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MECHANISM OF RESISTANCE TO TIAZOFURIN IN HEPATOMA 3924A*

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Abstract—Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC-286193) has shown potent cytotoxic and antitumor activity against hepatoma 3924A carried in the rat [Lui *et al. J. biol. Chem.* **259**, 5078 (1984)]. However, eventually the tumor emerged, proliferated and killed the host. To throw light on the factors that play a role in the resistance to this drug, a tiazofurin-induced resistant hepatoma 3924A line in culture was produced, and its biochemical and pharmacological pattern was examined. Resistance in hepatoma cells was expressed by a reprogramming of gene expression that entailed the display of a program of multiple biochemical alterations. In the resistant cells the activity of IMP dehydrogenase, the target enzyme of tiazofurin, was increased 2- to 3-fold. The steady-state guanylate pools were elevated 3-fold, and there was a decrease in the *de novo* synthesis of guanylate. There was an expansion of guanylate salvage, which could circumvent inhibition of *de novo* guanylate synthesis by tiazofurin. For the first time in studies on the resistance of different cell lines to tiazofurin, reduced tiazofurin transport (to 50%) in resistant hepatoma cells was identified which might account for the decreased concentration (50%) of the active metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD), in these cells. NAD pyrophosphorylase activity also decreased to 53% of that of the sensitive line, which was responsible, in part at least, for the decreased TAD concentration of the resistant cells. When resistant cells were cultured in the absence of tiazofurin, resistance to the drug gradually decreased, and by 50 passages sensitivity returned. Resistance to tiazofurin in hepatoma cells appears to be a drug-induced metabolic adaptation which involves alterations in the activity of the target enzyme, in the transport and concentration of the drug and the active metabolite, and an increase of guanylate concentration and guanine salvage capacity.

Tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide, has exhibited potent antitumor activity against murine tumors [1, 2]. In addition, the drug has shown curative activity against Lewis lung carcinoma, a tumor refractory to most oncolytic agents. This observation led to clinical trials on tiazofurin [3].

Studies by these and other laboratories have established that tiazofurin in susceptible tumors is metabolized to an analog of NAD, namely TAD§, wherein the nicotinamide moiety is replaced by thiazole-4-carboxamide. TAD is a potent inhibitor of IMP dehydrogenase, causing a depression of guanylate biosynthesis which leads to an inhibition of tumor cell proliferation [4–8].

Tiazofurin exhibits potent cytotoxicity against hepatoma 3924A in culture and *in vivo* [8, 9]. Treatment of rats bearing subcutaneously implanted hepatoma 3924A with tiazofurin every third day inhibited the proliferation of the tumor by 98%; at the end of the treatment schedule, however, the hepatoma emerged, proliferated and killed the host even though treatment was continued [10]. These results prompted us to produce a drug-induced resistant variant of hepatoma 3924A in culture and to examine the factors which contributed to the expression of resistance in these cancer cells.

MATERIALS AND METHODS

Materials. Tiazofurin was provided by Dr. Van Narayanan, National Cancer Institute, National Institutes of Health, Bethesda, MD. [^{14}C]Formic acid (specific radioactivity 53.0 mCi/mmole) and [8-^{14}C]guanine (specific radioactivity 55.5 mCi/mmole) were purchased from New England Nuclear, Boston, MA. [5-^{3}H]Tiazofurin was obtained from Research Triangle Institute, Research Triangle Park, NC. Chemically synthesized TAD was a gift from Dr. Victor Marquez, National Cancer Institute, National Institutes of Health, Bethesda, MD. All other chemicals were also of the highest quality available.

Cytotoxicity studies. For examining the inhibition of cell growth, cells in log phase of growth were exposed continuously to various concentrations of

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§ Abbreviations: TAD, thiazole-4-carboxamide adenine dinucleotide; DTT, dithiothreitol; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; TRMP, tiazofurin 5′-monophosphate; TRDP, tiazofurin 5′-diphosphate; TRTP, tiazofurin 5′-triphosphate; and TRXP, total tiazofurin 5′-phosphates (TRMP, TRDP and TRTP).

tiazofurin for 24 hr, and then cell number was determined in a Coulter counter. Control cells received saline. The effect of tiazofurin on hepatoma cell survival was examined by the procedure detailed earlier [8]. In short, 500 cells were seeded in flasks along with various concentrations of tiazofurin for 7 days; the colonies were stained with crystal violet, and the surviving fraction of treated cells was calculated in relation to the percentage of colonies formed by untreated ones. Under the conditions of the assay, sensitive (3924A/S) and resistant (3924A/TR) cells exhibited a doubling time of 11.1 and 12.6 hr, respectively, and a colony plating efficiency of about 60%.

Metabolism of tiazofurin. To study the metabolism of tiazofurin, sensitive and resistant cells growing in the log phase ($15–20 \times 10^6$ cells in 30 ml) were exposed to 10 or 100 μM [5-^{3}H]tiazofurin (specific radioactivity 95.5 mCi/mmole) for 2 hr at 37°. Cells were immediately centrifuged at 1500 g for 2 min, washed twice with 5 ml of cold PBS, extracted with 300 μl of cold 10% TCA, and promptly neutralized with 0.5 M tri-*n*-octylamine in freon. An aliquot was analyzed on a Partisil 10-SAX column using an ammonium phosphate buffer system as reported earlier [5].

Enzyme studies. Hepatoma 3924A/S and 3924A/TR cells in the log phase of growth were harvested by centrifugation at 1000 g for 10 min and washed twice with cold PBS. Cell pellets (10^8 to 10^9 cells) were homogenized (1:5 w/v) in 0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4. After centrifugation at 100,000 g for 60 min, the supernatant fraction was used for the determination of IMP dehydrogenase activity [11].

For measuring NAD pyrophosphorylase activity, cell pellets (see above) were homogenized (1:4, w/v) in 0.25 M sucrose containing 1 mM DTT and 20 mM Tris–HCl, pH 7.4. The pellet (3000 g) was washed twice and finally resuspended in 20 mM Tris–HCl, pH 7.4, containing 1 mM DTT. The suspension was used for measuring NAD pyrophosphorylase activity. The technique for measuring NAD pyrophosphorylase activity was essentially the same as reported [2].

Measurement of nucleotide concentrations. Hepatoma cells growing in log phase in McCoy's 5A medium containing 10% dialyzed fetal calf serum ($15–20 \times 10^6$ cells in 30 ml) were incubated at 37° with saline or tiazofurin (10 or 100 μM) for 2 hr. Cells were collected by centrifugation at 1000 g for 2 min, washed twice with cold PBS, and then extracted with 300 μl of cold 10% TCA. The TCA extracts were centrifuged at 18,000 g for 1 min and then quickly neutralized with 0.5 M tri-*n*-octylamine in freon. An aliquot of the neutralized TCA extract was analyzed for the nucleotide pools on a Partisil 10-SAX column of a Waters HPLC system using an ammonium phosphate buffer system as detailed earlier [5].

Studies of biosynthesis and salvage flux. Hepatoma cells growing in log phase (see above) ($15–20 \times 10^6$ cells in 30 ml) were incubated with saline or tiazofurin (100 μM) for 90 min at 37°; then [^{14}C]-formate (5 μCi, 0.09 μmole) or [8-^{14}C]guanine (5 μCi, 0.09 μmole) was added to the flasks, mixed, and further incubated for 30 min. Cells were quickly centrifuged at 1500 g for 2 min and washed twice with cold PBS. TCA extracts were prepared and analyzed for nucleotide pools as described above.

Transport studies. Hepatoma cells (3924A/S or 3924A/TR) growing in log phase in McCoy's 5A medium containing 10% dialyzed fetal calf serum were centrifuged at 1500 g for 2 min. Cells were washed twice with PBS containing 0.1% glucose and then resuspended in the same buffer at a cell density of 2.5×10^6 cells/ml. Cells were incubated at 37°. The techniques for performing uptake studies were essentially as reported earlier [12]. Briefly, to 2 ml of cell suspension at 37° various concentrations of [5-^{3}H]tiazofurin (7.8 to 500 μM, specific radioactivity 83 mCi/mmole) were added and mixed, and an aliquot of 0.5 ml was transferred to microcentrifuge tubes containing 600 μl of versilube F50 silicone oil. Incubations were terminated at various times (0.25 to 7.5 min) by centrifugation of cells through the oil at 12,000 g for 1 min in an Eppendorf centrifuge. The cell pellet was solubilized in 1.5 ml of 1 N NaOH and neutralized, and the radioactivity was determined by liquid scintillation spectrometry. Data were corrected for entrapped extracellular drug using [^{14}C]inulin as marker.

Examination of the stability of resistance to tiazofurin. Resistant cells of hepatoma (3924A/TR) growing in the presence of 10 mM tiazofurin were subcultured in the absence of drug selection pressure over a number of generations, and the cytotoxicity of tiazofurin towards the cells was determined periodically.

RESULTS

Development of a drug-induced resistant variant of hepatoma 3924A. Hepatoma 3924A cells in the log phase of growth were exposed to increasingly sublethal concentrations of tiazofurin (1 μM to 10 mM) over 115 generations. A resistant variant (3924A/TR) was selected which exhibited an IC_{50} (50% inhibition of cell growth) of 5 mM compared to an IC_{50} of 16 μM in the sensitive parent line (Fig. 1). In the clonogenic assay, the sensitive and resistant cells exhibited an LC_{50} (50% inhibition of colony formation) of 3 and 400 μM respectively (Fig. 1). These sensitive (3924A/S) and resistant (3924A/TR) lines were used to elucidate the pharmacologic and biochemical metabolic properties.

Metabolism of tiazofurin by hepatoma cells. Since the mechanism of action of tiazofurin involves activation of the drug to TAD which exerts its biochemical pharmacologic actions, the metabolism of tiazofurin in the two lines was examined. As indicated in Table 1, 3924A/TR cells formed less of the active metabolite, TAD, than the sensitive cells. Comparison of the concentration of TAD to tiazofurin nucleotides indicated a ratio of 1 in the sensitive cells, whereas in 3924A/TR cells the ratio was 0.5, showing that TAD was formed abundantly only in the sensitive line (Table 1).

IMP dehydrogenase activity. The mechanism of action of tiazofurin is thought to be due to the formation of TAD which potently inhibits IMP dehydrogenase activity [4–8]. Thus, biochemical expression of resistance to the drug could involve

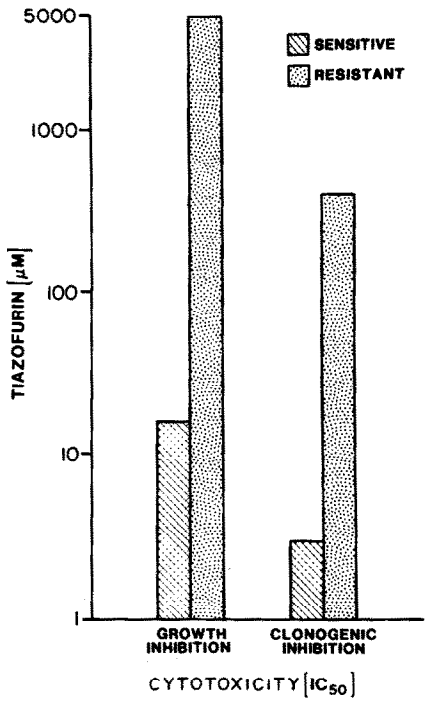

Fig. 1. Cytotoxicity of tiazofurin to hepatoma cells in culture. The techniques for the cytotoxicity measurements are cited in Materials and Methods. Cells in culture were exposed continuously to the drug for 24 hr at 37°, and the concentration of drug required to produce 50% inhibition of cell growth (IC_{50}) was expressed as the measure of growth inhibition. For studying clonogenic inhibition, cells were exposed to tiazofurin continuously for 7 days, and the concentration of drug required to produce 50% reduction in colony formation (LC_{50}) was expressed.

increased IMP dehydrogenase activity in the resistant line. Indeed, as shown in Table 2, IMP dehydrogenase activity in the resistant line was elevated 2.6-fold over that of the sensitive line.

NAD pyrophosphorylase activity. Expression of resistance in some cell lines was due to decreased TAD formation attributed to a decreased NAD pyrophosphorylase activity [9]. To test this possibility, the basal activity of NAD pyrophosphorylase was measured in the two lines. Table 2 shows that NAD pyrophosphorylase activity was 3.1-fold higher in the sensitive line than in the resistant cells.

Guanylate pools and the effect of tiazofurin. Since tiazofurin treatment depletes guanylate and dGTP concentrations in hepatoma 3924A [8], it was of interest to determine nucleotide pools. In the resistant line the basal concentrations of the guanylates were 2-to 3-fold elevated compared to the sensitive line (Table 3). Tiazofurin treatment depleted guanylate pools in the sensitive line without significantly decreasing guanylates in the resistant line (Table 3).

IMP pools and the effect of tiazofurin. Tiazofurin injection in rats bearing hepatoma 3924A causes a 15-fold enlargement of the IMP pools [8], and this rise can serve as a marker for tiazofurin action. The steady-state IMP concentrations were compared in the two lines along with their responses to tiazofurin challenge (Table 3). Basal pools of IMP were larger

Table 1. Metabolism of tiazofurin by 3924A/S and 3924A/TR cells in culture

Cell types	Tiazofurin (μM)	Tiazofurin metabolites (pmoles/g cells)				Tiazofurin metabolites		
		TAD	TR-MP	TR-DP	TR-TP	TRXP	Total metabolites	Ratio of TAD/TRXP
3924A/S	10	31.1 ± 0.1	28.5 ± 2.2	1.5 ± 0.3	0.6 ± 0	30.6 ± 2.5	61.7 ± 2.6	1.02
	100	192.6 ± 1.8	188.2 ± 10.2	5.1 ± 0.9	14.7 ± 0.9	208.0 ± 12.0	400.6 ± 13.8	0.93
3924A/TR	10	21.7 ± 0.6*	52.1 ± 17.4	<0.1*	<0.1*	52.1 ± 17.4	73.8 ± 18.0	0.42
	100	83.2 ± 8.7*	148.7 ± 10.0*	<0.1*	<0.1*	148.7 ± 10.0*	231.9 ± 18.7*	0.56

Means ± S.E. of four or more experiments are given. 3924A/S and 3924A/TR cells growing in log phase were incubated with 10 or 100 μM [5-^{3}H]tiazofurin (specific radioactivity 95.5 mCi/mmole; 0.1 mCi/ml) for 2 hr at 37°. Cells were processed, and tiazofurin metabolites were determined as cited in Materials and Methods; 200×10^6 cells were considered as 1 g of cells in all these studies.

* Significantly different from values of 3924A/S ($P < 0.05$).

Table 2. Activities of IMP dehydrogenase and NAD pyrophosphorylase in tiazofurin sensitive and resistant hepatoma cells

Cells	IMP dehydrogenase activity		NAD pyrophosphorylase activity	
	nmoles/mg protein/hr	% of 3924A/S	nmoles/mg protein/hr	% of 3924A/S
3924A/S	27.1 ± 1.6	100	8.9 ± 1.9	100
3924A/TR	70.1 ± 6.3	259*	2.9 ± 0.9	33*

IMP dehydrogenase and NAD pyrophosphorylase activities in the cell extracts were determined as cited in Materials and Methods. Activities are expressed as means ± S.E. of three or more assays.
* Significantly different from that of sensitive cells ($P < 0.05$).

in the resistant line (163%) than in the sensitive line. Tiazofurin (10 μM) incubation expanded the IMP pools to 759% and 460% compared to the control values in the sensitive and resistant lines. The absolute concentrations of IMP in the two lines following tiazofurin injection were similar due to initial high levels of IMP in the resistant line.

Adenylate pools and the effect of tiazofurin. Since tiazofurin decreased guanylate pools with a concurrent rise in IMP pools, it was of interest to determine whether IMP was channeled towards AMP synthesis. To examine this point, cells were incubated with tiazofurin for 2 hr and then analyzed for total adenylate pools. The steady-state concentrations of total adenylates were higher in the resistant than in the sensitive cells (169%) (Table 3). However, tiazofurin treatment did not alter significantly the adenylate pools.

Examination of activities of purine de novo *and salvage biosynthetic pathways in 3924A/S and 3924A/TR cells.* Expression of resistance in 3924A/TR cells could involve changes in the relationship of the activities of biosynthetic and salvage pathways, and hence it was relevant to determine the behavior of the activities of the *de novo* synthesis and salvage of guanylates. Flux of [^{14}C]formate into guanylates was taken as the measure of the activity of the biosynthetic pathway, and the flux of [^{14}C]guanine into guanylates was used as the index of salvage pathway activity (Table 4). The results indicate that in the resistant cell line biosynthesis of guanylates from [^{14}C]formate was decreased to 58%, whereas salvage of [^{14}C]guanine into guanylates was expanded to 277% as compared to activities of the sensitive cells. Thus, the ratio of the activity of the salvage versus *de novo* pathway in the resistant line was increased to 476% of that of the sensitive line.

Effect of tiazofurin on the de novo *biosynthesis of IMP in 3924A/S and 3924A/TR cells.* IMP pools were increased in both sensitive and resistant cells following tiazofurin incubation (Table 3). The flux of [^{14}C]formate into IMP in the resistant cells (control) was 22% of that of the sensitive line (Table 5) (3.2 ± 0.8 nCi/g compared to 14.3 ± 0.9 nCi/g in the sensitive line). By contrast, resistant cells have 164% higher IMP pools (36.3 ± 3.6 nmoles/g compared to 22.2 ± 1.2 nmoles/g in the sensitive line) and thus the specific activity of IMP in the resistant line (88 pCi/nmole) was 14% of that of the sensitive one (644 pCi/nmole). Tiazofurin incubation decreased the flux of formate into IMP only in the sensitive line (to 62%). Tiazofurin treatment decreased the specific activity of IMP in the sensitive line by 10-fold, whereas in the resistant line this decrease was only 2.7-fold. These studies indicate that sensitive cells depend on the *de novo* pathway 7-fold more than the resistant cells.

Salvage of [^{14}C]guanine into guanylates in 3924A/

Table 3. Effect of tiazofurin on guanylate pools in hepatoma 3924A cells

Nucleotides	Tiazofurin (μM)	Pools (nmoles/g cells)				
		3924A/S	% of Control	3924A/TR	% of Control	% of 3924A/S
GMP	Control	5.3 ± 0.7	100	12.6 ± 0.6	100	238†
	10	0.5 ± 0.1	9*	11.3 ± 0.1	90	2260†
GDP	Control	20.3 ± 0.7	100	62.3 ± 8.1	100	307†
	10	14.6 ± 0.8	72*	49.5 ± 8.2	80	339†
GTP	Control	69.1 ± 6.7	100	217.4 ± 43.5	100	315†
	10	37.9 ± 0.0	55*	154.8 ± 34.8	71	408†
Sum of guanylates	Control	94.7 ± 8.1	100	292.3 ± 52.2	100	309†
	10	57.5 ± 0.8	61	215.6 ± 43.0	74	375†
IMP	Control	22.2 ± 1.1	100	36.3 ± 3.6	100	163†
	10	168.4 ± 36.5	759*	167.1 ± 35.5	460*	99
Sum of adenylates	Control	493.3 ± 42.0	100	832.7 ± 148.8	100	169†
	10	552.1 ± 31.9	112	698.8 ± 120.4	84	127

Means ± S.E. of four or more experiments in each group are given. Cells growing in log phase were incubated with saline or 10 μM tiazofurin for 2 hr at 37°. Cells were processed and nucleotides were determined as cited in Materials and Methods.
* Significantly different from values of the control group ($P < 0.05$).
† Significantly different from values of the sensitive cells ($P < 0.05$).

Table 4. Flux of [^{14}C]formate into *de novo* synthesis and salvage of [^{14}C]guanine into guanylate synthesis in hepatoma cells

	Flux (nCi/g cells)					
	Biosynthesis		Salvage		Ratio of	
Cell lines	([^{14}C]formate)	% of 3924A/S	([^{14}C]guanine)	% of 3924A/S	([^{14}C]guanine)/([^{14}C]formate)	% of 3924A/S
3924A/S	26.7 ± 2.0	100	551.8 ± 19.6	100	20.7	100
3924A/TR	15.5 ± 2.1	58*	1529.0 ± 49.6	277*	98.6†	476†

Means ± S.E. of four or more determinations in each group are given. The measurements of flux of [^{14}C]-formate and [^{14}C]guanine in cells were performed as cited in Materials and Methods.

* Significantly different from values of controls ($P < 0.05$).

† Significantly different from values of sensitive cells ($P < 0.05$).

S and 3924A/TR cells. Expression of resistance could involve avid salvage of preformed guanine to guanylates, thereby circumventing the *de novo* biosynthetic blockage of guanylates by tiazofurin. In accordance with this hypothesis, the activity of salvage of guanine to guanylates was 3-fold higher in the resistant line (1529.0 ± 49.6 nCi/g compared to 551.8 ± 19.6 nCi/g in the sensitive cells) (Table 5). As a result of increased salvage, guanylate pools in the resistant line were also high. Thus, the specific radioactivity in the two lines was similar. Tiazofurin incubation markedly depressed the incorporation of guanine into guanylates in both lines. However, because of increased basal activity of the salvage pathway in the resistant line, there was still 2- to 3-fold more incorporation of guanine into guanylates, resulting in 4-fold higher levels of guanylate pools in the resistant line (196.0 ± 46.4 nmoles/g) compared to the sensitive one (48.1 ± 1.1 nmoles/g) even after tiazofurin treatment. Thus, in spite of similar responses to tiazofurin in the two lines, the resistant line succeeded in maintaining high levels of guanylates.

Transport of tiazofurin by 3924A/S and 3924A/TR cells in culture. Decreased transport of the drug could protect resistant cells from tiazofurin toxicity and thus could be an expression of the resistance phenomenon. To examine this facet, cells were exposed to [5-^{3}H]tiazofurin, aliquots were sampled from 0.2 to 7.5 min, and the radioactivity in the cell was determined. As indicated in Fig. 2, resistant cells exhibited a 50% reduced uptake of the drug with a V_{max} of 100 pmoles/10^6 cells/min, compared to sensitive cells (V_{max}, 200 pmoles/10^6 cells/min) with similar K_m (0.67 mM).

Stability of resistance. To test whether the resistant property expressed by the cells was due to cell mutation or adaptation, resistant cells were grown in the absence of drug-selection pressure (tiazofurin). Resistance to tiazofurin gradually decreased, and after 50 passages the cells exhibited an IC_{50} of 15 μM which was similar to that of the sensitive cells.

DISCUSSION

Tiazofurin is now undergoing phase I clinical trials,

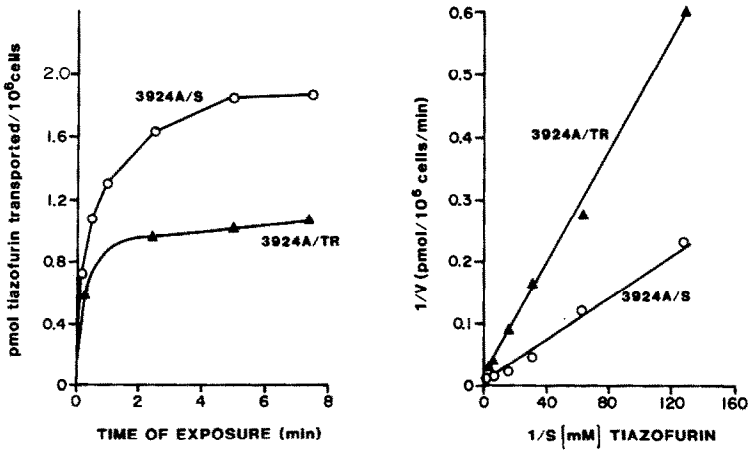

Fig. 2. Transport of tiazofurin into hepatoma cells in culture. Hepatoma cells in the exponential phase of growth were exposed to [5-^{3}H]tiazofurin (125 μM), and aliquots of cells were sampled from 0.25 to 7.5 min according to the procedures given in Materials and Methods (left panel). To determine the kinetics of transport, cells (3924A/S or 3924A/TR) growing in log phase were incubated with various concentrations of [5-^{3}H]tiazofurin (7.8 to 500 μM) for 0.25 or 0.50 min at 37°, and the reaction was terminated by quickly spinning the cells through oil according to the technique cited in Materials and Methods (right panel).

and it is important to elucidate all possible biochemical mechanisms of resistance in the hope that suitable modifications in therapy can be instituted either to overcome the resistance phenomenon or prevent it.

Tiazofurin metabolism to TAD in drug-induced resistant lines is substantially blocked due to very low activity of NAD pyrophosphorylase which catalyzes the conversion of tiazofurin monophosphate to TAD [13, 14]. In the case of the resistant hepatoma line (3924A/TR), unlike other drug-induced resistant cells, TAD formation was decreased only by 2-fold. Activity of NAD pyrophosphorylase was reduced (3.1-fold) in the resistant line but NAD pyrophosphorylase activity was not substantially deleted as was observed in other drug-induced lines [13, 14]. Small amounts of the other anabolites of tiazofurin, namely TR-DP and TR-TP, were found only in the sensitive line (3924A/S), the significance of which is not yet clear. Further studies are in progress to examine the roles of TR-DP and TR-TP in the mechanisms of sensitivity and resistance.

Since IMP dehydrogenase is the target of tiazofurin action, it is conceivable to predict an amplification of the IMP dehydrogenase gene in the resistant line. A 2.6-fold increase in IMP dehydrogenase activity in the resistant line is in accordance with this expectation. Such an increase in IMP dehydrogenase activity has not been observed in other drug-induced lines [13].

Guanylate pools were decreased significantly following tiazofurin incubation in the sensitive line; however, no such changes were observed in the resistant line. In fact, the basal pools of guanylates were significantly higher in the resistant line (3-fold). The reason for this increase in the pool might be due to an increased salvage activity of guanylates (2.8-fold).

Radioactive flux studies indicated that the sensitive line seems to substantially depend on the *de novo* pathway for its guanylate synthesis and this was inhibited by tiazofurin action. By contrast, the resistant line exhibited enhanced salvage capability of guanylates with reduced synthesis of guanylates via the *de novo* pathway. Since tiazofurin inhibits *de novo* guanylate synthesis more potently than salvage activity, the resistant line ought to be refractory to tiazofurin inhibition. However, such changes in the *de novo* and salvage activities have not been found in the drug-induced resistant line of P388 leukemia.*

Impeded transport of the drug by the resistant line can be a mechanism for drug resistance. In the hepatoma cells resistant to tiazofurin, the V_{max} of drug uptake was 2-fold reduced compared to the sensitive line. This is the first time that restricted transport has been implicated in the mechanism of resistance to tiazofurin in tumor cells.

Extrapolation of these findings to murine tumors naturally sensitive and spontaneously resistant to tiazofurin has indicated a good correlation between

Table 5. Effect of tiazofurin on the *de novo* biosynthesis of IMP and salvage of guanine by hepatoma 3924A cells

Cell type	Tiazofurin (μM)	[^{14}C]Formate flux (nCi/g)	IMP pool (nmoles/g cells)	Specific activity (pCi/nmole)	Specific activity % of Control	% of Control specific activity	[^{14}C]Guanine flux (nCi/g) into total guanylates	Total guanylates (nmoles/g)	Specific activity (nCi/nmole)	% of Control specific activity
3924A/S	Control	14.3 ± 0.9	22.2 ± 1.2	644	100	100	551.8 ± 19.6	94.7 ± 8.1	5.8	100
	100	8.9 ± 0.2*	142.2 ± 9.4*	63*	10*		149.1 ± 13.9*	48.1 ± 1.1*	3.1	53*
3924A/TR	Control	3.2 ± 0.8	36.3 ± 3.6†	88†	100	14†	1529.0 ± 49.6†	292.3 ± 52.2†	5.2	100
	100	4.3 ± 1.1†	130.4 ± 6.1*	33*	38*		344.6 ± 22.5*†	196.0 ± 46.4†	1.8†	35*†

Means ± S.E. of four or more determinations in each group are given. Procedures are as cited in Materials and Methods.
* Significantly different from values of controls ($P < 0.05$).
† Significantly different from values of sensitive cells ($P < 0.05$).

* D. A. Cooney, H. N. Jayaram, G. Ahluwalia, R. L. Dion, L. A. Zwelling, D. Kerrigan and D. G. Johns, presented at the *Thirteenth International Congress of Chemotherapy* (Eds. K. H. Spitzy and K. Karrer), Verlag H. Egermann, Vienna, Austria (1983).

TAD accumulation and the sensitive nature of the tumor [2]. Sensitive tumors exhibit higher specific activity of NAD pyrophosphorylase, which is responsible for TAD synthesis. On the other hand, resistant tumors show greater capacity to degrade TAD (higher TADase activity). The ratio of synthetic to degradative activity revealed a net synthetic activity for the sensitive tumors versus a net degradative activity for the resistant tumors [2].

To extend these observations to human tumor systems, Earle and Glazer [15] have examined the cytotoxicity of tiazofurin in four human lymphoid cell lines; the lines with higher sensitivity to tiazofurin formed somewhat larger amounts of TAD compared with the less sensitive ones.

Metabolism of tiazofurin and its pharmacologic actions have been examined in six human lung carcinoma cell lines, four of which are sensitive to the drug and two of which are less sensitive to the drug [16]. Overall, there was a positive correlation between drug sensitivity, increased formation of TAD, and reduced pools of guanylates [16].

The principal purpose of the present study was to elucidate the biochemical mechanisms of resistance in hepatoma 3924A cells. Tiazofurin has exhibited potent antitumor activity against rat hepatoma 3924A [8]; however, emergence of tumor cell population was noted even after continued drug treatment, indicating that either the cells have adapted to grow with altered biochemical parameters or a resistant cell population has emerged with altered gene expression. To examine this fact further, a drug-induced resistant line was selected.

Studies so far indicate that the resistance to tiazofurin in hepatoma 3924A/TR cells is a drug-induced adaptation or an unstable mutation, because the resistant cells revert to the sensitive state after removal of the drug-selection pressure.

Biochemical changes induced by resistance to tiazofurin in the hepatoma cells (3924A/TR) were decreased transport (2-fold), TAD synthesis (2-fold) and *de novo* synthesis of IMP (4.7-fold) compared to the sensitive line. In the resistant hepatoma cells there were also increases of IMP dehydrogenase activity (2.6-fold), guanylate pools (3-fold), guanylate salvage activity (2.8-fold) and the ratio of salvage to *de novo* synthetic activity favoring salvage (4.8-fold). Thus, these metabolic features are expressed as an integrated pattern of biochemical markers of tiazofurin resistance in hepatoma cells.

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