RESEARCH ARTICLE

Curcumin inhibits tumor growth and angiogenesis in glioblastoma xenografts

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Among the natural products shown to possess chemopreventive and anticancer properties, curcumin is one of the most potent. In the current study, we investigated the effects of this natural product on the growth of human glioma U-87 cells xenografted into athymic mice. We show here that curcumin administration exerted significant anti-tumor effects on subcutaneous and intracerebral gliomas as demonstrated by the slower tumor growth rate and the increase of animal survival time. While investigating the mechanism of its action *in vivo*, we observed that curcumin decreased the gelatinolytic activities of matrix metalloproteinase-9. Furthermore, treatment with curcumin inhibited glioma-induced angiogenesis as indicated by the decrease of endothelial cell marker from newly formed vessels and by the diminution of the concentration of hemoglobin in curcumin-treaded tumors. We also demonstrate, using an *in vitro* model of blood–brain barrier, that curcumin can cross the blood–brain barrier to a high level. These are the first results showing that curcumin suppresses tumor growth of gliomas in xenograft models. The mechanisms of the anti-tumor effects of curcumin were related, at least partly, to the inhibition of glioma-induced angiogenesis.

Keywords:
Anti-angiogenic / Anti-tumor / Blood–brain barrier / Curcumin / Glioblastoma

1 Introduction

Over the past decade, the incidence of primary brain tumors has rapidly increased. In the U.S., 18 000 patients are diagnosed with malignant primary brain tumors each year. More than half of them have glioblastoma multiforme, the most common type of malignant brain tumor in adults [1]. Despite advances in neurosurgical techniques and in radiation and drug therapies, glioblastoma multiforme have retained their dismal prognoses. Surgery and radiation are limited by the infiltration of tumor cells into healthy brain, leading to relapse even after treatment [2]. Adjuvant chemotherapy is thus essential for the treatment of glioblastoma multiforme.

Because of their smaller side effects compared with conventional treatment, there is increasing interest in the use of plant-derived chemicals that exhibit anti-tumor activity to combat human cancer. Curcumin, a natural phenolic compound found in the rhizomes of *Curcuma longa* (turmeric), gives specific flavor and color to curry. Tumeric is widely used in traditional Indian medicine to cure biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis. More recently, curcumin has been reported to display a series of biological activities important to human health, including antioxidant, anti-inflammatory and anti-microbial effects [3]. This natural product has also been demonstrated to efficiently induce

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Abbreviations: BBB, blood–brain barrier; **BBCEC,** bovine brain capillary endothelial cells; **i.p.,** intraperitoneal; **MEM,** minimum essential medium; **MMP,** matrix metalloproteinase; **P-gp,** P-glycoprotein; **RBE4,** rat brain capillary endothelial cells; **s.c.,** subcutaneous

growth inhibition and apoptosis in various cancer cell lines *in vitro* as well as decreased tumorigenesis *in vivo* [4]. Furthermore, this natural product inhibits both tumor initiation by some carcinogens and the tumor promotion induced by phorbol esters [3]. This polyphenolic compound has also been described as having anti-angiogenic activity [5].

Attempts to develop new, effective approaches for the prevention and treatment of cancer have lately focused on angiogenesis. Angiogenesis, a process that leads to the formation of new blood vessels from preexisting vascular networks, is a multistep process that occurs normally during a number of physiological processes, such as wound healing and embryogenesis. It also occurs in pathologic processes including arthritis, diabetes and cancer.

Malignant gliomas are one of the most highly vascularized human cancers and the growth and survival of these tumors are dependent on an adequate blood supply [6]. In fact, the neovascularization of gliomas is correlated with their biological aggressiveness, degree of malignancy and clinical recurrence [7]. Moreover, microvessel density in gliomas has become one of the most important criteria for histological grading of the malignancy and for prediction of prognosis [8]. Thus, the inhibition of tumor angiogenesis could be an efficient therapeutic strategy for the prevention and treatment of this disease.

In the current study, we assess the *in vitro* and *in vivo* anti-tumor activity of curcumin against human glioma U-87 cells using either cell proliferation assay or nude mouse xenograft model. We also determined the effects of curcumin on the growth of subcutaneous (s.c.) gliomas before and after establishment of tumors. In the literature, there are no reports on the effect of curcumin on gliomas *in vivo*, and only a few reports on the effect of curcumin on the angiogenesis of cancer. Thus, in this study, experiments were performed to explore the effect of curcumin on angiogenesis by measuring the concentration of hemoglobin in tumors. We also studied curcumin passage across the blood–brain barrier (BBB) using a well-established model of BBB, consisting of bovine brain capillary endothelial cells (BBCEC) co-cultured with newborn rat astrocytes [9]. This is the first report demonstrating the effects of curcumin on tumor growth and angiogenesis of gliomas *in vivo*. Our results confirm the potential utility of curcumin for both preventive and therapeutic applications in brain tumors.

2 Materials and methods

2.1 Chemicals

Curcumin was purchased from LKT Laboratories (St. Paul, MN, USA), whereas [^{14}C]sucrose (592 mCi/mmol) was provided by NEN (Boston, MA, USA). Cell culture media were obtained from GIBCO BRL (Burlington, ON, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT, USA). Type I collagen was extracted from rat tail tendons as previously described [10]. All other reagents were from Sigma-Aldrich Canada (Oakville, ON, Canada), unless otherwise specified.

2.2 Cell culture

The U-87 human glioma cell line was maintained in Eagle's minimum essential medium (MEM) containing 1 mM sodium pyruvate and 10% bovine calf serum. Rat brain capillary endothelial cells (RBE4) were kindly provided by Dr. Françoise Roux (Université Paris 7, Paris, France). RBE4 cells were plated on type I collagen-coated plates (200 μg/mL) and maintained in α-MEM/Ham's F12 (1:1) supplemented with 10% heat-inactivated fetal calf serum, 30 mg/mL geneticin, 50 mg/mL gentamycin and 100 ng/mL basic fibroblast growth factor from Upstate Biotechnology (Lake Placid, NY, USA). Establishment of an *in vitro* model of the BBB was performed as previously described [9]. All cells were grown at 37°C under a humidified atmosphere of 5% CO_2 in air.

2.3 *In vivo* studies in an s.c. glioma model

The *in vivo* chemopreventive and therapeutic effects of curcumin on U-87 derived xenografts were studied in athymic mice (Crl:CD-1 *nu*BR) from Charles River Laboratories (Wilmington, MA, USA). Athymic female mice received an s.c. injection of 2.5×10^6 U-87 cells, suspended in 100 μL of methyl-cellulose (1%) in serum-free MEM, into the right flank. This tumor cell inoculum resulted in the growth of tumors in all experimental animals with a highly reproducible growth rate. To determine dose response, daily intraperitoneal (i.p.) treatment with curcumin (at 30, 60 and 120 mg/kg) or with vehicle alone was initiated on day 5 after tumor implantation into groups of five mice *per* treatment. To determine the effect of curcumin before and after brain cancer establishment, athymic mice received i.p. treatment with curcumin, (60 mg/kg) or with vehicle alone either 7 days before cell inoculation (to evaluate the chemopreventive effect of curcumin) or on day 3 after tumor implantation (to test the therapeutic effect of curcumin) into groups of seven mice *per* treatment. The injection was repeated daily until the animals were sacrificed. Curcumin was dissolved in corn oil and freshly prepared every day. When s.c. tumors had reached a well detectable size of 40 mm^3, they were measured twice weekly, using a caliper, and volume was calculated by the formula: (length × width × height) until the end of the experiment. The body weights of the animals were recorded every 2 days. Mice were monitored daily for health status and when the control group tumor lengths exceeded 17 mm (Canadian Council on Animal Care recommended endpoint) all animals were killed. S.c. tumor from U-87 cells was confirmed postmortem in all mice. This protocol was evaluated and approved by the Institutional Committee for Good Animal Practices.

2.4 Treatment efficacy

Treatment efficacy was assessed in terms of curcumin's effects on the tumor volumes of treated mice relative to the tumor volumes of control mice. Tumor growth inhibition was calculated as the ratio of the median tumor volume in the treated group (T) *versus* the median tumor volume in the control group (C): T/C% = (median tumor volume of curcumin-treated group on day Y/median tumor volume of control group on day Y) × 100. The optimal value, being the minimal T/C ratio, reflects the maximal tumor growth inhibition achieved [11].

2.5 Cell proliferation assay

The effects of curcumin on U-87 and RBE4 cell proliferation was measured by [^{3}H]-thymidine incorporation assay. Briefly, cells were seeded onto 24-well plates. After 28 h, they were serum starved for 20 h, and then treated with various doses of freshly prepared curcumin, diluted in 0.1% DMSO for an additional 48 h. Then 1 μCi/mL thymidine was added to each well and the cells were further incubated for 4 h. The amount of [^{3}H]-thymidine incorporated into the cells was measured with a liquid scintillation counter.

2.6 Cell migration assay

Transwell migration of RBE4 was determined with a modified Boyden chamber assay. α-MEM/Ham's F12 media, with or without freshly prepared curcumin diluted in 0.1% DMSO, was added to the lower chamber. Media was separated from the upper chamber by type I collagen precoated onto the lower surface of polycarbonate filters (8 μm pore size) from Costar (Corning, NY, USA) at a concentration of 200 μg/mL for 2 h at 37°C. RBE4 were detached from culture flasks and suspended in fetal calf serum-free media at 5×10^5 cells/mL. Aliquots of 200 μL of the RBE4 cell suspension, containing either curcumin or vehicle alone, were added to the upper chamber. Migration was carried out at 37°C for 18 h, after which the filters were fixed in 3.7% formaldehyde in PBS for 20 min. After staining of the membranes, the upper surface of the filter was scraped with a cotton swab. For each treatment, five images were captured and the number of cells having migrated onto the lower side of the filter was quantified using Northern Eclipse software.

2.7 Tube formation assay

The tube formation assay was carried out using collagen as the extracellular matrix. The collagen gel was made as described by el Hafny [12]. Aliquots of 250 μL of the collagen gel were dispensed into each well of a 24-well plate and allowed to gel at 37°C. The RBE4 were plated onto the collagen at a density of 4×10^4 cells/well and incubated at 37°C for 16 h. The culture medium was removed, and 250 μL of collagen gel were poured on top of the first collagen gel and polymerized for 10 min at 37°C. The fresh medium containing curcumin diluted in 0.1% DMSO, at the stated concentrations (or vehicle alone), were added and replaced every day. After the cells were incubated at 37°C for 3 days, each well was photographed and the tubular length of the cells was quantified in five different areas using the Northern Eclipse software.

2.8 Zymography assay

Excised tumors were homogenized in five volumes of homogenate medium, composed of 250 mM sucrose and 10 mM Hepes-Tris, pH 7.5, with a Polytron at 4°C. Substrate gel zymography of the activities of homogenates (20 μg) from s.c. glioma tumors was performed as previously described [13]. The gels were incubated at 37°C for 17 or 21 h.

2.9 *In vivo* angiogenesis assay

Angiogenesis was quantitated in s.c. tumors by measuring the concentration of hemoglobin as previously described [14].

2.10 Quantification by RT-PCR

mRNA extracted from s.c. U-87 derived tumors previously excised from mice was reverse-transcribed into cDNA. Quantification by RT-PCR analysis of the relative expression of CD105 (5′-CCTTTGGTGCCTTCCTGATTG-3′ and 5′-TG TTTGGTTCCTGG-GACAAGTTC-3′), CD31 (5′-TATCCAA GGTCAGCAGCATCGTGG-3′ and 5′-GGGTTGTCTTTGA ATACCGCAG-3′) and 18S (5′-GATGGGCGGCGGAAAA TAG-3′ and 5′-GCGTGGATTCTGCATAATGGT-3′) were carried out using the LightCycler® DNA Master SYBR Green Kit on a LightCycler® 480 instrument (Roche, Mississauga, ON, Canada). 18S was used as an internal control and results were calculated relative to control mice.

2.11 Transcytosis of curcumin

The passage of curcumin across the BBB was measured as previously described [9]. Briefly, 300 μL of Ringer-HEPES containing freshly prepared curcumin (25 μM) and 74.5 nM [^{14}C]sucrose (0.01 μCi), with or without verapamil (25 μM), were added in the upper chamber of each insert. At the end of the experiment (90 min), the apical-to-basolateral fluxes of curcumin and [^{14}C]sucrose across the BBCEC monolayer

was evaluated. The endothelial permeability coefficient to [^{14}C]sucrose was calculated, with or without test compounds, to verify the integrity and the tightness of the barrier. In order to quantify the fluxes of curcumin, 500 μL from the 1 mL of each lower chamber were taken and immediately lyophilized overnight. After solubilization in DMSO, the suspensions were centrifuged at 30 000 × g and the concentration of curcumin in the supernatant was determined directly by absorbance at 450 nm and compared with a standard curve.

2.12 *In vivo* studies in an intracranial glioma model

Intracranial tumors were induced by implanting U-87 cells into the brains of athymic female mice (seven mice *per* treatment group) by stereotactic surgery. The mice received an injection of 5×10^5 suspended in 5 μL of methyl-cellulose (0.5%), *via* a Hamilton syringe, into the right caudate-putamen. I.p. treatment with curcumin (120 mg/kg/day or with vehicle alone) was initiated on day 1 after tumor implantation and the compound was dissolved as previously described. Animals were killed when they lost 20% of their body weight, which was monitored daily, or had difficulty ambulating, feeding or grooming. Intracranial tumor was confirmed postmortem in all mice that died. The Institutional Committee for Good Animal Practices approved this protocol.

2.13 Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between groups were performed using Student's *t*-test. Significance was accepted at a value of $p<0.05$. The experiments were repeated with similar results at least thrice. Comparisons of tumor volume were made using Student's *t*-test whereas comparisons of survival curves were made using the log-rank test.

3 Results

3.1 Anti-tumor effects of curcumin on s.c. gliomas

Tumors were induced in athymic nude mice by an s.c. inoculation of U-87 human glioblastoma cells into the flank regions of the animals. We first investigated whether curcumin could exert significant anti-tumor effects on glioma xenografts. The initial dosing strategy for curcumin treatment with daily i.p. injections employed 30, 60 and 120 mg/kg (curcumin treatment was started at day 5 after tumor cell inoculation). The results indicated that treatment with 60 mg/kg/day of curcumin was more effective than treatment with the lower dose, as shown by the faster tumor growth rate in the former, whereas there was no significant difference in the animal tumor growth rate between the 60 and 120 mg/kg/day groups (Table 1). Consequently, subsequent treatments were done with 60 mg/kg/day of curcumin.

Table 1. *In vivo* anti-tumor activity of curcumin given i.p. against s.c. implanted human malignant glioma

Dosage (mg/kg/day)	Tumor volume (mm^3)	T/C[a] (%)
0	537 ± 128	100
30	481 ± 59	89.6
60	307 ± 69	57.2
120	255 ± 74	47.5

Curcumin (30, 60 or 120 mg/kg/day) administration began 5 days after U-87 inoculation into the right flank of the nude athimic mice, and the injection was repeated until the animals were sacrificed.

a) T/C = (Median tumor volume of curcumin-treated group/ median tumor volume of control group) × 100.

In a second set of experiments, we evaluated the chemopreventive and therapeutic effects of curcumin on glioma xenografts. To test the chemopreventive effect of curcumin on tumorigenesis, we started the treatment 7 days before implantation of tumor cells whereas treatment with curcumin was started 3 days after tumor cell inoculation to evaluate the therapeutic effect. In the chemopreventive group, curcumin-treated mice had significantly smaller tumors than did the control mice from the beginning until the end of the experiment (Fig. 1A). In addition, in the therapeutic group, curcumin-treated mice exhibited significantly smaller tumors than did the control mice between the 25th day and the end of the experiment (Fig. 1B). The T/C values, which are used to determine tumor response, were similar between curcumin-treated mice in the chemopreventive group and in the therapeutic group at the end of the experiment (T/C values at the end of the treatment were 40.9 and 32.4%, respectively). Overall, the results presented in Table 2 indicate that curcumin was able to significantly suppress the growth of gliomas tumors before and after the establishment of tumor growth.

No obvious side effects of curcumin treatment were observed throughout the course of our investigations. During the treatment period, the animals showed good activity with normal food and water intake, and all mice demonstrated normal weight gain (unpublished observations), which indicated that mice tolerated the treatment well.

3.2 Anti-proliferative effects of curcumin on U-87 glioma cells and RBE4 brain endothelial cells

To determine the impact of curcumin on the growth of human glioma xenografts, a proliferation assay was

conducted on the U-87 glioma cell line and the RBE4 brain capillary endothelial cell line (Fig. 2A). Our data indicate that curcumin exerted a significant antiproliferative effect upon both cell lines in a concentration-dependent manner.

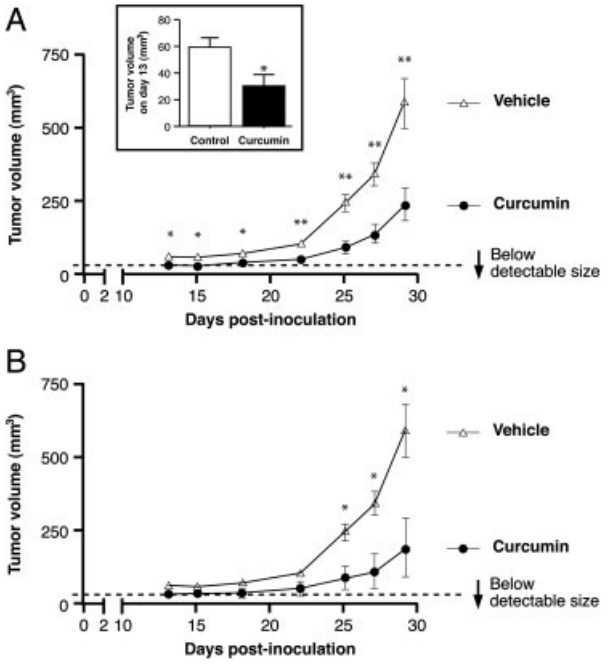

Figure 1. Chemopreventive and therapeutic effects of curcumin on human glioma tumor xenografts grown s.c. Curcumin (60 mg/kg/day) administration began 7 days before U-87 inoculation (chemopreventive effect) or 3 days after cell implantation (therapeutic effect). Curcumin (closed circles) or vehicle alone (open triangles) was injected into the i.p. area once a day, and the injection was repeated until the animals were sacrificed. The tumor size was measured twice a week. (A) Chemopreventive effect; (B) therapeutic effect. Results represent the means of seven mice in each group and are expressed as average volume (mm^3) $\pm$ SEM. Daily average tumor volumes for each group were compared throughout the course of the experiment using Student's *t*-test. $^{*}p<0.05$, $^{**}p<0.01$.

Table 2. I*n vivo* anti-tumor activity of curcumin given i.p. against s.c. implanted human malignant glioma

Treatment	Dosage (mg/kg/day)	Tumor volume (mm^3)	T/C[a)] (%)
Control	0	580 $\pm$ 88	100
Chemopreventive	60	237 $\pm$ 53	40.9
Therapeutic	60	188 $\pm$ 98	32.4

Curcumin (60 mg/kg/day) administration began 7 days before U-87 inoculation (chemopreventive effect) or 3 days after cell implantation (therapeutic effect) into the right flank of the nude athimic mice, and the injection was repeated until the animals were sacrificed.

a) T/C = (Median tumor volume of curcumin-treated group/median tumor volume of control group) $\times$ 100.

The concentrations of curcumin that inhibited 50% of cell proliferation (IC_{50}) were 11.6 $\pm$ 0.7 and 8.8 $\pm$ 0.2 μM for U-87 and RBE4 cells, respectively. These results suggest that RBE4 cells are slightly but significantly more sensitive to curcumin as compared with U-87 cells (Student's *t*-test, $p<0.05$). Our results indicated that curcumin might have an anti-angiogenic effect.

3.3 Inhibition of endothelial cell migration by curcumin

To evaluate the anti-angiogenic activities of curcumin, we examined its effect on endothelial cell migration, which is an essential step in the angiogenic process. The cell migration assay was conducted using a modified Boyden chamber system and collagen-coated polycarbonate filters. The ability of curcumin to inhibit RBE4 migration toward a chemoattractive source was determined by examining the number of cells that migrated onto the filter. RBE4 migration was blocked in a dose-dependent manner by curcumin with an IC_{50} of 1.3 μM, as shown in Fig. 2B.

3.4 Inhibition of endothelial tube formation by curcumin

The formation of tubular structures is another critical step in the angiogenesis process. We therefore investigated the effect of curcumin on the morphological differentiation of endothelial cells. In the tube formation assay, RBE4 were allowed to form tube cords in a three-dimensional collagen gel. The effect of curcumin was assessed by measuring the length of tube network created. As shown in Fig. 2D, treatment of RBE4 with curcumin affected tube formation compared with the control. Curcumin treatment diminished the width and length of endothelial tubular structures in a dose-dependent manner with an IC_{50} of 3.5 μM (Fig. 2C).

3.5 Inhibition of matrix metalloproteinase activity *in vivo* by curcumin

The release of matrix metalloproteinase (MMP) by endothelial cells represents an important step in neovascularization. For this reason, MMP activity was measured in s.c. tumor homogenates by gelatin zymography. After the staining and destaining of gels, the transparent bands observed in Fig. 3A correspond to the gelatinolytic activity of pro- and MMP-9 present in the various samples (typical levels of gelatinolytic activity are shown). In curcumin-treated mice, the gelatinolytic activities of both pro-MMP-9 and MMP-9 were decreased compared with the control group whereas pro-MMP-2 and MMP-2 levels and activation were unaffected by curcumin (unpublished observations). Zymograms were scanned by laser

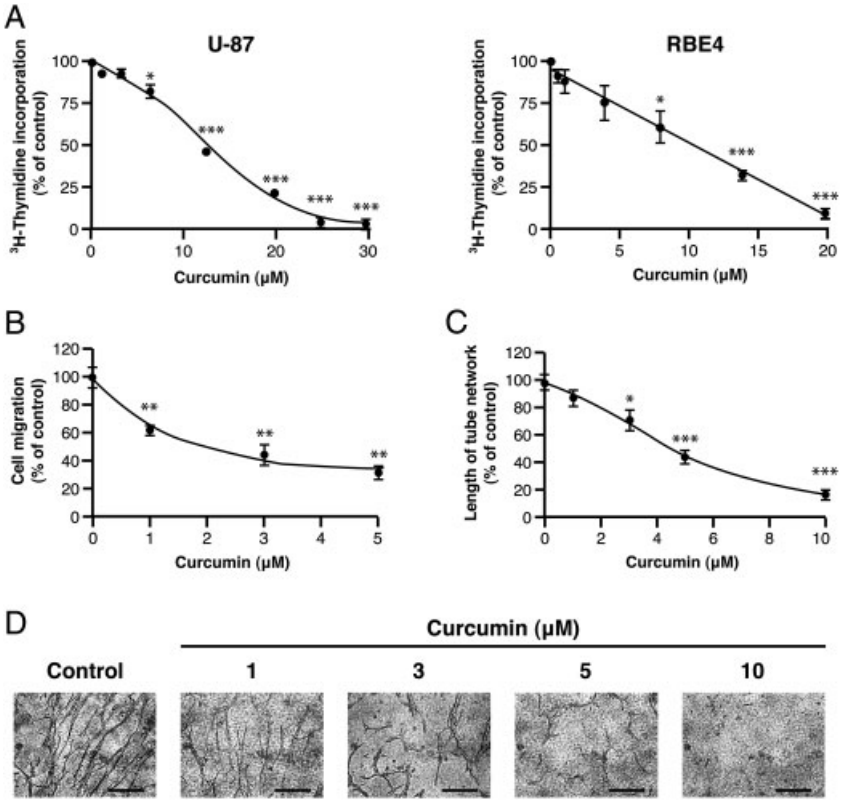

Figure 2. *In vitro* anti-angiogenic activities of curcumin. (A) Dose-dependent inhibition of human glioma cells (U-87) and RBE4 proliferation by curcumin. Cells were treated with various concentrations of curcumin and cell replication was measured by [^{3}H]-thymidine incorporation assay as described in Section 2. (B) Migration assay; curcumin was tested at a range of concentrations for its ability to inhibit endothelial cell migration. Cells were harvested and migration assessed on collagen-coated filters as described in Section 2. Data are presented as the mean number of cells migrated *per* field of microscope compared with that observed with untreated cells. (C) Tube formation assay; endothelial cells were harvested and curcumin was tested at a range of concentrations for its ability to inhibit three-dimensional, capillary-like structure formation on collagen as described in Section 2. Data are presented as the mean tube length *per* field of microscope compared with that observed with untreated cells. (D) Tube formation assay; typical pictures of capillary formation (bar = 50 μm). Results are representative of three independent experiments, and the means ± SEM are shown (*p<0.05, **p<0.01, ***p<0.001).

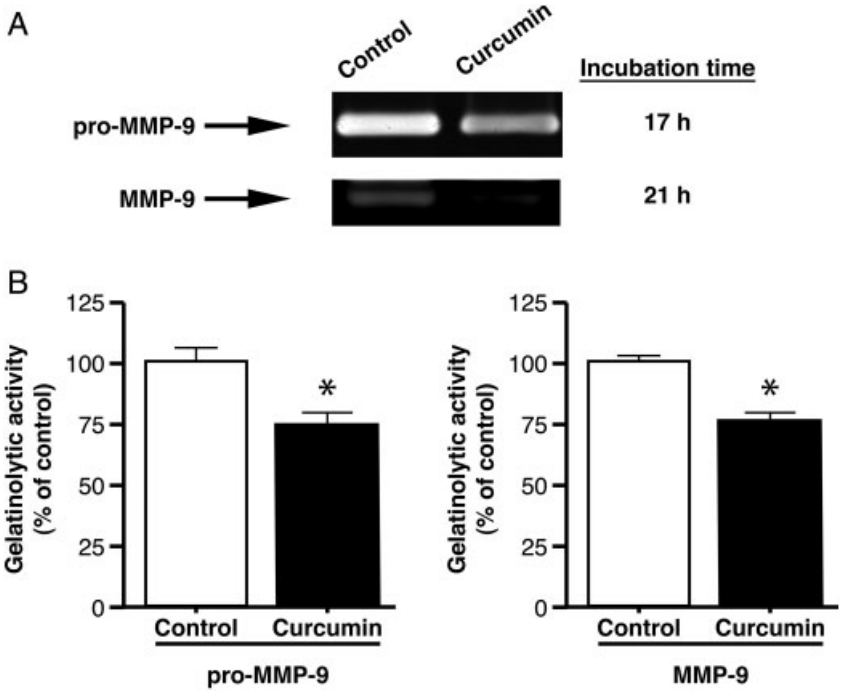

Figure 3. *In vivo* anti-angiogenic activities of curcumin; zymography assay. Zymography was performed on s.c. tumor homogenate proteins from curcumin-treated mice (60 mg/kg/day) and from a control group of mice. Curcumin administration began 3 days after cell implantation and the injection was repeated until the animals were sacrificed. Zymograms were scanned by laser densitometry to evaluate the levels of gelatinolytic activity. (A) Typical picture of gelatinolytic activity of tumors from curcumin-treated mice and from control mice is shown. (B) Enzymatic activities of pro-MMP-9 and MMP-9. For each condition, $n = 7$, and the mean ± SEM are shown (*p<0.05).

densitometry to quantitate the levels of gelatinolytic activity inhibition caused by curcumin. In curcumin-treated mice, the enzymatic activities of pro-MMP-9 and MMP-9 were both reduced by 25% (Fig. 3B) showing that the latent and active forms of the enzymes present in s.c. tumors were affected similarly by curcumin.

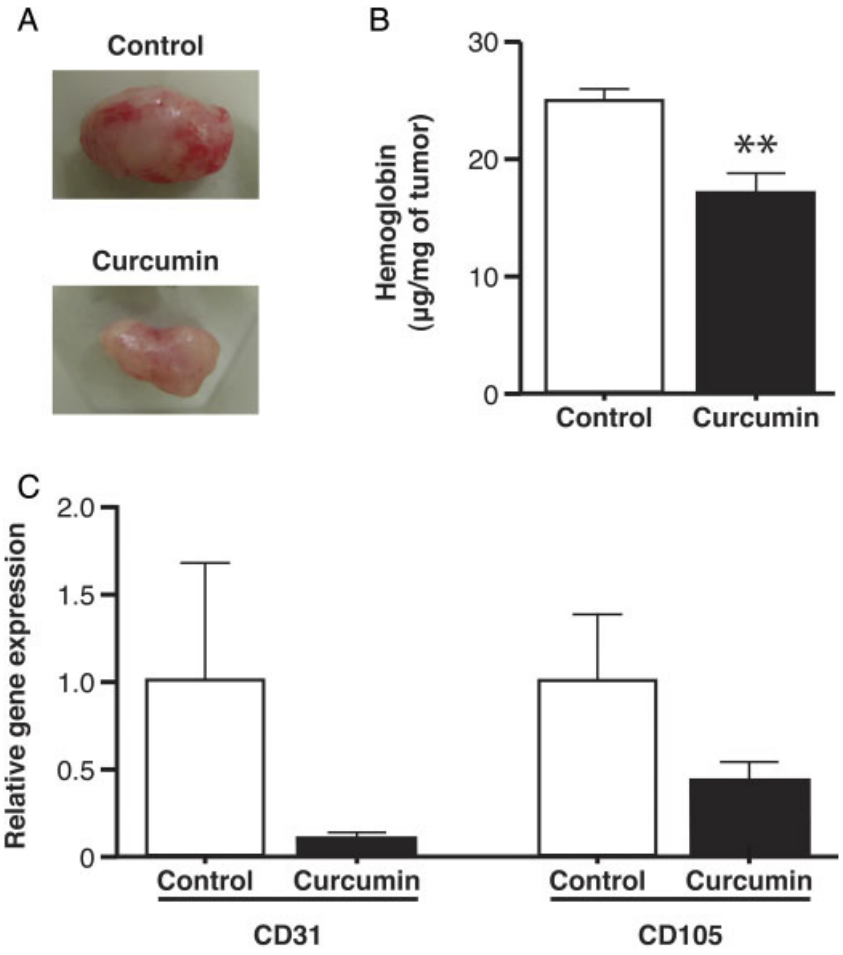

Figure 4. *In vivo* anti-angiogenic activities of curcumin; angiogenesis assay. S.c. tumors were induced in athymic nude mice and were excised and photographed 29 days later. Treatment with curcumin (60 mg/kg/day) or with vehicle alone was repeated daily until the animals were sacrificed. (A) Examples of s.c. gliomas after dissection are shown. (B) Quantification of neovessel formation by measurement of hemoglobin in tumors as described in Section 2. (C) mRNA expression of CD105 and CD31 in s.c. tumors. Results were calculated relative to control mice. For each condition, $n = 7$, and the mean $\pm$ SEM are shown ($^{**}p<0.01$).

3.6 Inhibition of glioma-induced angiogenesis *in vivo* by curcumin

To determine whether curcumin could suppress blood vessel formation *in vivo*, we measured the concentration of hemoglobin in s.c. tumors. Tumors were induced in athymic nude mice and were excised and photographed 29 days later. In control mice, tumors appeared dark pink. However, in curcumin-treated mice, tumors were pale pink, indicating less blood vessel formation as compared with the control group (Fig. 4A). To quantitate this effect, we measured the hemoglobin content of each tumor. Curcumin significantly inhibited the hemoglobin content (to 17.7 µg/mg) compared with the control group (25.2 µg/mg) (Fig. 4B). Additionally, mRNA expression of CD31 and CD105, two endothelial cell markers from newly formed vessels, were greatly downregulated. CD31 was downregulated by 90% compared with 55% for CD105, in curcumin-treaded s.c. tumors (Fig. 4C). However, due to the variation in the level of expression between the mice in the control group, this downregulation was not significant. Taken together, these results suggest that curcumin is able to inhibit glioma-induced angiogenesis *in vivo*.

3.7 High transcytosis of curcumin across the BBB *in vitro*

Since brain tumors are especially difficult to treat due to the lack of chemotherapeutic agent BBB transport or strong efflux through P-glycoprotein (P-gp) [15], we investigated the passage of curcumin across the BBB *in vitro*. Transcytosis of curcumin, from the apical-to-basolateral surface of BBCEC monolayers co-cultured with astrocytes, was measured with or without verapamil, a known P-gp inhibitor. After 90 min, more than 80% of the initial quantity of curcumin had crossed through the BBCEC monolayers, indicating that curcumin could cross the BBB to a high level. In addition, no significant augmentation in the transport of curcumin was observed in the presence of verapamil, suggesting that the transcytosis of curcumin is not affected by the presence of P-gp (Fig. 5A). During all experiments, apical-to-basal transport of [^{14}C]sucrose across monolayers of BBCEC cells

was assessed to confirm the integrity and the tightness of the barrier.

3.8 Anti-tumor effects of curcumin on the intracerebral gliomas

Because the effect of curcumin on s.c. gliomas does not represent its effect on the intracerebral gliomas, we studied the latter. Athymic nude mice inoculated orthotopically with 5×10^5 U-87 glioblastoma cells were treated i.p. with 120 mg/kg/day of curcumin. Curcumin treatment was started at day 1 after tumor cell inoculation and was continued until the animal died. Treatment with curcumin led to a significant increase in animal survival time of 12% compared with untreated mice (average survival of 23.4 days *versus* 20.9 days for control; log-rank test, $p < 0.05$). Figure 5B shows the survival rate of mice in this experiment.

4 Discussion

Glioblastoma multiforme continues to be the most common primary brain tumor in adults [1]; however, conventional treatments such as surgery, radiation and chemotherapy cure only a minority of these patients [2]. Therefore, significant effort has been made in researching the preventive effects of many micronutrients. In previous studies we and others have shown that many dietary products, including anthocyanidine and green tea polyphenol [16], have chemopreventive properties due to their anti-angiogenic and anti-tumor activities. Among the natural products that possess chemopreventive and anticancer properties, curcumin has emerged as one of the most powerful (reviewed in [17]).

In this study, we found that curcumin elicited a concentration-dependent inhibition of glioma cell proliferation, with an IC_{50} in the micromolar range. This IC_{50} is comparable to previously reported IC_{50} values for curcumin with other tumors such as lung, breast, oral and renal cancer [18–21], and suggests that prolonged treatment with curcumin at the micromolar serum level might be a possible treatment strategy for gliomas. Because angiogenesis has been considered to play a central role in the pathogenesis of gliomas [6–8] and since a critical step in angiogenesis involves the local proliferation of endothelial cells, we further studied the effect of curcumin on proliferation of brain capillary endothelial cells. We observed that curcumin inhibited proliferation of the endothelial cells in a concentration-dependent manner with a lower IC_{50} compared with the one found for glioma cells. Because curcumin inhibited proliferation in endothelial cells better that in glioma cells, we further investigated the anti-angiogenic effects of this natural product.

Our results suggest that curcumin has the ability to block *in vitro* angiogenesis. Extensive studies have shown that angiogenesis is a crucial event for the outgrowth of cancer cells and their spreading into other tissues. The process is complex and involves a cascade of events. These events include enzymatic degradation of basement membranes by proteolytic enzymes, endothelial cell migration, proliferation of these cells and formation of vascular loops [22]. *In vitro*

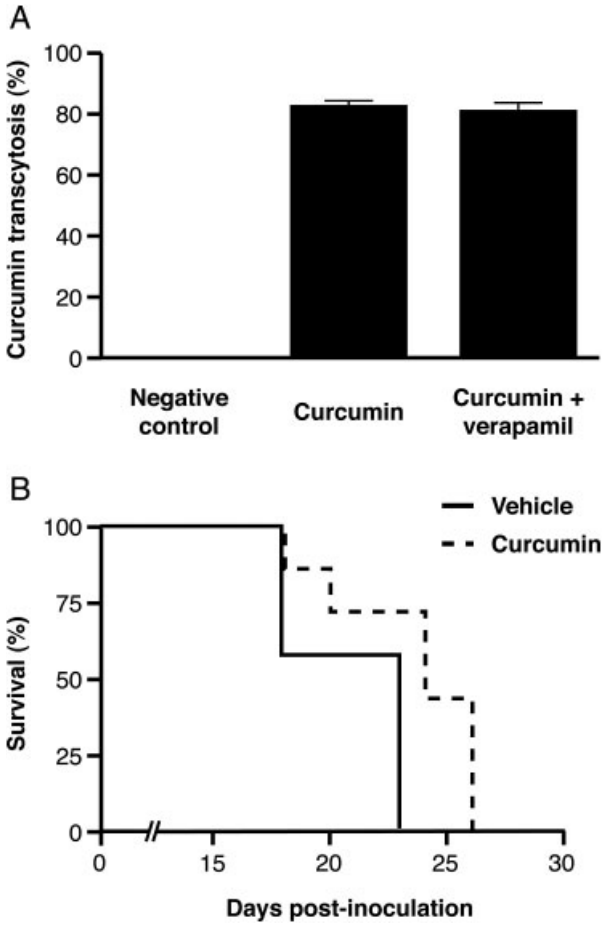

Figure 5. High transcytosis of curcumin across the BBB and its *in vivo* therapeutic effect on intracerebral gliomas. (A) Apical-to-basolateral passage of curcumin (25 μM) across BBCEC monolayers co-cultured with astrocytes was measured over 90 min with or without 25 μM verapamil. Negative control was done without curcumin. At the end of the experiment, curcumin passage was quantified as described in Section 2. The results of a representative experiment are shown; bars ± SEM ($n = 3$). (B) Percentage of survivors over time among mice that received intracranially implanted U-87 tumor cells. Curcumin (120 mg/kg) administration began 1 day after cell implantation. Curcumin (dotted line) or vehicle alone (solid line) was injected into the i.p. area once a day, and the injection was repeated until the animals were sacrificed. The average survival of animals treated with vehicle was 20.9 days, whereas the average survival of curcumin-treated mice was 23.4 days. Survival was statistically significantly prolonged in tumor-bearing ($n = 7$ in each group) nude mice treated with curcumin compared with survival in the group treated with vehicle (log-rank test, $p < 0.05$).

experiments revealed that the inhibitory effect of curcumin on these processes resulted partly due to the inhibition of two key events underlying angiogenesis, *i.e.* endothelial cell migration across the extracellular matrix and morphogenic differentiation of RBE4 cells into capillary-like structures. Furthermore, curcumin has been found to inhibit primary endothelial cell growth and proliferation [5]. These results indicate that the anti-angiogenic activity of curcumin is not restricted to specific endothelial cell lines, and suggest that curcumin may be an angiogenesis inhibitor. In this study, we demonstrated that curcumin exerted significant anti-tumor effects on s.c. gliomas at a dose of 60 mg/kg/day. Previous works have shown that i.p. administration of 100 mg/kg of curcumin to female mice achieved a peak plasma level of 2.25 μg/mL (~6 μM) after 15 min, which declined rapidly within the first 1 h [23]. Such a plasma level is in the range of concentrations tha inhibited angiogenesis *in vitro* in our experiments. In addition, the toxicology and pharmacokinetics of curcumin have been reported in human and high oral curcumin dosing appears safe as there was no treatment-related toxicity up to 8000 mg/day [24]. In human, the serum concentrations of curcumin usually peaked at 1–2 h after oral intake and gradually declined within 12 h. The average peak serum concentration after taking 8000 mg of curcumin was 1.77 ± 1.83 μM [24]. Curcumin-mediated inhibition of tube formation and cell migration in the RBE4 cell line was observed at concentrations (between 1 and 3 μM) that may be generated through dietary intake of the natural product. However, the BBB restricts the brain entry of many anticancer agents [15]. In this study, we demonstrated that curcumin could cross the BBB to a high level *in vitro*. These results are consistent with a previous *in vivo* report showing that 1 h after dosing 0.6 μg/mL (~1.6 μM) of curcumin in the plasma, a concentration of 0.41 μg/mL (~1.1 μM) was found in mouse brain [23]. Taken together, these results suggest that achievable brain levels may be close to blood levels.

Our results also suggest that curcumin has the ability to block *in vivo* angiogenesis. MMP-9 has been reported to play a major role in glioma angiogenesis and invasion *in vivo* [25]. In addition, MMP-9 has been shown to be significantly higher in human glioblastoma tissue samples than in normal brain or in low-grade brain tumors [25]. Thus, compounds that can inhibit MMP-9 could be part of an efficient therapeutic strategy for the prevention and treatment of this disease. In this study, we show for the first time that curcumin decreased the gelatinolytic activity of MMP-9 in gliomas *in vivo*. Our results are in agreement with a previous report showing that curcumin could inhibit the enzymatic activity of MMP-9 *in vitro* of U-87 and two other human glioma cell lines (U373MG and CRT-MG) [26]. These results suggest that the anti-angiogenic activity of curcumin is not restricted to specific glioma cell lines. Considering that glioma invasion and angiogenesis progress at least partly *via* MMP-9, the inhibition of this MMP by curcumin may possibly contribute to the anti-tumor effect of curcumin on gliomas *in vivo*. Moreover, curcumin was shown to downregulate CD31 and CD105 mRNA, two endothelial cell marker from newly formed vessels, and to decrease the hemoglobin content in glioma tumors. Taken together, these results strongly support the concept that, *in vivo*, curcumin may be an angiogenesis inhibitor.

In this study, we have also demonstrated that curcumin is capable of reducing the growth of s.c. gliomas in athymic mice before and after the establishment of tumors as demonstrated by slower tumor growth rate. Furthermore, curcumin affected intracerebral tumors prolonging animal survival time in mice bearing orthotopic intracranial gliomas. This is the first report showing the inhibitory effect of curcumin on primary brain cancer *in vivo*. The *in vivo* anti-tumor effect of curcumin had been studied in other reports. In murine models, curcumin suppressed carcinogenesis in skin, lymphomas/leukemias, forestomach, colon and liver (reviewed in [27]). In accordance with our results, these reports revealed that curcumin had a chemopreventive activity. In our study, the tumor-suppressive effect of curcumin was evident even when it was injected into mice after the tumor was established in both s.c. and intracerebral gliomas. Only a few studies have described the antitumoral activities of curcumin on tumor growth *in vivo* when administered as a therapeutic agent [27]. Here, we demonstrate that curcumin may be beneficial after the lesion has developed.

In conclusion, we have demonstrated that curcumin is capable of inhibiting the growth of glioma tumors in athymic mice. The mechanism of such anti-tumor effects of curcumin was found to be related at least partly to the inhibition of glioma-induced angiogenesis. The BBB is considered as a rate-limiting step for the penetration of drugs into the brain [15]. However, we show here that curcumin can cross a model BBB to a high level. This is the first report demonstrating the effects of curcumin on tumor growth and angiogenesis of gliomas *in vivo*. Because malignant gliomas are highly vascularized and the growth and survival of these tumors are dependent on an adequate blood supply [6], our results suggest the potential utility of curcumin for both chemopreventive and therapeutic applications in brain cancer.

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5 References

[1] Grossman, S. A., Batara, J. F., Current management of glioblastoma multiforme. *Semin. Oncol.* 2004, *31*, 635–644.

[2] Chintala, S. K., Tonn, J. C., Rao, J. S., Matrix metalloproteinases and their biological function in human gliomas. *Int. J. Dev. Neurosci.* 1999, *17*, 495–502.

[3] Maheshwari, R. K., Singh, A. K., Gaddipati, J., Srimal, R. C., Multiple biological activities of curcumin: a short review. *Life Sci.* 2006, *78*, 2081–2087.

[4] Duvoix, A., Blasius, R., Delhalle, S., Schnekenburger, M. *et al.* Chemopreventive and therapeutic effects of curcumin. *Cancer Lett.* 2005, *223*, 181–190.

[5] Arbiser, J. L., Klauber, N., Rohan, R., van Leeuwen, R. *et al.* Curcumin is an in vivo inhibitor of angiogenesis. *Mol. Med.* 1998, *4*, 376–383.

[6] Kesari, S., Ramakrishna, N., Sauvageot, C., Stiles, C. D., Wen, P. Y., Targeted molecular therapy of malignant gliomas. *Curr. Neurol. Neurosci. Rep.* 2005, *5*, 186–197.

[7] Leon, S. P., Folkerth, R. D., Black, P. M., Microvessel density is a prognostic indicator for patients with astroglial brain tumors. *Cancer* 1996, *77*, 362–372.

[8] Bian, X. W., Du, L. L., Shi, J. Q., Cheng, Y. S., Liu, F. X., Correlation of bFGF, FGFR-1 and VEGF expression with vascularity and malignancy of human astrocytomas. *Anal. Quant. Cytol. Histol.* 2000, *22*, 267–274.

[9] Cecchelli, R., Dehouck, B., Descamps, L., Fenart, L. *et al. In vitro* model for evaluating drug transport across the blood-brain barrier. *Adv. Drug Deliv. Rev.* 1999, *36*, 165–178.

[10] Silver, F. H., Trelstad, R. L., Type I collagen in solution. Structure and properties of fibril fragments. *J. Biol. Chem.* 1980, *255*, 9427–9433.

[11] Hendriks, H. R., Langdon, S., Berger, D. P., Breistol, K. *et al.* Comparative antitumour activity of vinblastine-isoleucinate and related vinca alkaloids in human tumour xenografts. *Eur. J. Cancer* 1992, *28A*, 767–773.

[12] el Hafny, B., Bourre, J. M., Roux, F., Synergistic stimulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities by retinoic acid and astroglial factors in immortalized rat brain microvessel endothelial cells. *J. Cell Physiol.* 1996, *167*, 451–460.

[13] Demeule, M., Brossard, M., Page, M., Gingras, D., Beliveau, R., Matrix metalloproteinase inhibition by green tea catechins. *Biochim. Biophys. Acta* 2000, *1478*, 51–60.

[14] McMahon, G. A., Petitclerc, E., Stefansson, S., Smith, E. *et al.* Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J. Biol. Chem.* 2001, *276*, 33964–33968.

[15] de Vries, N. A., Beijnen, J. H., Boogerd, W., van Tellingen, O., Blood-brain barrier and chemotherapeutic treatment of brain tumors. *Exp. Rev. Neurother.* 2006, *6*, 1199–1209.

[16] Lamy, S., Blanchette, M., Michaud-Levesque, J., Lafleur, R. *et al.* Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation. *Carcinogenesis* 2006, *27*, 989–996.

[17] Steward, W. P., Gescher, A. J., Curcumin in cancer management: recent results of analogue design and clinical studies and desirable future research. *Mol. Nutr. Food Res.* 2008, *52*, 1005–1009.

[18] Elattar, T. M., Virji, A. S., The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells *in vitro*. *Anticancer Res.* 2000, *20*, 1733–1738.

[19] Hong, R. L., Spohn, W. H., Hung, M. C., Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu. *Clin. Cancer Res.* 1999, *5*, 1884–1891.

[20] Martin-Cordero, C., Lopez-Lazaro, M., Galvez, M., Ayuso, M. J., Curcumin as a DNA topoisomerase II poison. *J. Enzyme Inhib. Med. Chem.* 2003, *18*, 505–509.

[21] Zhang, J., Qi, H., Wu, C., [Research of anti-proliferation of curcumin on A549 human lung cancer cells and its mechanism]. *Zhong Yao Cai* 2004, *27*, 923–927.

[22] Vacca, A., Ribatti, D., Pellegrino, A., Dammacco, F., [Angiogenesis and anti-angiogenesis in human neoplasms. Recent developments and the therapeutic prospects]. *Ann. Ital. Med. Int.* 2000, *15*, 7–19.

[23] Pan, M. H., Huang, T. M., Lin, J. K., Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab. Dispos.* 1999, *27*, 486–494.

[24] Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M. *et al.* Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* 2001, *21*, 2895–2900.

[25] Sawaya, R., Go, Y., Kyritisis, A. P., Uhm, J. *et al.* Elevated levels of Mr 92,000 type IV collagenase during tumor growth in vivo. *Biochem. Biophys. Res. Commun.* 1998, *251*, 632–636.

[26] Woo, M. S., Jung, S. H., Kim, S. Y., Hyun, J. W. *et al.* Curcumin suppresses phorbol ester-induced matrix metalloproteinase-9 expression by inhibiting the PKC to MAPK signaling pathways in human astroglioma cells. *Biochem. Biophys. Res. Commun.* 2005, *335*, 1017–1025.

[27] Sharma, R. A., Gescher, A. J., Steward, W. P., Curcumin: the story so far. *Eur. J. Cancer* 2005, *41*, 1955–1968.

