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COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

CRISPR gene editing technology – is there potential for genotoxicity?

Introduction

1. This discussion paper provides a brief overview of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology, its potential for use in disease research and therapy, and an overview of a paper where viral vector-mediated genotoxicity is discussed in general.

2. Clustered Regularly Interspaced Short Palindromic Repeats, commonly abbreviated to CRISPR, are a series of specific bacterial DNA sequences interspersed with viral DNA sequences integrated into the DNA following infection. The functions of CRISPR and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea as they are a form of acquired immunity, allowing the organisms to respond to and eliminate subsequent viruses or plasmid infection(Marraffini and Sontheimer, 2010; Moijica *et al*., 2010). These sequences are the basis of CRISPR technology.

3. A potential use of CRISPR technology that is currently being explored is as a gene editing tool in human medicine (refer to paragraphs 5 – 8). The potential for genotoxicity when CRISPR is used in this way may be of interest to COM.

4. CRISPR's were first identified in *E.Coli* but have since been found in approximately 50% of bacterial genomes and 90% of Archaea[1] (Ratan *et al*., 2018; Zhan *et al*., 2018). The palindromic (i.e. the same backwards and forwards) repeats are 20-40 base-pairs in length, which form RNA hairpin turns. Each has a spacer DNA sequence that matches a specific bacteriophage sequence. These sequences have been acquired during prior infections and have been acknowledged to be the bacterial immune system, as repeat infections trigger transcription of proteins which inactivate the specific viral DNA. Each spacer, principally transcribed to helicases and endonucleases, within CRISPR are thought to arise from different viral infections and therefore represent a library of infections. CRISPR associated proteins (Cas-) are linked to specific guide RNA's (gRNA) and, together, they locate and cut the invading viral DNA. Short DNA sequences, called protospacer adjacent motifs (PAMs), were identified as tags adjacent to the target sites which provide a built in 'safety mechanism' ensuring that only specific genes are transcribed. Therefore, the CRISPR/Cas complex is able to remove highly specific DNA regions, disabling the virus, and providing the bacteria with immunity to infection. The CRISPR/Cas system is diverse, with different Cas proteins providing a variety of helicases and nucleases

[1] Single cell prokaryotic organisms similar to bacteria but which have distinct biochemical characteristics.

classified broadly into 6 types (I to VI) (Zhan *et al*., 2018). One of the simpler CRISPR systems from *Streptococcus pyogenes* relies on the protein Cas9 (also known as Csn1) – illustrated in Figure 1 (se Annex 1)

5. The potential usefulness of CRISPR/Cas9 protein system as a gene editing tool was identified by a number of research groups and the first key attempts to harness the functionality were published in 2013 (Cong *et al*., 2013; Mali *et al*., 2013). The Cas9 endonuclease is a four-component system that includes two small RNA molecules named CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). Together these are referred to as the single guide RNA (sgRNA). These guide Cas9 to the target site, a section of DNA complimentary to the crRNA of approximately 20 nucleotides, and double strand breaks are generated. It was demonstrated that the crRNA can then be manipulated to provide a specific cutting tool so that selected sequences can be excised, and new DNA inserted (Gasiunas *et al* 2012). In this way genes can be altered to promote gene transcription; for example, transcriptional activators can be recruited to gRNA bringing RNA polymerase to target areas and increase transcription. Inactivation of transcription is also possible and fluorescence tags can be used to visualise the 3D architecture or to monitor gene activity. These characteristics have enabled researchers to adapt CRISPR for a number of molecular biology and genome editing applications.

6. Research initially focused on the development of genome editing techniques and proof-of principle investigations carried out *in vitro*. For example, controlled gene editing enables the construction of libraries of Cas9 variants which can induce targeted, specific single nucleotide base changes. CRISPR has also been used to generate gene knock out rodent models and human cell models for disease research (Zhan *et al*., 2018). Facilitated by the widespread use of open-information bioinformatic resources, its potential to elucidate and treat diseases has rapidly become apparent and the CRISPR research area is burgeoning (nearly 9000 publications between 2012 and May 2018).

CRISPR-Cas9 in disease research and therapy

7. Cancer research quickly applied the use of CRISPR/Cas9 related techniques. Its ability to modify DNA sequences, and to introduce epigenetic and transcriptional alterations to DNA can be utilised to explore the effects of gene expression deregulation during carcinogenesis. This gene editing functionality has the potential to be harnessed to develop cancer therapies. Modelling of the mutations and perturbations which define carcinogenesis (termed the ‘hallmarks of cancer’) has been made possible by using CRISPR to edit the genes associated with these changes (e.g. genes which inhibit apoptosis, activation of epigenetically silenced tumour suppressor genes and screening for genes which mediate cancer associated inflammation) (Moses *et al*., 2018). In another investigation a sgRNA library was transduced into cloned, murine lung cancer cell lines using CRISPR. When these cells were implanted *ex vivo* into immune-deficient mice, it was possible to identify

some genes involved in the tumour progression and metastasis of the resulting lung tumours (Chen *et al*., 2015).

8. Development of CRISPR/Cas9-based cancer therapeutics has provided a number of leads for potential treatments. Base editing techniques can introduce diverse point mutations and can be exploited for a wide variety of applications. Some specific examples of gene editing include; NANOG and NANOGP8 mutations in malignant prostate cancer cells *in vitro* were 'corrected' using CRISPR; editing of TP53 mutations in Arf-/-Eu-Myc lymphomas *in vitro* corrected over expression of Myc. In an additional *in vitro* model, Cas9 variants engineered to induce specific single nucleotide base-changes, induced targeted C-T and G-A transitions to correct Y163C hotspot mutations in a TP53 breast cancer cell line (Khan *et al*., 2016).

9. To date, most clinical trials using gene editing techniques have not used CRISPR but have used zinc finger nuclease (ZFN) or transcription activator-like effector nucleases (TALEN) technologies (Khan *et al*., 2016). The first clinical trial involving CRISPR was performed in China. In this, T-cells from lung cancer patients were removed, and CRISPR was used to 'edit-out' the gene expressed programmed cell death protein -1 (PD-1). Edited cells were then expanded and infused back to the patient (Zhan *et al*., 2018). The outcome of the trial has not yet been reported. The *ex vivo* approach is likely to be the technique most appropriate for CRISPR. To date trials using CRISPR appear to have only been initiated in China. Other trials include the use of PD-1 knockout T-cells as potential treatment for oesophageal cancer, bladder cancer, hormone refractory prostate cancer, renal cell carcinoma and non-small lung cancer; and CRISPR/Cas9 edited CAR-T cells to target CD19[2] in B-cell lymphoma/leukaemia (Zhan *et al* 2018).

10. CRISPR/Cas9-based genome modification is also being used to explore the treatment of human immunodeficiency virus (HIV) infection. CRISPR/Cas9 nucleases have been used to target the viral genome sequence with a view to eliminating the incorporated HIV genomic material from infected T cells *in vitro* (Khan *et al*., 2018).

Potential safety concerns with the use of viral vectors

11. The safe use of CRISPR *in vivo* will require high editing efficiency/specificity, low immunogenicity and the ability to precisely direct the cas9/sgRNA to the specific organ or cell. Safety concerns have arisen due to the fact that inconsistencies in gene insertion occur and the process is not yet completely efficient or accurate (Khan *et al*., 2016). Genetic toxicologists from the pharmaceutical industry have reviewed the potential use of such techniques and suggested that the use of viruses during ZFN, TALEN and CRISPR technologies, which allow permanent DNA integration, can theoretically result in genotoxic events (David and Doherty, 2017). These may include random insertion, proto-oncogene activation, insertional

[2] biomarker for B-cell development

mutagenesis or genetic instability, referred to by the authors as '*vector-mediated genotoxicity'.*

12. The review by David and Doherty explores a number of potential clinical risks posed by the introduction of viral vectors and lists some rogue insertions or proto-oncogene activations observed in clinical trials. For example; transcriptional activation of LMO-2 resulting in acute-T-cell lymphoid leukaemia in a male volunteer was noted when CD34[3] bone marrow cells were treated *ex vivo* with retrovirus therapy in X-SCID disease[4]. Specific factors which impact on potential genotoxicity include the design of the vector (e.g. the activity of the promoter and enhancer sequences), the integration site, the insertion profile and the nature of the target cell. (David and Doherty, 2017). Currently Cas9, when delivered as mRNA, is not considered stable enough to be useful as a gene therapy (Khan *et al*., 2016). However, the technology is moving rapidly forwards and there is potential for CRISPR/Cas9 to be delivered using a viral vector system.

13. The inability of current genotoxicity tests to detect vector-mediated mutagenesis is noted. Potential approaches, including the use of high-throughput insertional mutagenesis screens, are considered. It is suggested that viral-induced genotoxicity testing would require an assay capable of detecting insertional gene activation (David and Doherty, 2017). Therefore, safety issues associated more broadly with viral technologies as well as CRISPR itself may need to be considered.

Summary

14. CRISPR, identified as bacterial immune response, has been developed as a genome editing tool. The technology has the potential to be used therapeutically in man for the treatment of diseases such as cancer and viral infections including HIV. Mutagenesis has been observed with some viral vector technologies used clinically and therefore it is possible that CRISPR will also induce vector mediated genotoxicity.

[3] transmembrane phosphoglycoprotein protein encoded by the CD34 gene
[4] X-linked severe combined immunodeficiency (SCID) is an inherited disorder of the immune system that occurs almost exclusively in males. Boys with X-linked SCID are prone to recurrent and persistent infections because they lack the necessary immune cells to fight off certain bacteria, viruses, and fungi.

Questions for the Committee

- Do Members consider that CRISPR technologies have a potential for vector mediated genotoxicity?

- If so, how should this topic be further considered?

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Abbreviations

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats,
Cas	CRISPR associated proteins
CAR-T	Chimeric antigen receptor T-cells
CrRNA	CRISPR RNA
Grna	Guide RNA
PD-1	Programmed cell death protein -1
SgRNA	Single guide RNA
TALEN	Transcription activator-like effector nucleases
TracrRNA	Trans-activating CRISPR RNA
TP53	Tumour protein 53
ZFN	Zinc finger nuclease

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Figure 1. Cas9 *in vivo*: Bacterial Adaptive Immunity – Reference Jiang W., J., Bikard, D., Cox, D. et al. (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature Biotechnology, 31:233-239.

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