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To the Minister for the Environment
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DATE	11 March 2022
REFERENCE	CGM/220311-01
SUBJECT	Clinical application of AAV vectors containing targeted nucleases such as CRISPR-Cas9

Dear Ms Heijnen,

Further to a request from the GMO Office for advice on the use of AAV vectors containing targeted nucleases such as CRISPR-Cas9 as inserts (COG 21-008), COGEM notifies you of the following.

Summary:
COGEM was asked to advise on whether or not the use of targeted nucleases, such as CRISPR-Cas9, in AAV vectors in clinical trials meets the requirements of the generic environmental risk assessment for AAV vectors, and on the need for additional containment measures. COGEM was also asked if its answer also applies to future gene-editing applications in combination with AAV vectors. The term ‘targeted nucleases’ refers to certain enzymes (nucleases) which can induce breaks at specific sites in the DNA. With these applications, targeted modifications of DNA (gene editing) can be made. Targeted nucleases include CRISPR-Cas systems, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and meganucleases. CRISPR-Cas systems are currently the subject of much research. Besides bringing about desired effects in patients, targeted nucleases can also cause unwanted effects, both at the intended position in the DNA and at other positions (off-target effects). Such unwanted effects present a risk mainly to the patient, but shedding of vector particles can potentially lead to third-party exposure to these vectors. Although exposure of third parties to AAV vectors containing targeted nucleases will be limited, the possibility of this leading to unwanted effects cannot be ruled out for all possible cases. COGEM therefore advises that clinical applications involving AAV vectors containing targeted nucleases should be subject to a further condition requiring additional hygiene measures to be taken after administering the AAV vector. This condition may be

waived if an applicant provides a sound argument for doing so. COGEM points out that AAV vectors containing CRISPR-Cas sequences or sequences of other targeted nucleases have an increased risk of integration into the genome and therefore advises that to prevent any possible germline gene editing, such AAV vectors should not be administered in the sex organs of participants in clinical trials. Lastly, COGEM is of the opinion that future gene-editing applications in combination with an AAV vector should fall under the generic environmental risk assessment if these future applications have an effect comparable to the current known systems and the above-mentioned additional conditions are observed.

The attached report contains COGEM's advice and a discussion of the underlying reasoning.

Yours sincerely,

Professor Sybe Schaap
Chair of COGEM

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- Environmental Safety and Risks Directorate, Directorate-General for the Environment and International Affairs, Ministry of Infrastructure and the Environment
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Clinical application of AAV vectors containing targeted nucleases such as CRISPR-Cas9

COGEM Advisory Report CGM/220311-01

1. Introduction

COGEM was asked to provide generic advice on the use of targeted nucleases, such as CRISPR-Cas9, in AAV vectors in clinical trials (COG 21-008). The question concerns whether or not such vectors meet the requirements of the generic environmental risk assessment for AAV vectors and if additional containment measures are necessary. At the same time COGEM was asked if the above also applies to future gene-editing applications in combination with AAV vectors.

The term 'targeted nucleases' refers to nucleases which can induce breaks at specific sites in the DNA. These include CRISPR-Cas systems, in which the nuclease is directed to the cleaving sequences by the 'guide RNA' (gRNA), as well as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the meganucleases in which recognition of the cleaving sequences is based on a DNA–protein interaction (see also section 1.5 for more information on these applications). Several pre-clinical studies have been carried out into applications of AAV vectors containing ZFNs[e.g.,1,2,3,4] and meganucleases,[5,6] but currently most studies are on CRISPR-Cas applications in AAV vectors.

1. CRISPR-Cas9 gene editing

Clustered regularly interspaced short palindrome repeats (CRISPR) associated proteins (Cas) systems are derived from bacteria and archaea, where they function as defence mechanisms against viruses and other foreign (non-native) genetic elements. In 2012 a scientific paper was published which describes a relatively simple technique for using CRISPR-Cas9 to create double-strand breaks at specific positions in the DNA, thus allowing the genome to be modified.[7] This publication led to a breakthrough in gene-editing possibilities. Since then the use of CRISPR-Cas has grown rapidly, both in fundamental research and in various agricultural and medical applications, and the first clinical trials with CRISPR-Cas are now underway.[8] Gene editing with CRISPR-Cas9 makes use of a ribonucleoprotein (RNP) consisting of the Cas9 protein in combination with a guide RNA (gRNA). By means of base pairing, the sequence of the gRNA molecule determines where the two Cas9 nuclease domains induce the DNA break in the genome. A necessary condition for cleavage is the presence of a protospacer adjacent motif (PAM) sequence next to the gRNA binding site in the DNA: a short sequence of usually 2 to 6 nucleotides 'downstream' of the cleavage site.

The break in the DNA can be repaired in animal and human cells via the available repair routes. The commonest route is non-homologous end joining (NHEJ) in which both DNA strands are joined together, allowing deletions or insertions (indels) at the site of the break.[9] If the original sequence is preserved, the CRISPR-Cas9 system can induce another break. After NHEJ, genes can be inactivated by introducing frameshifts or the reading frame of a gene can be restored by altering splice sites.[10,11] If a donor sequence is provided with the CRISPR-Cas system, the double-strand break can be repaired by inserting a desired DNA sequence at the site of the break by homology-directed repair (HDR).[12] Via this

route it is possible, for example, to accurately repair point mutations or make large insertions or deletions. HDR is less error prone than NHEJ, but is only active during a few phases of the cell cycle and is less efficient.[13]

1.2 Developments in CRISPR-Cas techniques

Over the years the arsenal of possibilities and applications of CRISPR-Cas techniques has expanded enormously,[14] including modifications of the Cas9 protein itself, the gRNAs and the way the system is regulated. These modifications have made the outcomes of the original CRISPR-Cas system more efficient and reliable, including by creating Cas proteins with increased specificity (e.g. 'high fidelity' Cas9-HF1[15] and enhanced specificity eSPCas9[16]).[17] In addition to the original Cas9 protein isolated from *Streptococcus pyogenes*, there are now several orthologous Cas9 proteins in other organisms.[13,27] These differ, for example, in size or in the PAM sequence needed to induce the DNA break in the target sequence.[18,19] The Cas12a protein (originally called Cpf1) is also frequently used for gene editing. DNA cleavage by Cas12a produces a break with 5' overhang, in contrast to the 'blunt ends' produced by Cas9. The Cas protein can also process the necessary gRNAs itself, so that no separate promoters are needed to express more than one gRNA.[20] Cas13 can be used to cut RNA, for example as a potential treatment of infections with RNA viruses.[21,22]

The modified protein nickaseCas (nCas) cleaves just one of the DNA strands because one of the nuclease domains is inactivated. In combination with a pair of gRNAs targeting opposite DNA strands, a double-strand break can be created with greater precision and less off-target activity than with the wild type enzyme (see also section 1.4 Unwanted on- and off-target effects).[23] In deadCas (dCas) both nuclease domains are inactivated, but binding to the DNA is still possible. Linking these mutants to specific enzyme activities further expands the possible uses of CRISPR-Cas systems. In base editing, for example, dCas 9 is linked to cytidine deaminase or adenosine deaminase. This technique allows the mutation of C to T or A to G at a specific position.[24,25] In prime editing it is possible to induce deletions and make specific insertions, and introduce each of the 12 possible point mutations.[26] CRISPR-Cas techniques also play a major part in making epigenetic changes, which alter gene expression without changing the nucleotide sequence of the DNA itself.[27] Epigenetic changes are not covered by the Ministerial Regulation on Genetically Modified Organisms (GMO Regulation) and will therefore not be considered in this advice.

1.3 Administering CRISPR-Cas to test subjects

There are different ways to administer CRISPR-Cas9 to test subjects or patients. In ex vivo applications, somatic cells are removed and modified outside the body before being returned to the test subject or patient. Cells can be modified in one of many ways: via DNA plasmids or mRNA molecules, by administering RNP complexes consisting of Cas protein and the gRNAs, or by using viral vectors such as retroviral or lentiviral vectors. There are several possible methods for in vivo administration of the CRISPR-Cas system. Among the methods described are the use of lipid nanoparticles (LNPs) and the use of AAV vectors in which the CRISPR-Cas sequences have been inserted into the vector genome. In a clinical trial currently underway an AAV vector containing CRISPR-Cas9 is being used to treat the

eye disease Leber congenital amaurosis type 10 (LCA10),[11,56] and pre-clinical studies are ongoing for the treatment of Duchene muscle disease, among others.[28,29]

The Cas9 protein from *S. pyogenes* was the first to be used for gene editing and is still the most widely used protein. The size of the Cas9 protein (1,368 amino acids; the gene is 4.1 kb) makes inserting the coding sequences of the CRISPR-Cas system into AAV vectors quite challenging.[30] This problem can be circumvented by making use of two AAV vectors, one of which contains the Cas9 sequence as a transgene and the other expresses the gRNA sequences, either in combination with a donor sequence or without one,[31,32] or by using a split Cas system in which the Cas9 gene is split into two parts and the gene products in the cell then fuse together to form an active Cas protein.[33,34] However, gene editing requires that the cells are transduced by both AAV vectors, which makes such an approach inefficient. For this reason the use of smaller orthologues derived from other organisms, such as Cas9 from *Campylobacter jejuni* (CjCas9)[35] or *Staphylococcus aureus* (SaCas9)[36,29] is being investigated.

1.4 Unwanted on-target and off-target effects

Although CRISPR-Cas systems are very effective at inducing mutations in the genome, unwanted effects can also occur, both at the targeted sequence (on-target) and at other positions in the genome (off-target). These may result in the introduction of small insertions, deletions or point mutations, and even large deletions or reordering of chromosomes are possible.[37,38,39]

Because the unwanted effects can be harmful to test subjects and patients, research is being done into ways of making CRISPR-Cas systems safer.[40,41] This has resulted in more accurate Cas proteins, such as the previously mentioned nCas, Cas9-HF1 and eSPCas9. The design of gRNAs can influence the occurrence of potential non-specific effects during gene editing; prediction programs can help to identify off-target positions of the chosen gRNA sequences in advance. Unwanted harmful effects can also be prevented by regulating the expression of the Cas protein. For in vivo applications, this can be done, for example, by using tissue-specific promoters for the expression of CRISPR-Cas sequences, or by administering CRISPR-Cas only in a specific organ, such as the eye, thus restricting its further spread through the body.[13,56]

Usually the vector genome of AAV vectors remains in the cell nucleus in episomal form, but when double-strand breaks are made in the DNA the vector DNA can become integrated into the genome.[42] Increased integration of the AAV vector into the genome has been observed in CRISPR-Cas applications delivered by AAV vectors as a result of the breaks introduced by the Cas protein.[28,43] The prolonged expression of the Cas protein that can result from this may in turn lead to more off-target effects.[13,41]

Antibodies against the Cas protein may be present in test subjects or patients who have had bacterial infections in the past. Immune reactions to the components of CRISPR-Cas are then possible and these will have implications for the effectiveness of gene editing. In a recent study a large proportion of the subjects tested had antibodies against two commonly used Cas9 proteins: 78% possessed antibodies against SaCas9 and 58% had antibodies against SpCas9. T cells directed against the Cas proteins were

also detected in a majority of the blood samples.[44] The immunogenicity of the Cas protein has also been described in other studies.[45,46]

1.5 Other targeted nucleases

As indicated earlier, other applications besides CRISPR-Cas are included among the 'targeted nucleases', namely the ZFNs, TALENs and meganucleases. ZFNs are composed of 3 to 6 different DNA-binding zinc finger motifs fused to the endonuclease domain of the bacterial FokI restriction enzyme. Each zinc finger motif recognises a series of 3 nucleotides. As dimerisation of the FokI nuclease is necessary for cleavage activity, two ZFN constructs are used, each of which binds a DNA strand in opposing orientations, allowing dimerisation between the FokI cleavage domains. The double-strand break introduced by FokI has a 5' overhang of 3 nucleotides. To reduce the chance of off-target effects, use can be made of ZFNs with specific FokI domains that form obligate heterodimers.[47,48] Additional zinc finger motifs can also be added, and research is being done to increase zinc finger specificity.[49,50] As ZFNs are relatively small, various methods can be used to transfer them, including AAV vectors.[49]

The TALEN system also uses the FokI endonuclease. The FokI is linked to transcription activator-like effector (TALE) protein domains, which originate from phytopathogenic *Xanthomonas* bacteria. Here too, dimerisation is required to introduce a double-strand break. The TALE domain consists of 10 to 30 repeats of 33 to 35 amino acids, with the amino acids in positions 12 and 13 varying in each repeat (repeat variable diresidue (RVD)). The combinations of the RVDs correspond one to one with a base in the DNA. The DNA targeting range, and thus the specificity, is larger for TALENs than for ZFNs. Also, relatively few off-target effects have been reported for TALENs.[51,52] However, TALENs are often too big to be transferred by viral vectors with limited capacity, such as AAV vectors.[53]

Mega-nucleases are restriction enzymes with long target-recognition sites consisting of 14 to 40 base pairs. A distinction is made between homing endonucleases (HE), which occur naturally in the genome of bacteria, archaea and bacteriophages and in the mitochondrial or chloroplast genome of eukaryotes (particularly plants, algae, fungi and protozoa), and synthetic meganucleases, which are developed by exchanging different HE domains. The meganucleases most used for gene editing are I-SceI and I-CreI. The specificity of meganucleases is very high. Like TALENs, relatively few off-target effects have been reported for meganucleases.[49,54] However, adapting meganucleases to recognise specific nucleotide sequences is complex.[54] To circumvent this process, combinations of meganucleases and TALENs can be used, in which the TALE domain is fused to a meganuclease and a second DNA-binding domain is created. These mega-TALs also have a high specificity and low off-target activity.[49,55]

2. Previous COGEM advice

In 2019 COGEM drew up an advisory report on a generic environmental risk assessment for clinical applications with AAV vectors and came to the conclusion that the risks to human health and the environment from clinical trials with these vectors are negligible, provided a number of conditions are met.[57] These conditions were updated in 2020.[58] The conditions that apply to the transgene used in AAV vectors are that they must not code for sequences that are capable of complementing the vector, nor must they code for harmful gene products or for oncogenes or proto-oncogenes.

COGEM has recently issued an advice, for the first time, on a clinical trial with an AAV vector containing sequences from the CRISPR-Cas9 system to treat patients with a specific form of retinal degeneration and loss of vision (Leber congenital amaurosis type 10).[56] The method of administering this vector is by injection into the eye. The expression of the CRISPR-Cas9 sequences, including those of the gRNAs designed for repairing a sequence in one of the introns of the CEP290 gene, is controlled by a photoreceptor cell-specific promoter. COGEM considered that the probability of third-party exposure was very small. If third parties should, nevertheless, be exposed, no harmful effects were expected. For these reasons, COGEM considered the risks to human health and the environment associated with the application of this AAV vector to be negligible.

3. Answer to the present request for advice

COGEM was asked several questions regarding applications involving AAV vectors in which targeted nucleases, such as CRISPR-Cas9, are used as transgenes.

Question 1a. Is COGEM of the opinion that applications that make use of targeted nucleases as inserts in an AAV vector, which include CRISPR/Cas9 applications, satisfy the conditions of the generic environmental risk assessment for AAV vectors,[57] including the revised conditions,[58] and can these applications be processed under the VoV [an authorisation procedure with a set of standard licence conditions] for AAV vectors,[59] in particular the assumption that this insert is a harmless gene product? COGEM was also asked to explain its answer.

Question 1b. COGEM is asked to state any requirements that should be met by these applications, for example concerning the promoter or insert (such as the creation of unwanted gene drives).

Question 2. Is COGEM of the opinion that future gene-editing applications used in combination with an AAV vector should also fall under the generic environmental risk assessment[57,58] and can be processed under the VoV[59]? COGEM was asked to explain its answer and indicate any restrictions that should apply.

These questions are answered in the following sections.

3.1 Targeted nucleases as harmful or harmless gene products

The generic environmental risk assessment for clinical trials involving AAV vectors includes a condition concerning the insert which states that the transgene used must not code for sequences that are capable of complementing the vector, nor must it code for harmful gene products or for oncogenes or proto-oncogenes. Nucleases play an important part in repairing DNA when it is damaged and are not themselves considered to be harmful gene products. Gene-editing techniques involving targeted nucleases can be used to introduce single strand or double-strand breaks at specific sites in the DNA. These breaks can lead to unwanted and potentially harmful effects in test subjects or patients, such as those described in the section on unwanted on-target and off-target effects (section 1.4).

There are various ways to ensure that applications involving targeted nucleases lead to fewer unwanted effects. For example, in CRISPR-Cas applications the expression of the Cas protein and the gRNAs can be regulated by using tissue-specific promoters. The application can also be made safer by making use of 'high fidelity' Cas proteins with increased specificity. The gRNAs can also be altered in a such a way as to increase their specificity at the target site in the DNA. Although much research is being done to reduce the probability of unwanted effects occurring, these risks cannot yet be ruled out altogether. Moreover, these risks increase as sequences of the targeted nucleases are expressed for prolonged periods. This is the case for in vivo applications involving AAV vectors in which the AAV vector genome remains in the cell nucleus in episomal form or after integration resulting from the double-strand break made by the nuclease.

In the event of third-party exposure to the AAV vector, it cannot be concluded in advance that in all cases the intended modification by CRISPR-Cas or another targeted nuclease at the target site (on-target modification) or an unintended modification (unwanted effect) at an on-target or off-target position will be harmless. In addition, exposure may lead to undesired integration of AAV vector sequences into the genome of third parties. This could lead to prolonged expression of the nuclease in the third party or result in disruption (inactivation) of a tumour suppressor gene or in activation of a proto-oncogene.

Given the above, COGEM is of the opinion that the question of whether or not nucleases should be considered to be harmful gene products cannot be answered with a clear yes or no. The degree to which potential unwanted or harmful effects occur (on-target and off-target effects and integration of vector sequences) depends among other things on the specific construct combined with the specificity of the gRNAs or the DNA-binding domain of targeted nucleases other than CRISPR-Cas.

3.2 Third-party exposure to AAV vectors containing targeted nucleases

Although any unwanted effects of in vivo gene-editing applications involving AAV vectors containing targeted nucleases primarily present a risk to the patient or test subject, AAV vectors may be shed, for example via saliva, blood, faeces, semen and urine,[60,61] and for this reason third-party exposure to the AAV vector cannot be ruled out for all applications.[57] The amount that is shed, and therefore the likelihood of exposure, depends on the administration route of the vector used.[62] When administered intravenously, the concentration of shed vector DNA usually peaks within a week of administration. The time at which the vector DNA can no longer be detected in excretions varies between studies. In one study, vector DNA was still found in semen, saliva and faeces up to 52 weeks after administration.[63] However, COGEM points out that in almost all studies shedding of AAV vectors in bodily fluids was analysed by PCR and the presence of vector DNA does not necessarily mean that infectious vector particles are actually present. Some animal studies investigated the shedding of infectious vector particles, which were found in the faeces of sheep[64] and in the blood of macaques,[65] in both cases up to 48 hours after administration of the AAV vectors. To the best of COGEM's knowledge, no data are available on the shedding of infectious vector particles by participants in clinical trials.

COGEM notes that third-party exposure to AAV vectors will involve considerably fewer vector particles than the amounts that patients and test subjects are exposed to. Nevertheless, it is not possible to categorically rule out the possibility of third-party exposure and transduction, which could lead to unwanted effects. It should also be noted that further spread is impossible because the AAV vector is replication-deficient.

3.3 Preventing unwanted effects in third parties

To minimise the risk of unintended third-party exposure to AAV vectors containing targeted nucleases, COGEM advises prescribing additional hygiene measures that go further than the previously recommended conditions on the use of AAV vectors in clinical trials. As the extent, route and duration of shedding may vary, and depend in part on the method of administration and the vector used, COGEM advises taking the following generic measures as a precaution:

- During hospitalisation, COGEM advises the use of measures commensurate with those included in the 'WIP guidelines' on gene therapy (particularly Chapter 7 on isolation measures after administration), as published in 2011.[66]
- Regarding possible exposure via urine and faeces, COGEM advises the use of a private toilet (which no-one else is permitted to use) for 2 weeks after administration and/or combined with inactivation with a chlorine solution.
- Regarding possible exposure via saliva and semen, COGEM advises the use of effective contraception in the form of a physical barrier, such as a condom, for 2 weeks after administration and avoiding any exchange of saliva.
- Regarding exposure via blood, COGEM advises preventing any contact with blood for 2 weeks after administration and giving hygiene instructions to patients and third parties such as family members about the use of plasters, sanitary towels and tampons, and washing hands after their removal.

These measures are in line with the measures taken for patients with specific infectious diseases, such as Zika, to prevent further spread of the disease. COGEM notes that these measures may be waived for specific applications if the applicant can provide a sound argument for doing so. For example, methods of administration which restrict or rule out further distribution in the body (such as injection into the eye[56,67]) will require only short-lived and/or other control measures.

3.4 Germline modification

Question 1b refers to the creation of unintended gene drives. The creation of a gene drive requires the integration of the CRISPR-Cas construct into the genome of germline cells. COGEM points out that AAV vectors containing CRISPR-Cas sequences or sequences of other targeted nucleases have an increased risk of integration into the genome.[42,43] If the vector genome integrates into the genome of germline cells, this is considered germline modification, which is forbidden by law in the Netherlands. There are no indications that applications of AAV-CRISPR lead to germline transmission when the vector is not administered in the gonads. However, the number of pre-clinical trials of AAV vectors containing targeted nucleases that have examined germline transmission is very limited. In an animal study in which an AAV2/8 vector containing CRISPR-Cas9 sequences was administered intravenously

in a mouse model for phenylketonuria (PKU), a pair of genetically corrected mice did not produce any genetically corrected offspring, which indicates that no germline transmission had occurred.[68]

Given the increased chance of integration of all or parts of the AAV vector genome when using targeted nucleases, COGEM advises that AAV vectors containing CRISPR-Cas or other targeted nucleases should not be administered in the gonads of participants in clinical trials.

3.5 Future gene-editing applications

COGEM is of the opinion that future gene-editing applications in combination with an AAV vector may also be assessed in the same way if these future applications have a comparable effect to the current known systems, as described in section 1.2, and the additional conditions stated in section 3.3 are observed. COGEM takes 'applications with a comparable effect' to mean various Cas proteins that induce breaks in the DNA in the same way and optimised Cas systems, including prime and base editing systems.

4. Conclusion

In conclusion, COGEM is of the opinion that the possibility of AAV vectors containing CRISPR-Cas or other targeted nucleases as inserts causing unwanted on-target and off-target effects cannot be ruled out in advance. Because the vectors can be shed after administration to the patient, third parties may also be exposed to the vectors and could experience similar unwanted effects. COGEM therefore advises that to limit third-party exposure, additional hygiene measures be taken when using AAV vectors containing targeted nucleases. Taking into consideration the conditions described in the generic advice on the use of AAV vectors in clinical trials[57] and in the 2020 update,[58] and provided the additional hygiene measures proposed in this advice are applied, COGEM is of the opinion that the risks to human health and the environment of clinical trials with AAV vectors containing targeted nucleases are negligible.

COGEM also notes that AAV vectors containing targeted nucleases have an increased risk of integration into the host genome. COGEM therefore advises that AAV vectors containing targeted nucleases should not be administered in the gonads of participants in clinical trials.

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