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Citation for published version:

Digital Object Identifier (DOI):
10.1016/j.vetimm.2011.02.006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Veterinary Immunology and Immunopathology

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Targeted knockdown of canine KIT (stem cell factor receptor) using RNA interference

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Keywords: dog; mast cell tumor; KIT; small inhibitory RNA; RNA interference; stem cell factor

Abbreviations: CHO, Chinese hamster ovary; SiRNA, small inhibitory RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; B2M, beta-2 microglobulin; TRAIL, TNF related apoptosis-inducing ligand; XIAP,

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Abstract

Canine mast cell tumours often express mutations in KIT exon 11 that result in autophosphorylation and constitutive activation of the c-kit receptor, even in the absence of stem cell factor, its natural ligand. Such activating mutations have been associated with more aggressive neoplastic disease. The aim of the current study was to determine whether small inhibitory RNA (SiRNA) molecules could be used to specifically knock-down canine KIT expression. Canine beta-2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and KIT coding sequences were cloned downstream of the renilla luciferase element in the psiCHECK™-2 bi-cistronic vector, that also expresses firefly luciferase. SiRNA molecules were designed to target gene-specific sequences and were co-transfected with plasmid DNA into Chinese hamster ovary (CHO) cells. Renilla and firefly luciferase activity was subsequently measured using the Dual-GLO® Luciferase Assay (Promega). Using this luciferase reporter system, canine housekeeping gene-specific SiRNA molecules demonstrated knockdown of their targets (72.0% knockdown for B2M and 94.5% knockdown for GAPDH). An SiRNA molecule targeting exon 2 of canine KIT successfully knocked-down renilla luciferase expression of a KIT26-407 construct (90.8% knockdown). An SiRNA molecule targeting a common 48 base-pair in-tandem duplication mutation in KIT exon 11 selectively knocked down expression of the KIT1569-1966mutant construct (93.1% knockdown) but had no effect on the KIT1569-1918wild-type construct. The results show that RNA interference can be used to inhibit canine KIT mRNA expression and has the potential to selectively target the mutant version of KIT that is expressed by some malignant mast cells.
1. Introduction

Mast cell tumours (MCT) are the most common skin malignancy in dogs and are thought to arise from neoplastic transformation of tissue mast cells, although the precise aetiology is uncharacterised (reviewed by Dobson and Sease, 2007, Welle et al., 2008). In order to become malignant, mast cells must be able to resist those mechanisms associated with programmed cell death, to allow survival and proliferation. Developing a greater understanding of how such cells prevent apoptosis might enable us to design more rational treatment strategies for this type of cancer.

Mast cell viability in healthy tissues is regulated by stem cell factor (SCF) via its action on the KIT receptor (CD117), which has tyrosine kinase activity (Qiu et al., 1988). SCF binding to KIT is thought to provide survival signals, by inhibiting apoptosis through modulation of apoptosis-regulatory proteins (Kitamura et al., 1978, Kitamura & Go, 1979, Galli & Kitamura, 1987, Tsai et al., 1991, Galli et al., 1994, Serve et al., 1995, Linnekin et al., 1997, Vosseller et al., 1997, Timokhina et al., 1998, Brazis et al., 2000, Taylor & Metcalfe, 2000). In human mast cells, overexpression of Bcl-2 or Bcl-xL protects against programmed cell death and is thought to contribute to oncogenesis (Cerveró et al., 1999, Hartmann et al., 2003). Mutations in the KIT gene are found in 15-50% of canine MCT, which typically consist of in-tandem duplications, located in or around exon 11 (London et al., 1999, Ma et al., 1999, Reguera et al., 2000, Downing et al., 2002, Reguera et al., 2002, Zemke et al., 2002, Turin et al 2006, Webster et al., 2006 A & B). When expressed, such mutations often result in autophosphorylation of the cytoplasmic kinase domain of the receptor protein, leading to constitutive activation, even in the absence of SCF (London et al., 1999, Ma et al., 1999, Pryer et al., 2003). The C2 mastocytoma cell line, which is commonly used to study canine malignant mast cell biology in vitro, expresses a 48 base-pair in-tandem duplication (London et al., 1999, Ma et al., 1999), although the precise
location, nature and size of mutations can vary between different MCTs (Reguera et al., 2000, Downing et al., 2002, Pryer et al., 2003, Riva et al., 2005, Roskoski et al., 2005, Turin et al., 2006, Webster et al., 2006A, Letard et al., 2008).

KIT activating mutations have been shown to be associated with MCT of a more aggressive phenotype, which is likely due to increased proliferation and resistance to apoptosis (Hirota et al., 1998, Corless et al., 2004, Gleixner et al., 2007, Webster et al., 2007, Letard et al., 2008). This has led to several investigations into the therapeutic potential of receptor tyrosine kinase inhibitors (RTKi), designed to specifically target this molecule. There are currently two drugs (masitinib, Masivet®, AB Science; toceranib, Palladia®, Pfizer) that have recently been licensed for canine MCT. However, these compounds can also impact on other receptor tyrosine kinases (Rubin et al., 2001, Hayes & Thor, 2002, Humbert et al., 2009, London et al., 2009), potentially leading to adverse effects. More specific targeting of the mutant KIT receptor is likely to be more selective in having anti-cancer effects.

RNA interference can be used to target specific mRNA for degradation (Spee et al., 2006, Tsuchiya et al., 2006, Watanabe et al., 2009. Thus, gene expression can be selectively silenced, preventing production of the encoded protein, which allows experiments to be designed to determine the role of that specific gene product on cellular function. The aim of the current study was to determine whether small inhibitory RNA (SiRNA) molecules could be designed to specifically knock-down canine KIT expression. In particular we aimed to design novel SiRNA molecules that specifically targeted the mutant KIT expressed by C2 cells that would spare expression of the wild-type receptor. However, since C2 cells proved to be resistant to chemical transfection, we used a reporter gene assay to assess the ability of canine KIT-specific SiRNA molecules to knock-down their target.
2. Materials and Methods

2.1 Plasmid DNA constructs and SiRNA molecules

Partial coding regions for selected canine genes were amplified by PCR from cDNA prepared from the C2 canine mastocytoma cell line (a generous gift from Dr B. Helm, University of Sheffield; originally generated by Prof W. Gold, University of California (Lazarus et al., 1986), which expresses both wild-type KIT as well as a KIT exon 11 mutation (Figure 1). PCR products representing canine glyceraldehyde-3-phosphate dehydrogenase; GAPDH (sense primer: ACCACCGTCCATGCCATCAC; antisense primer: TCCACCACCAGGTGCTGTA; 452bp amplicon), beta-2 microglobulin; B2M (sense primer: TCCTCATCCTCCTCGCT; antisense primer: GTCAGTTGTCTCGGTCCCAC; 333bp amplicon), KIT (sense primer: ATTTTCTCTGCGTCCTGCTC; antisense primer: ACCAGCGTATCATTGCCTTC; 382bp amplicon), KIT (sense primer: CCTGTTCACACCTTTGCTGA; antisense primer: TAGGGCTTCTCGTTCGGTTA; 352bp amplicon for wild-type, 400bp amplicon for mutant) were initially cloned into the pSC-A vector (Stratagene, La Jolla, CA). Plasmid DNA from recombinant clones was sequenced to confirm the integrity of the inserts, which were then sub-cloned into the psi-CHECK™-2 bi-cistronic vector (Promega, Southampton, UK), downstream of the renilla luciferase element using NotI and XhoI restriction sites. Plasmid DNA was then extracted from recombinant E.coli using the GenElute™ Endotoxin-free Miniprep Kit (Sigma, Poole, UK) in preparation for transfection studies. The SiRNA molecules used in the study are shown in Table 1. In addition, a scrambled SiRNA molecule (AllStars Negative Control SiRNA, Qiagen, Crawley, UK) was used as a negative control.

2.2 Cell culture and transfections
Chinese hamster ovary (CHO) cells were maintained in 75 cm² flasks of culture medium consisting of Minimal Essential Medium (Sigma, Poole, UK) supplemented with 10 mM Glutamax-1 (Invitrogen) 10% foetal bovine serum (Serotec, Kidlington, UK) and 25 μg/ml gentamicin (Sigma). Cells for transfection studies were plated out in triplicate wells in Corning 96 well clear bottom, white microtitre plates (Sigma) at $1 \times 10^4$ cells in a 100 μl volume of culture medium lacking serum and antibiotics. Cells in each well were transfected with 200 ng plasmid DNA and 5 pmol SiRNA using Lipofectamine 2000™ (Invitrogen) according to the manufacturer’s instructions. Plates were incubated for 4 h then the complex-containing medium was replaced with culture medium lacking antibiotics.

2.3 Luciferase assay

Twenty four hours after transfection, cells were assayed for both firefly and renilla luciferase activity using the Dual-GLO® Luciferase Assay System (Promega). Briefly, cells were lysed and the firefly luciferase substrate added (50 μl per well Dual-GLO® Substrate/Buffer). After 15 min, luciferase activity was measured using a luminometer (Spectramax M2, Molecular Devices). Next the renilla luciferase substrate was added (50 μl per well Stop & GLO® Substrate/Buffer) and the luminescence measured after a further 15 min incubation. The renilla / firefly luciferase ratio was calculated from the mean luminescence values of triplicate wells, after blanking against values from untransfected cells. The percentage knockdown using test SiRNA molecules was calculated compared to the control scrambled SiRNA and data are shown as the mean of three experimental replicates.

3. Results

3.1 Knock-down in expression of canine housekeeping genes and KITwild-type
Since there are few studies using siRNA to specifically knock-down canine gene expression, several siRNA molecules were initially assessed that were designed to target housekeeping genes. Using a renilla luciferase siRNA molecule as a positive control, efficient knock-down of canine GAPDH was demonstrated, although siRNA molecules targeting beta-2 microglobulin were less efficient (Table 2). A validated human Kit siRNA molecule (which has complete identity to the target sequence in canine Kit) was evaluated and showed >90% knock-down efficiency (Table 2).

3.2 Knock-down in expression of the C2 Kit mutant

The mutation present in the Kit gene expressed by C2 cells consists of a 48 bp in-tandem duplication in exon 11. Since this generates a unique sequence at the 5’ junction of the insertion, an siRNA molecule was designed that spanned this region (Figure 1, Table 1). When tested against constructs containing either the wild-type sequence or the mutant version, this siRNA molecule demonstrated efficient knock-down when using the Kit2mutant construct (mean 93.1% knock-down) but had little effect on the Kit2wild-type construct (mean 2.98% knock-down) (Figure 2).

4. Discussion

Recent advances in treatment of canine MCT have focused on the use of RTki (e.g. masitinib and toceranib). However, these compounds are not specific for Kit and also inhibit other receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and vascular growth factor receptor (VEGFR2) (Rubin et al., 2001, Hayes & Thor, 2002, Humbert et al., 2009, London et al., 2009). Although the lack of specificity might enhance the anti-cancer efficacy of these drugs, this could also increase the risk of adverse effects in the patient. In addition, these RTki will not have specific effects on malignant mast cells, as they
will also inhibit wild-type KIT, expressed by tissue mast cells and other cell types including haematopoietic stem cells and melanocytes (Galli et al., 1994, Serve et al., 1995, Linnekin et al., 1997, Vosseller et al., 1997, Timokhina et al., 1998, Brazis et al., 2000) Molecular techniques, such as gene silencing by RNA interference, offer an alternative, more specific method for targeting mutant KIT that might be applicable for canine MCT, as well as for canine gastrointestinal stromal cell tumours that also commonly express KIT mutations in humans (Lasota et al., 2003, Steigen et al., 2007, Du et al., 2008).

There are relatively few published papers describing RNA interference in the canine system. In terms of studying the biology of canine malignant cells, siRNA targeting of apoptosis-modulator molecules including Bcl-2 (Watanabe et al., 2009), Bcl-xL (Tsuchiya et al., 2006) and XIAP (Spee et al., 2006) have previously been undertaken in canine cancer cell lines. Knockdown of canine (housekeeping) gene(s), not involved in malignancy, would be useful as negative controls for RNA interference studies, although there were no published reports of their use prior to commencing the current study.

To investigate the biological effect of KIT RNA interference, the C2 canine mastocytoma cell line was chosen for in vitro studies. The C2 cells used in the current study expressed both wild-type and mutant KIT mRNA (Figure 1), although wild-type KIT seems to be absent in the C2 cells used in some (Ma et al., 1999) but not all previous studies (London et al., 1999). Since no KIT siRNA molecules had been validated for the dog, several human KIT siRNAs, that had been shown to increase apoptosis in human cell lines (Lefevre et al., 2004; Li et al., 2007; Sikarwar & Reddy 2008) were assessed for sequence identity with the canine KIT sequence. One commercially available KIT SiRNA molecule (Silencer® SiRNA 35, Ambion), which demonstrated complete sequence identity between human and canine target sequences, was identified. For the other targets (GAPDH, B2M and KIT exon 11 mutation), novel SiRNA molecules were designed for evaluation.
Chemical transfection of C2 cells proved to be problematic, despite repeated attempts using various transfection reagents and protocols (data not shown). Therefore, to allow investigation of the activity of the siRNA molecules to specifically interfere with their target canine mRNA, an alternative assay was established. This assay was based on use of the psiCHECK-2™ reporter system in the CHO cell line, which had been shown to be readily transfectable with Lipofectamine™ 2000. This reporter assay allows rapid and relatively simple assessment of the activity of novel SiRNA molecules against their target sequences, prior to evaluation of RNA interference in the target cells. One disadvantage, however, is that it does not provide information as to any off-target effects of the SiRNA molecule.

Co-transfection of CHO cells with recombinant plasmid DNA containing canine target sequences and either scrambled or targeted siRNA molecules demonstrated that GAPDH and KIT^{exon2} siRNAs induced a knockdown efficiency of greater than 80%, although the canine B2M SiRNA molecules were somewhat less effective. The increase in firefly luciferase activity seen in CHO cells co-transfected with plasmid DNA with scrambled siRNA, compared to those cells transfected with plasmid DNA alone, might be explained by the greater amount of transfection reagent used, leading to increased transfection efficiency. A further increase in firefly luminescence was seen in CHO cells co-transfected with targeted siRNA compared to scrambled siRNA. This pattern of reactivity might be explained if firefly and renilla luciferase mRNA compete for translation. Thus, following successful targeting of the renilla-fusion mRNA for destruction, this could lead to enhanced firefly luciferase protein expression.

The KIT^{exon2} SiRNA molecule is not specific to the mutant KIT sequence and would be expected to knock-down both wild-type and mutant KIT mRNAs. Therefore, a novel canine KIT SiRNA molecule was designed, that targeted the 5’ junction of the insertion mutation to determine whether it might be possible to specifically knock-down the mutant
version, while sparing expression of wild-type KIT. Both firefly and renilla luciferase activity
were consistently lower when using the psiCHECK-2™ construct containing canine mutant
KIT, compared to the native vector or vector containing wild-type KIT sequences. Endotoxin
contamination of the plasmid DNA was considered, but a new batch of plasmid DNA
demonstrated the same response. The explanation for this reduced efficiency of luciferase
activity with this particular construct remains unclear. Despite this, the KIT\textsuperscript{exon11mut} SiRNA
molecule demonstrated specificity of activity against the mutant KIT construct, without
affecting expression from the construct containing the equivalent wild-type KIT sequence.

In the current study, we have validated several canine gene-specific SiRNA
molecules. These can now be assessed in canine cell lines and it is anticipated that alternative
delivery systems (e.g. electroporation) will allow evaluation of these molecules in the C2 cell
line. We have shown as “proof-of-concept” that SiRNA molecules can be designed to
specifically target KIT mutations, although the heterogeneity of mutations seen in canine
MCT means that it is unlikely that a single SiRNA molecule could be designed that would be
effective in all cases.
Acknowledgements

We would like to thank Petsavers for their generous support of this research project. We also thank the BBSRC and Merial Animal Health for funding RCE’s PhD studentship.

References
Fig. 1. The C2 cell line expresses both wild-type KIT and a KIT exon 11 mutant. (a) cDNA was prepared from C2 cells and used as the template for PCR using primers spanning KIT exon 11. PCR products were separated by 2% agarose gel electrophoresis. Anticipated amplicon size = 352bp for wild-type KIT and 400bp for the mutant version, which contains a 48bp in-tandem duplication. MWt = 200bp molecular weight marker. (b) Sequence of KIT mutation in C2 cells. Partial genomic DNA sequence is shown with exon 11 highlighted. The 48 bp mutation is shown in bold and the region targeted by the SiRNA molecule is underlined.

Fig. 2. Knockdown efficiency of an SiRNA molecule that targets the C2 KIT mutation. Recombinant psiCHECK-2 plasmid DNA was generated containing either the 5’ region of canine KIT (KIT1) or the region containing exon 11 (KIT2) of either the wild-type or mutant version of the gene. Triplicate wells of CHO cells were transfected with recombinant psiCHECK-2 plasmid DNA and selected wells were co-transfected with scrambled siRNA (negative control), renilla SiRNA (positive control) or siRNA targeted to KIT (either exon 2 or the in-tandem duplication mutation within exon 11). Both firefly and renilla luminescence were measured after 24 h incubation. The renilla/firefly luciferase ratio are shown which represents the knock-down capability of the SiRNA molecule on the target mRNA. A representative experiment is shown of three replicates.
<table>
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<tr>
<th>Target gene</th>
<th>Plasmid DNA construct</th>
<th>SiRNA molecule</th>
<th>SiRNA target sequence</th>
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<td>Silencer® SiRNA 35 (Ambion)</td>
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<td>KITexon11mut</td>
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Table 1. Plasmid DNA constructs and SiRNA molecules used in knock-down studies.
Table 2. Knockdown efficiency of canine housekeeping gene and KIT SiRNA molecules. Triplicate wells of CHO cells were transfected with native or recombinant psiCHECK-2 plasmid DNA and co-transfected with either scrambled siRNA, siRNA targeted to renilla luciferase, or SiRNA targeting the inserted canine sequence. Both firefly and renilla luciferase activity were measured after 24 h incubation. Mean luminescence data are shown, following subtraction of the luminescence values of untransfected CHO cells. Knockdown efficiency of targeted siRNA was calculated compared to scrambled siRNA. GAPDH = glyceraldehyde-3-phosphate dehydrogenase, B2M = β2microglobulin, Exp = experimental replicate, LU = luminescence units, KD (%) = percentage knockdown efficiency.

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<th>Exp #1 Firefly (LU)</th>
<th>Exp #1 Renilla (LU)</th>
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<th>Exp #2 Firefly (LU)</th>
<th>Exp #2 Renilla (LU)</th>
<th>KD (%)</th>
<th>Exp #3 Firefly (LU)</th>
<th>Exp #3 Renilla (LU)</th>
<th>KD (%)</th>
<th>Mean KD (%)</th>
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<td>550</td>
<td></td>
<td>407</td>
<td>372</td>
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<td></td>
<td>2662</td>
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FIGURE 1

a)

b)

TCTCTACCCTAGTGCTATAATGATCGAAATGTTATTCATTAAAAGATGATCTGTCTCTCTTTTCTCCCCCCACCAGAAACCATGTATGAAGTACAGTGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGACC

CAACACAGCCTCTCTTACGATACAAATGGGAGTTTCCCAGAA

AGCTTTGGTCAGTATGAAATAGGGGCTTTCCATGTAACCTTTTTGTGTAC

CGTGTAACAAATGACTTCTAGGGAACCATTAGCTTCTCTTCTGTCTGTC

CAAACAGACAATAAGTATTTTCTGTGAAAGTTCATCATATTTTGTATA

KIT\textsuperscript{mutant} (400 bp)

KIT\textsuperscript{wild-type} (352 bp)
FIGURE 2

Renilla / Firefly luciferase ratio
SiRNA molecule
pSI-CHECK2/KIT1
pSI-CHECK2/KIT2
pSI-CHECK2/KIT2
mutant
pSI-CHECK2/KIT2
wild-type

0
0.2
0.4
0.6
0.8
1
1.2
1.4
Renilla / Firefly luciferase ratio
SiRNA molecule
pSI-CHECK2/KIT1
pSI-CHECK2/KIT2
pSI-CHECK2/KIT2
mutant
pSI-CHECK2/KIT2
wild-type

0
0.2
0.4
0.6
0.8
1
1.2
1.4
Renilla / Firefly luciferase ratio
SiRNA molecule
pSI-CHECK2/KIT1
pSI-CHECK2/KIT2
pSI-CHECK2/KIT2
mutant
pSI-CHECK2/KIT2
wild-type