Role of the transcription factor Nrf2 and redox balance in Parkinson's disease
Two decades of cutting-edge research - Where do we go from here?

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Abstract: *Activating the transcription factor Nrf2 has been proposed as an effective method for combatting oxidative stress and inflammation in neurodegenerative diseases for 2 decades. We review the known mechanisms and evidence in favour of activating Nrf2 to stimulate the production of cytoprotective antioxidant enzymes and anti-inflammatory cytokines directly within neurons to break the vicious circle of oxidative stress and inflammation in Parkinson's disease. The bulk of this pre-clinical research has been carried out using sulforaphane, a potent and inexpensive Nrf2 activator, extracted from Brassica sprouts or seeds. Despite concerted efforts by leading researchers, this potentially disease-modifying therapy has not been tested in human trials for Parkinson's disease, largely because natural molecules which cannot be patented are unable to attract the huge funding required to finance such trials. Over the same 2 decades, synthetic anti-inflammatory molecules have been developed which are significantly more potent activators of Nrf2 than natural isothiocyanates. A selection of these synthetic molecules may become major drugs for Parkinson's and other neurodegenerative diseases, possibly in 10-15 years time. Meanwhile, the needs of Parkinson's disease sufferers in both rich and poor countries who could benefit from this potential low-cost therapy are ignored. Hopefully, this article will raise the awareness of Parkinson's sufferers to the existence of this potential natural therapy to treat Parkinson's disease and how current models for drug development favour high-cost therapies with considerably longer development times.*

Introduction

It is well accepted that up to 70% of neurons that produce the neurotransmitter dopamine are damaged or already dead at the moment of diagnosis of Parkinson's disease [1]. What causes this cellular damage and decline? Accumulating evidence extensively reviewed [2, 3] indicates that much of this cell damage can be ascribed to a cascade of three events operating in a vicious circle:

- Oxidative stress in neurons: the excessive presence of free radicals, H_2O_2, peroxides etc. that cause oxidative damage to cell membranes and mitochondria,
- Chronic inflammation of neurons induced by oxidative stress that prevents neurons from healing and is a major cause of neurological pain.
- Excessive damage to mitochondria (organelles in cells that generate energy) leads to reduced cellular energy production and more oxidative stress. This loss of cellular energy production may be a major cause of fatigue felt by patients.

A large survey of the dietary habits of Parkinson patients [4] demonstrated that the typical low-calorie Mediterranean diet, rich in polyphenols and omega-3 oils, fresh fruit, fresh vegetables, nuts, fish, olive oil and red wine is associated with a slower progression of Parkinson's disease, compared to a high-calorie western diet, based on red meat, fried foods, soft drinks and highly processed foods. Such an antioxidant diet does not however appear to be able to break the vicious circle described above and stop disease progression altogether. To do this we may also have to stimulate the Redox Balance system, a genetic process also called the Nrf2/Keap1/ARE pathway that regulates the natural antioxidant defence mechanism inside cells. For most people, this system functions very

well until mid-life but becomes progressively less effective with age. Oxidative stress in Parkinson's disease is believed to be due to the failure of Nrf2/Keap1/ARE processes to maintain the optimum balance between oxidising and reducing agents (Redox balance) in brain cells. This balance is normally tightly controlled by a multi-step process involving the action of a protein called the transcription factor Nrf2 on a gene promoter sequence called the Antioxidant Response Element (ARE). With advancing age, the activity of ARE declines leading to a higher oxidative imbalance, cellular damage, inflammation and reduced cellular function [5, 6]. Fortunately, a number of Nrf2/ARE activators have been identified in food sources [6,7]. The key is to be able to deliver a dose sufficient to maintain an adequate level Nrf2 to restore normal Redox balance.

The 1st decade: the scientific case for activating Nrf2 is established

Nrf2 was identified in 1996 about the same time as the discovery of sulforaphane by Paul Talalay. With the elucidation of the human genome, progress on the understanding of the Nrf2/Keap1/ARE pathway has been rapid in the decade up to 2010. NFE2L2 is a gene situated on human chromosome 2 which expresses a protein known as the transcription factor Nrf2 (nuclear factor-erythroid-2 p45-related factor 2). A highly-simplified schema [8] of how Nrf2 is involved in controlling oxidative stress and inflammation is shown in Fig. 1.

Under conditions of low oxidative stress, Nrf2 is captured in the cytoplasm of cells on docking sites of a protein called Keap1 (Kelch-like ECH-associated protein 1). The half-life of Nrf2 on the docking site of Keap1 is rather short (15-20 min) after which it is degraded. When chemically stimulated (oxidized) by oxidants, electrophiles or reactive oxygen species (ROS), Keap1 sites lose their ability to degrade Nrf2. Newly synthesized Nrf2 is then no longer held back by Keap1, migrates into the nucleus and binds to the enhancer sequence, ARE (Antioxidant Response Elements) in promoter regions of genes encoding more than 300 detoxifying enzymes and cytoprotective proteins [9].
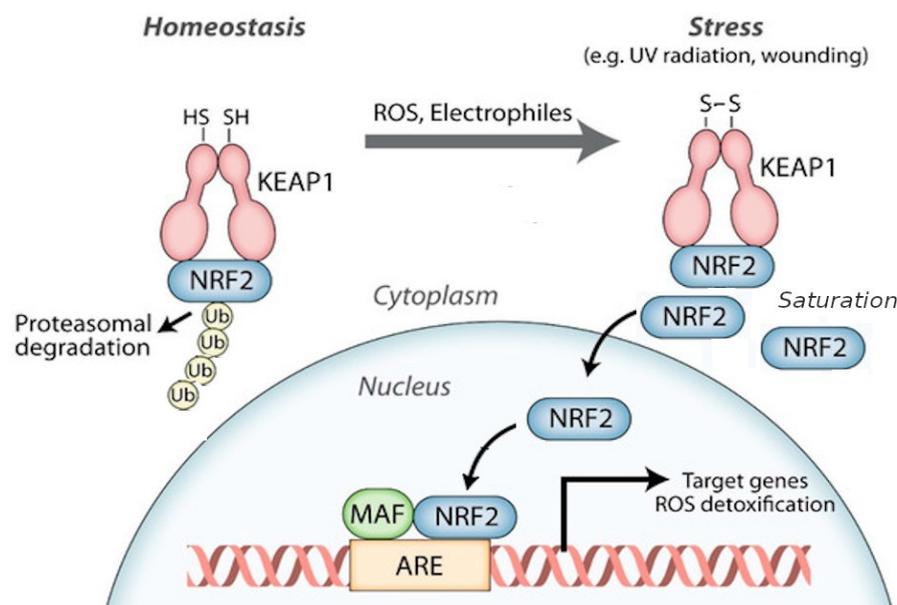

Fig. 1. When oxidative stress is low, Nrf2 protein binds to docking sites of the protein dimer Keap1 in the cytoplasm and is subsequently degraded. When Keap1 is activated (oxidised) by ROS or by electrophiles, Nrf2 is not degraded but saturates Keap1 sites. Newly synthesised Nrf2 then accumulates in the cytoplasm and migrates to the nucleus where it forms a hetero-dimer with MAF proteins and binds to ARE gene promoter sequences. This promotes the transcription of antioxidant and anti-inflammatory genes. Illustration adapted from P. Hiebert and S Werner. [8]

So we know the main players in the process:

- ARE is the operator that activates the mechanism to produce antioxidant proteins and enzymes and anti-inflammatory cytokines to combat oxidative stress and inflammation,

- Nrf2 are signalling molecules that bind to and switch on ARE,

- Keap1 is the gatekeeper that controls the flow of Nrf2 to ARE. It does this in response to instructions from sensors that measure the redox balance in every cell.

Whenever Nrf2 is bound to ARE, the gene promoter function of ARE initiates the production of antioxidant enzymes and anti-inflammatory cytokines and simultaneously suppresses inflammatory cytokines [10-11]. Cytokines are small messenger proteins that generate or suppress inflammation. The degree of production of these antioxidant and anti-inflammatory elements depends on the quantity of Nrf2 present in the nucleus. The expression of these genes has the capacity to restore redox balance and break the vicious circle of oxidative stress, inflammation and mitochondrial damage in brain cells.

During this period, research was intensified to identify substances which moderate the action of Keap1. Sulforaphane, an isothiocyanate extracted from broccoli seeds and sprouts became the cornerstone of research on chronic, age-related diseases such as neurodegenerative diseases and cancer. It is one of the main reasons why broccoli is recommended as a health-promoting food. Sadly, the quantity of isothiocyanate in vegetable broccoli, especially after cooking, is too small to have any significant effect on Parkinson's disease.

in 2009, a team lead by Dr Antonio Cuadrado, a leading neuroscience researcher in Madrid, investigated the induction ARE by activating the transcription factor Nrf2 as a way to stimulate the production of endogenous antioxidant enzymes and anti-inflammatory cytokines directly inside each cell. They are able to demonstrate the protective effect of the induction of ARE on Parkinson's disease in animal models thanks to a project, partially funded by the Michael J. Fox Foundation (MJFF) [12]. Then, in collaboration with Prof. Paul Talalay, the discoverer of the natural isothiocyanate, sulforaphane, they applied for a new grant from the MJFF for a phase 2 clinical trial. The study, called RASTOP (Rasagilin And Sulforaphane Therapy for Parkinsonism), was supported by 16 hospitals in several EU countries.

> **"This trial was not funded. The argument given was that in the case that it would show a relevant efficacy, no company would be interested in covering the huge expense of a Phase 3 trial for a natural compound that could not be patented"** (*A. Cuadrado, private communication*).

This statement is a damning indictment of the situation in which Parkinson's patients and researchers found themselves at the end of this first decade of Nrf2 research. A potential life-changing therapy for Parkinson's disease patients, backed up by outstanding research was blocked, not because of possible doubt about it's efficacy, but because the natural product proposed could not be patented. We have to understand the implication of the words "*could not be patented*". They mean that no private company would be granted a monopoly for about 20 years to sell the potential therapy to Parkinson's disease sufferers in rich countries. Without this option, no company is willing to finance the huge cost of clinical trials. Unless alternative sources of finance can be found, there is no way forward.

The 2nd decade: the neuroprotective role of ARE is proven

The scientific knowledge base of Nrf2 has considerably developed over the past decade [13, 14]. There is much greater understanding about the detailed mechanism, about the complex redox sensors on Keap1 and the key target sites. Research *in vivo* has confirmed the dual antioxidant and anti-inflammatory role of inducing Nrf2 and its impact on neurological disorders [6, 12-16]. ARE plays an important role in maintaining the cellular redox balance through the transcription of anti-oxidant (AO) genes and the synthesis of AO and detoxifying enzymes, notably glutathione reductase (GSR), quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). Induction of ARE increases the synthesis of glutathione (GSH) which scavenges ROS, whereas GSR is active in recycling oxidized GSH back to its reduced form, GSH. Nrf2 induces the Thioredoxin (Trx) enzyme system, a powerful system of defence against oxidative stress. Trx1 localises to the cytosol and Trx2 to the inner membrane of mitochondria. Trx/Trx reductase reduces cysteine disulphide bridges back to their unoxidised state. The anti-inflammatory effects of up-regulating Nrf2 are well established through the repression of pro-inflammatory cytokines (TNF-α, IL1, IL6 and MCP-1) in microglia, macrophages, monocytes and astrocytes [17-19]. Parkinson's disease, broccoli, Nrf2 and ARE was discussed by Simon Stott in his popular and very informative Parkinson Science blog, "The Science of Parkinson's" in 2017 - "*We need a clinical trial of broccoli. Seriously*! [20].

Critical elements of the Nrf2/Keap1/ARE pathway are now better understood

The Nrf2/Keap1/ARE pathway is immensely complex and it is beyond the scope of this article to cover all aspects of the process. Nrf2 is now regarded as the "Master Regulator of Redox homeo-stasis" To get a full vision of the process, we must consider not simply the action of Keap1 in regulating Nrf2, but all the upstream and downstream events and feedback processes involved in the Nrf2/Keap1/ARE pathway, both individually and collectively from the synthesis of each molecule right through to their final degradation and removal. As examples, we look at specific cysteine sites and their sensitivity to ROS and how redox balance is maintained through feedback loops.

C151 is the key cysteine ROS sensor of Keap1

Redox balance in mammals is dominated by the action of thiol (R-S-H) groups. In the reduced state, the hydrogen atom is weakly bonded and can be captured by electrophiles. In the oxidised state, the R-S group is unstable and reacts with a neighbouring R-S to produce a structurally different and more stable dimer R-S-S-R, folded at the sulphur positions. The antioxidant molecule glutathione therefore exists as a monomer in its reduced form GSH and as a dimer, glutathione disulphide (GSSG) in its oxidised form. In Keap1 protein, there are 27 different cysteine (thiol) residues which could be sensors for oxidising molecules and electrophiles, but 85% of the molecule is bundled up into a globular mass making many of the residues only accessible to certain toxins. Keap1 exists in the cytoplasm as a homo-dimer like a pair of cherries joined by their stems [21]. These stems are the sites of 2 cysteine sensors labelled C151, one on each stem, which are the most exposed and most reactive cysteine sites due to their particular structure. The thiol (R-S-H) group of C151 is located in a shallow trough surrounded by 5 basic amino acids which have the ability to deprotonate the thiol group [22]. This structure is therefore strongly electrically polarised creating a high affinity for electrophiles. Upon oxidation (removal of the hydrogen atom) Keap1 (in a similar way to glutathione) undergoes conformational changes to form a more stable dimer. In this new configuration, Keap1 can no longer degrade Nrf2 and is inactivated. Because of its site structure comprising the thiol group and amino acids, molecules which match the site in terms of size, shape and bonding capacity to both the thiol group and the surrounding basic amino acids will have the greatest affinity for C151. This has lead to the development of potent synthetic Keap1 inhibitors

which were subsequently found to match the whole C151 site, both physically and chemically [23]. The aim here is to firmly, but reversibly lock down C151 in its oxidised state which would have the effect of stopping Keap1 from degrading Nrf2 until the molecule is metabolised. Although sulforaphane is clearly strongly electrophilic, it is not ideally matched to the C151 site but still remains a potent activator of Nrf2. The more complex isothiocyanate, *4-(α-L-rhamnosyloxy)-benzyl-ITC* from *Moringa Oleifera,* a common tropical plant, may be better adapted to C151 than sulforaphane because of its size and chemical structure, but this remains to be established. 2 other sites, C273 and C288 situated on the perimeter of globe structure are also highly reactive.

Feedback mechanisms are important for redox balance

Various feedback mechanisms come into play when Nrf2 is activated.

- **Isothiocyanates are metabolised by glutathione (GSH).** Higher concentrations of GSH accelerate the metabolism and degradation of the isothiocyanates.

- **Thioredoxin reduces disulphide bridges of cysteine residues.** Consequently, when increased availability of Nrf2 leads to the increased the production of Trx and Trx reductase, there is greater competition to return cysteine sites on Keap1 to their reduced state.

- **Nrf2 is in competition with Bach1.** Once free of Keap1, Nrf2 migrates to the nucleus and forms a hetero-dimer with sMaf proteins prior to binding to ARE and activating antioxidant and anti-inflammatory genes controlled by ARE. Nrf2 is however in competition with the transcription factor Bach1 to form Bach1/Maf hetero-dimers which have similar affinity to bind to the same *cis* elements of ARE [23]. Bach1 is a potent repressor of antioxidant enzymes HO-1 and NQO1 [24]. The ARE mediated gene expression therefore depends on the dynamic nuclear balance between Nrf2 and Bach1. However Nrf2 overexpression also upregulates Bach1 synthesis, thus progressively increasing competition with Nrf2 which will tend to drive redox balance back towards its previous state. How does this play out over time? Could excessive overexpression of Nrf2 generate redox instability and an undesirable peak and trough response in terms of the production of antioxidant enzymes and anti-inflammatory cytokines? These are questions that still have to be answered. Given the relatively long half-lives (days) of antioxidant enzymes NQO1, γ-CGS and HO-1, we might expect that the initial impact of Nrf2 upregulation on Nrf2 drug-naive patients could be a powerful anti-oxidant and anti-inflammatory response, followed by a slow decline towards lower levels as negative feedback mechanisms kick in over time. However, a longer-term positive effect of reduced oxidative stress enabling better mitochondrial performance could have a significantly more sustained and cumulative effect than the actual duration of the Nrf2 activation. More research is needed to define the optimum degree of Nrf2 activation to create a significant positive effect without inducing a counter-reaction.

The Nrf2/Kaep1/ARE redox system – a simple analogue

The Nrf2/Kaep1/ARE pathway is a complex system to maintain redox balance within acceptable limits in all cells and at all times. It is a very-tightly regulated system with multiple sensors, amplifiers, controllers, actuators and feedback loops providing both short and long-term responses. A simple technical analogue is that of a closed loop system where part of the actual measured output is fed back to the controller in order to introduce a degree of proportionality of the output relative to the error between the measured and desired results. (*Illustration: Electronics Tutorials*). More refined control systems have multiple feedback loops.
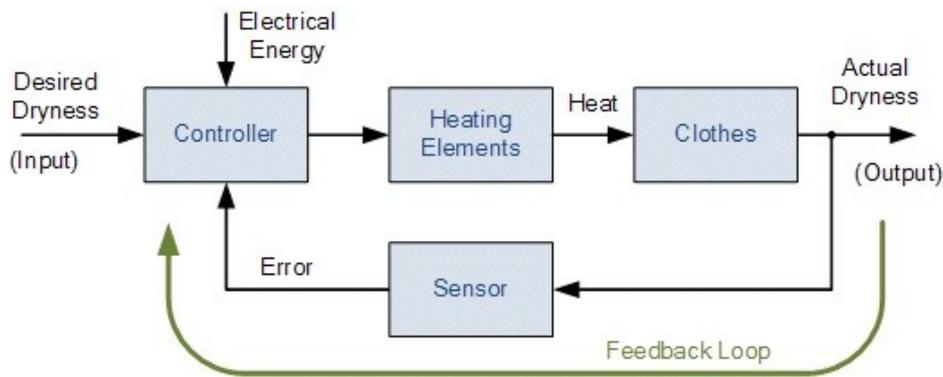
As the system ages, especially if it is not well maintained, its performance may decline. The causes can be multiple. On the input side, sensors may give false readings due to corrosion etc. On the output side, local power elements may give reduced performance. The performance of the controller may also decline as components age or feedback signals are weakened. The whole system also depends on an adequate and stable energy supply to the controller and the output systems. The result is that an ageing system may no longer have enough capacity to tightly regulate certain elements under certain conditions. Its response may be too slow or simply insufficient.

Applying this same thinking to our Nrf2/Kaep1/ARE redox process, we may be able to identify to which part of the overall system is causing the problems due to ageing: a) Modified input (ROS sensors), b) inadequate output production (the biological machinery that fights ROS and inflammation, c) the control system itself (modified feedback loops, changes in gain factors) or d) inadequate energy supply. If we can make this classification we may be able to better address how to respond to the failings.

The input gatekeeper

Keap1 is the gatekeeper of the system and is therefore the main regulator of the input flux. It is home to 27 cysteine-based ROS and toxicity sensors, some of which have affinity for specific ROS molecules such as H_2O_2 or toxins and some of which have affinity for a wide range of ROS species or electrophiles. Each of these sensors can partially unlock the gate and some (*e.g.* C151) may fully unlock it, freeing maximum Nrf2 to move on to the next stage of the process. Zhang *et al.* have reported that the synthesis of Keap1 increases with age, which will result in tighter gate-keeping activity, whereas Nrf2 activity is reported to be reduced with age [26-27]. The input flux is therefore progressively reduced with advancing age.

System changes with ageing

A second negative regulator of the Nrf2/Keap1/ARE process occurs in the nucleus. Here Nrf2 is in competition with transcription factor Bach1 (BTB and CNC homology 1) to form Bach1/Maf hetero-dimers which have similar affinity to bind to the same c*is* elements of ARE, resulting in potent repression of antioxidant enzymes HO-1 and NQO1. In contrast to Nrf2, Bach1 protein levels increase substantially with age such that older human and mice cells lose Nrf2-dependent signalling and adaptive homeostasis [19, 26, 27]. The ARE-mediated antioxidant gene expression is therefore subject to the dynamic nuclear balance between Bach1 and Nrf2. The ratio of Nrf2/Bach1 declines with age which reduces the gain factor in the signal driving the ARE antioxidant gene response. "*Keap1, Bach1, and β-CrTP form the negative arm of the main feedback loop of Nrf2/ARE signalling. These negative regulators either interact with Nrf2, cause its degradation or compete with Nrf2 for ARE binding, or repress its transactivation*". Zhang *et al.* [26].

Changes in output capacity

The output capacity of the ARE gene expression takes several forms, principally regulating antioxidant molecules and enzymes and detoxifying enzymes. Nrf2 induced ARE expression induces glutathione (GSH) synthesis, the rapid action scavenger of oxidants, and its regeneration through the production of glutathione reductase, GSR, the enzyme responsible for rapid recycling of GSH from the oxidized form GSSG, back to its reduced form GSH. It also induces the thioredoxin enzyme system (Trxs) which reduces disulphide bridges of proteins and, along with glutathione peroxidase removes H_2O_2 and peroxides by using NADPH as the electron donor [14].

Nrf2 regulates detoxifying enzymes quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). NQO1 catalyses the reduction of quinone to the redox-stable hydroquinone, preventing free-radical formation. HO-1 catalyses the breakdown of iron-containing heme molecules which are major sources of superoxide and ROS, thus protecting cells from further oxidation.

Changes in energy supply

All of the enzymes listed above are dependent on NADPH as their energy source. NADPH is a reducing agent which is generated from glucose, via the pentose phosphate pathway (PPP). In order to cope with increased demand of a co-ordinated antioxidant response, PPP enzymes are also regulated by Nrf2 [28]. In addition, oxidative stress damage to mitochondria contributes to reduced overall energy production in the cells of Parkinson's disease patients [29, 30].

Potential causes of failure and their location within the system

We can therefore identify 4 potential sources of decline in our control system with advancing age:

- increased synthesis of the gatekeeper protein Keap1 reduces the availability of free Nrf2 in the cytoplasm,
- increased synthesis of Bach1 reduces the ratio of Nrf2/Bach1 which intrinsically lowers the system gain factor for redox protection,
- reduced antioxidant enzyme capacity and reduced production of NADPH to recycle oxidised molecules back to their reduced form limits the long-term production of antioxidant molecules GSH and Txrs,
- oxidative stress damage of mitochondria contributes to reduced overall cellular energy.

According to current knowledge, upregulating Nrf2 by inhibiting Keap1 should have a positive impact on all of these factors sequentially. This cascade of events starting at Keap1 and progressing through to improved performance from mitochondria corresponds to operations with increasing processing and response times. Inhibiting Keap1 is a rapid process, whereas the synthesis of *de novo* enzymes takes hours and their lifetimes are counted in days. Repairing and replacing damaged mitochondria will take even longer. Only by cycling many times through this cascade will oxidative stress be progressively reduced and the overall health of neurons improved, hopefully leading to improved dopamine expression and better use of Parkinson's drugs. This suggests that inhibiting Keap1 is likely to have both a short-term impact due to improved antioxidant and anti-inflammatory activity and a long-term impact due to improved mitochondrial performance and neuronal health.

Sulforaphane, a natural molecule, has been the cornerstone of Nrf2 research

Nrf2 activators are best described as Keap1 inhibitors since their molecular targets are cysteine residues on Keap1. Although the vast majority of published research has been done on natural inhibitors, and particularly sulforaphane, there is now growing interest in synthetic molecules [23]. A thorough review of the many Nrf2 activators currently in clinical trials has recently been published [31]. There are currently no trials for Parkinson's disease based on Nrf2 activation. Several Keap1 inhibitors can be found in food. Common food sources include cruciferous vegetables, rosemary, turmeric and the leaves of the tropical tree, *Moringa Oleifera*. To be effective, the active molecules must be bio-available in sufficient quantity to enable a therapeutic dose to be absorbed. The most intensively studied activator of Nrf2 is an isothiocyanate called sulforaphane. This molecule (like other isothiocyanates) is produced by a reaction between a glucosinolate and an enzyme, both of which are present in cruciferous vegetables.

The glucosinolate precursor of sulforaphane, glucoraphanin is especially concentrated in the seeds of broccoli, cabbage or Brussels sprouts (the *Brassica* family). It can be converted to sulforaphane by the enzyme myrosinase which is released when the plant tissue is damaged or crushed. The vegetables themselves contain only small quantities, but the seeds and early seedlings are very rich in glucosinolates [32, 33]. *Each variety of the Brassica* family has a different profile of glucosinolates in the seeds [34]. It is therefore important to select the precise species, variety and cultivar, to know which glucosinolate is dominant because each specific glucosinolate will be transformed into a different isothiocyanate. Even varieties sharing the same Latin name may have different glucosinolate profiles. Common broccoli seeds are however a good source of sulforaphane.

Isothiocyanate profiles in Brassica varieties

Scientific research on isothiocyanates for medical use has been dominated by sulforaphane. The reasons for this are essentially due to the practical issues of its relative stability and ease of use. As a result of this extensive research, it is considered to be safe and forms the cornerstone of a very large number of research programmes. Apart from sulforaphane, several other isothiocyanates can be extracted from *Brassica* seeds. This field has been thoroughly researched. To review the glucosinolate profiles and related isothiocyanates of the most important *Brassica* varieties see refs. [34-36].

Some isothiocyanates found in *Brassica* seeds are harmful to health. The most important of these is goitrin which can block the uptake of iodine into the thyroid gland and reduce thyroid function if ingested over long periods. It is important that progoitrin, the precursor glucosinolate of goitrin is not present in significant quantities in the *Brassica* source.

Broccoli is the best source of glucoraphanin, the precursor of sulforaphane. Regular broccoli has many synonyms such as broccoli calabrese or sprouting broccoli. It is known to biologists as *Brassica oleracea var. Italica* or *Brassica oleracea botrytis var. cymosa* although that name can also be used for cauliflower. When buying broccoli as a vegetable, there is little risk of confusion. This is not the case when buying seeds as a food item because they are indistinguishable from each other.

Strains of broccoli resulting from cross-breeding with Chinese cabbage and/or turnip, known as Brocoletto and Broccoli Rabe respectively, are fast-growing varieties for seed production. The seeds are sold for growing sprouts or micro-greens and may simply be labelled Broccoli. Genetically, these are more closely related to the turnip group, *Brassica rapa* and have a totally different glucosinolate profile to that of true broccoli.
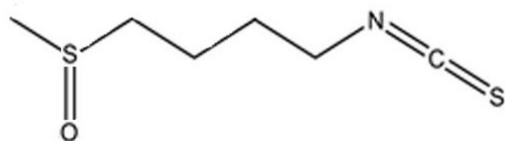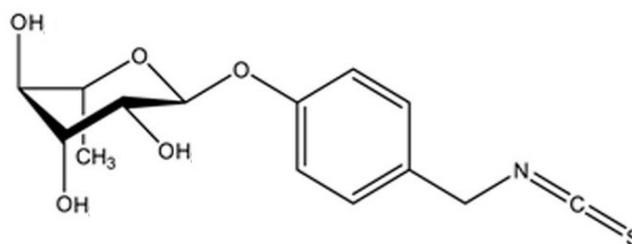
Fig 2. Sulforaphane from Broccoli   4-(α-L-rhamnosyloxy)-benzyl-ITC (4RBITC) from Moringa

Moringa isothiocyanates

In recent years, researchers have taken an interest in the medicinal properties of *Moringa Oleifera*, a distant relative of Brassica, native to NW India. The leaves of this tree are rich in protein, vitamins and antioxidants, and have long been used in traditional medicine [37]. Water extracts of *Moringa Oleifera* strongly attenuate inflammation *in vitro* [38]. *Moringa* leaves also contain glucosinolates of a more complex nature than their *Brassica* cousins due to the presence of a rhamnose-benzyl moiety. The isothiocyanates that can be readily extracted from *Moringa* leaves conserve this rhamnose-benzyl moiety and may be better matched to the C151 sensor in terms of size, shape and chemical affinity. The most abundant of these, 4-(α-L-rhamnosyloxy)-benzyl-ITC (4RBITC) has been shown to be significantly more potent at inducing the NQO1 enzyme compared to sulforaphane [39]. This apparent correlation between size, shape and chemical affinity in relation to the C151 site makes this molecule an interesting candidate for Nrf2 activation. In addition, 4RBITC is stable which facilitates its preparation and storage. More research is needed to fully appreciate the potential of 4RBITC.

Fahey *et al.* [40] have developed a simple method to extract 4RBITC into hot or cold teas and have evaluated the isothiocyanate contents of teas made from different varieties of *Moringa*. The common domesticated plant is a very good source of 4RBITC.

Extracting isothiocyanates from *Brassica* and *Moringa*

The relationship between the consumption of cruciferous vegetables and the beneficial effects on health is well established. It has been particularly well demonstrated to be protective against several forms of cancer and neurological disorders [41]. Fahey, Zhang and Talalay [32] pioneered the use of broccoli sprouts as a rich source of inducers of detoxifying enzymes as protection against carcinogenesis. 3-day broccoli sprouts contain 10-50 times more active glucoraphanin (the precursor of sulforaphane) than do mature plants. Trials relating to various conditions from cancer to autism have evaluated the therapeutic activity of sulforaphane using broccoli sprout extracts [33, 42, 43].

Isothiocyanates are formed by hydrolysis of their glucosinolate precursors through the action of the enzyme myrosinase, also present in *Brassica* plants [32, 33]. The process is temperature-sensitive and can take several routes, one leading to the formation of the biologically-active isothiocyanate and others leading to the formation of inactive compounds. The defining factor is the presence of a heat-sensitive protein called the epithiospecifier protein (ESP) which modifies the product of enzyme hydrolysis with the formation of a nitrile compound rather than the isothiocyanate [44]. Other researchers have reported even more complex pathways with lower yields of isothiocyanate [45]. Fortunately, ESP is inactivated when heated above 55°C, whereas myrosinase is stable up to about 65°C. The optimum conditions for isothiocyanate conversion would appear to be in the range 55-65°C. At lower temperatures, the inactive nitrile compound is formed and at higher temperatures the myrosinase enzyme is also inactivated. Interestingly, myrosinase present in white mustard or

Daikon radish seeds is much more efficient and more specific at producing isothiocyanates than that of other *Brassica* species. They can therefore be used in addition or as a replacement for endogenous myrosinase when using other *Brassica* seeds [32].

Isothiocyanates from *Moringa* are sourced from the dried leaves. These can be consumed as teas using crushed leaves or from powder made from ground leaves. As with *Brassica* sources, the myrosinase enzyme is inactivated at temperatures above 60°C. Cold teas made from crushed leaves conserve the enzyme activity which converts the glucosinolate to the isothiocyanate, but the optimum infusion time is long (more than 30 minutes) and inefficient. There is also a concern about possible bacterial or fungal contamination from cold teas although the isothiocyanate content also provides antibiotic protection. Extraction of the glucosinolate in boiling water is rapid and eliminates any bacterial or fungal contamination, but requires conversion to the isothiocyanate once the solution has cooled to below 60°C. This can be achieved in a few minutes by adding a 1-2% of ground white mustard seeds (synapsis alba) or ground Daikon radish seeds. When ground *Moringa* powder is used, the resulting soup is best left to cool before filtering through a coffee filter.

Commercially available Keap1 inhibitors

A number of sulforaphane supplements are commercially available. Of course, they have not been the subject of clinical trials for Parkinson's disease. Many of these are concentrated extracts of selected broccoli cultivars with a high glucoraphanin content. Since sulforaphane has a short shelf life, most supplements are composed of glucoraphanin extracted from broccoli and a myrosinase enzyme in a gel capsule. These are perfectly stable when dry. Although more concentrated, they are not fundamentally different from ground broccoli seeds with added myrosinase. After ingestion, the conversion of glucoraphanin to sulforaphane takes place in the aqueous environment of the intestinal tract. Some supplements are protected against the low pH of the stomach acids which would reduce the sulforaphane yield. Based on established data on the fractional excretion of sulforaphane metabolites in urine, conversion of glucoraphanin to sulforaphane in the intestinal tract rarely exceeds 35% whereas it can reach 70% under ideal aqueous conditions [46]. It is therefore more efficient to ensure that this conversion is accomplished just before ingestion by mixing the ingredients in warm water. Another Nrf2 activator based on Carnosic acid extracted from Rosemary leaf is also available.

Synthetic Nrf2 activators – 2 decades of development

The interest in synthetic electrophiles to suppress inflammation began at about the same time as the discovery of sulforaphane. These natural and synthetic routes have therefore been developed in parallel for more than two decades and have both been stimulated by growing understanding of the Nrf2/Kaep1/ARE pathway. During this period, the number and complexity of synthetic molecules has grown considerably [31, 47]. The most potent Nrf2 activators are built around a scaffolding of natural oleanane triterpenoids such as oleanic acid and ursolic acid, which were known to have anti-inflammatory and anti-carcinogenic properties. This work was pioneered by Michael Sporn *et al* [47] with a project to modify different functional units of the oleanane structure to improve its anti-inflammatory properties. It also lead to the establishment of a reliable anti-inflammatory test based on inhibiting the cellular synthesis of an enzyme that plays a key role in inflammation, namely, inducible nitric oxide synthase (iNOS) [48]. A linear correlation exists between the inhibition of iNOS and the activation of the Nrf2 target enzyme NQO1 [49].

Experience gained through the synthesis and evaluation of hundreds of molecules identified the interplay between various functional sites and has led to the discovery of highly active molecules

with a cyanoenone group (adjacent C≡N and =O groups on ring A or C). These highly electrophilic cyanoenones provoke a Michael reaction with cysteine resulting in reversible covalent bonding of the sulphur atom to the cyanoenone ring, accompanied by the transfer of an electron to the distant carbonyl group followed by protonation which stabilises the new configuration. Cyanoenones are strongly attracted to cysteines of Keap1 although their relative potency also depends on other elements of the molecule [50]. The high reactivity of cyanoenones for cysteine sensors is not however synonymous with selectivity for Keap1 sensors. A recent phase III clinical trial of the triterpenoid Bardoxolone-methyl, (CDDO-Me) a variant of CDDO, targeting Nrf2 to alleviate symptoms of chronic kidney disease was halted due to cardiovascular safety issues [51]. Proteomic analysis of CDDO-lm, a cyanoenone with an NQO1 inducer CD of 33 nM (see Fig. 3), interacts with 577 different proteins including many transcription factors [52]. CDDO derivatives could therefore be involved in a number of non-Nrf2 pathways. So far little is known of the potential side effects of these non-Nrf2-cyanoenone-protein interactions. Isothiocyanates such as sulforaphane are also involved in non-Nrf2 pathways, some of which are cytoprotective [40].
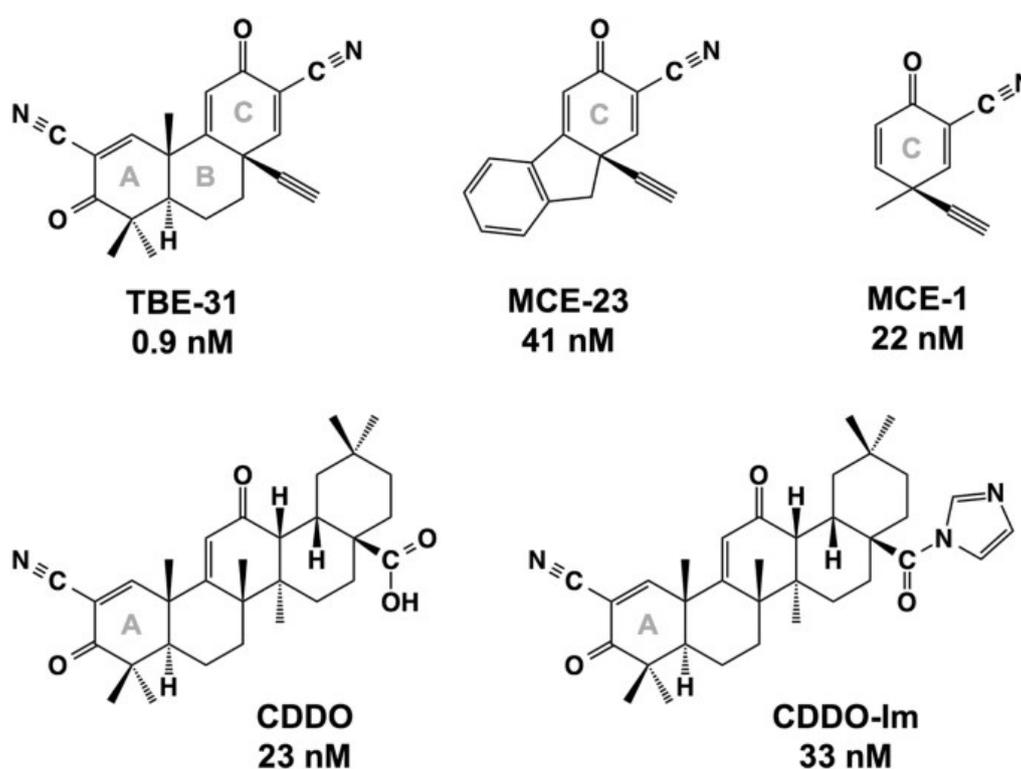

Fig 3. Examples of synthetic cyanoenone inhibitors of Keap1 and their NQO1 inducer potencies (nM = Concentration that Doubles (CD) the activity of NQO1 in murine Hepa1c1c7 cells). Smaller CD value means greater potency. For comparison, the CD value for sulforaphane is about 200 nM. (Illustration from ref. [23])

The structures of these molecules in relation to their relative activity has added considerably to our understanding of how cyanoenones react with cysteine sensors and helped identify the importance of C151 in Keap1 as the key regulator Nrf2 [22, 23]. However, until their capacity to activate non-Nrf2 pathways is fully researched and documented, their potential as safe molecules to treat oxidative stress and inflammation via the Nrf2 pathway in Parkinson's disease must remain hypothetical.

Conclusion - Where do we go from here?

Caution: Nrf2 expression in different tissues

Although Nrf2 is expressed in all tissues, it is most strongly expressed in brain, heart, urinary tract, gastrointestinal and endocrine tissues [53]. All of these tissues are therefore "On-target" for Nrf2 activation. Consequently, any therapy that targets the activation of Nrf2 in the brain, will also affect other tissues which strongly express Nrf2. Whereas dopaminergic brain cells of Parkinson's patients are known to suffer from high levels of oxidative stress and inflammation, cells in other organs may not be affected to the same degree. The starting conditions of our control system will therefore be different for each specific type of tissue. This raises the question of potentially undesirable effects occurring in other organs when activating Nrf2 to combat oxidative stress in brain cells. In general, Nrf2 activity is reduced with age in all tissues, so modest Nrf2 activation should also be beneficial in other tissues. Excessive activation of Nrf2 could however destabilise the redox balance in other tissues, for example by activating the Bach1 feedback loop. When considering Nrf2 activation, the response of all organs which strongly express Nrf2 should be taken into account.

After 2 decades of research into Nrf2 activation, there are now two clearly-defined pathways for developing a therapy for combatting oxidative stress and inflammation in Parkinson's disease:

- The natural-product route using isothiocyanates found in the seeds of *Brassica oleracea var. Italica* or the leaves of *Moringa oleifera*. Logically, this route should be the most rapid given the good safety profile of sulforaphane. The major problem is the difficulty posed by the high cost of clinical trials needed to pass the regulatory hurdles compared to the low intrinsic value of final product. If this barrier can be overcome, it could open up a *rapid and cheap* route. For low income countries with minimal drug regulations, it may be possible to develop this route through a programme of low-cost local trials or through traditional medicine. Another alternative might be to promote the development and testing of dietary supplements based on Broccoli or Moringa extracts. These are already sold in many countries, but are not recognised as delivering a therapeutic benefit for Parkinson's patients. This route puts its first priority as delivering "*Patient Benefit*" as quickly as possible to patients. Any reasonable programme that enables these natural products to prove their safety and efficacy and so become more available for Parkinson's patients should be considered. Hopefully, it will lead to the development of a "*Basic Antioxidant Therapy for Parkinson's";* cheap, natural and effective.

- The synthetic product route using patented cyanoenones and other synthetic electrophiles fits perfectly into the western model for drug development. The development of these molecules will most likely be pursued to meet the unmet medical need for Parkinson's disease, but only after selection of those proven to be safe also justify the huge cost of clinical trials. The decision about when to launch such products for clinical trials in Parkinson's disease may also depend on experience gained from ongoing trials of the same or similar molecules for other medical conditions. These will likely provide some information on the risks related to off-target side effects of these synthetic molecules. Many trials are already ongoing to prove the efficacy and safety of various molecules in other diseases based on inhibition of Keap1; diabetes, chronic kidney disease, hepatic impairment, pulmonary hypertension, ocular pain, ocular inflammation, melanoma, breast cancer, lymphoma, psoriasis … [31]. Since Parkinson's disease expresses itself in different ways, patients may need to be grouped into different phenotypes based on DNA testing. This could make clinical trials for Parkinson's disease even more expensive. Once all these factors have been researched and the risks eval-

uated, they will most-likely lead to a more "*Optimised Antioxidant Therapy for Parkinson's*". There is no doubt that this route will take considerably more time than the former and, to cover the enormous research effort, the medication will certainly be more expensive.

Until now, Parkinson's patients were probably unaware of these two possibilities. Clearly the two routes will be driven by different forces. The rapid and cheap route to a "*Basic Antioxidant Therapy for Parkinson's"* can only be driven by the collective action of Parkinson's Patients or public health authorities. For that to happen, many more patients will need to know that it exists and be persuaded that it can work despite its extremely humble origins. The actions of individuals and groups of patients will be critical in giving it a voice and advocating more action for its implementation.

The slow and expensive route to an "*Optimised Antioxidant Therapy for Parkinson's*" will of course be influenced by the clear unmet medical need for a disease modifying therapy for Parkinson's disease, but financial, financial-risk and medical risk considerations will also play a considerable role in the choice of the molecule(s) and the timing of the different stages to bring it to market.

The two routes are not, and hopefully will not, be mutually exclusive, although one could influence the other. Early development of a natural therapy based on isothiocyanates, could prove the benefit of the Nrf2 pathway for Parkinson's disease and actually facilitate more rapid development of an even better therapy based on synthetic molecules. Future synthetic molecules will almost certainly offer greater ease of use and be more widely marketed than the natural ones. However, they are unlikely to become available until the risks of off-target effects are fully evaluated and will most probably be too expensive for many patients in low income countries, as is already the case for standard Parkinson's disease medication [54].

This leaves a window of opportunity to act now in favour of a potential natural therapy that may respond to the urgent unmet medical need of Parkinson's sufferers worldwide.

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