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ORIGINAL ARTICLE

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Metronomic oral low-dose treosulfan chemotherapy combined with cyclooxygenase-2 inhibitor in pretreated advanced melanoma: a pilot study

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Abstract *Purpose*: The safety and efficacy of oral metronomic low-dose treosulfan chemotherapy in combination with the cyclooxygenase-2 (COX-2) inhibitor rofecoxib as a compound with antiangiogenic potential, a therapeutic regimen optimally targeting endothelial cells instead of tumor cells, were assessed in pretreated advanced melanoma patients. *Methods*: Endothelial cells were analyzed for proliferation, apoptosis and cytotoxicity in response to increasing concentrations of treosulfan, either in the absence or presence of COX-2 inhibitor, to determine whether inhibition of COX-2 enhanced the effect of treosulfan on cell function. In a clinical pilot study, 12 consecutive patients with pretreated advanced melanoma, meeting the eligibility criteria were enrolled. Patients received combined daily treosulfan chemotherapy (500 mg) with rofecoxib (25 mg). Metastatic lesions were assessed every 12 weeks. Patients with responsive or stable disease were eligible for a further 12-week treatment period. Response criteria according to the WHO handbook for reporting results of cancer treatment were applied. Side effects were classified according to the National Cancer Institute's Common Toxicity Criteria v2.0. *Results*: At noncytotoxic concentrations, treosulfan inhibited endothelial cell proliferation in a dose-dependent fashion. Simultaneous inhibition of COX-2 significantly increased the extent to which treosulfan suppressed cell proliferation, without inducing cytotoxicity. In the pilot study in which 12 patients were treated, toxicity was mild; only hematologic toxicity of grade II was seen. An objective response was noted in one patient, and four patients showed stabilization of their disease for 24 weeks (one) and 36 weeks (three). The patient who had a partial response subsequently showed stable disease for 48 weeks. The median survival time was 13 months. *Conclusions*: As increases in response rates following polychemo- or biochemotherapy have not been shown to correlate with prolongation in overall survival, more durable control of metastatic melanoma represents an attractive therapeutic goal. Thus, regimens scheduled to primarily target endothelial cells may potentially provide a palliative alternative that preserves quality of life in the absence of significant treatment-related toxicity.

Keywords Clinical trial · Melanoma · Antineoplastic combined chemotherapy protocol

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Introduction

Malignant melanoma is very resistant to systemic treatment and carries a poor prognosis in its metastatic stages. The treatment of advanced melanoma is mainly palliative, with a 5-year life expectancy of less than 10% and a median survival duration of 3 to 9 months, depending on the sites of distant metastasis [14]. In large randomized trials, polychemo- and biochemotherapies have not been shown to be superior to 'standard' single-agent dacarbazine chemotherapy as to overall survival. Although, at the expense of severe toxicity, combination chemotherapy and combined-modality regimens may considerably enhance antitumor activity in terms of response rates, no protocol has yet been proven to significantly impact on the long-term outcome for metastatic melanoma patients in multicenter randomized studies [1, 5, 6, 8].

Both dose-escalation of single agents and combinations of different cytotoxic agents and cytokines have failed to reproducibly result in sustained regression or stabilization of advanced melanoma disease. Therefore, in these patients a new paradigm for dosing of chemotherapy based on recent preclinical data revealing profound activity in drug-resistant tumors via the

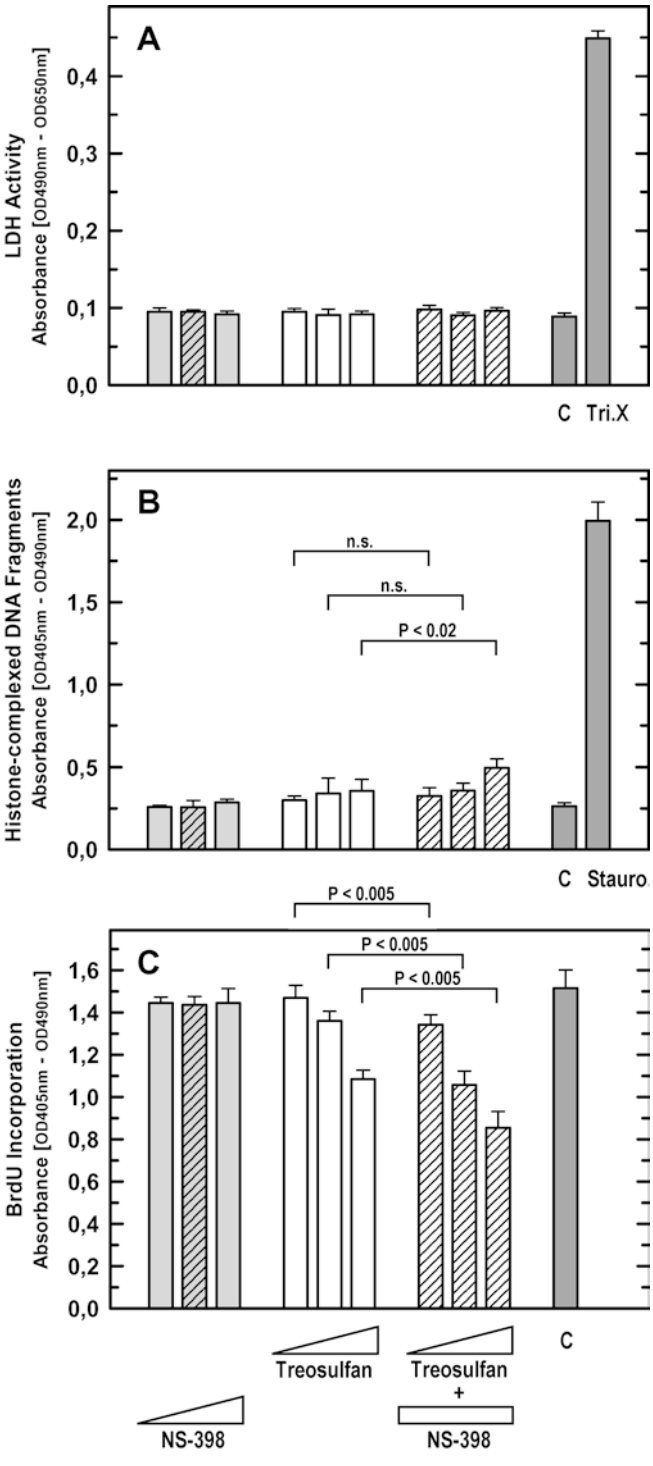

Fig. 1A–C Determination of the cytotoxic potential and the effects on apoptosis and cell proliferation of treosulfan and COX-2 inhibition on cultured HUVEC. **A** Colorimetric assay for quantification of plasma membrane damage, based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant (Cytotoxicity Detection Kit, Roche). After seeding HUVEC into 96-well plates, the cells were incubated with increasing concentrations of the COX-2 inhibitor NS-398 (1, 15, 30 μ*M*) or increasing concentrations of treosulfan (1, 12.5, 25 μg/ml) in the absence or presence of NS-398 (15 μ*M*) for 24 h as indicated (*Tri.X* Triton X). **B** Photometric enzyme immunoassay for quantification of apoptosis via analysis of cytoplasmic histone-associated DNA fragments (Cell Death Detection ELISA^{PLUS}, Roche). Cells were seeded into 96-well plates and were exposed to experimental the conditions outlined above and as indicated. **C** Colorimetric immunoassay for quantification of HUVEC proliferation, based on the measurement of BrdU incorporation during DNA synthesis (Cell Proliferation ELISA, Roche). The average absorbance values (means ± SD) from quadruplicate determinations per experimental condition were calculated (as detailed in Methods and materials). The data displayed are representative of the comparable results of three experiments. Student's *t*-test was used for statistical analysis. Significance was assumed for P values < 0.05 (*n.s.* not significant)

antiangiogenic properties of constant low-dose chemotherapy may be adopted [10]. As terminally differentiated and genetically stable endothelial cells are susceptible to much lower doses of cytostatic agents than tumor cells, therapeutic efficacy via vascular targeting may be accomplished at an acceptable level of toxicity by continuous 'metronomic' low-dose schedules. Since the combination of metronomic-like chemotherapy regimens with antiangiogenic drugs may amplify therapeutic efficacy, clinical trials are being initiated that integrate commercially available drugs with antiangiogenic potential, such as cyclooxygenase-2 (COX-2) inhibitors or thalidomide [17], into metronomic low-dose chemotherapy strategies [10].

In the study reported here, we showed that COX-2 inhibition can significantly enhance the effects of the chemotherapeutic agent treosulfan on endothelial cell proliferation in vitro, without inducing cytotoxicity. In a clinical pilot study, we evaluated the safety and efficacy of metronomic oral low-dose chemotherapy in 12 pretreated patients with stage IV melanoma using a treatment protocol that combined single-agent low-dose treosulfan chemotherapy with the COX-2 inhibitor rofecoxib.

Materials and methods

Cell culture and reagents

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and cultured at 37°C in an atmosphere containing 5% CO_2 in endothelial basal medium MV (PromoCell), supplemented with hydrocortisone (1 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 5% fetal calf serum (FCS) until the fifth passage. The COX-2 inhibitor NS-398 was purchased from BIOMOL (Hamburg, Germany); treosulfan was kindly provided by Medac (Hamburg, Germany).

Cell cytotoxicity, apoptosis and proliferation assay

The cytotoxic potential (Fig. 1A) was determined using a cytotoxicity detection kit (LDH) from Roche Diagnostics (Mannheim, Germany). This colorimetric assay analyzes cell cytotoxicity by measurement of lactate dehydrogenase (LDH) activity released from the cytosol of cultured cells as a result of plasma membrane damage. For these experiments, 2.0×10^4 HUVEC were seeded in a volume of 200 μl/well. Cells were exposed 24 h after seeding to increasing concentrations of NS-398 (1, 15, 30 μ*M*) or increasing concentrations of treosulfan (1, 12.5, 25 μg/ml) in the absence or

presence of NS-398 (15 μ*M*) for 24 h as indicated. After 6 h, BrdU was added for 18 h, and BrdU-DNA synthesis was determined according to the manufacturer's instructions (control, cells exposed to solvent, 0.3% DMSO). LDH activity in the supernatants was determined according to the manufacturer's instructions (low control, cells exposed to solvent, 0.3% DMSO; high control, cells lysed with 1% Triton X). Quantification of apoptosis (Fig. 1B) was performed using a Cell Death Detection ELISA^{PLUS} from Roche Diagnostics. Histone-complexed DNA fragments were analyzed in the wells of a microtiter plate after lysis according to the manufacturer's instructions. Briefly, 1.0×10^4 HUVEC were seeded in 96-well microtiter plates in a volume of 200 μl/well. Cells were exposed 24 h after seeding to different conditions as indicated (low control, cells exposed to solvent, 0.3% DMSO; high control, cells exposed to 500 n*M* staurosporine). The effect on cell proliferation (Fig. 1C) was measured by quantitating 5-bromo-2′-deoxyuridine (BrdU) incorporated into newly synthesized DNA of replicating cells, utilizing a colorimetric immunoassay from Roche Diagnostics (Cell Proliferation ELISA, BrdU). For these experiments, 1.0×10^4 HUVEC were seeded in 96-well microtiter plates in a volume of 200 μl/well. Cells were exposed 24 h after seeding to different experimental conditions for 24 h as indicated.

Patients

Eligibility criteria were previous treatment with chemotherapy for histologically confirmed malignant melanoma, measurable metastatic disease, a Karnofsky performance status ≥60%, and expected survival of more than 12 weeks. This protocol was approved by the Ethics Committee of J.W. Goethe-University, and voluntary written informed consent was obtained from each patient. Between December 2000 and November 2001, 12 patients were enrolled sequentially. Metastatic lesions were assessed every 12 weeks. Staging procedures included CT of chest and abdomen, and MRI of the brain. Toxicity was monitored by physical examination and regular blood and urine testing (weeks 1–4, once weekly; weeks 5–8, twice per month; thereafter, once monthly). Response criteria according to the WHO handbook for reporting results of cancer treatment were applied. Side effects were classified according to the National Cancer Institute's Common Toxicity Criteria v2.0 (CTC). Patients with responsive or stable disease were eligible for further 12-week treatment periods for up to 48 weeks. Duration of stable disease was calculated from the start of treatment to detection of progressive disease.

Study design and treatment

The treatment combined single-agent low-dose treosulfan chemotherapy (500 mg once daily; Ovastat, 250-mg capsules; Medac, Hamburg, Germany) with rofecoxib (12.5 mg twice daily; Vioxx, 12.5-mg tablets; MSD Sharp & Dohme, Haar, Germany). Dose adjustment was planned for grade 3 toxicities (250 mg treosulfan every second day until recovery to grade 2, thereafter 250 mg once daily until complete normalization). To alleviate pain or nausea/vomiting, concomitant administration of analgesics (e.g., tramadol) or antiemetics (e.g., metoclopramide, 5-H_3-receptor-antagonists) was approved.

Results

In order to determine whether inhibition of COX-2 enhanced the effect of treosulfan on endothelial cell function, we analyzed endothelial cells for cytotoxicity, apoptosis and proliferation in response to increasing concentrations of treosulfan, either in the absence or presence of COX-2 inhibitor (Fig. 1). NS-398 has been shown previously to inhibit COX-2 expression by cultured endothelial cells at the doses utilized in our studies [19]. The concentrations of treosulfan were selected to correspond to plasma concentrations detected in earlier investigations on the bioavailability of treosulfan as capsules in cancer patients [7]. However, our in vitro experiments with single treatments can only approximate the biologic effects that may occur during daily low-dose treosulfan in vivo. At concentrations that failed to exert cytotoxic effects, treosulfan inhibited endothelial cell proliferation in a dose-dependent fashion (Fig. 1A, C). Importantly, simultaneous inhibition of COX-2 significantly increased the extent to which treosulfan alone suppressed cell proliferation. The combined effects on induction of endothelial apoptosis were less pronounced (Fig. 1B), as only at the highest dose of treosulfan utilized in these experiments were significant effects regularly seen. Notably, combining treosulfan with NS-398 did not increase cytotoxicity. Together, these results are in line with those of previous studies, indicating that chemotherapeutics can impact endothelial function at low concentrations [3, 20]. Additionally, concurrent administration of a COX-2 inhibitor significantly enhanced the antiproliferative effects of treosulfan on endothelial cells, providing further support for the possibility that combining low-dose chemotherapy regimens with antiangiogenic drugs may amplify therapeutic efficacy.

The demographic and clinical characteristics of the 12 patients with progressive metastatic melanoma enrolled in the pilot clinical trial are summarized in Tables 1 and 2. All 12 patients and were assessable for toxicity and response.

Whereas only one objective response was detected (partial remission of widespread cutaneous involvement of the lower extremity; unchanged status on lymph node and lung metastases), four other patients showed stabilization of disease and therefore may also have benefited from the therapy. In one of these patients, stable disease lasted for up to 24 weeks, and in the three others

Table 1 Patient characteristics

Male/female	7/5
Age (years)	
Median	73
Range	45–82
Karnofsky index	
100/90	3/5
80/70	3/1
Site(s) of distant metastases (M classification[a])	
Distant skin, subcutaneous or nodal metastases (M1a)	1
Lung metastases (M1b)	5
Other visceral metastases/any distant metastasis with elevated serum lactate dehydrogenase (M1c)	6

[a]American Joint Committee on Cancer (AJCC) Staging System for Cutaneous Melanoma, 2001

Table 2 Baseline characteristics of study patients (*Ln* lymph node(s))

Patient no.	Sex	Age (years)	Prior therapy	Sites of metastases
1	M	77	Dacarbazine	Ln, lung, liver
2	F	45	Dacarbazine	Ln, bone, liver
3	M	55	Dacarbazine + IL-2	Ln, lung, skin
4	M	61	Dacarbazine + IFNα	Ln, lung
5	M	77	Dacarbazine	Ln, lung
6	F	77	Dacarbazine	Ln, lung, liver
7	M	76	Temozolomide	Lung
8	F	60	Dacarbazine, peptide vaccination	Ln, skin
9	F	59	Temozolomide	Ln, lung
10	M	80	Dacarbazine	Ln, skin
11	M	82	Dacarbazine	Ln, liver
12	F	70	Dacarbazine	Ln, lung, liver

Table 3 Response of pretreated patients with advanced melanoma to therapy (*PD* progressive disease, *PR* partial remission, *SD* stable disease)

Patient no.	Stage (M[a])	Week 0	Week 12	Week 24	Week 36	Week 48
1	M1c	PD	PD	–	–	–
2	M1c	PD	PD	–	–	–
3	M1b	PD	PR	PR	PR	PR
4	M1b	PD	SD	SD	PD	–
5	M1b	PD	PD	–	–	–
6	M1c	PD	SD	PD	–	–
7	M1b	PD	PD	–	–	–
8	M1a	PD	SD	SD	PD	–
9	M1b	PD	PD	–	–	–
10	M1c	PD	PD	–	–	–
11	M1c	PD	PD	–	–	–
12	M1c	PD	SD	SD	SD	PD

[a]According to the current AJCC classification

progression was seen only after 36 weeks. The patient who had a partial response continued to show a partial response for 48 weeks, until developing a single inguinal lymph node metastasis at week 60 (Table 3). The median survival time of the patients entered was 13 months. The protocol was well tolerated and administered in an outpatient setting (Table 4). CTC grade 3 or 4 toxicities were not observed.

Discussion

Considering the dismal prognosis of pretreated patients with advanced melanoma in the current absence of effective treatments, decisions on appropriate therapeutic options remain a challenge. Preservation of quality of life has to be balanced against treatment-related toxicity, as larger randomized trials are yet to show an impact on overall survival [1, 14]. Due to prognosis- and stage-dependent surveillance strategies, metastatic disease may be detected nowadays at earlier stages. Hence, many patients with metastatic melanoma disease have become eligible for second-line therapy protocols. Consequently, there is an indisputable need for initiation of new clinical studies that test alternative forms of treatment and take concerns about quality of life into account.

The idea of 'redefining' conventional chemotherapeutic agents as antiangiogenic drugs has been introduced recently [13], based on accumulating preclinical data revealing antivascular effects with 'metronomic' low-dose schedules of different cytotoxic agents [4, 11]. Rescheduling chemotherapeutic agents as a way of exerting antiangiogenic effects may potentially circumvent acquired drug resistance, as evidenced by efficacy even in drug-resistant tumors [4]. Irrespective of their particular mode of action, antiangiogenic properties have been demonstrated for different chemotherapeutic drugs, including alkylating agents, such as cyclophosphamide, and microtubule-interfering alkaloids, such as paclitaxel and vinblastine [10, 18]. In addition, preclinical studies have shown that effectiveness of uninterrupted low-dose antiangiogenic chemotherapy may be greatly improved by combination with antiangiogenic agents [2, 11]. Consequently, there is a compelling rationale for conducting clinical studies with metronomic-like chemotherapy regimens in combination with antiangiogenic drugs [10].

In order to assess a regimen scheduled to preferentially target endothelial cells instead of melanoma cells, we initiated a clinical pilot study in pretreated patients with advanced melanoma in which the safety and efficacy of metronomic low-dose chemotherapy in combination with a drug that carries an antiangiogenic potential were evaluated. Treosulfan, a prodrug of a bifunctional alkylating agent, was chosen as the cytostatic agent in this trial. Although the scientific rationale was not aimed at targeting melanoma cells primarily, a number of studies have indicated that single-agent treosulfan may be reasonably effective against melanoma cells in vitro and in vivo [15, 16]. Treosulfan is indicated for palliative treatment of advanced ovarian cancer in several European countries. The recommended daily dose of oral treosulfan is 400–600 mg/m^2 on days 1 to 28 every 8 weeks (750–1000 mg/m^2 with 28 days interruption). Previously, oral low-dose treosulfan (500 mg once daily) has been demonstrated to be well tolerated in platinum-resistant ovarian cancer, revealing

Table 4 Selected common toxicity criteria (National Cancer Institute's Common Toxicity Criteria v2.0, 1998)

Toxicity	Grade 1	Grade 2
Blood/bone marrow		
Hemoglobin	4/12	3/12
Leukocytes (total WBC)	2/12	2/12
Platelets		1/12
Constitutional symptoms		
Fatigue	4/12	2/12
Weight loss	2/12	
Fever	1/12	
Alopecia	3/12	
Gastrointestinal		
Constipation	2/12	3/12
Diarrhea	2/12	1/12
Nausea	3/12	
Pain	2/12	1/12

modest long-term side effects even after treatment for more than 12 months [9]. Taking our continuous antivascular approach and clinical experiences into consideration, we decided to administer treosulfan at a daily oral dose of 500 mg. As a commercially available drug with antiangiogenic potential, the COX-2 inhibitor rofecoxib was selected and administered at the maximal indicated dose. In our in vitro experiments on cultured endothelial cells, treosulfan was seen to inhibit proliferation at noncytotoxic concentrations (Fig. 1A, C). In addition, simultaneous COX-2 inhibition significantly enhanced the effects of treosulfan on endothelial cell proliferation in vitro at doses that did not induce cytotoxicity.

In terms of treatment-related toxicity, combination of single-agent low-dose treosulfan chemotherapy with rofecoxib induced only mild myelosuppression in patients. These observations suggest that hematologic side effects may not be significantly increased by the addition of rofecoxib compared to toxicities experienced with low-dose treosulfan therapy alone [9, 12]. This regimen can thus be considered a well-tolerated and safe treatment modality.

The results of this investigation indicate that metronomic therapy with low-dose treosulfan and rofecoxib may not lead to remarkable responses. However, our experience with this protocol may also suggest that partial control of pretreated, advanced melanoma disease can be achieved in a considerable proportion of affected individuals. Although our in vitro experiments revealed that COX-2 inhibition can enhance the effects of treosulfan on endothelial cells, the potential contribution of concomitant COX-2 inhibitor with treosulfan to disease stabilization in melanoma patients may only be resolved by a randomized trial comparing single-agent low-dose treosulfan therapy with treosulfan in combination with COX-2 inhibitor.

As increases in response rates to polychemo- or biochemotherapy have not been shown to correlate with prolongation in overall survival [1, 5, 6, 8], more durable control of metastatic melanoma represents an attractive therapeutic goal, considering the palliative capability of current treatment options in stage IV disease. Therefore, this oral regimen could potentially provide a palliative therapeutic alternative that preserves quality of life in the absence of significant treatment-related toxicity. In addition, efficacy of this concept may be improved by dose adjustments, incorporation of more potent antiangiogenic compounds (as they become available), or by selection of patients with advanced melanoma in a first-line situation [10]. Whereas proper skepticism is clearly warranted, pursuing the concept of low-dose chemotherapy may lead to more suitable treatments for advanced melanoma disease in the future, in which efficacy outweighs toxicity.

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