Bevacizumab plus Fotemustine as First-line Treatment in Metastatic Melanoma Patients: Clinical Activity and Modulation of Angiogenesis and Lymphangiogenesis Factors

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Abstract

Purpose: To assess the clinical and biological activity of the association of bevacizumab and fotemustine as first-line treatment in advanced melanoma patients.

Experimental Design: Previously untreated, metastatic melanoma patients (n = 20) received bevacizumab (at 15 mg/kg every 3 weeks) and fotemustine (100 mg/m2 by intravenous administration on days 1, 8, and 15, repeated after 4 weeks) in a multicenter, single-arm, open-label, phase II study. Primary endpoint was the best overall response rate; other endpoints were toxicity, time to progression (TTP), and overall survival (OS). Serum cytokines, angiogenesis, and lymphangiogenesis factors were monitored by multiplex arrays and by in vitro angiogenesis assays. Effects of fotemustine on melanoma cells, in vitro, on vascular endothelial growth factor (VEGF)-C release and apoptosis were assessed by ELISA and flow cytometry, respectively.

Results: One complete response, 2 partial responses (PR), and 10 patients with stable disease were observed. TTP and OS were 8.3 and 20.5 months, respectively. Fourteen patients experienced adverse events of toxicity grade 3–4. Serum VEGF-A levels in evaluated patients (n = 15) and overall serum proangiogenic activity were significantly inhibited. A significant reduction in VEGF-C levels was found in several post-versus pretherapy serum samples. In vitro, fotemustine inhibited VEGF-C release by melanoma cells without inducing significant cell death. Serum levels of interleukin (IL)-10 and IL-12p70 showed the highest levels in sera of PR patients, compared with patients with stable or progressive disease whereas IL-23 showed the opposite pattern.

Conclusions: The combination of bevacizumab plus fotemustine has clinical activity in advanced melanoma and promotes systemic modulation of angiogenesis and lymphangiogenesis factors. Clin Cancer Res; 16(23); 5862–72. ©2010 AACR.

Treatment of advanced melanoma remains unsatisfactory, because no therapy has demonstrated to affect overall survival (OS), with the exception of a recent immunotherapy study based on administration of the anti-CTLA-4 monoclonal antibody (mAb) ipilimumab with/without a gp100 vaccine (1). Moreover, there is no evidence that polychemotherapy is better than monotherapy, and no regimen can be considered of choice in metastatic disease. Nowadays, the CVD combination (cisplatin, vinblastine or vindesine, and dacarbazine) is widely used (2), as it can yield high response rates with manageable toxicity. However, all phase III trials have failed to show an effective advantage of the combined therapy over the single approaches (immunotherapy or chemotherapy; ref. 3, 4), with the exception of a phase III trial by Eton et al. (2). In such study, comparing CVD versus CVD plus interferon (IFN)-α and interleukin (IL)-2, in 190 melanoma patients, the response rate was 48% for the combination versus 25% for CVD (P = 0.001), time to progression (TTP) was 4.9 versus 2.4 months (P = 0.008), and the median survival was 11.9 versus 9.2
Translational Relevance

There is an urgent need for improved therapies in advanced cutaneous melanoma. So far only ipilimumab has shown significant impact on overall survival, although durable complete responses have been observed in some patients treated with high-dose interleukin 2. However, in randomized trials, biochemotherapy regimens have been shown to improve response rates in advanced disease, thus suggesting that this is a promising approach that could be further developed by new combinations of cytotoxic drugs and biological agents. In this study, we show that the combination of the antiangiogenesis monoclonal antibody bevacizumab with the nitrosourea fotemustine has clinical activity as first-line treatment in metastatic disease. Moreover, this biochemotherapy regimen significantly reduced systemic levels not only of vascular endothelial growth factor (VEGF)-A, but also of VEGF-C, suggesting that this therapeutic approach may contribute to the inhibition of both angiogenesis and lymphangiogenesis, 2 relevant processes in melanoma development and progression.

months ($P = 0.006$). Among the new chemotherapeutic treatments of advanced melanoma, fotemustine is a promising drug, and there is a growing interest for bevacizumab, a recombinant humanized mAb that binds to and inhibits the biological activity of human vascular endothelial growth factor (VEGF)-A (see ref. 5 for review). Bevacizumab is clinically active in different solid tumors (6–8): in colorectal cancer the addition of bevacizumab to irinotecan/fluorouracil/leucovorin has been shown to improve survival of metastatic patients (9). Bevacizumab-based approaches in advanced melanoma were reported in a randomized phase II trial comparing bevacizumab versus bevacizumab plus low-dose IFN-α2b (10). In that study, 8 of 32 patients (5 in the bevacizumab arm and 3 in the bevacizumab plus IFN-α2b arm) showed prolonged stabilization of disease (10). Additional evidence has been reported more recently, in a phase II biochemotherapy study based on paclitaxel, carboplatin, and bevacizumab (11). The authors reported that 17% of the patients achieved partial remission and 57% obtained stable disease (SD) for at least 8 weeks (11).

Fotemustine is a cytotoxic alkylating agent of the nitrosourea family showing activity in melanoma (12). In a randomized trial (13) comparing fotemustine with dacarbazine, as first-line chemotherapy in 229 patients, with or without brain metastases, overall response rate in the intent-to-treat population was respectively 15.5% versus 6.8% ($P = 0.043$). Interestingly, among patients without brain metastases at inclusion, median time to development of central nervous system relapses was 22.7 months in the fotemustine arm versus 7.2 months in the group receiving dacarbazine ($P = 0.059$).

On the basis of these clinical data, we carried out a phase II study to investigate the clinical and biological activity of the bevacizumab–fotemustine combination in untreated metastatic melanoma patients. On the basis of recent evidence (14, 15) of the relevance of serum biomarkers (detected by multiplex assays) as prognostic factors of response to therapy with biological agents such as IFN-α2b or IL-2, we designed multiplex ELISA arrays to detect 16 different angiogenesis, lymphangiogenesis, and immunomodulatory factors. The results of the study indicated that the combination of bevacizumab plus fotemustine has clinical activity and modulates both angiogenesis and lymphangiogenesis factors.

Materials and Methods

Patients

Eligible patients were at least 18 years of age, with histologically or cytologically confirmed measurable diagnosis of metastatic, inoperable nonchondroid melanoma; an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 1, no previous treatment of metastatic disease (exception for a vaccination with a wash-out period of at least 4 weeks), normal hematologic, hepatic, and renal functions; absence of brain metastases; and life expectancy of at least 3 months. Exclusion criteria were history of bleeding, diathesis, or coagulopathy, clinically significant cardiovascular disease, arterial thromboembolism less than 6 months before the first study treatment, uncontrolled hypertension, and a history of other malignancy within the past 5 years (except curatively treated nonmelanoma skin cancer or carcinoma in situ of the cervix). The trial was approved by the Institutional Review Board or ethics committee of each participating center and was conducted in accordance with the principles of the Declaration of Helsinki and with the Good Clinical Practice. All patients provided written, informed consent.

Study design and treatment

This was a multicenter, single-arm, open-label, phase II study. The primary endpoint was the best tumor response at any time during therapy. Secondary objectives were toxicity assessment, duration of response, TTP, and OS. Eligible patients were treated with a combination of bevacizumab and fotemustine. Fotemustine (Muphoran, Servier; Thissen Laboratories) dose was 100 mg/m² (intravenous infusion over 1 hour) and was given on days 1, 8, and 15 (induction phase), to be repeated after 4 weeks every 21 days (maintenance phase). Bevacizumab (Avastin; Roche Ltd.) was intravenously administered at a dose of 15 mg/kg every 3 weeks, after chemotherapy (i.e., at day 1, 21, 42, and so on), initially over 90 minutes, and then over 60 to 30 minutes according to degree of tolerance. Before starting each cycle, patients underwent physical examination, toxicity assessment, and complete biochemistry and hematologic tests. If the absolute neutrophil count (ANC) was less than 2,000/mm³, or if the platelets were less than
100,000/mm³, treatment was delayed for 1 week. If these low counts persisted, the dose of fotemustine was reduced by 25%. This dose reduction was maintained for the successive cycles. If the ANC was less than 1,500/mm³, the dose of fotemustine was reduced by 50%. The same dose was maintained for the successive cycles. No dose modifications of bevacizumab were done unless the subject’s weight changed by more than 10%, in which case the dose was recalculated. Further mAb administration was halted if the patient developed any of the following toxicities attributable to bevacizumab: grade 4 toxicity, grade 3 toxicity that did not resolve to grade 1 or less within 4 weeks, and arterial thromboembolic events, gastrointestinal perforation. If fotemustine was held or delayed for toxicity reasons, bevacizumab was delayed as well and was resumed with the chemotherapy. A treatment delay for toxicity reasons, bevacizumab was delayed as well and was resumed with the chemotherapy. A treatment delay was requested until ANC and platelet count recovery was resumed with the chemotherapy. A treatment delay toxicities reasons, bevacizumab was delayed as well and was resumed with the chemotherapy. A treatment delay was requested until ANC and platelet count recovery


greater than 1,500 and greater than 100,000/mm³, respectively). In the case of a dose delay of 1 of the 2 study drugs for more than 3 weeks, the patient withdrew from the study for toxicity.

Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (Version 3). All patients who received 1 dose of chemotherapy were assessable for toxicity.

Evaluation

Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST). The disease evaluation during treatment was performed every 9 weeks. In the case of objective response or SD, bevacizumab was continued until disease progression, unacceptable toxicity, patient refusal, or physician decision, and fotemustine was continued up to 6 cycles of maintenance. TTP was defined as the time from the beginning of the first cycle to the evidence of progressive disease (PD), or death, or the last date the patient was known to be progression free or alive. OS was calculated from the beginning of the first cycle to death from any cause, or the last date the patient was known to be alive. Biomarker analysis was carried out on serum samples obtained from the 15 patients enrolled in one of the participating centers (Istituto IRCCS Istituto Nazionale dei Tumori, Milan).

Serum biomarker analysis

Serum samples were obtained immediately before and 1 hour after administration of therapy (days 1, 21, 42, 63, and so on, as long as therapy continued). Blood samples were centrifuged at room temperature and serum samples were frozen and stored at −80°C until use. Serum samples were evaluated by custom-made, antibody-based chemiluminescent SearchLight multiplex arrays (Pierce SearchLight Proteome Arrays). Briefly, after plate incubation with biotinylated detecting antibodies, streptavidin–horseradish peroxidase was added, which then reacted with a chemiluminescent substrate (SuperSignal ELISA Femto Chemiluminescent Substrate) and the reaction produced a chemiluminescent signal that was detected by a charge coupled device (CCD) camera (SearchLight Plus CCD Imaging System). Analysis of SearchLight images was carried out with Array Analyst software (Pierce). Nonlinear regression analysis by Prism software (GraphPad) was then used to fit the following variable slope, 4-parameter equation of the standard curve: \[ Y = Bottom + (Top - Bottom)/(1 + 10^{(log EC_{50} - X)/Hill Slope}) \]

The arrays were designed to detect and quantitate the following molecules: VEGF-A, VEGF-C, VEGF-R1, VEGF-R2, s-E-selectin, P-selectin, soluble intercellular adhesion molecule (sICAM)-1, C-reactive protein (CRP), IL-8, IL-10, CXCL10, IL-12p70, IL-17, IL-23, IFN-γ, and tumor necrosis factor (TNF)-α. In addition to patients’ sera, a pool of sera from 5 healthy donors was also evaluated for each of the 16 molecules.

In vitro angiogenesis assay

Overall proangiogenic activity present in the serum of enrolled patients was evaluated by an in vitro angiogenic assay (AngioKit; CellWorks). AngioKit wells, containing human endothelial cells at the earliest stage of tubule formation, on a layer of human fibroblasts, were seeded at day 1 with dilutions of pre- and posttherapy serum samples from enrolled patients. Three replicate wells were set up for each serum sample. Negative and positive controls included wells fed with medium only, serum from healthy donors, VEGF-A (2 ng/mL), or suramin (20 μM/L). Medium from each well was replaced at days 4, 7, and 9 with aliquots of each test sample. Tubule formation was then assessed at day 11 after staining wells with CD31. Briefly, wells were stained with anti-CD31 antibody at 37°C for 60 minutes followed by the addition of alkaline phosphatase-conjugated secondary goat anti-mouse Ig. Colorimetric reaction was then developed by adding p-NPP buffered substrate. Digital images of each well were acquired (magnification × 5) on an Axiovert (Zeiss) microscope. Images were then processed with Angioquanto software to quantitate the extent of tubule formation (lengths, sizes, and number of junctions) in each replicate well.

Modulation of VEGF-C release by melanoma cells

Melanoma cell lines established from surgical specimens of neoplastic lesions of patients admitted to Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, were used. Cell lines Me4405P and Me26635P were isolated from primary melanomas of the right cheek and of the scalp of 2 patients in 1989 and 1995, respectively. Clone 2/21 was isolated by soft agar cloning from a subcutaneous metastatic melanoma cell line (Me 665/2) obtained in 1986. All the lesions were histologically confirmed to be cutaneous malignant melanomas. Molecular and biological characterization of these cell lines has been reported previously (16–18). Release of VEGF-C in melanoma supernatants after fotemustine treatment for 72 hours was evaluated by ELISA (Bender).

Apoptosis assay

Extent of apoptosis in melanoma cells treated with fotemustine or temozolomide (Temodal; Schering-Plough) was evaluated at 24 to 72 hours by staining tumor cells with
Annexin V-FITC and propidium iodide (PI; BD Biosciences) as described (19), followed by flow cytometric analysis with FACS calibur flow cytometer (BD Biosciences Pharmingen).

Statistical analysis

Data are reported as percentage of complete response (CR), partial response (PR), SD and PD. Data from all patients, who received at least 1 drug delivery and for whom at least 1 tumor evaluation was performed, were included in the response analysis. According to a typical 2-stage optimal design, if less than 10% is considered an unacceptable response rate and more than 30% acceptable, for 5% significance level and 90% power, then 18 patients are needed in the first stage. If patients \( n \leq 2 \) have an objective response, then the trial must be closed because of poor response. If 3 or more patients respond, the trial goes on to the second stage by recruiting 18 additional patients to reach a total of 36 patients. Exact binomial methods were used to estimate the response rates and their 95% confidence intervals. TTP or death was estimated by the product-limit method of Kaplan and Meier. All results of the serum marker analyses were evaluated by ANOVA, followed by the SNK multiple comparison test.

Results

Patient characteristics

Twenty patients entered the study and were included in the safety and efficacy analysis. The study recruited patients between December 2006 and November 2007 and was closed in March 2010. One patient (#16), initially with a diagnosis of M1b melanoma, was then confirmed to have histiocytosis. The patient characteristics are summarized in Table 1. Median age was 54 years, and all patients had a PS of 0. Eight patients had M1c disease; in particular, 5 of them had liver metastases.

Clinical activity

All the patients completed the induction phase and received a median of 5 (range, 2–8) administrations of bevacizumab. There was 1 CR in a patient (#13) with groin and pelvic lymph nodes, but such response developed after patient withdrawal from trial due to toxicity (Table 2). Two patients (#11 and #12), with M1a disease, showed a PR (Table 2). Ten patients obtained SD, yielding an overall disease control rate (clinical benefit) of 65% (13/20). The clinical benefit increased to 68.4% after excluding patient 16 from the analysis. Two patients (#8 and #17), bearing M1c stage disease for liver metastases, had SD but progressed after 7.1 and 25.8 months, respectively, from the beginning of therapy. All the 20 patients were assessable for TTP and OS. Median TTP was 8.3 months (range, 4.1–24.2) and median OS was 20.5 months (range, 9.9–26.5). These efficacy parameters did not change after excluding patient 16 from analysis. Nine patients are still alive, 2 of whom have liver disease.

Toxicity

Of the 20 enrolled patients, 18 experienced 1 or more adverse events: 14 of them developed at least 1 adverse event of grade 3–4 toxicity. The most frequently observed grade 3–4 hematologic toxicity was neutropenia, which occurred in 10 patients (Supplementary Table S1). Two patients (10%) developed febrile neutropenia. Nonhematologic toxicities were generally mild; 4 patients (20%) developed grade 3–4 hypertension. Treatment was discontinued because of toxicity in 5 of the 20 patients. In particular, therapy was interrupted for thrombocytopenia in 3 patients and for pulmonary thromboembolism in 1 patient. Serious adverse events included 1 case of metrorrhagia in a patient with thrombocytopenia, 1 case of pulmonary thromboembolism, and 1 case of dyspnea in a patient with pleural effusion. No treatment-related deaths were observed.

Serum VEGF-A analysis

To assess systemic levels and modulation of VEGF-A (bevacizumab target), serum samples obtained from 15 patients at several time points during therapy were evaluated by the SearchLight multiplex array approach. Among the 15 patients, 2 (#11, #12) showed a PR, 7 (#2, #4, #7, #8, #10, #17, #19) SD, and 6 (#3, #5, #6, #9, #15, #20) PD. In all assessed patients, pretherapy VEGF-A levels (average 419 ± 252 pg/mL, \( n = 15 \)) were about ~23-fold higher than those in serum from a pool of healthy donors (18 ± 7 pg/mL). Within 1 hour after the first administration of therapy (Fig. 1, black symbols), VEGF-A serum levels were significantly reduced compared with pretherapy values. In addition, by looking at all patients, a significant reduction in VEGF-A levels was also observed by comparing serum samples taken immediately before and 1 hour after administration of bevacizumab alone at day 21 (\( P = 0.0005 \)). In each of the patients, with only 1 exception (pt #2, third serum

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
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<tbody>
<tr>
<td>n</td>
</tr>
<tr>
<td>Median age (range), y</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>PS (ECOG): 0</td>
</tr>
<tr>
<td>Prior therapy</td>
</tr>
<tr>
<td>Adjuvant IFN-( \alpha )</td>
</tr>
<tr>
<td>Surgical resection of primary tumor</td>
</tr>
<tr>
<td>Sites of metastases( ^a )</td>
</tr>
<tr>
<td>M1a</td>
</tr>
<tr>
<td>M1b</td>
</tr>
<tr>
<td>M1c</td>
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</tbody>
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\( ^a \)M1a, subcutaneous, skin, and/or lymph nodes only; M1b, M1a, and/or lung only; M1c, other visceral organs involved and/or elevated lactate dehydrogenase.
sample), all VEGF-A serum values observed during therapy (66/67 posttherapy serum samples) remained significantly lower than pretherapy levels (see Fig. 1). Moreover, with the exception of some samples of patients 2, 7, and 11, VEGF-A levels dropped upon therapy administration to values similar to those found in serum from a pool of healthy donors (horizontal dotted line in Fig. 1). Interestingly, in one of the patients (patient 19), 2 cycles of therapy were skipped (due to intervening toxicity) between day 21 and day 77, but VEGF-A levels remained low for as long as 56 days after administration of therapy at day 21 (Fig. 1). These results indicate that administration of bevacizumab plus fotemustine was associated with profound and durable inhibition of systemic levels of VEGF-A in all assessed patients.

Modulation of proangiogenic activity at the systemic level in enrolled patients

To corroborate the evidence for VEGF-A inhibition after therapy, we carried out an in vitro angiogenesis assay (AngioKit) that allowed to test whether posttherapy serum samples had less growth-promoting activity for human endothelial cells than pretherapy samples. Using this 11-day in vitro assay, we could score the extent of development of a network of capillary-like tubules by human endothelial cells seeded on an underlying matrix of fibroblasts. Compared with the extent of tubule formation induced by a pool of pretherapy serum samples from 6 patients, posttherapy sera from the same patients showed a dramatic reduction in their proangiogenic activity as documented by the marked reduction in tubule size, length, and number of junctions (see Supplementary Fig. S1). Assessment of tubule lengths, sizes, and junctions indicated that posttherapy serum samples from treated patients had an overall proangiogenic activity reduced to that seen in control serum from healthy donors (Fig. 2). In addition, in agreement with the known half-life of bevacizumab (20), at the end of therapy, serum samples could still suppress the proangiogenic activity of recombinant VEGF-A. In fact,

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### Table 2. Characteristics of patients who achieved clinical benefit

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Best response</th>
<th>Age/Sex</th>
<th>Duration of study, mo*</th>
<th>Disease sites</th>
<th>Adjuvant therapy</th>
<th>TTPb, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SD</td>
<td>55/F</td>
<td>6.11</td>
<td>Skin</td>
<td>IFN-α2a</td>
<td>16.8</td>
</tr>
<tr>
<td>2</td>
<td>SD</td>
<td>33/F</td>
<td>3.65</td>
<td>Skin, lung</td>
<td>No</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>SD</td>
<td>59/M</td>
<td>4.73</td>
<td>Lymph nodes,</td>
<td>No</td>
<td>8.2</td>
</tr>
<tr>
<td>7</td>
<td>SD</td>
<td>35/F</td>
<td>2.73</td>
<td>Left iliac crest, lung</td>
<td>No</td>
<td>28.2</td>
</tr>
<tr>
<td>8</td>
<td>SD</td>
<td>66/M</td>
<td>5.32</td>
<td>Left axillary lymph nodes, liver, and pancreas</td>
<td>HD IFN-α</td>
<td>7.1</td>
</tr>
<tr>
<td>10</td>
<td>SD</td>
<td>69/M</td>
<td>5.19</td>
<td>Left axillary lymph nodes, left inguinal iliac lymph nodes, lungs</td>
<td>No</td>
<td>26.1</td>
</tr>
<tr>
<td>11</td>
<td>PR</td>
<td>67/M</td>
<td>4.57</td>
<td>Laterocervical lymph nodes, left supraclavicular lymph nodes, left mastoid region, left clavicular, and laterocervical region</td>
<td>No</td>
<td>14.8</td>
</tr>
<tr>
<td>12</td>
<td>PR</td>
<td>52/M</td>
<td>5.75</td>
<td>Right mammary region, right axillary lymph nodes</td>
<td>No</td>
<td>7.1</td>
</tr>
<tr>
<td>13</td>
<td>CR</td>
<td>75/F</td>
<td>10.84</td>
<td>Groin lymph nodes, pelvic lymph nodes</td>
<td>No</td>
<td>28.1</td>
</tr>
<tr>
<td>14</td>
<td>SD</td>
<td>68/M</td>
<td>0.76</td>
<td>Right axillary lymph nodes, mediastinum lymph nodes, right pleura</td>
<td>No</td>
<td>2.4</td>
</tr>
<tr>
<td>17</td>
<td>SD</td>
<td>38/F</td>
<td>4.17</td>
<td>Axillary lymph nodes, liver</td>
<td>IFN-α</td>
<td>25.8</td>
</tr>
<tr>
<td>18</td>
<td>SD</td>
<td>56/M</td>
<td>8.31</td>
<td>Laterocervical lymph node (right)</td>
<td>IFN-α</td>
<td>25.1</td>
</tr>
<tr>
<td>19</td>
<td>SD</td>
<td>49/F</td>
<td>5.49</td>
<td>Left inguinal lymph nodes, bilateral ovaries, lungs, and perineal region</td>
<td>No</td>
<td>23.9</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male.

*Duration of study was calculated as time elapsed between the date of baseline visit and the date of study discontinuation.

**TTP was defined as the time from the first treatment administration until objective tumor progression or death. If a patient had no events, TTP was censored at the date of the last adequate tumor assessment.
end-of-therapy serum sample from one of the patients (patient 4) could significantly inhibit tubule formation induced by recombinant VEGF-A (Fig. 2). Taken together, these results indicated that the treatment of patients with bevacizumab plus fotemustine leads not only to suppression of VEGF-A serum levels but also to profound inhibition of the overall proangiogenic activity detected in periphery of enrolled patients.
Modulation of the lymphangiogenesis factor VEGF-C upon treatment with bevacizumab plus fotemustine

The multiplex ELISA array was designed to include VEGF-C. Pretherapy serum samples from all patients contained high levels of the lymphangiogenesis factor VEGF-C (average pretherapy value: 2,458 ± 938 pg/mL, n = 15). In contrast, no VEGF-C could be measured in serum from a pool of healthy donors. Analysis of the VEGF-C serum levels indicated significant reductions (marked by asterisks, Fig. 3) compared with pretherapy levels (day 0) in 47 of 68 posttherapy samples. Only 6 posttherapy serum samples (patients 11, 4, and 6, samples marked by arrows, Fig. 3) showed significantly higher VEGF-C levels than pretherapy values. Collectively, comparison of end-of-therapy VEGF-C levels versus day 0 values provided evidence for significant reduction in 2 of 2 PR, 5 of 7 SD, and 5 of 6 PD patients (Supplementary Fig. S2). Taken together, these results indicate that treatment with bevacizumab plus fotemustine markedly inhibits serum levels of the lymphangiogenesis factor VEGF-C.

Fotemustine inhibits VEGF-C release by melanoma cells in vitro without inducing cell death

No available data indicate that bevacizumab can induce suppression of VEGF-C serum levels. Therefore, we hypothesized a role of fotemustine in the modulation of this factor. To address this possibility, culture supernatants from several melanoma cell lines established from patients were evaluated by ELISA for VEGF-C secretion (data not shown). Two cell lines producing VEGF-C (Me26635P and Me4405P) were selected for further analysis. Fotemustine-treated melanoma cells from one of these cell lines (Me26635P) showed a dose-dependent and significant decrease in VEGF-C secretion in a 72-hour assay (Fig. 4A). Similar results were obtained with Me4405P (data not shown). The inhibitory effect of fotemustine on VEGF-C release by these tumors was not due to induction of cell death. In fact, apoptosis assays showed that fotemustine did not induce significant cell death up to 72 hours in Me26635P and Me4405P tumors. However, the same tumors were susceptible to cell death induced by a different alkylating agent (temozolomide; Fig. 4B), and fotemustine could induce apoptosis of a different tumor (Me2/21; Fig. 4B). Gene-profiling experiments, by whole genome arrays under the Illumina platform, also showed that VEGF-C was among the genes significantly downmodulated in melanoma cells upon 6-hour treatment with fotemustine (data not shown). Taken together, these results indicate that fotemustine can impact on VEGF-C release by human melanoma cells.

Association of distinct cytokines with clinical response groups

We tested the hypothesis that the treatment with bevacizumab plus fotemustine could affect serum levels of additional molecules involved in the regulation of angiogenesis and of the immune response. To this end, the multiplex ELISA arrays were designed to allow detection of levels of VEGF-R2 and VEGF-R1, of endothelial adhesion molecules (E-selectin, P-selectin, and sICAM-1), of cytokines IL-8, IL-10, IL-12p70, IL-17, IFN-γ, and TNF-α, and of the chemokine CXCL10. CRP, a known biomarker of tumor load in melanoma (21), was also included in the analysis. Levels of CRP showed significant increases at the end of therapy compared with pretherapy values in 4 of 7 SD patients and in 5 of 6 PD patients but not in the 2 PR patients (Supplementary Fig. S2). A similar comparison of end-of-therapy versus pretherapy values of all factors showed changes in some of the patients but were not associated with the response groups (Supplementary Fig. S2). We then assessed whether an
association of any of the investigated factors with the 3 main response groups (PR, SD, and PD) could be found by analysis of all available serum samples from all patients rather than looking only at pre-versus end-of-therapy samples. By this approach, serum levels of 3 cytokines (IL-10, IL-12p70, and IL-23) showed a significant association with the 3 response groups (PR, SD, and PD; Supplementary Fig. S3). IL-10 and IL-12p70 showed the highest levels in sera of PR patients compared with SD and PD patients. IL-23 showed the opposite pattern, with the highest levels found in sera of PD patients compared with SD and PR patients (Supplementary Fig. S3).

Fig. 3. Modulation of serum VEGF-C levels in 15 patients upon treatment with fotemustine plus bevacizumab. Patients were identified by their randomization number and grouped according to response to therapy (PR, SD, or PD). VEGF-C levels were evaluated in serum samples taken immediately before (empty symbols) and 1 hour after (black symbols) therapy administration. Timing of therapy administration is indicated by vertical dotted lines. VEGF-C was also evaluated in serum from a pool of healthy donors (horizontal dotted line) but was undetectable. All VEGF-C values identified by asterisks (**) were significantly lower than day 1, pretherapy values (SNK test, $P < 0.01$). VEGF-C levels marked by arrows were significantly higher than pretherapy values (SNK test, $P < 0.01$).
Discussion

In this study, the choice of fotemustine, as chemotherapy agent, was based on 2 main reasons. The first one was that fotemustine, when compared in a randomized trial to dacarbazine (DTIC), a conventional chemotherapy drug for advanced melanoma, was shown to yield a higher overall response rate (13). The second one was that fotemustine, in contrast with DTIC, has the ability to cross the blood–brain barrier, thus potentially preventing the development of brain metastases (12). The choice of bevacizumab was initially based on the evidence for its clinical activity in different solid tumors (6–8) and on the emerging impact on OS, progression-free survival, and response rate found in colorectal cancer when adding this mAb to chemotherapy (9). The combination of bevacizumab and fotemustine has been recently investigated in recurrent malignant glioma patients in whom it showed good safety and efficacy (22). Patients received intravenous administration of bevacizumab 10 mg/kg on day 1 and day 15 and fotemustine 75 mg/m² on day 1 and day 8 as an induction phase; after 3 weeks, patients received bevacizumab 10 mg/kg and fotemustine 75 mg/m² every 3 weeks (maintenance phase). The rationale for using bevacizumab even in melanoma has been strengthened by preclinical studies (23) and early-phase clinical trials (10, 11). Moreover, the only clinical case reported so far of metastatic melanoma treatment with this biochemotherapy regimen showed an early tumor response in terms of extensive necrosis (24).

In this study, we found that a biochemotherapy regimen based on the combination of bevacizumab plus fotemustine has clinical activity as first-line treatment in metastatic melanoma and promotes suppression, at the systemic level, of soluble factors involved in angiogenesis and...

Fig. 4. Fotemustine inhibits VEGF-C release by melanoma cells without inducing cell death. A, melanoma cells (7.5 × 10⁶/mL) from a melanoma cell line (Me26635P) were cultured for 72 hours with the indicated concentration levels of fotemustine or in medium alone (−). VEGF-C levels were then evaluated in supernatants by ELISA. Statistical analysis by SNK test was annotated as follows: ***P < 0.001; **P < 0.01. B, melanoma cells from Me26635P and from 2 additional melanoma lines (Me4405P and Me2/21) were treated for 72 hours with the indicated concentrations of fotemustine (FTM) or of temozolomide (TMZ), or cultured in medium without drugs (Ctrl). Extent of early apoptosis (lower right quadrant in each dot plot) or of late apoptosis (upper right quadrant in each dot plot) was then assessed by flow cytometry after staining cells with Annexin V and PI. Numbers in each dot plot represent percentage of cells in each quadrant.
lymphangiogenesis. Moreover, given the relevance of angiogenesis and lymphangiogenesis in melanoma progression and in the development of metastases (25), the association of bevacizumab and fotemustine might be considered for testing in the adjuvant setting in high-risk stage III patients, in which, so far, only IFN-α2b has shown impact on OS (26). Of course, randomized trials comparing the biochemotherapy approach versus chemotherapy alone in a large series of stage IV melanoma patients should be carried out before moving to the adjuvant setting.

In agreement with previous bevacizumab-based studies in melanoma (11, 27, 28), our results confirmed the clinical benefit of this biochemotherapy approach. On the other hand, of possible concern for future trials involving the combination of bevacizumab plus fotemustine was the number of patients (n = 5) who discontinued treatment because of toxicity. In spite of the response rate (1 CR and 2 PR), clinical benefit (65% of patients), median OS (20.5 months), and good compliance with both drugs, based on the results of the safety analysis, we did not feel comfortable to proceed to the second stage of the study. Fourteen patients (70%) experienced adverse events of toxicity grade 3–4, although no treatment-related death was detected during the study. Therefore, the bevacizumab–fotemustine schedule used in brain tumors could be helpful, in terms of safety profile, in future trials in melanoma.

The serum biomarker analysis showed that this biochemotherapy was associated with a quick and highly significant drop in VEGF-A levels, which remained low, in all patients, throughout the whole duration of therapy. The reduction in VEGF-A levels was observed in all evaluated patients and therefore it did not correlate with the clinical response. In addition, an in vitro angiogenesis assay indicated that the overall proangiogenic activity detectable in peripheral blood was completely inhibited by the therapy. We also found that posttherapy serum from treated patients could completely inhibit the angiogenic activity of recombinant VEGF-A. Taken together, even though melanoma patients can have measurable levels of additional angiogenic molecules, such as IL-8 and bFGF (29–31), these results suggest that the therapeutic approach described in this study can durably revert the systemic “proangiogenic phenotype” of advanced melanoma patients to a condition similar to peripheral blood of healthy donors.

Unexpectedly, we found that bevacizumab plus fotemustine combination therapy also affected serum levels of VEGF-C, a molecule that together with VEGF-D plays a key role in the lymphangiogenesis process (32). Although we cannot rule out that both bevacizumab and fotemustine concur to VEGF-C inhibition in vivo, nevertheless, in vitro assays indicated that fotemustine, used alone, could inhibit VEGF-C gene expression (data not shown) in melanoma cells and significantly suppressed VEGF-C release from melanoma cells, a finding not due to induction of apoptosis. Therefore, these results suggest that a biochemotherapy based on the combination of bevacizumab and fotemustine may impact on both angiogenesis and lymphangiogenesis, 2 highly relevant processes in melanoma progression. In fact, strong evidence (see ref. 33 for review) indicates that VEGF-C expression in the tumor significantly correlates with lymph node metastasis. Moreover, experimental models have indicated that neoplastic cells can promote lymphangiogenesis even within draining lymph nodes and that this process promotes further metastases to distant organs (34).

Finally, investigation on the expression of several cytokines in enrolled patients showed a significant association of IL-10, IL-12p70, and IL-23 serum levels with the 3 response groups. The converse levels of IL-12p70 and IL-23 in the PR and SD versus PD patients seem to fit the recent model describing the contrasting immunoregulatory functions of these 2 cytokines under the control of STAT3 in the tumor microenvironment (35). According to this model, as well as to a wealth of previous evidence (see ref. 36 for review), IL-12p70 can promote adaptive T cell–mediated immunity and suppress angiogenesis whereas IL-23 can have a protumoral activity by stimulating regulatory T cells (35). The results obtained for IL-10 are unexpected, as this cytokine has been considered an anti-inflammatory immunosuppressive factor (37) and a marker of poor prognosis in advanced melanoma (38). However, IL-10 may also exert antitumor activity, thanks to its ability to inhibit MHC class I expression, thus favoring NK-mediated antitumor responses (37), and to its ability to inhibit MHC class I expression, thus favoring NK-mediated antitumor responses (37), and to its ability to inhibit MHC class I expression, thus favoring NK-mediated antitumor responses (37), and to suppress angiogenesis (39). In spite of the limited sample size, these results suggest that these immunomodulatory molecules may be evaluated in larger trials involving this drug combination as predictive biomarkers of response to therapy.

In conclusion, the good clinical impact of bevacizumab–fotemustine biochemotherapy, in terms of both disease control rate and median OS, suggests that this therapeutic approach should be investigated in future studies, possibly by modulating drug schedules (22) to improve the safety profile.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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15. Del Vecchio et al.
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In this article (Clin Cancer Res 2010;16:5862–72), which was published in the December 1, 2010 issue of Clinical Cancer Research (1), an affiliation for Prof. Mario R. Sertoli was missing. The correct affiliations for Prof. Sertoli are Department of Medical Oncology, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy and Department of Oncology, Biology, and Genetics, University of Genova, Genova, Italy.

Reference


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Bevacizumab plus Fotemustine as First-line Treatment in Metastatic Melanoma Patients: Clinical Activity and Modulation of Angiogenesis and Lymphangiogenesis Factors

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