SCIENTIFIC COMMUNIQUÉ III

CLINUVEL

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Following the positive response to SCIENTIFIC COMMUNIQUÉS I & II, released in April and May 2018, CLINUVEL will continue to publish a longer SCIENTIFIC COMMUNIQUÉ series throughout the course of 2018. The goal of the SCIENTIFIC COMMUNIQUÉS is to share the current understanding and progress in proopiomelanocortin (POMC) science and photomedicine with various interested audiences by conveying it in understandable and simple language. SCIENTIFIC COMMUNIQUÉ I provided an outline of the various physiological modifications taking place on proteins and the clinical relevance to our technology programs. In SCIENTIFIC COMMUNIQUÉ II we delved into how ligands bind to the various cellular receptors, signalling cascades and output to arrive at therapeutically meaningful applications. In SCIENTIFIC COMMUNIQUÉ III we review the effects of afamelanotide, CLINUVEL's lead drug, on the human genome, based on the existing scientific understanding. These three pieces will then be used as a basis for further COMMUNIQUÉS later in the year. After the first series of the three COMMUNIQUÉS we hope the reader will be able to grasp the, often opposing, opinions found in the abundance of literature on relevant topics. A library of scientific terms has been established on CLINUVEL's website clinuvel.com allowing the reader to follow up the key items discussed in time.

The later pieces in the series, SCIENTIFIC COMMUNIQUÉS IV, V and VI will review the topics of current sun protection consumer products, carcinogenesis related to skin, and photomedicine as a discipline.

UV RADIATION AND SKIN RESPONSE

Human skin serves us in miraculous ways. It shields the human organism from external stimuli and environmental threats. It acts as a physical barrier to heat, radiation and other toxins. And it releases and secretes various hormones and peptides to maintain a biological balance. Another gift to the human species is skin's ability to eliminate damaged cells *when required*, the so-called process of *apoptosis*.

It has only been a relatively short time since the scientific community realised that alpha-melanocyte stimulating hormone was actually being released by the top layer of the skin (epidermis), in contrast to the long-held view that POMC was released exclusively by the hypothalamus. Although both peptides are part of the POMC system, their target organs and functions are quite distinct. While it is beyond the scope of this COMMUNIQUÉ, one should keep in mind that the functions of hormones along the hypothalamic-pituitary-adrenal axis purport to elicit different reactions from the hormones released by our skin. Here it remains essential to realise that the endocrine and paracrine signals result from a homeostatic need, in other words the biological way to maintain a physiologic balance.

The organic response provoked by ultraviolet (UV) radiation is multi-fold in man, and differs from that in other mammals. The human response to UV is typically called an "SOS" response to localised stress induced by heat, electromagnetic radiation and, importantly, to prevent *further damage* to the nucleus of our cells.

As can be seen in Figure 1 in SCIENTIFIC COMMUNIQUÉ I, UV radiation on Earth is roughly divided into UVB (290-320 nm) and UVA (320-400nm), making up 5-10% and 90-95% of the

radiation absorbed by the skin respectively. Radiation of longer wavelengths, UVA, penetrates deeper in the dermis, whereas UVB is almost entirely absorbed by the epidermis. Factors such as the individual's constitution, the length and frequency of exposure, the endogenous protection mechanisms, and the immune status contribute to the skin's ability to repair UV damage and ultimately regenerate.

Broadly speaking, humans are divided in two categories: *melanocompetent* and *melanocompromised* (see Figure 3 of SCIENTIFIC COMMUNIQUÉ II). The former group defines those of us who can adequately respond to UV exposure by starting and completing the "tanning" cycle, leading to significant eumelanisation of the epidermis. The latter group consist of those individuals who burn and do not generate adequate and sufficient eumelanin, putting them at risk of chronic and faster photodamage and skin cancers. With regard to the visible effects of UV radiation, and in reference to CLINUVEL's website and SCIENTIFIC COMMUNIQUÉ I, the human skin goes through a number of stages which ultimately lead – that is in *melanocompetent* humans – to the complete cycle of melanogenesis.

The visible sign following UV exposure, however, is captured by the minimal erythemal dose (MED), reflecting the first reddening of the skin occurring from UVB at doses of 20-70 mJ/cm^2 and UVA at doses of 20-100 mJ/cm^2. One typically needs 2-6 hours of UV exposure to provoke one MED, but in melanocompromised individuals erythema can appear as fast as 5-10 minutes following radiation exposure. For evaluation and testing purposes, the CLINUVEL team replicates this kind of exposure under laboratory conditions to "phototest" light-sensitive patients. The latter should not be confused with *absolute photo-intolerant* patients, such as EPP patients. For these patients different radiation regimens, and at different wavelengths, are applicable to photoprovoke symptoms or reactions.

In healthy individuals **the first stage** of melanogenesis is called the initial photochemical reaction or *Immediate Pigmentary Darkening (IPD)*, which usually occurs within 90 minutes of UV and sun exposure and lasts up to 24 hours. The IPD stage is mainly attributed to UVA.

The **second stage** is called the *Delayed Pigmentary Darkening (DPD),* and occurs 24 to 72 hours after prolonged UV and sun exposure. The DPD is mainly ascribed to UVB exposure.

The biochemical mechanisms occurring when we expose skin to specific wavelengths of light thus require focused attention.

In SCIENTIFIC COMMUNIQUÉ I we discussed the various signalling pathways which are activated within the skin cell following UV exposure. Here we focus on specific proteins expressed by the skin following exposure to UV radiation and which play a part in the damage recognition and repair of the human genetic material within the cell.

Following UV insult, the skin (epi-/dermis) exhibits a stress response (see SCIENTIFIC COMMUNIQUÉ I) which is expected at biochemical level to protect us from further and irreversible damage.

The **p53 protein** is one of the most widely studied tumour suppressor proteins in cancer research. For the sake of this discussion it is presumed that p53 in its functional state is suppressing the formation of various cancers, but more specifically non-melanoma skin cancers. Back to the main theme of UV and sun exposure, p53 is activated via a number of routes within the cell. First, the enzyme tyrosinase – a key enzyme of the melanisation mechanism (pigment formation) – is provoked by UV exposure to initiate the process of eumelanin formation (see SCIENTIFIC COMMUNIQUÉS I & II) and p53 is one of the key regulators of this very enzyme. In experiments it has been shown that, following UV exposure, one would find increased levels of

p53 protein within the nucleus of the epidermal cells. Thus, p53 plays a part not only within the cytoplasm but also within the nucleus, the air traffic control of each cell.

Naturally, one must then ask what the precise function of p53 is in cellular repair, and why it is found in the nucleus of cells. Various research groups found in vitro and in pre-clinical in vivo models that nuclear accumulation of the protein served a number of purposes. In this discussion, we look at one particular research group, among many others, the photobiology group of the National Institutes of Health in Bethesda. In previous collaborations with the emeritus biologist Dr Hearing, CLINUVEL generated data on various scientific projects, which are part of the intellectual property and knowhow of the Company. This eminent scientist had focussed his professional career on understanding UV damage and melanisation of the skin in Caucasians and African-Americans. Ten years ago his group examined and analysed skin biopsies from human volunteers who had been irradiated with UV light sources. While a single piece of experimental research is not usually conclusive, results similar to the NIH findings have been reported globally and these particular outcomes have become part of contemporary scientific understanding.

Hearing's findings were significant on two key points. Firstly, the research led to the common understanding that UV penetrates deeper in individuals of Caucasian complexion compared with darker skinned volunteers. Secondly, the outcomes asserted that significantly more p53 accumulates in the nuclei of fair skinned individuals who have been exposed to UV radiation, compared to the nuclei of African-Americans. This strongly indicated that p53 was a central protein in damaged skin cells – mainly keratinocytes – as part of the body's stress response mechanism to UV radiation. From this point onwards, the scientific community furthered its research on the exact role of p53 and its various molecular sites.

The revered academic Ortonne and his group, with whom the CLINUVEL team had the privilege of collaborating during many years of his active academic career, mapped out the cellular mechanisms following acute and chronic UV radiation of human skin. Many more European, American and Australian research groups since then have focussed on this theme to unravel the risk of non-melanoma and melanoma skin cancer.

In decades of research by the group of Ortonne and Balotelli it became apparent that p53 as a proto-oncogene also played an important role in DNA repair mechanisms following UV damage. Generally, one can conclude from the analyses of human biopsies of skin cells that, following just minutes of sun exposure, p53 starts to form part of the cellular pathway which is meant to influence DNA replication. The latter is of importance when skin cells are UV and sun damaged, and in some instances following exposure to visible light sources (a theme to be discussed in SCIENTIFIC COMMUNIQUÉ VI).

In short, and essential to understanding this piece, UV causes photodamage, activating mechanisms within human physiology to eliminate photodamaged cells. This multicomplex process of **apoptosis** needs to occur swiftly and efficiently. The two other events taking place are the emergence of so-called cell death receptors provoked by UV and **cytochrome C** release following mitochondrial damage within the cell, the mitochondrion being the battery of the cell.

The former process involves apoptosis originating from fibroblast-associated, tumour necrosis factor receptor and tumour necrosis factor-related apoptosis-induced ligand (TRAIL) which all induce apoptosis to activate a caspase chain of events within the cell. In this radiation sequence of events the expression of **FasR** (apoptosis stimulating fragment receptor), simultaneously with **FasL** (its ligand, protein fitting the receptor) occurs quickly to provide us with another mechanism to regulate apoptotic activity. Outside the realm of this SCIENTIFIC COMMUNIQUÉ, one should be aware that pro- and anti-apoptotic proteins fight while UV damage is occurring.

The outcome of the battle determines how effective the human organism is in eliminating its damaged cells. Finally, **TRAIL** is expressed as a means to preserve skin integrity and is downregulated following UV exposure.

The mitochondrial response is very much regulated by the **Bcl-2 family** (B-cell lymphoma 2 gene/protein) of proteins, those which are pro-apoptotic and anti-apoptotic. Bcl-2 is pro-cell death and, for instance, **Bak** (Bcl-2 homologous antagonist killer), **Bax** (Bcl-2 Associated X)**,** and **Bid** (Bh3-interacting domain) proteins are expressed to inhibit apoptosis. Such is our human functioning, action versus required reaction, and the manner in which we maintain balance (homeostasis). The roles of Bak and Bax in ensuring human skin eliminates UV damaged cells are well understood. Bak is found in the outer membrane of the mitochondria, enabling the proteins to punctuate the membrane to accelerate cell death. Bax similarly moves from the cytosol to the outer membrane of the mitochondria following radiation, to be able to speed up apoptosis.

To summarise these biochemical events, all these processes eventually need to result in the activation of **caspases** 8, 9 and 3, which are cysteine-aspartic proteases, enzymes catalysing processes of apoptosis.

PHOTOPROTECTION & REPAIR

Before we delve into the subject of the types of UV skin damage one can incur, we first review the objectives for achieving photoprotection, and repair mechanisms to be initiated by our skin cells.

The search to develop a **systemic photoprotective agent** is based on a number of clinical objectives which have expanded over time as more knowledge has become available.

In this sense systemic photoprotection ought to take place in two main phases:

I preventative

II categorical

As part of the first phase, the objective has been to develop effective pharmaceutical agents to prevent the physical damage to the top and deeper layers of the skin, epidermis and dermis. By providing one or several physical barriers to UV and light, cellular damage may be prevented. Factors such as dose, frequency, time and length of exposure, constitutive and heritable traits, anatomy, and age all play a role in the level of photoprotection one is able to provide.

Figure 1: DNA in the nucleus resembles the shape of a spiral staircase

Categorically, one aims to arrive at a clinical solution to efficiently provide adequate biochemical signals or restore identified deficient ones within the affected target cells. Additionally, photoprotection provided throughout the human body aims to ensure an overall efficient ratio of elimination of *apoptotic cells* (see above *UV Radiation and Skin Response*), elimination of photoproducts within the nucleus of the cell, and, importantly, assist in effectively repairing DNA damage.

Without delving into a more extensive scientific review of the **human genome**, a few basic principles are unfolded for one to follow one research interest of CLINUVEL. It is perhaps useful to imagine deoxyribonucleic acid (DNA) within the nucleus of the cell as having the shape of a spiral staircase, with the rungs or steps connecting the two balusters of the staircase. Of particular interest to the research of our teams is not only how UV damage

occurs, but also where and how to fix the radiation lesion, the broken and distorted steps. In visualising the nitrogen bases and hydrogen bonds forming bridges between the strands, we focus here on the sequence of the nucleobases.

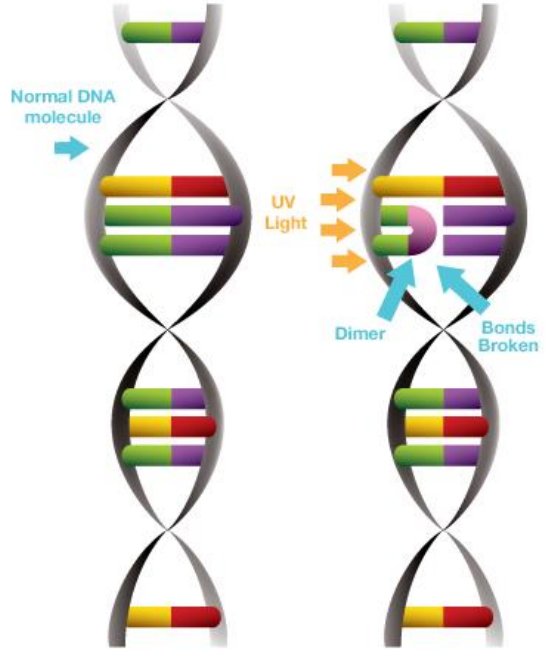

Figure 2: the impact of UV light on DNA pairs creates photoproducts

In terms of building blocks and bases of our genetic information we list adenine (A), cytosine (C), guanine (G) and thymine (T) as part of our **DNA**, and uracil as additional part of RNA (machinery required to synthesis proteins). The base pairs are formed by nucleobases chemically bonded together, and the unique sequence of all these base pairs ensures that no two human beings are identical (with the exception of monozygotic siblings). For further relevant understanding and revisiting knowledge most of us have gained, A and G are part of the purine bases while A, T and U are part of the pyrimidine bases. For completion, A-T and C-G pair within the DNA molecule.

UV (and in some instances visible light) exposure – within minutes of the appearance of erythema (see above *UV Radiation and Skin Response*) – causes a number of changes in the nucleus of human skin cells which are of concern not only for the affected individual but also for the next generation, as DNA aims to replicate itself as part of the regenerative processes we undergo. We provide an overview of the types of damage in the section below.

The human cell needs to contain abundant enzymatic capacity, **photolyases**, to be able to initiate and complete efficient and total repair of the DNA lesion caused by light. Separately, we also possess specific mechanisms to "cut and repair" UV lesions through nucleotide excision repair (**NER**) and base excision repair (**BER**). Both repair mechanisms may be required to recognise and act on the identified DNA damage within minutes. The NER, for instance, recognises specific **photoproducts** (see below). From longitudinal experiments it is thought that the BER system recognises other characteristics of nuclear damage. In studying the repair mechanisms, one also focuses on these disorders known to have defective DNA repair mechanisms. In subsequent SCIENTIFIC COMMUNIQUÉS the specific mechanisms of DNA repair will be discussed.

NER is the most resourceful DNA lesion repair mechanism we have, as it is able to remove CPDs, 6-4PPs (see next chapter) and other adducts (by-products). One can further categorise NER into global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). While further SCIENTIFIC COMMUNIQUÉS will review the various proteins and complement factors playing a role in NER and BER, here we review the various steps required for the body to recognise the UV damage in the first place.

DNA repair follows a sequence. There needs to be an immediate response following UV exposure and the occurrence of a DNA lesion. After this step the damage needs to be 'recognised' at molecular level, then recruitment of complement factors needs to take place, followed by incision complex, excision of the damaged DNA lesion, and restoration of the DNA duplex structure.

TYPE OF UV DAMAGE IN THE HUMAN DERMIS

Earlier we reviewed the difference between UVB and UVA, the most prominent parts of the solar spectrum for skin damage. The diffused energy absorbed by the exposed human skin leads usually to three classes of **DNA damage** in the exposed skin:

1. cyclobutane pyrimidine dimers (CPDs)
2. pyrimidine 6-4 pyrimidone photoproducts (6-4PPs)
3. Dewar valence isomers ('TpT3')

These three classes are summarised as **photoproducts**. The predominant photoproducts are cis-syn-configured CPD lesions found in our DNA. Although we will not provide greater detail of the photochemical reactions following UV radiation, it is good to remember that UVB and, in certain instances, UVA, are responsible for the induction of dimerisation reactions between T and C, so-called adjacent pyrimidine bases (see discussion above).

Of relevance, some nucleobases and sequences seem more photoreactive than others, such as T-T and T-C. Short-term UV radiation damage differs from longer and chronic insults, as seen in the sequence of photoproducts occurring. For instance, following prolonged radiation, 6-4 photoproducts may progress to Dewar isomers (formulaically similar but structurally different molecules), a photochemical process which severely affects DNA.

With a keen interest in photomedicine and decades of collecting preclinical and clinical data, our research teams have been keenly analysing the biopsies from UV exposed patients, some of them exhibiting "UV-signature mutations" which take place within the DNA structure of these cells. These then show up in about 60% of the biopsies as C→T mutations at a dipyrimidine site and in 5%-10% as CC→TT. The importance of the mutations is that these are also found in skin cancer lesions of sun-exposed patients. Hence, UV signature mutations of chronically sun exposed individuals indicate a risk profile for (non-melanoma) skin cancers, a topic of SCIENTIFIC COMMUNIQUÉ V.

RELEVANCE TO CLINUVEL

Of interest to these series is that both melanin and increased expression of proteins – like p53 – cause apoptosis of damaged skin cells. It is established that skin samples from individuals of darker complexion show fewer apoptotic cells compared to those of fair skin individuals. Therefore, simply put, darker (melanised) skin is more efficient at eliminating damaged cells. For the interested reader, it is a small step from here to understand that successfully and repetitively increasing eumelanin levels in the epidermis, as well as providing adequate molecular signalling, may result in more efficient elimination of damaged cells. CLINUVEL's teams have long known this concept as an advantageous effect of making afamelanotide available to patient populations affected by light and UV.

Damage and repair mechanisms are highly relevant to CLINUVEL's work to develop drugs in the field of photomedicine. Accent has been put on our scientific work by zooming in on the DNA repair mechanisms which take place in most 'photo-disorders'.

The relevance of these biological phenomena and the topics reviewed is high for CLINUVEL's team. As our research focus progresses towards the deeper understanding of light interacting with skin, photoprotection and repair mechanisms will eventually benefit our product offerings – initially for patients but eventually for individuals at risk and most in need of medical solutions.

CLINUVEL's areas of focus and progress are shown once more in the following diagram which serves as a compass while reviewing our SCIENTIFIC COMMUNIQUÉS.

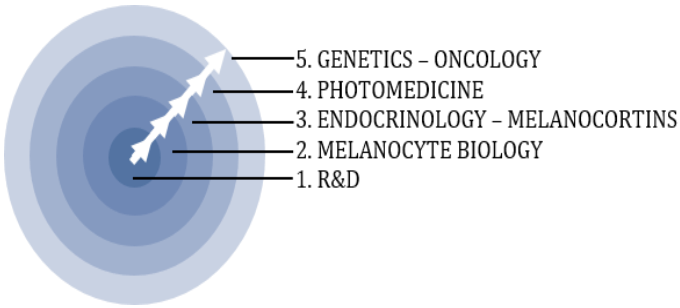

Figure 3 Concentric expansion of CLINUVEL's attention

APPENDIX: SCIENTIFIC COMMUNIQUÉ GLOSSARY

6-4 Photoproduct	molecular lesion within DNA following a photochemical reaction
Apoptosis	programmed cell death in order to regulate balance within the skin
Bak	Bcl2- homogenous antagonist killer, pro-apoptotic regulator
Bax	Bcl-2 Associated X
Bcl-2	B-cell lymphoma 2, regulator protein involved in apoptosis
Bid	BH3-interacting domain, a pro-apoptotic protein
BRN2	a transcription factor, belonging to homeodomain POU3F2, N-Oct-3
Caspase	cysteine-aspartic proteases, protease enzymes involved in apoptosis
Chromophores	a chemical group of atoms and electrons absorbing light of specific wavelength(s) and providing colour to a molecule
CPD	cyclobutane pyrimidine dimer, occurring as fast as 5-90 minutes following first UV and sun exposure
Dermis	mid layer of the skin, between the epidermis and hypodermis
Dewar	valence isomers, interrelated isomers (a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring (through pericyclic reactions)
Dimer	a molecular structure or complex comprising of two identical molecules linked together, in this context the pyrimidine dimers from thymine or cytosine
DNA	deoxyribonucleic acid, containing the genetic code
DPD	delayed pigmentary darkening, occurring after days of UV exposure
Endothelin-1	cell receptor on the melanocyte
EPP	erythropoietic protoporphyria: a rare metabolic genetic disorder in man which causes accumulation and storage of phototoxic protoporphyrin IX in the skin and liver and bile ducts
Fas	apoptosis stimulating fragment, Apo-1 or CD95
FasL	fas ligand
FasR	apoptosis stimulating fragment receptor
FEP	free-energy perturbation
FGF	fibroblast growth factor
Fitzpatrick skin type	first described by Fitzpatrick in 1975, classifies skin in six distinct types based on melanin density and tanning propensity
FOXD3	forkhead transcription factor D3

GG-NER	global genomic NER
GPCR	G-protein coupled receptors
GR	global repair, mechanism to repair UV damaged DNA
IPD	immediate pigmentary darkening
Keratinocyte	keratin producing cells of the epidermis
MAPK	mitogen activated protein kinase
MC1R	melanocortin-1 receptor, a 317 amino acid protein and a seven-pass transmembrane G protein coupled receptor Melanin
MD	melanin density
MED	minimal erythemal dose
Melanocompetent	individuals who can respond to UV exposure with a protective eumelanin response
Melanocompromised	individuals unable to generate sufficient eumelanin and burn as a result of UV exposure. These individuals are at a much higher risk of photodamage and skin cancers.
Melanocortin	peptide belonging to the group of proopiomelanocortin, such as ACTH, α-MSH, β-MSH, γ-MSH
Melanocyte	pigment producing cell
Melanogenesis	the process by which melanin is generated within the melanocyte and transferred to the keratinocyte
Melanoma	a malignancy originating from the melanocyte and now known to be linked to a variety of biochemical and genetic defects. Melanoma is an umbrella term for a variety of tumours with diverse biological behaviour.
MITF	microphthalmia-associated transcription factor: protein responsible for – among other activities – melanocyte development, differentiation, and survival
NER	nucleotide excision repair, mechanism to repair DNA damage
NF-κB	nuclear factor kappa beta
p38	delta protein kinase which, under normal conditions this protein kinase controls cell differentiation, but under stress it regulates a cellular distress response
p53	ubiquitous human tumour suppressor protein controlling the cellular response to DNA damage, cycle progression and apoptosis by regulating its targets transcriptionally. p53 plays a critical role in the normal UV stress response and activation of pigmentation by transcriptional activation of the POMC gene.

PAT	palmitoyltransferase
PAX3	paired box gene 3
PGE2	prostaglandin E2: a lipid signalling intermediate
Photodermatology	a sub-specialty of photobiology including all aspects of photobiology related to the skin ranging from sun exposure and its consequences (both short term and long term) to the therapeutic effects derived from exposure to natural or artificial radiation
Photolyase	DNA repair enzyme, belonging to the enzymatic class of flavoproteins
Photomedicine	deserves a broad definition spanning all aspects of photobiology, photophysics and photochemistry, investigating the interaction of light and human matter and tissues
Photophysics	concerned with processes that occur when light and sunlight, filtered through the Earth's atmosphere, interact with matter (atoms and molecules) present, with particular attention to the spectrum of solar radiation striking the organic matter
Photothermolysis	thermal damage following a photochemical reaction
POMC	proopiomelanocortin
Purine	a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring, such as the case in adenine, guanine
Pyrimidine	a heterocyclic aromatic organic compound similar to benzene and pyridine, containing two nitrogen atoms at positions 1 and 3 of the six-member ring
RHC	Red Hair Colour (phenotype)
RNA	ribonucleic acid
SLUG	SNAI2 transcription factor
SNAII	SNAI1 transcription factor
SOX9/10	HMG-box of the sex-determining gene SRY on the Y-chromosome
Squamous Cell Carcinoma	epidermal tumours (skin cancers) caused by chronic sun damage
TC-NER	transcription-coupled NER
TCF	transcription factor
TI	thermodynamic integration
TpT3	Dewar valence isomers
TRAIL	Tumour Necrosis Factor-related apoptosis-induced ligand
USF-1	transcription factor

UV/UVR	ultraviolet radiation, electromagnetic radiation from 10-400nm wavelength, further divided into UVA (320-400nm), UVB (280-320nm) and UVC (100-280nm).