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Original Paper

The Antimetabolite Tiazofurin (TR) Inhibits Glycoconjugate Biosynthesis and Invasiveness of Tumour Cells

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We investigated the effect of Tiazofurin (TR-2-β-D-furanosylthiazole-4-carbamide) on tumour cell invasion using metastatic 3LL-HH murine lung carcinoma and HT168-M1 human melanoma as experimental models. TR pretreatment of 3LL-HH cells, in a dose range of 15–60 μM, caused inhibition of cell proliferation, adhesion to plastic and extracellular matrix proteins. The TR-induced altered matrix interactions of 3LL-HH cells were reflected in decreased migration through matrix-covered filters. Analysis of the expression of certain invasion markers indicated that TR suppressed the expression of αvβ3 integrin and MMP2 metalloproteinase. Biochemical studies indicated that 24 h 60 μM TR treatment of 3LL-HH cells inhibited glycosylation of a wide range of glycoproteins with the most pronounced effect on proteoglycans. TR pretreatment of 3LL-HH tumour cells resulted in the loss of lung colonisation potential *in vivo*. Furthermore, *in vivo* TR treatment inhibited the formation of liver metastases of 3LL-HH murine carcinoma. TR treatment also induced inhibition of integrin and MMP2 expression, migration and liver colonisation of the human melanoma HT168-M1 cell line. Since the TR concentration which inhibited various cellular functions was much lower for cell adhesion and lung colonisation than for cell proliferation, we suggest that the predominant effect of TR is the inhibition of metastasis in these model systems. We also suggest that both the effect of TR on tumour cell proliferation and on extracellular matrix interaction contribute to its remarkable antimetastatic potential *in vivo*.

Key words: tiazofurin, integrin, MMP2, glycoconjugates, metastasis, 3LL carcinoma, human melanoma

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INTRODUCTION

FOR SEVERAL antimetabolites, such as 5-fluorouracil (5FU), arabinofuranosyl cytosine (AraC), methotrexate (MTX) and 6-mercaptopurine monohydrate (6MP), the antiproliferative activity has been found to be associated with stimulation of tumour cell invasiveness [1,2]. New antimetabolites which do not promote tumour cell invasiveness are therefore required. Tiazofurin (TR, 2-β-D-furanosylthiazole-4-carbamide, NSC 286193) is a chemotherapeutic agent efficient in the treatment of leukaemia [3–6]. TR is also a powerful inhibitor of solid tumours such as rat hepatomas [7], murine lung carcinoma 3LL [8] and human colon carcinoma xenograft model system [9]. Experimental data suggest that TR also has a significant inhibitory effect on both induced and spontaneous metastases of murine tumour variants 3LL [8,10] and 3LL-HH [11]. Studies on the mechanism of the antitumoral effect of TR have demonstrated the reduction of *de novo* purine biosynthesis by inhibiting inositol monophosphate (IMP) dehydrogenase activity [12], resulting in a sharp decrease in the concentration of guanine triphosphate (GTP) [13]. Interestingly, TR has been shown to downregulate *C-MYC* and *C-KI-RAS* oncogenes and to induce erythroid differentiation [14,15].

Tumour cell dissemination involves tumour cell—extracellular matrix and cell–cell interactions where the non-proliferative events, such as adhesion to, degradation of and migration in the surrounding matrix, are equally important [16]. These interactions are mediated by cell surface receptors which are frequently glycoconjugates. Besides uridine triphosphate (UTP), GTP also acts as a carrier of sugar precursors in synthetic pathways. It has been reported that TR inhibits precursor incorporation into glycolipids and glyco-

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This paper provides new experimental data on the simultaneous inhibition of glycoconjugate biosynthesis and invasiveness (adhesion, microinvasion and organ colonisation) in TR treated metastatic murine and human tumour cells.

MATERIALS AND METHODS

Tumour cells

Primary culture of murine 3LL-HH cells was prepared from the liver metastases of a 3LL-HH tumour [18], characterised by preferential liver colonisation ability [19], and maintained *in vitro* as described previously [20,21]. HT168-M1, a cell line developed from a human melanoma liver metastasis from a HT168 melanoma xenograft, was cultured *in vitro* as previously described [22].

TR treatment

TR (2-β-D-furanosylthiazole-4-carbamide) (NSC 286193) was kindly provided by N.R. Lomax (Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, U.S.A.); 10^7 3LL-HH cells derived from liver metastases were incubated for 24 h in 10 ml RPMI 1640/10% fetal calf serum (FCS) medium, the culture dishes were washed and the adherent cells were treated with TR 5–240 μM) diluted in complete culture medium. After 24 or 48 h, the non-adherent cells were discarded and the adherent cells were detached with 0.2% EDTA, washed in complete medium and used for the experiments ("TR-pretreated").

Cell cycle analysis

Cells were fixed in 70% ethanol at −20C°, overnight. The cell suspension was washed in Hank's Balanced Salt Solution, and the cellular RNA was digested with 50 μg/ml ribonuclease-A for 30 min. The low molecular weight DNA was extracted by 0.1 M citrate/0.2 M phosphate buffer pH 7.8 with 0.1% Triton X-100; 10^6 cells/ml were stained with propidium iodide (50 μg/ml) for 20 min at room temperature, and filtered through a silk gauze prior to flow cytometry. The analysis was performed on a FACStar flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountains View, California, U.S.A.) in conjunction with a HP-200 computer and Consort 30 data acquisition package. Excitation was at 488 nm (with 200 mW of a 2 W argon ion laser source the emission was at 585/42 nm). The instrument was optimised every day by chicken red blood cells (fixed by glutaraldehyde). Ten to fifteen thousand cells per sample were scanned using low flow rates (100–500/s) to maximise resolution. Simultaneous gating on both forward and 90° light scatter was used to exclude signals from debris. The cell cycle distribution was estimated using Rabinovitch's Multicycle (Phoenix Flow Systems Inc., San Diego, California, U.S.A.) software.

Radioactive isotope labelling of cellular glycoconjugates including proteoglycans

3LL-HH tumour cells (5×10^6) were cultured in 75 cm^2 dishes and were treated with 60 μM TR for 24 h. In the last 12 h of the treatment, cells were labelled with 30 μCi/ml [^{14}C] glucosamine(GlcN; spec. act. 197 Ci/mM in 50% EtOH; UVVVR).

Separation of glycoconjugates

Cells were scraped into 1.5 ml of medium, and centrifuged at 2000 rpm for 10 min. Cellular radioactivity was measured and the pellets were extracted at room temperature with guanidine–HCl (4 M guanidine–HCl, 0.1 M NaCOOH, 2% Triton X-100) containing protease inhibitors [8 mM N-ethyl maleimide (NEM), 2 mM phenyl-methyl sulphonate (PMSF), 5% Gordox] for 2 h. The lysates were centrifuged at 10 000*g* for 10 min and the supernatant containing the glycoconjugates was dialysed overnight at 4°C in 7 M urea containing protease-inhibitors as above.

Applying DEAE-52 ion exchange chromatography, the glycoconjugates were eluted stepwise (8 M urea and 0, 0.2, 0.8 M NaCl) to obtain the positively charged glycoconjugates (eluent 8 M urea), neutral glycoconjugates (eluent, distilled water), weakly negative glycoconjugates (eluent; 0.2 M NaCl) and heavily charged proteoglycans (eluent; 0.8M NaCl). Hyaluronic acid was identified in the weakly negative glycoconjugate fraction according to its sensitivity to *Streptomyces* hyaluronidase, while proteoglycans were identified by their sensitivity to chondroitinase ABC and heparinase [23]. Radioactivity was measured in a Beckman scintillation counter as previously reported [20].

Adhesion assay

Five microgrammes per well of ECM proteins; fibronectin (Sigma), collagen IV (UBI, Lake Placid, New York, U.S.A.), collagen I-III (isolated as described; [19]) all diluted in 200 μl phosphate-buffered saline (PBS), were dried on to plastic 24-well plates overnight at 37°C under UV light. 10^5 TR-pretreated tumour cells in 500 μl serum-free RPMI were incubated on the matrix surface for 1 h at 37°C, washed twice with medium and fixed with 1% paraformaldehyde for 10 min. The number of adherent cells/unit area was counted under phase contrast microscope.

Detection of integrin expression by flow cytometry

In vitro cultured tumour cells were detached by EDTA, suspended and fixed in MetOH for 10 min. After washing in PBS, cells were incubated in non-immune goat serum diluted 1:2 in PBS for 30 min to block non-specific antibody binding. Integrin αvβ3 expression was detected by incubating the cells for 60 min with mouse monoclonal anti-αvβ3 antibody (IgG) (Chemicon, Temecula, California, U.S.A.) diluted 1:100 in PBS. After washings in PBS (3×), the bound mouse IgG was detected by goat anti-mouse IgG–biotin complex (Amersham) diluted 1:200 in PBS and the bound biotin was later detected by Streptavidin-FITC diluted 1:100 in PBS. After washings in PBS, cells were measured for fluorescence in FACStar Plus flow cytometer (Becton Dickinson). Background fluorescence of negative control cells, where the labelling process was performed in the absence of the primary antibody, was determined. The mean relative fluorescence was calculated as previously described [24].

Matrix invasion

The assay was performed in EHS matrix-coated invasion chambers (Biocoat; Collaborative Research, Bedford, Massachusetts, U.S.A.) equipped with an 8 μ polycarbonate filter. The inserts were placed into a 24-well plate; 10^5 cells in 200 μl serum-free RPMI-1640 were transferred into the upper chamber which was placed into 500 μl of RPMI containing 10% FCS (lower chamber) separated by an 8 μ polycarbonate filter. Cells were incubated at 37°C in CO_2 for 6 h, and the adherent and non-adherent cells in the upper chamber were discarded. The bottom of the filter was stained with Diff

Quick solution (American Scientific Products, McGraw Park, Illinois, U.S.A.), before being removed from the insert and mounted on to glass coverslips using 50% gelatin/PBS. The total number of tumour cells which migrated/filter was counted under a light microscope. Each experimental point contained three parallel samples.

Detection of the MMP2 collagenase IV expression in tumour cells by flow cytometry

Cultured tumour cells were detached by EDTA, suspended and fixed in absolute MetOH for 10 min. After washing in PBS, cells were incubated in non-immune goat serum diluted 1:2 in PBS for 30 min to block non-specific antibody binding. MMP2 collagenase IV expression was determined by incubating the cells for 60 min with polyclonal anti-MMP2 rabbit antibody (IgG) (Research Genetics, Huntsville, Alabama, U.S.A.), diluted in 1:100 in PBS. This antibody was developed against the unique MMP2 sequence APSPIIKFPDGDVAPKTD and was extensively characterised by the manufacturer for specificity. After washings in PBS (3×), the bound rabbit IgG was detected by goat anti-rabbit IgG–biotin complex (Amersham) diluted 1:200 in PBS and the bound biotin was later detected by Streptavidin-FITC diluted 1:100 in PBS. After washings in PBS, cells were measured for fluorescence as described for integrin (above).

Proliferation assay

Control or TR-pretreated 3LL-HH cells were harvested from T75 dishes by EDTA (viability was between 82 and 97% by Trypan Blue exclusion test), washed and placed into 24-well plates at a concentration of 5×10^4 viable cells/well for up to 72 h in 1 ml RPMI + 10% FCS. After 24, 48 and 72 h incubation, cells were scraped into medium and the total and viable cell number was determined.

Lung colonisation assay

Adherent 3LL-HH tumour cells (5×10^4 viable cells/animal) from control and TR-pretreated cultures were injected into the tail vein of C57B6 mice as previously described [19], the cell number normalised according to viability. Twenty-one days after tumour cell injection, animals were killed by overdose of Nembutal and the number of colonies on the lung surface was counted under a stereomicroscope. There were 10 animals per group.

Liver metastasis assay—murine cell line

A single cell suspension of 3LL-HH cells (3×10^3 cells/animal) was inoculated into the spleen of C57/B16 mice and the spleen was removed on day 3. TR, diluted in physiological saline, was administered i.p. as a daily dose of 100, 200 or 400 mg/kg-body-weight from day 4 for 8 days. Control animals were treated with saline alone. The animals were killed on day 12. The liver and spleen were analysed under a stereomicroscope.

Liver metastasis assay—human cell line

A single cell suspension was prepared from a human melanoma xenograft growing in the spleen; 10^6 viable tumour cells were inoculated into the spleen of immunosuppressed CBA/CA mice in a volume of 50 µl as previously described [22]. Animals were killed by Nembutal overdose on the 27th day after inoculation and the spleen and liver were analysed under a stereomicroscope. TR treatment began on day 7 after inoculation as a daily dose 200 or 400 mg/kg body weight administered i.p. Control animals were treated with PBS alone. Treatment was continued for 20 days.

Statistical analysis

Data were evaluated by the χ^2 test, Student t-test or single factor ANOVA.

RESULTS

In vitro pretreatment of 3LL-HH cells with TR at 15–60 µM concentrations for 24 h resulted in inhibition of cell proliferation in the following 24–48 h incubation period, without the presence of the drug (Figure 1). However, there was no significant alteration in cell viability (82–97% in controls versus 76–87% in treated samples) after TR treatment. Analysis of the cell cycle parameters of adherent tumour cells treated for 24 h (5–30 µM) showed a slight accumulation of cells in G1 phase with a simultaneous decrease of S phase cells; there was no indication of apoptosis (data not shown).

During these studies, we found that TR pretreatment for 24 h (in the presence of serum) decreased the adhesiveness of tumour cells dose dependently (5–30 µM) (Figure 2a). The TR effect on tumour cell adhesiveness in serum-containing culture media indicated significant alteration in tumour cell phenotype. On examining the extracellular matrix (ECM)–tumour cell interactions affected, it was shown that the most pronounced reduction in adhesion of tumour cells was to collagen type IV, followed by collagen type I and fibronectin (Figure 2b). We also measured the expression of αvβ3 integrin in TR-pretreated (30 µM, 24 h) 3LL-HH cells, since β3 integrins are the most commonly expressed matrix receptors [25]. The majority of the 3LL-HH cells expressed this integrin (data not shown) and after TR pretreatment, there was a significant reduction in the intensity of αvβ3 integrin expression measured by flow cytometry (Figure 2c).

The microinvasive potential of TR-pretreated tumour cells was investigated using Engelberth–Holm–Swarn (EHS)-matrix covered Transwell-membranes. Twenty-four hour TR

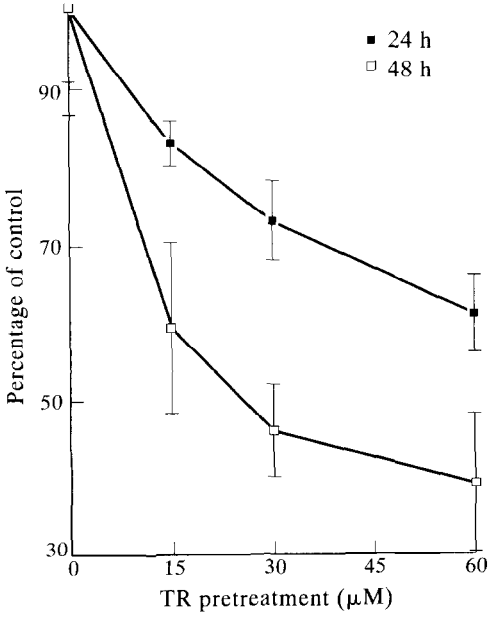
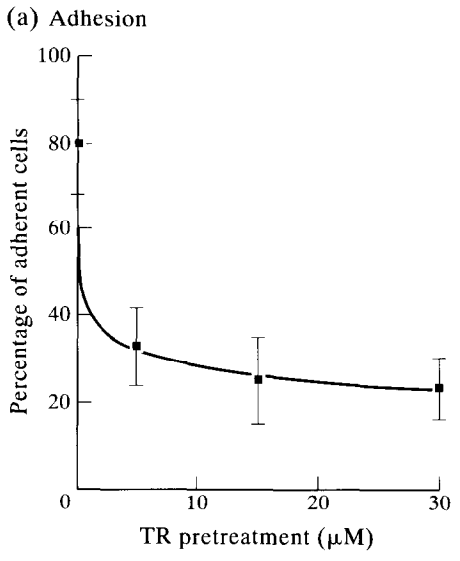
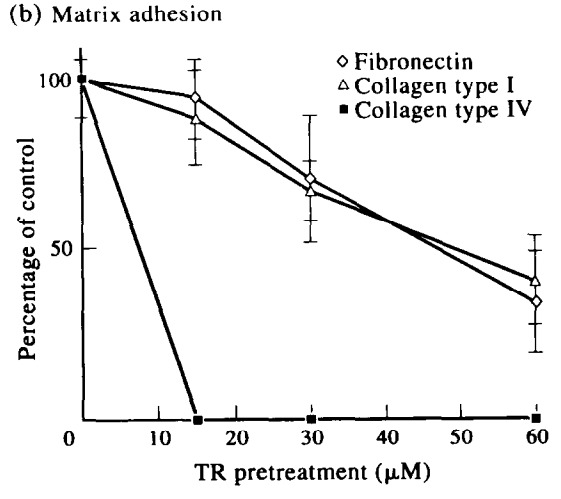


Figure 1. Effect of TR pretreatment on 3LL-HH cells *in vitro*.





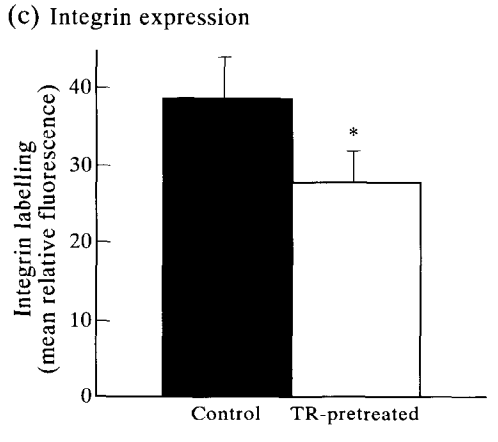
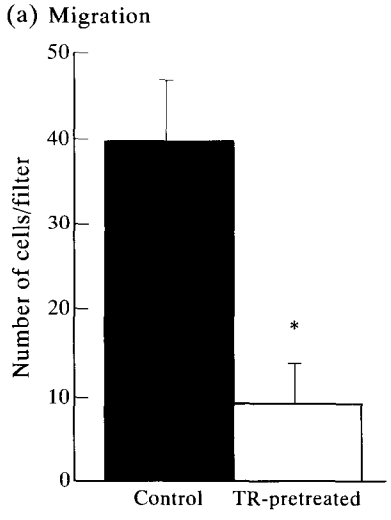


Figure 2. Effect of TR pretreatment on adhesion and integrin expression of 3LL-HH cells *in vitro*. (a) Data are expressed as % of viable adherent cells of the cell population and are means ± S.D. (n = 3). (b) Data are expressed as a percentage of the control and represent means of three parallel samples (±S.D.). (c) Data are means of three parallel measurements ± S.E. * $P < 0.05$.

pretreatment at 30 μM concentration significantly decreased the migration of 3LL-HH cells (Figure 3a). Since one of the predominant component of the EHS matrix is collagen type IV [16] and microinvasion of tumour cells through collagen type IV requires at least the function of matrix metalloproteinase 2 (MMP2) [26], we also measured the expression of MMP2 in TR-pretreated 3LL-HH cells using flow cytometry. These studies indicated a significant decrease in cellular expression of MMP2 in TR-pretreated (30 μM, 24 h) 3LL-HH cells (Figure 3b) suggesting that the decreased invasiveness of the EHS matrix can be explained, at least in part, by the decreased metalloproteinase expression.

Glycoconjugate biosynthesis of 3LL-HH cells after TR pretreatment was analysed since heavily glycosylated surface molecules have been shown to be important in cell adhesion [27,28]. TR inhibited entry of the radiolabelled precursor into the cells by 56% and inhibited the incorporation of the precursor into glycoconjugates by 41% (Table 1). However, incorporation of the precursor into positive, neutral and negative glycoconjugate fractions (glycolipids and glycoproteins)



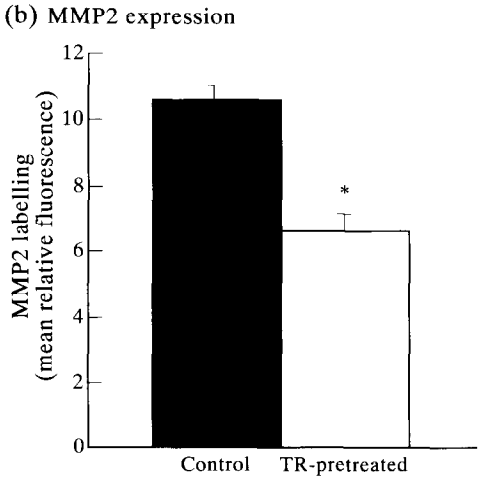


Figure 3. Effect of TR pretreatment on migration and MMP2 expression in 3LL-HH cells. (a) Data represent the mean number of cells/filter ± S.E. (n = 3). Data were analysed by single factor ANOVA; * $P < 0.005$. (b) Data are means of three parallel measurements ± S.E. Data were analysed by single factor ANOVA; * $P < 0.05$.

Table 1. *Effect of* 24 *h TR pretreatment on* [^{14}C]*GlcN incorporation into cellular glycoconjugates in* 3*LL-HH cells* in vitro

[^{14}C]GlcN incorporation	Control	TR (60 μm, 24 h)	Inhibition (%)
Cellular precursor	78075 ± 4885	34121 ± 2957*	56
Cellular glycoconjugates (GC)	109466 ± 11811	64952 ± 4417*	41
Positively charged GC	47159 ± 4619	32441 ± 1778†	31
Neutral GC	43741 ± 4779	24052 ± 1759*	45
Negatively charged GC	13449 ± 1577	6885 ± 990*	49
Proteoglycans	5117 ± 556	1574 ± 273*	69

Data are expressed in cpm/10^6 cells and are means of three parallel samples (±S.D.). *P* values were determined by χ^2 test; * $P < 0.01$; † $P < 0.05$.

was variable, with the positively charged glycoconjugates relatively resistant to the TR effect, and the proteoglycans, the most negatively charged glycoconjugate, the most sensitive, with inhibition of the precursor incorporation exceeding that observed for total cellular precursor incorporation (Table 1).

We analysed the effect of TR pretreatment on lung invasion of 3LL-HH cells *in vivo*, since tumour cell interactions with ECM and their surface glycoconjugates [27–29] have been shown to be involved in metastasis. Pretreatment of 3LL-HH cells with TR (from 5 to 240 μM) *in vitro* resulted in a significant decrease and the disappearance (120–240 μM) of lung colony forming potential of 3LL-HH cells, expressed both as incidence of metastasis (Figure 4a) and number of lung colonies (Figure 4b). This effect was not due to the toxic effect of the TR pretreatment because the viability was only 10% lower in the highest TR concentration compared with that of the control cells (88% control versus 78% TR) and the cell number injected into animals was normalised for viability. This indicates that the TR pretreatment is interfering with cell proliferation, matrix adhesion, migration and glycoconjugate biosynthesis of 3LL-HH cells also has a profound effect on the invasive potential of the tumour cells. In addition, *in vivo* TR administration at 200–400 mg/kg completely inhibited the occurrence of liver metastases from 3LL-HH murine tumour cells (Table 2) indicating that TR is also able to suppress tumour cell invasiveness *in vivo*.

TR pretreatment on the invasive phenotype of a highly metastatic human melanoma cell line HT168-M1 was examined. The pretreatment significantly reduced integrin expression (Figure 5a), MMP2 protein expression (Figure 5b) and the invasive potential of melanoma cells on EHS matrix (Figure 5c), suggesting a similar effect of TR on human melanoma cells as seen in murine 3LL-HH carcinoma. *In vivo* TR treatment of immunosuppressed mice inoculated with this human melanoma cell line (HT168-M1) reduced the incidence of liver metastasis and the number of liver colonies, whereas there was no alteration in the incidence of the primary tumour, indicating a predominantly antimetastatic effect of the drug (Table 3).

DISCUSSION

Neoplastic cells are characterised by increased IMP dehydrogenase activity [26] providing a target for chemotherapy [13]. The primary effect of TR is the inhibition of DNA and RNA synthesis and cell proliferation [4]. However, TR not only inhibits the growth of the primary tumour but is a powerful inhibitor of experimentally induced and spontaneous metastasis [8,10,11]. This effect cannot be fully explained by

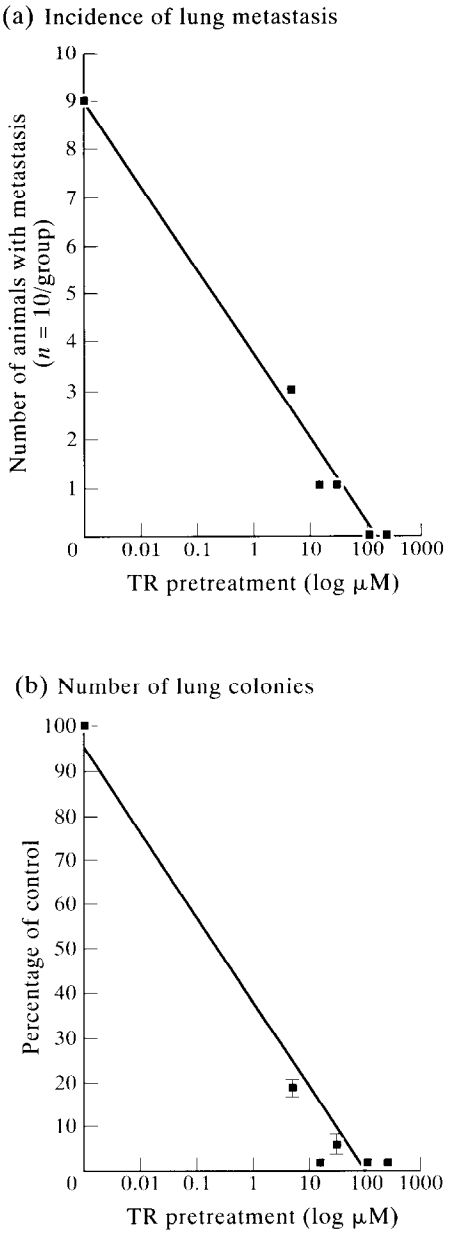


Figure 4. Effect of 24 h TR pretreatment on lung colonisation of 3LL-HH cells. (a,b) Individual numbers of metastases were counted and data expressed as a percentage of the control (100% = 162 ± 32). Each point is the mean of 10 samples (±S.D.).

Table 2. Effect of in vivo *TR treatment on the liver metastasis formation of 3LL-HH cells injected into the spleen*

TR (mg/kg)	Primary tumour (incidence)	Liver metastasis (incidence)	Number of metastases (mean ± S.E.)	*P*
Control	9/9	9/9	105 ± 52.5	
100	9/9	5/9	9 ± 7	<0.002
200	9/9	0/9	0 ± 0	<0.001
400	8/8	0/8	0 + 0	<0.001

Evaluation on the 12th day after tumour cell inoculation. *P* value was determined by Student *t*-test.

Table 3. Effect of in vivo *TR treatment on liver metastasis formation of HT168-M1 human melanoma cells in immunosuppressed mice*

TR (mg/kg)	Primary tumour (incidence)	Liver metastasis (incidence)	Number of metastases (mean ± S.E.)	*P*
Control	9/9	9/9	64.6 ± 28.9	
200	7/7	5/7	30.6 ± 17.6	<0.5
400	8/8	5/8	22.1 ± 10.9	<0.05

P value was determined by single factor ANOVA.

the inhibition of cell proliferation. Here we provided evidence that TR inhibits cell adhesion to collagen type IV and to a lesser extent to collagen type I and fibronectin, and inhibits migration through extracellular matrix proteins *in vitro*, without significantly decreasing cell viability.

Studies on tumour cell interactions with the host tissues indicate that cell adhesion molecules are of critical importance. Several cell–cell adhesion molecules (CAMs) are heavily glycosylated where the ligand is frequently a glycoconjugate on the target cell membrane. In cell–matrix interactions, both integrins [30,31] (less heavily glycosylated membrane proteins) as well as proteoglycans [29] play critical roles. Consequently, sugar moieties of glycoconjugates may be important in tumour progression. This hypothesis has been tested in various experimental models, where both β1-6 branched Asn-linked oligosaccharides [32] as well as proteoglycans were shown to be overexpressed in metastatic variants [20,21]. Accordingly, surface glygoconjugates proved to be new therapeutic targets to prevent tumour progression and metastasis [32–34].

Chemotherapeutic modifications of glycoconjugate biosynthesis can be achieved by the inhibition of sialyltransferase [35] or glycosyltransferase [36,37], and the use of tunicamycin [38] or 2-deoxy-glucose [39] while the glycanation of proteoglycans is sensitive to β-xyloside [40], ethane-1-hydroxy-1,1-diphosphonate (ETDP) [41] and 5′ hexyl-2′ deoxyuridine (HUdR) [20]. It seems that chemical modification of tumour cell surface glycoconjugates frequently inhibits invasiveness *in vitro* and metastasis *in vivo* [1,2].

It has been observed that non-lethal concentrations of antimetabolites induce profound alterations in the glycosylation of cellular proteins [42]. 5FU, AraC, MTX and 6MP but not 6-thio-guanosine monophosphate (6TG), increase glucosamine (GlcN) incorporation into cellular glycoconjugates [42] irrespective of their effects on the sugar-nucleotide pool. *In vitro* studies indicate that these antimetabolites may increase the invasiveness of treated tumour cells, which is considered to be associated with modifications in glycoconjugates [43]. Earlier studies indicate that the antimetabolite, TR, inhibits GTP-requiring biosynthetic pathways of some glycoconjugates in tumour cells [17]. We have shown that, unlike other antimetabolites, TR inhibits precursor incorporation into various glycoconjugate fractions of 3LL-HH cells by inhibiting cellular uptake of the radioactive precursor. In addition, a considerable decrease in the biosynthesis of proteoglycans was observed in TR pretreated tumour cells, which was more pronounced than the inhibition of precursor uptake suggesting a particular TR-sensitivity for the glycanation process.

Such a profound alteration in glycosylation of various glycoconjugates may not fully explain the sharp decrease in tumour cell invasiveness induced by TR. We measured the effect of TR pretreatment on integrin expression, since αvβ3 is one of the most common adhesion molecules, and is able to mediate cell adhesion to various matrix substrates [25]. In human melanoma, the expression of αvβ3 integrin is associated with an invasive phenotype [44]. Our analysis of integrin expression in murine or human tumour cells (3LL-HH and HT168-M1) indicated that TR pretreatment reduced the expression of this multifunctional integrin suggesting that TR modulates the expression of adhesion molecules. TR also reduced the expression of MMP2 metalloproteinase, which is one of the most important enzyme of tumour cells, responsible for collagen type IV, and consequently basement membrane, degradation [26]. The complex phenotypic alterations induced by TR in murine and human tumour cells (i.e. in the expression of glycoconjugates, integrin and metalloproteinase) was associated with the inhibition of microinvasion of artificial matrix *in vitro* and specific antimetastatic effects *in vivo*. It appears that, in addition to antiproliferative effects, TR has significant anti-invasive potential. This statement is further

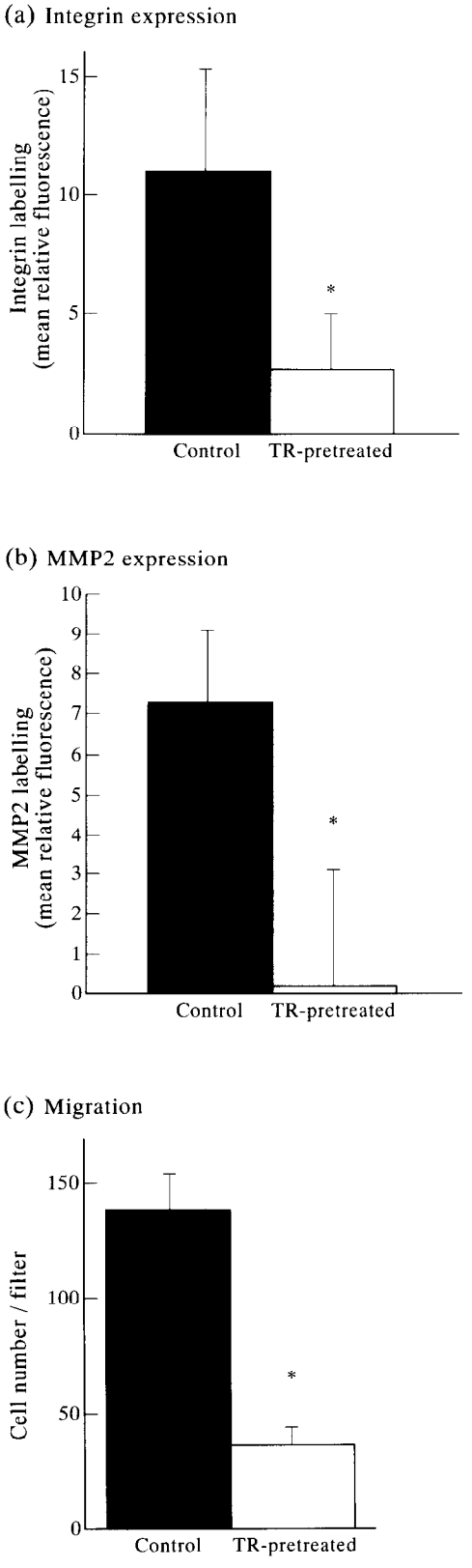


Figure 5. Effect of TR pretreatment on the integrin and MMP2 expression and migration of HT168-M1 human melanoma. (a) Data are means of three parallel measurements ±S.E. Data were analysed by single factor ANOVA; * $P < 0.05$. (b) Data are means of three parallel measurements ±S.E. Data were analysed by single factor ANOVA; * $P < 0.005$ (c) Data represent the mean number of cells/filter ±S.E. ($n = 3$). Data were analysed by single factor ANOVA; * $P < 0.005$

supported by the fact that the IC_{50} of TR for 3LL-HH cells for the inhibition of proliferation is >60 μM, while being 1–7.5 μM for cell adhesion and is <1 μM for the inhibition of lung metastasis.

Interference with adhesion mechanisms of tumour cells by RGDS (a peptide-ligand for integrins [45]), laminin-peptides (ligands for laminin-receptor [46]) or anti-integrin receptor antibodies [24] can be used to inhibit experimental metastasis *in vivo*. Such interference with adhesive function of tumour cells may not affect cell proliferation. However, proliferative events have significance in specific phases of metastasis, particularly in local growth after extravasation [47]. Therefore, drugs which can inhibit both adhesive and proliferative properties of disseminating tumour cells may have particular therapeutic significance. TR seems to be a promising new antimetabolite and antimetastatic candidate. It is a powerful inhibitor of cell proliferation of various tumour cell types [6–9] and it has been introduced into clinics in the treatment of chronic myelogenous leukaemia in blast crisis [5,13]. Since the subcellular target of TR is IMP dehydrogenase and consequently the GTP biosynthesis, several biosynthetic pathways can be affected including DNA, RNA [4], glycolipid [17] and inositol lipid synthesis [48]. Cell proliferation is the primary target of TR, although it has also caused downregulation of oncogene expression (*C-MYC* and *C-HA-RAS* [14]) and induction of differentiation [15]. The results obtained in this study reveal new effects of TR, that is, inhibition of tumour cell adhesion to and migration through ECM at a dose range which has minimal effect on cell viability. These effects appear to be the result of TR's profound effect on biosynthesis of a wide range of glycoconjugates, including proteoglycans, as well as the decreased expression of integrin(s) and metalloproteinase(s). These actions of TR on the adhesive and migratory properties of tumour cells explain our present results and earlier reports indicating significant *in vivo* antimetastatic action [8,10,11]. Since the antimetastatic action of TR has only been reported in 3LL murine tumour variants [8,10,11], evaluation of its effectiveness against various metastatic human tumours is needed. Our preliminary data showing significant *in vivo* antimetastatic effect of TR against a human melanoma liver metastasis, HT168-M1, are particularly promising in this regard.

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