Canine osteosarcoma cells exhibit resistance to aurora kinase inhibitors

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Abstract

We evaluated the effect of Aurora kinase inhibitors AZD1152 and VX680 on canine osteosarcoma cells. Cytotoxicity was seen in all four cell lines; however, half-maximal inhibitory concentrations were significantly higher than in human leukaemia and canine lymphoma cells. AZD1152 reduced Aurora kinase B phosphorylation, indicating resistance was not because of failure of target recognition. Efflux mediated by ABCB1 and ABCG2 transporters is one known mechanism of resistance against these drugs and verapamil enhanced AZD1152-induced apoptosis; however, these transporters were only expressed by a small percentage of cells in each line and the effects of verapamil were modest, suggesting other mechanisms contribute to resistance. Our results indicate that canine osteosarcoma cells are resistant to Aurora kinase inhibitors and suggest that these compounds are unlikely to be useful as single agents for this disease. Further investigation of these resistance mechanisms and the potential utility of Aurora kinase inhibitors in multi-agent protocols is warranted.

Keywords

chemotherapy, comparative oncology, in vitro models, oncology, osteosarcoma

Introduction

Aurora kinases A and B are highly conserved serine/threonine protein kinases that play essential roles in eukaryotic cell mitosis.1 Aurora kinase A (AURKA) and Aurora kinase B (AURKB) have different roles, with AURKA being primarily involved in mitotic spindle assembly and AURKB involved in the spindle assembly checkpoint, correct chromosome segregation and cytokinesis.2,3 Aurora kinases also appear to be involved in tumourigenesis and may be important for malignant transformation, invasion and metastasis.1–4 Their overexpression has been documented in various human cancers and AURKB overexpression has been correlated with poor prognosis in ovarian, prostate, liver and thyroid cancers. Thus, Aurora kinases (and especially AURKB) are attractive targets for new cancer therapies.4–8 Small molecule Aurora kinase inhibitors that selectively block the ATP-binding pocket and inhibit kinase function have demonstrated efficacy in vitro and in rodent xenograft models of human cancers, and several have been evaluated in early phase clinical trials in advanced solid malignancies in humans.9–15 Aurora kinase inhibitors have also been shown to increase sensitivity of human colon cancer cells to other chemotherapeutics and to radiation.16,17 To the authors’ knowledge, there are no published data on the use of these drugs in any naturally occurring cancer in species other than humans.

Canine osteosarcoma has a high rate of metastasis and a poor prognosis.18 Recent gene expression profiling data from our laboratory showed that canine osteosarcomas could be stratified into two groups according to tumour biological behaviour.
Dogs with ‘worse’ or ‘better’ outcome were distinguished according to expression of two inversely related gene clusters. AURKA and AURKB were among the components of a highly expressed gene cluster that was linked to more aggressive behaviour of canine osteosarcomas. The remaining genes in this cluster exclusively encoded proteins that participate in mitosis, mitotic spindle assembly and chromosome segregation. When applied to human osteosarcomas this gene expression signature revealed molecularly homologous subgroups. Considering that Aurora kinases, and particularly AURKB, play central roles in mitosis, we examined the sensitivity of four canine osteosarcoma cells lines representing diverse molecular phenotypes to the cytotoxic effects of Aurora kinase inhibition. Testing the hypothesis that cultured osteosarcoma cells are sensitive to pharmacologic abrogation of Aurora kinase activity was the first step to explore development of Aurora kinase inhibitors as novel therapeutic agents for canine osteosarcoma.

Materials and Methods

Cell lines

Canine osteosarcoma cells (OSCA 8, OSCA 32, OSCA 30 and OSCA 78) were grown in DMEM (Gibco/BRL, Grand Island, NY, USA) containing 5% glucose, supplemented with 10% foetal bovine serum (Atlas Biologicals, Fort Collins, CO), 0.1% Primocin (Invivogen, San Diego, CA, USA) and 10 mM 4-[(2-hydroxyethyl)-1-piperazine ethanesulphonic acid buffer (HEPES) (Mediatech, Manassas, VA, USA) as described. CLBL1 is a canine B cell lymphoma cell line. Cells were maintained in IMDM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 20% foetal bovine serum, 2 mM L-glutamine and 0.1% Primocin. HL-60 is a human myeloid leukaemia cell line. Cells were maintained in RPMI1640 (Gibco/BRL) supplemented with 10% foetal bovine serum (Atlas Biologicals), 2-mercaptoethanol (Gibco/BRL), 10 mM HEPES, 2 mM L-glutamine, 2 mM sodium pyruvate (Mediatech) and 0.1% Primocin (Invivogen). All cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

Aurora kinase inhibitors

AZD1152-HQPA (AZD1152) and VX680 were purchased from Selleck Chemicals (Houston, TX, USA). Both drugs were reconstituted to 10 mM stock solutions in DMSO and further diluted to the desired concentration using cell culture medium at the time of application to cell cultures.

Viability assay

OSCA 8, OSCA 30, OSCA 78, CLBL-1 and HL-60 cells were incubated in 96 well plates (5000 cells per well) for 24 h before addition of AZD1152 or VX680 (0–10 μM), with 1% DMSO added to the control wells. Viability was measured 72 h later using the MTS assay for all cell lines (CellTiter Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI, USA) as recommended by the manufacturer. All measurements were done in triplicate and experiments were repeated at least three times for the osteosarcoma cells and twice for CLBL-1 and HL-60 cells. Half maximal inhibitory concentrations (IC50’s) were determined from the dose–response curve for each experiment using the equation of the line of best fit generated in Excel (Microsoft, Redmond, WA, USA). The mean and SD of 72-h IC50 were determined.

Apoptosis assays

OSCA 8 and OSCA 32 cells were seeded in 6-well plates (250 000 cells/well) and incubated for 24 h before the addition of AZD1152 or controls. AZD1152 concentrations were 1 μM (for OSCA 8) or 5 μM (for OSCA 32). AZD1152 concentrations spanned the range of half-maximal inhibition seen in the cell lines used for these experiments. The same volume of DMSO was added to negative control wells, and doxorubicin (1 μM) was used as a positive control.

For caspase activity assays, cells were incubated for 4–6 h as described above. Activity was measured using a poly-caspase detection assay (Carboxyfluorescein FLICA™ Apoptosis Detection Kit Caspase Assay, AbD Serotec, UK) as per the manufacturer’s instructions.
Loss of membrane asymmetry and integrity were measured by flow cytometry through binding of Annexin V and uptake of 7-AAD, respectively. Cells were incubated as described above for 20 h with Aurora kinase inhibitors or controls, with additional conditions where verapamil (10 μM) was added to inhibit ABC transporter proteins. Cells were harvested, suspended in solution and stained with Annexin V and 7-AAD (eBioscience, San Diego, CA, USA) per manufacturer’s recommendation. Flow cytometry was performed using a FACSCalibur cytometer (BD Immunocytometry Systems, San Jose, CA, USA) and results were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

**ABC transporter protein expression**

Adherent cells (OSCA 8, OSCA 32 and OSCA 78) were detached from culture flasks using Accutase to prevent trypsin hydrolysis of transmembrane proteins, placed in suspension, and incubated with normal mouse serum (ThermoFisher Scientific, Waltham, MA, USA) to prevent non-specific antibody binding to Fc receptors. Cells were stained using phycoerythrin (PE)-conjugated antibodies to CD243 (ABCB1) and CD338 (ABCG2) (eBioscience). Both antibodies recognize the canine homologue (BH Gorden and EB Dickerson, unpublished data). Flow cytometry was performed using a FACSCalibur cytometer (BD Immunocytometry Systems) and results were analysed using FlowJo software (Tree Star).

**Western blotting**

Cells (OSCA 8, OSCA 32 and OSCA 78) were seeded in T25 flasks (1 × 10^6/flask) and incubated for 1 h with AZD1152 at concentrations of 1 or 5 μM with or without verapamil (10 μM). DMSO was added to control flasks. Cells were harvested and lysed using 300 mM sodium chloride buffer with protease inhibitor and phosphatase inhibitor cocktails (Halt Protease Cocktail and Halt Phosphatase Cocktail, Thermo Fischer Scientific, Rockford, IL, USA), per manufacturer’s recommendations.

Protein concentration of each sample was determined using the Bradford method using a commercial protein assay per manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). For each sample, 10μg of total protein was loaded on an SDS-polyacrylamide gel (8%) and electrophoresis was performed at 90 V for 2 h using the Bio-Rad Criterion Cell System (Bio-Rad). Proteins were transferred to a nitrocellulose membrane using the Bio-Rad semi-dry system (Bio-Rad) per manufacturer’s instructions. Primary antibodies against the protein targets were as follows: 1:833 dilution of Rabbit polyclonal anti-AURKB (Acris, San Diego, CA, USA), 1:833 dilution of Rabbit polyclonal anti-AURKB pThr232 (Acris), 1:1666 dilution of Mouse monoclonal anti-β-actin (Sigma-Aldrich). Secondary antibodies were donkey anti-rabbit or donkey anti-mouse antibodies (dilution 1:10 000) conjugated to infrared dyes (Li-Cor, Lincoln, NE, USA). Proteins were visualized using the Odyssey imaging system per manufacturer’s instructions (Li-Cor). Densitometry was evaluated using ImageJ software (http://rsbweb.nih.gov/ij/)

**Gene expression**

For quantitative real-time polymerase chain reaction (qRT-PCR), RNA was isolated and purified using the RNaseasy Mini kit (Qiagen, Valencia, CA, USA) as per the manufacturer’s directions. Total RNA was quantified using the NanoDrop® spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA). Reverse transcription and elimination of genomic DNA were carried out using the QuantiTect Reverse Transcription kit (Qiagen). Concentration and quality of cDNA were determined using the NanoDrop spectrophotometer. qRT-PCR reactions were performed using the SYBR Green method (FastStart SYBR Green Master, Roche Applied Science, Indianapolis, IN, USA) as described. ABCB1 and ABCG2 primer sequences were designed using the Primer3 online software. GAPDH primer sequences have been published. Primer sequences are provided in Table 1. GAPDH was used as the reference standard for normalization and comparison, and relative levels of mRNA were established using the delta Ct relative quantification method. The variance among cell lines for GAPDH was 2 cycles (12.9–14.9), for ABCG2 it was 3.1 cycles...
(22.5–25.6) and for ABCB1 it was 5.6 cycles (21.8–27.4).

Results

Effect of Aurora kinase inhibitors on viability of cultured canine osteosarcoma cells

Both AZD1152 and VX680 inhibited viability in all four cell lines tested in a dose-dependent manner (Fig. 1). However, the concentrations required to significantly inhibit viability (i.e., to achieve >50% cell death) were higher than anticipated, based on previous data from human cancer cell lines using comparable 72-h cytotoxicity assays.9,10,22,23 The IC50 for all four cell lines was within the same order of magnitude, and each of the cell lines showed similar sensitivity to AZD1152 and to VX680 (Table 2). To confirm the apparent resistance in canine osteosarcoma cell lines, we performed the same experiment with AZD1152 in the canine lymphoma cell line CLBL1 and the human leukaemia cell line HL-60, which proved much more sensitive to AZD1152 than any of the canine osteosarcoma cell lines tested, with IC50s in the low nanomolar range (Fig. 2).

Expression of ABC transporters in canine osteosarcoma cells

One possible explanation for the apparent resistance to Aurora kinase inhibition was drug efflux by ABC transporters; previous work has shown that either ABCB1 or ABCG2 can mediate resistance against Aurora kinase inhibitors,24 thus, we evaluated expression of these transporters in our osteosarcoma cell lines. Messenger RNA for both ABCB1 and ABCG2 were detectable in each of the cell lines (Fig. 3). For the remainder of the experiments, we used OSCA 8, OSCA 32 and OSCA 78. We confirmed surface expression of ABCB1 and ABCG2 proteins by flow cytometry. The data show that <8% of the cells in each of the three cell lines expressed surface ABCB1 or ABCG2 (Fig. 4), suggesting that these proteins were unlikely to account for the observed resistance to AZD1152 and VX680.

Apoptosis in canine osteosarcoma cell lines exposed to Aurora kinase inhibitors

Caspase activation as a marker for apoptosis was evaluated in OSCA 8 and OSCA 32 (each representative of one of the previously described molecular phenotypes) after exposure to AZD1152 (1 or 5 µM) or doxorubicin (1 µM). There was a slight increase in caspase activation in cells treated with AZD1152 (Fig. 5). In contrast, doxorubicin induced greater caspase activity in both cell lines. To complement the caspase assays, Annexin V binding and 7-AAD uptake were evaluated as markers of early and late apoptosis, respectively (Fig. 6). AZD1152 or verapamil used alone only increased apoptosis by ~2% over baseline in both cell lines (up to a maximum of ~5%). AZD1152 and verapamil used in combination showed incrementally greater apoptosis (8–15%); however, this did not reach the levels seen in response to doxorubicin (>80%). Interestingly, treatment with AZD1152 resulted in an increase in mean fluorescence intensity for Annexin V binding and 7-AAD uptake in both cell lines, which seemed to be potentiated by verapamil (Table 3).

Expression of Aurora kinase B and target modulation by Aurora kinase inhibitors in canine osteosarcoma cells

Our previous studies documented that canine osteosarcoma cells generally express robust levels of AURKA and AURKB mRNA;19 however, the observed resistance to Aurora kinase inhibition might have been the result of relatively low expression of the target protein or absence of target modulation. To examine this, we evaluated expression and phosphorylation of AURKB protein in the three cell lines by immunoblotting. Our results confirmed that each of the cell lines tested expressed AURKB protein (Fig. 7). The levels of AURKB showed only modest variability among the cell lines; treatment with AZD1152 and/or verapamil did not appear to alter expression of total AURKB protein in any of the cell lines, but the levels of phospho-AURKB were reduced in OSCA 8 and OSCA 32 cells exposed to AZD1152. Active phospho-AURKB was virtually undetectable.
Table 1. Primer details for gene expression analysis by qRT-PCR

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Figure 1. Effects of AZD1152 and VX680 on viability of cultured canine osteosarcoma cells (OSCA 8, OSCA 30, OSCA 32 and OSCA 78). Cells were incubated with Aurora kinase inhibitors at concentrations of 0–10 μM for 72 h. Cell viability was determined using the MTS assay and is expressed as a fraction of viability in vehicle treated (negative control) cells and each plot point represents the mean ± SD from one representative example of three independent experiments.

in OSCA 78 cells, so predictably, no significant change was perceptible when this cell line was treated with AZD1152 (Fig. 7). Consistent with our observations on the limited role of ABCB1 and ABCG2 transporters to mediate resistance to this drug, the addition of verapamil did not further reduce phospho-AURKB expression in these cell lines.

Discussion

This study was the first step to assess the potential to include targeted inhibition of Aurora kinases in the treatment of canine osteosarcoma. Previous work in our laboratory showed a dichotomy in expression of ~150 mitosis-associated genes, including AURKA and AURKB, in canine bone tumours with differential biological behaviour.19
This finding, and the availability of small molecule Aurora kinase inhibitors, prompted the in vitro investigation described herein to assess sensitivity of canine osteosarcoma to two Aurora kinase inhibitors with favourable activity and toxicity profiles that have progressed to clinical trials in humans. We found that both a pan-Aurora kinase inhibitor (VX680) and a selective AURKB inhibitor (AZD1152) exert dose-dependent anti-proliferative effects in canine osteosarcoma cell lines. However, when compared to previously published results and to our results in human and canine haematopoietic cancer cell lines,9,10,23,26,27 all canine osteosarcoma cell lines we tested were relatively resistant to Aurora kinase inhibition, requiring micro-molar doses to reach an IC50.

The similar sensitivity of canine osteosarcoma cells to AZD1152 and VX680 fits with the previously recognized phenomenon that cells treated with pan-Aurora kinase inhibitors have a phenotype consistent with AURKB inhibition, and so those inhibitors specific for AURKB tend to be as effective as pan-Aurora kinase inhibitors.8,13,28,29

Our data show that AZD1152 can achieve effective target modulation in canine osteosarcoma
Autophosphorylation at Thr323 is indispensable for AURKB function in cytokinesis. As has been shown with another targeted small molecule inhibitor (toceranib) in canine mast cell tumours, the cell line (OSCA 78) with the lowest baseline levels of target expression and activity also was the one that showed the least target modulation. Despite evidence of effective target modulation by AZD1152, treatment using micro-molar concentrations did not lead to irreversible apoptosis of osteosarcoma cell lines. The alteration in mean fluorescence intensity for Annexin V binding and 7-AAD uptake suggests that AZD1152 might affect the metabolic machinery of the cells, reducing their capacity to retain membrane asymmetry and increasing their membrane permeability albeit not irreversibly leading to death.

Aurora kinases are considered necessary for normal cell division, and it may be that despite significant reduction in AURKB activity (based on reduction of autophosphorylation), there remains sufficient residual activity to prevent mitotic catastrophe and apoptosis induction in these cells. It is also possible that these cells have altered apoptotic or checkpoint pathways, and can continue to survive even under conditions of abnormal mitosis.
Aurora kinase inhibitor resistance in osteosarcoma

Figure 5. Caspase activity in AZD1152-treated osteosarcoma cell lines. OSCA 8 and OSCA 32 cells were incubated with AZD1152 (1 and 5 µM, respectively) for 4–6 h. Doxorubicin (1 µM) was used as a positive control. Caspase activity was determined using a fluorometric pan-Caspase activity kit. Data are expressed as mean fold increase in activity over negative controls (vehicle-treated cells) ± SD from three independent experiments.

which might be a mechanism of resistance to various chemotherapeutics.32

Several other possibilities might account for the relative resistance of canine osteosarcoma cells to Aurora kinase inhibition (compared to other human tumour types). It is possible that sarcomas have inherent properties that confer resistance to these compounds. Aurora kinase inhibitors have been most studied in haematopoietic and epithelial tumours with, to our knowledge, no previous published work in sarcomas. Furthermore, similar resistance has been identified in canine osteosarcoma cells treated with inhibitors of c-Met,33 Src,34 Stat334 and HSP-90 35 all of which had IC50’s in the micro-molar range in comparable 72-h assays. This suggests that osteosarcoma cells may have overlapping mechanisms of resistance to a variety of small molecule targeted inhibitors.

The genes encoding P-glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) have been shown to be upregulated in cell lines resistant to AZD1152,24 and P-glycoprotein has been previously shown to be a mechanism of resistance to doxorubicin in canine osteosarcoma.36,37 Although each of the cell lines in our study expressed mRNA for ABCB1 and ABCG2, only a minority of cells (<10%) expressed either of these ABC transporter proteins in the two cell lines we tested using flow cytometry. Verapamil incrementally enhanced apoptosis induced by AZD1152; however, given the relatively small increase, we do not believe that ABCB1 or ABCG2 transporter-mediated efflux is a dominant mechanism of resistance in these cells.

c-Myc also has been evaluated in cells treated with Aurora kinase inhibitors. This proto-oncogene has been shown to be overexpressed in many canine and human cancers, including sarcomas.38–41 The pleotropic, and sometimes opposite, functions of Myc proteins are especially evident in certain tumours. In human osteosarcoma, for example, Myc expression is an indicator of negative prognosis and contributes to drug resistance,40 but Myc also contributes to apoptosis induced by cytotoxic drugs in the same cells.42 A recent study showed the cytotoxic effects of VX680 were restricted to cells expressing c-Myc.43 Evaluation of c-Myc in canine osteosarcoma cells may offer insight into determinants of response to Aurora kinase inhibitors in this disease.

Other genes and proteins probably influence sensitivity to Aurora kinase inhibitors. Specifically, p53 deficient cells are more sensitive to AURKB inhibition, though it is not clear if this represents a causal relationship or if Myc is the major determining factor.43–46 Mutations in p53 are commonly associated with canine osteosarcoma.47–49 In a previous study in our laboratory, the p53 gene was not among those identified in the differential gene expression signature seen in canine osteosarcoma, although a number of DNA damage checkpoint genes in the signature were p53 targets.19 At present, the role of p53 in stratification of canine osteosarcoma and resistance to Aurora kinase inhibitors remains to be further investigated.

Finally, point mutations in AURKB that confer resistance to multiple Aurora kinase inhibitors have been described.50 These mutations are likely present in some cells before treatment, and are selected for by prolonged treatment. Target mutation is also a mechanism of resistance to other small molecule inhibitors.51,52 Additional work will be required to assess the frequency of AURKB mutations in these cell lines, as well as the potential role such mutations might have in resistance to Aurora kinase inhibitors in canine osteosarcoma.

In summary, we examined for the first time the effects of Aurora kinase inhibitors in canine cancer cells. We found evidence of target modulation.
Figure 6. Induction of apoptosis by AZD1152 in osteosarcoma cell lines. OSCA 8 and OSCA 32 cells were treated with AZD1152 (1 or 5 μM, respectively) with or without verapamil (10 μM) as indicated for 20 h. Doxorubicin (1 μM) was used as a positive control and vehicle treated-cells served as negative controls. Apoptotic cells were identified on the basis of staining with Annexin V-FITC (X-axis) and 7-AAD uptake (Y-axis) using multi-parameter flow cytometry. The lower left box represents live cells and the polygonal region represents apoptotic cells. The region was excluded in the doxorubicin conditions to avoid exclusion of late apoptotic cells (Annexin V-negative and 7-AAD-positive) from the analysis. Numbers within the gates indicate the percent of events within the gate (positive cells); the mean fluorescence intensity of the populations in the lower left box for each condition is shown in Table 3.

Figure 7. AZD1152 inhibits Aurora kinase B autophosphorylation at Thr232 in osteosarcoma cells. Exponentially growing OSCA 8, OSCA 32 and OSCA 78 cells were incubated with AZD1152 (AZD) with or without verapamil (V) for 1 h. The levels of Aurora kinase B and phospho-Aurora kinase B in whole cell lysates were determined by Western blotting. β-actin levels were determined to ensure equal loading. On the basis of densitometry, the ratio of phosphorylated to total AURKB following AZD1152 treatment was reduced by 62.7% in OSCA 8, 94.4% in OSCA 32 and 15% in OSCA 78.

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References


52. Rosenzweig SA. Acquired resistance to drugs targeting receptor tyrosine kinases. *Biochemical Pharmacology* 2012; 83: 1041–1048.