) concentrations were as described in "Materials and Methods." The values indicated are the average of determinations from duplicate flasks. The control value for GTP was 763 pmol/10<sup>6</sup> cells and for TAD was 532 pmol/10<sup>6</sup> cells.

Table 2 Comparative metabolism of 3-deazaguanine and 3-deazaguanosine by CHO cells after treatment with tiazofurin

Procedure was as described for metabolic experiments. Cultures were all extracted at the same time, after 3 h incubation with analogues. Results shown are representative of several experiments carried out.

Additions during 1st 3 h incubation (500 μM)	Additions during 2nd 3 h incubation (200 μM)	Nucleotides/10 <sup>6</sup> cells (nmol)		
		GTP	3-DeazaGTP	3-DeazaGTP/GTP
None	None	0.67		
None	3-DeazaGua <sup>a</sup>	0.53	1.38	2.6
None	3-DeazaGuo	0.58	1.34	2.3
Tiazofurin	None	0.11		
Tiazofurin	3-DeazaGua	0.11	0.51	4.6
Tiazofurin	3-DeazaGuo	0.11	1.36	12.4

<sup>a</sup> 3-DeazaGua, 3-deazaguanine; 3-deazaGuo, 3-deazaguanosine.

The accumulation of 3-deazaGTP from the nucleoside, however, appeared to be essentially the same both with and without tiazofurin treatment. Since 3-deazaguanosine appears to be metabolized by two routes in CHO cells (6) (direct phosphorylation as well as via purine nucleoside phosphorylase degradation to the base and subsequent phosphoribosylation by HGPRTase) this probably represents increased metabolism by the direct phosphorylation route and less via the HGPRTase route. The ratio 3-deazaGTP/GTP was increased greater than 5-fold in cells incubated with tiazofurin and 3-deazaguanosine compared with those treated with 3-deazaguanosine alone.

To verify the assumption that HGPRTase activity is not necessary to obtain a substantial increase in 3-deazaGTP/GTP, a similar experiment was carried out using the HGPRTase-deficient cell line TG<sup>R</sup>-3 (Fig. 4). In this case, 3-deazaGTP and GTP levels were determined at various times during incubation of TG<sup>R</sup>-3 cells with 3-deazaguanosine. In the tiazofurin-treated cells the 3-deazaGTP rose to a significantly higher level than in those cells that were not exposed to tiazofurin. The 3-deazaGTP/GTP ratio, shown graphically in Fig. 5, dramatically illustrates the effectiveness of this treatment.

Since the GTP pool is so profoundly affected by incubation with tiazofurin it follows that the guanine deoxyribonucleotide pools would probably be reduced as well. This was tested by comparing the utilization of [<sup>3</sup>H]deoxyguanosine and [<sup>3</sup>H]deoxycytidine by the HGPRTase-deficient TG<sup>R</sup>-3 cells pretreated both with and without tiazofurin (Fig. 6). Incorporation of [<sup>3</sup>H]deoxyguanosine into perchloric acid-insoluble material was increased 5–6-fold suggesting that guanine deoxyribonucleotide levels are depressed by incubation with tiazofurin. [<sup>3</sup>H]Deoxycytidine incorporation into perchloric acid-insoluble material

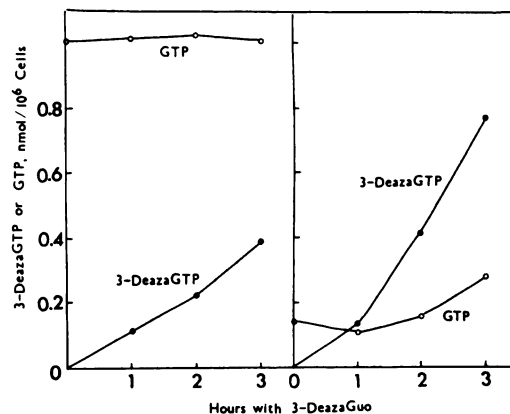


Fig. 4. Comparison of 3-deazaGTP formation from 3-deazaguanosine (3-deazaGuo) in TG<sup>R</sup>-3, HGPRTase-deficient, cells preincubated with (right) and without (left) 500 μM tiazofurin. The 3-deazaguanosine concentration was 200 μM. Perchloric acid extract equivalent to 2 × 10<sup>6</sup> cells was used for each determination as described in "Materials and Methods." 3-DeazaGTP (●); GTP (○). The data shown are representative of three experiments that were carried out.

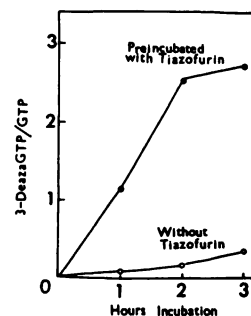


Fig. 5. Effect of preincubation with tiazofurin on the ratio 3-deazaGTP/GTP during incubation with 3-deazaguanosine. The values for 3-deazaGTP and GTP were those obtained in Fig. 3 from TG<sup>R</sup>-3 cells preincubated with (●) and without (○) 500 μM tiazofurin.

was markedly reduced in the tiazofurin-treated cells, presumably reflecting the slower rate of DNA synthesis resulting from limiting dGTP. Accordingly, there was a 3-fold increase in the accumulation of label in the acid-soluble fraction from these cells which was found by HPLC analysis to reflect primarily accumulation of dCTP (not shown). The failure of [<sup>3</sup>H]dGTP to accumulate in tiazofurin-treated cells probably reflects the rapidity with which it was incorporated into DNA since it was presumably the rate-limiting nucleotide for DNA synthesis. These observations suggested that analogues of deoxyguanosine might be more effective if used in sequential combination with tiazofurin.

The relevance of enhanced metabolism to cytotoxic activity of 3-deazaguanosine and some deoxynucleoside analogues was assessed by determining viability of the cells after exposure to drug (Table 3). Tiazofurin itself had little, if any, effect on survival under these conditions, 500 μM for 3 h. 3-Deazaguanosine, which alone demonstrates minimal toxicity under these conditions (0.5–1 mM for 12 h) showed somewhat enhanced cytotoxic activity after pretreatment with tiazofurin. The most pronounced effect, however, was seen with the deoxyguanosine analogues, 2'-deoxy-3-deazaguanosine and the arabinosyl derivative, arabinosylguanine. The failure of tiazofurin treatment to enhance the activity of β-2'-deoxythioguanosine may reflect different routes of metabolism of these compounds (or the involvement of different enzymes).

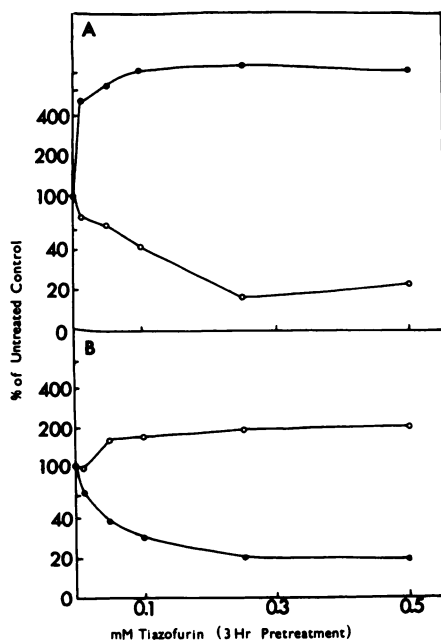


Fig. 6. Effect of tiazofurin pretreatment on utilization of deoxynucleosides by HGPRTase-deficient cells (TG<sup>-</sup>-3). TG<sup>-</sup>-3 cells,  $2.6 \times 10^5$  per vial were incubated as described in "Materials and Methods." Actual values for the untreated controls with [<sup>3</sup>H]deoxyguanosine were 753 cpm in the soluble fraction and 773 cpm in the insoluble. Values for [<sup>3</sup>H]deoxycytidine were 29,131 cpm in the soluble fraction and 34,076 in the insoluble. Incorporation of [<sup>3</sup>H]deoxyguanosine (●) and [<sup>3</sup>H]deoxycytidine (○) into perchloric acid insoluble (upper graph) and soluble (lower graph) fractions was measured.

Table 3 Effect of tiazofurin pretreatment on survival of HGPRT-deficient (TG<sup>-</sup>-3) cells with guanosine and deoxyguanosine analogues

Procedure was as described in "Materials and Methods." Plating efficiency of control of cells (no additions) was 0.6. Preincubation was 3 h; incubation with analogues, 12 h. This experiment is representative of three that were carried out.

Analogue	Concentration (mM)	Relative plating efficiency	
		Preincubation without tiazofurin	Preincubation with 500 μM tiazofurin
None		1.00 ± 0.07 <sup>a</sup>	0.98 ± 0.09
3-DeazaGuo <sup>b</sup>	0.5	0.95 ± 0.08	0.75 ± 0.08
3-DeazaGuo	1.0	0.80 ± 0.10	0.48 ± 0.05
Deoxy-3-deazaGuo	0.5	0.88 ± 0.07	0.21 ± 0.05
DeoxythioGuo	0.5	1.02 ± 0.09	0.81 ± 0.08
ArabinosylGua	0.5	0.98 ± 0.11	0.25 ± 0.06

<sup>a</sup> Mean ± SD; N = 4.

<sup>b</sup> 3-DeazaGuo, 3-deazaguanosine; deoxy-3-deazaGuo, 2'-deoxy-3-deazaguanosine; deoxythioGuo, β-2'-deoxythioguanosine; arabinosylGua, arabinosylguanine.

## DISCUSSION

Although tiazofurin has been somewhat disappointing as a single agent in cancer therapy, its ability to rapidly and effectively impair cellular guanylate synthesis provides a useful tool for modulating guanylate pools and thus altering the metabolism and activity of certain guanosine analogues. The series of experiments described here was designed to test the hypothesis that this combination of nucleoside analogues may be a rational therapeutic approach and one that can be monitored biochemically.

Smejkal *et al.* (12) have presented suggestive evidence for synergism between tiazofurin and 3-deazaguanosine when administered together. Sequential combination, in which tiazofurin is given first, was chosen for the experiments described here for several reasons: (a) there is suggestive evidence in the literature (13) indicating that tiazofurin may interfere with nucleoside transport; (b) the identities of the enzyme(s) that phosphorylate tiazofurin and 3-deazaguanosine are unknown,

thus, the two compounds may possibly compete for the same one; (c) higher levels of cellular 3-deazaGTP are achieved when cells are incubated first with tiazofurin as opposed to after or at the same time (not shown). The observations presented demonstrate enhanced (approximately 2-fold) synthesis of 3-deazaGTP from 3-deazaguanosine after treatment with tiazofurin. This phenomenon, coupled with the depleted guanylate pool, results in increased toxicity. The guanosine analogues that appear most likely to be effective in combination with tiazofurin are those that can be phosphorylated directly, rather than those that are metabolized exclusively by purine nucleoside phosphorylase-mediated degradation with subsequent phosphoribosylation by HGPRTase. The rationale for this was illustrated by the decreased cellular metabolism of the base, 3-deazaguanine, after treatment with tiazofurin, presumably reflecting inhibition of HGPRTase activity by accumulated IMP and/or competition by resulting hypoxanthine. In spite of the decreased metabolism of 3-deazaguanine, however, the ratio of analogue-triphosphate to GTP was nearly doubled by tiazofurin treatment suggesting that the sequential combination of tiazofurin and 3-deazaguanine might still result in a therapeutic advantage. This might be true for other guanine analogues as well, such as 6-thioguanine. Although Kovach and McGovern (14) tried this combination and were unable to detect synergism between tiazofurin and such agents as 6-thioguanine, 6-thioguanosine, and deoxy-6-thioguanosine they did not utilize sequential administration of drugs which might have produced different results for the reasons discussed above.

Tiazofurin enhancement of cytotoxic activity was considerably greater with 2'-deoxy-3-deazaguanosine than with 3-deazaguanosine. This could be interpreted to suggest that the deoxynucleotide may be the primary active form of 3-deazaguanine as suggested by the data of Pieper and Mandel (15). Thus tiazofurin enhancement of guanosine analogue metabolism may be a useful tool in clarifying the primary mechanisms of action of this class of nucleoside analogues as well as a potentially useful tool in therapy.

In this communication, we have demonstrated that these drug combinations can be manipulated advantageously. There are, however, many variables in these experiments (*i.e.*, concentrations of both agents, times of incubation, sequence of exposure, etc.) that are deserving of closer investigation to determine optimal conditions for synergism.

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