

# RNA Interference and its potential use in the treatment of Canine Parvovirus

BY

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## **Abstract**

In this project I have looked at how RNA interference works and have discussed its possible uses in medicine and veterinary medicine, along with the ethical concerns arising from such uses.

I have specifically focused on the use of RNAi in combating canine parvovirus (CPV), a highly contagious viral disease which results in acute gastrointestinal disease or heart disease in young dogs. My research has demonstrated the way in which RNAi could be a feasible method in treating the viral disease, using our current knowledge of the mechanism and existing technology.

## **Introduction**

DNA, i.e. deoxyribonucleic acid was discovered in 1953 by James Watson and Francis Crick (1) and has greatly influenced the scientific world as we have broadened our knowledge on genetics and hence, genetic diseases such as Cystic Fibrosis and Huntington's disease. Our intelligence on genetics was deepened further in 1956, just 3 years later, when Elliot "Ken" Volkin and Lazarus Astrachan's discovered 'DNA-like-RNA,' which in 1960 was called 'messenger RNA' by François Jacob and Jacques Monod (2). This gave an explanation as to how ribosomes received information from the nucleus to synthesise the polypeptides required by living organisms. However it was not until fairly recently that short interfering RNA (siRNA) came to the attention of scientists, yet now it is regarded as extremely significant as its potential uses in medicine and veterinary medicine are extraordinary; these shall later be discussed.

Firstly, before any suggestions can be made as to how ribonucleic acid can be manipulated to benefit us, it is important to understand its function. Proteins are synthesised from just 20 amino acids, within the cytoplasm, and different sequences of these amino acids code for different proteins. The way in which these proteins are manufactured is dependent on information provided by the DNA which is present in the cell's nucleus. Transcription, the process of making pre-mRNA begins when DNA helicase breaks the hydrogen bonds between the bases of the DNA molecule; this causes the strands to separate, exposing the nucleotide bases. A second enzyme, RNA polymerase, then causes the nucleotides on one of the strands, known as the template strand, to join with free, individual, complimentary nucleotides within the nucleus. These individual nucleotides build up to form a strand of pre-messenger RNA, which is modified, after transcription, into messenger 'm' RNA during a process called splicing in which base sequences copied from introns (portions of DNA within a gene that do not code for a polypeptide i.e. are non-functional) are removed. The mRNA leave the nucleus through a nuclear pore, into the cytoplasm where they attract ribosomes; the mRNA attaches to ribosomes, beginning a process called translation in which the mRNA attaches to complementary transfer-RNA molecules, resulting in the amino acids, which the tRNA carry, linking to form a polypeptide (3). It is this stage, translation, which is particularly significant and fascinating when contemplating the future of veterinary medicine. This is because, if translation was stopped i.e. if the mRNA never arrived at the ribosomes, certain genes could be stopped or 'silenced' resulting in what may be massive breakthroughs such as cures for cancer, or hereditary diseases such as cystic fibrosis. In essence we could use the technology to turn off any monogenic disease requiring the cleaving of one particular gene and perhaps polygenic diseases if each part of the disease was to be silenced individually.

The 'silencing' of genes is known as RNA interference, as it is the RNA that is influenced to create the desired effects. RNA interference was first described by scientists looking at plants in 1990. Rich Jorgensen at the University of Arizona wanted to create petunias with a deeper purple colour by inserting an extra copy of the gene that coded for the flower's purple colour. However, instead of resulting in a darker colour, the petunia came out variegated with white areas where there was no pigment at all, as seen in Figure 1. The gene he wanted to over-express was turned off as the DNA

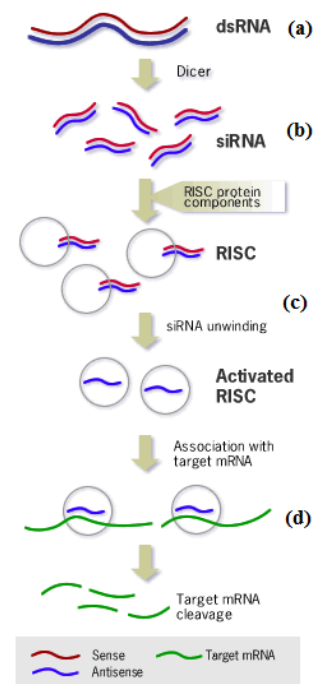


**Figure 1: Experimentation on petunias (4)**

inserted was recognised as non-self by the cell; believing it to be a virus, the cell destroyed and blocked the mRNA responsible for sending the information to the ribosomes, hence the gene was not expressed at all. This effect was named co-suppression (5).

The mechanism for the gene silencing due to RNA interference, as shown in Figure 2, is as follows. When a foreign dsRNA (double-stranded) molecule enters the cell (a), it is, as afore mentioned, recognised as a virus, hence an interference response is triggered. Firstly, the dsRNA is cleaved by an enzyme called DICER (b) to form small interfering double-stranded RNA (siRNA); these are 20-30 base pairs in length. The siRNA activate another enzyme called the RNA-induced silencing complex (RISC) which separates the dsRNA strands (c). One of these is used to target complementary base pairs of mRNA molecules, cleaving them in the middle of the complementary region (d). This cleavage does not require the presence of ATP, however ATP makes numerous cleavages more efficient. The remaining sections of the mRNA are further broken down into single nucleotides which are reused by the cell, meaning the proteins previously synthesised by the RNA after transcription, will no longer be produced (6-7).

Co-suppression was seen in plants and fungi other than petunias, however when dsRNA was injected into mammals, anti-viral inflammatory based response was triggered due to the molecules being of lengths greater than 30 nucleotides, causing all protein synthesis to terminate.; In 2006, Andree Fire and Craig C. Mello shared the Nobel Prize in Physiology Or Medicine for their work on RNA interference in the nematode worm *C. elegans*, which they published in 1998 (7-8), showing that gene silencing was not just restricted to plants, when using shorter chains of dsRNA. At present, research is being done in looking at how RNAi can target some of the world’s largest killers such as cancer, HIV and genetic orders such as Huntington’s Disease.



**Figure 2: The mechanism of RNAi (9)**

Cancer is not a single disease but is the term given to a large group of approximately one hundred diseases, involving mutant genes that result in uncontrolled cell growth and the migration of cancerous cells to distant sites within the body. Cancer kills; one out of every four deaths in the United States is from cancer (10). Recently scientists have silenced over twelve known cancer causing genes and have evaluated specific genes that maintain tumour cell survival. For example in 2007, scientists at Abbot Labs used RNAi to establish that *Ran* and *TPX2* reduced the survival of cells that had been transformed by oncogene ‘*K-Ras*’, hence these two genes could be targeted for therapeutics in the future. (11) Furthermore, Greg Hannon and his group at Cold Spring Harbor Laboratory are currently attempting to determine the function of 15,000 genes in a variety of human cancer cells. This research could possibly identify specific genes, previously unknown to be related to cancer, offering proposals for new innovative treatments (12).

The possible uses of RNAi in targeting HIV are also being researched. However HIV is a more difficult target as it is constantly mutating and is ‘escape-prone’ (12); as a result, more than one RNAi strategy must be used to prevent viral escape. By 2002, Phillip Sharp and his colleagues at MIT announced they could use RNAi to interrupt various steps in the HIV life cycle. Nevertheless, these, along with experiments carried out regarding cancer cells, are largely done in cell cultures, not human patients. John Rossi and colleagues at Colorado State University used RNAi to target several HIV genes to stop HIV replicating and targeting the immune system. Rossi’s group is hoping to create a way in which the RNA can be delivered, by extracting stem cells from a patient’s bone marrow, altering the genes in these cells with the RNA therapy, then transfecting them back into the patient, where they would develop into healthy immune-system cells safeguarded against HIV.

**Discussion**

I have chosen to focus my discussion on Canine Parvovirus (CPV), a highly contagious viral disease which mainly affects dogs, taking on two forms; the intestinal form results in severe vomiting and haemorrhagic diarrhoea, whilst the cardiac form can lead to cardiovascular and respiratory failure in young puppies (13). The virus invades and

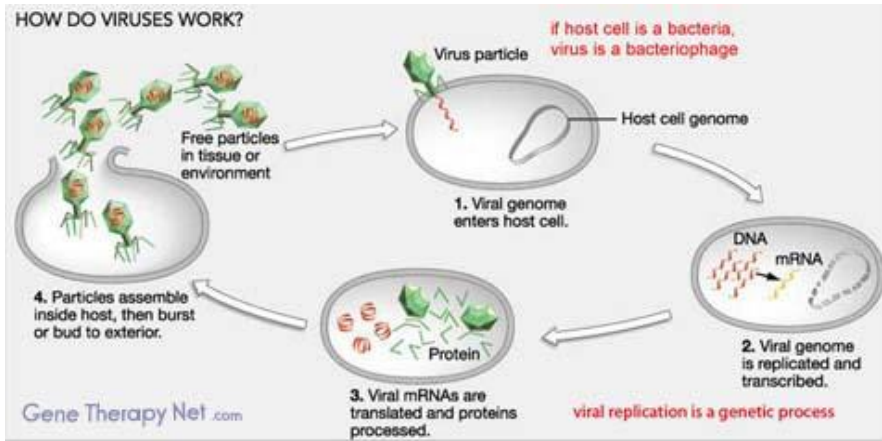
destroys growing cells in the intestine, bone marrow and lymphoid tissue; the invasion of the bone marrow cells results in a decrease in the number of white blood cells causing an increased vulnerability to bacterial infections (14) and on occasions, endotoxemia which may cause haemorrhages, necrosis of the kidneys, and shock (15). It is primarily spread from dog to dog by contact with their faeces, however it can also be spread on infected items such as clothing, and it is believed that rodents and insects may also be vectors for the disease. (16). The virus can be fatal; death can occur within 48-72 hours of contracting it, plus mortality rates can be as high as 91% in untreated cases of the cardiac form of the disease. Breeds susceptible to the disease include Doberman pinschers, Rottweilers, German shepherds, Staffordshire terriers, black Labrador retrievers and dachshunds (14). Dogs of all ages are at risk but puppies are even more susceptible to the virus as they are not protected by both vaccinations and maternal antibodies.

There are two types of canine parvovirus – CPV1, also known as ‘canine minute virus’ and CPV2, discovered in the late 1970s, which is the virus that causes the disease in dogs on which this discussion is based. This CPV2 virus has three different known strains, CPV-2a, CPV-2b and CPV-2c. CPV-2c has only been recently discovered hence some have claimed that vaccinations against the parvovirus may now be ineffective; however this is a controversial issue as studies have been undertaken, showing that the vaccines remain adequate protection against the 2c strain of the virus. (13). These vaccines are designed to treat a variety of symptoms.

Canine parvovirus causes different symptoms depending on what form is taken. The intestinal form, otherwise known as ‘diarrhoea syndrome’, or enteritis, is diagnosed by symptoms of depression, lack of energy, vomiting, high fever, loss of appetite (anorexia), and severe bloody diarrhoea; the vomiting and diarrhoea can continue until the death of the animal, therefore it is vitally important to rehydrate the patient (17). The second, cardiac form, or myocarditis, is identified by the following symptoms: difficulty in breathing, an irregular heartbeat and a racy, weak pulse (18)

Research has already been committed to RNAi in Veterinary Medicine; Work is currently being done in researching the use of RNAi in fighting foot and mouth disease (FMD) as the disease is extremely contagious and an outbreak of it is potentially devastating to the economy. For example in October 1967, a farmer in Shropshire reported a lame sow, which was later diagnosed with FMD. The virus spread and as a result approximately 442,000 animals had to be slaughtered; the total cost of the outbreak was £370 million. Foot and mouth disease or ‘hoof-and-mouth’ disease is a viral disease of cloven-hoofed animals, most commonly seen in cattle and pigs though it can also occur in deer, sheep, goats and, more surprisingly, hedgehogs and elephants. Furthermore, an animal does not need to be susceptible to the disease in order for them to spread it e.g. Canada had a case of foot and mouth disease in 1952 when dogs had carried away bones left from dead animals that were victims of a previous infection. The disease is caused by a virus of which there are seven serotypes (groups of microorganisms or cells classified together due to the cell surface antigens they present), all of which can only be distinguished in a laboratory, and produce the same symptoms. These seven are: O, A, C, SAT-1, SAT-2, SAT-3, and Asia-1, the most common of which is the O serotype (19). According to Dr Tim Doran of CSIRO Livestock Industries’, “FMD is known to be one of the most infectious animal viruses known” (20), affecting both humans and animals due to its rapid growth if not contained and controlled. Symptoms include blistering of the mouth and feet which if ruptured, may cause lameness, a high fever, weight loss in adults of the species and swelling of the testicles in males. Tests have been carried out across the world using RNAi and studies by Weizao Chen et al have shown that co-transfection of siRNA, which target specific sequences in the VP1 virus of the FMDV genome, results in a significant reduction level of viruses transcribed (21); this is a promising sign for the veterinary world. Also, work is being done to target internal parasites that are becoming resistant to drenches (20) and against the influenza virus, which is not only a danger to animals, but is a threat to the human race after mass outbreaks of the zoonotic disease; the H1N1 strain i.e. ‘swine flu’ resulted in a human pandemic in 2009, causing thousands of deaths across the globe (22). Results from studies by Qing Ge et al, show that some siRNAs can strongly inhibit the influenza virus reproduction in specific cells (MDCK - Madin–Darby canine kidney cells) (23). This again, is demonstrates the great potential of RNA interference.

However, research has not been committed to the canine parvovirus. As CPV is a virus, *there is no cure* for it. Treatments are provided which aim to maintain the comfort and composition of the infected dog. Fluid therapy is fundamental as once 12-15% of fluid is lost from the body, a dog will die; intravenous fluids are used to rehydrate the patient and provide electrolytes and nutrients to nourish the dog. Many dogs, however, must be hospitalised, enabling veterinary surgeons to provide medicine and constant attention; most dogs are obliged to stay in hospital for at least 2-4



**Figure 3: How viruses work (24)**

days, yet necessitate further treatment at home, provided by the owner. At this moment in time, the best way to deal with CPV is to employ preventative measures by sanitising inanimate objects such as kennels and floors with disinfectant, yet it is impossible to do so in all areas with which a dog will come into contact such as streets and parks. Therefore, if a dog is infected, it is important that it is isolated in order to prevent further spread of the virus. These measures will greatly reduce the amount of virus present in the environment, but only a full series of

vaccinations, supported by boosters, will control the source of an infection and even these are not 100% effective (25). I therefore have dedicated my research to CPV and believe the process of RNAi could possibly be used to prevent replication of the virus, hence the symptoms of the disease would not be expressed and the majority, if not all of the animals contracting the disease would survive. To prevent replication of the virus, the CPV2 would need to be silenced. This would be done by targeting messenger RNA which codes for the CPV2 virus; hence, siRNA would need to be produced containing complementary base pairs of the virus. The siRNA would activate the RNA-induced silencing complex, causing the siRNA to associate with the CPV2 gene. The mRNA would then be cleaved therefore translation, shown by stage 3 in Figure 3, would not occur. As a result, the ribosomes would never receive the code for the synthesis of the proteins containing the CPV2 disease, also shown in stage 3; hence the virus would not multiply to an extent at which the infected dog would be affected such as that in stage 4. Nevertheless, as previously mentioned, there are three strains of the CPV2 virus therefore a way in which all three of the strains can be targeted must be established.

Essentially, there are two ways in which siRNAs can be delivered ‘in vivo’ i.e. directly into the living organism. Firstly, viral vectors can be used to immediately deliver the siRNA to the relevant cells; many gene therapies use retroviruses or adenoviruses to deliver the necessary gene. Retroviruses are a group of enveloped RNA viruses that contain an enzyme that causes genetic transcription to reverse, from RNA to DNA, instead of DNA to RNA. The viral DNA transcribed integrates into the host cell’s DNA strand for the production of new RNA retroviruses (26-27). Using retroviruses allows tissue-specific silencing to occur. Alternatively, the reagent could be chemically synthesised; this too causes the siRNA to become more stable and allows it to survive, undamaged, for longer periods of time in the bloodstream. It also changes the way in which the siRNA are taken up, hence there would be greater uptake into the required cell (28). I suggest that viral vectors should be used in order to deliver the siRNA into a dog containing the CPV2 virus.

However, there are many ethical concerns to be dealt with in regards to the use of RNA interference. Predominantly there is the risk of side effects including vomiting, weakness, an increased susceptibility to infections and the need for high risk operations. Also the patient could receive vector induced oncogenesis, development of abnormalities formed by the expression of transgenic proteins, and if the siRNA are not delivered to the correct cell type at the correct time, it is possible that there would be detrimental effects on the immune system. Moreover, some people oppose retroviruses due to the chance that they may mutate back into a dangerous form of the virus. Four ethical measures have been created to ensure that gene RNAi and other gene therapies are moral and reasonable. These include: do no harm, avoiding the causation of harm, do good, respect the fundamental self-worth, dignity and decision-making capacity of individuals, and to distribute the benefits, risks and costs fairly’ (29). RNAi treatments will also be judged on the same criteria as other drugs available: potency, stability, and safety (30).

Many argue that RNAi is comparable to ‘playing God’, interfering with nature. It can be reasoned that RNAi will disrupt the process of Natural Selection by which weaker individuals within species die out, maintaining a strong, controlled population. Without diseases, which may be eradicated by RNAi, species may become overpopulated.

Conversely, it can be argued that surely it is better to prevent any further pain and suffering; for example chemotherapy can kill a patient before the cancer does, causing hurt and upset in the process, whereas RNAi targets specific genes, therefore if delivered correctly, this would not be a problem.

In order to develop RNAi therapeutics, animal testing must be carried out, causing great controversy in itself. For example, so as to silence a specific gene, that gene must first be identified; large numbers of animals would be left with disfigurements due to the process of finding the specific gene, in which individual genes are silenced and the embryo is grown in order to determine the effects. Animal testing, however, generally provides more reliable results than those produced in cell cultures and using computer generated testing, yet it should only be used when absolutely necessary, as it may result in permanent damage to cells, accidental deaths or the need for euthanasia. Tests can either be done on animals already suffering with the condition or by inflicting the specific disorder onto an animal. The first option means that no additional animals must suffer as a consequence of research into RNAi; there are, however, less animals in such a situation therefore the procedure would be slow, but this method is more realistic, natural and considerate, whereas the induced disease method would be strongly opposed by animal rights activists and may not produce such dependable results. On the contrary, it can be argued that using animal testing whilst researching RNAi will ultimately benefit not only humans, but animals, as previously discussed, therefore the number of animals saved by the 'end product' i.e. treatments developed, will greatly outweigh the number of animals harmed in the process. Using the previously mentioned measures of ethics, these points must be evaluated before testing takes place. Methods could also be refined with the intention of easing the pain and stress caused by testing.

## **Conclusion**

In conclusion, I believe that the use of siRNAs and the mechanism of RNAi should be considered in regards to the treatment of Canine Parvovirus. This is because, although I have not proven scientifically how this would work, my research and development of ideas has shown that the procedure could be possible if delivery methods were improved and specific genes i.e. the CPV2 gene, could be targeted. RNAi would greatly benefit the world of medicine and veterinary medicine if used effectively, as viruses currently cannot be cured, but only treated, hence if mRNA could be disengaged and protein synthesis could be halted, diseases caused by viruses could be a thing of the past.

Our knowledge on genetics has been significantly broadened in the past century and even more so in just the last decade, so it is extremely likely that problems arising from our current awareness of RNAi, such as the delivery of siRNAs to the correct cells, will soon be solved.

Finally, the ethical issues arising from RNA interference must be addressed in order to pay respect to both animals and humans, before RNAi can be taken to the next level. The use of RNAi is an extremely exciting topic as advancements have already been made when focusing on some of the world's major killers such as cancer and HIV; new applications of the system are being discovered continually and its potential, including the use against canine parvovirus, is extreme; one day almost any viral disease may be cured thanks to RNA interference.

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