Bortezomib induces schedule-dependent modulation of gemcitabine pharmacokinetics and pharmacodynamics in non-small cell lung cancer and blood mononuclear cells

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Abstract

Bortezomib combination with gemcitabine/cisplatin in patients with advanced tumors, predominantly non-small cell lung cancer (NSCLC), showed an unexpected transient drop in the deoxycytidine plasma levels, a marker for gemcitabine activity. This study investigates the pharmacokinetic/pharmacodynamic effect of bortezomib on gemcitabine in NSCLC and peripheral blood mononuclear cells (PBMC).

Gemcitabine metabolites, including difluoro-dCTP (dFdCTP), were studied in PBMCs from bortezomib/gemcitabine/cisplatin-treated patients and from volunteers and NSCLC cells (H460 and SW1573) exposed to 4 h simultaneous or sequential treatments of gemcitabine (50 μM/L, 4 h) and bortezomib (100 nmol/L, 2 h). Gemcitabine total phosphate levels measured by liquid chromatography-tandem mass spectrometry in PBMCs from bortezomib/gemcitabine/cisplatin-treated patients were strongly reduced after 90 min (−82.2%) up to 4 h post-gemcitabine infusion compared with gemcitabine/cisplatin-treated patients. Accordingly, bortezomib/gemcitabine combinations reduced dFdCTP in PBMCs treated ex vivo. Surprisingly, differential effects were observed in NSCLC cells. dFdCTP decreased after 4 h following gemcitabine removal in H460 but continued to increase for 24 h in SW1573. However, dFdCTP significantly increased (2-fold) in both cell lines in the bortezomib—gemcitabine exposure, coinciding with a major reduction in cell growth compared with single drugs, and the highest increase of deoxycytidine kinase expression, possibly mediated via E2F-1. Bortezomib affects differently gemcitabine pharmacokinetics/pharmacodynamics in PBMCs and NSCLC cells, suggesting that PBMCs are not adequate to evaluate the anticancer activity of bortezomib/gemcitabine combinations. The bortezomib—gemcitabine/cisplatin schedule appeared a safe and active combination for the treatment of advanced NSCLC and the bortezomib—gemcitabine was the most cytotoxic combination in NSCLC cells. The increase of deoxycytidine kinase and dFdCTP might contribute to this synergistic interaction and supports its further clinical investigation. [Mol Cancer Ther 2009;8(5):1026–36]

Introduction

The ubiquitin-proteasome pathway is the major system for the degradation of intracellular proteins, including those involved in proliferation and survival of cancer cells. In recent years, the proteasome pathway has been identified as a key target for cancer therapy (1).

Bortezomib has been the first proteasome inhibitor tested in clinical trials. It potently inhibits the chymotryptic-like as well as caspase-like activity harbored in the 26S proteasome. Proteasome inhibition prevents proteolysis of polyubiquitin-tagged proteins, thus affecting signaling cascades at different cellular levels, including alteration of gene transcription, modulation of cell cycle, and induction of apoptosis (2). Thus far, bortezomib has been approved for second-line treatment of patients with multiple myeloma (3) and mantle cell lymphoma (4).

Preclinical studies have shown that bortezomib displays cytotoxic effects against a broad variety of cancer cells, including non-small cell lung cancer (NSCLC) cell lines (5). Early clinical studies showed promising activity of bortezomib monotherapy in NSCLC patients (6, 7) Disappointingly, in a recent phase II study, single-agent bortezomib showed only modest activity as second-line therapy in relapsed or refractory advanced NSCLC (8). As early studies also showed notable activity of bortezomib monotherapy in the histologic subtype of bronchioloalveolar cancer, a phase II study has been initiated in this specific patient subgroup (9).
Further preclinical studies showed a synergistic or additive effect when bortezomib was used in combination with gemcitabine (10, 12) and/or platinum compounds against different tumors (13), including NSCLC (14, 15). Inhibition of proteasome activity is hypothesized, for example, by inhibition of nuclear factor-κB activation and induction of p53 stabilization, to increase the cytotoxic effects of conventional chemotherapeutics, acting as a chemosensitizing agent.

Therefore, recent clinical studies focused on the combination of bortezomib and standard chemotherapeutic drugs (16). In several preclinical investigations, a sequence-specific interaction of bortezomib and chemotherapeutic agents was observed, suggesting that administration of chemotherapy before bortezomib increases apoptosis induction compared with chemotherapy administration after bortezomib (10, 15). Nevertheless, further data are needed to elucidate the underlying mechanism and to determine whether drug sequencing might be of clinical relevance. Furthermore, in the clinical setting, a schedule of a (twice-weekly) weekly i.v. pulse administration of bortezomib is used, compared with prolonged high-dose continuous exposure of most in vitro studies, whereas other in vivo studies did not clearly show this sequence-dependent effect (17, 18).

In our previous phase IB clinical study, bortezomib was administered immediately before gemcitabine/cisplatin infusion (19). The main plasma pharmacokinetic variables of cisplatin and gemcitabine were not affected by the addition of bortezomib. However, the endogenous deoxycytidine plasma levels showed a transient drop. As this drop was never observed before in patients treated with cisplatin/gemcitabine (20, 21), it was hypothesized to be a bortezomib-induced effect on the gemcitabine metabolism.

Gemcitabine (2′,2′-difluorodeoxycytidine) is an effective agent against NSCLC, and the cisplatin-gemcitabine combination is one of the standard regimens for advanced NSCLC (21). Gemcitabine is a prodrug that needs cellular uptake and intracellular phosphorylation to be active. The drug is phosphorylated to its mononucleotide by deoxycytidine kinase (dCK) and subsequently by nucleotide kinases to its active metabolites gemcitabine diphosphate (difluoro-dCDP) and gemcitabine triphosphate difluoro-dCTP (dFdCTP) (22). Gemcitabine has several targets. The main mechanism of action is dFdCTP incorporation into the DNA, for which it competes with deoxycytidine (dCTP). Furthermore, difluoro-dCDP inhibits its ribonucleotide reductase resulting in a decrease of competing deoxyribonucleotide pools necessary for DNA synthesis and increasing the probability of successful incorporation of gemcitabine into nucleic acids. In addition, dCTP is a feedback inhibitor of dCK and its decrease will stimulate gemcitabine phosphorylation. Other self-potentiating activities of the gemcitabine metabolites have been described, including inhibition of CTP synthetase and the inhibition of deoxycytidylate (dCMP) deaminase by dFdCTP (22).

A strong correlation has been found between the extent of dFdCTP formation, its DNA incorporation, and the inhibition of DNA synthesis (23, 24). The extent of dFdCTP accumulation in the cell was related with cell sensitivity (25), and previous pharmacokinetic studies analyzed dFdCTP content in peripheral blood mononuclear cells (PBMC) from treated patients as surrogate marker for gemcitabine (26).

The aim of this study was to investigate the interaction of bortezomib on gemcitabine pharmacokinetics and pharmacodynamics. For this purpose, we measured the active metabolite of gemcitabine, dFdCTP, and the levels of nucleoside triphosphates (NTP) in samples from the above-mentioned phase IB study, in human PBMCs, and in two NSCLC cell lines using different administration schedules.

Materials and Methods

Clinical Study

Patients, Treatment, and Pharmacokinetic Analysis. Chemotherapy-naive patients with histologically or cytologically proven NSCLC and measurable or evaluable clinical stage IIIB/IV disease, for whom gemcitabine and cisplatin were acceptable therapeutic option, were enrolled in a phase IB, open-label, dose-escalation study of two different schedules of bortezomib in combination with gemcitabine and cisplatin, increasing doses of bortezomib on days 1 and 8 (weekly schedule) or days 1, 4, 8, and 11 (twice-weekly schedule), followed by 1,000 mg/m² gemcitabine on days 1 and 8 and 70 mg/m² cisplatin on day 1 every 21 days. In particular, planned bortezomib dose levels were 1.0, 1.3, and 1.6 mg/m² for the weekly schedule and 0.7, 1.0, and 1.3 mg/m² for the twice weekly schedule.

Blood samples were drawn for pharmacokinetic analysis as described previously (22). In a limited number of patients (n = 12), PBMCs were isolated by Ficoll-Isopaque density gradient centrifugation (Pharmacia) following the manufacturer’s instructions. In these samples, dFdCTP (n = 12) and total gemcitabine phosphate (n = 6) accumulation was analyzed by either high-performance liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry method, respectively. The quantification of dFdCTP was done after extraction in cell pellets with TCA using a gradient HPLC system with a Partisphere SAX column (27). The analysis of total gemcitabine phosphate was achieved with a validated liquid chromatography-tandem mass spectrometry method (28) in samples before and after dephosphorylation with 1 unit alkaline phosphatase (Sigma). Briefly, 3 μL of 1.0 mol/L ammonium bicarbonate (Merck) and 3 μL of 0.1 mol/L ammonium acetate (pH 7.0) were added to 30 μL sample extracts. Then, 4 μL alkaline phosphatase was added, and after mixing, the samples were incubated at 37°C for 90 min in a 5% CO₂ atmosphere. At the end of the incubation, each sample was transferred to a HPLC vial inserts and 4 μL were injected in the system.

Ex vivo Studies

dFdCTP Accumulation Studies after Ex vivo Incubation of Mononuclear Cells with Gemcitabine and Bortezomib. PBMCs from healthy volunteers were exposed to both...
compounds to further investigate pharmacokinetic and pharmacodynamic interactions between gemcitabine and bortezomib. PBMCs were isolated by Ficoll-Isopaque and 2.5 × 10^6 cells were suspended in 1 mL RPMI 1640 and incubated at 37°C either with bortezomib (100 nmol/L, 2 h) followed by gemcitabine (50 μmol/L, 4 h) or with gemcitabine alone or with a 4 h simultaneous bortezomib-gemcitabine treatment. After drug exposure, cells were washed with PBS and counted and the pellets were frozen at -80°C. For retention experiments, cells were cultured for an additional 4 and 24 h in drug-free medium. Cellular nucleotides and dFdCTP were extracted with TCA and separated by HPLC as described above.

**In vitro Studies**

**Drugs and Chemicals.** Bortezomib was a gift from Millennium Pharmaceuticals and Johnson & Johnson Pharmaceutical Research & Development, whereas gemcitabine was a gift from Eli Lilly. The drugs were dissolved in DMSO and sterile water and diluted in culture medium before use. RPMI 1640, DMEM, fetal bovine serum, penicillin (50 IU/mL), and streptomycin (50 μg/mL) were from Life Technologies. All other chemicals were from Sigma.

**Cell Lines.** Cell lines were maintained as monolayer cultures in RPMI 1640 or DMEM (containing 2 mmol/L l-glutamine) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The human NSCLC cell line NCI-H460 (H460) was cultured in RPMI 1640, whereas SW1573 cells were cultured in DMEM. H460 cells originate from a patient with a large cell carcinoma. SW1573 cells originate from a bronchioalveolar carcinoma. Cells were grown in 75 cm² culture flasks (Costar) at 37°C in 5% CO₂ and 95% air and harvested with trypsin-EDTA when they were in exponential growth.

**Characterization of Cell Lines for Determinants of Drug Activity.** The NSCLC cells used in this study have been characterized previously for proteins involved in gemcitabine metabolism and bortezomib activity (29, 30). Furthermore, we evaluated genetic variations that may influence gemcitabine metabolism and sensitivity, such as the polymorphism of Lys²⁷Gln, resulting from a A→C substitution in exon 79 of the cytidine deaminase (CDA) gene, as well as the gene expression of dCK, CDA and ribonucleotide reductase subunit 1 and 2 (RRM1 and RRM2), determined by TaqMan probe-based assays (31, 32).

**Procasparase-3/9, Apaf-1, dCK, RRM1, and RRM2 protein expression was evaluated by Western blot analysis as described previously (29, 32, 33). Densitometric analysis of the images captured on the VersaDoc3000 instrument (Bio-Rad) was done with the Kontron Analysis Image software (Kontron-Elektronik).**

**CDA activity was measured in lysed cells using 250 μmol/L gemcitabine as substrate. A reverse-phase ion-pair HPLC method using an AquaC18 (Phenomenex) column with PIC-B7 (Waters Chromatography) plus 3.5% acetonitrile (pH 2.8), flow 1 mL/min, was used for quantification of the product, dFdU (31). Finally, dCK catalytic activity was determined by using a radioactive assay, with [³H]chlorodeoxyadenosine (29).**

**Cytotoxicity Studies.** The cell growth-inhibitory effect of bortezomib and gemcitabine was studied using the MTT assay. Cells were plated at 10⁴ per well in 96-well plates (Costar). After 24 h, cells were treated with bortezomib (0.001-0.5 μmol/L) or gemcitabine (0.001-100 μmol/L) for 72 h. At the end of the incubation, cells were incubated with 50 μL MTT (final concentration, 0.42 mg/mL), formazan crystals were dissolved in DMSO, and the absorbance was measured at 540 nm. Growth inhibition was expressed as the percentage of control (vehicle-treated cells) absorbance corrected for absorbance before drug addition. The IC₅₀ was calculated by nonlinear least squares curve fitting (GraphPad PRISM, Intuitive Software for Science).

**Drug Combination Studies.** Preclinical studies have shown a more efficient interaction when cytotoxic chemotherapeutic agents are given before bortezomib (15). Our studies were focused on simultaneous 4 h treatment and sequential treatments with an initial exposure to gemcitabine for 4 h followed by bortezomib for 2 h and vice versa. SW1573 and H460 cells (10⁴ per well in 96-well plates) were exposed to 50 μmol/L gemcitabine with or without 100 nmol/L bortezomib. Drug exposure was followed by culture in drug-free medium for 72 h and growth inhibition was assessed by MTT. The interaction between the drugs was evaluated using the fractional approach, in which the relative cytotoxicities of each drug were multiplied to calculate a theoretical value ("expected value"); when the experimental value was lower than the expected value, the combination was synergistic (34).

**dFdCTP Accumulation Studies in NSCLC Cells.** The accumulation of dFdCTP was measured by HPLC as described previously. SW1573 and H460 cells were seeded at a cell density of 10⁵ per well and treated as described above for combination studies. Pellets were collected at 0, 4, 6, 8, and 24 h after the beginning of drug exposure. Control pellets were collected after similar times from cells treated with gemcitabine alone.

Because altered dFdCTP accumulation might be related to altered metabolism of cofactors involved in its synthesis (27), we also measured the ribonucleotide (NTP) pools, such as ATP and UTP, which are cofactors in gemcitabine phosphorylation, whereas CTP and UTP may regulate dCK. The HPLC method also enabled the measurement of endogenous nucleotides (ATP, UTP, CTP, and GTP) in one run and thus the assurance of the different nucleotides not interfering with each other.

**Modulation of dCK Expression.** The rate-limiting step of dFdCTP synthesis is catalyzed by dCK. To evaluate the mechanisms underlying the differential effects of gemcitabine-bortezomib combination in PBMCs and NSCLC, we evaluated the modulation of dCK expression in both NSCLC cells and PBMCs. Cells were treated with gemcitabine (50 μmol/L, 4 h exposure), bortezomib (100 nmol/L, 2 h exposure), and gemcitabine-bortezomib combinations, as reported above. Cells were harvested at the end of drug treatments and protein extracts were prepared, separated, and transferred to nitrocellulose membranes as described previously (32). dCK expression was also studied at the mRNA level in the cancer.
cell lines using the PCR method described above. Finally, because previous studies have reported a binding site for the transcriptional regulator E2F-1 in the promoter of dCK (35), and proteasome inhibitors affected E2F-1 degradation (36), we also evaluated the protein expression of E2F-1 in both NSCLC cells and PBMCs.

Statistics. All experiments were done in triplicate and repeated three times. Data were expressed as mean ± SE and analyzed by Student’s t test or ANOVA followed by the Tukey’s multiple comparison, setting the level of significance at P < 0.05.

Results

Clinical Study

In the clinical trial, the addition of bortezomib did not seem to alter the plasma concentrations of gemcitabine and its metabolite dFdU compared with historical controls generated in our laboratory (20, 21). However, we observed an unexpected drop in the level of deoxycytidine in all the samples regardless of schedule type (19). The HPLC analysis showed the expected dFdCTP accumulation at the end of the gemcitabine infusion and after 60 min. However, thereafter, no dFdCTP could be detected, although in later samples dFdCTP was again detectable (Fig. 1A). Other samples from a different clinical study with gemcitabine were analyzed simultaneously and showed the expected dFdCTP profile, excluding technical errors. Because the dFdCTP levels were below detection limit of HPLC, the remaining samples from 6 of the 12 patients were analyzed with a more sensitive liquid chromatography-tandem mass spectrometry method (28). This analysis showed a significant drop in the total gemcitabine phosphate levels in the samples taken after 90 to 150 min from the start of gemcitabine infusion (Fig. 1B), similar to the data obtained with HPLC. In particular, we found a ~82.3% decrease after 90 min from the start of gemcitabine infusion. These data are in agreement with results obtained in the same samples with HPLC analysis of dFdCTP levels, which were not detectable because of the lower sensitivity of HPLC with respect to the mass spectrometry method.

Ex vivo Studies

In the ex vivo studies, we incubated PBMCs with bortezomib and gemcitabine in various schedules. This analysis showed that the dFdCTP accumulation was also significantly reduced in PBMCs treated in vitro with the gemcitabine-bortezomib combinations. The absolute values of dFdCTP after 4 and 8 h were 79.8% and 49.8% with respect to those observed after treatment with gemcitabine alone in PBMCs treated with concurrent bortezomib/gemcitabine. Similarly, the dFdCTP levels in cells treated with bortezomib→gemcitabine combination after 4 and 8 h were 61.4% and 41.1% of the values observed with gemcitabine alone. After 24 h retention, we did not find any difference among dFdCTP values in cells exposed to the two combinations and gemcitabine alone (Fig. 2A).

In vitro Studies

Characterization of the Cell Lines. H460 and SW1573 NSCLC cell lines have been used extensively for mechanistic studies of drugs commonly used in NSCLC (29, 33). First, we characterized differences between the two cell lines that might influence gemcitabine metabolism. The evaluation of CDA A79C polymorphisms revealed that SW1573 cells are homozygotes for the A allele, whereas H460 have the CC genotype. In agreement with previous data on the CDA A79C polymorphism (31), mRNA expression and enzyme activity of CDA were ~10-fold lower in SW1573 cells. The PCR analysis also showed slight differences in dCK expression between the two NSCLC cell lines, similar to the dCK activities which differed 1.4-fold, being lowest in H460 cells (Table 1). Regarding other enzymes involved in gemcitabine catabolism and activity, 5-NT expression was 7-fold lower...
in H460 cells, whereas RRM1 mRNA expression was 3-fold lower in SW1573, and RRM2 mRNA expression levels were comparable. Similarly, as shown in Fig. 3A and B, the cell lines showed no relevant differences in protein expression of the RRM2 subunit, but that for RRM1 subunit was ~2.5-fold lower in SW1573 cells. Secondly, as gemcitabine and bortezomib induce apoptotic cell death in NSCLC cells, we compared cells for differences in apoptotic machinery components. The Western blots revealed a 2-fold higher expression of procaspase-9 in H460 and a 1.5-fold higher expression of procaspase-3 and Apaf-1 in SW1573 cells (Fig. 3).

**Cytotoxicity of Gemcitabine and Bortezomib.** SW1573 was the most sensitive cell line to gemcitabine 72 h exposure, whereas H460 cells were slightly more resistant (Table 1). A concentration-dependent inhibition of cell growth was also observed after 72 h exposure to bortezomib, with IC_{50} values of 10.7 ± 1.2 and 69.0 ± 6.4 nmol/L in SW1573 and H460 cells, respectively. The sensitivity of SW1573 and H460 cells to a 4 h exposure to gemcitabine followed by 72 h incubation in drug-free medium are reported in Fig. 3C and D. The simultaneous and the sequential treatments with bortezomib significantly decreased cell growth in both cell lines. Bortezomib—gemcitabine combination was clearly more effective in H460 cells (P < 0.05 versus concurrent gemcitabine/bortezomib and gemcitabine—bortezomib schedules). However, in H460 cells, the expected growth inhibition was lower than that observed for the concurrent gemcitabine/bortezomib and gemcitabine—bortezomib combinations. In contrast, in SW1573 cells, all the schedules were more effective than expected, whereas no significant differences in cell growth inhibition were found among the three combinations.

**Figure 2.** Effect of bortezomib on dFdCTP levels in PBMCs from healthy volunteers and in NSCLC cells. Modulation of dFdCTP content in PBMCs from 9 healthy volunteers (A) and in H460 (B) and SW1573 (C) cells treated in vitro with gemcitabine-bortezomib combinations as described in Materials and Methods. For PBMCs, the average ± SE values of dFdCTP from three experiments are shown, whereas for the NSCLC cells the graphs show the dFdCTP absolute values ± SD from one representative experiment. Average ± SE values of dFdCTP after 8 h in NSCLC cells treated with gemcitabine, gemcitabine + bortezomib, gemcitabine—bortezomib, and bortezomib—gemcitabine were 5.6 ± 1.3, 4.3 ± 1.4, 3.6 ± 1.7, and 5.6 ± 1.4 pmol/μg protein in H460 and 5.7 ± 2.2, 5.4 ± 1.5, 6.7 ± 1.9, and 4.3 ± 1.4 pmol/μg protein in SW1573 cells, respectively. *, P < 0.05, significantly different from gemcitabine-treated cells.
Effects of gemcitabine and bortezomib on NTP pools. RRM1 and dCK gene expression and protein levels in H460 and SW1573 cells treated with gemcitabine and bortezomib. IC50 values have been calculated as described in Materials and Methods. dCK and CDA activity values have been calculated as pmol/h/mg protein.

Table 1. Characterization of CDA gene status, dCK, CDA, and RRM1 mRNA gene expression, dCK and CDA activity, and gemcitabine and bortezomib cytotoxicity in H460 and SW1573 cells

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<thead>
<tr>
<th></th>
<th>H460</th>
<th>SW1573</th>
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<tr>
<td>RRM1 gene expression*</td>
<td>12.9 ± 2.7</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>RRM2 gene expression*</td>
<td>13.9 ± 3.1</td>
<td>10.8 ± 1.0</td>
</tr>
<tr>
<td>5-NT gene expression*</td>
<td>9.3 ± 1.1</td>
<td>68.5 ± 3.2</td>
</tr>
<tr>
<td>dCK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene expression</td>
<td>142.7 ± 26.5</td>
<td>152.1 ± 1.2</td>
</tr>
<tr>
<td>Activity</td>
<td>3.1 ± 0.5</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>CDA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A79C polymorphism</td>
<td>CC</td>
<td>AA</td>
</tr>
<tr>
<td>Gene expression</td>
<td>24.3 ± 5.2</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Activity</td>
<td>18.6 ± 3.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>IC50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>4.9 ± 0.1</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>69.0 ± 6.4</td>
<td>10.7 ± 1.1</td>
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*Mean values calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene β-actin.

**Effect on dFdCTP Accumulation and Normal Nucleotide Pools in NSCLC Cells.** In H460 cells, we previously observed a paclitaxel-induced increase of dFdCTP (37) associated with the additive interaction of these drugs. Therefore, we measured the accumulation of dFdCTP in two different NSCLC cells exposed to gemcitabine and bortezomib in three different schedules.

The accumulation of dFdCTP after exposure to 50 μmol/L gemcitabine was comparable in SW1573 and H460 cells (2.58 versus 2.97 pmol/μg protein), and growth inhibition at that (4 h) exposure did not show substantial differences. However, a major difference was found in the retention of dFdCTP. In SW1573 cells, the dFdCTP levels continued to increase after drug removal, whereas in H460 cells dFdCTP concentrations were reduced with respect to those observed after 4 h retention (Fig. 2B and C). Surprisingly, levels of dFdCTP were significantly increased in both cell lines after bortezomib preincubation. In the SW1573 cell line, the dFdCTP accumulation in bortezomib—gemcitabine-treated cells was 1.8-fold higher with respect to dFdCTP values observed in cells treated with gemcitabine alone or with the other combinations. Moreover, in line with the synergistic effect of all the combinations in SW1573 cells, dFdCTP continued to increase in the three schedules. In contrast, in H460 cells, the dFdCTP levels were increased only after the bortezomib—gemcitabine treatment, whereas the antagonistic schedules (simultaneous exposure and gemcitabine—bortezomib combination) were associated with a decrease in dFdCTP similar to gemcitabine alone (Fig. 2B).

Because gemcitabine inhibits enzymes involved in ribonucleotide metabolism, differences in intrinsic NTP pools and changes in NTP pools during drug exposure may affect drug sensitivity (25). Therefore, we studied the NTP pool content of untreated and treated NSCLC. In agreement with previous results obtained in vitro and in patients, in both NSCLC cells, all NTP levels clearly increased after gemcitabine exposure (Table 2). The NTP pools were additionally increased by most gemcitabine-bortezomib combinations in both cell lines, although GTP pools were not changed by the gemcitabine bortezomib treatments (4 h) or (4 + 4 h) and UTP concentrations were not elevated compared with control in any of the SW1573 cell treatments, except the bortezomib (2 h)-gemcitabine (4 + 4 h) group.

**Modulation of dCK Expression.** The rate-limiting step of dFdCTP synthesis is catalyzed by dCK. To gain further insight into the mechanisms involved in regulating the interaction between gemcitabine and bortezomib, we examined changes in dCK protein expression in both H460 and SW1573 cells and PBMCs.

Representative Western blots are shown in Fig. 4A. These analyses revealed that most treatments affected dCK protein expression in cancer cells. In particular, a marked induction was detected in H460 cells after bortezomib—gemcitabine exposure, whereas the faintest bands were observed in the extracts of H460 cells treated with gemcitabine alone (Fig. 4A, top). Similar results were observed in the blots of SW1573 cells, showing relevant increase of dCK expression with respect to both control and gemcitabine-treated cells after all the gemcitabine/bortezomib combinations (Fig. 4A, middle). In contrast, no modulation of dCK protein expression was detected in PBMCs (Fig. 4A, bottom). The mean values obtained in the densitometric analysis of the Western blots are reported in Fig. 4B, showing the significant modulation of dCK expression with respect to both control and cells treated with gemcitabine alone after all the combinations in SW1573 and after the bortezomib—gemcitabine combination in H460 cells.

To further investigate the causes of the increase in dCK protein expression after drug exposure, we also evaluated dCK gene expression. Gemcitabine, bortezomib, and the drug combinations significantly enhanced dCK mRNA levels in the cancer cells as shown in Fig. 4C. In particular, the dCK mRNA levels in cells treated with the bortezomib—gemcitabine combination were significantly increased with respect to both control and cells treated with gemcitabine alone. However, the dCK mRNA induction by bortezomib was time dependent (Fig. 4C).

Finally, we studied the protein levels of E2F-1, as reported in the blots in Fig. 4D, showing a slight increase in E2F-1 protein expression in cells treated with gemcitabine and bortezomib, whereas a significant enhancement of E2F-1 was observed after bortezomib—gemcitabine combination in H460 cells (Fig. 4D, top). In contrast, no modulation of E2F-1 expression was detectable in PBMCs (Fig. 4D, bottom).

**Discussion**

The present study shows that bortezomib and gemcitabine have very different interactions and a discongruent pharmacokinetic interaction profile in PBMCs and NSCLC.

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Table 2. Effects of gemcitabine and gemcitabine-bortezomib combinations on NTP levels H460 and SW1573 NSCLC cells (pmol/10^6 cells)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control*</th>
<th>Treatments</th>
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<tr>
<td></td>
<td>Gemcitabine</td>
<td>Gemcitabine +</td>
</tr>
<tr>
<td></td>
<td>(4 h)†</td>
<td>(4 + 4 h) retention</td>
</tr>
<tr>
<td>ATP H460</td>
<td>1,136 ± 273</td>
<td>172†</td>
</tr>
<tr>
<td>SW1573 21</td>
<td>2,292 ± 557</td>
<td>114</td>
</tr>
<tr>
<td>GTP H460</td>
<td>251 ± 65</td>
<td>126†</td>
</tr>
<tr>
<td>SW1573 21</td>
<td>643 ± 162</td>
<td>99</td>
</tr>
<tr>
<td>UTP H460</td>
<td>463 ± 151</td>
<td>127†</td>
</tr>
<tr>
<td>SW1573 21</td>
<td>1,511 ± 476</td>
<td>78</td>
</tr>
<tr>
<td>CTP H460</td>
<td>202 ± 57</td>
<td>160†</td>
</tr>
<tr>
<td>SW1573 21</td>
<td>319 ± 34</td>
<td>157†</td>
</tr>
</tbody>
</table>

*Control values (mean ± SE; pmol/10^6 cells) from three independent experiments.
†Percentages with respect to control values.
†P < 0.05, significantly different from untreated cells.
concentrations in H460 cells were lower than the values reported after 8 h in all treatments groups, with the exception of the bortezomib—gemcitabine combination. Indeed, the bortezomib—gemcitabine sequence significantly increased the levels of dFdCTP and was synergistic in both cell lines.

Reports of the sequence-dependent effects of bortezomib are limited. Fahy et al. (10) showed that the optimal apoptotic effect in pancreatic cancer cells occurred with the gemcitabine—bortezomib sequence and similar results were reported in the NSCLC cell line A549 treated for 12 h to gemcitabine followed by 12 h exposure to bortezomib (15). These results suggested that the effect of bortezomib may not be the direct sensitization of cancer cells to the apoptotic effect of gemcitabine but that it may modulate the cellular response to the cytotoxic activity of gemcitabine, thereby facilitating cell death. However, the timing between the two drugs was arbitrarily chosen and it may play a critical role on the pharmacologic interaction. Indeed, after 24 h, bortezomib treatment resulted in a marked increase in the number of cells in the G2-M phase, possibly preventing cells from entering in the part of the cell cycle during which gemcitabine is more active (15).

Bortezomib is among the most potent cytotoxic compounds tested in the 60 cell lines included in the National Cancer Institute panel (2). Most preclinical studies were done with different drug concentrations with respect to those used in the clinical setting, and cells were treated for longer exposure times, 24, 48, and 72 h (10, 12, 44). In contrast, the in vitro studies in the present work were done using a schedule that may be more comparable with the clinical setting. Cells were exposed for 4 h to 50 μmol/L gemcitabine, which is the peak concentration in NSCLC patients administered a dose of 1,000 mg/m² over 30 min (32), with or without bortezomib (2 h exposure) at a

Figure 3. Protein expression of gemcitabine and apoptosis determinants and cytotoxicity of bortezomib and gemcitabine in NSCLC cells. Representative blot of at least three independent Western blot analyses done as described in Materials and Methods (A). β-Actin was included as a loading control and to calculate the mean expression values in the densitometric analysis (B). Growth-inhibitory effects of gemcitabine, bortezomib, and their combinations in H460 (C) and SW1573 (D) cells. Dashed line, expected inhibition in the combined treatment calculated by multiplying the relative cytotoxicities of each drug. *, P < 0.05, significantly different from gemcitabine-treated cells; #, P < 0.05, significantly different from gemcitabine + bortezomib and gemcitabine—bortezomib-treated cells.
concentration of 100 nmol/L, which is the peak concentration in patients receiving 1.6 mg/m² (45).

In agreement with previous studies showing an enhancement of dFdCTP in cells exposed to synergistic combinations of gemcitabine and other chemotherapeutics (37), the increase in dFdCTP was associated with a synergistic interaction of bortezomib and gemcitabine. Similarly, we observed a major increase in dCK protein expression after all the combinations in SW1573 cells and after the bortezomib→gemcitabine combination in H460 cells. In contrast, no modulation of dCK expression was observed in PBMCs. These results suggested that the modulation of dFdCTP levels and dCK expression might be used to predict the synergistic interaction of bortezomib and gemcitabine in cancer cells.

The increase in dCK expression was also observed after bortezomib exposure. Previous studies reported similar data suggesting that dCK may be considered as a stress protein, because its activity increased after exposing cells to several stress such as UV, radiation, DNA-damaging agents, and antimetabolites (46). Because protein expression was often not affected, recent studies suggested that dCK activation might be explained by post-translational regulation of dCK possibly via protein phosphorylation (47). However, the present data showed for the first time that bortezomib increased dCK mRNA and protein expression, which may affect its enzymatic activity and its role as possible marker of drug sensitivity in the clinical setting. No data are available on proteasomal degradation of dCK, whereas previous studies reported that E2F-1 degradation is mediated by the ubiquitin-proteasome pathway (36). Because the dCK promoter has a binding site for this transcriptional factor (35), we can hypothesize that the increased levels of E2F-1 following bortezomib inhibition of proteasomal degradation enhance the transcription of dCK. In agreement with this hypothesis, increased levels of E2F-1 protein and dCK mRNA were detected in the cancer cells treated with bortezomib and the
bortezomib-gemcitabine combinations. The lack of E2F-1 and dCK modulation in PBMCs might be explained by previous studies showing the tight control of E2F-1 levels in normal cells because of its important function in cell proliferation (48). In cancer cells, E2F-1 is one of the main regulators connecting checkpoints and apoptosis, and distinct expression patterns have been reported in several tumor types (49). However, the proteasome controls a plethora of survival factors in all mammalian cells analyzed to date (2). Several other mechanisms, including cell proliferation status, sensitivity to anticancer drugs, and cytokines and other factors involved in transcriptional and translational regulation as well as in protein catabolism, might play a role in the differential effects of bortezomib on gemcitabine metabolism in PBMCs and cancer cells.

Further, better defined studies are also needed to determine whether drug sequencing might be of clinical relevance. As for our clinical trial on the bortezomib and gemcitabine/cisplatin combination, we administered gemcitabine after bortezomib, achieving comparable progression-free survival and median overall survival duration compared with two recent clinical studies done with the reverse schedule (19, 50).

In conclusion, our results show that bortezomib affects differently gemcitabine pharmacokinetics and pharmacodynamics in PBMCs and NSCLC cells. Furthermore, comparing NSCLC cells, interactions are also different, reflecting the heterogeneous nature of this disease. However, the bortezomib–gemcitabine/cisplatin schedule appeared as a safe and active combination in the first-line treatment of advanced NSCLC patients, and the bortezomib–gemcitabine combination showed a synergistic interaction in both NSCLC cells. These data warrant further phase II studies on this schedule as well as on the modulation of dFdCPT levels and dCK expression as possible factors to predict the synergistic interaction of bortezomib and gemcitabine in cancer cells.

Disclosure of Potential Conflicts of Interest
G. Giaccone received financial support and speaker’s honoraria from Millennium Pharmaceuticals.

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