CLINUVEL

TECHNICAL NOTE

CUV151 – DNA REPAIR PROGRAM

30 July 2024

In this Technical Note, four chapters are discussed:

i. Executive summary
ii. DNA, RNA and proteins
iii. RNA sequencing (RNA-seq) technique
iv. Clinical study results

i. Executive summary

In-vitro and early in-vivo studies have shown that melanocortins – peptides and their analogues which bind to melanocortin receptors, including afamelanotide – can assist in the repair of DNA that has been damaged by ultraviolet (UV) and visible light[1–7].

CLINUVEL's DNA Repair Program seeks to confirm these results in a broader clinical program, focusing initially on xeroderma pigmentosum (XP), a group of disorders characterised by deficient DNA repair (and thus extreme rates of skin cancer). Healthy volunteer studies – including the recently completed CUV151 study – serve as controls for XP where placebo-controlled studies may be deemed unethical. If CLINUVEL can demonstrate that melanocortins are of clinical benefit in the most extreme disorders of DNA damage and repair, these learnings may be applicable to some two billion individuals who – due to genetic variation – are at increased risk of skin cancer.

The results obtained so far from CLINUVEL's program support the hypothesis that SCENESSE® (afamelanotide 16mg) can protect the nucleus of skin cells from the deleterious effects of UV and visible light and assist DNA repair mechanisms.

CUV151 is the first study to use RNA sequencing (RNA-seq) analysis techniques to deepen the understanding of the processes at play. This Technical Note explores the RNA-seq technique used and presents the results that demonstrate a broader role of afamelanotide in preventing and assisting repair in UV-induced skin damage than has been previously reported.

ii. DNA, RNA and proteins

Deoxyribonucleic acid (DNA) contains genetic information, often referred to as the biological blueprint for all living cells. Collectively, the human genome is composed of 30,000 genes, with each gene carrying the information needed to produce, or synthesise, different proteins. In the simplest sense, expressing a gene means manufacturing its corresponding protein comprising one or more chains of amino acids. The sequence of amino acids determines each protein's unique structure and functions (examples of proteins: antibody, enzymes, hormones etc.).

The journey from gene to protein is complex and tightly controlled within each cell. It consists of two major steps: transcription and translation.

The first step of DNA decoding is the transcription, in which the information stored in a gene's DNA is copied to a similar molecule called ribonucleic acid (RNA) and more precisely to a messenger RNA (mRNA).

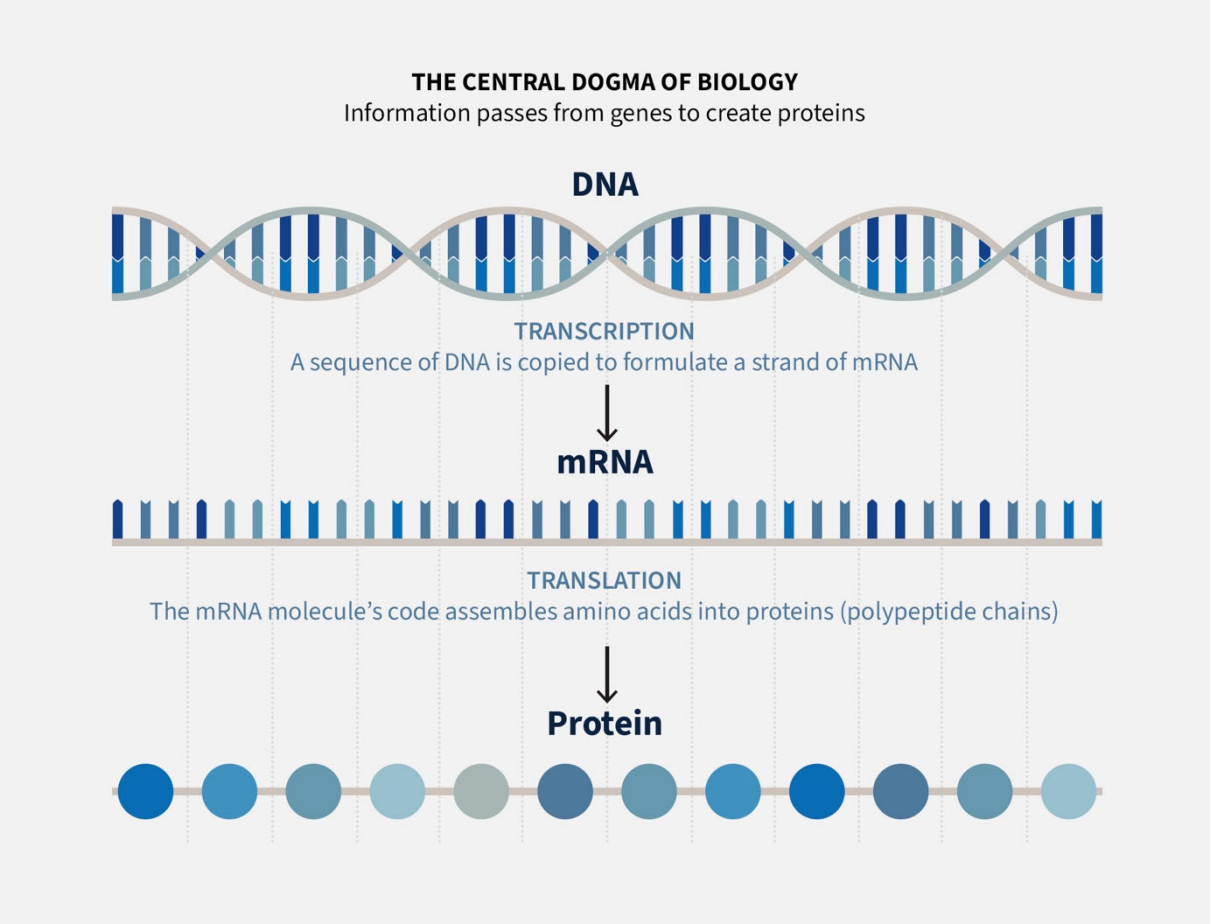

Figure 1: From DNA transcription to RNA translation into protein

The second step is the translation. mRNA is "read" by a group of molecules, according to a certain code. This code translates the mRNA sequence into amino acids, the building block of proteins.

iii. RNA sequencing (RNA-seq) technique

Each cell expresses (or ''turns on'') only a fraction of its genes at a given time and environment. The rest of the genes are repressed (or ''turned off''), a process known as gene regulation. Cells are allowed to adapt to environmental changes. For example, after sun exposure, skin cells react to UV radiation penetrating the nucleus by expressing genes involved in melanin production and inflammation.

RNA-seq provides a snapshot of gene expression in a cell. This specialised laboratory technique examines the quantity and sequences of RNA to identify and quantify the expression and repression of specific genes as well as allowing for the comparison of gene regulation under certain experimental conditions.

In order to understand the impact of both UV and afamelanotide treatment, RNA-seq was performed with skin biopsies taken from healthy volunteers under four different states:

i. prior to treatment with SCENESSE® without UV-irradiation;
ii. prior to treatment with SCENESSE® following a controlled UV radiation dose 24 hours earlier;
iii. 7 days following a single SCENESSE® implant without UV-irradiation; and
iv. 7 days following a single SCENESSE® implant following a controlled UV radiation dose 24 hours earlier.

The differences in gene expression were then quantified; this is referred to as Differentially Expressed Genes (DEGs).

More detailed analyses, involving bioinformatics tool like the Ingenuity Pathway Analysis (IPA), can be conducted to understand the implications of the DEGs seen. IPA uses the knowledge of interactions between genes and other molecules, which are categorised by pathways and functions, and classifies the identified DEGs within those (example of a function: inflammation). This allows to determine which pathways are

enriched (over-represented) or depleted (under-represented), highlighting potential key biological processes involved under the experimental condition(s) being studied.

iv. Clinical study results

Differentially Expressed Genes (DEGs) analysis using RNA-seq

Untreated skin samples (i.e. without afamelanotide) demonstrated an increase in 625 DEGs when comparing non-irradiated and irradiated skin. Put simply, there was considerable genetic activity in response to UV insult.

Following afamelanotide treatment, the number of DEGs between irradiated and non-irradiated skin was reduced to 183, a factor 3.4 less DEGs ($p<0.05$). This suggests that the level of genetic activity in response to UV insult was reduced following afamelanotide treatment.

By analysing which genes are expressed or repressed, one begins to understand the impact of both UV and, subsequently, afamelanotide treatment. Many of the genes repressed or reduced following afamelanotide treatment are crucial in the regulation of UV-induced DNA repair and inflammatory reactions; their reduction indicates a decreased need to express genes involved in DNA repair due to less damage, which consequently leads to reduced inflammation. These included genes coding for tumour necrosis factor (TNF) receptors and interleukins, histones and proteins involved in Extracellular Matrix (ECM) function.

ECM proteins such as matrix metalloprotease-1 (MMP-1) and MMP-3 are UV-responsive and are involved in tissue repair[8]. In injured skin, MMP expression is induced by keratinocytes, leading to the degradation of elastin and collagen components of the skin, which is subsequently repaired. However, persistent UV exposure may lead to permanent damage to the ECM, resulting in photoaging[8,9]. Results from CUV151 demonstrate a reduction in MMP-1 and MMP-3 expression in samples treated with afamelanotide, suggesting afamelanotide helps to protect against ECM degradation and, thus, photoaging.

Histones are damage-associated proteins that, when modified, enable DNA accessibility by repair machinery. For example, in the event of DNA double strand breaks, post-translational modification of histones leads to the unravelling of chromatin and propagation of DNA damage signalling. This process ensures that the cell can appropriately repair the damage, therefore reducing risk of mutagenesis[10]. It was found that expression of histones such as H3C10 and H2BC9 is decreased following afamelanotide treatment, suggesting a reduction in DNA damage. This is consistent with the significant reduction in phosphorylated histone variant H2AX (γH2AX) observed, a modification that signals the presence of DNA lesions to facilitate repair.

Ingenuity Pathway Analysis (IPA): enriched pathways

To identify enriched pathways in UV-irradiated skin on untreated vs treated samples, IPA was performed. Results show that the four key DNA damage/repair pathways below were only enriched in the untreated samples.

- Oxidative stress induced senescence – this pathway is activated through oxidative stress and reactive oxygen species (ROS) production to limit oxidative DNA damage[11].
- DNA damage/telomere stress induced senescence – ROS are implicated in telomere-dependent senescence, due to induction of telomere stress and the progressive loss of telomere which is a driver of ageing. When the end of the telomere becomes exposed, this is recognised by DNA repair machinery as a double strand break, leading to repair, cell cycle arrest and apoptosis (programmed cell death)[12].
- DNA Double Strand Break Response – DNA double-strand breaks are the most cytotoxic DNA lesions. The repair of double strand breaks is divided into two main sub-pathways: non-homologous end joining (NHE) and homologous recombination (HR). The choice between these two pathways is determined at an early stage, and is regulated by many factors[13].
- Senescence-associated secretory phenotype (SASP) – senescent cells often exhibit changes in their secretory profiles, which reinforces the senescent state and promotes tissue repair through DNA damage signalling pathways and activation of p53[14].

Enrichment of these pathways in untreated skin only suggests increased DNA damage and oxidative stress after UV exposure in these samples and thus a greater need for repair mechanisms to be activated. After

afamelanotide treatment, those pathways were not enriched, neither depleted, testifying a reduction of UV-induced DNA damage, as shown with the concomitant diminution of cyclobutane pyrimidine dimers (CPDs), presented earlier.

Overall, the RNA-seq and IPA results demonstrate that treatment with afamelanotide significantly reduces genetic expressions provoked by UV radiation, including oxidative stress and the inflammatory responses. This is of clinical relevance for the general population, and specifically individuals with a fair skin type who easily sun burn, since these processes are known play a role in acute and chronic skin damage (photoageing) and the development of skin cancers.

– END –

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About CLINUVEL PHARMACEUTICALS LIMITED

CLINUVEL (ASX: CUV; ADR LEVEL 1: CLVLY; Börse Frankfurt: UR9) is a global specialty pharmaceutical group focused on developing and commercialising treatments for patients with genetic, metabolic, systemic, and life-threatening, acute disorders, as well as healthcare solutions for specialised populations. As pioneers in photomedicine and the family of melanocortin peptides, CLINUVEL's research and development has led to innovative treatments for patient populations with a clinical need for systemic photoprotection, assisted DNA repair, repigmentation and acute or life-threatening conditions who lack alternatives.

CLINUVEL's lead therapy, SCENESSE® (afamelanotide 16mg), is approved for commercial distribution in Europe, the USA, Israel, and Australia as the world's first systemic photoprotective drug for the prevention of phototoxicity (anaphylactoid reactions and burns) in adult patients with erythropoietic protoporphyria (EPP). Headquartered in Melbourne, Australia, CLINUVEL has operations in Europe, Singapore, and the USA. For more information, please go to https://www.clinuvel.com.

Head of Investor Relations

Mr Malcolm Bull, CLINUVEL PHARMACEUTICALS LTD

Investor Enquiries

https://www.clinuvel.com/investors/contact-us

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Contact:

Tel: +61 3 9660 4900
Fax: +61 3 9660 4909
Email: mail@clinuvel.com
Australia (Head Office), Level 22, 535 Bourke Street, Melbourne, Victoria, 3000, Australia