

A study into RNAi- a way of silencing viruses for good?

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Research Paper Based on Pathology Lectures at Vet-Medlink 2009.

Abstract.

In this research paper I plan to study the concept of RNA interference. RNAi is responsible for turning genes off or silencing them but is not found in humans. After first hearing about the idea of using siRNA-short double strands of RNA- at the Vet Med-Link Pathology Conference I wanted to discover the possible advantages that RNAi could have to the world of medicine in a solution for HIV, a disease which has cost the lives of millions of people all over the world. During my investigation I wanted to discover whether or not it was realistic to deduce that in the future RNAi could be used to treat the HIV virus. I also wanted to delve into the ethics of RNAi and see what possible barriers prevented this potentially world changing scientific development from occurring. After thorough research into this idea I concluded that the prospect of RNAi interference being used as a solution for HIV is a definite possibility in years to come after further experimentation and developments have taken place.

Introduction.

Proteins are made in two processes, transcription and translation. Transcription is the process by which the sense strand of the DNA is copied onto single stranded mobile RNA molecules that are called messenger RNA molecules (mRNA). Free nucleotides made up of a sugar, phosphate and base are found in the cell. An enzyme called DNA polymerase forces the nucleotides to bond alongside their opposite base pair (cytosine and guanine pair together and adenine and uracil which replaces thymine pair together). The mRNA separates from the DNA and diffuses out of the nucleus through a nuclear pore leaving the DNA behind. Found in the cytoplasm are ribosomes and tRNA molecules. At the top of the tRNA a specific amino acid is found, and an anticodon is found at the base of the tRNA.

The anti-codon at the tRNA corresponds with the codon on the mRNA, for example if the first codon on the mRNA was AUG that means the anti-codon on the tRNA would be GAC, which corresponds to Methionine. The ribosome moves along the mRNA systematically fusing together the amino acids as it goes along. The chain of amino acids is complete when the ribosome comes across a stop codon and a polypeptide is created. These polypeptides can become proteins.

Therefore this means that there is the possibility of two places where the process of protein synthesis could be stopped-before translation or before transcription. So far scientists have been researching the possibility of halting the transcription process. The cellular complex Dicer cleaves a dsRNA molecule which produces double stranded duplexes. The production of these 21-23 nucleotide RNA duplexes called small interference RNA (siRNA) is a way of halting protein production in eukaryotic cells before translation takes place. It is a way of stopping the harmful effects of the proteins that these mutant genes would encode for e.g cancer before the problem arises. RNAi first came to light after the *Caenorhabditis elegans* genome project was completed in 1998. The process of RNAi was found to be naturally occurring in the fruit fly *Drosophila melanogaster* as well as in some plant species (Alvarado 1999). Further extensive research was carried out by Andrew Fire and Craig Mello on the nematode worm, *C. elegans* and they came up with a way of silencing precise genes by double stranded RNA (dsRNA) - a technology called RNA interference (RNAi). They shared the Nobel Prize for Medicine in 2006 for their ground breaking studies into RNAi and its potential benefits.

At the moment the most popular application of RNAi would be to protect organisms from viruses for example HIV by suppressing the activity of transposons. Transposons are segments of DNA that can move around to different positions within the cell and can cause mutations. Other uses would be the ability to turn off cancer cells which arise from a mutation and have no Hayflick limit- so have the ability to keep dividing unchecked.

Small interfering ribonucleoprotein complexes (siRNPs) either contain sense or anti-sense siRNA which have the ability to cut nucleic acids. Sense siRNA are called anti-sense cleaving and anti-sense siRNA are called sense-cleaving. If gene silencing was to take place the mRNA transcribed from the gene would have to be cleaved through an antisense siRNA preventing it from being translated into a protein.

At first it was believe that by introducing dsRNA to mammal cells it would halt the production of the proteins they correspond with. However a problem arises when the dsRNA is longer than 30 base pairs as it causes an interferon based inflammatory response causing all of protein production to be halted due to a second RNAi pathway. The way scientists have overcome this problem is by using siRNA which instead of causing a nonspecific response as dsRNA does it only targets the particular problem gene for example it could target the gene for cancer.

Discussion.

Whilst much has been discovered about the use of RNAi in combating viruses and gene defects that lead to problems such as cancer and HIV there is still much to be uncovered and many problems that need solutions. Scientists hope that RNAi will be able, in the future to be used alongside more conventional methods such as chemotherapy to help combat cancers. There is also the major obstacle of overcoming ethical hindrances such as whether it is morally acceptable to meddle with cells to such an extent that the process of translation can actually be stopped. During this discussion I intend to go into depth about the future developments of RNAi in relation to HIV and also discover whether the advantages outweigh any potential negatives to this new technology.

One of the major advantages RNAi offers is that it targets a short section of a viral protein meaning even a small viral genome could result in a large number of prospective areas for the RNAi to target. So theoretically once a viral genome sequence is discovered the use of RNAi could begin straight away. In comparison, in highly active antiretroviral therapies (HARRT) the anti-HIV small molecule drugs used can only target one or two viral enzymes. However there are still a large number of problems to overcome for example viruses replicate very quickly and a large build up of mutations can result in the virus becoming resistant to the RNAi suppression. In HIV type 1(HIV-1) the transcriptional activator protein (TAT) can prevent the RNAi from

working by inhibiting Dicer. One way this could be overcome is by catching the virus early enough in the virus cycle or focusing on restraining the suppressor RNA. This would mean having to identify the suppressor RNA and then making that the focus of what is being targeted which could prove difficult.

Another possibility that I would like to consider in more depth would be to actually see whether RNAi could be used to build up a resistance to the virus before any symptoms are actually identified. J N Leonard and D V Schaffer wrote in their paper that hematopoietic stem cells(a stem cell from which all red and white blood cells evolve) could be produced with a virus *ex vivo* that could mean anti- HIV RNAi constructs would protect T cells and macrophages against HIV. An element regarding this I would like to consider is whether the amount of RNAi-inducing nucleic acid delivered has any effect on the results. Is there a dosage that is too low to have a noticeable effect in protecting the T cells and macrophages? Also can too much RNAi inducing nucleic acid be delivered that causes problems for instance damaging the T cells and the macrophages rather than protecting them? The way I could carry out this experiment is by using varying amounts of RNAi inducing nucleic acids and seeing what amount has the most beneficial effect. Of course a large sample would have to be carried out and the experiment would have to be carried out repeatedly to make sure I could make a valid conclusion from my results. Also I would have to consider that whilst a certain dosage of RNAi inducing nucleic acids may be most beneficial in the treatment of protecting T cells and macrophages against HIV in other viruses and in other types of cells varying amounts of the acids may be needed depending on the strength and damage that the virus causes. Also if the diagnosis is caught early then potentially less dosage is required than in a case where the virus is further developed. When a study was carried out with anti-influenza siRNAs the higher siRNA doses was more effective in protecting mice who had influenza. However another report stated that a high delivery of anti-hepatitis shRNA expression constructs to the murine liver “using vectors based on adeno-associated virus serotype could induce significant liver toxicity”. This shows that whilst the RNAi may be effective in silencing the gene it is causing others problems such as liver toxicity. These factors have to be weighed up to see whether it is beneficial to carry out treatment even with the potential side effects or whether it causes more damage. If there is a treatment for the side effect or it is mild compared to the problem trying to be treated then perhaps it beneficial but if the side effect is life threatening then it has to be considered if the use of RNAi in that particular case is really worth it. The ideal solution would be a dosage that is strong enough to silence the gene but does not cause further problems. Only by carrying out many different experiments with varying dosages to see the outcomes would this be possible.

A large amount of ATP is used by the RNAi pathway so too much siRNA or shRNA could put extra pressure on the host cells and therefore reduce the effectiveness of the siRNA and shRNA. A way this problem could be overcome is by making sure that RNAi is only delivered to the cells that are infected with the pathogen therefore reducing unnecessary ATP consumption. However if an infected cell was ignored by mistake it would lower the chances of

the RNAs success. Therefore there would need to be highly specialised sensitive monitors that could indicate the specific cells that were infected by a certain pathogens so that they were the only ones treated and none were ignored.

Another problem to overcome is the fact that genes are able to mutate out of the target sequence and therefore they become resistant to the RNAi meaning that it fails to silence the gene. If just one of the cells is able to resist the RNAi it leads to further problems as the virus replicates very rapidly so one cell quickly becomes many. A possible way of overcoming this problem is to apply multiple RNAi-inducing species that would be able to target a wide range of target sequences. However potential problems resulting from this would be the expense of using more than one type of RNAi-inducing species. Also the mixture of these could have a negative impact on the body and maybe the RNAi species are not as effective when mixed together. However it could have the opposite outcome and when a cocktail of RNAi-inducing species are used it could in fact make the treatment more effective against the virus. Many viruses have built up a resistance to vaccinations meaning that scientists have to keep turning out new vaccines that can kill the vaccine which takes up a lot of time and money. This is also a worry when just one type of RNAi-inducing species is used as over time the virus may be able to build up a resistance to the RNAi as occurs with vaccines. If different types of the RNAi inducing species are used it could potentially make it harder for the viruses to build up a resistance to a wide range of species. If one allele is resistant to one type of the RNAi inducing species then the likelihood is that it is not resistant to another and therefore can still be silenced.

Many RNAi based therapies have reached the stage where they can be tested on humans in clinical trials after positive results have come from laboratory studies in test tubes, cell cultures and animal models. These lab results allow scientists to gain preliminary efficacy, toxicity and pharmacokinetic information before humans are tested and therefore hopefully prevent damage to humans. It has however come to light that siRNA's can have off-target effects. These off-target effects have been recognised and scientists are coming up with solutions so that the benefits of RNAi can outweigh the risks in a risk-benefit analysis and it can be deemed ethically safe to test RNAi based treatments on humans in clinical trials. The first key problem I have already mentioned is that dsRNAs can bring about the interferon response. This occurs even when dsRNAs as small as 11 bp in length are used though to a lesser extent than when dsRNAs 30bp in length are used. The way scientists hope to bring a solution to this problem is by using siRNA's which target the specific gene. However when high levels of siRNA are used activation of the interferon response can still occur. Therefore studies in the laboratory are needed to find the minimum amount of siRNA duplex that will give the RNAi response that you are looking for without bringing about the interferon response.

One of the other problems identified is that the siRNAs can saturate the cell's RNAi machinery which would prevent the miRNAs from functioning properly and lead to non-specific effects in the body. Studies have shown that this is most likely to occur when high levels of siRNA are used so again it is a case of experimenting to find the minimum amount of siRNA that is effective in gene silencing.

siRNAs are meant to correspond to just one mRNA transcript however problems have arisen when they have caused an effect on other non-target mRNAs as well. This has led scientists to wonder about how specific the sequence match between the siRNA and the target mRNA have to be in order to achieve gene silencing in the target gene. In the report delivered by Elbashir et al it stated that a single difference between the siRNA and the mRNA it is corresponding to has a negative impact on the effectiveness of the RNAi activity. However Boutla et al found that a mutated siRNA was still able to achieve successful gene silencing in the target mRNA sequence in that of the fruit fly *Drosophila*. It seems that the ability to cope with mutations depends on the siRNA and to what degree the siRNA and the mRNA have to correspond. siRNAs seem to be able to deal with mutations in the 5' end but mutations in the 3' end they are less successful in coping with.

When dealing with ethical issues it is not only the risks against the benefits to the patient that have to be considered but also whether it is morally right to tamper with a human's genes even if it is in their best interests and could ultimately result in a better standard of life for them. Many religious groups do not believe in new scientific technologies such as RNAi as it means changing the human body that God created. They believe that it is not up to humans to interfere with genes as God has created the human body and humans do not have the right to change it. However many religious people say that God is Omnibenevolent and, as we should try to be more like God RNAi is a positive breakthrough as saving someone's life is a loving action and something God would approve of.

Some people oppose animal experimentation as they say it is risking the animal's life with no positive outcome for them. Many campaigners against animal testing believe that as the animal is unable to stand up for itself and prevent the experimentation it is their moral obligation to give the animals a voice. However it must be considered that testing on animals is necessary with new scientific breakthroughs such as RNAi as it means any potential problems come to light and can be dealt with before they are used on humans and potentially endanger their lives rather than save their lives. Also testing on animals in this case is different to cosmetic testing as RNAi has the potential in the future to save hundreds of lives whereas a new cosmetic is only for beauty purposes not health purposes. If animal experimentation was not used then new scientific discoveries would never come to anything as there would be no way of finding out the drawbacks and changing them so that they are fit to use on humans.

One of the other ethical issues involved with RNAi interference is the use of hematopoietic stem cells which could be produced with a virus *ex vivo* that could mean anti- HIV RNAi constructs would protect T cells and macrophages against HIV. Stem cell research comes up against a lot of opposition as one of the main ways of getting stem cells is from human embryos. The Catholic church particularly oppose this as they

believe in the sanctity of life where all life is sacred and that every embryo is a potential human life and has the right to live- therefore stem cell research is the murder of another human life. However the argument against this is many of these embryos would never become develop into foetuses so isn't it better to get some benefit out of them rather than simply disregarding them completely? There are also other ways of extracting stem cells for instance from umbilical cords and from the bone marrow of adults. However the bone marrow in adults is only able to differentiate into a limited range of cells- white and red blood cells for example but not neurones. The stem cells in an embryo on the other hand can differentiate into several kinds of special cells. Stem cells are particularly useful as they are unspecialised and have no Hayflick limit so can divide more than 52 times- as can cancer cells which is what makes them so dangerous.

Conclusion.

From my study into RNAi it is has become clear that since the discovery of RNAi in 1998 it now has the potential to be very effective in the treatment of diseases. A lot of research has gone into its potential benefits and ways of overcoming the problems in order for it to become a treatment in both the human and animal world. With further experimentation happening and the start of human clinical trials not far away it seems that it will not be long before RNAi is an option in the treatment of many viruses such as HIV that have, for years killed hundreds of human lives with their being no effective treatment.

Of course there are many hurdles still to overcome however what is evident is that scientists have come up with many solutions or alternatives to these problems. A major problem identified with RNAi in the treatment of HIV is that the transcriptional activator protein (TAT) can inhibit Dicer and prevent the RNAi from working. Though this may make the use of RNAi in HIV treatment harder the solution is to catch the virus early enough in its cycle and to target the suppressor RNA.

Another factor that still requires experimentation to make RNAi more effective is to only deliver RNAi to cells that are infected with the pathogen as this lessens the amount of ATP consumed and lowers the chances of potentially dangerous side effects. A particular dosage needs to be found that is strong enough to silence the gene without causing further problems and using up huge amounts of ATP. The only way that this can be overcome is by further experimentation with differing amounts of dosage until the most effective dosage for that particular virus is found. This may take time and money but once it is discovered the advantages would be huge.

Resistance of cells to the RNAi treatment is another problem that has surfaced. Using multiple RNAi-inducing species would deal with this problem as it is harder for cells to build up a resistance to a large number of RNAi-inducing species.

Having looked at the ethical perspective it seems to me that any experimentation on animals and the use of stem cells is worthwhile if the outcome is a lifesaving treatment. People may oppose the use of animals in the research but it must be remembered that mammals stand to benefit from RNAi as well if it become a treatment. For those who argue that manipulating genes is wrong as God has created the human body I simply think that we should embrace scientific breakthroughs as it is for the benefit of the human race and the Bible teaches us to do good during our time on earth.

After studying the concept of RNAi and the studies surrounding it I do believe that RNAi has the potential to silence viruses for good once the necessary problems I have identified in the above have been dealt with. Though more work has gone into the treatment of human diseases such as HIV it will have wider implications for the veterinary world as if RNAi has the potential to silence viruses then it can be used to treat disease epidemics such as Foot and Mouth which has in the past resulted in the mass culling of cloven hooved livestock.

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