Safety & Environmental Assurance Centre

A Next Generation Risk Assessment (NGRA) case study for the bioactive food component sulforaphane

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Introduction

We previously developed a workflow for a 'tier 1' systemic toxicity assessment based on integrating *in vitro* points of departures (PoDs) from a cell stress panel (CSP), *in vitro* pharmacological profiling (IPP) and high-throughput transcriptomics (HTTr) with Physiologically-based Kinetic (PBK) Modelling predictions of human exposure to calculate a bioactivity exposure ratio (BER)[1] [Fig 1]. This approach shows promise in a capacity that protects consumers, however, it may be conservative, as PoDs are based on bioactivity in *in vitro* assays which may not necessarily translate into adverse effects in humans, and many substances (especially food ingredients), display bioactivity at consumer relevant exposures.

This was exemplified for sulforaphane (SFN), a component of cruciferous vegetables [Fig 2]. Numerous studies have linked *Cruciferae* intake with beneficial effects e.g., decreased risk of cancer, with SFN widely hypothesised as a plausible agent for this protection[2]. Given dietary exposure to SFN may have benefits, it is unsurprising that bioactivity occurs at equivalent *in vitro* exposures, illustrating a challenge for the assessment of bioactive substances under the current NGRA paradigm.

Under scenarios where the tier 1 safety assessment cannot enable a safety decision, a tier 2 assessment may be required to elucidate whether the bioactivity would ultimately cause adaptative or adverse effects in humans. The composition of such an assessment is bespoke, however is informed by the hypothesised mode of action (MoA) indicated through tier 1 testing and pre-existing literature knowledge [Fig 1].

In this study, we have conducted a tier 2 hypothetical assessment for SFN to inform a safety decision, focusing on the potential for systemic toxicity using 2 different SFN exposure scenarios that are known to be low risk to humans.

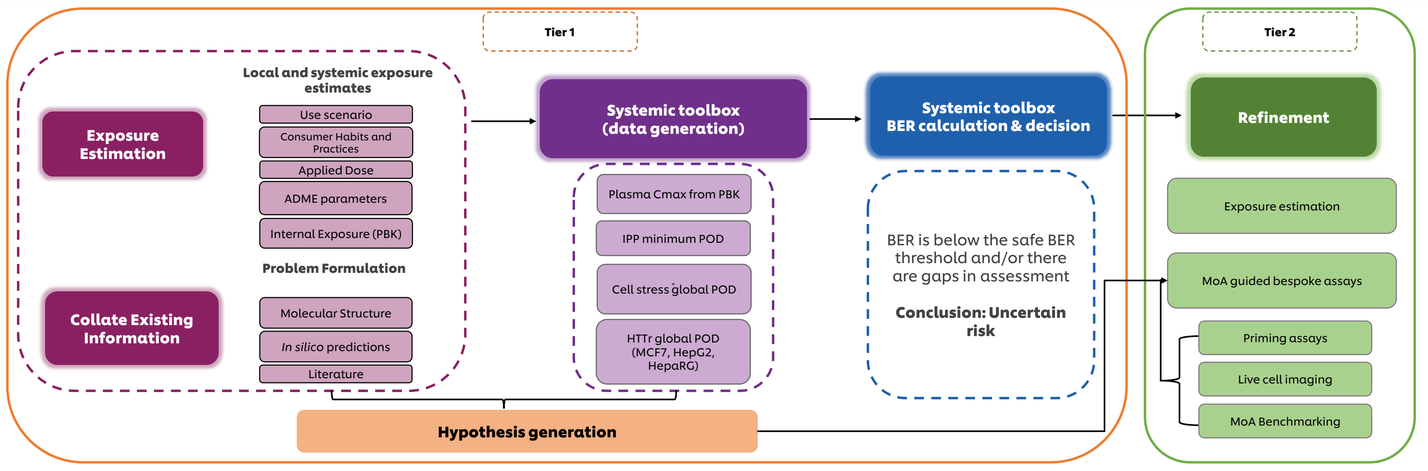

Figure 1. Tier 1 safety assessment framework implemented in previous evaluation and possible areas for refinement in the context of data available for SFN as part of a tier 2 assessment using NAMs[1].

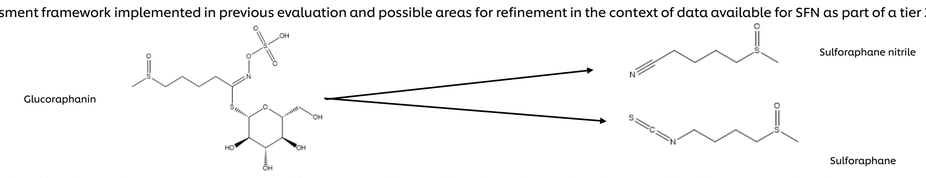

Figure 2. Simplified sulforaphane formation process in cruciferous vegetables. Sulforaphane is not found at considerable concentrations in unprocessed *Cruciferae*. Instead is stored as a precursor, glucoraphanin. Upon damage to the plant, glucoraphanin is converted to an unstable intermediate (not shown) via the enzymatic action of myrosinases, either present in the plant or in the gastrointestinal tract. This unstable intermediate is converted to sulforaphane or sulforaphane nitrile, with the proportion of conversion to either depending on e.g., temperature, pH etc.

Tier 1 data

Exposure Estimation	**External exposure and risk classification** Two 'low risk' exposure scenarios were selected for sulforaphane[1]: Scenario 1: 3.9 mg/day (oral) – Considered representative of 'normal' consumption of Broccoli. Scenario 2: 60 mg/day (oral) – A clinical trial comprising intake of SFN at 20 mg 3 x daily.	**Internal exposure** To enable comparison with *in vitro* PoDs, external exposures are converted to internal exposures (as Cmax) using PBK Modelling [Fig 4].
***In Vitro* Biological Activity Characterisation**	**IPP** SFN showed no hits at a screening concentration of 10 µM. **HTTr** Three cell lines (HepG2, HepaRG, and MCF-7) are included and two different methods, BIFROST (a NOTEL) and the minimum BMDL (the lower bound of the pathway-average Benchmark concentration) are used to analyse the transcriptomics concentration response data and estimate a PoD. Across the different methods/cell lines, the lowest PoD was from the BIFROST method at 0.072 µM (HepG2)[1,3].	**CSP** Across the CSP, several biomarkers were perturbed at concentrations prior to cytotoxicity, with glutathione (GSH) content, and oxidative stress the lowest responding biomarkers. The global (lowest) PoD was determined as 0.51 µM for GSH content[1,4] [Fig 3].

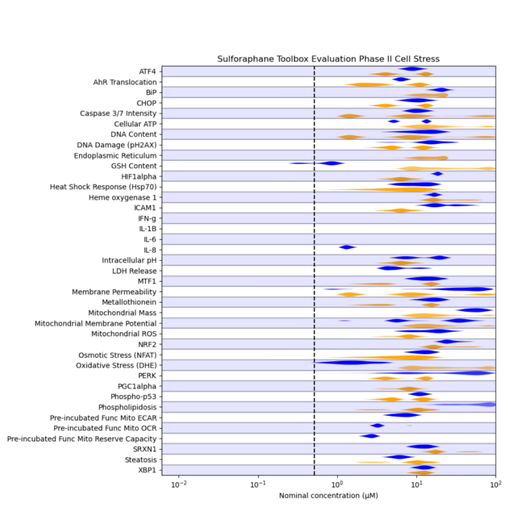

Figure 3. Summary of CSP bioactivity for Sulforaphane. Blue densities indicate PoDs for assay-specific biomarkers and orange densities indicate pooled PoDs for assay-specific cell health biomarkers. Vertical line at 0.51 µM represents the best estimate/'Global PoD' across all biomarkers (corresponding to change in GSH content)[4].

Risk characterisation

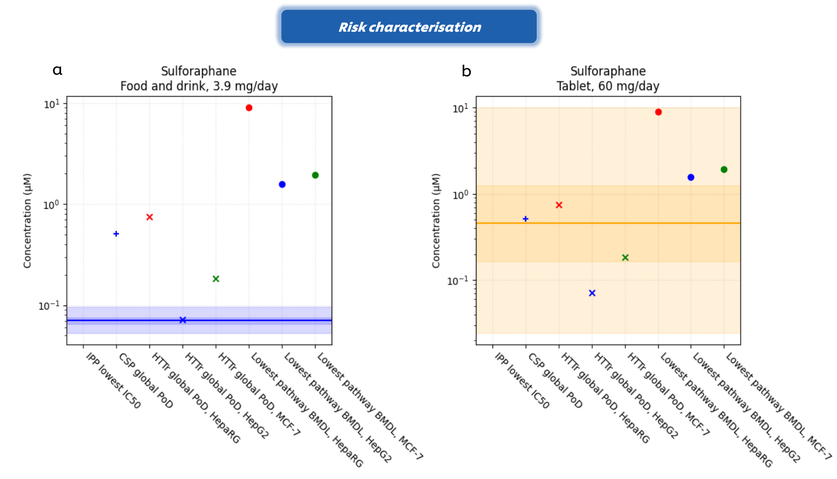

Figure 4. PoDs from Tier 1 *in vitro* assays and internal exposure (Cmax) estimates (blue/orange line) for both SFN scenarios. A.) for food and drink exposure scenario (3.9 mg/day) b.) for 6-month clinical study involving intake of 60 mg/day SFN. Blue and orange lines in a/b represent exposure estimates with shaded areas representing uncertainty in Cmax estimate, with darker shaded area representative of 50th percentile and lighter area representative of 95th percentile[1,5].

Tier 2 hypothesis generation

Literature knowledge:

- Soft electrophiles such as SFN form covalent adducts with nucleophiles of similar softness, for example cysteine residues on proteins and GSH.
- One protein susceptible to SFN is KEAP1, the negative regulator of Nrf2 [Fig 5]. Nrf2 is responsible for the transcriptional regulation of >200 genes containing the antioxidant response element (ARE) which have a range of functions such as redox balance/inflammation [Fig 5].
- Although Nrf2 induction by SFN has a potentially beneficial, cytoprotective impact against sources of oxidative stress, at higher exposures, soft electrophiles are well known for causing GSH depletion, oxidative stress and consequently cytotoxicity[6].

Tier 1 data:

- The above information is supported by tier 1 data e.g., *in silico* profiling (not shown), where positive alerts were returned for protein binding and from the bioactivity assays e.g., the CSP [Fig 4], where the lowest PoDs primarily relate to GSH depletion and reactive oxygen species (ROS) accumulation (eventually causing cytotoxicity).

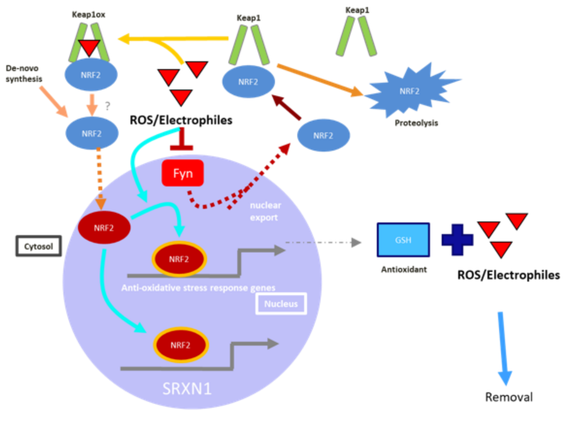

Figure 5. Schematic of NRF2 pathway

Tier 2 testing strategy:

Based on tier 1 data/literature informed hypothesis, characterisation of SFN's effect on the oxidative stress pathway is necessary to identify a 'tipping point' between adaptation and adversity. Further assays investigating this include:

- Priming assays: Pre-treatment of cells with SFN prior to challenge with an oxidative stress inducer (menadione). Analysis to compare SFN + menadione PoDs with menadione only PoD [Fig 6].
- Live cell imaging: Live monitoring of Nrf2 pathway dynamics after single/repeat dosing in 2D and 3D cells with SFN and 'similar' substances (e.g., CDDO-Me, Andrographolide, ethacrynic acid) [Fig 7].
- Benchmarking: Comparing tier 1 bioactivity profile for SFN to other substances with similar MoAs.
- Pathway analysis: Understanding toxicological relevance of differentially expressed genes at consumer relevant exposures.

Tier 2 data

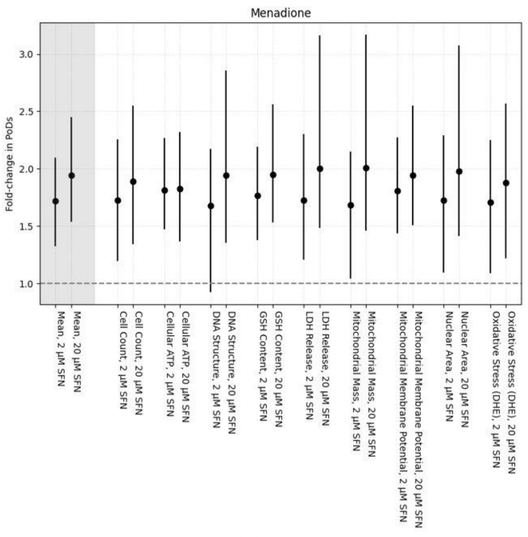

Figure 6. Estimates of the shifts in biomarker-specific PoDs for menadione following 2 or 20 µM SFN pre-treatment. Lines represent 95% credible ranges.

Priming studies

- To investigate the potential cytoprotective effect of SFN, cells were treated for 24 hours with sulforaphane (0, 2, 20 µM) followed by 24 hour challenge with the oxidative stress inducer menadione.
- Several relevant biomarkers from the CSP were analysed including Cell Count, Cellular ATP, DNA Structure, GSH Content, LDH Release, Mitochondrial Mass, Mitochondrial Membrane Potential, Nuclear Area & Oxidative Stress (DHE).
- For menadione/sulforaphane treatment, minor increases in PoDs (up to 2-fold) for most biomarkers was observed, illustrating a potential cytoprotective effect.

Figure 7. Nrf2 nuclear accumulation in 2D HepG2 cells following 24-hour single exposure with Andrographolide, CDDO-Me, Ethacrynic acid and Sulforaphane. Dashed and solid pink lines refer to 95% credible range and median of the control data, while dashed and solid black lines refer to 95% credible range and median of the mean response.

- Across the 4 substances, the dynamics and potency of Nrf2 expression differs, with Sulforaphane, Ethacrynic acid and Andrographolide showing a rapid peak and return to baseline expression, whereas CDDO-Me shows a lower peak but slower return to baseline expression. Further analysis with Nrf2 downstream targets and other stress response pathways across 2D and 3D cell lines and following multiple exposures is required to understand the significance of these differences.

Next steps:

Priming:
- SFN dose response to determine dose range where priming is cytoprotective vs the dose that tips the cells towards adversity.
- Complete data generation for further priming substances with different MoAs.

Live cell imaging:
- Complete analysis on live cell imaging 2D, 3D, single and multiple dose studies to understand Nrf2 pathway dynamics for SFN and other substances with high/low risk scenarios.

Benchmarking:
- Generating tier 1 data for low/high risk substances with similarity in MoA (Nrf2 modulators): Astaxanthin, CDDO-Me, Dimethyl fumarate, Allyl isothiocyanate, Alpha-lipoic acid, resveratrol, oleanolic acid, andrographolide.
- Compare similarity in bioactivity profile SFN (e.g., in transcriptomic profile, IPP alerts etc).
- If substance is considered sufficiently similar to SFN, tier 1 data may be used as a 'benchmark'.

Safety decision making:
- Weight of evidence approach integrating several lines of evidence to enable safety decision making.

Conclusions

- From the data presented above, based on a tier 1 safety assessment, neither SFN exposure scenario would be supportable as a result of BERs <1[1].
- Such a decision from tier 1 does not mean that an exposure scenario constitutes a safety risk *per se*, instead, SFN-induced bioactivity is predicted following such exposures. Under such scenarios, a tier 2, bespoke assessment is required to understand whether the bioactivity predicted would ultimately cause adaptative or adverse effects in humans.
- The areas of focus for a tier 2 assessment will be case-dependent and could focus on either refining exposure estimates (e.g., through generating further in vitro ADME data or generating human PK clinical data) or refining the hazard characterisation element, where follow-up assays will depend on the bioactivity/hypothesised MoA concluded following tier 1 testing/review.
- For SFN, from the tier 1 data and literature knowledge, further characterisation of the oxidative stress response was chosen as the bioactivity area to focus on.
- Several tier 2 approaches are under way, including priming studies, pathway analysis, live cell imaging, and benchmarking studies. Following the completion and analysis of these studies, a weight of evidence decision may be possible to enable safety decision making.

References:
1. Middleton et al., 2022, Toxicological Sciences, 189 (1): 124–147
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