Inhibition of angiogenesis by non-toxic doses of temozolomide

Hjalmar Kurzen[a], Stefan Schmitt[a], Helmut Näher[a] and Thomas Möhler[b]

It is well established that certain chemotherapeutic agents have potent antiangiogenic properties which may be part of their antitumor activity. Temozolomide (TMZ) is a lipophilic methylating agent used in the therapy of malignant melanoma and other tumors. We sought to determine whether TMZ is capable of inhibiting angiogenesis or influencing endothelial function. We used the *in vivo* chorioallantoic membrane (CAM) assay, and HUVEC-based *in vitro* Matrigel, adhesion and proliferation assays to determine the antiangiogenic effects of different doses of TMZ. In the CAM assay, angiogenesis was significantly inhibited by 5 μM TMZ, a concentration also found to be effective in interfering with *in vitro* angiogenesis as measured by the Matrigel assay. For the inhibition of basic fibroblast growth factor (bFGF)-, vascular endothelial growth factor (VEGF)- or β-phorbol 12-myristate-13-acetate (PMA)-induced endothelial cell proliferation or endothelial cell adhesion to fibronectin, TMZ concentrations of at least 25 μM were necessary, indicating that bFGF-, VEGF- or protein kinase C-mediated pathways may not primarily be involved in the observed antiangiogenic effect. Thus, we could demonstrate that TMZ inhibits angiogenesis at low, non-toxic doses that correspond to the plasma concentrations achieved by an oral application of 20 mg/m^2 every 8 h. This 'metronomic' scheduling has already been used in phase I studies and has produced antitumor effects. Therefore, the antitumor activity of TMZ may, at least in part, be due to its antiangiogenic properties. The precise mechanism of its antiangiogenic action remains to be elucidated.

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Departments of [a]Dermatology and [b]Medicine, University of Heidelberg, Heidelberg, Germany.

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Correspondence to H. Kurzen, Department of Dermatology, University of Heidelberg, Voss-strassse 2, 69115 Heidelberg, Germany.
Tel.: +49 6221 5639532; fax: +49 6221 568509 or +49 6221 564996;
e-mail: Hjalmar_Kurzen@med.uni-heidelberg.de

Introduction

Angiogenesis, i.e. growth of new vessels, is a main prerequisite for tumors to grow beyond a certain size and to metastasize, and considerable progress has been made in recent years in understanding its molecular mechanisms [1,2]. That chemotherapeutic agents cause endothelial damage has been known for a long time and has been extensively studied since then [3–5]. For example, it has been shown previously that thrombomodulin, an indicator of endothelial cell damage, can be detected after chemotherapy. This effect was primarily seen under the aspect of adverse effects of chemotherapy, like thrombosis and veno-occlusive disease (VOD), or lung damage, like the 'bleomycin lung' [3,6–8].

In recent years, several groups observed antiangiogenic activity of different chemotherapeutic agents and provided evidence that this can contribute to the therapeutic antitumor effect. Induction of apoptosis and inhibition of proliferation and migration of endothelial cells were recognized as mechanisms for the antiangiogenic effects of cytostatic drugs [9–13]. Further investigation of the antiangiogenic effect of cyclophosphamide in preclinical models revealed that the best inhibition was achieved by 'high-frequency' application of lower doses of cyclophosphamide in contrast to the 'standard' cyclic therapy [11]. This antitumor effects of the so-called 'metronomic' scheduling of chemotherapy was confirmed by others and synergistic activity was shown in combination with an antiangiogenic vascular endothelial growth factor (VEGF) receptor inhibitor [12]. Metronomic scheduling was recently reviewed by Gasparini [14].

Another advantage of optimizing chemotherapy to target endothelial cells participating in the angiogenesis process is their genetic stability. In contrast, acquired drug resistance is one of the major mechanisms for failure of cancer chemotherapy due to the genetic instability of tumor cells [15].

Temozolomide (Temodal®; TMZ) is a methylating agent used in the therapy of malignant glioma and malignant melanoma. It is a lipophilic analog to the triazene dacarbazine and dissolves in watery solutions spontaneously to the active metabolite MTIC [16]. Due to its lipophilic properties, it is orally available and readily crosses the blood–brain barrier. Apart from its methylating properties, an inhibition protein kinase C (PKC) has

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been implicated as a possible mechanism of action [17]. It has been used in the 'traditional' cyclic fashion in various regimens for different tumor entities. Interestingly, in phase I studies, TMZ has also been used in continuous low-dose schedules, which have led to the regression of several tumors [16].

We therefore sought to determine whether TMZ has antiangiogenic properties that might be responsible for the observed clinical effects.

Material and methods

Source of TMZ

TMZ was obtained from Essex Pharma (Munich, Germany). It was dissolved in DMSO with final DMSO concentrations not exceeding 0.5%. In all instances DMSO control experiments were performed in order to exclude toxic effects of DMSO [18]. TMZ concentrations were in the range 0.5–100 μg/ml (2.5–500 μM).

Chicken chorioallantoic membrane (CAM) assay

Fertilized white chicken eggs (at least 17 per group) were incubated at 37°C at constant humidity. On incubation day 7, a small square window was opened in the shell and a piece of sterile Whatman paper disks, 6 mm in diameter, loaded with 20 μl conditioned medium from 3T3 mouse fibroblasts (3T3 CM) or 40 ng/20 μl basic fibroblast growth factor (bFGF; R & D Systems, Wiesbaden, Germany), was placed onto a big vessel of the CAM. The Whatman paper disks for the negative control were loaded with dilution buffer (2 M NaCl, 10 mM Tris, pH 6.0) only. On day 8, TMZ at final concentrations ranging from 2.5 to 50 μM was pipetted onto the Whatman paper disks. This was repeated on days 9 and 10. On day 11, the membranes were harvested, and the numbers of branch points of blood vessels and capillaries underlying the Whatman paper disks were counted using a stereomicroscope (Wilovertl Will, Wetzlar, Germany). The angiogenesis index was calculated by counting all branch points of visible capillaries and blood vessels on the CAM tissue beneath the paper disk [19].

Cells and culture conditions

Human umbilical chord vein endothelial cells (HUVEC) were isolated from umbilical chords of newborns as described by Jaffe [20] and cultivated on gelatinized Primaria cell culture dishes in basal growth medium (ECBM; Promocell, Heidelberg, Germany) containing 1% L-glutamine (Gibco/BRL, Paisley, UK), 20% fetal calf serum (FCS; CC-Pro, Neustadt, Germany), 0.32%. ECGS, 0.08 ng/ml human epidermal growth factor, 40 ng/ml Amphotericin B and 40 μg/ml Gentamicin (Sigma, St Louis, MO). Cells were assayed between passages 3 and 12.

3T3 Swiss albino mouse fibroblasts were obtained from the German Collection of Microorganisms and Culture cells (DSMZ, Braunschweig, Germany) and were cultured in RPMI containing 10% FCS. 3T3 CM, which contains several angiogenic factors [21], was prepared by incubating subconfluent cells in serum-free RPMI for 24 h. The supernatant was then collected under sterile conditions, centrifuged at 13000 r.p.m. for 20 min at 4°C and stored at −20°C [5].

Matrigel assay

Unpolymerized Matrigel (Becton Dickinson, San Jose, CA) was placed in the wells (150 μl/well) of a 48-well microtiter plate and allowed to polymerize for 1 h at 37°C. HUVEC were plated at 30 000 cells/well in serum-free medium containing 50% 3T3 CM alone (positive control) or, in addition, TMZ at a final concentration of 2.5–50 μM. For the negative control, serum-free medium without 3T3 CM was used. After 24 h of incubation in a 5% CO_2 humidified atmosphere at 37°C, endothelial cell alignment was quantified by counting the intersections of the newly formed capillary-like structures.

Cytotoxicity assay

In order to determine the cytotoxic effects of TMZ on proliferating endothelial cells, we plated HUVEC as described above (4000 cells/well) in basal growth medium. TMZ at final concentrations in the range 1–100 μg/ml (5–500 μM) was added to the culture medium. For each TMZ concentration, eight wells were used. The experiment was repeated twice. After 72 h the cells were washed with HEPES buffer, trypsinized and counted after Trypan blue staining in a Neubauer chamber. No living cells could be detected in the culture supernatant. LD_{50} was defined as concentration of TMZ that led to a 50% cell reduction [22].

Proliferation assay

All experiments were repeated at least twice in order to obtain 24–36 values for each condition. In a first experiment, proliferation of HUVEC was induced by standard growth medium (positive control). Serum-free ECBM without growth factors was used as negative control. On day 1, cells were seeded on a 96-well microtiter plate at 4000 cells/well. TMZ was added to reach final concentrations of 0.5–50 μM on day 2. The medium was changed every day, in order to exclude degradation artifacts. In a second series of experiments, in order to antagonize specific angiogenic pathways, HUVEC proliferation was induced by the addition of 2 ng/ml bFGF, 0.01 ng/ml VEGF (both R & D Systems) or 50 nmol/l β-phorbol 12-myristate-13-acetate (PMA; Sigma) to basal growth medium with reduced serum concentrations (5% for bFGF and VEGF, and 2% FCS for PMA), and without ECGS and human endothelial growth factor (positive control). HUVEC maintained in

basal growth medium with respective serum concentrations was used as negative control. TMZ was added on day 2 in the same concentrations as before. In all cases, cell numbers were determined on day 5. The cells were washed with HEPES buffer, fixed with 4% buffered formaldehyde, stained with 1% methylene blue and air-dried. After thorough washing, methylene blue concentration in the extraction buffer (0.1 N HCL) was determined using an ELISA reader at 620 nm. Methylene blue is incorporated into cells in a linear proportion to the cell number. After normalization to a standard curve, relative cell density could be assessed [23].

Adhesion assay on fibronectin

Plates (96-well) were coated with a fibronectin solution (20 μg/ml) at 4°C overnight. HUVEC were plated (5000 cells/well) and incubated in quadruplicate in basal, serum-free culture medium alone (positive control) or containing different TMZ concentrations from 2.5 to 50 μM at 37°C in a 5% CO_2 humidified atmosphere as described [5]. Non-attached cells were removed after 30 or 90 min depending on the experimental conditions. The cell numbers were determined as described for the proliferation assay. After 30 min the first part and after 90 min the second part of the seeded cells were fixed and stained with methylene blue. After having performed this original adhesion assay, a modified one was performed. Cells were seeded on 96-well plates coated with 1% gelatine in PBS on day 1. The following day, they were incubated with basal culture medium alone as positive control or with TMZ in the same concentrations as described above. One day later the cells were transferred to a 96-well plate coated with fibronectin. After 30 and 90 min of incubation cells were fixed, and the cell number was determined as described above.

Statistics

To compare and analyze results of all experiments the data were calculated as follows. For each experiment the mean of the positive control (induction of angiogenesis or proliferation) was set at 100%. All individual data of a single experiment were now expressed as percentage of the mean of the positive control of the same experiment. After this, all data available for each treatment condition were pooled and used for the statistical analysis.

Statistical analysis was performed according to standard procedures. For the Matrigel and proliferation assays, a normal distribution of the obtained values could be demonstrated. Hence, the unpaired Student's *t*-test was used to determine the level of significance. In the case of the CAM assay, a normal distribution could not be demonstrated. Therefore, the Wilcoxon two-sample test was used to determine the p level of statistical significance. A value of $p < 0.05$ was considered statistically significant for both tests.

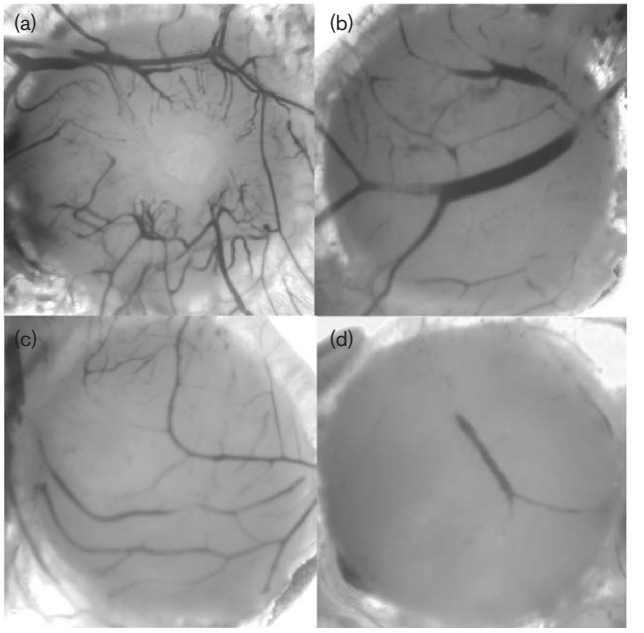
Results

The inhibitory activity of TMZ on angiogenesis and endothelial cell function was determined using an *in vivo* assay and three *in vitro* assays.

CAM assay

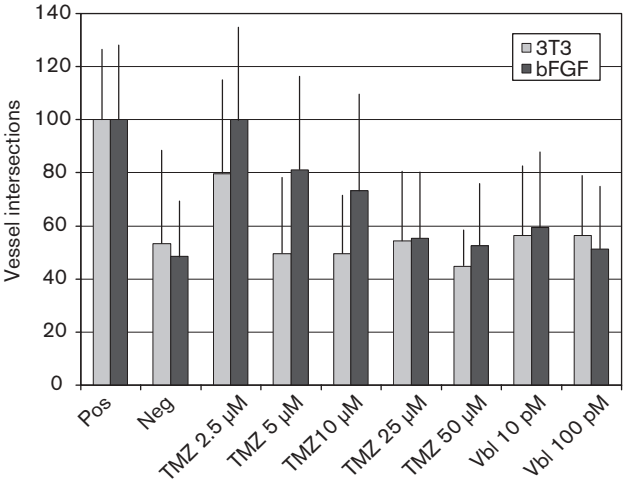
Angiogenesis was significantly induced on the CAM in six different, independent experiments. A mean of 103 branch points was observed if 3T3 CM was used to induce vessel growth ($n = 61$). In the negative control ($n = 27$), the mean vessel count was 57% of positive control and thus significantly lower ($p < 0.0001$). We noted a considerable range in the vessel count (10–214) which is reflected by rather high SD values. We think that an increase in humidity that could be observed on some of the CAMs during the experiment may have accounted for variations of pro-angiogenic cytokine and also TMZ concentration. Addition of TMZ led to a dose-dependent inhibition of vessel formation (Figs 1 and 2). At a TMZ concentration of 2.5 μM, significance was not reached, but from 5 ($n = 20$) to 50 ($n = 22$) μM statistical significance was evident ($p < 0.001$). If angiogenesis was induced on the CAM by bFGF ($n = 32$), vessel growth was comparable to induction by 3T3 CM and significantly above negative control (about 50% of positive control, $n = 20$, $p < 0.0001$). To inhibit bFGF-induced angiogenesis, a 5-fold higher TMZ concentration was necessary as compared to 3T3 CM. Significant reduction of the

Fig. 1



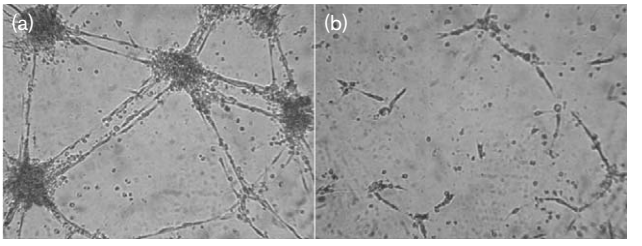
Photomicrographs of CAMs overlying Whatman filter disks. Sprouting angiogenesis is induced after addition of 3T3 CM (a). Addition of 5 (b), 25 (c) and 50 (d) μM TMZ inhibits vessel formation dose dependently.

Fig. 2



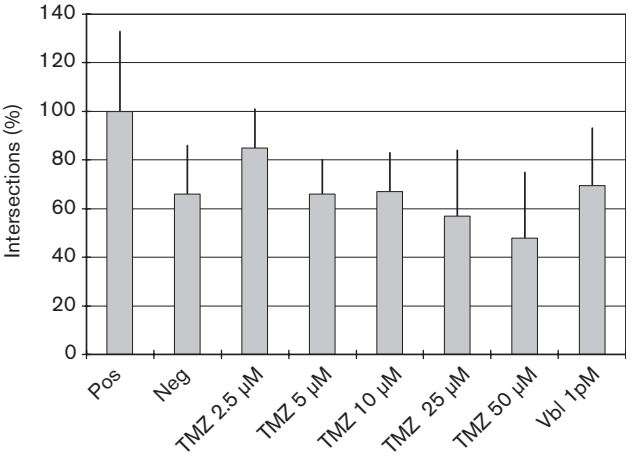
Data shown represent pooled data of independent experiments with the mean of each positive control set as 100%. If TMZ was added, vessel formation was inhibited dose dependently. A slight but not quite significant reduction could be observed ($p=0.07$) already at a TMZ concentration of 2.5 μM and a significant reduction of the vessel count to the level of negative control was evident from 5 to 50 μM if 3T3 CM was used as angiogenic stimulus ($p<0.001$). If vessel growth was induced on the CAM by bFGF, TMZ concentrations needed to obtain significant reduction of vessel growth were slightly higher (10–50 μM). Inhibition of angiogenesis by 5 μM corresponded to that obtained with 10 or 100 pM vinblastine ($p=0.0006$). Columns and bars indicate mean + SD.

Fig. 3



Endothelial alignment in Matrigel after 12 h stimulation by 3T3 CM.

Fig. 4



TMZ at 25 μM leads to a significant reduction of vessel intersections. Without angiogenic stimulus, significantly fewer intersections could be observed (negative control, $p<0.0001$). Using 2.5 μM TMZ, the mean intersection count was reduced to 86% of the positive control. Statistical significance was not reached ($p=0.0597$). Higher concentrations of TMZ (5–50 μM) reduced endothelial alignment significantly ($p<0.007$). Pooled data from a series of four different, independent experiments. Columns and bars indicate mean + SD.

angiogenic index to the level of negative control was observed from 25 to 50 μM ($p < 0.002$), whereas 2.5–10 μM failed to reach significance (Fig. 2).

Matrigel assay

A series of four different, independent experiments was performed. For the positive control ($n = 32$) the mean count ranged between 10 and 50 vessel intersections per five high power fields, and was set 100%. The median was 98.5%. In each experiment, the vessel count in the negative control was significantly lower than in the positive control. The mean of the negative control ($n = 42$) was 66%, the median was 63%. Since for both positive and negative control, mean and median were very close, the distribution of values was considered to be normal.

Using 2.5 μM TMZ, the mean 'alignment index' ($n = 26$) was 86% of he positive control. Statistical significance was not reached ($p = 0.0597$). Using higher concentrations of TMZ (5–50 μM), endothelial alignment could be significantly inhibited ($p < 0.007$, see Figs 3 and 4).

Determination of cytotoxicity (LD_{50}) in HUVEC

In order to determine cytotoxic effects of TMZ on endothelial cells, we exposed HUVEC to increasing concentrations of TMZ, ranging from 5 to 500 μM, in standard growth medium for 3 days. Concentrations up to 150 μM had no significant cytotoxic effects. Higher concentrations led to significantly decreased cell numbers. The LD_{50} was determined to be around 50 μg/ml, corresponding to 250 μM (data not shown).

Proliferation assays

Unspecific inhibition of HUVEC proliferation

HUVEC proliferated significantly in standard growth medium as compared to negative control ($p < 0.0001$). Between 2.5 and 10 μM TMZ did not lead to significant inhibition of HUVEC proliferation after 4 days, while 25 and 50 μM led to slightly reduced cell numbers with 91 and 86% of the positive control ($p = 0.025$ and 0.0008)

Inhibition of bFGF-induced proliferation of HUVEC

Using 2 ng/ml bFGF as stimulus, proliferation of HUVEC could be induced significantly ($p < 0.0001$) in a series of four independent experiments ($n = 36$). Inhibition of HUVEC proliferation could only be demonstrated using 50 μM TMZ in the culture medium ($p < 0.0001$). Between 2.5 and 10 μM TMZ led to significantly enhanced proliferation ($p < 0.5$) (Fig. 5).

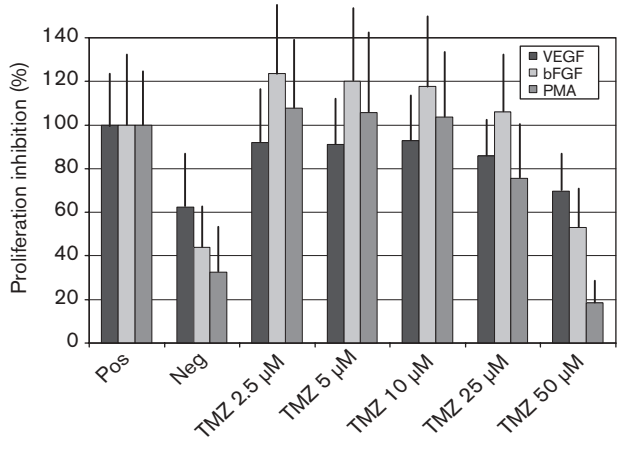
Inhibition of VEGF-induced proliferation of HUVEC

Significant proliferation of HUVEC, compared to negative control (62%), was induced with 0.01 ng/ml VEGF ($n = 35$, $p < 0.0001$). Between 2.5 and 10 μM TMZ, proliferation could not be inhibited significantly, whereas from 25 to 50 μM, a significant inhibition could be observed ($p = 0.024$ and $p < 0.0001$, respectively, Fig. 5).

Inhibition of PMA-induced proliferation of HUVEC

In order to determine whether inhibition of protein kinase C (PKC) might be involved in the observed antiangiogenic effects of TMZ, HUVEC were stimulated by PMA, a potent activator of PKC [24]. HUVEC proliferation could be significantly induced by 50 nmol/l PMA in each series ($n = 36$, $p < 0.0001$). Between 0.05 and 10 μM TMZ, endothelial cell proliferation could not be significantly inhibited. Using 25–50 μM TMZ, inhibition of HUVEC proliferation was significant ($p < 0.0005$, Fig. 5).

Fig. 5



Inhibition of HUVEC proliferation. Proliferation of HUVEC was significantly induced with 2 ng/ml bFGF, 0.01 ng/ml VEGF or 50 nmol/l PMA ($p < 0.0001$). Proliferation was most pronounced after addition of PMA (3-fold), while bFGF and VEGF led only to a approximately 2.3- and 1.6-fold increase, respectively. Values shown are normalized to the positive control which was set 100%. Hence, values for the negative control differ, even though conditions were similar. Using 2.5–10 μM TMZ, endothelial cell proliferation could not be significantly inhibited regardless of the stimulus used. VEGF ($p < 0.025$ and $p < 0.0005$)- and PMA ($p = 0.0005$ and $p < 0.0001$)-induced proliferation could be significantly inhibited by 25–50 μM TMZ, while 50 μM TMZ was needed to inhibit bFGF-induced proliferation significantly ($p < 0.0001$). Columns and bars indicate mean + SD.

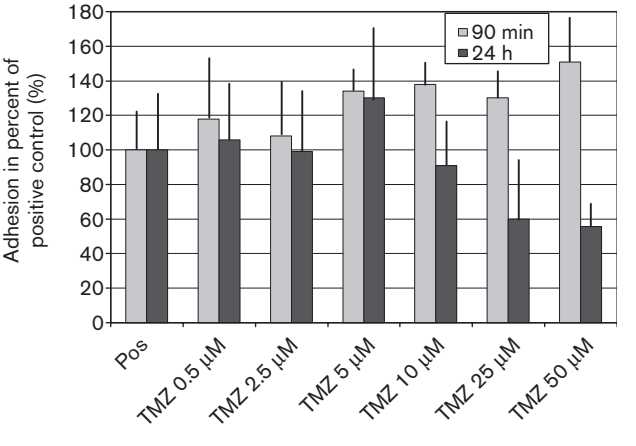
Adhesion assay on fibronectin

Adhesion of endothelial cells to the extracellular matrix is a prerequisite for new vessels to grow. We therefore examined the influence of TMZ on HUVEC attachment to fibronectin, one of the ligands of integrins expressed by HUVEC. Using the protocol as described by Vacca *et al.* [5] we found no significant influence of TMZ on attachment rates after 30 min (not shown). However, after 90 min, attachment was significantly enhanced if TMZ at 5–50 μM was present. If HUVEC were preincubated for 24 h on gelatin in the presence of TMZ and then transferred, attachment to fibronectin after 30 min again showed no significant difference (not shown) at all concentrations tested, while after 90 min attachment was significantly diminished from 25 to 50 μM ($p < 0.03$), whereas 2.5–10 μM showed no change (Fig. 6).

Discussion

In order to determine whether the novel methylating chemotherapeutic drug TMZ is able to inhibit angiogenesis we performed a set of experiments, starting with the well-established *in vivo* CAM assay [25]. In this assay, the complete process of angiogenesis is assayed, including endothelial cell detachment, dissolution of the extracellular matrix, proliferation, migration, alignment, reattachment and maturation [26]. First, we were interested in whether angiogenesis could be inhibited

Fig. 6



Adhesion of HUVEC to fibronectin. After 90 min incubation, attachment of HUVEC to fibronectin was significantly enhanced if 5–50 μM TMZ was present ($p < 0.013$). If HUVEC were preincubated for 24 h on gelatin in the presence of TMZ and then transferred, attachment to fibronectin was significantly diminished from 25 to 50 μM ($p < 0.03$), whereas 2.5–10 μM showed no change. Data are shown as percent of positive control. Columns and bars indicate mean + SD.

by TMZ at all. We therefore used as stimulus 3T3 CM, which has been reported to contain an array of angiogenic factors and thus simulates the tumor microenvironment [21], and bFGF, which is a potent inducer of sprouting angiogenesis [27]. At low concentrations of TMZ (5 μM) we could already demonstrate a highly significant reduction in vessel growth if 3T3 CM was used to induce angiogenesis. Inhibition of angiogenesis was less pronounced and reached significance only at 25 μM TMZ if bFGF was used. bFGF is considered to be one of the main angiogenic components contained in 3T3 CM. The concentration of bFGF reached in 3T3 CM is much lower (22.55 pg/ml; S. Manns, Heidelberg, pers. commun.) than the dose needed if bFGF alone is used to induce angiogenesis in the CAM (2 μg/ml). Thus, either higher amounts of TMZ are necessary to compensate for the surplus of bFGF or other factors present in the 3T3 CM contribute to vessel growth and may be affected by low-dose TMZ.

In subsequent *in vitro* experiments we first identified the toxic dose range of TMZ on proliferating endothelial cells. Decreased endothelial cell survival *in vitro* was found for concentrations of 150 μM and higher. The LD_{50} was determined to be 250 μM. This toxic dose range in endothelial cells corresponds, with few exceptions [18], to the toxic dose range of TMZ on human tumor cells *in vitro* [28] and is 3- to 50-fold higher than the effects of this drug on endothelial cells in subsequently performed functional endothelial cell assays as well as angiogenesis assays. This finding indicates that TMZ in the non-toxic dose range has inhibitory effects on endothelial cell functions necessary for angiogenesis that most probably will not be influenced by the development of drug resistance as can be observed for most cancer cells.

The Matrigel assay is a morphogenesis assay that measures cell functions necessary for angiogenesis [29]. In order to align to capillary-like structures in the basement membrane-like matrix of the Matrigel, endothelial cells have to adhere to and move on the extracellular matrix, a process which is mainly dependent on various integrins [19]. We could demonstrate that at low concentrations (5 μM) endothelial cell alignment to form branching, anastomozing tubes was already significantly reduced (Figs 3 and 4). The remaining cells were mostly isolated and spherical. The results obtained for 5 μM TMZ were comparable to those obtained for 1 pM vinblastine as described by Vacca *et al.* [5]. Fibronectin is a component of the extracellular matrix and the ligand of integrins expressed in endothelial cells [30,31]. We therefore used a previously described attachment assay [5] to study the influence of TMZ on endothelial cell adhesion to fibronectin. We could not demonstrate any influence on adhesion to fibronectin after 30 min. After 90 min we found a significantly increased adhesion in the presence of 5–50 μM TMZ. We hypothesized that TMZ needed more time to influence endothelial cell function and therefore preincubated HUVEC for 24 h on gelatin with TMZ. After this prolonged pretreatment, adhesion to fibronectin was significantly reduced after 90 min for 25–50 μM TMZ. This indicates that TMZ might influence complex transcription or translation processes leading to altered integrin-mediated adhesion. Alternatively, TMZ may have to be processed intracellularly in order to influence endothelial cell function. However, in contrast to vinblastine [5], the lowest antiangiogenic dose of TMZ, 5 μM, did not influence endothelial cell adhesion to fibronectin.

As a next step, we examined, whether inhibition of endothelial cell proliferation could be one of the mechanisms leading to reduced angiogenesis. HUVEC proliferating in standard growth medium could be slightly inhibited in their growth by 25–50 μM TMZ. Hypothesizing that complex medium conditions with a high content of serum (20%) and an undefined proliferation stimulus (ECGS) might disguise observable effects, we used bFGF and VEGF that have been shown to induce HUVEC proliferation via distinct signaling pathways [32]. Both cytokines led to a significant proliferation of the endothelial cells. However, again, TMZ was not able to antagonize this at a concentration of 2.5–5 μM. For bFGF-induced HUVEC proliferation, only relatively high doses of TMZ (50 μM) produced a significant effect, while VEGF-induced proliferation was significantly inhibited at 25–50 μM TMZ (Fig. 5). Recently, it has been shown that other antiangiogenic chemotherapeutic agents like doxorubicin also inhibit angiogenesis at low doses without influencing endothelial cell proliferation, whereas vinblastine, paclitaxel and others already significantly reduce HUVEC proliferation at very low doses [33].

Since, in addition to its well-known methylating activity, inhibition of PKC is a suggested mechanism of action of TMZ [17], and PKC has been implicated in the regulation of angiogenesis *in vitro* and *in vivo* [34–38], we wanted to test whether inhibition of PKC could be involved in the observed antiangiogenic effects. We induced HUVEC proliferation with the phorbol ester PMA, a well-known, unspecific activator of PKC [24,39]. Again, TMZ did not produce significant inhibitory effects at 5 μM, but only at 25–50 μM. The observed effects obtained Tentori *et al.* [16], i.e. inhibition of metastasis formation, impaired attachment of tumor cells to an endothelioma cell line, inhibition of α_6 integrin phosphorylation and inhibition of PKC, were observed at much higher concentrations of TMZ (250 μM), that we found to be cytotoxic for endothelial cells (see above). Interestingly, in the work by Tentori *et al.* metastasis formation and tumor cell–endothelial cell adhesion were also reduced at 25 μM TMZ, but not at 2.5 μM. It is not

clear, however, whether these effects were statistically significant [17]. Taken together, these data indicate that TMZ inhibits PMA-induced PKC activation at medium (25 μM) and high (250 μM), but not at low (2.5–5 μM), concentrations. It has been proposed that the VEGF-induced signaling cascade is dependent upon activation of PKC [19]. This is well in line with our finding that VEGF-induced proliferation is inhibited by the same concentration range of TMZ (25–50 μM) as PKC-dependent proliferation. However, angiogenesis could already be inhibited at 5-fold lower concentrations (5 μM) of TMZ. Even though a comparison between *in vivo* and *in vitro* assays may be problematic, our data indicate that PKC inhibition by TMZ is probably not the main mechanism responsible for its antiangiogenic properties at low concentrations.

Conclusion

Altogether, we could demonstrate that TMZ inhibits angiogenesis at low, non-toxic doses. Inhibition of PKC, endothelial cell proliferation and inhibition of endothelial cell adhesion to fibronectin do not seem to be mainly involved in the observed antiangiogenic effects. Further studies are underway to address the exact mechanism of action of TMZ in angiogenesis. The lowest concentration that exerted inhibitory activity on vessel growth (5 μM = 1 μg/ml) can be achieved *in vivo* after oral administration of 20 mg/m^2 body surface. Taking into consideration the pharmacokinetic profile of TMZ, an oral application of this dose every 8 h would lead to a constant serum level [40]. Interestingly, up to 75 mg/m^2 body surface of TMZ has already been used in phase I studies on a daily basis and has produced antitumor effects [16]. Therefore, the antitumor activity of TMZ may, at least in part, be due to its antiangiogenic properties, which opens the door for the design of new, well-tolerated orally available treatment options of malignant melanoma and other tumor entities.

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