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Temozolomide Reduces the Metastatic Potential of Lewis Lung Carcinoma (3LL) in Mice: Role of α-6 Integrin Phosphorylation

L. Tentori, C. Leonetti and A. Aquino

The involvement of protein kinase c (PKC) in the mechanism underlying the antimetastatic properties of triazenes was studied in C57BL/6 mice bearing Lewis lung carcinoma (3LL). *In vivo* and *in vitro* treatment with temozolomide, an *in-vitro* active analogue of dacarbazine, or calphostin c produced a concentration-dependent reduction of spontaneous and artificial metastases. Both agents reduced the ability of 3LL cells to adhere to endothelium. Diethylaminoethyl (DEAE)-sepharose chromatography of cell extracts revealed that incubation of 3LL cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a rapid translocation of protein kinase c activity from cytosol to the membrane fraction. Membrane PKC activity induced by TPA was reduced by 60% after treatment with temozolomide. Coincident with these changes, TPA induced phosphorylation of α-6 integrin, whereas temozolomide or calphostin c abolished the appearance of this phosphoprotein. These results suggest that temozolomide reduced metastatic potential by interfering with α-6 phosphorylation induced by PKC activation.

Key words: PKC, temozolomide, metastasis, calphostin c, integrins, cell adhesion, phosphorylation
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INTRODUCTION

THE CELLULAR signalling systems have recently come to the fore as potential targets for the development of new anticancer drugs. Because of its pivotal importance for signal transduction, protein kinase c (PKC) is undoubtedly a logical target for drug intervention. PKC, a family of structurally related isoforms, may play a critical role in the regulation of tumour cell invasion and metastasis. In fact, tumour cells showing high PKC activity have enhanced ability to invade and metastasise. This association has been found for mouse B16 melanoma [1], mouse Lewis lung carcinoma [2], mouse mammary adenocarcinoma [3] and murine fibrosarcoma [4]. 3LL cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) display an enhancement of adhesion to endothelial cells. This event is associated with increased phosphorylation of cell proteins [2], as well as PKC membrane translocation. Tumour cell adhesion is mediated by a large array of cell surface adhesion molecules, including P- and E-selectins [5, 6], cadherins [7, 8], integrins [9, 10], intercellular adhesion molecules and vascular adhesion molecules [11]. In particular, α-6 subunit integrin is known to be present in higher amounts in 3LL cells which possess higher capacity to metastasise to the lung [12]. However, it is not clear how cell adhesion is regulated during tumour progression to metastatic stage. Recent data suggest that adhesion molecules, in addition to their function in mediating cell–cell adhesion, play a crucial role in signal transduction [11, 13]. PKC activators, such as TPA, have been shown to activate adhesion receptors by promoting phosphorylation of integrins [14, 15], which can affect binding characteristics for the ligands [16]. Studies using various PKC inhibitors such as H-7 and calphostin c, developed against PKC at its catalytic and regulatory domain [17, 18], also provide evidence that PKC is associated with metastasis. Specificity seems more likely to be associated with agents which inhibit predominantly at the regulatory site [18]. Moreover, anticancer drugs in clinical use, such as tamoxifen [19] and doxorubicin [20–21], have been described as possessing PKC inhibitory properties.

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Triazene derivatives are alkylating agents which appear to inhibit the development of spontaneous metastasis by a specific mechanism different from cytotoxicity and independent from their inhibitory effects on primary tumour [22]. The mechanisms whereby these compounds reduce the metastatic potential of the tumour are not yet well defined. Temozolomide, an *in vitro* active analogue of dacarbazine, is readily cleaved in aqueous solution to form the linear triazene 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC), the putative active metabolite of dacarbazine, generated by host metabolism [23, 24]. Thus, a prodrug form of MTIC, which does not depend on host metabolic activation to an unstable species but relies instead on chemical transformation, may provide advantages compared to dacarbazine.

Since phosphorylation of integrins seems to be important in the events that lead to 3LL adhesion and metastasis, we were interested in determining whether temozolomide can modulate α-6 phosphorylation and tumour cell adhesion. The present study indicates that both calphostin c and temozolomide reduce spontaneous metastasis in mice bearing 3LL carcinoma. Both agents reduce the ability of 3LL cells to adhere to endothelium and to form lung colonies. Moreover, the present data show that TPA treatment of 3LL cells induces phosphorylation of α-6 integrin subunit via membrane translocation of PKC. This phenomenon is abolished by temozolomide and calphostin c pretreatment. The results suggest the temozolomide reduced metastatic potential, interfering with α-6 phosphorylation induced by PKC activation.

MATERIALS AND METHODS

Materials

Temozolomide was kindly provided in part by M.G.F. Stevens (Pharmaceutical Sciences Institute, Aston University, Birmingham, U.K.) and in part by Schering Plough (Kenilworth, New Jersey, U.S.A.) MTIC was kindly provided by Drug Synthesis and Chemistry Branch (National Cancer Institute, National Institute of Health, Bethesda, Maryland, U.S.A.). Calphostin c was purchased from Biomol (Plymouth, Pennsylvania, U.S.A.); TPA, ethyleneglycol (β-aminoethyl ether)-*N*-*N*-tetracetic acid (EGTA), phosphatidylserine (PS), ATP, phenylmethane sulphonyl fluoride (PMSF) and Triton-X100 from Sigma (St Louis, Missouri, U.S.A.); H-7 from Seikagaku America (Rockville, Maryland, U.S.A.); [γ-^{32}P]ATP (3000 Ci/mmol), $H_3[^{32}PO_4]$, [^{51}Cr] and [^{125}I]IUdR [^{3}H]TdR from Amersham (Amersham, U.K.).

Animals and cell lines

Male C57BL/6, weighing 20–22 g, were obtained from Charles River Laboratories (Calco, Italy).

The 3LL carcinoma was maintained as solid intramuscular (i.m.) tumours by serial transplantation, at 2-week intervals, of 2.5×10^5 viable cells (trypan blue negative). The single cell suspension was obtained by a standard mechanical procedure and injected i.m. into hind legs of mice. Tumour cells were cultured overnight in RPMI 1640 (Gibco, Paisley, U.K.) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco), 2 mM glutamine, 5 mM Hepes and antibiotics (complete medium (CM)). Adherent cells were then used for *in vitro* experiments. The mouse endothelioma cell line tEnd [25] was obtained through the courtesy of Dr A. Mantovani (Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 15% FCS (Gibco). Endothelioma cells were grown to confluence in 96-multiwell plates, washed twice with fresh medium before the adhesion assay.

In vivo *studies*

To assess the therapeutic effect of temozolomide, 2.5×10^5 viable tumour cells were implanted i.m. and drug treatment was started when tumours became palpable (on day 4 after tumour implantation). Drug was administered intraperitoneally (i.p.) in a volume of 0.01 ml/g of body weight, in a solution made by dissolving the drug in dimethylsulphoxide (DMSO) prior to dilution with saline solution, as previously reported by Stevens [23]. Mice were injected according to the schedules described in Table 1. Experiments were also performed to evaluate the tumorigenicity and metastatic ability of 3LL tumour cells after *in vitro* treatment with graded concentrations of temozolomide or calphostin c. Briefly, single cell suspensions were obtained by mechanical dissociation of 3LL tumours, washed and cultured overnight. 3LL cells at a concentration of 5×10^5/ml were then incubated for 3 h at 37°C in complete medium containing temozolomide or calphostin c at the concentrations specified in Table 2. Treatment with temozolomide was performed in the dark. Calphostin c was dissolved in DMSO as a stock solution (1 mM) and stored in the dark at −20°C. Due to the light-dependent activation of calphostin c, treatment was always carried out under ordinary fluorescent lighting. Cells (1×10^5) were washed and injected i.m. in a volume of 0.2 ml. Control mice received tumour cells maintained in drug-free CM. Each experimental group consisted of at least 25 animals, 10 for primary tumour controls and 15 for lung metastases evaluation. Experiments were repeated three times. Tumour growth was monitored by measuring maximal and minimal diameters by calliper three times a week and tumour weight was evaluated according to Geran and associates [26]. Mouse survival was checked for at least 90 days. The spontaneous metastasising ability of 3LL carcinoma was evaluated on days 15 and 21 after tumour implant. Mice were killed, lungs were removed, fixed in Bouin's solution and nodules counted.

Artificial metastasis

3LL cells were treated with graded concentrations of temozolomide or calphostin c for 3 h. C57BL/6 mice were given intravenous (i.v.) injections, via the tail vein, of 1×10^5 viable cells in 0.2 ml. Two weeks later, the lungs were excised and the numbers of metastatic nodules were counted. Experiments were repeated twice using 10 animals/group.

Adhesion assays

Adhesion of 3LL cells to endothelioma cell line tEnd, originally derived from a thymic haemangioma, was evaluated as previously described [25]. Briefly, 3LL cells were labelled for 45 min with 5 μCi [^{51}Cr] (400 mCi/mgCr), treated as indicated in Figure 1, and then washed twice with PBS containing 5 mM EGTA. For adhesion experiments, 1×10^6 cell/ml were resuspended in DMEM supplemented with 10% FCS. Cell suspensions were then added to each well (final volume 0.2 ml). After 15, 30 and 60 min of incubation at 37°C in 5% CO_2 non-adherent cells were removed by washing three times with PBS. The remaining cells were solubilised with 0.2 ml of 1 N NaOH—1% sodium dodecylsulphate (SDS). Radioactivity of adherent cells was counted with a gamma counter (Compugamma, LKB-Pharmacia, Turku, Finland).

Adhesion of 3LL cells to immobilised proteins was performed using six-well precoated plates (Becton Dickinson, Mountain

Table 1. Anti-tumour effects of temozolomide against 3LL cells: in vivo *treatment*

Treatment* (mg/kg/day × 5)	T/C%†	MST (days) (range)	ILS% (days)	Drug deaths‡	Median number of metastases Day 15	Day 21
Control	—	29 (28–35)	—	—	18 (4–21)	47 (19–59)
5	94	30 (29–36)	3	0/10	11 (6–21)	27 (14–51)
25	93	30 (28–34)	3	0/10	5 (3–9)§	22 (12–41)
50	85	32 (29–37)	10	1/10	4 (2–10)§	11 (7–21)*
100	70	32 (29–39)	10	1/10	4 (3–8)§	5 (3–10)*

* Treatment was started on day 4 following intramuscular implantation of 2.5×10^5 3LL tumour cells; † T/C%, tumour weight of treated compared to control mice 1 week after the end of treatment; ‡ Mortality occurring in treated mice before death of the first control mouse was considered as due to drug toxicity. § $P < 0.05$ according to Wilcoxon test comparing the number of metastases found in treated mice versus those observed in control animals. MST, median survival time in days; ILS%, increase in life span in days.

Table 2. Tumour growth and metastases in mice inoculated with 3LL cells treated in vitro *with temozolomide or calphostin c*

Treatment*	Concentration (μM)	T/C%†	MST (range)	ILS%	Median number of metastases Day 15	Day 21
Control		—	38 (32–46)	—	23 (12–31)	47 (19–59)
Temozolomide	2.5	92	39 (32–48)	3	12 (1–21)	27 (12–41)
	25	72	44 (36–50)	16	7 (0–9)§	22 (14–21)
	50	44	52 (41–59)	37	6 (0–8)‖	11 (7–17)§
	250	2	>90‡	>137	0‖	5 (3–10)§
Calphostin c	0.02	89	39 (31–50)	3	17 (10–22)	48 (31–76)
	0.2	86	42 (30–48)	10	7 (3–10)§	22 (21–35)
	2	6	>90	>137	0‖	6 (0–10)

* 2.5×10^5 3LL cells, treated with temozolomide or calphostin c for 3 h, were injected intramuscularly into C57B1/6 mice; † T/C%, tumour weight of treated compared to control mice 1 week after the end of treatment; ‡ On day 90 after tumour implantation, mice were tumour free and considered long-term survivors. § $P < 0.05$; ‖ $P < 0.01$ according to Wilcoxon test comparing the number of metastases of control mice versus those obtained by injecting 3LL treated cells. MST, medium survival time in days; ILS%, increase in life span in days.

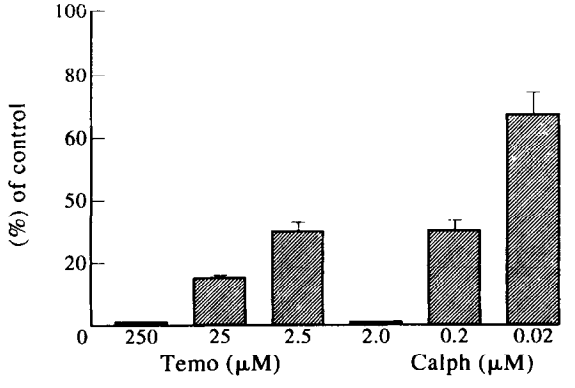

Figure 1. Effects of temozolomide and calphostin c on artificial metastasis. 3LL were treated with temozolomide (Temo) or calphostin c (Calph) as described in Materials and Methods. Results are expressed as per cent of control of lung metastases following intravenous injection (mean of 10 animals ± S.E.). Data shown refer to a representative experiment of two performed independently, both of which produced comparable results.

View, California, U.S.A.). 3LL cells were labelled with [^{3}H]TdR (1 μCi/ml) for 24 h. Cells were treated with temozolomide (250 μM) for 3 h, washed and incubated for 1 h at 37°C (cell density 1×10^6/ml) into collagen I, collagen IV, fibronectin or laminin precoated plates. Wells were washed 10 times with PBS containing 0.1% bovine serum albumin (BSA), and attached cells were removed from plates by 10-min incubation with RPMI containing 20 mM Hepes, 0.05% BSA and 2 mM EDTA. Cells were then harvested and radioactivity was determined by β-scintillation counting.

Quantitation of [^{125}I]IUdR-labelled 3LL cells in lungs

The procedure was performed as described previously [27–29]. Tumour cells (2×10^6/ml) were incubated in CM for 18 h at 37°C in a CO_2 incubator in the presence of 5–10 μg/ml of FUdR (Sigma), in order to prevent endogenous thymidine synthesis, and 0.3 μCi/ml of [^{125}I]IUdR (specific activity, 2200 Ci/nmol). The labelled cells were washed three times in RPMI 1640 and treated as indicated in Figure 2. 3LL cells were counted by the trypan blue exclusion test and then 2×10^5 viable cells in 0.2 ml were injected i.v. into mice via the tail vein. At different times (1 and 30 min), the animals (at least six mice per group) were killed, the lungs were excised and placed in 70% ethanol, and then radioactivity associated with the lungs was quantitated in a gamma counter (LKB Pharmacia). Results were expressed as percentage of recovery (geometric mean of the results of the individual mice in each group) of the injected radioactivity.

Flow cytofluorimetric analysis

For immunofluorescence staining, cultured tumour cells were washed in PBS containing 0.1% sodium azide, and incubated with 135-13C MAb, anti-α-6 [11, 30] (kindly provided by Dr A.

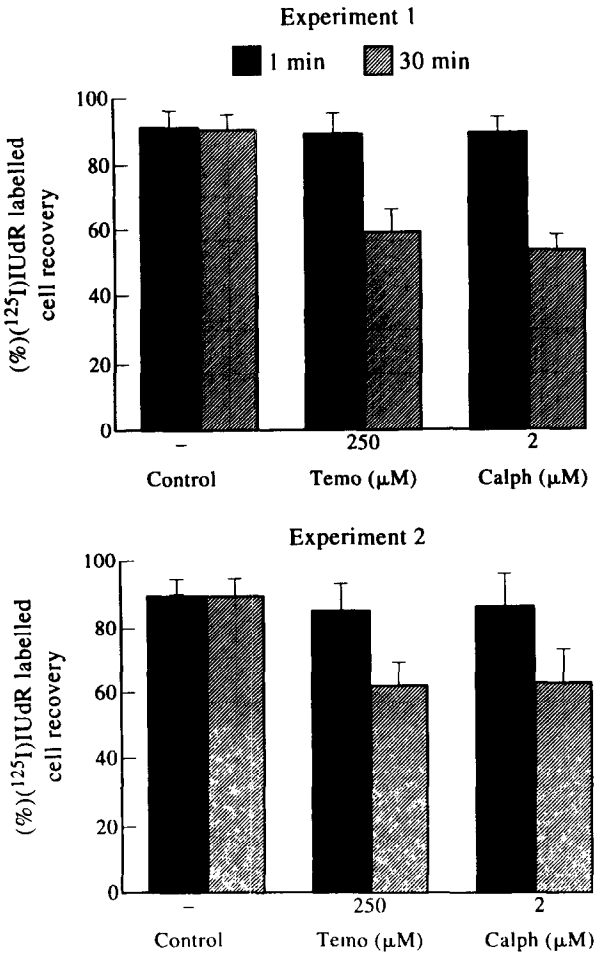

Figure 2. Effects of temozolomide and calphostin c on 3LL cell recovery in lungs. [^{125}I]IUdR-labelled 3LL cells, treated for 3 h with temozolomide (Temo) or calphostin c (Calph) at the concentrations indicated in the figure, were injected intravenously in mice. At 1 and 30 min, lungs were excised and radioactivity measured in a γ-counter. One minute after injection, the lungs of mice inoculated with untreated 3LL cells retained 1.85 × 10^5 ± 7900 cells whereas lungs from animals injected with cells pre-treated with temozolomide and calphostin c retained 1.8 × 10^5 ± 9500 and 1.76 × 10^5 ± 8700, respectively. Data in the figure are expressed as per cent of control (mean of six animals per group ± S.E.) and show two representative experiments out of three performed independently, all of which produced comparable results.

Sacchi) integrin subunit for 30 min. After additional washings, the cells were stained with fluorescein-conjugated rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark) for 30 min, washed and analysed on a FACScan (Becton Dickinson). Controls to detect non-specific binding of the fluorochrome-coupled reagent were always included.

Preparation of cell extracts

Cells were washed with PBS. The cell pellet was suspended in 500 μl of lysis buffer (25 mM HEPES pH 7.5, 2.5 mM $MgCl_2$, 2.5 mM EGTA, 50 mM 2-mercaptoethanol, 200 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 400 μg/ml soybean trypsin inhibitor) sonicated for 5 s at 4°C and centrifuged for 1 h at 100 000 *g* at 4°C. The supernatant was designated as the cytosol fraction. The pellet was resuspended in lysis buffer containing 1% Triton X-100, sonicated for 5 s and centrifuged for 10 min at 15 000 *g* at 4°C in an Eppendorf microcentrifuge. The supernatant was defined as membrane fraction. Protein concentration was determined by Coomassie plus protein assay (Pierce, Rockford, Illinois, U.S.A.) with BSA as the standard.

Protein kinase c assays

Cytosol and membrane fractions were adsorbed in a 1.5 ml microcentrifuge tube to 0.2 ml of DEAE-Sepharose fast flow equilibrated in buffer A (20 mM HEPES pH 7.5, 10 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA), washed three times with buffer A, and eluted with 0.2 M NaCl in buffer A. The 0.2 M NaCl eluate was incubated in a 50-μl reaction mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 40 μg of histone H1, 100 μg/ml of PS, 200 nM PDBu (phorbol dibutyrate), 500 μM $CaCl_2$ or 200 μM EGTA in place of $CaCl_2$, PS and PDBu. Assays were carried out as previously described [31].

Phosphorylation of α-6 integrin subunit

Cells were incubated in 1 ml of phosphate-free RPMI medium for 60 min before labelling for 90 min with 0.5 mCi/ml $H_3{}^{32}PO_4$. Cells were treated with 1 μM of calphostin c or with 250 μM of temozolomide for 3 h at 37°C alone and/or with 0.1 μM TPA for 30 min. Following the incubations, cells were washed twice with ice-cold PBS and lysed in buffer containing 1% Nonidet-P-40, 0.1% SDS, 0.15 M NaCl, 10 mM EDTA, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 0.6 M sucrose, 10 mM EDTA and 2 mM PMSF. α-6 was immunoprecipitated with monoclonal antibody 135-13C previously complexed with protein A-sepharose plus rabbit anti-rat Ig (Dakopatts). The immunoprecipitates were washed four times with buffer containing 50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 0.25% Nonidet-P40, 0.1% Na-deoxicolate, 0.15% SDS, and solubilised in SDS–PAGE sample buffer (0.063 mM Tris–HCl, pH 6.8, 5 mM diothiothreitol (DTT), 2% SDS, 0.01% bromophenol blue). Samples were heated for 3 min in boiling water and separated in SDS–7.5% polyacrylamide gels as described by Laemmli [32]. Labelled proteins were detected by autoradiography with Kodak X-Omat AR films.

RESULTS

Effects of temozolomide on 3LL tumour growth and spontaneous metastases

The results summarised in Table 1 show that treatment with temozolomide for 5 days, right after the appearance of a palpable tumour, reduced the metastatic potential of 3LL carcinoma cells in a concentration-dependent manner, whereas the growth of primary tumour was not significantly affected. Treatment with 100 mg/kg of temozolomide caused 78% and 90% reductions of the number of spontaneous metastases on days 15 and 21, respectively.

Effects on tumour growth and spontaneous metastases were also evaluated by treating 3LL cells *in vitro* with graded concentrations of temozolomide (Table 2). Exposure of tumour cells to 250 μM of temozolomide for 3 h inhibited tumour growth and the appearance of metastases. On day 90, mice were still tumour free. Treatment of tumour cells with temozolomide at lower concentrations produced a statistically significant reduction in the number of metastases 15 days after injection, whereas tumour weight was not significantly reduced. Table 2 shows that treatment with 0.2 μM of calphostin c was needed to obtain a 70% reduction in the number of metastases without affecting tumour growth. Higher concentrations (2 μM) inhibited tumour growth and abolished metastasis formation on day 15. Cell

viability was not affected by treatment with either temozolomide or calphostin c at the concentration tested, as assessed by the trypan blue exclusion test. Moreover, the proliferation assay performed 48 h after treatment of 3LL cells with 250 μM of temozolomide did not show a significant difference of [^{3}H]TdR uptake between control and treated groups (i.e. 19100 ± 1450 versus 18770 ± 1800).

Effects of temozolomide on artificial metastases

3LL cells treated with temozolomide or calphostin c *in vitro* were also tested for their ability to form experimental metastases. Figure 1 shows that both agents inhibited the metastatic potential of the tumour cells in a concentration-dependent manner.

Arrest of 3LL cells in lungs

To ensure that comparable amounts of control, temozolomide or calphostin c treated cells reached the lungs, the distribution of tumour cells inoculated i.v. in the lungs of C57BL/6 mice was examined using [^{125}I]IUdR-labelled cells. One minute after injection, 3LL cells (Figure 2) exposed to temozolomide or calphostin c reached the lungs, as well as untreated cells. Figure 2 also shows that 30 min after injection, lungs from animals inoculated with treated cells retained a significantly lower amount of radioactivity than those from animals inoculated with untreated cells.

Adhesion of 3LL cells to endothelial cells

The experiment summarised in Figure 3 shows that the adhesion of 3LL cells to the endothelioma cell line was markedly diminished by treating 3LL cells with temozolomide or PKC inhibitors (calphostin c or H-7). After 15 min of incubation, adhesion of 3LL cells, treated with high concentrations of temozolomide or PKC inhibitors, ranged between 45 and 55% of controls. As incubation times increased and concentrations diminished, the inhibitory effect tended to disappear. Maximal adhesion was observed by 60 min, at which time adhesion of 3LL cells treated with the lowest concentrations of drug did not differ from adhesion of controls. Concentrations of the drugs used and time points of the experiments were therefore crucial.

Adhesion of 3LL cells to immobilised proteins

3LL cells treated with 3 h with 250 μM of temozolomide showed decreased adhesion to both collagen IV and laminin, a slight reduction of adhesion to fibronectin, while adhesion to collagen I was not affected (Table 3).

Effect of temozolomide of PKC

The activity of PKC in the cytosolic and membrane fraction of 3LL cells treated with temozolomide and/or TPA is shown in Figure 4. More than 85% of PKC activity was found in the

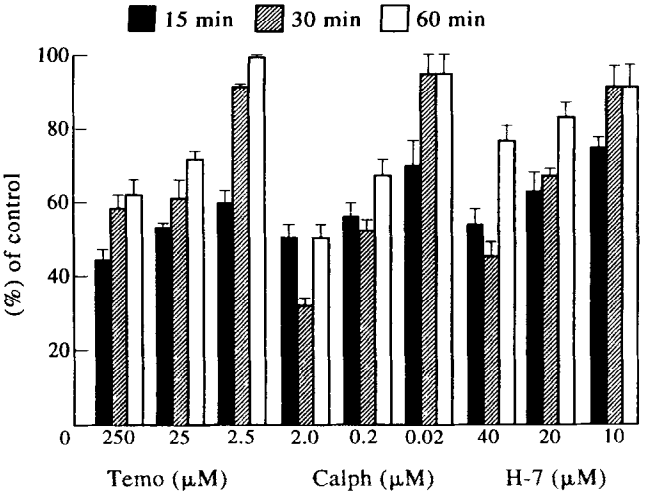

Figure 3. Effects of temozolomide (Temo) and PKC inhibitors on 3LL cell adhesion to endothelioma. ^{51}Cr-labelled cells treated with the drugs at the indicated concentrations were added to confluent endothelioma cells as described in Materials and Methods. At the times indicated, radioactivity of adherent cells was measured with a γ-counter. Results are expressed as per cent of control (mean of quadruplicates ± S.E.). Data shown refer to a representative experiment (n = 3, all of which produced comparable results). Calph, Calphostin c.

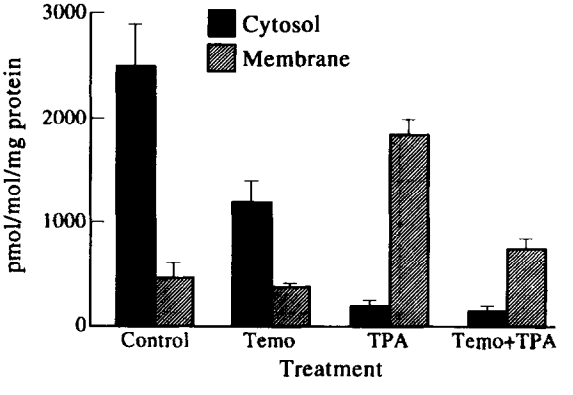

Figure 4. PKC activity in cytosol and membrane fractions of 3LL cells. Cells were treated with 250 μM temozolomide (Temo) for 3 h or/and 0.1 μM TPA for 30 min. After cell lysis, assays were carried out with the 0.2 M NaCl fraction eluted from DEAE-Sepharose. Each PKC value represent the mean of three independently performed experiments ± S.E.

Table 3. Adhesion of 3LL cells to immobilised proteins

	Controls	Temozolomide	% of controls
Collagen I	6450 ± 500	6300 ± 560	97
Collagen IV	4700 ± 490	3200 ± 600	68
Fibronectin	6755 ± 755	5155 ± 255	76
Laminin	10900 ± 450	5740 ± 440	56

3LL cells were labelled with [^{3}H]TdR, treated with 250 μM temozolomide for 3 h, added to protein-coated wells and then incubated for 1 h. The wells were washed, and adherent cells were quantitated by liquid scintillation counting. Values represent cell adhesion to matrix proteins expressed as cpm (± S.E.) and as percentage of controls.

cytosolic fraction of 3LL cells. After 3 h treatment with 250 μM temozolomide, cytosolic PKC activity was reduced by 50%, whereas no change was observed in the membrane fraction. Incubation of 3LL with 0.1 μM TPA for 30 min induced translocation of Ca^{2+}, PS-dependent protein kinase activity from the cytosol to the membrane fraction. TPA-induced PKC activity in the membrane fraction was depressed more than 50% after pretreatment with temozolomide. *In vitro* experiments confirmed the inhibitory effects of triazene on PKC activity. Partially purified PKC activity from rat brain in the presence of MTIC was measured as described in the Materials and Methods. The enzyme was incubated for 10 min in the presence of PS, PDBu, CaCl at graded concentrations of MTIC (range 2.5–1000 μM). Under the conditions used, half-maximal inhibition was obtained at 250 μM of the compound. SDS–PAGE analysis of lysate from 3LL cells showed that MTIC inhibited a Ca^{2+}–PS dependent phosphorylation of 80-kD protein (data not shown).

Phosphorylation of α-6 integrin

Following treatment of $H_3{}^{32}PO_4$ labelled cells with temozolomide, calphostin c or TPA, phosphorylation of α-6 was measured by immunoprecipitation of cellular lysate with MAb 135-13C. Incubation of 3LL cells with 0.1 μM TPA at 37°C for 30 min produced the phosphorylation of α-6 integrin (116 kDa; Figure 5, lane 2) which was absent in control cells. In addition to the 116-kDa band, a 180-kDa band, which is probably non-specific, was also noted in all the lanes. After treatment with 1 μM of calphostin c or 250 μM temozolomide for 3 h, phosphorylation of α-6, induced by TPA, was completely blocked (Figure 5). Analysis by flow cytometry, performed after exposure to temozolomide or calphostin c for 3 h or TPA for 30 min, indicated that the percentage and fluorescence intensity of α-6 integrin subunit surface expression in cells treated with temozolomide, calphostin c and TPA were identical to those of control cells (Figure 6). Comparable results were obtained 4 and 18 h after treatment (data not shown).

DISCUSSION

Metastasis is a multistep process including tumour cell intravasion, haematogenous and/or lymphatic dissemination, arrest, adhesion to endothelium, adhesion to and degradation of subendothelial matrix and finally extravasion [33, 34]. Adhesion to endothelium or to matrix components could be a rate-limiting step in the metastatic cascade [34]. Hence, modulation of tumour cell adhesion by chemotherapeutic agents may affect metastatic potential of tumours.

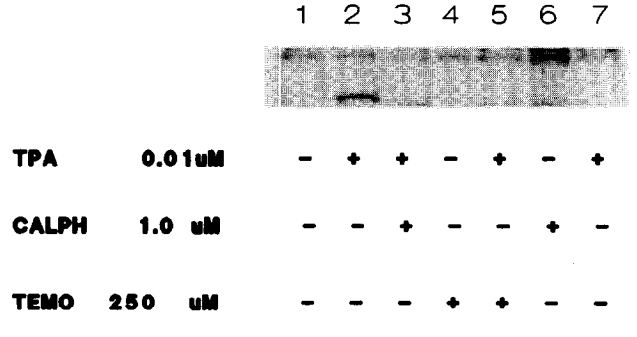

Figure 5. Autoradiograph of α-6 phosphorylation in 3LL cells. Cells were labelled with $H_3{}^{32}PO_4$ in the presence of calphostin c (CALPH) or temozolomide (TEMO) for 3 h or TPA for 30 min. Lysates were then immunoprecipitated using 135-13C Mab to α-6 integrin subunit analysed by SDS–PAGE and detected by autoradiography as described in the Materials and Methods. Lane 1, untreated cells; lane 2, TPA 0.1 μM; lane 3, calphostin c 1 μM and TPA 0.1 μM; lane 4, temozolomide 250 μM; lane 5, temozolomide 250 μM and TPA 0.1 μM; lane 6, calphostin c 1 μM; lane 7, lysate from TPA-treated 3LL cells immunoprecipitated with protein A-sepharose complexed with rabbit anti-rat Ig in the absence of Mab to α-6 integrin subunit.

The involvement of PKC in the metastatic phenotype has been implied by previous studies that have demonstrated that (a) more highly metastatic tumour cell lines have high levels of PKC [35, 36] and (b) PKC inhibitors decrease both tumour invasion [37–39] and adhesion to endothelium [35]. However, most of these compounds showed poor specificity in inhibiting PKC. Downregulation of PKC by long-term treatment with phorbol esters [40] has been shown to decrease metastases and to reduce the adherence of tumour cells [1]. Alternatively, short-term exposure to TPA produced translocation of PKC from cytosol to the membrane fraction [41], resulting in enhanced metastasis formation [1]. Consistent with these reports, the present study shows the inhibitory effect of calphostin c [18] on experimental metastases and on 3LL cell adhesion to endothelium. Triazenes possess, in addition to several biological and pharmacological properties, a rather selective anti-metastatic action, dependent on their chemical structure [22, 42–46]. Triazene compounds cause increased expression of some tumour-associated antigens (chemical xenogenisation [47]) and their anti-tumour and anti-metastatic activity might be partially due to an increased susceptibility to immunological mechanisms [48]. Our study has addressed the role of phosphorylation of α-6 integrin subunit in the inhibition of metastasis induced by temozolomide. In addition, evidence is provided that α-6 phosphorylation is catalysed by PKC. Moreover, inhibition of metastases induced by treatment with temozolomide was associated with a reduction of PKC. Lower levels of PKC activity in the cytosol of cells treated with temozolomide seem to render the cells less susceptible to PKC activation by TPA.

Incubation of 3LL cells with temozolomide reduced both cell adhesion to endothelium or to matrix components and lung colonisation. The decreased lung colonisation, following treatment of mice with temozolomide, cannot be accounted for only by a decreased proliferative capacity since tumour growth was not significantly affected by treating 3LL cells with the agent at low concentrations. The decrease of tumour weight observed in the primary tumour in some of the treated groups could be explained by different mechanisms, such as immunological factors. In addition, viability and [^{3}H]TdR incorporation by drug-treated cells during a 48-h period was equivalent to those of controls. Therefore, the anti-metastatic properties of temozolomide may be due, at least in part, to the reduced ability of 3LL cells to adhere to the endothelium and to matrix components. It is not clear whether tumour cell adhesion to the endothelium is mediated by integrins or selectins [5, 6]. It has been described that the appearance of an α-4/β-1 integrin in tumour cells may represent a mechanism for tumour cell arrest on endothelial cells, favouring the metastatic process [49]. Circulating or locally released cytokines can activate endothelial cells to express or modulate adhesive structures for tumour cells. A murine tumour surface protein complex which includes α-6 integrin subunit has been associated with the metastatic phenotype of 3LL clones [30]. Furthermore, antibodies to α-6 integrin stimulate 3LL cell metastatic capacity [30]. In our model, α-6 integrin phosphorylation, possibly induced by contact between tumour cells and the endothelium, is inhibited by temozolomide. Cells pretreated with temozolomide produce a significant inhibition of TPA-

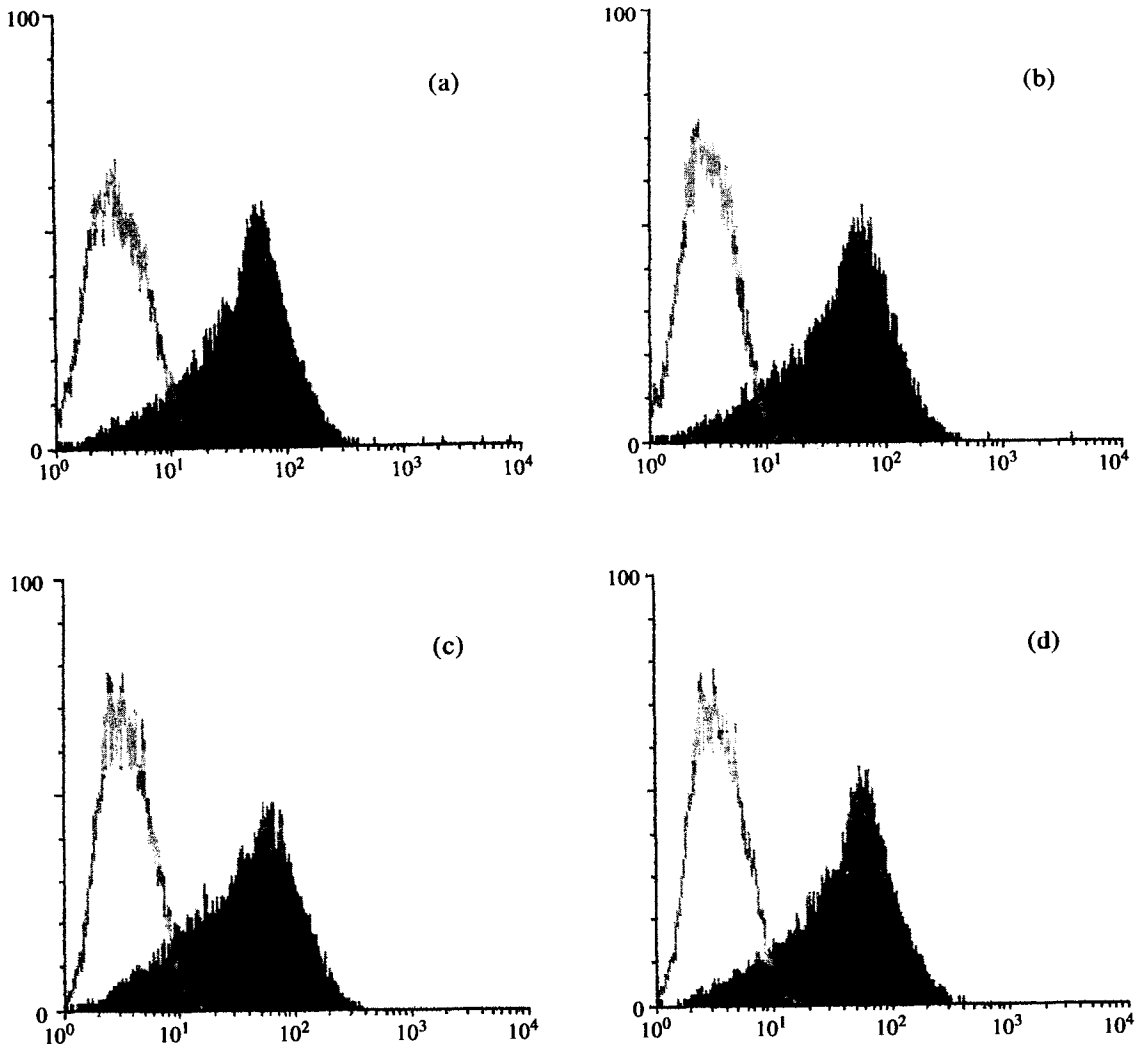

Figure 6. Flow cytometry analysis of α-6 integrin subunit in untreated and temozolomide and calphostin c treated 3LL cells. Flow cytometry analysis was performed with 135-13C monoclonal antibody as described in the Materials and Methods. Control profiles (FITC-rabbit anti-rat Ig). (a) untreated cells; (b) 250 μM temozolomide (3 h); (c) 1 μM calphostin c (3 h); (d) 0.1 μM TPA (30 min).

induced translocation of PKC activity to the membrane fraction. The biological activity of many integrins depends on the activation process. Stimulation of cells promoted by various factors (agonists), such as TPA or cell-to-cell contact, leads to conformational change in integrin receptors, without significant alterations of the levels of expression, rendering these adhesion receptors competent for ligand binding. The ability of cell surface receptors to adhere to specific cell or protein targets has been shown to be related to the phosphorylation state of integrin subunits [14, 50, 51]. Factors modulating the expression of these molecules in tumour cells can be important regulators of metastases. In the present model, the expression of the α-6 integrin subunit (Figure 6) and of the β-4 integrin subunit, which is also increased in the highly metastatic 3LL subline [11–30], was not modified by temozolomide treatment (data not shown). Phosphorylation experiments using 135-13C MAb to immunoprecipitate α-6 integrin revealed that this protein was phosphorylated by stimulation of 3LL cells with TPA. This event is consistent with the observation that cell membrane-enhanced adhesiveness correlates with TPA-induced phosphorylation of the α-6 integrin subunit [15]. It is possible that translocation of PKC on membrane fractions induced by TPA might be responsible for α-6 integrin subunit phosphorylation. Blockage of PKC activity by temozolomide or PKC inhibitor abolished α-6 phosphorylation promoted by TPA. Since α-6 integrin has been shown to bind to laminin [11], the inhibition of phosphorylation of this integrin might be one of the causes of the reduction of the adhesion capacity of 3LL cells to matrix components. Phosphorylation of the β-4 integrin subunit was not modified by TPA or temozolomide treatment. Although there is no direct evidence, it can be speculated that tumour cell interaction with the extracellular matrix involves a multistep process with initial adhesion of tumour cells to the endothelium, which can be mediated by different integrins or selectin [5, 6, 49]. This event is followed by activation of α-6 integrin and finally strong adhesion of phosphorylated α-6 integrin to the matrix. Consistent with this model, drug-induced prevention of α-6 phosphorylation is associated with reduced adhesion of tumour cells to laminin which has been shown to be α-6 dependent by blocking experiments, using anti-α-6 antibody [52]. The binding between α-6 and laminin has been described as playing a crucial role in tumour cell invasion through the basement membrane [52], which is an essential step in the metastatic process [53, 54]. It is possible that modulation of

other integrins, which bind to laminin or other matrix components, could be involved in the drug-mediated inhibition of metastases. Temozolomide treatment also decreased adherence of 3LL cells to fibronectin and collagen IV. Studies are currently under way to look at the effects of temozolomide on other integrins involved in binding to these substrates. Furthermore, we are currently studying whether the contact between tumour cell and endothelium induce α-6 phosphorylation, and whether this phenomenon could also be inhibited by treatment with temozolomide. Alternatively, post-translational modifications of integrins could trigger an intracellular signal which may regulate gene expression and modify adhesion events. Tumour cell adhesion to endothelial cells or extracellular matrix mediated by integrins has been shown to transmit signals which regulate cell growth, differentiation and gene expression [11, 13].

In conclusion, this study suggests that temozolomide induces a reduction of tumour cell adhesion properties which in turn may be mediated by a downregulation of PKC activity. Inhibition of PKC appears to abrogate α-6 integrin phosphorylation which is involved in tumour cell adhesion to laminin. Therefore, it is conceivable that temozolomide could be considered for anti-metastatic therapy in designing cancer chemotherapy strategies.

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The Effect of Different Routes of Administration of 5-Fluorouracil on Thymidylate Synthase Inhibition in the Rat

C.L. van der Wilt, A. Marinelli, H.M. Pinedo, J. Cloos, K. Smid, C.J.H. van de Velde and G.J. Peters

A rat colon tumour model of liver metastases was used to administer 5-fluorouracil (5FU) by intraperitoneal (i.p.) bolus injection (50 mg/kg), isolated liver perfusion (ILP, 150 mg/kg) and hepatic artery infusion (HAI, 50 mg/kg). The biochemical effect of 5FU, delivered by different routes, on its target enzyme thymidylate synthase (TS) was studied in both tumour and normal tissues of the rat. In tumour tissue, only small differences were observed in the extent of TS inhibition. A pronounced inhibition of TS was observed 3 h after 5FU administration by all routes, but was followed by a recovery of TS activity within 24 and 48 h. Effects of 5FU on normal tissues were diverse. In liver, TS activity increased 6-fold after ILP and HAI administration of 5FU, and a 2-fold increase of FdUMP binding to TS was seen for all routes of administration. In intestinal mucosa, both induction of TS activity (by ILP) and inhibition of TS activity (by HAI) were observed, while i.p. injection did not cause major changes. TS activity and FdUMP binding to TS in bone marrow was strongly inhibited after administration of 5FU by all routes, but administration by ILP seemed slightly advantageous, since a smaller extent of TS inhibition was observed compared to the other routes of administration. 5FU given by ILP had a small antitumour effect in this colon tumour model, while HAI administration had no antitumour activity. Since this difference in antitumour activity could not be related to differences in TS inhibition in the tumour, the RNA-directed mechanism of action of 5FU could be involved. Focusing on the effects of TS, we may conclude that the ILP administration of 5FU offered the important advantage of a lack of severe TS inhibition in normal tissues, which corresponds with the low systemic toxicity observed.

Key words: thymidylate synthase inhibition, 5-fluorouracil, route of administration, animal model, rat, liver carcinogenesis

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