

**THE USES OF RNAi TECHNOLOGY IN THE VETERINARY PROFESSION,
WITH EMPHASIS ON ITS TREATMENT OF CANINE ALPHA 1-
ANTITRYPSIN DEFICIENCY**

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PASS WITH DISTINCTION

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ABSTRACT

In 2009 it was estimated that the dog population in the UK was approximately 8 million under the ownership of 6 million households (1). Using this as a representation of the popularity of the canine species in today's society and combined with my personal interest in canine welfare, my research was naturally drawn to monogenic diseases in the canine species. I discovered a rare but severe monogenic disease called Alpha 1-Antitrypsin Deficiency (A1ATD), which affects both humans and animals. This is a recessive autosomal (i.e. not on an X or Y chromosome) genetic disorder in which the sufferer fails to produce enough alpha 1-antitrypsin in the blood and lungs and excessive amounts of abnormal A1AT in the liver, which can lead to chronic pulmonary obstruction disorder, emphysema and, in rare cases, primary biliary cirrhosis. In this paper I hope to prove how this disease and similar diseases could potentially be eliminated by using RNAi to silence this gene.

INTRODUCTION

The first discovery of RNAi was by Fire et al. in 1998, following their work with the *Caenorhabditis elegans* roundworm. They discovered that, by introducing double stranded RNA (dsRNA) into a cell a defence sequence was put into action that resulted in any matching RNA being destroyed (2). (see Figure 1). Two years later in 2000 it was discovered by Zamore et al. that the long strands of dsRNA could be broken into smaller fragments by an enzyme called Dicer (3). The first suggestion of the use of RNAi in the mammalian world was by Song et al., who proposed the idea that it could be used therapeutically in mice in order to protect them from fulminant hepatitis (4), a disease which causes severe loss of liver function due to viral infection. It was, however, in 2006 when the term "gene silencing" really came into play as Mello and Fire won the Nobel Prize for their work in the discovery of the RNAi mechanism, which showed how a gene could be "turned off" by post-transcriptional gene silencing (5). However, there was a problem. By introducing the long chain dsRNA into a mammal an anti-viral inflammatory response was triggered, called the interferon response. This prevents the synthesis of any proteins in the cell and hence prevents the use of dsRNA to induce the RNAi response in the cells of most mammalians. However this interferon response does not affect strands of short double stranded RNA (siRNA) less than 30nanoteslas – the optimum length of siRNA being 21 nanoteslas. (6)

This of course led scientists and both medical professions to consider the prospect of RNAi as a step closer to the medicine of the future. However as of yet the mechanism is not foolproof. Currently the only way scientists have to transfer the siRNA to the cells intended is by injecting then transfecting them with dsRNA. There is also the longevity issue. Although the effects of the gene silencing do appear to be passed to the next generation, its effects seem to be lessened. This would mean that, should it be injected into a dog suffering from A1ATD, the effect of silencing the gene would not reach all cells as its effects would be reduced. To combat this, a method would have to be devised in which the siRNA was constantly being released into the organism concerned. Recently scientists have created expression vectors as a way of continually expressing siRNA into stable transfected mammalian cells (7 -13) and this may be a way to prevent this issue. There is also the possibility of using nanotechnology to create molecules similar to lipids that could be used as delivery agents to allow siRNA to reach cells ten times more effectively (14).

In short, RNAi is the mechanism in which dsRNA suppresses the expression of a gene bearing a sequence to which it is complementary. Examples of research being done in this area include the "development of gene silencing (RNAi) as a therapeutic for inflammatory diseases" (15) and the "use of gene knockout and RNAi to investigate gene function and, very recently, on the genetics of drug resistance" (16). However the main area in which scientists are using RNAi is in cancer treatments. There is a possibility that, if the target mRNA was the cancer gene, the cancer cells could be "killed off" (17). (see Figure 1)

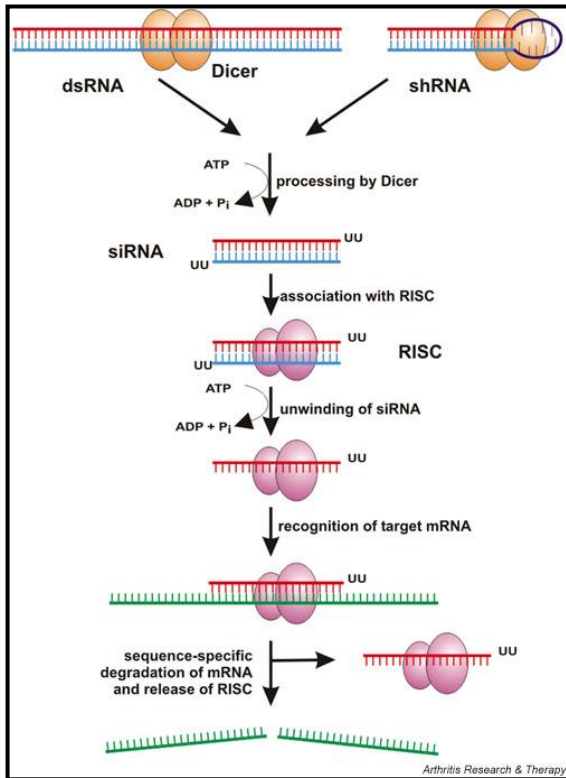


Figure 1

If this were the case, there are an infinite number of possibilities for disease treatment in both humans and animals. Any monogenic disease could be cured relatively easily as it only requires the cleavage of one gene. Obviously polygenic diseases would prove more complex as there is more than one gene needing to be silenced. However by isolating each different part of the disease and silencing each gene in turn, it could be possible to treat polygenic diseases in this way as well.

There are a number of monogenic diseases which affect dogs: Exocrine Pancreatic Insufficiency, in which the dog suffers a loss of acinar cells in the pancreas and as a result does not produce enough digestive enzymes (can also be caused in humans as a secondary disease from diseases such as cystic fibrosis – Collies and German Shepherds are often sufferers of this. Meningo-encephalitis, a fatal disease affecting Greyhounds under the age of one where the brain and spinal cord become inflamed.

Dogs can also suffer from Alpha 1-Antitrypsin Deficiency (A1ATD) which is the disease I would like to consider in this paper.

DISCUSSION

Alpha 1-Antitrypsin Deficiency is a monogenic disease which affects both dogs and humans. I believe it would lend itself nicely to being a new area of RNAi research and gene silencing as it is monogenic. It would also be a good place to start as curing or beginning to alleviate the symptoms of this disease would affect the worlds of both medicine and veterinary medicine.

The disease itself is not necessarily fatal, however it would greatly worsen the quality of life for the sufferer and the prolonged effect of its secondary diseases might indeed contribute to death. It is caused by defective production of a protease inhibitor (serpin) alpha 1-antitrypsin (A1AT), which protects tissues from enzymes from inflammatory cells (e.g. neutrophil granulocytes), most specifically their enzyme elastase. This results in the uncontrolled breakdown of the connective tissue elastin, especially in the lungs. This causes emphysema (see Figure 2) which can chronic pulmonary obstruction disorder (COPD), reducing air flow to and from the lungs. A1AT is produced in the liver and, in some rare cases, the genetic mutation can cause problems in the secretion of A1AT in the liver. This leads to cirrhosis, most commonly in humans. Research shows that dogs also can suffer from chronic liver disease as a result of A1AT. (18)

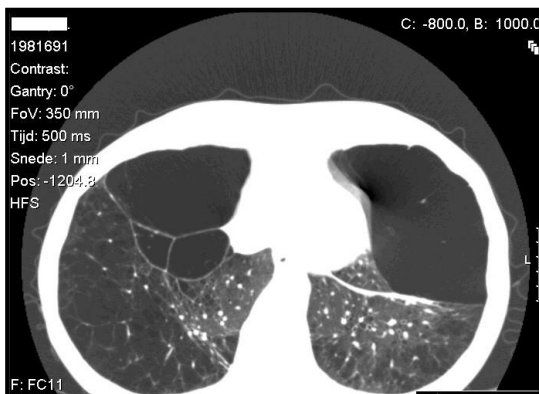


Figure 2

As a result of the COPD and emphysema that is secondary to A1ATD, many sufferers will die of respiratory or

cardiac failure, pulmonary infections or cardiac arrhythmias (19). At the moment there is no way of preventing A1ATD, short of not allowing two dogs who are both carriers of the gene to breed. This is unlikely to happen as A1ATD is a relatively rare condition that the majority of dog breeders are unaware of. The same is true of human carriers, as only 1 in 50,000 to 70,000 people in North America suffer from A1ATD. (20).

Research has already been undertaken by the veterinary profession into how RNAi technology can help animals. For example, in 2006 it was discovered that by injecting siRNA which was targeted to silence the apolipoprotein B (ApoB), responsible for the secretion and assembly of the “bad” cholesterol low density lipoprotein, the expression for this gene was greatly reduced over a course of 11 days in the monkeys tested (21). However I feel it is theoretically possible to cure any monogenic disease in the canine species by using the RNAi pathway as shown in Figure 1. Indeed, I believe it is possible to use this technology to silence certain disease causing genes and therefore eradicate numerous monogenic diseases in the canine species.

In order to effectively silence the A1ATD gene you would have to produce dsRNA that contained complementary base pairs to the target mRNA which contained the gene for A1ATD. This technique could be adapted to any other monogenic disease in dogs, or indeed any other mammalian species. This would mean that when the enzyme Dicer fragmented the dsRNA into siRNA. The siRNA would contain these complementary base pairs, and therefore when RISC was activated this siRNA would associate with the A1ATD gene. As a result when the target mRNA was cleaved and therefore taken out of the mRNA, the base pairs that coded for the A1ATD gene would not be able to be translated. This in turn would mean the gene for A1ATD would not be coded into amino acids and therefore proteins as the mRNA would be removed from every part of the body. This is because the genetic code for the proteins containing the undesirable A1ATD gene would never get past the stage of translation.

If disease treatment in this way was achieved its impact on the medical and veterinary professions would be phenomenal. Dog breeders and humans alike would never need to worry if they were carriers of the recessive A1ATD gene causing the disease. Any dogs that breeders were wishing to breed could be injected with the dsRNA that contained the complementary base pairs for the A1ATD gene in its target mRNA. By the use of the expression vectors or nanotechnology mentioned earlier (6-14) the siRNA could be continually expressed and so the A1ATD gene would be eliminated. This would be because the siRNA would continually seek the complementary base pairs that exist on the A1ATD gene's mRNA. This could be proved by the neonatal testing of new puppies or babies (21). If the parents had been treated with RNAi technology there would prove no need for this as the effect of the gene silencing would continue.

Human A1ATD sufferers can experience pulmonary and liver disease to such an extent that an organ transplant is the only viable option of treatment (23). If a dog with A1ATD was presented to its veterinary surgeon with chronic lung or liver failure euthanasia would be recommended to its owner as there would be no treatment that would be viable financially or with regards to the animal's deteriorating quality of life– liver and lung animal to animal organ transplants are not authorised in the UK (24). By using RNAi technology the disease could be averted before reaching this stage, hence protecting the dog from suffering and the owner from financial and moral dilemmas regarding the animal's quality of life.

However the use of RNAi does raise many ethical and safety issues that must be addressed before considering the technology fit to use. There is a risk that by silencing one gene you may inadvertently cause other defects in the animal if the gene being silenced is in any way linked

to another gene in the canine species. This would then put you in a situation with a difficult decision to make – do you allow this new scientifically caused disease to continue, risk it mutating and becoming out of control, hence making the situation worse? Or do you stop the gene therapy and hence allow the disease you were initially trying to prevent continue, with the possibility of allowing many more future generations of dogs to suffer from it? In this situation you would have to consider both sides of the argument and make an impartial decision as to which would be the lesser of two evils. Hopefully future research will prevent this ever being anything more than an ethical debate, but it is definitely a worrying prospect and some thought must be given to such possible events.

A safety issue that also must be considered is the risk of infecting the animal or human being infected with HIV, if HIV lentiviruses were going to be used as vectors for the RNA. This being the case there would be a risk of the HIV self-replicating when the vectors were produced (25) and hence infecting the organism with HIV. If RNA were to be transported using this method this risk must be considered and necessary precautions must be put in place to prevent this happening.

Another issue to consider is that, in order for RNAi to be used to silence a gene and hence cure a disease, the specific gene in question must be located. In the case of A1ATD, research has found the gene responsible and therefore enabling the neonatal testing mentioned earlier (21), but for other diseases this is not yet known. To enable scientists to locate these genes they must first establish “which gene does what” in the animal. This would involve silencing each gene and letting an embryo develop to see what aspect of its genetic make-up is, in effect, “missing”. There is concern over the number of animals such investigation would leave deformed or disfigured; again it is a matter of weighing up the “pros and cons” of each option and choosing the lesser of two evils.

There is also the question of when to stop with RNAi technology. There is a risk that, as scientists become more confident in using RNAi to treat monogenic diseases and any problems are overcome with this aspect, the next natural “step up” will be to use RNAi to treat polygenic diseases. This could lead to science using RNAi to combat every single genetic disease, leading to the possible complete elimination of disease (that can be treated with RNAi). Although this initially seems like a brilliant prospect, it does raise numerous issues which must be considered and thought through carefully. By reducing diseases we are in effect playing havoc with natural selection. This could lead to certain populations of animals spiralling out of control as they are not being “killed off” by diseases that would keep numbers at a reasonable level. If this was applying to a situation where breeding is closely controlled, for example, pedigree Daschunds, this would not be a problem. However if such animals were released into the wild or any of these disease free animals were allowed to breed with wild animals a problem could arise rapidly.

Dartmoor ponies are already infamous for their rapidly multiplying numbers. If you take out the effect of disease on population of these ponies, their numbers will increase even more, aggravating an already problematic issue. As a direct result of this people could turn to mass culling of such species in order to keep the numbers down – this again presents you with the issue of man-made culling versus death from natural selection and the ever constant theme of which is the lesser of two evils.

However this could be beneficial in some sectors as it could be used to help increase the number of endangered species in the wild. For example, if red squirrels were treated with RNAi to protect them against some diseases, they would be able to compete with the grey squirrel, numbers-wise. The same principle could be applied to many other endangered

species such as sea otters, Iberian lynx, Giant Pandas and many others, possibly preventing the extinction of many animals. If the animals from conservation centres which were released were disease-free following RNAi treatment they would stand a better chance of surviving to breed and hence increasing their numbers.

In the canine species the RNAi pathway (as outlined in Figure 1) could be used to combat many monogenic diseases. Exocrine Pancreatic Insufficiency (EPI) is a disease which results in the degeneration of the pancreas and hence a lack of digestive enzymes leading to maldigestion and malabsorption of nutrients . It mainly affects dogs, specifically Eurasians, Collies and German Shepherds, but can also be present in cats and humans, especially as a secondary condition to cystic fibrosis and Shwachmann-Diamond Syndrome. Similar to A1ATD as it is an autosomal recessive gene that causes the disease, and also similar to A1ATD RNAi technology could be used to potentially eliminate the disease from the canine species in a method similar to that I outlined earlier. Due to the “autoimmune nature of the disease together with the clinical characteristics lending some resemblance to diabetes mellitus” (26) there is also a possibility that a breakthrough in the treatment of EPI could lead to a further understanding of diabetes. This in turn could potentially lend itself to the cure of diabetes mellitus– another disease which affects numerous species – by using RNAi technology.

CONCLUSION

In theory at least, the application of the RNAi pathway in the canine species is relatively simple. Obviously it is not that simple in practice, but essentially there is scope for it to be used to cure various monogenic diseases in canines and, following on from that, humans. By identifying the gene responsible for a particular disease we can silence it and hopefully cure that particular animal of it. It has been proved that the silencing effect lasts over one generation, and so if an animal is treated any of its offspring will not suffer from the disease. By using technology such as expression vectors (7-13) and lentiviruses (25) this would mean that the gene silencing effect could continue indefinitely down a species.

However a crucial issue is that the effect of gene silencing could potentially diminish over time. Current evidence suggests otherwise; this does not take into account the effect of expression vectors and lentiviruses continually expressing the siRNAs. Therefore this is an area of RNAi research which needs to be considered to reach a definitive conclusion in this matter. If the gene silencing effect was not reduced over time the possible uses of RNAi in medicine is unlimited; if the effect was reduced then obviously some solutions must be found to alleviate this problem.

I believe one such solution would be to monitor the offspring of treated animals to see if the effectiveness of treatment against the disease had continued. This would enable one to see at which generation the gene silencing would become ineffective i.e. when the offspring tested positive for the disease. One would then be able to conclude that the gene silencing effect became ineffective at the generation before the generation that tested positive, and then one could take preventative measures to increase the gene silencing effectiveness. One way of doing this would be to administer a “top up” of sorts containing the dsRNA with the complementary base pairs to the target mRNA, in essence starting the whole process again.

In theory the use of RNAi in the canine species is limited only to that of the diseases the dogs are affected by and the vision of the many scientists working globally on this new, essentially profession-changing treatment. It seems to me this will definitely be the medicine of the future by disease treatment along the lines of the methods I have outlined earlier for A1ATD treatment. However I do believe that the progress and use of RNAi need to have strict parameters set. It is one thing to improve gene therapy for the good of the medical community

and in the interest of pushing the frontiers of science, however due to human nature I do not believe everyone will necessarily be motivated purely by the goodness of their hearts. Once you introduce gene therapy such as RNAi into society there will be a risk of crossing the line between gene therapy for medical uses and gene therapy for purely aesthetic reasons. Parameters must be set, as mentioned earlier, and these must be closely regulated and monitored. In the wrong hands the technology could be dangerous, becoming a eugenics tool and potentially leading to scientists “playing God”.

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Figure 2: CT scan of lower lung lobes showing emphysema and bullae. Subject was a sufferer of alpha 1-antitrypsin deficiency

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