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A GEDANKEN EXPERIMENT IN RELATION TO RNA  
INTERFERENCE AND HOW IT COULD PROVIDE A CURE TO  
EQUINE INFECTIOUS ANAEMIA

BY  
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PASS WITH MERIT

RESEARCH PAPER  
BASED ON  
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AT VET-MEDLINK 2009

## ABSTRACT

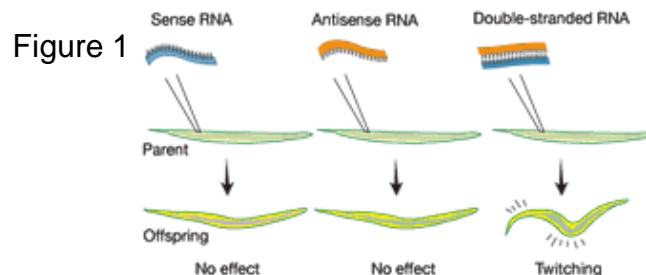
This paper is about RNA interference and the possibilities of how new research could find a cure for Equine Infectious Anaemia. There is currently great media coverage after two horses were recently imported into the UK from Romania and found to have this highly infectious disease. There is currently no effective treatment, despite the outbreak in Ireland in 2006 that led to 38 confirmed cases of the disease. I believe that using RNAi could provide an effective treatment for horses, mules and donkeys infected and provide an alternative to euthanasia which a majority of infected animals face. There are however, many scientific, as well as ethical issues, which need to be addressed and overcome before a cure can be found.

## INTRODUCTION

The possible phenomenon of gene silencing occurred to US and Netherland scientists in 1990 whilst developing Petunias. It is now known that there is vast potential in RNA Interference in a multitude of different areas and rapid scientific research and progression reflects this. Potentially, every gene that encodes for a protein could be switched off using this technique.

RNAi works as a natural defence mechanism in the following way. Firstly the double strand of RNA that enters the cell is cleaved into sections that are smaller than 23 nucleotide pairs in length, by the enzyme 'Dicer'. This then enables RISC, the RNA induced silencing complex, to bind with one of the pair. This acts as a probe to detect and stop messenger RNA molecules coming from the cells nucleus and therefore preventing the mRNA from reaching its desired location and thus it is silenced. In terms of viruses, when they inject their RNA into the cells they are attacking, it is shortened by the enzyme Dicer. The RISC complex then continues by destroying the mRNA, allowing the cell to survive the virus as the proteins required by the virus are not provided for.

The most significant progressions of gene silencing discoveries were that of Craig C. Mello and Andrew Fire, who later received the Nobel Prize in 2006 for their work on "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*". (Nature1998). They found that when the 'muscle sense RNA' and the 'muscle anti-sense' RNA were injected separately, no effect occurred. However, when the two RNA strands were injected together, forming a double helix, the worms displayed the same movements as worms that had completely lost the gene for the muscle protein. Further experiments confirmed that when specific siRNA for a gene were injected into the worms, that specific gene was silenced, ceasing to function.



The implications of this research in medicine is now becoming even more apparent as until recently this effect has only been shown in much simpler organisms, for example fruit flies and prokaryotic organisms such as bacteria. With larger more complex organisms, an inflammatory response is triggered when double stranded RNA enters the cell, therefore not having the desired effect. However if sections that are smaller than 23 nucleotide pairs in length are used, the interferon response can be achieved, blocking the messenger RNA.

More recently is the work of Professor Robert Langer and research scientist Daniel Anderson, whose work has proven the viability of transferring the known techniques into more complex eukaryotic organisms, successfully managing to access monkey and mice heart and liver tissue with siRNA. This has been possible by making use of the phospholipid bilayer which surrounds cells, allowing lipid soluble substances to pass through whilst maintaining a different internal environment inside the cell itself. This has been achieved using substances called 'lipidoids'. A February 2010 article in New Scientist, written by Linda Geddes, also highlighted further research in this area. She describes how "Khvorova's team chemically modified RNA molecules to reduce their negative charge, and make them smaller." This also overcame the problem of getting the siRNA compounds inside the required cells successfully.

Other research on gene silencing relating specifically to Equines is that of the University of Glasgow led by Professor Lubna Nasir. They have successfully managed to use RNAi techniques to reduce growth of sarcoid cells in vitro in a laboratory, eventually causing the cancerous cells to die. This however, has not yet progressed to a clinical trial and Professor Lubna Nasir states that "we are currently seeking funding to use this technique in clinical trials of horses that have sarcoids." Sarcoids are caused due to Bovine Papillomavirus, similarly Equine Infectious Anaemia is also caused by a virus. "One of the challenges with gene silencing is administering it within clinical setting - as you need to get molecules into every cell. As sarcoids are on the surface of a horse, we think administration should be relatively easy - potentially by injecting or applying a cream to the sarcoid," explained Lubna Nasir.

Equine Infectious Anaemia, or EIA, is a Retrovirus affecting horses, donkeys and mules. According to Oxford English Dictionary a Retrovirus is, "a group of RNA viruses which insert a DNA copy of their genetic material into the host cell in order to replicate, e.g. HIV." EIA is a lentivirus and is transferred via the blood; this can occur through a number of different ways predominately insect bites. It can also be spread by contaminated surgical equipment and is also passed from mare to foal via the placenta. Due to the connection with insects, this is also known as 'swamp fever' as it is especially rife in waterlogged areas close to trees, which provide a good habitat for the biting insects.

The symptoms of EIA depend on whether the disease is acute or chronic; animals can survive the disease but will be carriers for the rest of their lives, with the possibility of transferring the disease to other animals in close proximity. The symptoms of the disease in acute cases are "high fever, anaemia, weakness, swelling of the lower abdomen and legs, weak pulse and an irregular heartbeat." Horses suffering from the Chronic Disease have the following symptoms; "Horse tires easily and is unsuitable for work. May have a recurrent fever and anaemia, may relapse to the acute form even several years after the original attack." (Reference from Wikipedia)

The virus is widespread in some parts of Europe, America, Middle and Far East, Russia and South Africa. American regulations give a good indication of the endemic this disease is currently causing. It states that horse owners must "Obtain the required certification of negative EIA test status for horse shows, county fairs, race tracks, and other places where many animals are brought together." The test used to identify the presence of EIA in an animal is known as the Coggins test. "The Coggins test (agar immunodiffusion) is a sensitive diagnostic test for Equine Infectious Anaemia developed by Dr. Leroy Coggins in the 1970s." (Wikipedia)

## DISCUSSION

There would be many advantages of curing an animal that was a carrier of EIA. Crucially, it would prevent the spread of the disease to other animals surrounding them. It also would allow a horse to be transported into other countries and States. Furthermore, mares could be bred from, without the risk of transferring the antigens to their young via the placenta. A method of curing an animal with the acute or chronic symptoms would also be highly beneficial, potentially saving their lives.

There are two ways in which RNAi could be used to help prevent EIA from taking over the cells. One way would be to use gene silencing to alter the virus itself, making it unable to replicate. Alternatively, RNAi could be used to alter the cell in the equine, making it more resistant to the virus.

If it was decided that altering the virus would be the best approach, then it could be experimented on an agar plate in a laboratory. The virus would have to be altered, making it unable to damage the cells it normally attacks. If this was to be carried out, then the specific siRNA from the Equine Infectious Anaemia virus would first need to be isolated. The siRNA would need to be no longer than 23 nucleotides long with an overhang of two nucleotides on one or both ends to prevent the inflammatory response. The siRNA from the virus would also have to be the specific strand of the virus that takes over the cell and uses it for rapid division, known by Lieberman (2002) as the "gag." The transcription process would then be halted, preventing the virus from using the host cell for replication.

More recent research suggests that additional siRNA as well as the 'gag' would also need to be silenced. Retroviruses like HIV, according to B. Berkhout, (2010) are "especially hard to target through RNAi attack as they are escape prone, making it necessary to use a combination of RNAi strategies to prevent viral escape." This therefore would have to be combated through providing possibly large numbers of different siRNA to the infected organism. Despite the fact in a laboratory the viruses "gag" genes could be silenced, possible providing a solution in vitro. It would be hard to put this technique into practice in real life situations. This is because it would be nearly impossible to access the EIA virus population in order to silence the 'gag' genes inside it.

Due to the fact that it is difficult to alter the virus itself, it may be more appropriate to concentrate on using RNAi on the cells of the organism under attack. Lieberman, in 2002, had success in removing the CD4 protein receptor on the surface of the human cell. She found that without this particular protein the HIV was up to four times less likely to enter the cell. There could be a similar receptor on an equine cell. It hence stands to reason that silencing the gene that produced the receptor could prevent the EIA from entering a horse's cell. If this could be found and isolated then EIA would be a reduced threat to equines. There is quite possibly other ways in which altering the genetic makeup of the horses cells could make them less susceptible to attack from the EIA virus. This would therefore need to be further researched with other genes and through further examination an alternate possibility could also be achieved.

After looking at both possible options, concentrating on the cells in the organism is most probably the most viable option for the siRNA, and shows great promise in finding a solution. However, the problem still remains as to how to provide every cell in an organism with the necessary siRNA.

In order for the treatment to be successful, the siRNA complexes must reach all the cells in the organism that have been or could be infected by the Virus. Then the cells response would be to activate RISC, the RNA Induced Silencing Complex, opening the strand and copying the fingerprints allowing matching messenger RNA to be destroyed, thus preventing the virus from taking over the cell. I believe there are two main ways in order to provide all necessary cells with the siRNA. One way to achieve this would be to provide cells with siRNA via an external means, for example by infection with an additional virus with the siRNA attached. Alternatively the cells in the organism could be reached by the siRNA via an alternative passive manner, by allowing the siRNA to reach the cells whilst the body is acting of its own accord.

The first option of using an additional virus to infect the cells with siRNA is popular in similar situations, for example gene therapy. However, I believe that it is not wise to employ a method with so many risks attached. 'Infecting' the Animals cells with a siRNA attached to a virus could be unsuccessful as a resistance could be built up against the virus providing the siRNA. The organism immune system could also destroy the virus long before the siRNA reaches the cells. Moreover, there is also a great risk attached if the virus mutates. These risk factors contribute to the fact that there would be many ethical objections to administering the siRNA by means of this method. Some may also say that it is wrong to try and cure a disease with an additional virus that could potentially cause equal damage itself.

The alternative method to using a virus would be to provide the animal with the siRNA disguised as a substance that is taken up by the cell normally from the bloodstream. One option, similar to the work of Professor Robert Langer and research scientist Daniel Anderson, would be to provide the siRNA by attaching it to a lipid or by making it lipid soluble by reducing its negativity, allowing it to be transported into the cells via the phospholipid bilayer. Therefore all the cells could be reached by diffusion, simply and effectively without causing damage to the cells themselves. Also this method would also be effective as the substances would not be limited to channel and carrier proteins. This could then be administered intravenously or possibly orally, either way a very large volume of the substance would be required in order for it to reach the millions of cells in a horse.

If the "lipoid" was to be given orally, then the "lipoid" would need to be tested in order to determine whether it would be denatured by the acidity of the horse's stomach. The acidity varies depending on the location, from being around 6-7pH near the oesophagus to being 1-2pH near the pyloric sphincter. Due to the variance of the pH, depending on the location in the digestive system, it would also be necessary to determine the location where the horses would absorb the lipid into their blood stream. Generally speaking horses absorb lipids into their blood stream at their stomach or small intestine. This would need to be further investigated, as if this could be administered orally, this would greatly reduce the cost of treating an animal, as well as reducing the risk of blood contamination. Oral administration would also be of a great advantage as treatment would probably need to be continuous.

The other way in which the siRNA could enter the blood stream would be to allow it to enter the cells via another substance. One way would be to attach the siRNA to glucose or amino acids; this would first have to be tested as the cells carrier and channel proteins may not accept this. The siRNA would also predominately reach the metabolically active cells. Cells that do not require glucose or amino acids in large amounts may not be reached by the siRNA. Other substances or elements in addition to amino acids and glucose could also be trialled; however the same problems will apply. The siRNA, despite being attached to required elements, may

not be accepted into the cell. Also, only cells requiring that particular substance will receive the siRNA, this could be advantageous if the virus only affects certain cells.

When the siRNA had reached the organisms cells, its effect would not be permanent after the cell had undergone mitosis and the silencing effect would not remain. However this technique could still be of considerable use. For example mares that are carriers of the disease could still be bred from. Furthermore, immunity could be provided on a short term basis using siRNA protection.

There are many ethical issues surrounding this recent development of scientific knowledge. In some people's opinion, the risks may outweigh the advantages of using RNAi as it is such a new technique in its infancy. There are a multitude of things that could possibly go wrong resulting in it being a very high risk strategy. One example of the possible problems that it could cause is if the siRNA causes an additional unexpected response in the animal's cells. An example of this would be if the animal stopped producing a particular protein as the siRNA had silenced a gene that is necessary for normal function. This could have a devastating impact, especially if the problem only arises after the veterinary treatment has been approved. This could occur if the effect was on a metabolically slow cell, then it may take many months for the problem to become apparent. Despite the fact that this risk could be greatly minimised through intensive clinical experimentations, the risk would still remain.

In addition, some may say that it is unethical to use resources and time researching an animal retrovirus and not concentrating on finding cures for humans with HIV. However due to their similarities, further research into EIA may help with finding a cure for HIV.

There may also be ethical issues as a considerable number of equines will need to be used in clinical trials in order for a treatment to be successfully developed. Some people may state that it is inhumane to deliberately inflict suffering upon an animal in order for the 'Greater Good.' The amount of animals required for research could also be reduced by experimenting on cells on an agar plate and using simpler organisms first, for example rats or mice.

**Figure 2 EQUINE INFECTIOUS ANEMIA TEST RECORD**

Print name and address legibly for window envelope use

I hereby certify that the blood specimen submitted with this form was drawn by me from the animal described below on the date indicated.

Dr. \_\_\_\_\_ DATE BLED \_\_\_\_\_ SIGNATURE \_\_\_\_\_

Address \_\_\_\_\_ City \_\_\_\_\_ State \_\_\_\_\_ Zip \_\_\_\_\_

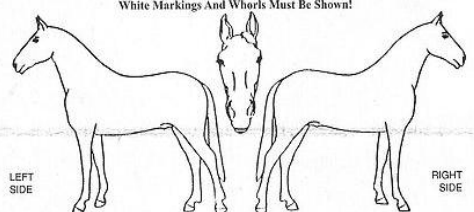
Owner \_\_\_\_\_ Reason for Test: 1. Clinical \_\_\_\_\_ 2. Involved \_\_\_\_\_ 3. Exposed \_\_\_\_\_ 4. Show \_\_\_\_\_ 5. Sale \_\_\_\_\_ 6. Routine \_\_\_\_\_ 7. Other \_\_\_\_\_ Animal Stabled At \_\_\_\_\_ Address \_\_\_\_\_ County \_\_\_\_\_ TOWN \_\_\_\_\_

Address \_\_\_\_\_ Zip \_\_\_\_\_ Farm No. (DBSP) \_\_\_\_\_

Phone \_\_\_\_\_

TUBE NUMBER	NAME	COLOR	BREED	SEX* Check One	AGE	TEST RESULTS
				S M G		

**White Markings And Wherls Must Be Shown!**



LEFT SIDE \_\_\_\_\_ RIGHT SIDE \_\_\_\_\_

Date and condition of samples received \_\_\_\_\_

The result of the test for Equine Infectious Anemia on the above specimen is as indicated

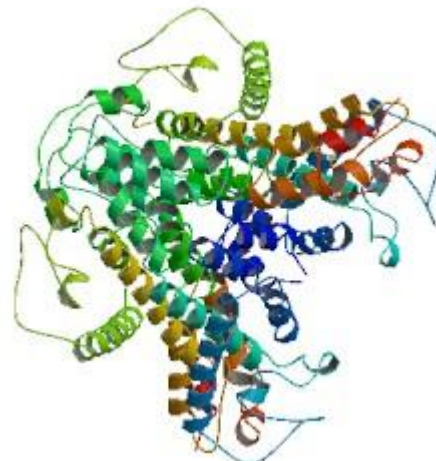
Signature \_\_\_\_\_

Accession No. \_\_\_\_\_

\*Please Use Legend: S -- Stallion/Steale  
M -- Mare/Female  
G -- Gelding

WHITE COPY -- VETERINARIAN  
YELLOW COPY -- OWNER  
PINK COPY -- FILE  
GOLDENROD COPY -- STATE VETERINARIAN

Figure 3: The EIA virus



## CONCLUSION

In conclusion I believe that a cure will be found for Equine Infectious Anaemia and that RNAi is a likely source of this cure. I do however also recognise the fact that there are many scientific hurdles that need to be overcome before this can be achieved. Although there has been a great deal of success with RNAi at a cellular level, developments need to be made before the techniques can move from agar to animal.

The first decision to make was to decide whether siRNA should be focused on the EIA virus itself, or on the cells in the organism for which it causes a problem. It was decided that currently the only possible option would be to send siRNA to the cells in an organism. If the cells in an organism were genetically altered, they then could have greater resistance to the virus itself.

Another potential problem identified was how to administer such a large amount of substance in order for it to reach every necessary cell in an organism. There were a number of possible options leading from this. One way would be to make the siRNA lipid soluble or by increasing the siRNA's negative charge. Doing this would allow siRNA to gain access to the organism's cells via the phospholipid bilayer. An alternate way in which the cells could be accessed would be to use a technique based on the principle of co-transport, however this has limitations due to the finite number of channel and carrier proteins in an organism's membrane. An option, which after further discussion was deemed inappropriate, was to attach the siRNA to a virus to then 'attack' the organism. This could also cause further problems as the immune system would be likely to attack the virus, preventing a majority of the cells from being reached and provided with siRNA. All of the possible options in the paragraph above would have to be tested in order to determine the best method or combination of methods.

The ethical issues surrounding this subject were also explored. Such as should we be tampering with organisms at a cellular and genetic level and could we possibly cause more problems than we are solving? For example, if we removed the protein receptor, what other affects could this have on the animal. There is also the ethical issue of whether animals should be tested on for clinical experimentation. Significantly, if the success rate is not 100% are we taking an unnecessary risk by not carrying out a euthanasia programme?

In conclusion, I believe that a cure will be found for retroviruses such as EIA and that RNAi is a likely source of this. However there are scientific advances that need to be made before this can be achievable and safe. Berkhout stated that "The future of antiviral RNAi therapeutics is very promising, but it remains of critical importance to include many controls in pre-clinical test models to unequivocally demonstrate sequence-specific action of the RNAi inducers."

## References and articles used for research

Andrew Fire and Craig Mello won the Nobel Prize in Physiology or Medicine for discovering RNAi mechanism:

[http://nobelprize.org/nobel\\_prizes/medicine/laureates/2006/press.html](http://nobelprize.org/nobel_prizes/medicine/laureates/2006/press.html)

Also figure 1 & 2.

General Information on RNA interference:

<http://web.mit.edu/newsoffice/2009/explained-rna.html>

Quote from Berkhout:

[http://en.wikipedia.org/wiki/RNA\\_interference](http://en.wikipedia.org/wiki/RNA_interference)

New scientist article written by Linda Geddes:

<http://www.newscientist.com/article/mg20527494.200-disease-gene-blocker-sneaks-past-cell-defences.html>

Article about the new Sarcoid treatment being developed for horses by the University of Glasgow led by Professor Lubna Nasir :

<http://www.horsetalk.co.nz/news/2010/01/069.shtml>

Information on EIA:

<http://www.dardni.gov.uk/code-of-practice-for-equine-infectious-anaemia.pdf>

Oxford English Dictionary: Definition of a retrovirus.

American Regulations regarding EIA:

[http://www.aphis.usda.gov/animal\\_health/animal\\_diseases/eia/downloads/fs\\_eia.pdf](http://www.aphis.usda.gov/animal_health/animal_diseases/eia/downloads/fs_eia.pdf)

Symptoms of the disease:

[http://en.wikipedia.org/wiki/Equine\\_infectious\\_anemia](http://en.wikipedia.org/wiki/Equine_infectious_anemia)

pH of a Horses Digestive System:

<http://www.extension.org/faq/138>

HIV RNAi:

<sup>^</sup> Berkhout, B; ter Brake, O (2010). "RNAi Gene Therapy to Control HIV-1 Infection". *RNA Interference and Viruses: Current Innovations and Future Trends*. Caister Academic Press. ISBN 978-1-904455-56-1.

Shoot the Messenger (2008) Rachael Moeller Gorman:

[http://rachaelgorman.com/article\\_full.php?article\\_stamp=1222362529](http://rachaelgorman.com/article_full.php?article_stamp=1222362529)

Figure 3: [http://www.rcsb.org/pdb/images/2eia\\_bio\\_r\\_250.jpg](http://www.rcsb.org/pdb/images/2eia_bio_r_250.jpg)