Regulating RNA interference by modifying RNA backbone with amides

Professor Eriks Rozners and colleagues at Binghamton University in New York, USA, are using innovative nucleic acid chemistry to modify RNA-based technologies such as RNA interference (RNAi) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to enhance their utility in molecular biology. These technologies suffer from off-target effects that limit their clinical utility. By replacing phosphates in the backbone with amides, the team aims to improve the stability, specificity, and uptake of these technologies by cells to make them more suitable for in vivo applications.

Gene expression is the process by which the information encoded in a gene is turned into a product, such as a protein. This manifestation of genes is fundamental to cell function and implicated in many diseases. RNA-based technologies are now widely used to regulate gene expression for therapeutic benefit. Controlling processes such as RNA interference (RNAi) are being increasingly studied as they give scientists the ability to influence gene expression. CRISPR-Cas9, a technology that acts like scissors enabling sequences to be cut out and genes to be edited, is another powerful tool to regulate gene expression.

The problem is: both RNAi and CRISPR-Cas9 suffer specificity problems, meaning they have undesired off-target effects that hamper their therapeutic benefit. Building on over two decades of research in the field of nucleic acid chemistry and using cutting-edge technology, Professor Eriks Rozners and his team at Binghamton University in New York, USA, recently demonstrated that chemical modifications – specifically, amide linkages in short interfering RNAs (part of RNAi) – reduce their undesired off-target activity. Furthermore, new preliminary results indicate such modifications may be applied to CRISPR-Cas9. The researchers hope this might kick-start further research in this area and, one day, lead to CRISPR-Cas9 optimisation.

THE RNA INTERFERENCE PATHWAY

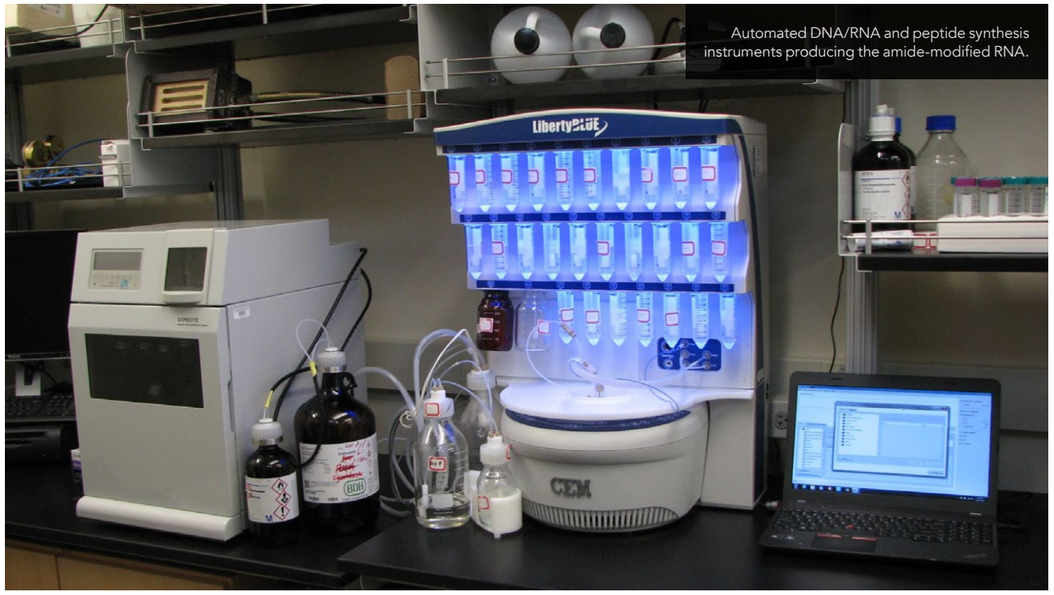
RNA interference is a pathway where small RNAs are used in the silencing of RNA to reduce gene expression. Because of this role, RNAi is used in research and increasingly explored for therapeutic applications. Two types of small RNAs are involved in RNAi: short interfering RNAs (siRNAS) and microRNAs.

siRNAs, regulatory molecules formed from double-stranded RNA, interfere with gene expression. During the process of RNAi, one strand of the RNA duplex is selected as a guide strand and is actively incorporated into the RNA-induced silencing complex (RISC), while the other strand (known as the passenger strand) does not have an active role and is degraded. siRNAs are investigated for pharmacological use and manipulated to improve delivery to various organs of the body. In fact, in 2018 the first RNAi drug, an siRNA, was approved for use. Since then, four others have been approved to treat disease.

The other type of small RNA involved in RNAi and gene silencing is microRNAs. With an overlap in the gene silencing machinery they use, siRNA can mimic microRNAs causing similar off-target activity (known as microRNA-like off-target activity). As siRNAs are used as a research tool, such off-target activity can lead to false experimental results and cause toxicity when used therapeutically in clinical trials.

FOUNDATION STUDIES

The natural phosphate backbone of RNAs can be chemically modified in different ways, giving rise to backbone-modified RNA. Rozners and collaborators have focused their efforts on replacing phosphate in RNA with amide linkages, a bond native to proteins. Phosphates make siRNAs inherently negatively charged. By replacing these phosphates with internucleoside amide linkages, the team aims to enhance the cellular uptake of siRNAs and improve their in vivo application.



Automated DNA/RNA and peptide synthesis instruments producing the amide-modified RNA.

Early experiments that replaced phosphates with modified linkages in DNA caused destabilisation, the opposite effect to that noted in RNA. The team's earlier studies laid the groundwork for using amide linkages by illustrating that amide linkages are well tolerated as replacements of phosphates in the A-form of RNA. Importantly, they showed that the use of amide linkages has little effect on the structure of RNA and does not thermally destabilise it. This led to the foundation knowledge that amides mimic phosphates in RNA and offer promise for use in manipulating siRNAs.

The researchers investigated amide-linked RNA, specifically siRNAs and their RNAi activity. Experimentally, they painstakingly changed the phosphate linkage of four guide strands of a specific gene, which enabled them to pinpoint the exact locations where replacements had maximum RNAi activity-enhancing effects. These early studies found if amides were strategically placed in certain positions of the strands, RNAi activity could be increased. In fact, a mixture of amide modifications in both passenger and guide strands can enhance siRNA activity, which will be useful for use in vivo. Furthermore, an important discovery was that positioning just one amide linkage at a particular location in a guide or passenger strand greatly reduced its off-target activity, the primary issue hindering clinical application.

AMIDE MODIFICATIONS IN siRNA IMPROVE SPECIFICITY

Chemical modifications of siRNAS (modified siRNA) are used to increase stability and improve specificity and other characteristics of siRNA. Inspired by their earlier studies and the need to optimise RNA-based technologies, the researchers explored how amide modifications in the guide strand affect off-target activity. They analysed a section of mRNA from the PIK3CB gene – a section known for its off-target mRNAs, namely YY1 and FADD. Off-target activity was measured in experimental assays by cleverly inserting copies of YY1 and FADD into reporter plasmids.

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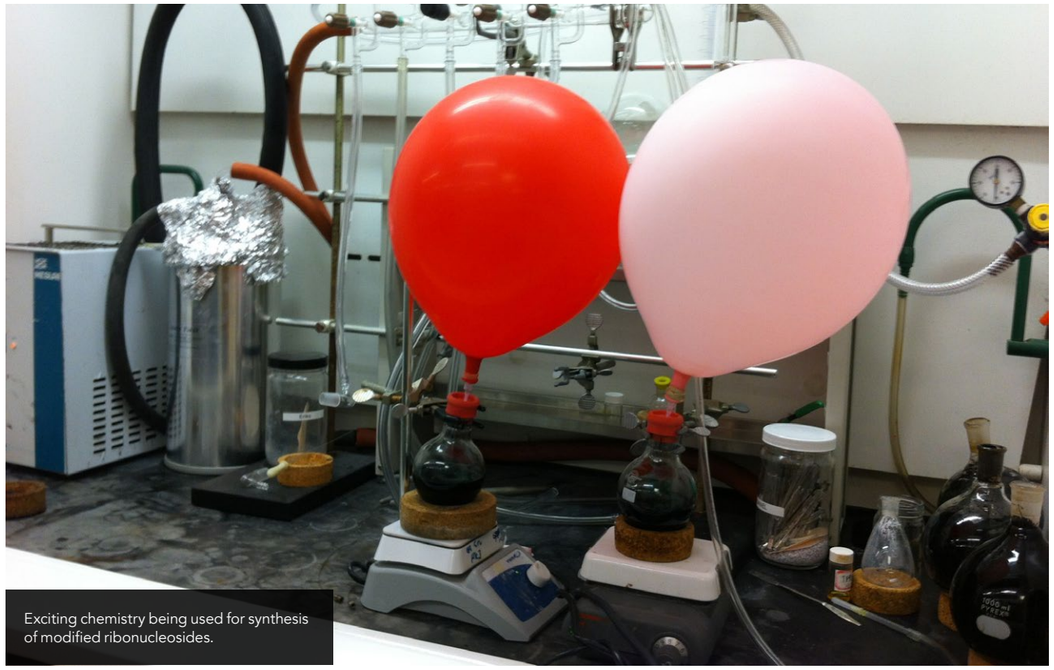
Positions G2 to G20 were analysed with the team detailing the effects of amide replacements on off-target activity for each position. Replacement with an amide at G1 reduces on-target activity, which is undesirable; thus, this position is not considered an option for modification.

G3 showed the most potential as it significantly reduced off-target effects and only slightly reduced on-target effects. Amide modifications may affect RNAs differently; for example, the effect of G2 on FADD was opposite to its effect on YY1. The study focused on particular siRNAS, and the researchers acknowledge the most optimal positions for reducing off-target effects may differ for other siRNAS and that more research is needed to establish this. By identifying the exact location where phosphate replacement with an amide significantly reduces off-target effects but keeps on-target specificity offers promise that the undesired off-target effects that limit therapeutic use can be controlled.

EARLY SIGNS OF SUCCESS

Encouraged by the success with siRNAs amide modifications, the team turned their attention to the gene editing



Exciting chemistry being used for synthesis of modified ribonucleosides.

technology CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). First identified in bacteria, CRISPR-Cas9 is a nuclease serving as a natural defence system. It recognises specific sequences and can be used genome-wide to alter protein and gene expression. Consequently, it holds enormous potential for treating genetic diseases in biomedical research. Like siRNAS and other RNA technologies, CRISPR would benefit from optimisation to improve specificity through chemical modifications. Other studies have successfully chemically modified the protospacer adjacent motif (PAM) region, while others have demonstrated that modifications at certain positions in CRISPR RNA decreased off-target DNA activity.

In their latest paper published in the journal *ACS Chemical Biology,* Rozners and colleagues are the first to investigate whether amide linkages are tolerated in CRISPR RNA (crRNA). They replaced phosphates with amide linkages in crRNA of two genes, namely human vascular endothelial growth factor A (VEGF-A) and hypoxanthine phosphoribosyl-transferase 1 (HPRT1). Modifications at specific locations in the PAM-distal region maintained CRISPR activity but, if modified at locations nearer to the seed region, decreased activity.

Positioning just one amide linkage at a particular location in a guide or passenger strand greatly reduced its off-target activity, the primary issue hindering clinical application.

However, there were exceptions, such as at location H16, where a modification maintained CRISPR activity even though it was in the seed region. Coupled with previous studies in which modifications at the same location increased CRISPR specificity, this site holds promise and should be further explored. More experiments are needed to examine the effects of amide linkages on other positions to see if they could improve specificity. In particular, the researchers suggest that phosphates 12, 14, and 16 might have potential.

These promising preliminary results reveal CRISPR-Cas9 activity remains unchanged following amide modifications in the PAM-distal region of CRISPR RNA. Conversely, if modified in the seed region, DNA cleavage can be lost at some positions. Overall, amide modifications were well tolerated, which will open an avenue for further exploration of amide linkage modifications in CRISPR. These initial results are highly relevant considering the immense therapeutic potential of CRISPR. Rozners and his team are hopeful that further studies of amide linkages could advance CRISPR technology.

FUTURE DIRECTIONS

More structural studies are needed to fully elucidate all possible benefits of modifications with amides to the seed region of siRNAs. Furthermore, the team's research on amide linkages in CRISPR-RNA provides a stepping stone to progress study in this field, with the ultimate aim of optimising these powerful RNA-based technologies that are at the heart of gene therapy. Watch this space.

Behind the Research

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Research Objectives

Professor Rozners and his team use nucleic acid chemistry to improve RNA-based technologies such as RNAi and CRISPR.

Detail

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Bio

Eriks Rozners received his chemistry degrees from Riga Technical University in Latvia. After postdoctoral stays at Stockholm University and Karolinska Institute (Sweden) and University of Wisconsin, Madison and University of Michigan (USA), he started his independent research career in 2001 as Assistant Professor at Northeastern University in Boston. Dr Rozners moved to Binghamton University as Associate Professor of Chemistry in 2008 and was promoted to Full Professor in 2015. He currently serves as a Chair of Chemistry Department.

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- Professor Martin Egli, Vanderbilt University
- Dr Scott D Kennedy, University of Rochester School of Medicine and Dentistry
- Present and former colleagues at DharmaconII, and all students and postdocs whose names appear on the papers

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Personal Response

Are further studies underway to determine if amide-modified crRNAs can improve CRISPR specificity? Where to next?

" Currently, Rozners' group is optimising synthetic chemistry to expand the scope of CRISPR RNA sequences that can be modified with amide linkages. Experiments are also underway to adopt modern next-generation sequencing approaches for rigorous evaluation of how amide modification improves the off-target activity of siRNAs and CRISPR RNA. Moving forward, Rozners group will take advantage of the nucleic acid chemistry expertise gained with amides and include other synthetic linkages in specificity and activity studies of siRNAs and CRISPR RNAs. "