The proteasome inhibitor bortezomib inhibits the growth of canine malignant melanoma cells in vitro and in vivo

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Abstract

Canine malignant melanomas are highly aggressive and fatal neoplasms. In the present report, 21 drugs that target specific signalling pathways were screened for their growth inhibitory activity on three canine malignant melanoma cell lines. The proteasome inhibitor bortezomib inhibited the growth of these cell lines. The growth inhibitory properties of bortezomib were then examined using nine canine malignant melanoma cell lines. Bortezomib demonstrated potent growth inhibitory activity in all cell lines with calculated IC50 values of 3.5–5.6 nM.

Because suppression of the NF-κB pathway by preventing proteasomal degradation of IκB is an important mechanism of the anti-tumour activity of bortezomib, the activation status of and the effect of bortezomib on the NF-κB pathway were examined using a canine malignant melanoma cell line, CMM-1. The NF-κB pathway was constitutively activated in CMM-1 cells and bortezomib efficiently suppressed this activated pathway. Using a CMM-1 xenograft mouse model, bortezomib also significantly inhibited tumour growth via suppression of tumour cell proliferation.

Collectively, these findings suggest that bortezomib has growth inhibitory activity against canine malignant melanomas potentially through suppression of the constitutively activated NF-κB pathway. Targeted therapy using bortezomib could therefore be beneficial in the management of canine malignant melanomas.

Introduction

Canine melanomas occur most commonly in the skin, mouth, and nail bed (Smith et al., 2002). The majority of skin melanomas are benign, while the majority of oral and nail bed melanomas are highly malignant with local tissue invasion and distant metastasis (Smith et al., 2002). Because of the aggressive nature of canine malignant melanomas, chemotherapeutic agents such as platinum-based drugs have been used for treatment. Although some clinical benefit has been reported using these drugs in dogs with gross disease (Rassnick et al., 2001), in an adjuvant setting (Dank et al., 2013), and in combination with radiation therapy (Freeman et al., 2003), melanomas are generally refractory to chemotherapy (Proulx et al., 2003; Murphy et al., 2005; Brockley et al., 2012).

Immunotherapy using DNA vaccination with xenogeneic human tyrosinase has been attempted for the treatment of canine malignant melanoma with reports of potential benefit in terms of survival time, although the objective tumour response was rare (Bergman et al., 2003). Therefore, new therapeutic strategies are needed for the successful treatment of malignant melanoma in dogs.

Targeted therapy uses compounds to block the growth of tumour cells by interfering with molecular targets necessary for tumourigenesis and tumour growth. This approach has been shown to be effective in the treatment of many types of malignancies, including malignant melanomas in humans (Chapman et al., 2011; Keeffe and Bateman, 2011) and mast cell tumours in dogs and cats (Hahn et al., 2008; Isotani et al., 2008, 2010; London et al., 2009). Despite the promising potential of targeted therapy, no compound has been identified to treat canine malignant melanomas.

Bortezomib (Velcade, Janssen Pharmaceutical), a modified dipeptidyl boronic acid analogue, is a first-in-class proteasome inhibitor. It inhibits the 26S proteasome, a large protease complex that degrades ubiquitinated proteins (McConkey and Zhu, 2008). Bortezomib has been approved for the treatment of multiple myeloma and mantle cell lymphoma in humans. A possible central
mechanism of bortezomib associated with its anticancer activity is suppression of the nuclear factor-kappa B (NF-κB) signalling pathway (McConkey and Zhu, 2008; Kojima et al., 2013). NF-κB is a transcription factor that can orchestrate complex biological processes such as regulating cell proliferation, differentiation and survival, mediating inflammatory, immune, and stress responses. In most unstimulated cells, NF-κB exists in the cytoplasm in an inactive form; upon stimulation, it is activated by phosphorylation and subsequent proteasome degradation of its inhibitor protein IκB. Degradation of phosphorylated IκB by proteasomes leads to translocation of NF-κB to the nucleus, where it binds to a specific DNA sequence of several target genes to promote transcription (Karim et al., 2004). Constitutive activation of NF-κB has been shown to be crucial for the development and progression of various types of malignancies, including myelomas and lymphomas. Preventing the degradation of IκB by bortezomib suppresses the translocation of NF-κB into the nucleus, resulting in reduced cell proliferation and increased apoptosis in these tumour cells (Hideshima et al., 2001; Pham et al., 2003; An et al., 2004).

In the current study, we screened for compounds that have growth inhibitory potency in canine malignant melanoma cells using 21 drugs developed for targeted therapy in humans and dogs. On the basis of the screening, a candidate compound, bortezomib, was selected and its growth inhibitory property against malignant melanoma cells was examined in vitro and in vivo.

Materials and methods

Compounds

Compounds used for the experiment are listed in Table 1. All substances, except for vemurafenib, were purchased from LC Laboratories. Vemurafenib was purchased from Selleck. These compounds were dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM and stored at –30 °C until used.

Cell lines

Five canine oral melanoma cell lines (CMM-1, CMM-2, ChMC, KMeC, and LMeC; kindly provided by Dr. Nakagawa, University of Tokyo) and four newly established canine melanoma cell lines (OMJ, OMS, and OMK from oral melanoma; NML from kindly provided by Dr. Nakagawa, University of Tokyo) and four newly established melanoma cells was examined in vitro and in vivo.

Table 1 Compounds used for the screening.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Targets</th>
</tr>
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<tbody>
<tr>
<td>Axitinib</td>
<td>VEGFR, PDGFR, KIT</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>Proteasome</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>BCR-ABL</td>
</tr>
<tr>
<td>Canertinib</td>
<td>EGFR, HER2, ErBr-4</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>BCR-ABL, SRC family, KIT, EphA2, PDGFR</td>
</tr>
<tr>
<td>Dovitinib</td>
<td>EGFR, VEGFR, PDGFR, KIT, CSF-1R</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>EGFR</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>EGFR</td>
</tr>
<tr>
<td>Imatinib</td>
<td>BCR-ABL, PDGFR, KIT</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>EGFR, HER2</td>
</tr>
<tr>
<td>Lestaquinib</td>
<td>FLT3</td>
</tr>
<tr>
<td>Masitinib</td>
<td>KIT, PDGFR, FGF, FAK</td>
</tr>
<tr>
<td>Mubritinib</td>
<td>HER2</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>BCR-ABL, PDGFR, KIT, CSF-1R</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>VEGFR, PDGFR, FGF, KIT, ITK, LCK, CSF-1R</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>CRAF, BRAF, KIT, FLT3-3, RET, VEGFR, PDGFR</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>PDGFR, VEGFR, KIT, FLT3, CSF-1R, RET</td>
</tr>
<tr>
<td>Tandetinib</td>
<td>FLT3, KIT, PDGFR</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>VEGFR, VEGFR, RET, BRK, TIE2, EphR, SRC family</td>
</tr>
<tr>
<td>Vatalanib</td>
<td>VEGFR, PDGFR, FGF, KIT, CSF-1R</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>BRAF</td>
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Screening of compounds

Three canine melanoma lines (CMM-1, ChMC, and KMeC) suspended in cDMEM were placed in 96-well plates (2 × 10^4 cells/well) and incubated for 24 h. The medium was then replaced with cDMEM containing 0.1 μM of each compound or carrier control (DMSO; final concentration of 0.1%) and cultured for 72 h. Cell viability was evaluated using a WST-1 cell proliferation assay kit (Takara).

Cell growth inhibition assay

Nine canine melanoma cell lines (CMM-1, CMM-2, ChMC, KMeC, LMeC, OMJ, OMS, OMK, and NML) were cultured in 96-well plates (2 × 10^4 cells/well) for 24 h in cDMEM and treated with different concentrations of bortezomib (0–100 nM) for 72 h. Cell viability was then measured by a WST-1 cell proliferation assay kit (Takara) and the half maximal inhibitory concentration (IC50) of bortezomib for each cell line was calculated using the GraphPad Prism software (GraphPad Software).

Detection of phospho-IκB, IκB, and NF-κB

CMM-1 cells suspended in cDMEM were seeded in 6-well plates (2 × 10^5 cells/well). After 24 h in culture, the cells were treated with vehicle or 5 mM of bortezomib for 12 or 24 h. Cytoplasmic and nuclear extracts from both control and bortezomib-treated cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The extracts were applied to a 12% SDS–PAGE gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with rabbit anti-phospho-IκBα antibody (1:1,000, Cell Signaling Technology), rabbit anti-IκBα antibody (1:200, Santa Cruz), rabbit anti-NF-κB p65 antibody (1:200, Santa Cruz), goat anti-GAPDH antibody (1:200, Santa Cruz), or mouse anti-histone H1.0 antibody (1:500, Abcam), followed by biotin-conjugated goat anti-rabbit immunoglobulin (Invitrogen), biotin-conjugated rabbit anti-goat immunoglobulin (Invitrogen), or biotin-conjugated rabbit anti-mouse immunoglobulin (Invitrogen). After incubation of the membranes with peroxidase-conjugated streptavidin, immunoreactive bands were visualized with an enhanced chemiluminescence system (GE Healthcare) and the LAS-4000 imaging system (Fujifilm), and semi-quantified with ImageQuant TL software (Fujifilm).

In vivo tumour growth inhibition studies

In vivo experiments using mice were conducted after approval from the Committee for Animal Experimentation of the Nippon Veterinary and Life Science University (approval 11-2). CMM-1 cells maintained in cDMEM were trypsinized, washed in phosphate-buffered saline (PBS), and suspended in PBS at 1.5 × 10^7/mL. An aliquot of 200 μL PBS containing 3 × 10^5 CMM-1 cells was injected subcutaneously into one flank of 5-week-old female BALB/c nu/nu mice (n = 12, CLEA Japan). After palpable subcutaneous tumours reached 100–200 mm^3, mice were randomized to a bortezomib-treatment (n = 6) and a control (n = 6) groups. For the bortezomib-treatment group, bortezomib was administered as intravenous (IV) injections at a dose of 0.8 mg/kg in 100 μL PBS containing 4.2% DMSO (vehicle) twice weekly. The dose and injection interval were determined according to a previous report (Williamson et al., 2009). The control group received an injection of 100 μL vehicle without bortezomib.

Tumour size was measured every day during the treatment period in two dimensions using callipers and the tumour volume was estimated using the following formula: V = (L × W^2)/2 (V: volume, L: length, W: width). The first day of administration was set at Day 0 and treatment was continued until Day 22. Mice were euthanized on Day 22 and their xenograft tumours were excised. The mice then underwent necropsy and pathological examination of vital organs.

Proliferation and apoptosis analysis of tumour xenografts

Tumour xenografts were fixed in 10% buffered formalin, embedded in paraffin blocks and sections (5 μm thick) were prepared. Cell proliferation was evaluated using the mitotic and Ki67 indices. The mitotic index was measured with sections stained with haematoxylin and eosin (H&E). For the Ki67 index, sections were subjected to immunohistochemistry using a mouse anti-human Ki-67 (1:100, Dako) combined with a Dako EnVision HRP Kit. Sections were also stained using an isotype-matched mouse IgG as negative control. A terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay using an In situ Apoptosis Detection Kit (Takara) was used for detection of apoptotic cells. The mitotic, Ki67, and apoptotic indices were calculated by counting morphologically identifiable mitotic cells, Ki67 positive cells, and TUNEL positive cells, respectively, in three non-contiguous fields per section at a x400 magnification and were expressed as the percentage of positive cells/total number of cells in the same fields.

Statistical analysis

Statistical analysis was performed with an unpaired two-tailed Student t test. P = 0.05 was considered significant.
Results

Identification of bortezomib as a potent growth inhibitor of canine malignant melanoma cells in vitro

The effects of compounds on the viability of CMM-1, ChMC, and KMeC cells are shown in Fig. 1. Of the 21 drugs, bortezomib potently suppressed the growth of the three cell lines examined, while other compounds had no or minimal effect on cell growth. We thus focused on bortezomib and examined its growth inhibitory properties against nine canine malignant melanoma cell lines (CMM-1, CMM-2, ChMC, KMeC, LMeC, OMJ, OMS, OKM, and NML). Bortezomib inhibited the growth of all cell lines (Fig. 2A) with calculated IC50 values of 3.5–5.6 nM (Fig. 2B).

Constitutive activation of NF-κB and its inhibition by bortezomib in CMM-1 cells

To evaluate whether the growth inhibitory effects of bortezomib on canine malignant melanoma cells is associated with inhibition of NF-κB activation, the activation status of IkB and NF-κB in CMM-1 cells and the effect of bortezomib on their activation status was examined by western blotting. Phosphorylated IkB and NF-κB were detected in cytoplasmic and nuclear extracts, respectively, of control CMM-1 cells (Fig. 3A). Increases of both phosphorylated IkB and IkB in the cytoplasmic extract were observed following treatment with bortezomib (Fig. 3A), with a significant increase in phosphorylated IkB at 12 and 24 h (P < 0.05 vs. control cells) and in IkB at 24 h (P < 0.05 vs. control cells) (Fig. 3B). Although no obvious changes in cytoplasmic NF-κB were detected following treatment with bortezomib, NF-κB was apparently decreased by treatment with bortezomib (Fig. 3A), with a significant difference at 24 h (P < 0.05 vs. control cells) (Fig. 3B).

Tumour growth inhibition of bortezomib in vivo

The in vivo growth inhibitory activity of bortezomib against CMM-1 cells was evaluated using a xenograft mouse model (Fig. 4). Bortezomib significantly suppressed the growth of tumours after Day 4 of treatment (P < 0.01, control vs. bortezomib) (Fig. 4A). Tumours from the bortezomib-treated mice showed a significant decrease in mitotic index compared to controls (P < 0.01) (Fig. 4B). Similarly, the Ki67 index was significantly decreased in tumours excised from the bortezomib-treated mice when compared to controls (P < 0.01) (Fig. 4B). The apoptotic index was low in both bortezomib-treated and control mice (around 0.5–1.0%) and no significant difference was found between the two groups (Fig. 4B).

At necropsy and on the following pathological evaluation of vital organs, no metastasis of tumour cells was observed in both bortezomib-treated and control mice and no drug-related toxicity was identified.

Discussion

In the current study, we screened 21 oncologic drugs and identified bortezomib to be a potent growth inhibitor of canine malig-
nant melanoma cells in vitro. The IC50 values were 3.5–5.6 nM, i.e. lower than the reported therapeutic plasma ranges of bortezomib in human patients with multiple myeloma (Cmax for a 1.3 mg/m2 IV dose of approximately 200–300 nM) (Reece et al., 2011). Moreover, our IC50 values were in a range similar to the IC50 values reported for bortezomib in human multiple myeloma cell lines (IC50 of five cell lines, 1.5–30 nM) (Hideshima et al., 2001) and mantle cell lymphoma cell lines (IC50 of two cell lines, 4.9 and 6 nM) (Pérez-Galán et al., 2011), suggesting that canine malignant melanoma cells have a response to bortezomib similar to these human tumour cells.

Because the NF-κB pathway is an important target for bortezomib, we examined the activation status of the NF-κB pathway and the effect of bortezomib on this status using CMM-1 cells. Cytoplasmic IκB was phosphorylated and a high level of nuclear NF-κB was detected in control CMM-1 cells, suggesting constitutive activation of NF-κB in CMM-1 cells. Treatment of CMM-1 cells with bortezomib resulted in cytoplasmic accumulation of both phosphorylated IκB and IκB as well as a decrease in nuclear NF-κB. These changes may be linked to the inhibitory effect of bortezomib on the proteasomic degradation of phosphorylated IκB and the subsequent prevention of NF-κB nuclear translocation. Collectively, the findings suggest that the NF-κB pathway plays an important role in the growth of canine malignant melanoma cells and that its suppression by bortezomib contributes to the inhibition of tumour cell growth.

We further examined the in vivo effects of bortezomib on canine malignant melanoma cells using a CMM-1 xenograft mouse model. Bortezomib significantly inhibited tumour growth with decreased cell proliferation as evaluated by mitotic and Ki67 indices. In contrast, no difference in apoptotic index was observed between bortezomib-treated and control mice. Although bortezomib has been reported to suppress tumour growth by inducing apoptosis in a mouse xenograft model of human multiple myeloma (LeBlanc et al., 2002), our results indicate that bortezomib suppressed tumour growth in vivo due to a cytostatic, rather than an apoptosis-mediated cytotoxic effect.

The apoptosis-inducing activity of bortezomib has been attributed to alterations in the expression of several Bcl-2 family proteins in many tumour types (Fennell et al., 2008). However, as previously reported by Qin et al. (2006), bortezomib is not always sufficient to induce apoptosis in tumour cells because it may ineffectively downregulate the anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 in human melanoma cells. Similar mechanisms could explain the resistance of canine malignant melanoma cells to bortezomib-induced apoptosis. Bortezomib has been shown to result in cell cycle arrest in G1 and G2/M in many preclinical tumour models (Pham et al., 2003; Coquelle et al., 2006; Colado et al., 2008; Lioni et al., 2008). The cell cycle arrest effects of this agent could lead to cytostasis in CMM-1 cells, resulting in tumour growth inhibition in the xenograft mouse model.

In humans, approximately 50% of cutaneous melanomas possess a BRAF mutation at amino acid position 600 (V600E) and vemurafenib, which targets BRAF with this mutation, has shown potent anti-tumour activity in vitro and in the clinic (Bollag et al., 2012). Moreover, approximately 20% of mucosal, acral, and
cutaneous melanomas that originate in chronic sun-damaged skin possessed a mutated c-kit gene, preferentially in exon 11 (Curtin et al., 2006), and the inhibitory activity of the tyrosine-kinase inhibitors imatinib and dasatinib against melanomas with the c-kit mutation has been reported (Woodman et al., 2009; Carvajal et al., 2011; Guo et al., 2011).

Although vemurafenib, imatinib, and dasatinib were included in our initial screening, they did not show any growth inhibitory activity in the three selected canine malignant melanoma cell lines. For these compounds, we also examined their effect on cell growth for the six additional canine malignant melanoma lines and no inhibitory effect was observed (data not shown). This result may reflect previous findings that no mutations corresponding to the human BRAF V600 mutation were present in BRAF exon 15 in canine malignant melanoma tissues and cell lines (Shelly et al., 2005) and that no or very few missense mutations were identified in c-kit exon 11 in canine melanomas (Murakami et al., 2011; Chu et al., 2013). The entire BRAF nucleotide sequence was examined in the nine canine malignant melanoma lines and no mutation was identified. Hence, canine malignant melanoma cells appear to differ from human melanomas in terms of mechanisms of tumorigenesis/tumour growth and susceptibility to kinase inhibitors.

Conclusion

Bortezomib has potent growth inhibitory activity in canine malignant melanoma cells in vitro. In CMM-1 cells, NF-κB was constitutively activated and bortezomib inhibited this constitutive activation. Moreover, bortezomib showed efficacy in a canine malignant melanoma xenograft mouse model with inhibition of tumour cell proliferation. Canine malignant melanoma cells may be dependent on the NF-κB pathway for their growth, and suppression of this pathway by bortezomib could result in growth inhibition. Bortezomib represents a potential agent for targeted therapy of canine malignant melanoma.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

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References

Hahn, K.A., Ogilvie, G., Rusk, T., Devauchelle, P., Leblanc, A., Legendre, A., Powers, B., Leventhal, P.S., Kinet, J.P., Palmerini, F., et al., 2008. Masitinib is safe and constitutively activated and bortezomib inhibited this constitutive activation. Moreover, bortezomib showed efficacy in a canine malignant melanoma xenograft mouse model with inhibition of tumour cell proliferation. Canine malignant melanoma cells may be dependent on the NF-κB pathway for their growth, and suppression of this pathway by bortezomib could result in growth inhibition. Bortezomib represents a potential agent for targeted therapy of canine malignant melanoma.


