Published in final edited form as:

Invest New Drugs. 2014 October ; 32(5): 1017–1027. doi:10.1007/s10637-014-0111-8.

Phase II trial of bortezomib plus doxorubicin in hepatocellular carcinoma (E6202): a trial of the Eastern Cooperative Oncology Group

Kristen K. Ciombor,

Division of Medical Oncology, Department of Internal Medicine, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, The Ohio State University, A445A Starling Loving Hall, 320 West 10th Avenue, Columbus, OH 43212, USA

Yang Feng,

Dana Farber Cancer Institute, Boston, MA, USA

Al Bowen Benson III,

Northwestern University, Chicago, IL, USA

Yingjun Su,

Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, China

Linda Horton,

Vanderbilt University, Nashville, TN, USA. Department of Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, USA

Sarah P. Short,

Vanderbilt University, Nashville, TN, USA

John Sae Wook Kauh,

Emory University, Atlanta, GA, USA

Charles Staley,

Emory University, Atlanta, GA, USA

Mary Mulcahy,

Northwestern University, Chicago, IL, USA

Mark Powell,

New York, NY, USA

Katayoun I. Amiri,

Celgene Corporation, Summit, NJ, USA

Ann Richmond, and

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Correspondence to: Kristen K. Ciombor, kristen.ciombor@osumc.edu.

Ethical standards The experiments described in this manuscript comply with the current laws of the United States of America.

Conflict of interest The authors declare that they have no conflicts of interest.

Vanderbilt University, Nashville, TN, USA. Department of Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, USA

Jordan Berlin

Vanderbilt University, Nashville, TN, USA

Kristen K. Ciombor: kristen.ciombor@osumc.edu

Summary

Purpose—To evaluate the efficacy and tolerability of bortezomib in combination with doxorubicin in patients with advanced hepatocellular carcinoma, and to correlate pharmacodynamic markers of proteasome inhibition with response and survival. **Experimental Design** This phase II, open-label, multi-center study examined the efficacy of bortezomib (1.3 mg/m² IV on d1, 4, 8, 11) and doxorubicin (15 mg/m² IV on d1, 8) in 21-day cycles. The primary endpoint was objective response rate.

Results—Best responses in 38 treated patients were 1 partial response (2.6 %), 10 (26.3 %) stable disease, and 17 (44.7 %) progressive disease; 10 patients were unevaluable. Median PFS was 2.2 months. Median OS was 6.1 months. The most common grade 3 to 4 toxicities were hypertension, glucose intolerance, ascites, ALT elevation, hyperglycemia and thrombosis/embolism. Worse PFS was seen in patients with elevated IL-6, IL-8, MIP-1 α and EMSA for NF- κ B at the start of treatment. Worse OS was seen in patients with elevated IL-8 and VEGF at the start of treatment. Patients had improved OS if a change in the natural log of serum MIP-1 α /CCL3 was seen after treatment. RANTES/CCL5 levels decreased significantly with treatment.

Conclusions—The combination of doxorubicin and bortezomib was well-tolerated in patients with hepatocellular carcinoma, but the primary endpoint was not met. Exploratory analyses of markers of proteasome inhibition suggest a possible prognostic and predictive role and should be explored further in tumor types for which bortezomib is efficacious.

Keywords

Hepatocellular carcinoma; Bortezomib; Doxorubicin; Proteasome inhibition

Introduction

Bortezomib is a potent and reversible proteasome inhibitor with proven efficacy in hematologic malignancies such as multiple myeloma and mantle cell lymphoma [1, 2]. It selectively inhibits the 26S proteasome, an adenosine triphosphate-dependent, multicatalytic protease that selectively degrades polyubiquitinated proteins. The ubiquitin-mediated proteasome is the final degradative compartment involved in an important catabolic pathway for many intracellular regulatory proteins including inhibitor of κ B (I κ B), an important regulator of NF- κ B [3, 4], p53 [5], and the cyclin-dependent kinase inhibitors p21 [6] and p27 [7]. A proteasome inhibitor such as bortezomib has potential for significant antitumor effect through disruption of cell cycle checkpoints and apoptosis pathways. In several hepatocellular carcinoma cell lines specifically, bortezomib has been shown to suppress cellular proliferation and induce apoptosis [8–10].

Given that proteasomal inhibition by bortezomib has a major effect on NF- κ B activation [11–14], it is important that several lines of evidence also demonstrate specific roles of NF- κ B in HCC. NF- κ B activation is a frequent and early event in HCC and likely contributes to the acquisition of a transformed phenotype in hepatocarcinogenesis [15, 16]. The degree of tumor differentiation in HCC appears to be related to NF- κ B activation [17]. NF- κ B may mediate TGF- β -induced upregulation of fibronectin expression in hepatoma cell lines [18], where fibronectin plays an important role in cell adhesion and migration and may contribute to oncogenic transformation and metastasis. NF- κ B also induces expression of the multidrug resistance transporter protein Mdr1b in rat hepatocytes and hepatoma cells [19] and induces the expression of a host of chemotactic cytokines (chemokines) [20–22]. Perhaps most important, NF- κ B regulates the expression of a host of survival factors, which enable stressed hepatocytes to escape apoptosis [23, 24]. However, the NF- κ B activation has different results on cancer progression depending upon whether it is the hepatocyte, Kupffer cells, or hepatic myofibroblasts that are activated. Indeed, deletion of the kinase that activates NF- κ B, IKK β , in hepatocytes promotes chemical carcinogenesis in the liver in a mouse model [25].

Despite this preclinical rationale, proteasome inhibitors such as bortezomib used as single-agent therapy for solid tumors in general have not demonstrated efficacy, including in HCC [26]. However, there is preclinical evidence that bortezomib has additive activity with other therapies and can confer chemosensitizing effects when combined with other agents [9, 11]. This has been demonstrated with the use of bortezomib and doxorubicin in multiple myeloma cell lines [12, 27, 28], among others. In this instance, the combination of bortezomib and doxorubicin was additive and/or synergistic in every sequence tested, with the best results obtained when doxorubicin was given prior to bortezomib [27]. Bortezomib has also been shown to confer renewed sensitivity to chemotherapy such as doxorubicin in cell lines that previously developed resistance to their cytotoxic effects [27].

HCC is an attractive tumor type to consider therapy with bortezomib and doxorubicin given this preclinical rationale and the limited efficacy of current chemotherapeutic regimens for advanced and unresectable HCC. Prior to the Food and Drug Administration's approval of sorafenib for patients with HCC in 2007, doxorubicin was considered a standard chemotherapeutic option for patients with HCC [29]. Subsequent randomized trials have shown a survival advantage not only in HCC patients treated with sorafenib [30], but also in those treated with the combination of doxorubicin and sorafenib [31]. However, novel agents and combination therapies are still needed for HCC.

Given this preclinical data in support of bortezomib in HCC and demonstrating potential synergy between bortezomib and doxorubicin, there was rationale for testing this combination clinically in HCC. A phase I trial was conducted previously in patients with advanced malignancies utilizing bortezomib in combination with doxorubicin in a 21-day cycle [32]. Bortezomib was administered on days 1, 4, 8 and 11 along with doxorubicin on days 1 and 8 of each cycle. The maximum tolerated dose for bortezomib was 1.3 mg/m² and for doxorubicin was 15 mg/m². Dose-limiting toxicities included neutropenia, thrombocytopenia and neuropathy. On the basis of this safety data and the rationale for utilizing bortezomib and doxorubicin in combination in HCC, we initiated this phase II trial

in the pre-sorafenib era, with emphasis on correlation of pharmacodynamic markers of proteasome inhibition with response and survival of patients with HCC.

Material and methods

This was an open-label, non-randomized phase II study (NCT00083226) undertaken by the Eastern Cooperative Oncology Group (ECOG) to evaluate the efficacy of bortezomib in combination with doxorubicin for patients with advanced hepatocellular carcinoma and no prior systemic chemotherapy. The trial was conducted in accordance with good clinical practice guidelines and the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board (IRB) of each participating institution.

Patient selection

All included patients provided written informed consent and fulfilled the following criteria: age ≥ 18 years, microscopically confirmed hepatocellular carcinoma not amenable to curative resection, at least one measurable site of disease defined by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0, documented progression of at least one lesion, and Eastern Cooperative Oncology Group (ECOG) performance status 0–2. Prior embolization without chemotherapy for HCC was allowed, as was radiofrequency ablation, cryosurgery and ethanol injection.

Exclusion criteria included absolute neutrophil count (ANC) $<1,500/\text{mm}^3$ in the absence of splenomegaly or $<1,000/\text{mm}^3$ in the presence of splenomegaly, platelet count $<100,000/\text{mm}^3$ in the absence of splenomegaly or $<75,000/\text{mm}^3$ in the presence of splenomegaly, aspartate aminotransferase (AST) or alkaline phosphatase >5 times the institutional upper limit of normal, serum bilirubin >2 mg/dL, Child Pugh Scale Grade C cirrhosis, serum creatinine >2.0 mg/dL, ejection fraction (EF) $<50\%$ by echocardiogram or Multi Gated Acquisition Scan (MUGA), prior systemic chemotherapy, octreotide or tamoxifen as therapy for hepatocellular carcinoma, baseline peripheral neuropathy $>$ grade 1, or history of untreated malignancy other than HCC or history of malignancy treated within the past 5 years. Pregnant or lactating females were excluded, and patients had to agree to use adequate contraception. Patients with known allergy to boron, mannitol or bortezomib were not eligible, as were patients with a history of active collagen vascular disease, including systemic lupus erythematosus, rheumatoid arthritis and scleroderma. Patients on verapamil who could not be switched to an alternative medication were not eligible, due to the interaction with doxorubicin. Patients must not have had psychiatric illness or continued substance abuse that might impair the ability to provide informed consent or prevent safe administration of bortezomib.

Study design

Bortezomib was administered at a dose of 1.3 mg/m^2 IV push over 3–5 s on days 1, 4, 8, 11 of a 21-day cycle. Doxorubicin was administered at a dose of 15 mg/m^2 IV over 5–15 min on days 1, 8 of each 21-day cycle. The first dose of doxorubicin was administered on day 8 of cycle 1 after the first two doses of bortezomib (cycle 1, day 8). On days when both bortezomib and doxorubicin were administered (days 1 and 8), doxorubicin was

administered before bortezomib. Patients would continue to receive chemotherapy until progression. Patients would discontinue doxorubicin after receiving 12 cycles, regardless of response. Patients who had stable disease could continue on single agent bortezomib when doxorubicin was removed due to reaching the 12-cycle maximum or decreased ejection fraction. Doses were based on actual body weight. Patients had to maintain adequate organ function as defined by the eligibility criteria, or dose would be delayed. If a dose was delayed for >2 weeks, the patient was considered for removal from study unless there was evidence of symptomatic or radiographic benefit and approval was obtained through the medical monitor.

While on study, patients had weekly complete blood counts and blood chemistry panels with every cycle. In addition to standard laboratory evaluations, performance status, history and physical examination, alpha fetoprotein (AFP) measurements were obtained at the beginning of each cycle (every 3 weeks). Radiographic assessment of disease and evaluation of cardiac ejection fraction were obtained every 9 weeks. Blood samples for correlative studies were drawn on days 1 and 8 of cycles 1 and 2, and again on days 1 and 8 of cycles 9 and 10, if applicable. For all correlative assays, duplicate samples were analyzed from each patient sample at each time point.

Study objectives

The primary endpoint of this study was objective response rate (ORR) in patients with hepatocellular carcinoma treated with bortezomib and doxorubicin, as defined by RECIST. Planned secondary endpoints included overall survival (OS), time to progression (TTP), toxicity, progression-free survival (PFS) at 4.5 months, and effect of bortezomib on 26S proteasome activity in tumor tissue, peripheral white blood cells, and serum. Correlative studies were undertaken to assess the pharmacodynamics of bortezomib and to ensure its target, the proteasome, was being affected.

Assessment of safety

Adverse events (AE), serious AEs (SAE), clinical laboratory evaluations, vital signs, and echocardiograms or MUGA scans were collected from provision of informed consent until 30 days after discontinuation of study treatment as specified in the study protocol. Adverse events were collected using Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In addition, interim analyses of toxicity were performed twice yearly as per ECOG guidelines.

Correlative studies

Assays were performed to evaluate correlations between bortezomib treatment, inhibition of proteasome activity, reduction in NF- κ B activity and chemokine/cytokine levels. Specifically, the effect of bortezomib on 26S proteasome activity in patients' serum was evaluated by changes in levels of nuclear p65/RELA based on EMSA (Electrophoretic Mobility Shift Assay) for the detection of nuclear NF- κ B from pre-treatment to those on cycle 1, day 8. Since many key chemokines and cytokines that are important in inflammation associated with cancer progression are regulated at the transcriptional level by NF- κ B, we examined the serum levels of several of these key cytokines and chemokines

[33]. Levels of IL-6, IL-8, MGSA/Gro- α (Melanoma Growth Stimulatory Activity Factor/Growth-Regulated Oncoprotein- α)/CXCL1 (Chemokine (C-X-C Motif) Ligand 1), MIP-1 α (Macrophage Inflammatory Protein-1 α)/CCL3 (Chemokine (C-C Motif) Ligand 3), VEGF (Vascular Endothelial Growth Factor), RANTES (Regulated Upon Activation, Normal T-cell Expressed and Secreted)/CCL5 (Chemokine (C-C Motif) Ligand 5) were determined and comparisons between pre-treatment levels to those on cycle 1, day 8 were made.

Patient serum was analyzed for determination of circulating levels of IL-6, IL-8, MGSA Gro- α , MIP1- α , VEGF and RANTES using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems. Protein analyses were first determined on plasma samples and chemokine levels were normalized to total mg/ml of protein. A standard curve of purified cytokine/chemokine was run with each assay, and determination of the concentration of cytokine/chemokine in blood samples was based upon this standard curve. In addition, a reference sample from blood from a patient treated or not treated with bortezomib was run with each assay to normalize for day effects on the assays.

Electrophoretic mobility shift assays (EMSA) were performed on isolated proteins from nuclear extracts prepared from peripheral blood leukocytes [34], with a radiolabeled oligonucleotide probe encoding the NF- κ B DNA binding element as previously described [36]. Nuclear protein-DNA binding assays were conducted with an EMSA Kit (Promega, Madison, WI) and the procedures were following the manufacturer's instructions. As nuclear translocation of p65/p50 heterodimer or p65 homodimers and the binding of these NF- κ B elements to DNA consensus promotor sequence are the critical steps for gene transactivation, the major nuclear NF- κ B elements, p65 and p50, were determined by supershift assay with anti-human p65 and anti-human p50 antibodies in the EMSA. Appropriate controls were utilized as previously described [35].

Of note, evaluation of proteasome 26S inhibition in tumor tissue via analysis of p21, p27, p53, Bax, Bcl-2 and phosphorylation of I κ B was also preplanned as part of these studies; however, the number of biopsy samples with adequate tumor tissue received in this trial was inadequate to analyze with statistical significance, so this endpoint and the request for tissue-based studies were withdrawn on November 17, 2006.

Statistical and analytic methods

A single-stage design was utilized in this study where a total of 42 patients were accrued (38 eligible patients). A true response rate of 27 % or more was taken as evidence of activity in this patient population. The null hypothesis was that the true response rate was 10 % or lower. Therefore, if there were seven or more responses, the treatment would be considered promising. This test had 91 % power under the alternative hypothesis if the true response rate was 27 % assuming a 1-sided significance level of 0.07. Assuming a 1-sided significance level of 0.07, there was 84 % power to detect a difference of 21 % in 4.5 month PFS (50 % versus 71 %).

Descriptive statistics were used to characterize patients at baseline. Best response rate was reported and its confidence interval was computed by the method of exact binomial confidence interval. Overall survival and progression-free survival curves were obtained

using the Kaplan-Meier method [36], with 95 % confidence intervals calculated using Greenwood's formula. The associations between changes in natural logs of serum levels (differences of natural logs of values between pre-treatment and cycle 1, day 8) due to bortezomib and OS, PFS were explored using Cox regression models [37]. Since the lab values for serum markers were not normally distributed, non-parametric methods were utilized to compare the changes in serum levels of IL-6, IL-8, MGSA Gro- α , MIP-1 α , RANTES, VEGF and nuclear p65/RELA based upon EMSA (pre-treatment versus cycle 1, day 8) due to bortezomib administration. The Wilcoxon Signed Rank test was used to determine change at the two time points. The log-rank test [38] was used to explore OS, PFS by pre-treatment serum marker levels split at median values, and also to explore OS, PFS by whether a patient had an increase serum levels at cycle 1, day 8 versus those patients who had a decrease serum marker levels at cycle 1, day 8.

Efficacy analysis (response, overall survival, progression-free survival, correlative) included the eligible patients who started treatment (N=38) and the safety analysis (toxicity) included all the patients who started treatment (N=39). Data cutoff point was February 15, 2011.

Results

Demographics and other patient characteristics

A total of 42 patients were enrolled from March 2004 to February 2007 at four U.S. institutions: Mayo Clinic, Northwestern University, Vanderbilt University and Emory University, and 38 patients received therapy on protocol. Four patients were excluded because of ineligibility (n=1), physician decision (n=1), disease progression before treatment initiation (n=1), and patient withdrawal of consent (n=1). Selected baseline characteristics of the 38 eligible and treated patients are shown in Table 1. Median age was 59 (range: 23–76.5), and the majority were Caucasian (71.1 %) and male (71.1 %). The majority of patients (71.1 %) had an ECOG performance status of 0 or 1 (15.8 % and 71.1 %, respectively). Only 13.2 % of patients had active or chronic hepatitis B, but a significant number of patients (44.7 %) were positive for hepatitis C. A minority (26.3 %) of patients had ascites, and the majority (73.7 %) had Child Pugh Class A cirrhosis. Patients received a median of 3 cycles of therapy (range 1 to 12).

Response evaluation

A total of 38 patients were evaluable for response assessment, and Table 2 summarizes the best response of all eligible and treated patients. One patient (2.6 %) had a partial response, 10 (26.3 %) patients had stable disease, and 17 (44.7 %) patients had progressive disease as their best response. Eleven patients (28.9 %) had clinical benefit, defined as either partial response or stable disease. No patients had a complete response. Ten patients (26.3 %) were unevaluable. As of February 2011, 33 out of 38 eligible and treated patients had died. A total of 36 cases were available for progression-free survival (PFS) analysis. The median progression-free survival was 2.2 months (95 % CI [2.0, 3.4]) (Fig. 1) and 4.5 month progression-free survival rate was 28 % (95 % CI, [13 %, 42 %]). The median overall survival was 6.1 months (95 % CI, [4.4, 8.5]) (Fig. 1).

Safety

Table 3 summarizes post-baseline grade 3 and 4 toxicities for all treated patients (n=39). There were eight grade 3 toxicities, and one grade 4 toxicity. The most common grade 3 toxicity was hypertension (8 %), and the only grade 4 toxicity was thrombosis/embolism. No patient experienced lethal toxicities. The most common grade 1 and 2 toxicities, the only ones which were seen in at least half of the patients, included increase in AST (69 %), increase in alkaline phosphatase (59 %), and anemia (54 %).

Correlative studies

In order to evaluate the effect of bortezomib on 26S proteasome activity, serum samples were collected prior to treatment (cycle 1, day 1) and on cycle 1, day 8 for analysis of IL-6, IL-8, MGSA Gro- α , MIP-1 α , RANTES, VEGF levels and nuclear p65/RELA based upon EMSA and supershift analyses. Statistically significant differences in mean and median values of RANTES were seen in these time points (Table 4). Mean pre-treatment RANTES was significantly higher than that of cycle 1, day 8 RANTES (45,378.2 pg/mL vs. 38,224.7 pg/mL), as was median pre-treatment RANTES compared to cycle 1, day 8 RANTES (47,556.1 vs. 34,358.6 pg/mL, $p=0.02$ by Wilcoxon-Signed Rank test). Differences in the other correlates between these two time points were not statistically significant.

In the analysis of progression-free survival (PFS) by pre-treatment levels split at median values, significantly worse median PFS was seen in patients with pre-treatment IL-6 greater than or equal to 18.2 pg/mL (2.1 vs. 4.6 months, $p=0.019$), EMSA less than 15 pg/mL (2.1 vs. 4.3 months, $p=0.046$), IL-8 greater than or equal to 286.5 pg/mL (2.3 vs. 5.0 months, $p=0.017$), and MIP-1 α greater than or equal to 21.5 pg/mL (2.1 vs. 4.6 months, $p=0.036$) (Table 5). Patients who had a decrease in serum MIP-1 α levels at cycle 1, day 8 compared to pre-treatment levels had significantly worse median progression-free survival (2.0 vs. 4.3 months, $p=0.039$) (Table 6).

In the analysis of overall survival (OS) by pre-treatment serum levels split at median values, significantly worse median overall survival was seen in patients with IL-8 greater than or equal to 286.5 pg/mL (3.4 vs. 12 months, $p=0.04$) and VEGF greater than or equal to 465.9 pg/mL (3.9 vs. 12 months, $p=0.045$) (Table 5). Patients who had a decrease in serum MIP-1 α levels at cycle 1, day 8 compared to pre-treatment levels had significantly worse median overall survival (3.1 vs. 16 months, $p=0.014$) (Table 6).

Using Cox regression analyses, changes in natural logs of blood leukocyte levels of nuclear p65/RELA (NF- κ B) based upon EMSA between pre-treatment and cycle 1, day 8 correlated with a decreased median PFS (hazard ratio 2.17, $p=0.04$; 95 % CI [1.05, 4.49]). Changes in natural logs of serum levels of MIP-1 α between pre-treatment and cycle 1, day 8 correlated with an improved median OS (hazard ratio 0.62, $p=0.01$; 95% CI [0.42, 0.90]) (Table 7).

Discussion

In this trial of doxorubicin and bortezomib, the primary endpoint of objective response rate of at least 27 % in patients with advanced HCC was not met. Only 2.6 % of patients had a partial response, and 26.3 % of patients had stable disease as their best response. Low

objective response rates are common in therapeutic HCC trials and reflect the difficulty in obtaining substantial tumor responses in HCC. Contrary to the survival benefit seen in the SHARP trial, however, this study regimen failed to substantively delay tumor growth, as evidenced by the median PFS of 2.2 months, 4.5 month PFS of 28 % and median overall survival of 6.1 months. The combination of doxorubicin and bortezomib did appear to be safe, with fewer grade 3 and 4 toxicities than even seen in a phase II study of single-agent bortezomib in HCC [26]. This favorable safety profile seen with the combination of a cytotoxic such as doxorubicin and a targeted agent is consistent with that seen in other studies, including that of doxorubicin and sorafenib in HCC⁴, and is an encouraging finding in light of the need for combination therapies to provide synergism and/or mechanisms to overcome resistance in the treatment of these tumors. However, given the lack of efficacy seen with doxorubicin and bortezomib in this trial, it is possible that the dose of doxorubicin was too low to be effective, and this could have contributed to its tolerability. If so, it remains to be seen whether these agents are combinable at effective doses. Furthermore, it is possible that doxorubicin and bortezomib had antagonistic effects, limiting potential efficacy in HCC. Additional limitations to this study include its small sample size, non-randomized design and relatively large proportion (26.3 %) of unevaluable patients, lessening our ability to discern the true effects of this regimen in HCC.

Despite the lack of significant clinical activity with this combination, analysis of the pharmacodynamic markers of 26S proteasome activity in this trial demonstrated interesting findings also potentially pertinent to other tumor types treated with bortezomib. The pharmacodynamic markers analyzed in this trial were chosen on the basis of their known relationship to proteasomal inhibition, inflammation and malignancy.

Paradoxically, patients with low nuclear p65/RELA binding based upon electrophoretic mobility shift assay (EMSA) levels at the start of treatment, indicating relatively low NF- κ B activation, had relatively worse progression-free survival than those with higher levels. While the mechanism for this is unclear, perhaps this is indicative of other confounding variables, given the nonrandomized nature of our data. Indeed the low NF- κ B activity based upon EMSA is a reflection of the activation state of circulating leukocytes, while the circulating cytokine and chemokine levels are a reflection of secretion of these factors by tissues throughout the body. Moreover, NF- κ B activity in hepatocytes can be protective, based upon reports that hepatocyte specific deletion of the kinase that activates NF- κ B (IKK β) results in sustained activation of JNK1, with enhanced hepatocyte proliferation and enhanced chemical hepatocarcinogenesis [25]. However, in work performed here, NF- κ B activity is a reflection of leukocyte and not hepatocyte activity. Alternatively, patients with lower levels of NF- κ B activity would be less likely to show a response to a proteasomal inhibitor that was exhibiting its major therapeutic effect based upon inhibition of proteasomal driven NF- κ B activity. However, patients with a change in natural log of EMSA levels between pre- and post-treatment did demonstrate worse progression-free survival, consistent with the tenet that NF- κ B activation results in part through proteasomal degradation of I κ B, and this results in enhanced expression of survival factors by tumor cells [23, 24].

IL-6 is an important cytokine that helps regulate the immune and inflammatory response, and it is involved in cell growth and anti-apoptotic signaling in tumorigenesis, particularly in hepatobiliary cancers [39]. It enhances tumor angiogenesis by way of VEGF expression in different tumor types [40, 41]. In several studies, high serum IL-6 levels in patients with cancer was linked with poorer prognosis [42, 43], which is consistent with our findings of worse progression-free survival among patients with relatively high IL-6 levels at the start of therapy. Similarly, patients with high IL-8 levels at the start of our study had worse progression-free and overall survival. This is consistent with the above, especially given that IL-8 is an NF- κ B-regulated, macrophage-derived chemokine shown to promote angiogenesis [44].

While patients with high VEGF, IL-6 and IL-8 levels at the start of treatment had worse survival in our study, statistically significant changes in these levels were not seen in patients after treatment with doxorubicin and bortezomib. It had been hypothesized that decreases in these cytokine levels might be seen, given that blockade of NF- κ B signaling (as with bortezomib) is known to inhibit angiogenesis and tumorigenicity of some cancer cell lines, one mechanism being through suppression of VEGF and IL-8 expression [45]. It is possible that statistically significant cytokine level decreases were not seen due to the relatively small sample size of the trial, as well as the overall lack of clinical efficacy seen by the treatment. In contrast, levels of RANTES/CCL5, a chemokine important in inflammation and whose promoter activity is potently up-regulated by NF- κ B [46], did decrease with one cycle of doxorubicin and bortezomib, consistent with the results seen previously in the treatment of human monocyte-derived dendritic cells with bortezomib [47].

In our study, patients with high MIP-1 α /CCL3 levels at the start of therapy had worse progression-free survival than those with lower levels. It has been previously shown in a preclinical model that CCL3 is an essential contributor to hepato-cellular carcinoma progression [48], though the mechanism has not been fully elucidated, and some cancer cell-derived chemokines are alternatively known to positively and negatively regulate cancer progression [49]. Interestingly, in our study, patients with a decrease in CCL3 level with treatment had both poorer progression-free and overall survival but improved overall survival if a change in the natural log of serum CCL3 levels was seen between pre- and post-treatment.

Clearly, the mechanisms of these cytokines and chemokines must be examined further, particularly as to how they affect the tumor microenvironment and recruitment of pro-tumor or anti-tumor leukocytes to the tumor. Our study indicates that these molecules could be important indicators of the underlying pathophysiology of inflammation as it relates to cancer, as well as the mechanism of proteasomal inhibition in the treatment of certain malignancies. Given the small sample size and lack of efficacy for doxorubicin and bortezomib in this study, however, these correlative studies are hypothesis-generating and in no way conclusive. Our results do provide impetus, however, for further study of chemokines and cytokines such as these as possible prognostic and/or predictive biomarkers for tumors in which proteasomal inhibitors such as bortezomib are known to be clinically effective.

Acknowledgments

This study was conducted by the Eastern Cooperative Oncology Group (Robert L. Comis), and supported in part by Public Health Service Grants CA23318, CA66636, CA21115, CA49957, CA17145, CA116021, CA9060625, an SRCs award from the Department of Veterans Affairs, and from the National Cancer Institute, National Institutes of Health, and the Department of Health and Human Services. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute. Laboratory correlatives were supported by Public Health Service Grant 5R21 CA099269-02 from the National Cancer Institute.

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Fig. 1. Kaplan-Meier curves of progression-free and overall survival for all evaluable patients treated with doxorubicin and bortezomib

Table 1

Baseline patient demographics and treatment characteristics

Characteristic	Total (n=38)
Age, years	
Mean (SD)	57.1 (11.1)
Median (Range)	59 (23.0–76.5)
Sex [n (%)]	
Male	27 (71.1)
Female	11 (28.9)
Race [n (%)]	
Hispanic	1 (2.6)
Non-Hispanic White	27 (71.1)
Non-Hispanic Black	8 (21.1)
Other	2 (5.3)
ECOG performance status [n (%)]	
0	6 (15.8)
1	27 (71.1)
2	5 (13.2)
Child Pugh class [n (%)]	
A	28 (73.7)
B	10 (26.3)
Liver biopsy [n (%)]	
No	10 (26.3)
Yes	28 (73.7)
Prior RT [n (%)]	
No	37 (97.4)
Yes	1 (2.6)
Prior surgery [n (%)]	
No	25 (65.8)
Yes	13 (34.2)
Treatment cycles received	
Mean (SD)	3.8 (3.0)
Median (Range)	3.0 (1–12)

SD standard deviation

Table 2

Best treatment response

Best response	Total (n=38)
Best response by RECIST [n (%)]	
Complete response (CR)	0 (0.0)
Partial response (PR)	1 (2.6)
Stable disease (SD)	10 (26.3)
Clinical benefit (PR + SD)	11 (28.9)
Progressive disease (PD)	17 (44.7)
Unevaluable	10 (26.3)

Table 3

Treatment-related grade 3/4 adverse events

Adverse event	Grade 3 No. (%)	Grade 4 No. (%)
Hypertension	3 (8)	0 (0)
Glucose intolerance	1 (3)	0 (0)
Ascites	2 (5)	0 (0)
ALT elevation	1 (3)	0 (0)
Hyperglycemia	1 (3)	0 (0)
Thrombosis/embolism	0 (0)	1 (3)

Table 4

Correlative studies: pre-treatment versus cycle 1, day 8

Variable	Time point		P-value (n for each paired analysis) ^a
	Cycle 1 day 1	Cycle 1 day 8	
EMSA			0.31 (n=22)
Median (Range)	15 (0–182.8)	18 (0–98.8)	
Mean (SD)	31.4 (46.4)	25.0 (24.8)	
IL-8			0.50 (n=21)
Median (Range)	287 (0–185,961.2)	3,173.4 (0–26,336.6)	
Mean (SD)	12,721.8 (35,933.5)	6,866.6 (9,304.7)	
IL-6 (pg/ml)			0.38 (n=22)
Median (Range)	18 (1.6–24,888)	27 (1.9–2,521)	
Mean (SD)	890.3 (4,457.2)	369.8 (680)	
MGSA Gro- α (pg/ml)			0.20 (n=22)
Median (Range)	341 (42.1–20,000)	365 (81.6–2,466.9)	
Mean (SD)	1,425.7 (3,822.1)	640.0 (695)	
MIP-1 α (pg/ml)			0.64 (n=20)
Median (Range)	21 (0–18,787.5)	31 (0–4,245.9)	
Mean (SD)	910.4 (3,418.4)	432.0 (996.6)	
RANTES (pg/ml)			0.02 (n=23)
Median (Range)	47,556.1 (6,497.1–150,023.4)	34,358.6 (1,713.5–121,441.4)	
Mean (SD)	45,378.2 (31,896.1)	38,224.7 (32,094.7)	
VEGF (pg/ml)			0.11 (n=20)
Median (Range)	466 (88.8–1,424.9)	542 (124.4–1,190.6)	
Mean (SD)	564.0 (313)	604.9 (317.7)	

SD standard deviation

^aWilcoxon Signed Rank test

Table 5

Survival by pre-treatment serum levels split at median values

	Median Survival (mos)	95 % CI	Log-rank P-value
Progression-free survival			
IL-6 (pg/ml)<18.2	4.6	(2.0–15)	0.019
IL-6 (pg/ml) 18.2	2.1	(1.3–2.8)	
EMSA<15	2.1	(1.3–2.5)	0.046
EMSA 15	4.3	(2.1–9.3)	
IL-8<286.5	5.0	(2.1–15)	0.017
IL-8 286.5	2.3	(1.5–2.8)	
MGSA Gro- α (pg/ml)<340.5	2.5	(2.0–9.2)	0.674
MGSA Gro- α (pg/ml) 340.5	2.7	(1.5–4.3)	
MIP-1 α (pg/ml)<21.5	4.6	(1.9–15)	0.036
MIP-1 α (pg/ml) 21.5	2.1	(1.3–3.4)	
RANTES (pg/ml)<47,556.1	2.4	(1.6–9.2)	0.803
RANTES (pg/ml) 47,556.1	2.8	(1.3–4.3)	
VEGF (pg/ml)<465.9	5.0	(2.0–9.3)	0.139
VEGF (pg/ml) 465.9	2.3	(1.5–3.7)	
Overall survival			
IL-6 (pg/ml)<18.2	12	(4.5–19)	0.063
IL-6 (pg/ml) 18.2	3.6	(2.5–5.7)	
EMSA<15	4.4	(2.5–7.7)	0.229
EMSA 15	9.9	(3.9–21)	
IL-8<286.5	12	(4.5–19)	0.039
IL-8 286.5	3.4	(1.5–5.0)	
MGSA Gro- α (pg/ml)<340.5	9.7	(5.0–16)	0.254
MGSA Gro- α (pg/ml) 340.5	3.8	(1.5–7.9)	
MIP-1 α (pg/ml)<21.5	12	(5.0–19)	0.084
MIP-1 α (pg/ml) 21.5	3.9	(1.5–5.7)	
RANTES (pg/ml)<47,556.1	5.7	(3.4–12)	0.545
RANTES (pg/ml) 47,556.1	5.1	(2.8–14)	
VEGF (pg/ml)<465.9	12	(4.5–NR)	0.045
VEGF (pg/ml) 465.9	3.9	(2.8–7.2)	

Table 6

Survival by increase versus decrease in post-treatment serum levels

	Median (mos)	95 % CI	Log-rank p-value
Progression-free survival			
IL-6 decrease (n=10)	2.2	(0.39–3.4)	0.460
IL-6 increase (n=11)	2.8	(2.0–12)	
EMSA decrease (n=11)	3.4	(1.6–15)	0.355
EMSA increase (n=10)	2.5	(0.46–9.2)	
IL-8 decrease (n=5)	2.5	(1.5–12)	0.776
IL-8 increase (n=15)	2.1	(0.79–4.3)	
MGSA Gro- α decrease (n=7)	2.8	(1.5–15)	0.545
MGSA Gro- α increase (n=14)	2.3	(0.79–4.3)	
MIP-1 α decrease (n=8)	2.0	(0.39–2.8)	0.039
MIP-1 α increase (n=11)	4.3	(1.3–16)	
RANTES decrease (n=16)	2.4	(1.5–9.2)	0.802
RANTES increase (n=6)	2.5	(1.3–16)	
VEGF decrease (n=6)	2.4	(1.5–15)	0.346
VEGF increase (n=13)	2.8	(2.0–12)	
Overall survival			
IL-6 decrease (n=10)	4.7	(0.39–16)	0.862
IL-6 increase (n=12)	6.4	(2.5–21)	
EMSA decrease (n=12)	7.4	(3.7–19)	0.760
EMSA increase (n=10)	4.7	(0.46–.)	
IL-8 decrease (n=5)	3.1	(1.5–.)	0.747
IL-8 increase (n=16)	6.4	(2.8–16)	
MGSA Gro- α decrease (n=7)	3.1	(1.5–.)	0.583
MGSA Gro- α increase (n=15)	5.7	(0.79–14)	
MIP-1 α decrease (n=9)	3.1	(0.39–14)	0.014
MIP-1 α increase (n=11)	16	(4.5–.)	
RANTES decrease (n=17)	5.0	(1.5–19)	0.868
RANTES increase (n=6)	6.1	(2.5–.)	
VEGF decrease (n=6)	6.5	(1.5–21)	0.342
VEGF increase (n=14)	6.4	(3.0–.)	

Post-treatment=Cycle 1, day 8

Table 7

Cox regression models for survival for correlatives

	Hazard ratio	95 % CI	P-value
Overall survival			
Model 1 (n=22):			
Change in natural logs of IL-6	0.87	(0.65–1.18)	0.36
Model 2 (n=19):			
Change in natural logs of EMSA	1.20	(0.57–2.50)	0.63
Model 3 (n=17):			
Change in natural logs of IL-8	0.87	(0.61–1.23)	0.42
Model 4 (n=22):			
Change in natural logs of MGSA Gro- α	0.90	(0.45–1.80)	0.76
Model 5 (n=14):			
Change in natural logs of MIP-1 α	0.62	(0.42–0.90)	0.01
Model 6 (n=23):			
Change in natural logs of RANTES	0.79	(0.40–1.55)	0.49
Model 7 (n=20):			
Change in natural logs of VEGF	0.39	(0.10–1.60)	0.19
Progression-free survival			
Model 1 (n=21):			
Change in natural logs of IL-6	0.86	(0.65–1.14)	0.30
Model 2 (n=18):			
Change in natural logs of EMSA	2.17	(1.05–4.49)	0.04
Model 3 (n=17):			
Change in natural logs of IL-8	0.88	(0.65–1.21)	0.43
Model 4 (n=21):			
Change in natural logs of MGSA Gro- α	0.91	(0.53–1.57)	0.73
Model 5 (n=13):			
Change in natural logs of MIP-1 α	0.74	(0.53–1.02)	0.07
Model 6 (n=22):			
Change in natural logs of RANTES	0.79	(0.35–1.74)	0.55
Model 7 (n=19):			
Change in natural logs of VEGF	0.38	(0.10–1.55)	0.18