Thalidomide Enhances the Anti-Tumor Activity of Standard Chemotherapy in a Human Melanoma Xenotransplatation Model

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It has been demonstrated that thalidomide's anti-angiogenic properties result in clear anti-tumor activity in a number of human malignancies. We studied thalidomide in a human melanoma severe combined immunodeficiency mouse xenotransplantation model. Thalidomide as a single agent showed a significant tumor reduction of 46% compared with the control group. Thalidomide combined with dacarbazine treatment markedly enhanced the anti-tumor effect of chemotherapy and showed a significant tumor reduction relative to the dacarbazine-only group (61%) and even more tumor reduction (74%) compared with the control group. We also measured clearly reduced levels of tumor necrosis factor-α in the thalidomide-treated group. A significantly lower microvessel density was encountered in the thalidomide treatment groups (thalidomide alone or combined with DTIC), underscoring the anti-angiogenic effect of thalidomide as a single agent as well as in combination with chemotherapy in this model. In line with these results, we observed a nearly 3-fold increase of apoptosis for the combination of thalidomide and DTIC compared with the rate of apoptotic cells in DTIC-only-treated melanoma xenotransplants. These data underline the rationale for combining dacarbazine—a cytotoxic agent—and thalidomide—an anti-angiogenic cytostatic agent—as a promising strategy for the treatment of melanoma.

Key words: anti-angiogenesis/apoptosis/chemotherapy/melanoma/microvessel density/thalidomide/TNF-α
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Growth of tumors beyond a few millimeters requires the induction of new capillary blood vessel. The ability of solid tumors to induce new blood vessel formations, i.e. angiogenesis, is therefore crucial for tumor growth and development of metastasis. Ample evidence exists that inhibition of angiogenesis prevents tumor growth; therefore, anti-angiogenic therapy seems to be a rational and promising modality for therapy of human malignant disease (Carmeliet and Jain, 2000; Folkman, 2001).

Thalidomide (α-*N*-phtalimidoglutarimide) is a derivative of glutamic acid. It has a broad spectrum of pharmacological immunological and other effects, including its well-known teratogenicity (Franks *et al*, 2004). The mechanism of action of thalidomide is complex and not yet completely understood. It has been shown to suppress tumor necrosis factor-α (TNF-α) production by macrophages *in vitro* due to enhancement of the degradation of tumor-necrosis-factor-α mRNA (Porter and Jorge, 2002).

Recent attention focused on the possibility of using thalidomide as an anti-angiogenic agent (Hales, 1999; Kumar *et al*, 2002). D'Amato *et al* (1994) first demonstrated that thalidomide exerted anti-angiogenic property in a rabbit corneal neovascularization assay (D'Amato *et al*, 1994). Subsequently, animal studies demonstrated also that thalidomide inhibits tumor-induced angiogenesis in several cancers (lung cancer, colon cancer, sqamous cell carcinoma, esophageal cancer, myeloma) and produces a significant anti-tumor effect (Kotoh *et al*, 1999; Myoung *et al*, 2001; Ruddy and Majumdar, 2002; Yaccoby *et al*, 2002; Fujii *et al*, 2003; Li *et al*, 2003a, b). In clinical trials, the most pronounced anti-tumor results were observed in patients with refractory multiple myeloma (Singhal *et al*, 1999). But thalidomide was also studied in further advanced solid tumors in phase II such as recurrent gliomas, renal cancer, and hepatocellular carcinoma, showing promising significant anti-tumor effects (Fine *et al*, 2000; Singhal and Mehta, 2002).

Melanoma is a prime example of a tumor with a poor response to adjuvant therapeutic strategies modalities like chemotherapy or radiation (Bajetta *et al*, 2002). Melanomas are highly vascularized and it has been reported that increased vascularity correlates with poor prognosis (Ribatti *et al*, 2003; Valencak *et al*, 2004). Thalidomide as a single agent did not show any objective responses in melanoma patients, but 35% of the patients experienced stable disease for a duration of 12–32 wk (Eisen *et al*, 2000; Pawlak and Legha, 2004). A phase II trial combined treatment of thalidomide with temozolomide showed promising results.

Abbreviation: DTIC, dacarbazine; SCID, severe combined immunodeficiency; TNF-α, tumor necrosis factor α

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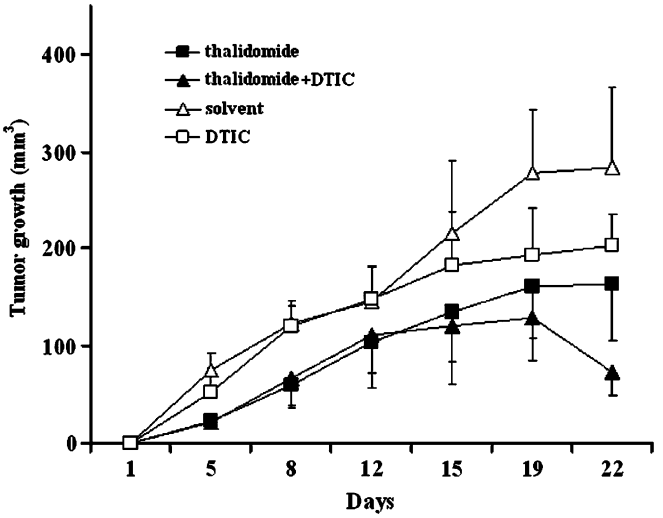

Figure 1
Inhibition of human melanoma growth in svere combined immunodeficiency (SCID) mice by the combination of thalidomide and dacarbazine (DTIC). SCID mice were injected subcutaneously with 2.5×10^7 human melanoma cells (518 A2) into the lower left flank ($n = 10$ animals per group). Starting one day after cell inoculation, thalidomide (administered daily at a dose of 400 mg per kg per day) or carrier solution (control) was administered for 22 d via the intraperitoneal route. From day 12 to 16, two of four groups also received daily dacarbazine (80 mg per kg per day i.p.). On day 22, the experiment was terminated.

In this study, 32% of the melanoma patients had an objective tumor response (Hwu *et al*, 2003). This open-labeled phase II trial, however, was conceptualized as a single-arm study and did not compare the treatment groups within the same patient population. The mechanism of thalidomide's anti-tumor activity in melanoma remains unclear.

Marriot *et al* (2003) showed in proliferation assays *in vitro* that a panel of tumor cells (colon, pancreatic, prostatic, and melanoma cells) treated with thalidomide did not exhibit reduced cell growth. It was also reported that thalidomide showed no growth inhibition on human colon cells *in vitro*, but a growth inhibition of over 40% in a mouse model *in vivo* (Kinuya *et al*, 2002). Only recently, Ansiaux *et al* (2005) showed that thalidomide radiosensitizes fibrosarcoma tumors in mice, whereas the *in vitro* studies did not demonstrate any growth inhibition or sensitizing effect on fibrosarcoma cells.

We therefore focused our studies of thalidomide effects in melanoma on a well-established *in vivo* model system. In this study, we investigated the efficacy of thalidomide in a xenotransplantation model of human melanoma grown in severe combined immunodeficiency (SCID) mice. The aim of this study was to compare the effects of treatment on melanoma growth with thalidomide, control (carrier solution), dacarbazine (DTIC), and the combination of thalidomide and DTIC. We could demonstrate a clearly more pronounced effect on tumor growth by the combination of thalidomide and DTIC over single-agent treatment. This effect is associated with enhanced induction of apoptosis. Also, thalidomide treatment led to a significant decrease in microvessel densitity associated with a decrease of TNF-α levels.

Results

Tumor growth is reduced by the combination of thalidomide and DTIC Subcutaneous injection of human melanoma cells into SCID mice resulted in tumor-take in all animals inoculated.

Figure 1 shows the tumor volumes of mice treated either with thalidomide, carrier solution (control), DTIC, or the combination of DTIC and thalidomide over 22 d (see also Table I). The growth curve shows a clear difference in the reduction of tumor volume between the thalidomide and the control group. The mean tumor volume of the thalidomide group reached 155.9 ± 59.4 mm^3 (mean $\pm$ SD), which was 46% smaller than the mean tumor volume of the control group on day 22 ($p = 0.014$).

On day 22, the tumor volume of the DTIC (only)-treated group showed a clear tumor reduction (34%) compared with the control (carrier solution) group (187.8 ± 32.0 mm^3). The difference of the mean volume of this group compared with the control group was also significant ($p = 0.003$). Notably, the single-agent thalidomide and DTIC groups showed no significant differences in tumor volume ($p =$ NS).

The combination of thalidomide and DTIC (73.2 ± 23.8 mm^3) led to a significant reduction in tumor size on day 22 when compared with all other groups ($p < 0.05$). The combination of thalidomide and DTIC showed a 74% tumor size reduction compared with the control group ($p = 0.018$).

All the animals tolerated the treatments well. There was no difference in body weight (data not shown) among the animals in the various treatment or control groups.

Inhibition of TNF-α plasma level by thalidomide It had been shown *in vitro* that thalidomide inhibits TNF-α in sti-

Table I. Results of tumor development, microvessel density and apoptosis index of mice ($n = 10$ animals per group), which received carrier-solution (control), thalidomide, dacarbazine + carrier-solution, or DTIC + thalidomide at the end of the described 22-d experiments

Treatment groups ($n = 10$)	Mean tumor volume (mm^3 $\pm$ SD)	TNF-α Levels (pg per mL $\pm$ SD)	Microvessel densitiy (area per 0.74 mm^2)	Rate of apoptosis (%)
Carrier-solution	286.0 ± 81.2	34.4 ± 17.0	119.8 ± 11.2	0.5 ± 0.2
DTIC	187.8 ± 32.0	13.9 ± 6.2	108.3 ± 11.2	1.8 ± 0.4
Thalidomide	155.9 ± 59.4	41.2 ± 21.1	45.4 ± 23.4	0.8 ± 0.3
Thalidomide + DTIC	73.2 ± 23.8	19.8 ± 1.9	45.3 ± 23.4	5.2 ± 1.2

DTIC, dacarbazine; TNF-α, tumor necrosis factor-α.

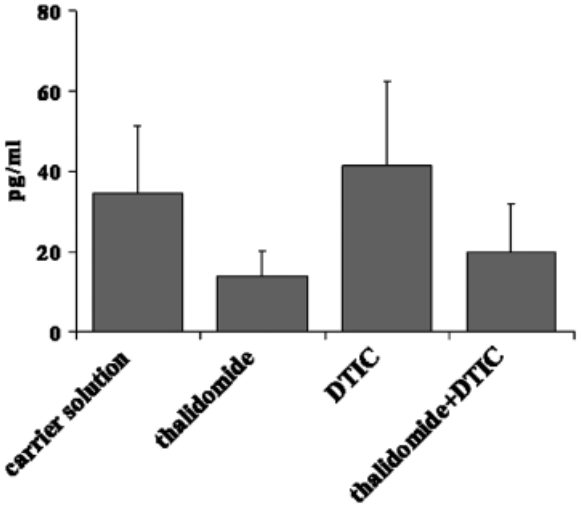

Figure 2
Decrease of tumor necrosis factor-α (TNF-α) level in mouse serum by thalidomide. Peripheral blood serum levels of TNF-α were measured after 22 d of treatment (carrier solution (control); thalidomide; dacarbazine (DTIC); thalidomide + DTIC). Mean TNF-α level ± SD, n = 10.

mulated human peripheral monocytes (Sampaio *et al*, 1992). This inhibitory action was thought to be due to the enhancement of TNF-α mRNA degradation. Thalidomide has also been shown to act in the clinic as an anti-TNF-α agent during TNF-α-mediated diseases (Moreira *et al*, 1993). We therefore focused on assessing TNF-α and measured TNF-α plasma levels after 22 d of treatment in all treatment groups. In accordance with the literature, we found a clear inhibition (59%) of total TNF-α levels in the peripheral blood of thalidomide-treated animals compared to vehicle-treated animals (Fig 2). This difference was not significant ($p = 0.074$). TNF-α is known to be elevated by DTIC in melanoma (Wack *et al*, 2002). Not surprisingly, we did observe a clear elevation of TNF-α level in the DTIC single-agent group (17%) compared to the carrier solution (control) group. The differences of TNF-α plasma levels between the DTIC and thalidomide group were over 66% ($p = 0.06$). The combination group showed higher TNF-α plasma levels than the thalidomide group; however, the values measured were still clearly lower than the ones in the control group.

Inhibition of microvessel density by thalidomide in human melanoma in SCID mice To gain further insight into the mechanism of action of thalidomide, we examined sections of the generated tumors for microvessel density (Fig 3*a*). CD31 staining was used as a well-established marker for microvessels (Myoung *et al*, 2001; Valencak *et al*, 2004). The mean number of microvessels of tumors treated with thalidomide (alone or in combination with DTIC) was significantly reduced compared with the control (carrier solution) group and the DTIC group ($p \leqslant 0.05$) (Fig 3*b*). This significant reduction of microvessel density by thalidomide indicates the anti-angiogenic potential of thalidomide in this model system.

Induction of apoptosis by the combination of thalidomide and DTIC It is well accepted that DTIC exerts its anti-tumor activity by induction of apoptosis (Wack *et al*, 2002). For thalidomide, the evidence is less clear and somewhat conflicting. Very recently, it was shown that thalidomide evokes apoptosis in human blood monocytes in a time- and concentration-dependent matter (Gockel *et al*, 2004). In line with these observations, we determined the number of apoptotic cells in xenograft tumors treated with thalidomide, DTIC, or the combinations of both agents. Thalidomide alone as well as carrier solution (control) treatment showed no clear change in the rate of apoptotic cells compared with the control group (see Fig 4). Single-agent DTIC resulted in a moderate increase of apoptosis by 1.8% ± 0.4% SD compared to the control group. The combination group of thalidomide and DTIC, however, showed a clear increase of 5.2% ± 1.2% SD of apoptotic cells compared with the control group. Detailed results are summarized in Table I.

Discussion

This study investigates the mechanism of action and the anti-tumor potential of thalidomide as a single agent and in combination with DTIC in a xenotransplantation model of human melanoma.

Even when administered as a single agent, thalidomide showed a clear tendency to inhibit tumor growth. In consideration of thalidomide's potential as an adjuvant to chemotherapy, our data document a clear advantage of the thalidomide combination therapy over DTIC single-agent treatment.

A Phase II study of temozolomide plus thalidomide showed significant clinical activity (Hwu *et al*, 2003). On the other hand, temozolomide, a active metabolite of DTIC, did not show significant differences in the overall response rate compared with DTIC (Middleton *et al*, 2000). The results of Hwu *et al* (2003) are in line with our findings. Thalidomide has been reported to use its anti-tumor properties via inhibition of angiogenesis in a variety of models of myeloma, esophageal, lung, colon cancer, and oral squamous cell carcinoma (Kotoh *et al*, 1999; Myoung *et al*, 2001; Yaccoby *et al*, 2002; Fujii *et al*, 2003; Li *et al*, 2003b). The mechanism of action in melanoma, however, is only poorly understood. This study appears to clearly demonstrating the inhibition of angiogensis by thalidomide in human melanoma. It was the aim of the study to explore the anti-angiogenic potential of thalidomide against non-established melanomas resembling the clinical situation of an adjuvant treatment against micro-metastases after excision of a high-risk primary melanoma. Therefore, we started the treatment with thalidomide one day after melanoma cell inoculation. It is of interest to note that thalidomide mono-therapy using a comparable administration schedule led to clearly reduced numbers in lung and liver metastases in a murine xenotransplantation model of hepatocellular carcinoma (Zhang *et al*, 2005). Several reports link the inhibition of growth factors like VEGF and bFGF to the anti-angiogenic effect of thalidomide (Myoung *et al*, 2001; Li *et al*, 2003a). In our study, however, immunohistochemical staining of VEGF and bFGF did not show any regulation of these factors by thalidomide (data not shown). In line with our results, no detectable VEGF or bFGF regulation has been reported

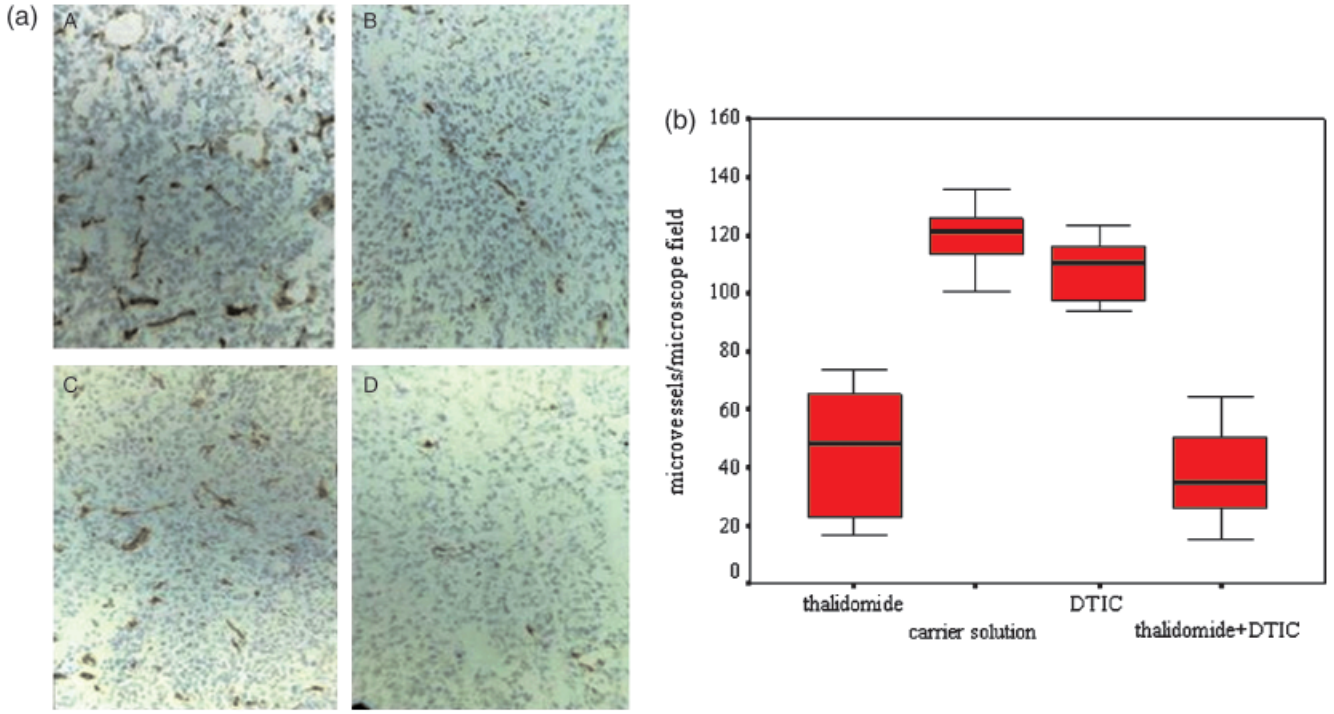

Figure 3
(*a*) **Reduction of microvessel density by thalidomide.** (*a*) Immunohistochemical staining of microvessels in tumor sections with CD31. A, carrier solution; B, thalidomide; C, dacarbazine (DTIC); D, thalidomide/DTIC. Figure 2 shows a representative example of tumor sections stained with CD31. The number of stained discrete microvessel in field was counted at × 200 magnification. (*b*) Significant reduction of microvessel in melanoma tumors by thalidomide. Statistical evaluation of the effect of thalidomide compared with carrier solution (control) on the number of microvessels in melanoma tumors grown in severe combined immunodeficiency mice. The mean number of microvessels per microscope field (0.74 mm^2) was significantly reduced in the tumors treated with thalidomide and thalidomide combined with DTIC compared with the control group and the DTIC-only group.

in other animal studies despite a clear anti-tumor effect (Yaccoby *et al*, 2002; Fujii *et al*, 2003). It is also reported that thalidomide blocks the action of VEGF or bFGF on endothelial cells during angiogenesis rather than blocking VEGF production/regulation (Amato, 2002; Gelati *et al*, 2003).

Thalidomide has also been described as an inhibitor of TNF-α, probably by enhancing the degradation of TNF-α

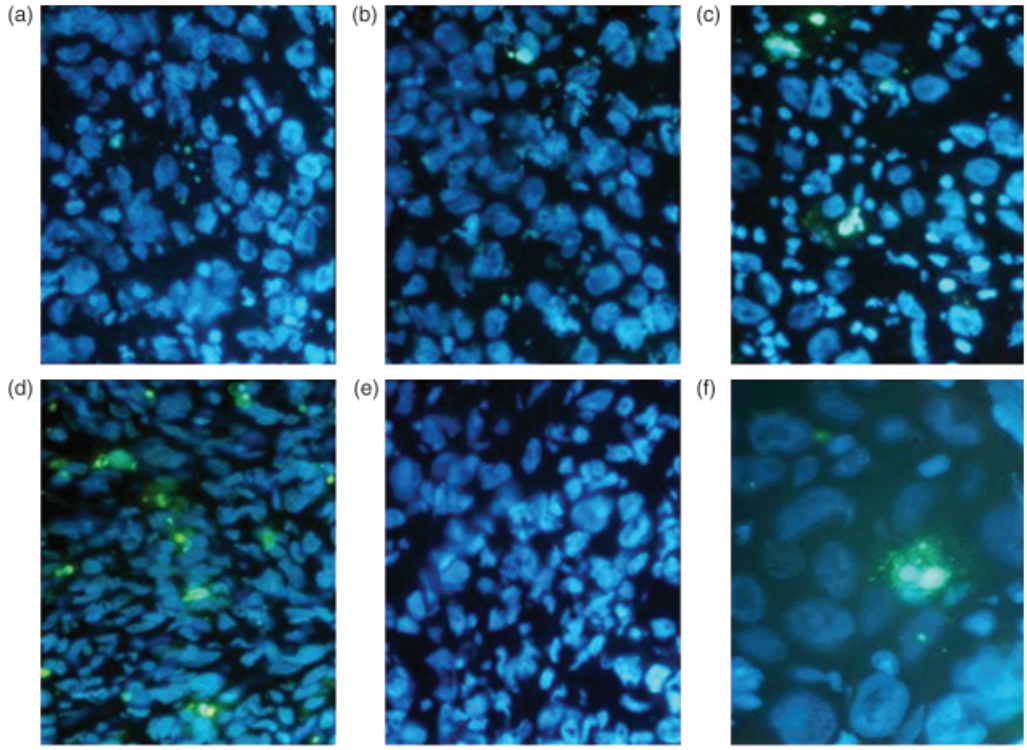

Figure 4
Induction of apoptosis by the combination of thalidomide and dacarbazine (DTIC). TUNEL staining of melanoma tumors grown in severe combined immunodeficiency (SCID) mice (apoptotic cells appear fluorescent green; counterstain is 4′-6′-diamidino-2-phenyllin-dole). Representative photographs of 518A2 tumors treated with (*a*), carrier solution (*b*) thalidomide, (*c*) DTIC, (*d*) thalidomide/DTIC, and (*e*) negative control for 22 d. Original magnification × 200. (*f*) Higher magnification (× 1000) to confirm apoptotic morphology.

mRNA (Moreira *et al*, 1993; Ching *et al*, 1995; Argiles *et al*, 1998). Serum of mice mice treated with single-agent thalidomide showed a clear reduction of TNF-α compared with serum of mice in the control group. This finding was somewhat surprising since most anti-tumor strategies using the death receptor–ligand TNF tend to facilitate a local increase of TNF around the tumor. An increased level of mRNA of TNF-α in melanoma was found after treatment with DTIC (Wack *et al*, 2002).

Stebbing *et al* (2001) showed a statistically significant decrease of TNF-α level in serum in patients with renal cancer who received high doses of thalidomide and responded with stable disease or objective response. In our study, we observed a direct correlation between the decrease of TNF-α level in mice serum and the anti-tumor effect of thalidomide in melanoma. The detailed mechanism and the significance of this regulation, however, remain to be elucidated. Recently, it was shown that thalidomide induces apoptosis in monocytes by using a cytochrome *c*-dependent pathway (Gockel *et al*, 2004). Also, thalidomide treatment resulted in an increase of apoptosis in human T leukemic cells as evidenced by an increase of caspases-3 activity (Ezell *et al*, 2003). Furthermore, a novel subclass of thalidomide analogue (SelCID-3) induced apoptosis in various tumor cell lines through the induction of G_2-M cell cycle arrest, which in turn led to caspase 3-mediated apoptosis (Marriott *et al*, 2003). Surprisingly, in our study, we could not detect a clear increase of apoptotic cells in the tumors that were treated with thalidomide alone. The combination of thalidomide and DTIC, however, resulted in a nearly 3-fold increase of apoptotic cells (5.2%) compared with tumors treated with DTIC alone (1.8%). These data indicate that thalidomide clearly facilitates the induction of apoptosis induced by DTIC in a supra-additive manner. Similarly, the combination of paclitaxel and thalidomide showed significant tumor growth retardation in a human colon cancer xenograft model. Furthermore, this study noticed a trend of increased apoptotic index in the combination group (Fujii *et al*, 2003).

The concept of either lowering the dose of a given chemotherapeutic agent or supplementing a particularly side-effect-prone agent in a combinatorial regime with anti-angiogenic agents is aimed at improving the effect and tolerability of cancer therapy (Verheul *et al*, 1999). The blocking of vasculature of tumors can be expected to cause the death of cells farthest from capillaries, ultimately leading to a decreased tumor mass and an increased efficiency of administered chemotherapy drugs. The angiogenesis inhibitors could also prevent further growth and progression to metastatic disease after a chemotherapeutic regimen (Teicher *et al*, 1992; Amato, 2002).

The ability to achieve better anti-tumor responses by combining dacarbazine—a cytotoxic agent—and thalidomide—an anti-angiogenic cytostatic agent—may qualify as a rational treatment approach to improve the outcome of melanoma.

Materials and Methods

Thalidomide Thalidomide powder was kindly provided by Grunenthal (Aachen, Germany). It was suspended in 1% carboxymethylcellulose (CMC), 0.1% Tween 80, and phosphate-buffered saline (PBS, Gibco BRL, Paisley, Scotland) by extensive stirring.

Cell culture The human melanoma cell line 518 A2 (Jansen *et al*, 1998) was maintained in basal tissue culture medium (DMEM, Gibco BRL) supplemented with 8% fetal calf serum (FCS) and antibiotics, in a humidified 5% CO_2, 95% ambient air atmosphere of 37°C.

Experimental animals and tumor treatment in SCID mice Pathogen-free female C.B-17 scid/scid (SCID) mice, 4–6 wk old, were obtained from Bomholtgard Breeding and Research Center (Bomice, Denmark) and randomly assigned to the experimental groups. The animals were housed in laminar flow racks and microisolator cages under specific pathogen-free conditions and received autoclaved food and water, but no antibiotic prophylaxis.

SCID mice ($n = 40$) were injected subcutaneously with 2.5×10^7 human melanoma cells (518 A2) into the lower left flank. Four groups ($n = 10$ animals per group) were stratified to receive either (1) thalidomide + carrier solution; (2) carrier solution; (3) dacarbazine + carrier solution; and (4) dacarbazine + thalidomide + carrier solution. One day after cell inoculation, groups 1 and 4 received thalidomide via the intraperitoneal route at a dose of 400 mg per kg per day (Ruddy and Majumdar, 2002) for 22 d. Groups 2 and 3 also received daily injections for 22 days, but of the carrier solution (control). From day 12 to 16, groups 3 and 4 additionally received dacarbazine (80 mg per kg per day) intraperitoneally on a daily basis. The length and width of the subcutaneous tumors were measured using a caliper twice a week (volume (mm^3) = length $\times$ width^2). On day 22, blood was collected and the experiment was terminated. This study was approved by the Medical University of Vienna's animal welfare committee.

Quantitative estimate of human TNF-α by ELISA Levels of TNF-α in serum were determined using an ELISA (Quantikine Mouse TNF-α kit; R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. Briefly, serum samples were added to microwells coated with monoclonal antibodies against the cytokine measured. After incubation, wells were washed and then an enzyme-linked polyclonal anti-TNF-α antibody was added. Following a new incubation and a subsequent wash that removed any unbound antibody-enzyme reagent, substrate was added. After incubation, an amplifier solution was added, leading to color development. Absorbance was measured at 450 nm, and the results were calculated from a standard curve produced by serial dilutions of a given cytokine sample.

Histopathological examination For histopathology studies, macroscopically normal tissues (brain, lung, heart, liver spleen, kidney, and small intestine) and tumor tissue were obtained from all animals, and then fixed in 7.5% neutral-buffered formalin, dehydrated, and embedded in paraffin using routine methods. Glass slides with 5 μm tissue sections were prepared and stained with hematoxylin and eosin. Additional tumor tissue was frozen and used for immunohistological stainings that were performed with the use of the avidin–biotin–peroxidase complex method. Hamster anti-mouse polyclonal antibody (Endogen, Boston, Massachusetts) was used for CD31 (PECAM). 5 μm frozen tumor sections were incubated with the primary antibody (dilution 1:75) at room temperature for 1 h. The subsequent steps were carried out using LSAB Kit (DAKO, Glostrup, Denmark) with a second biotin-conjugated anti-hamster antibody (DAKO).

Microvessel count The CD31-stained sections were scanned at a low magnification to identify the highest vascular area, and three fields were chosen for further assessment. The numbers of stained discrete microvessels/field were counted at $\times$ 200 magnification (0.74 mm^2 per area). Vessel counting was performed in a blinded manner. Average vessel numbers in three fields as well as the mean vessel number and SD of each group were calculated.

Evaluation of apoptosis TdT-mediated dUTP nick end-labeling (TUNEL) immunohistochemistry was performed in tumor xenotransplants according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Only comparable sections of the outer layers of the tumors without signs of necrotic areas were analyzed. The apoptotic index was determined by fluorescence microscopy blinded to the treatment groups.

Statistical analysis Statistical significance of differences between treatment groups was calculated by using one-way ANOVA and post-test comparing means using the Bonferroni's test for *post hoc* testing (SPSS, Chicago Illinois). p-Values of $\leqslant 0.05$ were considered to be of statistical significance.

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