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# Growth Inhibition and Induction of Phenotypic Alterations by Tiazofurin: Differential Effects on MCF-7 Breast Cancer and HBL-100 Breast Cell Lines

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**Abstract**—*The effect of the nucleoside anti-metabolite tiazofurin (TR) was examined on the growth and phenotypic alterations of MCF-7 breast cancer and HBL-100 normal breast cell lines. TR was shown to inhibit MCF-7 cell growth. This inhibition could be reversed by exogenous addition of guanosine. The anti-proliferative effect of TR is accompanied by phenotypic alterations that include lipid accumulation and an increase in alkaline phosphatase activity. In contrast to MCF-7 cells, the HBL-100 breast milk derived cell line is relatively resistant to inhibition by TR. Alkaline phosphatase is not affected by TR and untreated cells accumulate lipid droplets, similar to TR-treated MCF-7 cells. Determination of GTP and ATP pools in both cell lines revealed that TR markedly reduces GTP content in MCF-7 cells. In HBL-100 cells, TR induces only a small decrease in GTP and does not affect ATP levels. The prototypic IMP dehydrogenase inhibitor, mycophenolic acid (MA), markedly inhibits HBL-100 cell growth, similarly to its effect on MCF-7 breast cancer cells. These findings may suggest differential metabolism of TR in MCF-7 and HBL-100 cells.*

## INTRODUCTION

THE c nucleoside analog tiazofurin (TR) is a potent inhibitor of IMP dehydrogenase, a key enzyme in the synthesis of guanine ribonucleotides (GuRN) [1]. GuRN play a major role in multiple cellular functions such as DNA, RNA and protein synthesis [2]. The activity of IMP dehydrogenase was shown to be markedly increased in transformed cells [3]. IMP dehydrogenase inhibitors were found to be cytotoxic towards several cancer cell types [4, 5]. TR was shown to exert anti-proliferative effects on several cancer cell types, including leukemia, hepatoma and lung cancer cells [1, 6–8]. This compound showed marked *in vivo* antitumor activity against Lewis lung carcinoma [6, 9] and has been introduced into clinical trials [10]. TR has also been shown to induce differentiation in the promyelocytic cell line HL-60, similar to the prototypic IMP dehydrogenase inhibitor, mycophenolic acid (MA) [11, 12].

Our recent studies have shown that MA inhibits MCF-7 breast cancer cell growth and induces phenotypic alterations that are in part associated with cell differentiation [13]. In the present study we examined the effects of TR on cell growth and phenotypic alterations of MCF-7 breast cancer cells in comparison to HBL-100 cells, originating from normal breast milk [14]. It is shown that TR selectively inhibits cell growth and induces phenotypic alterations in the MCF-7 cells, whereas HBL-100 non-cancer cells are relatively resistant.

## MATERIALS AND METHODS

Reagents and fine chemicals for enzyme assay were purchased from Sigma Chem. Comp. Media and tissue culture supplements were obtained from Biol. Industries, Israel.

### *Cell cultures*

MCF-7 breast cancer cells were cultured in RPMI-1640, supplemented with 10% fetal calf serum and antibiotics. HBL-100 breast cells were

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cultured in RPMI 1640 supplemented with 15% fetal calf serum, insulin (0.5 U/ml) and antibiotics. In selected experiments the MCF-7 cells (that do not require the addition of insulin for growth) were incubated in a medium containing 15% fetal calf serum and insulin (0.5 U/ml), similar to the HBL-100 cell line. Cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Cells were passaged every 3–4 days, using a trypsin/EDTA solution (Biol. Industries) for detachment.

*Cell growth experiments*

For cell growth measurements $10^5$ cells were plated in 1 ml culture medium in culture dishes (3.5 cm). Three hours later, after most cells had attached to the bottom of the culture dish, TR or MA were added. Seventy-two or 96 h later, cells were detached and counted in a Coulter counter. Cell detachment was done by incubating the cells with 1 mM EDTA in Ca/Mg free phosphate buffered saline. Cell clumps were dispersed mechanically by intensive pipeting. Cell viability was assessed by the trypan blue exclusion test.

*Lipid staining*

Cell culture dishes were washed with phosphate buffered saline, fixed overnight with formol-calcium and stained by oil red O method [15]. The stained cell cultures were covered with cover slips using the glycerine jelly mount.

*Extraction and determination of alkaline phosphatase activity*

$10^6$ cells were plated in 10 ml culture medium and incubated in the presence of TR or MA for 72 h. Cell extracts and alkaline phosphatase activity determinations were done as previously described [13, 16]. Enzyme activity was expressed as nmoles *p*-nitrophenol formed/h/mg protein. Protein was determined by the method of Lowry *et al.* [17] with bovine serum albumin as a standard.

*Determination of GTP and ATP pools*

Cells were plated at $10^5$ cells/ml in 10 ml culture medium in culture dishes (9 cm) for 72 h. Nucleotides were extracted as follows: Cells were washed with ice cold phosphate buffered saline and rapidly frozen in liquid nitrogen. The frozen cells were scraped into ice-cold $HClO_4$ (1 M) for 2 min. Extracts were neutralized with KOH and the supernatant obtained following centrifugation was used for nucleotide determination. GTP and ATP were measured by HPLC using a SAX column as previously described [13, 18]. Prior to extraction with $HClO_4$ a sample of lyzed cells was taken for protein determination.

*Statistical analysis*

Student's *t* test was used to evaluate the significance of differences between untreated and treated cells. A *P* value of 0.05 is considered to be significant.

## RESULTS

*Effects of TR on cell growth*

The effect of TR on MCF-7 breast cancer and HBL-100 breast cancer growth was examined. MCF-7 cells were incubated for 72 h with TR and HBL-100 cells for 96 h in order to obtain similar cell numbers. The results depicted in Fig. 1 show that TR markedly inhibits MCF-7 cell growth at the micromolar concentration range. Incubation of MCF-7 cells for 72 h in the presence of 5 μM TR results in a 50% decrease in cell number. Complete growth inhibition is achieved at 10 μM TR. Viability of the cells as assessed by the trypan blue exclusion test is not reduced at these concentrations. Growth inhibition can be reversed by the addition of exogenous guanosine (Table 1). In contrast to MCF-7 breast cancer cells, the non-tumorigenic HBL-100 cells are relatively resistant to the action of TR. A dose as high as 50 μM reduced cell number by only about 30% (Fig. 1). The differences

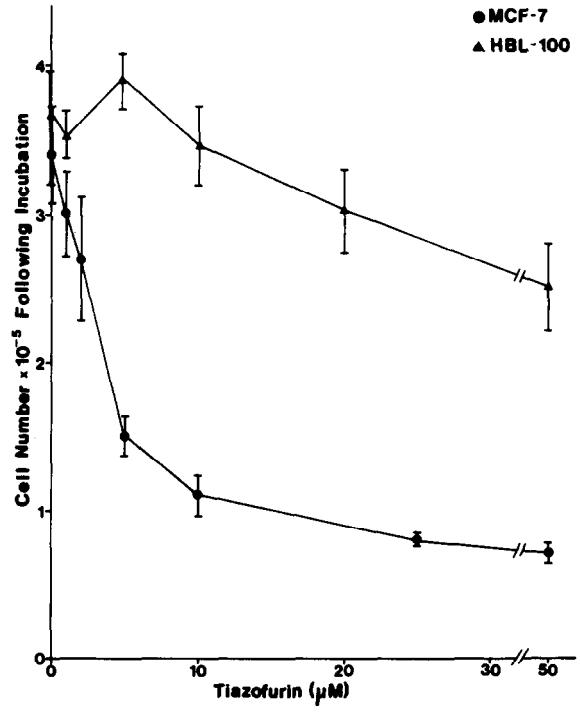


*Fig. 1. The effect of TR on MCF-7 breast cancer and HBL-100 cancer cell growth. MCF-7 cells were incubated in the presence of the specified concentrations of TR for 72 h and HBL-100 cells for 96 h. Initial cell number for both cell lines was 1.5 × $10^5$. Cells were detached and counted as described in Materials and Methods. Values are means ± S.E. of 4–11 replicates done with different cell preparations. HBL-100: TR 50 μM vs. control* $P < 0.05$. *MCF-7: TR 5, 10, 25, 50 μM vs. control* $P < 0.001$.

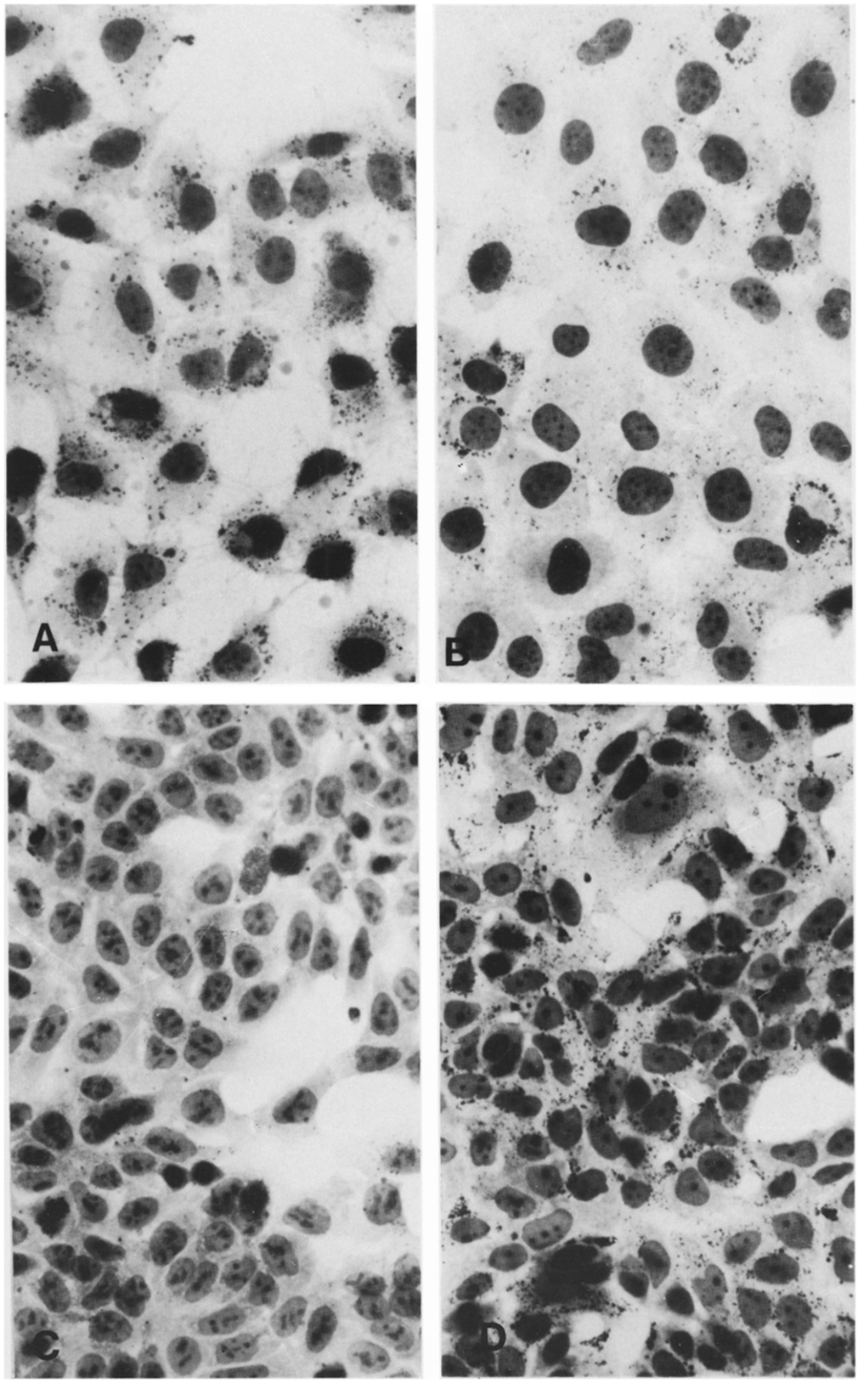


*Fig. 2. Lipid content in untreated and TR-treated MCF-7 breast cancer and HBL-100 breast cells. 10⁵ MCF-7 and HBL-100 cells were incubated in the absence or presence of TR for 72 and 96 h respectively. Cells were stained by the oil red O method and visualized by light microscopy. A. Untreated HBL-100 cells. B. HBL-100 TR-treated cells. C. Untreated MCF-7 cells. D. TR-treated MCF-7 cells. × 200.*

*Table 1. The effect of exogenous addition of guanosine on untreated or TR-treated MCF-7 cell growth*

| Treatment | Cell number × $10^5$ after 72 h incubation |
|---|---|
| None | 4.3 ± 0.1 |
| Guanosine | 4.3 ± 0.5 |
| TR (5 μM) | 1.7 ± 0.2 |
| Guanosine + TR (5 μM) | 3.4 ± 0.4 |
| TR (10 μM) | 1.0 ± 0.1 |
| Guanosine + TR (10 μM) | 3.5 ± 0.4 |

Cells were incubated for 72 h in the absence or presence of TR, guanosine or TR + guanosine. Values are means ± S.E. of 5–6 experiments.
TR (5, 10 μM) vs. None $P < 0.01$.
Guanosine + TR (5 μM) vs. TR (5 μM) $P < 0.02$.
Guanosine + TR (10 μM) vs. TR (10 μM) $P < 0.001$.

in sensitivity to TR are not due to the addition of insulin and 15% fetal calf serum, since MCF-7 cells retained their sensitivity to TR under these conditions. Incubation of MCF-7 cells for 96 h in the presence of insulin and 15% fetal calf serum with 10 μm TR resulted in a decrease of cell number by 79%, from $8.69 \times 10^5 \pm 0.71 \times 10^5$ to $1.81 \times 10^5 \pm 0.24 \times 10^5$. This inhibition is similar to that obtained in the absence of insulin (Fig. 1).

*Phenotypic expression of MCF-7 and HBL-100 cells following TR treatment*

The anti-proliferative effect of TR on MCF-7 breast cancer cells is accompanied by phenotypic alterations. TR induces an increase in the activity of the plasma membrane bound enzyme alkaline phosphatase in MCF-7 breast cancer cells. Addition of guanosine reverses the effect of TR on the activity of this enzyme to that of untreated values (26 ± 4). In contrast, alkaline phosphatase in HBL-100 cells is not affected by TR (Table 2). Accumulation of lipid droplets in breast cancer cells following treatment with chemical inducers of differentiation has been suggested to be a differentiated feature

*Table 2. Alkaline phosphatase activity in untreated and TR-treated MCF-7 and HBL-100 cell lines*

| Cell line | Alkaline phosphatase (nmoles/mg protein/h) | |
|---|---|---|
| MCF-7 | 26 ± 4 | 51* ± 12 |
| HBL-100 | 24 ± 7 | 20 ± 5 |

MCF-7 cells were incubated for 72 h and HBL-100 cells for 96 h in the absence and presence of TR (10 μM). Values are means ± S.E. of 5 independent experiments.
*TR vs. untreated $P < 0.05$.

[19, 20]. Figure 2 reveals that the non-cancerous HBL-100 cells contain lipid droplets, whereas untreated MCF-7 breast cancer cells do not. Treatment of MCF-7 cells with TR results in accumulation of lipid droplets.

*Effect of TR on intracellular pools of GTP and ATP*

We studied the possibility that the differential sensitivity to TR results from different effects of TR on intracellular pools of GTP and ATP. The results described in Table 3 show that TR markedly reduces GTP levels in MCF-7 cells (6-fold) and also reduces ATP levels. In HBL-100 cells, TR induces only a small decrease in GTP.

*Growth inhibition of HBL-100 cells by MA*

The possibility that the differential effects of TR on MCF-7 and HBL-100 cells result from altered metabolism of TR in both cell lines is supported by the finding that MA acid (a prototypic IMP dehydrogenase inhibitor) inhibits HBL-100 cell growth at micromolar concentrations (Table 4), similarly to its effect on MCF-7 cells [13].

## DISCUSSION

IMP dehydrogenase inhibitors have been shown to exert anti-tumor activity, both *in vitro* and *in vivo* studies [6–9]. These agents also have the ability to induce cell differentiation in the HL-60 cell line [11, 12]. We have recently shown that MA, the prototypic IMP dehydrogenase inhibitor, reduces cell growth and induces several differentiated features in MCF-7 breast cancer cells [13]. The present study extends our previous observations to TR. This compound, like MA inhibits markedly the growth of MCF-7 cells and induces lipid accumulation and an increase in alkaline phosphatase activity. It should be noted that T47D breast cancer cells are also inhibited by TR (unpublished data). The present data suggest that the anti-tumor effects of TR on MCF-7 cells result from inhibition of IMP dehydrogenase by this agent. This assumption is based on the fact that the anti-tumor effects of TR were accompanied by GTP depletion and could be reversed by addition of exogenous guanosine, which repletes GTP pools.

The chemical inducer of differentiation, sodium butyrate has previously been shown to induce lipid accumulation and an increase in alkaline phosphatase activity in MCF-7 breast cancer cells [13]. It should be mentioned that similar to the effect of sodium butyrate on cancer cells, TR was reported to cause a $G_1$-phase block in lung cancer cells [8, 21].

HBL-100 non-cancer breast milk derived cell line is shown to be relatively resistant to the action of TR. Interestingly, MA does inhibit the growth of

*Table 3. The effect of TR on ATP and GTP pools of MCF-7 and HBL-100 cell lines*

| | GTP | | ATP | |
|---|---|---|---|---|
| | (nmoles/mg protein) | | | |
| Cell lines | Untreated | TR | Untreated | TR |
| MCF-7 | 4.3 ± 1.5 | 0.8 ± 0.3 | 17.0 ± 5.6 | 10.6 ± 6.7 |
| HBL-100 | 3.0 ± 0.3 | 2.3 ± 1.1 | 16.1 ± 2.6 | 17.5 ± 5.1 |

MCF-7 cells were incubated for 72 h and HBL-100 cells for 96 h in the absence or presence of TR. Values are means of 3 independent determinations ± S.D.

this cell line. It should be noted that TR, in contrast with MA, has to be phosphorylated to TR-5-phosphate and anabolized to the NAD analog thiazole 4-carboxamide adenine dinucleotide (TAD) in order to inhibit IMP dehydrogenase [6, 22]. It has been suggested in previous studies that resistance of different cancer cell lines to TR results from decreased formation of the active metabolite TAD [7]. The fact that HBL-100 cells growth is inhibited by the prototypic IMP dehydrogenase inhibitor MA may suggest that the relative resistance of HBL-100 cells to TR is due to failure of these cells to metabolize TR to its active metabolite. The property of selective activity of TR on relatively immature cells has been demonstrated previously in hepatoma bearing rats. TR was shown to deplete GTP markedly in the hepatocellular carcinoma cells, whereas only mild changes occured in normal liver cells [1].

TR has been introduced into clinical trials, based on its anti-tumor effects on murine lung cancer *in vivo* [9, 10]. The differential sensitivity of cancer cells and relative resistance of differentiated cells, as reflected in the present study, might be of importance in future clinical use of TR. It remains to be clarified whether selective sensitivity of malignant and non-malignant cells exists in multiple cell types.

*Table 4. The effect of MA on HBL-200 cell growth*

| Treatment | Cell number × $10^5$ |
|---|---|
| None | 1.05 ± 0.10 |
| MA (1 μM) | 0.38* ± 0.02 |
| MA (2 μM) | 0.19* ± 0.03 |

$5 \times 10^4$ cells were incubated in 0.5 ml growth medium in the presence and absence of MA in multiwell plates for 96 h. Values are means ± S.E. for 4 experiments.
*MA vs. None $P < 0.001$.

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