Cancer Letters 344 (2014) 291–298

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

NVP-BEZ235, a dual PI3K/mTOR inhibitor, inhibits osteosarcoma cell proliferation and tumor development *in vivo* with an improved survival rate

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ARTICLE INFO

Article history:
Received 29 August 2013
Received in revised form 17 October 2013
Accepted 19 November 2013

Keywords:
PI3K Inhibitor
mTOR Inhibitor
Survival rate
Pre-clinical model
Osteosarcoma

ABSTRACT

Despite recent improvements in chemotherapy and surgery, the problem of non-response osteosarcoma to chemotherapy remains, and is a parameter that is critical for prognosis. The present work investigated the therapeutic value of NVP-BEZ235, a dual class I PI3K/mTOR inhibitor. NVP-BEZ235 inhibited osteosarcoma cell proliferation by inducing G0/G1 cell cycle arrest with no caspase activation. In murine pre-clinical models, NVP-BEZ235 significantly slowed down tumor progression and ectopic tumor bone formation with decreased numbers of $Ki67^+$ cells and reduced tumor vasculature. Finally, NVP-BEZ235 considerably improved the survival rate of mice with osteosarcoma. Taken together, the results of the present work show that NVP-BEZ235 exhibits therapeutic interest in osteosarcoma and may be a promising adjuvant drug for bone sarcomas.

1. Introduction

Primary bone tumors are part of a large family of bone tumors including malignant and benign entities [1]. Bone sarcomas are malignant primary bone tumors with a common cellular origin. Osteosarcoma, which is the most frequent malignant bone tumor, originates from mesenchymal stem cells and has a common origin with Ewing's sarcoma and chondrosarcoma [1,2]. The current standard therapeutic approach to osteosarcoma is based on preoperative chemotherapy with a cocktail of 3–4 drugs (generally adriamycin, platinum-related drugs and methotrexate) followed by delayed en-bloc wide resection for histologic assessment. After preoperative chemotherapy, patients are categorized as good and poor responders depending on the residual tumor cells in the resected tumor tissue [3]. This protocol was initially proposed by Rosen et al. at the end of the 1980s and made it possible to improve patient life expectancy and to avoid amputation of the limb in question [4]. Despite recent improvements in surgery and the development of different regimens of multi-drug chemotherapy over the last 3 decades, the long-term survival rate remains around 55–70% at 5 years according to published series [5,6].

Unfortunately, the natural history of osteosarcoma is still mostly unknown, despite the fact that considerable efforts have been made by the medical community to identify new biomarkers that will be used to predict the therapeutic response of patients prior to the initiation of therapy or to adapt the drug regimen in case of recurrent disease [7]. Nevertheless, in the last few years, various therapeutic targets have been suggested and are at the origin of customized therapies [8,9]. Drugs against protagonists of signaling pathways have been developed extensively and entered very quickly into clinical trials. Almost cell signaling induced by cytokines/growth factors involves the PI3K/mTOR pathway, small synthetic inhibitors of these targets have been produced [10–13]. However, resistance mechanisms to mTOR inhibitors and the high frequency for mutation and/or gain in function of PI3K catalytic subunits in cancer cells has led to the development of dual PI3K/mTOR inhibitors such as NVP-BEZ235 [14]. NVP-BEZ235 is an imidazol [4,5] quinolone derivative which specifically targets the ATP-binding sites of PI3K and mTOR enzymes. Although NVP-BEZ235 has shown promising therapeutic activity in carcinomas [15,16] and lymphomas [17], only a few reports are available for bone sarcomas [18,19], and, more specifically, osteosarcomas. For this report, the efficiency of NVP-BEZ235 was studied *in vitro* in 4

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http://dx.doi.org/10.1016/j.canlet.2013.11.017

human and murine cell lines and in two models (syngenic and xenogenic) of osteosarcoma where tumor growth, bone histomorphometry and histology were assessed.

2. Materials and methods

2.1. Reagent

NVP-BEZ235 was kindly provided by Pharma Novartis (Basel, Switzerland). Stock solution (10 mM) was prepared in 100% of DMSO for the *in vitro* experiments. For mice, NVP-BEZ235 was dissolved in 10% of 1-Methyl-2-pyrrolidinone (NMP; Sigma) + 90% of PEG300 (Sigma).

2.2. Cell lines and culture conditions

Human osteosarcoma MG-63 and HOS-MNNG (HOS) cells purchased from ATCC (USA) and rat osteosarcoma OSRGA cells [20] were cultured in DMEM (Lonza, Belgium) supplemented with 5% of Fetal Bovine Serum (FBS; Hyclone Perbio, France). Murine osteosarcoma POS-1 and MOS-J cells were provided by Kamijo [21] and Shultz [22] respectively and cultured in RPMI (Lonza, Belgium) supplemented with 5% of FBS.

2.3. Cell growth and viability

Two thousand cells were seeded into 96-well plates and cultured for 72 h with or without NVP-BEZ235 (1–200 nmol/L). Cell growth and viability were determined by a colorimetric assay using the XTT reagent assay kit (Roche Molecular Biochemicals, Germany). Cell viability was also determined by means of trypan blue exclusion assay; viable and non-viable cells were counted manually after 24 and 48 h of treatment.

2.4. Caspase activity

Two hundred thousand cells were seeded in 6-well plates and cultured with or without NVP-BEZ235 for 3–48 h (25 μM). Caspase activity was assessed on 10 μl of whole cell lysates using the CaspACE Assay System kit (Promega), in accordance with the manufacturer's recommendations. The results were expressed in arbitrary units, and corrected for protein concentration quantified by Bicinchoninic acid assay (Sigma). The lysate of cells treated with 1 μg/ml of Staurosporine (Invitrogen) overnight was used as the positive control.

2.5. Cell cycle analysis

Subconfluent cultures were incubated with or without 200 nM of NVP-BEZ235 for 24 h, trypsinized, washed and incubated in PBS containing 0.12% of Triton X-100, 0.12 mM of EDTA and 100 μg/ml of ribonuclease A DNase-free (Sigma). Then, 50 μg/ml of Propidium Iodide (IP; Promega) were added for 20 min. Cell cycle distribution was determined by means of flow cytometry (Cytomics FC500; Beckman Coulter), based on 2 N and 4 N DNA content, and analyzed using DNA Cell Cycle Analysis Software (Phoenix Flow System, San Diego, CA, USA).

2.6. Cell signaling analysis

Two hundred thousand cells were treated with 50–1000 nM of NVP-BEZ235 for 3 h or with 200 nM NVP-BEZ235 for 1–24 h and then lysed in RIPA buffer (150 mmol/L of NaCl, 5% of Tris, pH 7.4, 1% of NP-40, 0.25% of sodium deoxycholate, 1 mmol/L of Na_3VO_4, 0.5 mmol/L of PMSF, 10 mg/mL of leupeptin, and 10 mg/mL of aprotinin). Lysates were cleared of debris by centrifugation at 12000 $\times g$ for 10 min. Total cell lysate (40 μg), determined using the BCA kit, was run on 10% of SDS–PAGE and electrophoretically transferred to Immobilon-P membranes (Millipore). The membrane was blotted with antibodies (Supplementary Table 1) in PBS, 0.05% of Tween 20, and 3% of BSA. The membrane was probed with the secondary antibody coupled to horseradish peroxidase. Antibody binding was visualized with the enhanced chemiluminescence system (Roche Molecular Biomedicals).

2.7. In vivo experiments: mouse models of osteosarcoma

Mice (Elevages Janvier) were housed in pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. Two groups of 8 C57Bl/6J and two groups of 8 Rj:NMRI-nude mice were assigned randomly to receive either placebo [oral administration of NMP-PEG (0.1%)] or NVP-BEZ235 (oral administration, 45 mg/kg/day).

2.7.1. Syngenic model of osteoblastic osteosarcoma

Five-week-old male C57Bl/6J mice were anesthetized by inhalation of an isoflurane/air mixture (2%, 1L/min) before receiving an i.m. injection of 1×10^6 mouse MOS-J osteosarcoma cells in close proximity to the tibiae, rapidly leading to tumor growth in the soft tissue with secondary contiguous bone invasion. Tumors appeared at the injection site 8 days later and led to osteoblastic lesions reproducing the osteoblastic form of human osteosarcoma [23]

2.7.2. Xenogenic model of osteoblastic osteosarcoma

Five-week-old female Rj:NMRI-nude mice were anesthetized as previously described before an i.m. inoculation of 2×10^6 human HOS osteosarcoma cells.

The mice were sacrificed by cervical dislocation for ethical reasons when the tumor volume reached 2000 mm^3. For both models, tumor volumes were calculated by measuring two perpendicular diameters using calipers, according to the following formula: $V = 0.5 \times L \times (S)^2$, in which L and S are, respectively, the largest and the smallest perpendicular diameters as previously described [23]. The bone microarchitecture was analyzed using the high-resolution X-ray micro-computed tomography (μCT) system for small animal imaging Sky-Scan-1076 (SkyScan, Belgium). Analyses were performed at necropsy (tumor volume 2000 mm^3, according with the recommendations of the Ethical Committee). All tibiae were scanned using the same parameters (pixel size 18 μm, 50 kV, 0.5 mm aluminum filter and 0.8 degree of rotation step). Three-dimensional reconstructions were made using CTvox software (Skyscan). Two-dimensional analyses of the bone parameters were performed on 200 layers (cortical aera) using the CTan software (Skyscan). Analysis of the specimens involved the following bone measurements: tissue volume (TV, mm^3), bone volume (BV, mm^3), percentage of bone volume (BV/TV,%), tissue surface (TS, mm^2), bone surface (BS, mm^2), bone surface/bone volume ratio (BS/BV, mm^{-1}), bone surface density (BS/TV, mm^{-1}), trabecular spaces (TbSp, mm) and cortical or trabecular thickness (CTh, TbTh, mm).

2.8. Bone histology and immunohistochemistry

After euthanasia, the tibiae were preserved and fixed in 10% of PFA, decalcified with 13% of EDTA, and 0.2% of PFA in PBS using a microwave tissue processor (KOS, Mikron Instruments, USA) for 5–7 days and embedded in paraffin. 3 μm sections were cut and stained using the Masson-trichrome method [23]. For TRAP staining, serial 4 μm-thick sagittal sections were prepared and stained to identify osteoclasts following1hour of incubation in a solution composed of 1 mg/mL of naphthol AS-TR phosphate, 60 mM of NN-dimethylformamide, 100 mM of sodium tartrate, and 1 mg/mL of Fast red TR salt solution (Sigma) [24]. The staining surface was quantified using ImageJ (NIH, USA). Immunohistochemistry was carried out on 3 mm-thick deparaffinized sections as described previously. The sections were incubated with diluted goat corresponding primary antibody (Supplementary data 1) for 1 h. Positive immunostaining was quantified using Qwin software (Leica).

2.9. Statistical analysis

Each experiment was repeated independently 3 times. The results are given as a mean ± standard deviation (SD) (*in vitro* experiments) and mean ± SEM (*in vivo* experiments) and were compared using the Unpaired t test or ANOVA followed by the Bonferroni post test using Graph Pad InStat v3.02 software. Results with $p < 0.05$ were considered significant.

3. Results

3.1. NVP-BEZ235 is a powerful drug that inhibits the proliferation of osteosarcoma cells

We first assessed the ability of NVP-BEZ235 (Fig. 1A) to modulate the signaling pathway in osteosarcoma cells. As expected, NVP-BEZ235 considerably decreased the levels of both AKT and mTOR phosphorylation in a dose – (Fig. 1B) and time-dependent (Fig. 1C) manner in all the cell lines assessed, thus confirming the functional activity of NVP-BEZ235 on osteosarcoma cells. We then performed an XTT assay and manual counting of viable cells to determine the effects of NVP-BEZ235 on cell growth (Fig. 1D). After 72 h of treatment, and in a dose-dependent manner, NVP-BEZ235 had significantly inhibited the growth of all the cell lines tested with an IC_{50} ranging from 8 to 38 μM and IC_{90} from 44 to 98 μM at 72 h (Supplementary Table 2). In addition, manual counting of viable cells revealed that NVP-BEZ235 had significantly decreased the number of viable cells without inducing apoptotic cell death (data not shown). Flow cytometry investigations revealed that 200 nM of NVP-BEZ235 increased cell numbers in the