Bortezomib is ineffective in an orthotopic mouse model of pancreatic adenocarcinoma

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Abstract
The purpose of the present study was to evaluate the potency of the proteasome inhibitor bortezomib ± gemcitabine in vitro and in vivo in pancreatic carcinoma. It could be shown that bortezomib induced apoptosis and inhibited proliferation of pancreatic carcinoma very efficiently in vitro. In contrast, in an orthotropic pancreatic adenocarcinoma mouse model, gemcitabine treatment inhibited tumor growth, whereas bortezomib promoted it. Bortezomib-treated animals showed significantly higher tumor burden compared with gemcitabine-treated and control animals, although bortezomib was locally active and induced a decrease of proteasome activity, which was most pronounced following the simultaneous administration of gemcitabine. Also, tumor progression was not caused by immunosuppression as a result of proteasome inhibition. Interestingly, anti-CD31 staining of tumors showed that angiogenesis was significantly increased in the tumors of bortezomib-treated mice compared with the tumors of control animals. In addition, bortezomib resulted in an increase of pericytes, vascular endothelial growth factor, RGS-5, and hypoxia-inducible factor-1α in the tumor. Although this study supports efficacy of bortezomib against pancreatic carcinoma in vitro, it strongly indicates that bortezomib therapy has a significant tumor-promoting effect in vivo by induction of angiogenesis. The data are in accordance with the complete failure of bortezomib in a phase II trial for this indication. Choosing the right schedule of gemcitabine and bortezomib showed some synergistic effects, but the gain might not be big enough to compensate the potentially detrimental effects.


Introduction
Patients with carcinoma of the exocrine pancreas have an especially poor prognosis with a 5-year survival rate of <1% and a median survival of 4 to 6 months. At present, chemotherapy with gemcitabine is recommended as standard for advanced pancreatic carcinoma resulting in a median survival rate of ~6 months (1). Accordingly, new treatment modalities are worth being investigated. One of the promising approaches is inhibition of the proteasome.

The 26S proteasome is a multicatalytic intracellular protease expressed in eukaryotic cells. It is a multisubunit, cylindrical complex consisting of a 20S core catalytic component and 19S regulatory particles (2–4). The 19S particle contains polyubiquitin-binding sites and isopeptidase activity necessary for the cleavage and release of ubiquitin from the protein substrate (2, 5). It contains three active enzyme sites with trypsin-like, chymotrypsin-like, and postglutamyl peptide hydrolase-like activities (3, 5). The protein substrate is degraded progressively, resulting in the release of short peptides. The proteasome is responsible for selective degradation of intracellular proteins that are essential for cell proliferation, growth, regulation of apoptosis and transcription of genes involved in execution of key cellular functions (6). Treatment with proteasome inhibitors results in stabilization and accumulation of proteasome substrates, a phenomenon that may result in cell cycle arrest and activation of apoptotic programs (7, 8). Bortezomib inhibits chymotrypsin activity and induces apoptosis by releasing cytochrome c from mitochondria and by activating caspase-9 and c-Jun NH2-terminal kinases and the Fas-caspase-8-dependent apoptotic pathway (9). The inhibition of the transcriptional factor NF-κB activation was found to be a crucial mechanism in inducing apoptosis and overcoming resistance mechanisms (10). Also, bortezomib has been shown to inhibit angiogenesis and the IL-6-induced proliferation of myeloma cells (11). Angiogenesis is a prerequisite for tumor growth, invasion, and the development of metastases. Oxygen deprivation leading to hypoxia is a common feature of solid tumors and expression of angiogenic factors and microvessel density correlate with a poor prognosis in patients with pancreatic cancer (12–15). Therefore, strategies to inhibit angiogenesis are of special interest.

Bortezomib has been investigated in a variety of cell lines derived from solid tumors. There, growth inhibition and induction of apoptosis has been reported (7, 8, 16–32). Although there were differences among cell types, bortezomib-mediated cell death involved cell cycle arrest, alteration of antiapoptotic and/or proapoptotic factors, and activation of apoptotic caspase pathways. The activity of bortezomib in solid tumors in vivo has been evaluated in a variety of xenograft models. I.v. bortezomib at the maximal tolerated dose (MTD) of 1 mg/kg given weekly or twice weekly

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reduced tumor growth in nude mice bearing palpable prostate tumors by 50% to 80% (18, 20). Bortezomib and gemcitabine exhibited additive tumor growth inhibition in mice bearing MiaPaCa-2 pancreatic xenograft tumors (21).

The clinical feasibility of using bortezomib for treating solid malignancies has been explored in phase I and II studies (19, 22–25, 30). The phase II trial of the North Central Cancer Treatment Group investigated bortezomib monotherapy versus bortezomib plus gemcitabine in patients with metastatic pancreatic carcinoma. Patients who received bortezomib monotherapy had a median survival of just 2.4 and of 4.8 months when gemcitabine has been added (33).

The purpose of the present study was to evaluate the potency of bortezomib ± gemcitabine in vitro and in vivo in pancreatic carcinoma. In particular, the study focused on the effect of bortezomib on angiogenesis.

**Materials and Methods**

**Cell Lines and Treatment Scheme**

The pancreatic carcinoma cell lines Panc-1 and MiaPaCa, Capan-1, AsPC (DSMZ), and Panc 02 (murine pancreatic carcinoma) were cultured in RPMI 1640 with 10% FCS and incubated at 37°C and 5% CO₂ in a humidified atmosphere. Cells were treated in vitro with two cycles of either 20 nmol/L bortezomib on days 1 and 3 or with 20 μmol/L gemcitabine on day 1 or in various combinations; each cycle lasted 1 week. Cells were investigated 1 day after the last treatment.

**Determination of Apoptosis and Proliferation**

Apoptotic cells were detected using the Annexin V/propidium iodide staining kit (BD Pharmingen) according to the manufacturer’s instructions. Cells were analyzed immediately after staining. The nonradioactive proliferation assay “EZ4U” kit (Biomedica) was used according to the manufacturer’s instructions. The proliferation rate was determined after 5 h of incubation.

**Purification and Detection of the Proteasome**

Standardized lysates from cells or tumors were incubated for 30 min with proteasome 20S and incubated at 37°C for 10% FCS and incubated at 37°C and 5% CO₂ in a humidified atmosphere. Cells were treated in vitro with two cycles of either 20 nmol/L bortezomib on days 1 and 3 or with 20 μmol/L gemcitabine on day 1 or in various combinations; each cycle lasted 1 week. Cells were investigated 1 day after the last treatment.

**Determination of Proteasome Activity**

Standardized amounts of tumor lysate were loaded on μMACS columns as described above. The fluorescence-labeled peptide substrate Z-Suc-Leu-Leu-Val-Tyr-MCA (Bachem) was applied to the column at a concentration of 200 μmol/L. After 30-min incubation at 37°C, the products were eluted. Fluorescence intensity was determined at an excitation wavelength of 360 nm and emission wavelength of 465 nm in a photometer.

**Animal Model**

Male C57BL/6 mice (Charles River) were kept and treated in accordance with the principles laid down in the European Community Council Directives and approved by the local administration (reference 35-9185.81/G-125/04). Eight-week-old male mice were used for the experiments. To investigate s.c. tumors, 5 × 10⁵ Panc 02 cells in 200 μL PBS were administered s.c. in the back and tumor size was determined in two dimensions by vernier caliper.

Orthotopic injection was done as described previously (34). Therapy was started 5 days after pancreatic tumor cell inoculation. Animals were sacrificed blinded when they became moribund or developed specific signs such as ascites, scabby coat, and loss of motion. Tumor volume was measured once a week after relaparotomy using a vernier caliper and applying the formula: (length × width) / 6. Female CB/Scid/CrL mice (Charles River) were used to investigate the effect of the immune system to antitumor response.

Animals were treated with two cycles of either 325 μg/kg bortezomib i.p. in 200 μL of 0.9% NaCl on days 1 and 3 or 150 μg/g gemcitabine i.p. in 200 μL of 0.9% NaCl on day 1 or in various combinations; each cycle lasted 1 week. Control animals were treated with equal amounts of saline.

**Immunohistochemistry**

Sections (7 μm) from tumor were stained by indirect three-step immunohistochemistry using anti-rat immunoglobulin horseradish peroxidase detection kit. Tissue samples were incubated with a 1:100 dilution of a monoclonal rat anti-CD31 antibody (Pharmingen). The quantitative analysis was done by computer-assisted image analysis. For this aim, four microscopic fields with highest neovascularity were chosen by light microscope in a high resolution (×250, Leica DMRB; Leica), digitalized by a color video camera (CF 20/4DX; Kappa) to histologic images, and saved on a computer.

**Vascular Endothelial Growth Factor Determination**

Vascular endothelial growth factor (VEGF) levels were determined in sera obtained from animals sacrificed on day 21 after tumor inoculation. Amounts of VEGF were determined by using the Quantikine Immunoassay Mouse VEGF Kit (R&D Systems). The assay was done in duplicates according to the manufacturer’s instructions.

**Quantification of mRNA**

Snap-frozen tumors were homogenized and total RNA was extracted using RNeasy Mini Kit 50 (Qiagen) following the manufacturer’s instructions. An aliquot of the extracted RNA was used for cDNA generation using Transcriptor First-Strand cDNA Synthesis Kit, which generates cDNA from mRNA fragments with oligo(dT) primers in the synthesis reaction, using LightCycler FastStar DNA MasterPLUS SYBR Green I (Roche) for real-time PCR. The real-time PCR was done in LightCycler Capillaries using the LightCycler FastStar DNA MasterPLUS SYBR Green I Kit (all from Roche) and gene specific primers for RGS-5 (MWG-Biotech AG; forward-5'-GCTTTGACTTGGCCCAGAAA-3' and reverse-5'-GCTCAGCACCAGATCTATTGATTTGCT-3'). Melting curves were plotted as indicated in the LightCycler.
instructions. For quantification, we used a glyceraldehyde 3-phosphate dehydrogenase housekeeping gene set (QuantiTect Primer Assays) under the same PCR settings as described.

**Statistical Analysis**

Fisher’s exact t test, Student’s t test, and log-rank test on SPSS 11.5 were used to analyze statistical significance where appropriate. \( P < 0.05 \) was considered as significant.

**Results**

**In vitro Analysis**

Three different human cell lines (derived from primary tumor, metastases, and ascites) and the murine cell line Panc 02 were treated with bortezomib and/or gemcitabine over two cycles (2 weeks) and analyzed for proliferation rate, apoptosis induction, and proteasome activity. All treated cell lines presented a clear induction of apoptosis, which was the highest in the bortezomib-containing groups (84 ± 3% apoptosis and necrosis after gemcitabine monotherapy and at least 97 ± 5% after bortezomib treatment; \( P < 0.01 \), compared with gemcitabine monotherapy; Fig. 1A).

After two cycles of gemcitabine treatment, alive human and murine cells showed no altered proliferation rate. Two cycles of bortezomib resulted in a decreased proliferation rate (−57 ± 15% for human cells and −35 ± 4% for the murine Panc 02 cells; \( P < 0.05 \), compared with control and gemcitabine monotherapy). Two cycles of gemcitabine with one concurrent cycle of bortezomib showed a less pronounced effect (−23 ± 26% for human cells and −26 ± 12% for Panc 02 cells). The most prominent inhibition of proliferation was induced by two cycles of bortezomib with one concurrent cycle of gemcitabine (−98 ± 23% for human cells and −58 ± 17% for Panc 02 cells; \( P < 0.01 \), compared with control and gemcitabine monotherapy; Fig. 1B).

Because bortezomib acts on the chymotrypsin activity, Panc 02 cells were analyzed for chymotrypsin activity after two cycles of treatment. Gemcitabine monotherapy resulted in a moderate increase of proteasome activity (21 ± 15%), whereas bortezomib induced a decrease of proteasome activity, which was particularly pronounced when used as monotherapy (−42 ± 8% for bortezomib monotherapy with \( P < 0.001 \), −21 ± 16% after two cycles bortezomib with one concurrent cycle of gemcitabine, and −7 ± 6% after two cycles gemcitabine with one concurrent cycle bortezomib; \( P > 0.05 \); Fig. 1C). Proteasome was no longer visible in silver stains of proteasome-purified cell lysates after bortezomib treatment (data not shown).

**In vivo Analysis**

Mice were divided into groups and randomly treated with gemcitabine or bortezomib. The treatment started 5 days after tumor implantation (when the tumor was well established). In the s.c. model, gemcitabine showed the strongest antitumor effect: here, the tumor size was significantly lower than in control animals from the third week after tumor implantation onwards (37.8 ± 6.5 mm\(^2\) in control animals versus 3.6 ± 9.6 mm\(^2\) in gemcitabine treated mice; \( P < 0.05 \)). In contrast, bortezomib had no significant effect on tumor growth, although there was a trend toward a smaller tumor size compared with controls (26.3 ± 0.7 mm\(^2\); \( P > 0.05 \); Fig. 2A).

In the orthotopic model, the two regimens either using gemcitabine alone or administering two cycles of gemcitabine with one concurrent cycle of bortezomib showed the strongest antitumor effect (in week 3: 1,459 ± 269 mm\(^3\) in control animals versus 490 ± 99 mm\(^3\) in gemcitabine-treated mice versus 45 ± 0 mm\(^3\) in gemcitabine with one concurrent cycle of bortezomib treated mice; in week 4: 5,040 ± 0 mm\(^3\) in control animals versus 497 ± 172 mm\(^3\) in gemcitabine-treated mice versus 629 ± 157 mm\(^3\) in gemcitabine with one concurrent cycle of bortezomib-treated mice; \( P < 0.01 \)). The results with respect to bortezomib monotherapy were completely unexpected. Instead of getting the expected tumor growth reduction or, at worst cases, no or only a slight effect, a significant tumor growth promotion was observed. Bortezomib-treated animals had a significantly higher tumor burden than gemcitabine-treated or control animals (in week 2: 160 ± 25 mm\(^3\) in control animals versus 342 ± 112 mm\(^3\) in bortezomib-treated mice; week 3: 1,459 ± 269 mm\(^3\) in control animals versus 2,055 ± 303 mm\(^3\) in bortezomib-treated mice; \( P < 0.05 \); Fig. 2B). They also died within the first 3 weeks after tumor implantation. Mice treated with the MTD of 1 mg/kg had tumor volumes comparable with gemcitabine-treated animals (data not shown). However, severe side effects as lethal hemorrhage were observed.

Tumors of treated animals were analyzed for proteasome and proteasome activity after purification of the proteasome by 
MACS technique. Proteasome was not detectable in silver stains of purified tumor lysates from bortezomib-treated animals (Fig. 3A). Proteasome (chymotrypsin) activity was determined in tumors after two cycles of treatment. Gemcitabine monotherapy had no effect on proteasome activity (−2 ± 11%), whereas bortezomib combinations induced a decrease of proteasome activity, which was particularly pronounced when gemcitabine was administered with concurrent bortezomib (−32 ± 5% after two cycles gemcitabine with one concurrent cycle bortezomib, −28 ± 2% for bortezomib monotherapy, and −8 ± 1% after two cycles bortezomib with one concurrent cycle of gemcitabine; Fig. 3B).

**Tumor Growth in Immunodeficient Mice**

Because bortezomib was active in mice with respect to proteasome inhibition, we investigated whether the unexpected increase in tumor growth in bortezomib-treated mice could be explained by bortezomib-induced immunosuppression and loss of immunosurveillance. Therefore, murine Panc 02 cells and human MiaPaCa cells were inoculated s.c. or orthotopically in immunodeficient CB/Scid/Crl mice and treated according to the protocol. No effect of bortezomib could be documented in mice with s.c. growing tumors. Meanwhile, gemcitabine monotherapy and particularly gemcitabine in combination with bortezomib induced a clear reduction in tumor growth (Fig. 4A).
Orthotopically xenografted mice again had a significantly larger tumor volume on day of sacrifice when treated with bortezomib (13 ± 7 mm$^3$ in control animals versus 75 ± 10 mm$^3$ in bortezomib-treated animals on day 21; Fig. 4B).

Analysis of Angiogenesis

The failure to explain the observed phenomena by bortezomib-induced immunosuppression as a result of proteasome inhibition and apoptosis in immune cells led to

Figure 1. In vitro effect of bortezomib. Human and murine cell lines were treated with bortezomib ± gemcitabine and induction of apoptosis (A), proliferation rate (B), and proteasome activity (C) was determined. Mean ± SE of three independent experiments. Controls from untreated cells were set to zero. * P < 0.05.
further investigations seeking for a plausible explanation. Thus, the effect of bortezomib on angiogenesis was the next step to focus on. Interestingly, anti-CD31 immunohistochemistry showed that tumor-induced angiogenesis microvessel density was significantly less within the tumors of control animals (17 ± 1 vessels) than those treated with bortezomib (31 ± 3 vessels; P < 0.01; Fig. 5A). This was also true in xenografted mice (data not shown). To evaluate an increase of VEGF secretion as a possible cause, mice were sacrificed on day 21 after tumor inoculation and VEGF serum level was measured. Bortezomib-treated mice had a significantly higher level of serum VEGF (53 ± 1 pg/mL in control animals versus 130 ± 4 pg/mL in bortezomib treated mice versus 37 ± 4 pg/mL in gemcitabine with one concurrent cycle of bortezomib-treated animals; P < 0.05; Fig. 5B). In addition, numbers of RGS-5 mRNA copies determined by quantitative real-time PCR decreased only after the treatment with gemcitabine, whereas bortezomib alone had an inducing effect on RGS-5 mRNA (115,900 ± 33,000 copies in bortezomib-treated animals versus 40,400 ± 7,000 copies in control animals versus 16,600 ± 7,000 copies in gemcitabine-treated mice versus 51,200 ± 11,000 copies after treatment with two cycles of gemcitabine with one concurrent cycle of bortezomib; P < 0.05; Fig. 5C). Immunofluorescent staining showed that α-smooth muscle actin and platelet-derived growth factor-β were overexpressed in bortezomib-treated tumors (Supplementary Data). Additionally, it could be shown that these proangiogenic effects are tumor derived as VEGF and hypoxia-inducible factor-1α (HIF-1α) mRNA levels were much higher in tumors of bortezomib treated animals (see Supplementary Data).

Discussion

Our study showed that bortezomib induced apoptosis and inhibited proliferation in vitro. Also, bortezomib treatment resulted in a significant reduction of proteasome activity. However, in a s.c. syngeneic mouse model, it was not possible to reproduce the encouraging in vitro data. Whereas gemcitabine efficiently inhibited tumor growth inhibition, bortezomib had no significant effect on tumor size. To simulate the biological situation more precisely, the tumor cells were subsequently injected directly into the pancreas. Again, treatment with gemcitabine showed the expected tumor growth inhibition, whereas bortezomib treatment did not only result in a completely unexpected failure to inhibit tumor growth but also appeared to promote tumor progression. Bortezomib-treated animals exhibited a significantly higher tumor burden compared with gemcitabine-treated and control animals. They also died within the first 3 weeks after tumor implantation.
Interestingly, two cycles of gemcitabine with one concurrent cycle of bortezomib showed a strong antitumor effect as the tumor volume was significantly lower than in control animals.

This is in contrast to data from literature. However, nearly all animal experiments described were done with the MTD of bortezomib (1 mg/kg). We decided against this approach as the MTD for mice is much higher than MTD in humans. Therefore, bortezomib dosage was calculated from the human dose by a standard formula (35, 36); consequently, 325 µg/kg were given twice a week. As this dose was effective with regard to proteasome inhibition, the observed tumor-progressive effect could hardly be explained by too low dose. However, mice treated with the MTD showed, in our hands, also decreased tumor growth but associated with severe side effects.

Our animal data are in contrast to not only the encouraging in vivo data from other groups (21) but also from the randomized phase II trial of the North Central Cancer Treatment Group (33). They randomized patients with metastatic pancreatic carcinoma to receive 3-week cycles of either arm A (bortezomib 1.5 mg/m² i.v. bolus on days 1, 4, 8, and 11) or arm B [bortezomib 1.0 mg/m² (same as arm A otherwise) + gemcitabine 1,000 mg/m² i.v. on days 1 and 8; ref. 33]. Forty-two evaluable patients enrolled in arm A had a confirmed response rate of 0% and a median survival of 2.5 months. Twelve of 43 (28%) evaluable patients experienced at least one grade 4+ adverse event. In contrast, 39 evaluable patients enrolled in arm B had a confirmed response rate of 10% and a median survival of 4.8 months. Again, 11 of 43 (26%) evaluable patients experienced at least one grade 4+ adverse event. Unfortunately, the trial had no control arm with gemcitabine monotherapy. However, the literature data from numerous trials testing gemcitabine monotherapy show a median survival of 6.0 months for patients with metastatic pancreatic adenocarcinoma (37–39). Likewise, a casuistic reported about 2 patients with multiple...
myeloma is to mention. During bortezomib treatment, they had a very good response in the serum monoclonal protein level, but extramedullary lesions appeared in the central nervous system and s.c. (40).

The right schedule for proteasome inhibition and chemotherapy seems to be crucial. Fahy et al. reported that gemcitabine treatment of pancreatic carcinoma cells followed by bortezomib induced the greatest induction of apoptosis and long-term inhibition of cell growth (41). Bortezomib treatment led to accumulation of p21 and p27 and decreased Bcl-2; gemcitabine decreased p27, induced Bcl-2, and had no effect on p21. This observation would indicate that a major function of bortezomib in the chemosensitization of cancer cells is the blockage of the endogenous cell survival response after exposure to chemotherapy. Thus, inhibition of the 26S proteasome following chemotherapy appears to be the most effective regimen. This is in accordance to the observed antitumoral effect in mice treated with two cycles of gemcitabine with one concurrent cycle of bortezomib.

To exclude pharmacodynamic reasons for in vitro bortezomib failure, tumors of treated animals were analyzed for proteasome and proteasome activity. Proteasome was not detectable in silver stains of purified tumor lysates from bortezomib-treated animals. Gemcitabine monotherapy had no clear effect on proteasome activity, whereas bortezomib combinations induced a decrease of proteasome activity, which was strongest pronounced when gemcitabine was administered with concurrent bortezomib.

Because bortezomib was active in mice with respect to proteasome inhibition, we investigated whether the unexpected increase in tumor growth in bortezomib-treated mice could be explained by bortezomib-induced immunosuppression and failure of immunosurveillance. To exclude immunologic interactions, tumor cells were inoculated in immunodeficient mice and animals were treated according to the protocol. Again, no effect of bortezomib could be documented in mice with s.c. growing tumors, whereas gemcitabine monotherapy and especially gemcitabine in combination with bortezomib induced a clear reduction in tumor growth. Also, orthotopically xenografted immunodeficient mice had a significant larger tumor volume on day of sacrifice when treated with bortezomib.

Immunosuppression as a result of proteasome inhibition in immune cells failed to explain the observed phenomenon. Therefore, the effect of bortezomib on angiogenesis was investigated. Interestingly, anti-CD31 immunohistochemistry showed that tumor-induced angiogenesis microvessel density was significantly less within the tumors of control animals than those treated with bortezomib. This was also true in xenografted mice. Angiogenesis induced by tumor-associated oxygen deprivation is a prerequisite for tumor growth, invasion, and the development of metastases. Under hypoxic conditions, a signaling pathway involving a key oxygen response regulator, termed the HIF, is switched on. HIF is a transcription factor, which, in hypoxia, drives the induction or repression of genes controlling multiple cell functions such as angiogenesis, metabolism, invasion/metastasis, and apoptosis/survival (42). HIF is degraded after ubiquitination by the proteasome. Inhibition of the proteasome may cause accumulation of HIF, thus resulting in proangiogenic effects. Bortezomib resulted in an increase of VEGF, pericytes, and markers as RGS-5 (43, 44). Intratumoral HIF-1α and platelet-derived growth factor-β were found to be stimulated by bortezomib. The differences in tumor growth kinetics between the s.c. and the orthotopic model (small effect versus promoting effect) could be explained by the differences in the vascular microenvironment because HIF-1α has differential roles in tumor progression depending on the microenvironment of the tumor (45).

In this study, we show that bortezomib inhibits growth and induces apoptosis in pancreatic cancer cells in vitro but exhibits a significant tumor-promoting effect in an orthotopic ductal adenocarcinoma mouse model. This is in accordance with the complete failure of bortezomib in a randomized phase II trial for pancreatic adenocarcinoma. We hypothesize that the bortezomib-induced tumor progression is mediated by the induction of angiogenesis. Nonetheless, we found tumor response in animals treated with MTD and a minor synergistic effect when combining gemcitabine with bortezomib, which appeared to depend on the right treatment schedule. However, in our opinion, the gain is not big enough to deal with such a complex substance in patients with pancreatic carcinoma as variations of the treatment schedule and dose reductions as needed and recommended in the SPC might induce tumor progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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