NVP-BEZ235 as a New Therapeutic Option for Sarcomas

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Abstract

Purpose: To evaluate the in vitro and in vivo effects of NVP-BEZ235, a dual pan-phosphoinositide 3-kinase–mammalian target of rapamycin inhibitor in the three most common musculoskeletal tumors (osteosarcoma, Ewing's sarcoma, and rhabdomyosarcoma).

Experimental Design: Antiproliferative activity as well as the effects on migration and metastasis were evaluated in a panel of osteosarcoma, Ewing's sarcoma, as well as rhabdomyosarcoma cell lines. Moreover, simultaneous and sequential treatments were done in association with two of the most important conventional drugs in the treatment of sarcoma, doxorubicin and vincristine.

Results: NVPBEZ235 effectively blocked the pathway in in vitro and in vivo settings. Under the experimental conditions tested, the compound induced disease stasis, by arresting cells in G1 phase of cell cycle, without remarkable effects on apoptosis. As a consequence, to obtain the maximum exploitation of its therapeutic potential, NVP-BEZ235 has been evaluated in combination with conventional cytotoxic agents, thus showing promising efficacy with either doxorubicin and vincristine. Inhibition of the phosphoinositide 3-kinase/mammalian target of rapamycin pathway increased activation of extracellular signal-regulated kinase 1/2, likely due to the presence of autocrine circuits shifting growth factor signaling toward the mitogen-activated protein kinase pathway. This supports the combined use of NVP-BEZ235 with other small signaling inhibitors. Here, we showed synergistic effects when the compound was associated with an anti–insulin-like growth factor-I receptor tyrosine kinase inhibitor. NVP-BEZ235 also inhibited cell migration and metastasis. Combination with vincristine further potentiated the antitumoral effects.

Conclusions: NVP-BEZ235 displays the features to be considered for sarcoma therapy to potentiate the activity of other anticancer agents. The drug is currently undergoing phase I/II clinical trials in advanced cancer patients. Clin Cancer Res; 16(2); 530–40. ©2010 AACR.

Rhabdomyosarcoma, Ewing's sarcoma, and osteosarcoma, here collectively called musculoskeletal sarcomas, share several fundamental biological and clinical features. Unlike carcinomas, which affect the elderly and are preceded by a long natural history of preneoplastic lesions, musculoskeletal sarcomas arise abruptly in infants and adolescents (1, 2). The progression of carcinomas through a sequence of preneoplastic and neoplastic stages allowed the molecular definition of multiple carcinogenic events, whereas the natural history of musculoskeletal sarcomas is still mostly unknown. Genes causally involved in cancer onset and progression are being progressively turned into therapeutic targets, and a bonanza of selective inhibitors, ranging from small molecules to monoclonal antibodies, is revolutionizing the therapeutic approach to carcinomas while therapy of musculoskeletal sarcomas is substantially still firmly entrenched in conventional cytotoxic drugs. This is partly the consequence of an insufficient knowledge of relevant therapeutic targets. However, in the last years, with an increasing number of molecular pathways being actively investigated, new systemic treatments have been proposed and targeted therapies have been successfully proved for some soft tissue sarcomas (for a review, see ref. 3) or are on the horizon for others. Regarding rhabdomyosarcoma, Ewing's sarcoma and osteosarcoma preclinical data convincingly supported the development of insulin-like growth factor-I receptor (IGF-IR) inhibitors in the therapy of sarcoma (for a review, see ref. 4, 5). The antitumor and antimetastatic effects against Ewing's sarcoma and osteosarcoma xenograft models with anti-IGF-IR–targeted drugs have been shown (6–8). Recently,
Translational Relevance

Data show a broad antiproliferative activity of the new dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 on a panel of sarcoma cell lines. The inhibitor induced cytostatic effects, with no induction of apoptosis. As a consequence, to obtain the maximum exploitation of its therapeutic potential, NVP-BEZ235 has been evaluated in combination with conventional cytotoxic agents, thus demonstrating promising efficacy with either doxorubicin and vincristine. NVP-BEZ235 also inhibited cell migration and metastasis. Combination with vincristine further potentiates the antmitotic effects. In addition, the drug may be of interest also in prevention of resistance to some new targeted drugs, such as the anti–insulin-like growth factor-IR HAbs and the allosteric and specific mTORC1 inhibitor (e.g., rapamycin and its analogues).

phase I clinical studies with human neutralizing antibodies to IGF-IR showed stabilization of disease (9, 10), and three of eight patients with Ewing's sarcoma treated with R1507, a humanized monoclonal antibody to IGF-IR, developed partial response to treatment in a phase I trial.7 Thus, phase II trials of IGF-IR inhibitors in Ewing's sarcoma, osteosarcoma, rhabdomyosarcoma, and other sarcomas are under way. In these trials, neutralizing antibodies to IGF-IR have been generally preferred. However, the small molecular mass inhibitors maintain several potential advantages, including easier administration and potential higher flexibility in combination therapy. Because most of the IGF-IR actions are mediated by the activation of phosphatidylinositol-3 kinase 10-Akt and mitogen-activated protein kinase (MAPK) pathways, a possible alternative to the use of specific small molecular mass tyrosine kinase IGF-IR inhibitors is represented by the new generation of phosphoinositide 3-kinase (PI3K) or MAPK pathway modulators that mostly overcome earlier problems of poor selectivity, unfavorable pharmacokinetic profiles, and unacceptable toxicity (for a review, see refs. 11–13). A number of these agents have entered early-phase clinical trials. NVP-BEZ235 is a synthetic small molecular mass compound belonging to the class of imidazoquinolines that potently and reversibly inhibits class 1 PI3K catalytic activity by competing at its ATP-binding site. NVP-BEZ235 also inhibits mammalian target of rapamycin (mTOR) catalytic activity (14), and this makes the compound particularly interesting for sarcomas (15). The mTOR signaling pathway is abnormally activated in many human tumors that have multiple mTOR alterations both upstream and downstream, leading to its dysregulation. In rhabdomyosarcoma, high levels of phosphorylation in several components of the pathway, such as Akt, 4E-BP1, S6K1, and elf-4G, and low levels of 4E-BP1 expression are associated with poor overall and disease-free survival (16). Thus, the mTOR pathway is considered an important therapeutic target. Currently, the allosteric and specific mTORC1 inhibitor rapamycin and its derivatives CCI-779, RAD001, AP23573 are being evaluated in cancer clinical trials. For targeted therapies of sarcomas, AP23573 has shown promising clinical efficacy and low toxicity profiles in patients (17). However, resistance to rapamycin is a frequent event and involves cross-talk with IGF-IR signaling and Akt activation (18–20). Some patients treated with rapamycin analogues showed an increase in p-Akt in tumors and this effect is thought to be one of the reasons of their still limited clinical activity (19, 21). Therefore, a dual PI3K/mTOR catalytic inhibitor, such as NVP-BEZ235, may be of great help. In this report, we show that NVP-BEZ235 is active against the three most common musculoskeletal sarcomas by inhibiting tumor cell growth, migration, and metastasis.

Materials and Methods

Cell lines. A panel of nine osteosarcoma, nine Ewing's sarcoma, and five rhabdomyosarcoma cell lines was analyzed. The osteosarcoma cell lines Saos-2, U-2OS, and MG-63 and the Ewing's sarcoma cell lines SK-ES-1, SK-N-MC, and RD-ES were all obtained from the American Type Culture Collection. The alveolar rhabdomyosarcoma cell lines SJ-RH30 and SJ-RH4 were provided by Dr. A. Rosolen (University of Padua, Padua, Italy) and Dr. D.N. Shapiro (St. Jude Children's Hospital, Memphis, TN). Ewing's sarcoma cell lines TC-71 and 6647 were kindly provided by T.J. Triche (Children's Hospital, Los Angeles, CA). All other osteosarcoma and Ewing's sarcoma cell lines were obtained from the Rizzoli laboratories and were previously described (22). The alveolar rhabdomyosarcoma cell line RMZ-RC2 (named RC2) and the embryonal rhabdomyosarcoma cell line CCA were established in the Cancer Research Section, Department of Experimental Pathology, University of Bologna, Bologna, Italy (23, 24). The RD/18 cell line is a clone, obtained at the Cancer Research Section, University of Bologna, Bologna, Italy, of the commercially available human embryonal rhabdomyosarcoma cell line RD (Flow Laboratories). Cells were routinely cultured in Iscove's modified Dulbecco's medium supplemented with 20 U/mL penicillin, 100 µg/mL streptomycin (Sigma), and 10% heat-inactivated fetal bovine serum (Lonza).

Drugs. NVP-BEZ235 and NVP-AEW541 were kindly provided by Novartis Pharma. Stock solution of this drug was prepared in DMSO and was stored at −20°C. Doxorubicin and vincristine were purchased from Sigma.

7 Benjamin R., et al., CTOS 13th annual meeting, Seattle, WA, abstract 932.
In vitro treatments with NVP-BEZ235 alone or in combination with other drugs. Cells were plated into 6-well (200,000 cells per well) or 96-well plates (2,500 cells per well) in Iscove's modified Dulbecco's medium plus 10% fetal bovine serum. After 24 h, various concentrations of NVP-BEZ235 (1 nmol/L to 10 μmol/L) were added and cells were exposed up to 72 h. Cell proliferation was evaluated either by trypan blue vital cell count or by MTT assay (Roche) according to the manufacturer's instructions. Cells were also treated with DMSO-containing medium as a control. The final concentration of DMSO in the medium was <0.001%, and it had no effect on cell growth inhibition. For combined treatments, cells were treated for 72 h with varying concentrations of doxorubicin (0.3-100 ng/mL) or vincristine (0.01-30 ng/mL), or NVP-AEW541 (30 nmol/L to 3 μmol/L), a selective tyrosine kinase inhibitor against IGF-IR (6), without (control) or with NVP-BEZ235 (50 nmol/L for TC-71 and 100 nmol/L for U-2 OS, corresponding to the dose that gives ~30% growth inhibition in these cell lines). In sequential treatments, cells were exposed to doxorubicin or vincristine for 12 h (a time that corresponds to the in vitro doubling time of these cell lines) and then to NVP-BEZ235 for 72 h. Otherwise, cells were treated for 6 h with or without NVP-BEZ235 and then with doxorubicin or vincristine for 72 h.

Analysis of cell cycle and apoptosis was done on cells treated with NVP-BEZ235 for 24 or 48 h accordingly to procedures previously described (6).

Parameters of in vitro malignancy. Anchorage-independent growth was determined as previously described (6) in 0.33% agarose (SeaPlaque, FMC BioProducts) with a 0.5% agarose overlay. The number of cells per dish was as follows: 3,300 for TC-71, SK-N-MC, 6647, RD-ES, and SKES-1; 10,000 for U-2 OS and Saos-2; and 33,000 to 100,000 for the other cell lines. Motility assay was done using Trans-well chambers (Costar) as previously described (7). In addition, cells were pretreated with NVP-BEZ235 for 12 h, were counted, and 100,000 viable cells were seeded in the upper chamber for migration analysis to rule out possible effects of drug treatment on cell vitality that might affect cell migration. The wound healing assay was done using a pi-tor against IGF-IR (6), without (control) or with NVP-BEZ235 (50 nmol/L for TC-71 and 100 nmol/L for U-2 OS, corresponding to the dose that gives ~30% growth inhibition in these cell lines). In sequential treatments, cells were exposed to doxorubicin or vincristine for 12 h (a time that corresponds to the in vitro doubling time of these cell lines) and then to NVP-BEZ235 for 72 h. Otherwise, cells were treated for 6 h with or without NVP-BEZ235 and then with doxorubicin or vincristine for 72 h.

In vivo treatments with NVP-BEZ235 alone or in combination with vincristine. Athymic Crl:CD1-Foxn1tm1 (nude) mice were purchased from Charles River Italy. BALB-Rag2<sup>−/−</sup>/γ<sup>−/−</sup> breeders were kindly provided by the Central Institute for Experimental Animals. Mice were bred in the Animal Care Facility of Section of Cancer Research, Department of Experimental Pathology, University of Bologna (Prof. Pier-Luigi Lollini), under sterile conditions. Seventeen- to 34-wk-old female BALB-Rag2<sup>−/−</sup>/γ<sup>−/−</sup> mice or 4- to 5-wk-old female nude mice were used. Tumorigenicity was determined after the s.c. injection of 5 × 10<sup>6</sup> cells in nude mice; metastatic ability was evaluated after the i.v. injection of 2 × 10<sup>6</sup> cells in BALB-Rag2<sup>−/−</sup>/γ<sup>−/−</sup> mice. For the evaluation of treatment effectiveness, mice were randomized into controls (seven animals) and treated groups (five animals/group) when tumors started to be measurable (7 d after cell inoculation). In the group treated with NVP-BEZ235 alone, each mouse orally received 25 or 50 mg/kg dissolved in NMP 10% (1-methyl-2-pyrrolidone)/PEG300 90% (Fluka) daily, 5 d weekly, for 3 to 4 wk. A group received vincristine alone i.p. (1 mg/kg/d) on days 0 and 1 of treatment. Another group received either NVP-BEZ235 and vincristine following the time schedule mentioned above. The control group was treated with NMP10%/PEG300 90% orally only. Tumor volume was calculated as π([1.346 × a × b - 0.0183 × a<sup>2</sup> × b<sup>2</sup>])<sup>3/2</sup> where a is the maximal tumor diameter and b is the tumor diameter perpendicular to a. Lung, kidney/adrenal, and liver metastases were counted using a dissection microscope. All animal experiments were authorized by the local ethics committee, and mice were treated according to institutional and European Union guidelines.

Immunohistochemistry. Avidin-biotin-peroxidase procedure was used for immunostaining, as previously described (7). For immunohistochemical detection of all the antigens, sections were pretreated with a citrate buffer solution [0.01 mol/L citric acid and 0.01 mol/L sodium citrate (pH 6.0)] in a microwave oven at 750 W for three cycles of 5 min each. The primary antibodies used were as follows: anti-phosphorylated Akt (Ser473; Cell Signaling Technology, Inc.; dilution, 1:1,000), anti-AKT (Cell Signaling Technology; dilution, 1:1,000), anti-phospho-mTOR (Ser2448; Cell Signaling Technology; dilution, 1:1,000), anti-mTOR (Cell Signaling Technology; dilution, 1:1,000), anti-extracellular signal-regulated kinase (ERK; Cell Signaling Technology; dilution, 1:2,000), anti-phospho-S6 (Ser240/244; Cell Signaling Technology; dilution, 1:3,000), anti-S6 (Cell Signaling Technology; dilution, 1:2,000), and anti-phospho-ERK (Tyr202/Tyr204; Covance, Princeton; dilution, 1:1,000). Anti-rabbit and anti-mouse antibody conjugated to horseradish peroxidase (GE Healthcare) was used as secondary antibody.
Statistical analysis. Differences among means were analyzed using a two-sided Student’s *t* test. When data were not normally distributed, the nonparametric Mann-Whitney rank-sum test was used. IC\textsubscript{50} values were calculated from linear transformation of dose-response curves. To define drug-drug interactions (in terms of synergism, additivity, or antagonism), the combination index (CI) of each two-drug treatment was calculated with

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**Fig. 1.** NVP-BEZ235 inhibits PI3K/mTOR pathway mediators. A, dose-dependent effects on PI3K/mTOR family members in TC-71 Ewing’s sarcoma, U-2OS osteosarcoma, and RD/18 rhabdomyosarcoma cells after 24 h of exposure to NVP-BEZ235. B, time course inhibitory effects of NVP-BEZ235 on PI3K/mTOR pathway members in U-2 OS cells. In A and B, phosphorylation level of Akt, mTOR, S6, and Erk1/2 was revealed by Western blotting. Total proteins were detected as controls. C, absorbance readings related to the amount of p-Akt in time course experiments. Once normalized to the amount of total protein, the phosphorylation level is directly related to the extent of activation. Data from two separate cell-based ELISA tests (three wells per test).
Fig. 2. NVP-BEZ235 inhibits proliferation but it does not induce apoptosis in sarcoma cells, independently from the basal level of p-Akt or p-mTOR. 
A, in vitro sensitivity of nine Ewing’s sarcoma, nine osteosarcoma, and five rhabdomyosarcoma cell lines to NVP-BEZ235. Cells were exposed for 72 h to different doses of the compound and IC50 doses were calculated. Columns, mean of three independent experiments; bars, SEM. 
B, constitutive activation of PI3K/mTOR pathway on a representative panel of sarcoma cell lines maintained in standard culture conditions. 
C, cell cycle analysis of NVP-BEZ235 effects after 24 h of treatment in three representative cell lines. Data as percentages of mean of two independent experiments. Percentage of cells in the G1 phase are significantly different after treatments compared with controls (P < 0.01 by Student’s t test). 
D, Annexin V Fluorescent test was used to identify cells in apoptosis or necrosis after 24 h of NVP-BEZ235 treatment. Data as percentages of mean of two independent experiments.
Fig. 3. NVP-BEZ235 inhibits tumor cell growth in anchorage-independent conditions and cell migration. A, effects of NVP-BEZ235 on sarcoma cells in anchorage-independent conditions. Columns, mean of three experiments; bars, SEM. *, $P < 0.05$; **, $P < 0.01$ by Student’s $t$ test. Representative pictures of NVP-BEZ235 effects on colony formation from RD/18 rhabdomyosarcoma cells. B, migration ability of TC-71 and U-2OS cells after treatments with NVP-BEZ235 for 18 h. Columns, mean of three experiments; bars, SEM. *, $P < 0.05$, Student’s $t$ test. C, effects of NVP-BEZ235 on cell migration, as determined by wound assay. Representative pictures after 6 and 12 h of treatments are shown.
the isobologram equation (25) by using the CalcuSyn software (Biosoft).

**Results**

**NVP-BEZ235 inhibits PI3K/mTOR signaling and efficiently affect growth, migration, and adhesion of sarcoma cells.** Dose-response experiments showed that NVP-BEZ235 was able to inhibit the phosphorylation of Ser473 Akt, Ser2448 mTOR, and Ser240/244 S6 in two representative cell lines, without significant differences among them (Fig. 1A). Time course experiments showed transient inhibition of pAkt, which progressively regained its basal phosphorylation levels, whereas mTOR and pS6 seemed to be more sensitive to the drug and remained inhibited for at least 24 h starting from the dose of 100 nmol/L.

**Fig. 4.** NVP-BEZ235 shows positive association with conventional and anti-IGF-IR drugs. A, effects of simultaneous combined or sequential treatments of NVP-BEZ235 in association with doxorubicin (DXR) or vincristine (VCR). In sequential treatments, cells were first exposed to doxorubicin and vincristine for 12 h and then to NVP-BEZ235. Isobologram analysis is shown. The individual doses of NVP-BEZ235, and doxorubicin or vincristine to achieve 90% (straight line) growth inhibition (ED90), 75% (dotted line) growth inhibition (ED75), and 50% (hyphenated line) growth inhibition (ED50) were plotted on the X- and Y-axes. CI values calculated using CalcuSyn software are represented by points above, on, or below the lines, indicating antagonism, additive, or synergy, respectively. B, isobologram analysis of combination of NVP-BEZ235 with the anti-tyrosine kinase IGF-IR inhibitor, NVP-AEW541. CI values are represented by points above, on, or below the lines, indicating antagonism, additive, or synergy, respectively.
The transitory effects on pAkt were also confirmed by cell-based ELISA assay (Fig. 1C). Inhibition of PI3K pathway induced an increased activation of pERK1/2 (Fig. 1B).

Effects of NVP-BEZ235 on cell proliferation were examined in a panel of nine Ewing’s sarcoma, nine osteosarcoma, and five rhabdomyosarcoma cell lines (Supplementary Fig. S1; Fig. 2A). Rhabdomyosarcoma was the

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**Fig. 5.** NVP-BEZ235 inhibits tumor growth and increases the effects of vincristine (VCR). A, inhibition of TC-71 tumor growth in nude mice by NVP-BEZ235 in association or not with vincristine. Mice were orally treated for 5 d weekly, once per d with NVP-BEZ235 (25 or 50 mg/kg) or with vehicle alone (controls). In combined treatments, vincristine (1 mg/kg/d i.p.) was added on days 0 and 1 of treatment [P < 0.05, Student’s t test compared with controls (CTR)]. Point, tumor size mean; bars, SEM. B, immunohistochemical evaluation of p-Akt, p-mTOR, p-Erk-1/2, and Ki-67 in controls or in tumors derived from mice treated with NVP-BEZ235 (50 mg/kg orally). Representative figures are shown (magnification, ×200). C, representative H&E-stained lung sections of controls and mice treated with NVP-BEZ235 plus vincristine (magnification, ×100 or ×200 for inserted small figures; lungs were previously perfused with black India ink to count metastases). D, representative livers and kidneys from controls and mice treated with NVP-BEZ235 plus vincristine.
most sensitive tumor (IC_{50} mean values: rhabdomyosarcoma versus Ewing’s sarcoma or versus osteosarcoma, P < 0.05; Student’s t test). However, all cells tested were potentially inhibited by NVP-BEZ235, with IC_{50} values ranging from 6 nmol/L to approximately 500 nmol/L. The differences in sensitivity did not correlate with the basal level of phosphorylation of Akt or mTOR (Spearman’s correlations in sensitivity did not correlate with the basal level of phosphorylation of Akt or mTOR (Spearman’s correlation test; Fig. 2B). Cell cycle progression was inhibited and the proportion of cells in the G_{1} phase was substantially increased after treatment with the inhibitor (P < 0.01; Student’s t test; Fig. 2C). In contrast, no induction of apoptosis was observed (Fig. 2D).

Growth inhibitory effects were confirmed in anchorage-independent conditions. NVP-BEZ235 treatment reduced both the number and the size of cell colonies in soft-agar as shown in Fig. 3A. Cell migration was also inhibited by NVP-BEZ235 (Fig. 3B and C). Effects were dose- and time-dependent.

**NVP-BEZ235 effects in combination therapy and against chemoresistant cells.** Simultaneous and sequential treatments were made in association with two of the most important conventional drugs in the treatment of sarcomas, doxorubicin and vincristine. Synergistic or additive effects were observed with both drugs (synergism: CI < 0.9; additive: 0.90 ≤ CI ≤ 1.10 according to ref. 25; Fig. 4A). In sequential treatments, chemotherapeutic agents should be administered before NVP-BEZ235 to obtain advantageous effects (Supplementary Fig. S2; Fig. 4A). The administration of NVP-BEZ235 before the chemotherapeutic agents induced subadditive effects (CI ≥ 1.1, according to ref. 25; Supplementary Fig. S3), likely because of the NVP-BEZ235–induced G_{1} blockade that reduced the inhibitory cell cycle dependent effects of vincristine and doxorubicin. Considering that Akt is only a part of the IGF-IR signaling and that inhibition of PI3K pathway induces compensatory activation of p-ERK1/2 in sarcoma cells (Fig. 1), we evaluated the possible combination of NVP-BEZ235 with more specific anti-IGF-IR–targeted drugs. Systematic analysis of relationship between NVP-BEZ235 and components of different signaling pathways, including IGF-IR–mediated pathway, was done by Maira et al. (14). In this report, we show synergistic (U-2 OS cells) or additive (TC-71 cells) effects when NVP-BEZ235 is combined with the TK inhibitor NVP-AEW541 (synergism: CI < 0.9; additive: 0.90 ≤ CI ≤ 1.10 according to ref. 25; Supplementary Fig. S2; Fig. 4B). This advantageous effect may also be due to the ability of IGF-I to revert p-Akt inhibitory activity of NVP-BEZ235 at doses as low as 50 to 100 nmol/L (Supplementary Fig. S4). Because sarcoma cells show autocrine activation of the IGF-IR pathway (26, 27) and are constantly exposed to high levels of IGF-I stored in bone tissue, simultaneous inhibition of both MAPK and PI3K pathways may be required to obtain a significant antiproliferative effect.

**Activity of NVP-BEZ235 against tumor growth and metastasis.** The antitumor activity of NVP-BEZ235 was analyzed in a xenograft model from TC-71 Ewing’s sarcoma cell line. The model is very aggressive with a latency time of 7 days after s.c. injection of 5 × 10^{6} cells. When tumors were measurable and reached an average volume of 0.1 cm^{3}, the animals were treated with two doses of NVP-BEZ235 (25 or 50 mg/kg). The inhibitor reduced tumor growth rate (Fig. 5A), but no statistical significance was obtained when tumors were treated with NVP-BEZ235 alone or with vincristine. The positive and necessary association of NVP-BEZ235 with vincristine was confirmed in vivo because only combined treatments induced a significant shrink in tumor size. No significant changes in the weight of the tumor-bearing mice were observed after treatments with NVP-BEZ235 (data not shown). Specific effects of NVP-BEZ235 on the PI3K/Akt pathway in vivo were confirmed by immunohistochemistry on tumor tissues at the end of treatments (Fig. 5B). Consistently with the cytostatic effects induced by NVP-BEZ235, KI-67 immunostaining was also reduced following treatments with the inhibitor (Fig. 5B).

Activity against metastasis was studied in RD/18 rhabdomyosarcoma experimental model because this cell line gave the broadest spectrum of metastasis (lungs, liver, and kidneys) when injected in BALB-Rag2^{−/−};γc^{−/−} mice, which

Table 1. Activity of NVP-BEZ235 against RD/18 metastasis in Rag2^{−/−};γc^{−/−} mice

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<th>Treatment</th>
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<td>50 mg/kg NVP-BEZ235</td>
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<td>VCR</td>
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Note: Number of lung and kidney/adrenal metastases were obtained using a dissection microscope. Liver metastases were uncountable so that liver weight was used as a measure of the treatment efficacy. All the treatments gave significant differences compared with controls (P < 0.05, Mann-Whitney or Student’s t test). Combined treatments gave additional benefits although no statistical significance was achieved with respect to single treatments.
lack both the recombinase activating gene 2 (Rag2) and the common cytokine receptor γ chain (γc), and show a complete absence of T, B, and natural killer response (28). NVP-BEZ235 significantly inhibited metastasis, either alone or in combination with vincristine (Table 1; Fig. 5C and D).

**Discussion**

In this study, we analyzed the therapeutic potential of the novel dual inhibitor of PI3K/mTOR NVP-BEZ235 against the most common musculoskeletal tumors (Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma). NVP-BEZ235 is an ATP competitor that reversibly reduces the kinase activity of both PI3K and mTOR in wild-type and mutated p110 (29), as well as in cells with PTEN loss (12), thus offering a valid therapeutic agent for the treatment of solid tumors with mutated PI3K.

In sarcomas, aberrant activation of PI3K is mainly due to the existence of redundant autocrine circuits rather than mutations. IGF-IR signaling is commonly activated (4), and constitutive activation of the two major signaling mediators, MAPK and PI3K, have been described (6, 30). Here, we confirmed that PI3K and mTOR are generally active in cell lines and showed the specificity of action of NVP-BEZ235 to inhibit several PI3K pathway effectors, including Akt, mTOR, and the ribosomal protein S6. Consistent with the data recently obtained in carcinoma (29), we also observed differential time course effects on Akt or mTOR and S6. Although cell exposure to NVP-BEZ235 resulted in a sustained inhibition of mTOR and S6 for at least 24 hours, the effects on pAkt are transient and the mediator regained its activity after 6 to 24 hours, depending on the dose. This effect may be due, at least partly, to the loss of negative p70 S6K-IRS1 feedback (15) on pAkt, as a consequence of the disruption of pS6 activity by NVP-BEZ235. In our hands, inhibition of the PI3K pathway induced the activation of ERK1/2 phosphorylation, likely due to the presence of redundant autocrine circuits in sarcoma cells (26), mainly IGF-IR mediated, that may shift growth factor receptor signaling on MAPK when PI3K is inhibited. Thus, concomitant inhibition of MAPK pathway should be emphasized. Accordingly, we observed synergistic effects when NVP-BEZ235 was combined with NVP-AEW541, presumably by concomitant inhibition of MAPK pathway and full suppression of IGF-I actions (6, 31), suggesting that one may achieve improved antitumor activity by targeting two components of the integrated receptor–signaling pathways.

NVP-BEZ235 blocked cell proliferation, by inducing G1 arrest, in all sarcoma cell lines (23 cell lines), independently of their basal levels of activation of the PI3K/mTOR pathway. Among the three histotypes, rhabdomyosarcoma was the most sensitive (IC_{50} values between 1-30 nmol/L), likely due to its higher sensitivity to mTOR inhibitors (15). However, the drug was active at doses lower than 1 μmol/L in all cell lines considered here and may find application in the treatment of osteosarcoma and Ewing’s sarcoma patients refractory to conventional drugs. In addition, NVP-BEZ235 may be of interest also in the prevention of resistance to some new targeted drugs, such as the allostERIC and specific mTORC1 inhibitor rapamycin and its analogues or anti–IGF-IR HAbs. In fact, recent studies have shown that rapamycin and its analogues induced feedback activation of Akt in several human cancers, including rhabdomyosarcoma (20), thus weakening antitumor activity. Cao et coworkers (30) have recently shown how the neutralizing anti–IGF-IR antibody h7C10 was initially capable of downregulating IGF-IR and inhibiting AKT pathway in sarcoma cells with elevated IGF-IR but p-AKT levels eventually recovered after tumors resumed growth in the presence of h7C10. Accordingly, the use of NVP-BEZ235 may result in a dual benefit of preventing the tumor escape seen after anti–IGF-IR treatment alone, and limiting the potential problem of mTOR-induced upregulation of AKT.

In our hands, the inhibitor induced cytostatic effects, with no induction of apoptosis. As a consequence, to obtain the maximum exploitation of its therapeutic potential, NVP-BEZ235 should be combined with conventional cytotoxic agents. Advantageous drug-drug combinations were obtained either with doxorubicin or vincristine. In combining treatments, NVP-BEZ235 should be administered simultaneously or after the chemotherapeutics to obtain maximum advantages with respect to single agents. The association with vincristine was found to be effective also in vivo, both against tumor growth and metastasis. NVP-BEZ235 alone reduced the rate of tumor growth and the number of experimental metastasis. Both effects were potentiated by vincristine. Recently, another dual PI3K/mTOR inhibitor, PI-103, was found to be active against T-cell acute lymphoblastic leukemia cells and was found to synergize with vincristine (32). The positive drug-drug combination may find a rationale in previous reports indicating that mTOR activation specifically confers resistance to chemotherapeutic agents that target the microtubules, including vincristine (33).

In conclusion, our investigation could pave the way for using dual PI3K/mTOR inhibitors to improve the therapeutic outcome of sarcoma patients. Activation of PI3K/mTOR signaling appears as a common feature of Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines. Its inhibition reduces cell proliferation, migration, and metastasis. However, as a single agent, the drug induces only disease stasis, due to the lack of apoptotic effects and increased activation of MAPK pathway. Thus, association with conventional drugs (vincristine or doxorubicin) or with other signaling inhibitors (anti–IGF-IR drugs or other specific MAPK inhibitors) is highly recommended to obtain therapeutic improvements in the cure of sarcoma patients.

**Disclosure of Potential Conflicts of Interest**

S.-M. Maira and C. Garcia-Echegut, employment and ownership interest, Novartis Pharma AG. The other authors disclosed no potential conflicts of interest.
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