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Identification of anti-proliferative kinase inhibitors as potential therapeutic agents to treat canine osteosarcoma

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ABSTRACT

Osteosarcoma is the most common primary bone tumour in dogs but various forms of therapy have not significantly improved clinical outcomes. As dysregulation of kinase activity is often present in tumours, kinases represent attractive molecular targets for cancer therapy. The purpose of this study was to identify novel compounds targeting kinases with the potential to induce cell death in a panel of canine osteosarcoma cell lines. The ability of 80 well-characterized kinase inhibitor compounds to inhibit the proliferation of four canine osteosarcoma cell lines was investigated in vitro. For those compounds with activity, the mechanism of action and capability to potentiate the activity of doxorubicin was further evaluated.

The screening showed 22 different kinase inhibitors that induced significant anti-proliferative effects across the four canine osteosarcoma cell lines investigated. Four of these compounds (RO 31-8220, 5-iodotubercidin, BAY 11-7082 and an erbstatin analog) showed significant cell growth inhibitory effects across all cell lines in association with variable induction of apoptosis. RO 31-8220 and 5-iodotubercidin showed the highest ability to potentiate the effects of doxorubicin on cell viability. In conclusion, the present study identified several potent kinase inhibitors targeting the PKC, CK1, PKA, ErbB2, mTOR and NF-κB pathways, which may warrant further investigations for the treatment of osteosarcoma in dogs.
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Introduction

Osteosarcoma (OS) is the most common primary bone tumour in dogs. It arises spontaneously in various skeletal locations and is highly metastatic. Currently, therapy includes amputation or limb sparing surgery at the site of the primary tumour, and chemo- and/or radiation therapy to target micrometastases and control local recurrence. Despite advancements in diagnostic approaches as well as the availability of multiple platforms for therapy, the prognosis and survival rates of dogs with OS have not changed dramatically over the past decades (Langdon, 2012).

While the exact pathogenesis of OS development and progression is still poorly understood, several prognostic factors in canine appendicular OS have recently been elucidated (Boerman et al., 2012) and it has been suggested that various signalling pathways and genetic alterations may be involved (Selvarajah and Kirpensteijn, 2010). In particular, several protein kinases are potential interesting drug molecular targets, as these are often overexpressed or dysregulated in cancer cells. Individual kinases have been found to be expressed in canine OS and OS cell lines, including HER2 (Flint et al., 2004), EGFR (Selvarajah et al., 2012), cMET (De Maria et al., 2009; Fieten et al., 2009), PDGFR (Fahey et al., 2013; Maniscalco et al., 2013), cKIT (Fahey et al., 2013), and PKC (Hong et al., 2011). This suggests that kinases represent potential targets for canine OS adjuvant therapies.

To the best of our knowledge no kinase inhibitor is yet in clinical trials for dogs with OS. However, a recent case study reported treatment of a dog with OS, having established ex vivo tumour sensitivity to a panel of kinase inhibitors (Davis et al., 2013). This approach appears promising, since at the time of publication the animal was alive without recurrent disease. Overall, kinase inhibitors appear to be well tolerated as a class of drugs, although adverse effects still occur (Hartmann et al., 2009). Combination chemotherapy rather than monotherapy may lead to a better therapeutic index with reduced side effects and lower risk for inducing resistance.

There are various approaches used in the discovery of novel small molecule anti-cancer agents (Hoelder et al., 2012). High-throughput methods like screening multiple compounds in multiple tumour

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Table 1
Compounds in the library of kinase inhibitors and their targeted pathways.

Compound name	Target/pathway	Compound name	Target/pathway
PD-98059	MEK	KN-93	CaMK II
U-0126	MEK	ML-7	MLCK
SB-203580	p38 MAPK	ML-9	MLCK
H-7	PKA, PKG, MLCK, and PKC	2-Aminopurine	p58 PITSLRE beta1
H-9	PKA, PKG, MLCK, and PKC	N9-Isopropyl-olomoucine	CDK
Staurosporine	Pan-specific	Olomoucine	CDK
AG-494	EGFRK, PDGFRK	iso-Olomoucine	Negative control for olomoucine
AG-825	HER1-2	Roscovitine	CDK
Lavendustin A	EGFRK	5-Iodotubercidin	ERK2, adenosine kinase, CK1, CK2
RG-14620	EGFRK	LFM-A13	BTK
Tyrphostin 23	EGFRK	SB-202190	p38 MAPK
Tyrphostin 25	EGFRK	PP2	Src family
Tyrphostin 46	EGFRK, PDGFRK	ZM 336372	Craf
Tyrphostin 47	EGFRK	SU 4312	Flk1
Tyrphostin 51	EGFRK	AG-1296	PDGFRK
Tyrphostin 1	Negative control	GW 5074	cRAF
Tyrphostin AG 1288	Tyrosine kinases	Palmitoyl-DL-carnitine Cl	PKC
Tyrphostin AG 1478	EGFRK	Rottlerin	PKC delta
Tyrphostin AG 1295	Tyrosine kinases	Genistein	Tyrosine kinases
Tyrphostin 9	PDGFRK	Daidzein	Negative control for Genistein
HNMPA	IRK	Erbstatin analog	EGFRK
PKC-412	PKC inhibitor	Quercetin dihydrate	PI 3-K
Piceatannol	Syk	SU 1498	Flk1
PP1	Src family	ZM 449829	JAK-3
AG-490	JAK-2	BAY 11-7082	IKK pathway
AG-126	IRAK	DRB	CK II
AG-370	PDGFRK	HBDDE	PKC alpha, PKC gamma
AG-879	NGFRK	SP 600125	JNK
LY 294002	PI 3-K	Indirubin	GSK-3beta, CDK5
Wortmannin	PI 3-K	Indirubin-3’-monoxime	GSK-3beta
GF 109203X	PKC	Y-27632	ROCK
Hypericin	PKC	Kenpaullone	GSK-3beta
Ro 31-8220	PKC	Terreic acid	BTK
Sphingosine	PKC	Triciribine	Akt signalling pathway
H-89	PKA	BML-257	Akt
H-8	PKA, PKG	SC-514	IKK2
HA-1004	PKA, PKG	BML-259	Cdk5/p25
HA-1077	PKA, PKG	Apigenin	CK-II
HDBA	EGFRK, CaMK II	BML-265 (Erlotinib analog)	EGFRK
KN-62	CaMK II	Rapamycin	mTOR

samples from a particular tumour type increase the chances of discovering novel broad spectrum inhibitors. To this end, we screened a kinase inhibitor library on a panel of canine OS tumour cell lines. The anti-proliferative effect of that library (composed of 80 kinase inhibitors) on cell viability of the canine OS cell lines was evaluated. Inhibitors that displayed significant inhibition of cell proliferation across four OS cell lines were investigated further to determine their half maximal inhibitory concentration (IC50) in vitro, their ability to induce apoptosis and to evaluate their potential to work synergistically with doxorubicin, a currently widely applied cytotoxic chemotherapeutic drug against canine OS.

Materials and methods

Cell lines and culture conditions

Four canine OS cell lines HMPOS (Barroga et al., 1999), D17 (ATCC CCL-183), KOS-003 and KOS-004 (identical to MCKOS and SKKOS, respectively; kind gifts from Dr. C. Khanna) (Hong et al., 2011) were used in this study. D17 and HMPOS are derived from a pulmonary metastatic lesion established from a primary OS of unknown location of a female poodle and from the left proximal femur of a male mixed-breed dog, respectively; KOS-003 and KOS-004 are derived from primary tumours of the right distal radius of a Labrador cross and the left distal radius of a mixed breed, respectively. All cells were grown in Dulbecco’s minimal essential medium (DMEM, Lonza) supplemented with 10% fetal bovine serum, FBS), 2% L-glutamine and 0.1% gentamycin, and maintained at 37 °C in a 5% CO_2 humidified chamber.

Kinase inhibitor compound library

A kinase inhibitor library containing 80 compounds with well-characterised activity (Screen-Well Kinase Inhibitor Library, Enzo Life Sciences BML-2832) was kindly provided by Dr. E. de Vries. The complete list of compounds and their targeted kinases and/or signalling pathways are shown in Table 1. Compounds were reconstituted in 10% dimethylsulfoxide (DMSO) (v/v) to obtain 100 µM stocks in a 96-well plate format.

Single kinase inhibitors and doxorubicin

The compounds identified from the kinase inhibitor library that resulted in a significant reduction of cell viability were subsequently obtained from Sigma Aldrich (BAY 11-7082 B5556, RO 31-8220 R136, 5-iodotubercidin I100, Erbstatin Analog D2667). Staurosporine (S4400) served as a positive control, while doxorubicin (D1515) was used for the combination treatment. Dose response curves were performed by treatment of cells with serial, twofold dilutions of each drug.

Cell viability assay

Three thousand cells from each cell line were seeded in 96-well plates, allowed to attach overnight, and subsequently incubated with the kinase inhibitors at a final concentration of 10 µM. After 72 h, cell proliferation was established using the WST-1 cell proliferation/viability assay (Roche Diagnostics). In brief, 10% WST-1 reagent was added to the culture medium, and cells were incubated for 45 min at 37 °C. The optical density (OD) at 450 nm was measured in an enzyme-linked immunoassay (ELISA) microplate reader (BioTek). The viability of the compound-treated cells was calculated relative to that of the vehicle control wells containing 1% DMSO (v/v). All experiments were performed in triplicate in two independent experiments.

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