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Short communication

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

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ABSTRACT

Canine mast cell tumours often express KIT mutations that result in constitutive activation of the c-kit receptor and which are associated with more aggressive disease. The aim of the current study was to determine whether small inhibitory RNA (SiRNA) molecules could specifically target canine KIT mRNA for knock-down. Canine beta-2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and KIT sequences were cloned into the psiCHECKTM-2 vector. SiRNA molecules, designed to target gene-specific sequences, were co-transfected with plasmid DNA into Chinese hamster ovary (CHO) cells. Renilla and firefly luciferase activity was measured using the Dual-GLO[®] Luciferase Assay (Promega). Using this 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 reporter gene expression of a KIT^{26–407} construct (90.8% knockdown). An SiRNA molecule targeting a 48 base-pair in-tandem duplication mutation in KIT exon 11 selectively knocked down expression of the KIT^{1569–1966mutant} construct (93.1% knockdown) but had no effect on the KIT^{1569–1918wild-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.

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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 (reviewed by Dobson and Scase, 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 (Galli and Kitamura, 1987; Taylor and Metcalfe, 2000). In human mast cells, overexpression of Bcl-2 or Bcl-x_L, protects against programmed cell death

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, X-linked inhibitor of apoptosis protein.

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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; Downing et al., 2002; Webster et al., 2006). When expressed, such mutations often result in autophosphorylation of the intracellular 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). Thus, canine mast cells seem to be capable of developing independence from growth/survival signals, one of the key features of malignancy (Hanahan and Weinberg, 2000). 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 (Downing et al., 2002; Webster et al., 2006; 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 (Gleixner 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 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 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 and 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; Linnekin et al., 1997). Molecular techniques, such as gene silencing by RNA interference, offer an alternative, potentially more specific method for targeting mutant KIT that might be applicable for canine MCT, as well as for human gastrointestinal stromal cell tumours that commonly express similar KIT mutations (Lasota et al., 2003; Steigen et al., 2007; Du et al., 2008).

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 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. Chemical transfection of C2 cells proved to be problematic, despite repeated attempts using various transfection

reagents and protocols. Therefore, to allow investigation of the activity of the siRNA molecules to specifically interfere with their target canine mRNA, we investigated use of 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 (Fig. 1). PCR products representing canine glyceraldehyde-3-phosphate dehydrogenase; GAPDH^{520–971} (sense primer: ACCACCGTCCATGCCATCAC; antisense primer: TCCACCACCCGGTTGCTGTA; 452 bp amplicon), beta-2 microglobulin; B2M^{47–379} (sense primer: TCCTCATCTCCTCGCT; antisense primer: GTCAGTTGTCTCGGTCACC; 333 bp amplicon), KIT^{26–407} (KIT1: sense primer: ATTTCTCTGCGTCCTGCTC; antisense primer: ACCAGCGTATCATTGCCCTTC; 382 bp amplicon), KIT^{1569–1920}wild-type and KIT^{1569–1968}mutant (KIT2: sense primer: CCTGTTACACCTTTGCTGA; antisense primer: TAGGGCTTCTCGTTCGGTGA; 352 bp amplicon for wild-type, 400 bp 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 psiCHECKTM-2 vector (Promega, Southampton, UK). This vector contains both firefly and renilla luciferase reporter genes under the control of separate promoters. Each canine gene-specific sequence was inserted downstream of the renilla luciferase element using *NotI* and *XhoI* restriction sites, which will result in expression of the target sequence in the 3' UTR of the renilla luciferase mRNA. Following transfection, any changes in renilla luciferase activity, which might result from siRNA binding and knockdown of the fusion mRNA, can thus be normalized against firefly luciferase activity. Plasmid DNA was then extracted from recombinant *E. coli* using the GenEluteTM 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 × 10⁴ cells in a 100 µl volume of culture medium lacking serum and antibiotics. Cells in each

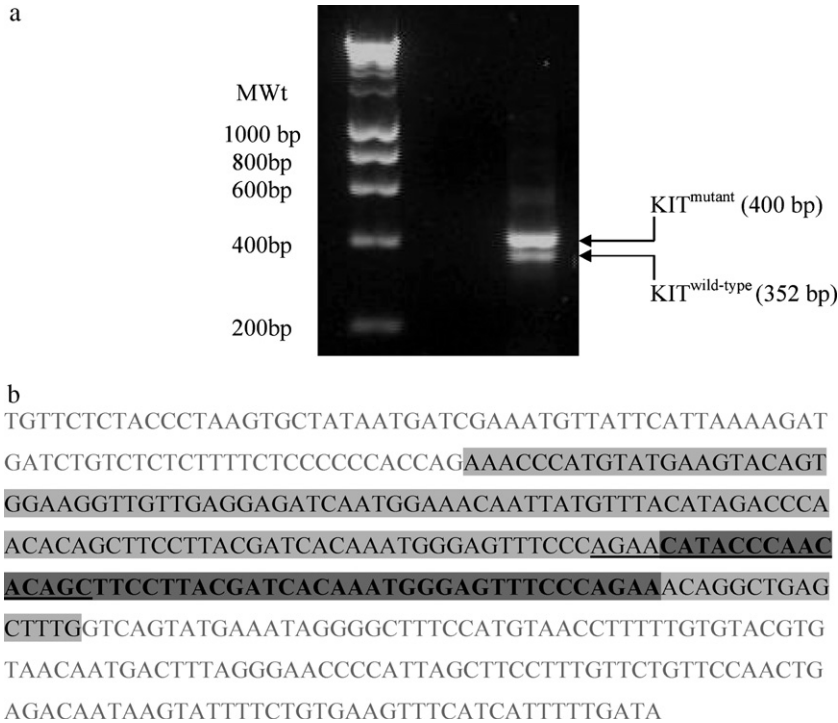


Fig. 1. The C2 cell line expresses both wild-type KIT and a KIT exon 11 mutant. (a) cDNA was prepared from C2 cells and used as the template for PCR using primers spanning KIT exon 11. PCR products were separated by 2% agarose gel electrophoresis. Anticipated amplicon size = 352 bp for wild-type KIT and 400 bp for the mutant version, which contains a 48 bp in-tandem duplication. MWt = 200 bp 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.

well were transfected with 200 ng plasmid DNA and 5 pmol siRNA using Lipofectamine 2000TM (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 μ l 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 μ l 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 & discussion

3.1. Knock-down in expression of canine housekeeping genes

Few studies have been performed using siRNA to specifically knock-down canine gene expression. In terms of studying the biology of canine malignant cells, siRNA targeting 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

Table 1
Plasmid DNA constructs and siRNA molecules used in knock-down studies.

Target gene	Plasmid DNA construct	siRNA molecule	siRNA target sequence	siRNA source
Renilla luciferase	psiCHECK-2	Renilla	tggccttctactactcctacg	Custom synthesized (Ambion)
GAPDH	psiCHECK-2/GAPDH ^{520–971}	GAPDH#1 ^{exon8a}	ccactttgtcaagctcatt	Custom synthesized (Ambion)
GAPDH	psiCHECK-2/GAPDH ^{520–971}	GAPDH#2 ^{exon8b}	cgacatcaagaagtagtg	Custom synthesized (Ambion)
B2M	psiCHECK-2/B2M ^{47–379}	B2M#1 ^{exon2a}	ctccaatgagcaggatga	Custom synthesized (Sigma)
B2M	psiCHECK-2/B2M ^{47–379}	B2M#2 ^{exon2b}	ccgtgtaaagcatgttact	Custom synthesized (Sigma)
KIT	psiCHECK-2/KIT ^{26–407}	KIT ^{exon2}	ggctcttcaaccatctg	Silencer [®] siRNA 35 (Ambion)
KIT	psiCHECK-2/KIT ^{1569–1966mutant}	KIT ^{exon11mut}	agaacataccaacacagc	Custom synthesized (Ambion)

canine cancer cell lines. In the current study, several SiRNA molecules were initially assessed that were designed to target canine 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 effective (Table 2). 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.

3.2. Knock-down in expression of canine KIT

To investigate the biological effect of KIT RNA interference, the C2 canine mastocytoma cell line was chosen for *in vitro* studies. The mutation present in the KIT gene expressed by C2 cells consists of a 48 bp in-tandem duplication in exon 11. The C2 cells used in the current study expressed both wild-type and mutant KIT mRNA (Fig. 1), with the latter seeming to dominate. Since C2 cells are heterozygous for the mutation, it is possible that the mutant allele is transcribed at a higher level, or that the mutant transcript is more stable than the wild-type transcript. Expression of wild-type KIT seems to be variable in C2 cells with expression reportedly absent in 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 be effective (Lefevre et al., 2004; Li et al., 2007; Sikarwar and Reddy, 2008) were assessed for sequence identity with the canine KIT sequence. A commercially available and validated KIT SiRNA molecule (Silencer® SiRNA 35, Ambion), which demonstrated complete sequence identity between human and canine target sequences, was selected which demonstrated >90% knock-down efficiency (Table 2). 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. The presence of the mutation generates a unique sequence at the 5' junction of the insertion (Fig. 1). Therefore, a novel canine KIT SiRNA molecule was designed, that targeted this region to determine whether it might be possible to specifically knock-down the mutant, while sparing expression of wild-type KIT. When tested against constructs containing either the wild-type sequence or the mutant version, this SiRNA molecule demonstrated efficient knock-down when using the KIT^{mutant} construct (mean 93.1% knock-down) but had little effect on the KIT^{wild-type} construct (mean -2.98% knock-down) (Fig. 2).

Due to the relatively large size of the KIT coding sequence (almost 3kB) and the problems associated with cloning this in entirety, we opted to clone only the specific

Table 2 Knockdown efficiency of canine housekeeping gene and KIT SiRNA molecules.

Transfection conditions	Exp #1			Exp #2			Exp #3			Mean KD (%)
	Firefly (LU)	Renilla (LU)	KD (%)	Firefly (LU)	Renilla (LU)	KD (%)	Firefly (LU)	Renilla (LU)	KD (%)	
psiCHECK TM -2	2981	1493		1023	550		407	372		
psiCHECK TM -2+scrambled siRNA	7528	3307		2662	1474		1536	1316		
psiCHECK TM -2+renilla siRNA	10474	240	94.8	2794	91	94.1	3014	19	99.3	96.1
psiCHECK TM -2/B2M	2505	1586		2137	1305		1873	998		
psiCHECK TM -2/B2M+scrambled siRNA	9952	5278		7329	3733		7001	3509		
psiCHECK TM -2/B2M+B2M#1 siRNA	6175	1858	43.3	5657	1296	55.0	5593	1206	57.0	51.8
psiCHECK TM -2/B2M+B2M#2 siRNA	8532	1300	71.3	7452	1126	70.3	7478	958	74.4	72.0
psiCHECK TM -2/GAPDH	369	1045		535	896		474	703		
psiCHECK TM -2/GAPDH+scrambled siRNA	1236	2620		843	1034		1102	1133		
psiCHECK TM -2/GAPDH+GAPDH#1 siRNA	1503	101	96.8	1354	174	91.6	1774	90	95.07	94.5
psiCHECK TM -2/GAPDH+GAPDH#2 siRNA	2162	767	83.3	2052	489	80.6	2225	466	79.63	81.2
psiCHECK TM -2/KIT1	524	1105		506	751		619	682		
psiCHECK TM -2/KIT1+scrambled siRNA	1512	2580		1130	1199		1390	1325		
psiCHECK TM -2/KIT1+KITexon2 siRNA	1933	241	92.7	1526	202	87.5	2368	174	92.28	90.8

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 = beta2microglobulin. Exp = experimental replicate, LU = luminescence units, KD (%) = percentage knockdown efficiency.

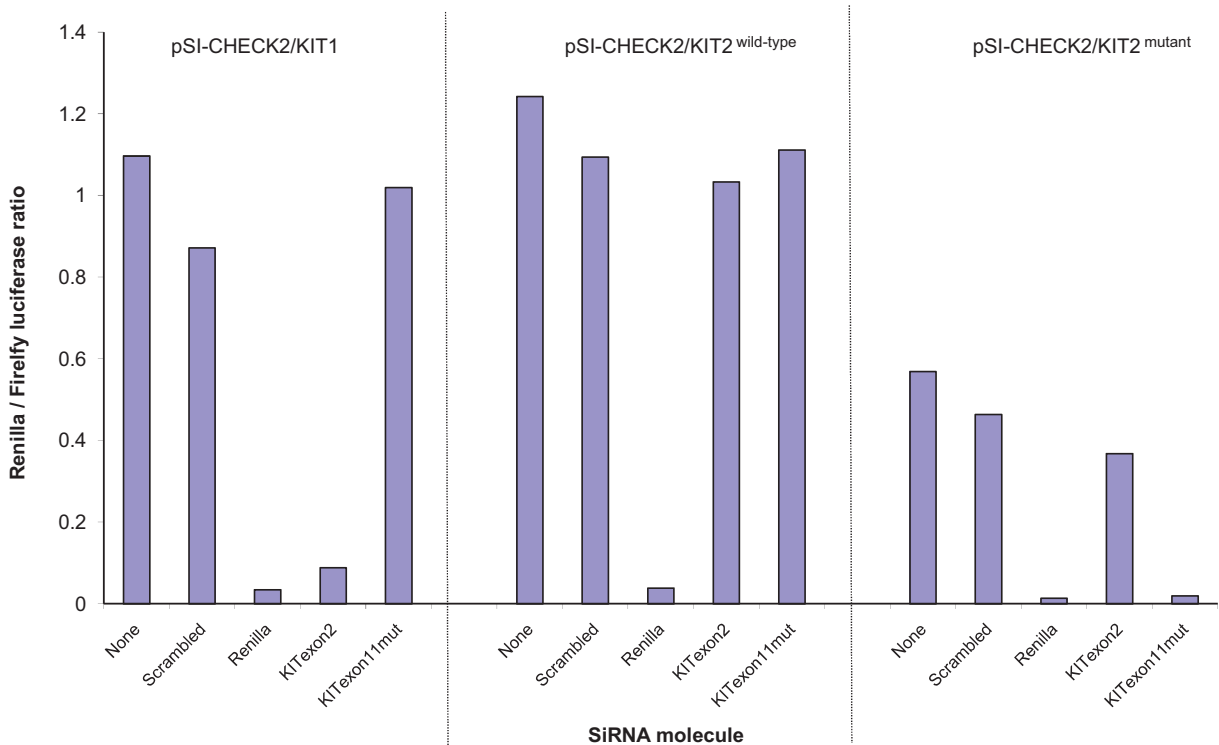


Fig. 2. Knockdown efficiency of an SiRNA molecule that targets the C2 KIT mutation. Recombinant psiCHECK-2 plasmid DNA was generated containing either the 5' region of canine KIT (KIT1) or the region containing exon 11 (KIT2) of either the wild-type or mutant version of the gene. Triplicate wells of 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.

regions of the KIT coding sequence targeted by the SiRNA molecules selected/designed. This strategy has its disadvantages, since the secondary structure of the native KIT mRNA is lost, which might influence the efficiency of SiRNA silencing. This also precludes assessment of the effect of SiRNA on KIT protein expression, although the reporter gene assay is based on evaluation of luciferase activity, rather than mRNA expression. Both firefly and renilla luciferase activity were consistently lower when using the psiCHECK-2 construct containing 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^{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 attempted to validate 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, viral vectors) 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.

Conflict of interest statement

No conflict of interest.

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