RESEARCH ARTICLE

Stabilized sulforaphane for clinical use: Phytochemical delivery efficiency

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Scope: The isothiocyanate sulforaphane (SF) from broccoli is one of the most potent known inducers of the cytoprotective phase 2 response. Its role in a host of biochemical pathways makes it a major component of plant-based protective strategies for enhancing healthspan. Many nutritional supplements are now marketed that purport to contain SF, which in plants exists as a stable precursor, a thioglucoside hydroxysulfate. However, SF in pure form must be stabilized for use in supplements.

Methods and results: We evaluated the stability and bioavailability of two stabilized SF preparations—an α-cyclodextrin inclusion (SF-αCD), and an SF-rich, commercial nutritional supplement. SF-αCD area-under-the-curve peak serum concentrations occurred at 2 h, but six of ten volunteers complained of mild stomach upset. After topical application it was not effective in upregulating cytoprotective enzymes in the skin of SKH1 mice whereas pure SF was effective in doing so. Both of these "stabilized" SF preparations were as potent as pure SF in inducing the cytoprotective response in cultured cells, and they were more stable and as bioavailable.

Conclusion: Our studies of a stabilized phytochemical component of foods should encourage further examination of similar products for their utility in chronic disease prevention and therapy.

Keywords:
Chemoprevention / Chemoprotection / Glucoraphanin / Myrosinase / Phytochemical

1 Introduction

Sulforaphane (SF; 4-methylsulfinylbutyl isothiocyanate; 1-isothiocyanato-4-methylsulfinylbutane) is a dietary phytochemical that is present in plants as its biologically inactive precursor. This precursor, glucoraphanin, is a member of a large family of phytochemicals called glucosinolates which are all rapidly converted to their cognate isothiocyanates by an enzyme called myrosinase, upon mastication of the plant tissue by humans (or pathogens or predators) [1, 2]. Myrosinase is also present in human gut microbiota [3]. Glucoraphanin occurs in particularly high concentration in broccoli seeds and sprouts (young plantlets [4]). We have extensively studied the effects of SF on a variety of biochemical and molecular biomarkers including those associated with oxidative stress, antioxidant capacity, defects in reduced glutathione synthesis, mitochondrial dysfunction and low oxidative phosphorylation, increased lipid peroxidation, and neuroinflammation [5]. Further, SF can cross the blood–brain barrier and can exert its protective effects in the central nervous system [6–8]. It has

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Abbreviations: **αCD**, α-cyclodextrin; **CD**, concentration required for doubling the NQO1 specific activity in Hepa1c1c7 cells; **DTC**, dithiocarbamate; **GMP**, good manufacturing practice; **IND**, investigational new drug; **NQO1**, NAD(P)H quinone acceptor oxidoreductase 1 (EC 1.6.99.2); **SF**, sulforaphane

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potent and selective antibiotic activity, in particular, against *Helicobacter pylori*, a risk factor for gastric cancer [9, 10].

Since the re-discovery of SF in 1992 [11], at which time the primary interest was in its potency for cancer prevention, it has been tested in a variety of cancer prevention, cancer therapy and other chronic disease prevention and treatment bioassays. This work has been extensively reported and reviewed [12–15] and we are aware of no other natural product that is as potent an inducer of the so-called phase 2 cytoprotective, antioxidant/detoxification system [16, 17], which is primarily regulated by the Keap1/Nrf2/ARE pathway [18, 19]. We have long been interested in SF as a prototypical phytochemical component of foods that might be utilized either by prescribing increased consumption of the foods containing it, given as a nutritional supplement or applied topically [13, 20, 21, 26, 30] and we have administered an extract of broccoli sprouts in many small clinical studies (discussed further, below).

SF is an inherently reactive small molecule that is temperature sensitive and degrades in many solvents including water [22, 23]. Due to the high chemical reactivity of its –N=C=S or isothiocyanate moiety with organics (e.g. macromolecules such as proteins, carbohydrates, or nucleic acids) SF elicits a wide spectrum of biological activities [24], but the fate of the molecule in food and supplement preparations is not well understood. A variety of approaches to stabilize SF and plant extracts containing SF have been examined in order to facilitate its administration to animals and to human volunteers. We initially delivered boiling water extracts of broccoli sprouts to volunteers [13, 25], but ultimately utilized freeze-dried powders of similarly prepared extracts for both topical and systemic protection of animals and humans [26–44]. The disadvantage of all of these SF-rich broccoli sprout extracts is that they are extraordinarily hygroscopic and degrade rapidly. We report on the stability of SF herein, as have others [22, 23, 45, 46]. We were initially attracted by the methods for preparing inclusion complexes of SF in α-cyclodextrin (αCD) posted in the patent literature [47], but which to our knowledge have not appeared in the peer-reviewed literature. Similarly, a group in Zhejiang, China has utilized standard methodologies for encapsulating SF in hydroxypropyl-β-cyclodextrin [48, 49] and for spray drying those inclusion complexes [50]. We thus produced an inclusion complex of SF-αCD from a purified SF-rich broccoli sprout extract and we report here on its stability in solid phase and in solution, as well as the pharmacokinetics of this complex when applied to mouse skin, and when delivered systemically to mice and to ten human volunteers under an IND from the US Food and Drug Administration (FDA).

While examining the SF-αCD inclusion complex, a nutritional supplement became available on the retail market in Europe. This supplement, Prostaphane® (Nutrinov, Noyal sur Vilaine Cedex, France), was used in a well-documented multicenter prostate cancer prevention study [51]. Although details regarding precisely how SF is stabilized in this supplement are proprietary, it appears not to be stabilized as a cyclodextrin inclusion product [52]. We therefore evaluated the stability and bioavailability of Prostaphane® in a separate pilot study of ten volunteers, under IND from the FDA, and those data are included in this report.

2 Materials and methods

2.1 Reagents and supplies

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), JT Baker (Center Valley, PA, USA), Fisher (Hampton, NH, USA), or Pierce (Waltham, MA, USA) unless otherwise indicated. Cyclodextrin was purchased from Wacker Chemical Corp. (Adrian, MI, USA), and Prostaphane® was provided by Nutrinov (Rennes, France).

Cultures of Hepa1c1c7 murine hepatoma cells used for the Prochaska bioassay [53, 54] were originally gifts to our laboratory and have been maintained at JHU for over 25 years.

2.2 SF-αCD preparation and analysis

Material used to evaluate temperature stability, biological activity in vitro, and in mouse skin was prepared from pure SF (LKT Labs, St. Paul, MN, USA) which was complexed with food grade αCD (Cavamax W6 from Wacker Chemical Corp.). For studies of stability in solution, SF and Cavamax were mixed directly together in water in a stoichiometric ratio according to the following rationalization: the anhydrous molecular weight (MW) of αCD is 973, and it is common to assume that the commercial material is hydrated ($6{\cdot}H_2O$) and that the hydrated MW is 1081. Since the MW of SF is 177, a stoichiometric complex will contain $(177)/(1081 + 177) \times 100\% = 14.1\%$ SF by weight.

The material used for clinical study was prepared by making an SF-rich broccoli sprout extract, as described previously [34, 35, 37, 38]. In brief, 3-day-old broccoli sprouts were grown commercially using seeds that had been pre-analyzed for glucoraphanin content. These sprouts were plunged into boiling water, boiled, cooled, filtered, treated with myrosinase (the enzyme which catalyzes conversion of glucoraphanin to SF), and lyophilized. The lyophilized extract was redissolved in water at a concentration of 200 mg/mL, stirred at 37°C for 75 min, cooled to ambient (~22°C), pH adjusted to about 7.2 with solid KOH, frozen and thawed, centrifuged at $4300 \times g$ for 50 min at room temperature (~22°C). The clarified supernatant was applied to conditioned, and rinsed (water) PCA-433 (a strong basic anion exchange resin) in 2.5 cm columns poured to a height of 6 cm (30 mL bed volume). Twenty mL of supernatant (containing about 800 μmol SF) were loaded to each column and eluted sequentially with water, 50% ethanol, and 100% ethanol. Fractions eluted immediately after switching to 100% ethanol contained about 80% of the SF and these were lyophilized in the dark to

an oily brownish liquid. Stoichiometric quantities of αCD (Cavamax W6) in water (145 mg/mL) were added to the combined lyophilized fractions, incubated in the dark with shaking, first at ~22°C for 4–5 h, then overnight at 4°C. This product was finally evaporated to dryness by lyophilization, and the powder was stored at 4°C.

All steps were performed in good manufacturing practice (GMP) facilities—the first step (broccoli sprout extract production) at Oregon Freeze Dry (Albany, OR, USA) and subsequent steps in a GMP compliant facility in Baltimore, MD, USA. Pilot runs using essentially this protocol were performed in a non-GMP laboratory and produced material used only for temperature stability testing of the SF-αCD powder, or topical application to mouse skin.

Measurement of SF and its metabolites by cyclocondensation reaction was performed as previously described [28, 55, 56]. This method has been shown to quite accurately measure the sum of SF and its major metabolites (N-acetyl-SF, cysteinyl-SF, cysteinyl-glycine-SF, and glutathione-SF) [37]. In cases where SF alone was measured chromatographically by HPLC, methods previously described were used [27,57,58]. The SF-αCD preparations contained no fiber or other additives, whereas Prostaphane® is reported by its manufacturer to contain 342 mg of microcrystalline cellulose per two tablets.

2.3 In vitro efficacy

NAD(P)H quinone acceptor oxidoreductase 1 (EC 1.6.99.2) (NQO1) inducer activity of redissolved SF-αCD, and of extracts made from Prostaphane® tablets, was measured by the Prochaska assay [53, 54]. Briefly, samples are serially diluted in a microtiter plate containing Hepa1c1c7 cells. After 48-h incubation, the enzyme activity was determined spectrophotometrically. One unit of inducer activity is defined as the *concentration* that *doubles* the NQO1 specific activity in a microtiter well containing 150 μL of medium. This concentration has been designated the concentration required for doubling the NQO1 specific activity in Hepa1c1c7 cells (CD) value. Hence, a compound with a CD of 1.0 μM has a potency of 6667 units of inducer activity per micromole.

2.4 Mouse skin application

Ten female SKH1 immunocompetent hairless mice (Charles River Laboratories, Wilmington, MA, USA) were treated as follows: five animals were treated bilaterally by applying 50% acetone to a 1 × 5 cm (5 cm^2) rectangle of dorsal skin on the right side of the centerline, and 200 nmol/cm^2 of SF (100 μL of 10 mM SF) in 50% acetone to the contralateral side. Five separate animals were treated as above, with αCD in 50% acetone on the right side and 200 nmol/cm^2 SF-αCD on the left side. These treatments were repeated once a day for each of 3 days. Twenty-four hours after the final treatment, mice were euthanized and sections of skin were taken to compare NQO1 activity in homogenates of the treated skin. Animal studies were approved by the JHU Animal Care and Use Committee (Protocol no. MO08M469).

2.5 Human studies

Ten healthy adult participants between the ages of 27 and 68 years, not taking any antibiotics or other medications, were recruited for each study by word-of-mouth and posted flyers. Accordingly, the ten volunteers for the SF-αCD substudy were of mean age 51.9, range 45–61 years, three males and seven females, six African Americans and four Caucasians. The ten volunteers for the Prostaphane® substudy were of mean age 46.5, range 27–68 years, two males and eight females, four African Americans and six Caucasians. Subjects were screened after providing written informed consent based on whether they were able to comply with the dietary restrictions and medication exclusions. They were asked to refrain from consuming cruciferous vegetables and condiments (e.g. mustard, horseradish, wasabi) that might contain isothiocyanates or glucosinolates for 3 days before and during the study. The participants fasted overnight and received at 8 a.m., by mouth, their oral doses of SF either in the form of SF-αCD or Prostaphane®. The participants provided a predosing urine sample, and the entire urine excreted during the first 8 h and for the following 16 h were also collected. The total dithiocarbamate (DTC) excretion was measured [55, 56] in each urine sample (three per subject) and an aliquot of each urine was sent to Meritus Medical Laboratories (Hagerstown, MD, USA) for determination of creatinine concentration. Both clinical studies were approved by the Johns Hopkins University Institutional Review Board (NA_00045538 and IRB00060447), and each study was the subject of an "Investigational New Drug" (IND) application to the US FDA.

For the SF-αCD substudy, 200 μmol of SF was contained in about 350 mg of SF-αCD powder dissolved in 25 mL of distilled water, which subjects were given to drink upon arrival at the clinic. Subjects then drank another 50 mL of water. In addition to urine collection, venous blood samples (2–3 mL each of clotted and anticoagulated blood) were collected immediately before and at 2, 4, 6, 8, and 24 h after administration of the SF-αCD preparation. All blood samples were collected in syringes that were entirely made of polyethylene in order to avoid contamination by small amounts of DTC present in rubber septa (as vulcanization accelerators). Blood sera were obtained from clotted blood, and their DTC concentrations were determined [56].

For the Prostaphane® substudy, the dose (two Prostaphane® tablets containing 100 μmol of SF; lot PO50V, expiration 02/2017) was given to participants with water as above. Only urine was collected from subjects (no venipuncture) for this study.

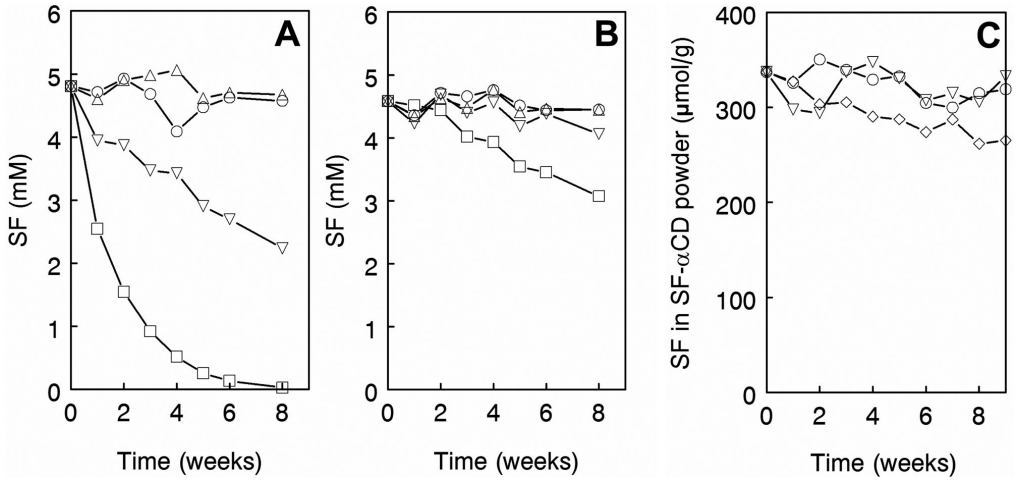

Figure 1. Stability of sulforaphane in aqueous solution, and of SF-αCD in solution and as a dry powder. (A) Stability of pure synthetic (R,S)-SF (LKT Laboratories, St. Paul, MN, USA) in dilute aqueous solutions at −20°C (Δ), 4°C (O), 22°C (▽), and 37°C (□). Solutions all contained 1 mg (5.65 μmol) SF in 1.0 mL of water, and were incubated for 8 weeks, and assayed weekly for SF. Half-life was less than 1 week at 37°C. (B) Stability of pure synthetic (R,S)-SF in dilute aqueous solution in the presence of α-cyclodextrin (αCD). The solutions all contained 1 mg (5.65 μmol) SF and 103 μmol αCD in 1 mL of water, and were incubated at −20°C (Δ), 4°C (O), 22°C (▽), and 37°C (□) for 8 weeks. About 75% of the SF remained after 8 weeks at 37°C. (C) Stability of dry SF-αCD powder at 4°C (O), 22°C (▽), and 50°C (◇). SF concentration was essentially unchanged after 8 weeks at 4 and 22°C, and declined by only about 20% when stored at 50°C.

3 Results and discussion

In our previous studies of bioavailability, participants consumed a self-monitored crucifer-free diet for the 3 days preceding a single dose of the challenge agent that consisted of either glucoraphanin or SF in the form of a broccoli sprout or seed extract, or a nutritional supplement containing glucoraphanin [29, 37, 59]. The present study was conducted in a similar manner.

3.1 SF-αCD

3.1.1 SF-αCD stability

The stability of SF-rich broccoli sprout extracts (freeze-dried powders) that we have made over the years for use in clinical trials is excellent when these powders are maintained under freezing conditions and protected from humidity. For example, one lot that was measured 21 times over 5 years maintained a concentration of 228 ± 19 μmol/g (1 SD). However, their hygroscopicity and the need for rigorous maintenance at cold temperatures limit their utility in population-based interventions. SF-αCD was evaluated over a range of temperatures, and it was more stable than pure SF when maintained at either room temperature or elevated temperatures (22 or 37°C, respectively) for any extended period (Fig. 1A and B). SF is rapidly degraded in aqueous solution (~5 mM starting concentration) such that after 1 week at 37°C only half of the original concentration remains, whereas the half-life of pure aqueous SF at 22°C is 8 weeks (Fig. 1A). In contrast, after 8 weeks incubation of SF-αCD in aqueous solution at 37°C, about 70% of starting SF remains and there is essentially no loss of SF concentration after 8 weeks at 22, 4, or –20°C (Fig. 1B). An "accelerated aging" storage protocol (50°C) is required to promote appreciable loss in concentration (about an 11.5% per month reduction) of SF-αCD from the dry, powdered, nonhydrated material (Fig. 1C).

3.1.2 In vitro efficacy of SF-αCD

The induction by SF of NQO1, the prototypical phase 2 cytoprotective enzyme, resulted in a CD of 0.22 μM, whereas two separate preparations of SF-αCD inclusion product were measured in the same assay to have CDs of 0.18 and 0.22 μM, respectively, thus confirming equivalent in vitro bioavailability and potency to the unprotected parent compound.

3.1.3 Mouse skin efficacy: Topical application of SF-αCD

We have previously demonstrated that by application of broccoli sprout extracts rich in SF to the skin of hairless, immunocompetent, female SKH-1 mice, the NQO1 activity could be induced in the skin of those mice, and that applying once per day for 3 consecutive days led to an enhanced induction of the response compared to a single application [30]. We used this protocol to investigate the topical efficacy of SF-αCD inclusions. Control values (solvent alone and solvent containing only αCD) were virtually identical to our previously published values with this model, and NQO1 activity

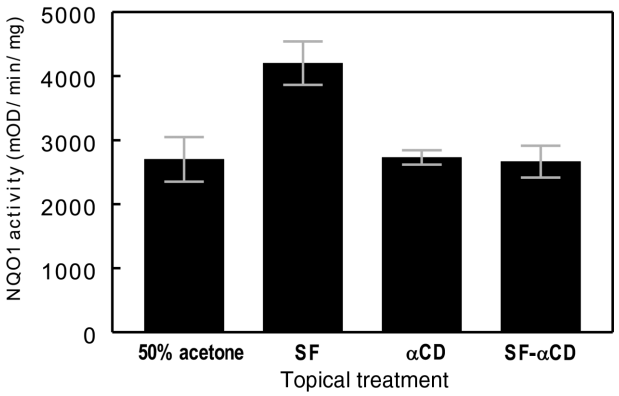

Figure 2. NQO1 activity following topical treatment of mouse dorsal skin with SF formulations. The dorsal area of each SKH-1 hairless mouse was topically treated bilaterally either with 200 nmol/cm^2 SF in 50% acetone (vol/vol) on one side and solvent only on the other side ($n = 5$), or with 200 nmol/cm^2 SF-αCD in 50% acetone on one side and αCD in 50% acetone on the other side ($n = 5$) over a 5.0 cm^2 area for three doses at 24-h intervals. Mice were euthanized 24 h after the last dose, and dorsal skin was harvested. NQO1 specific activity was measured in supernatant fractions of homogenates of skin sections treated with solvent (control), SF, αCD, or SF-αCD. Means ± SD are shown.

induced by SF alone was also similar to our reports [30]. In contrast, SF-αCD inclusions whereby SF was at an equimolar concentration to SF alone as used in this experiment (200 nmol/cm^3 × 3 daily applications) were completely ineffective in inducing NQO1 (Fig. 2), thus suggesting that forming the cyclodextrin inclusion product somehow occludes or prevents SF from penetrating the stratum corneum and entering the epidermis.

3.1.4 Human study: SF-αCD pharmacokinetics

Ten healthy volunteers consumed a single dose of about 200 μmol SF-αCD (Table 1). The 24 h excretion of urine DTCs varied between 19.5 and 86.9% of dose, with a mean of 62.3% of dose (Fig. 3A), which was higher than the intersubject range observed in other studies [29, 37, 59]. Total (24 h) urinary creatinine measurements ranged from 1032 to 3671 mg with a mean of 1649 mg, and there was no correlation between creatinine levels and DTC. The half-life of SF in the body was 2.07 ± 0.26 h (Table 1) as calculated from serum area-under-the-curve determinations (Fig. 4). Serum and total urinary (24 h) levels of SF metabolites or DTCs were highly correlated (pwcorr = 0.757; Fig. 5), as expected. Six of the ten subjects (participants 5–10, Table 1) complained of mild stomach upset.

3.2 Prostaphane®

3.2.1 Prostaphane® stability

Prostaphane was obtained directly from its manufacturer (Nutrinov), and was immediately analyzed. Initial measurements confirmed that there was about 55 μmol of SF per tablet—in accordance with the manufacturer's claim of 10 mg SF (56.5 μmol) per tablet. We did not measure SF in these tablets over a range of storage conditions such as temperature and humidity, but used the conditions we typically use for storing broccoli seed and sprout extracts (note: the manufacturer's recommendations are actually to store at a less-stringent temperature of 4–8°C—a household refrigerator). The ingredients that the manufacturer lists as being used in the manufacture of Prostaphane® tablets gives no indication as to the use of anything particularly unique in their composition, though they claim that their process of stabilization is covered by two patents (http://www.prostaphane.com/prostaphane/what-is-prostaphane.html). One of these patents clearly suggests that preservation of SF (i.e. shelf-life of a tabletized product) is in part governed by the encapsulation (a film-coating) applied to that tablet [52]. In their posted composition (http://www.prostaphane.com/buy-prostaphane/prostaphane.html), the manufacturers list a film-coating agent. Although they do not clearly indicate what the "film" is, they also list

Table 1. Age, gender, and urine and serum DTC measurements for ten volunteers who consumed a single dose of about 200 μmol SF-αCD prepared from an SF-enriched broccoli sprout extract

Participant	Age	Gender	Half-life (h)	Percentage of dose excreted in urine	Peak serum DTC (μM)
1	58	M	1.919	38.4	1.110
2	46	F	2.709	19.5	0.359
3	45	M	2.186	70.3	0.952
4	47	F	1.915	62.7	1.581
5	55	M	1.932	76.5	1.837
6	46	F	1.808	65.8	1.792
7	61	F	2.018	56.3	1.232
8	58	F	1.965	86.9	2.032
9	48	F	2.024	78.2	1.212
10	55	F	2.190	68.4	1.233
Average	51.9		2.067	62.3	1.334
SD	6.1		0.255	20.0	0.468

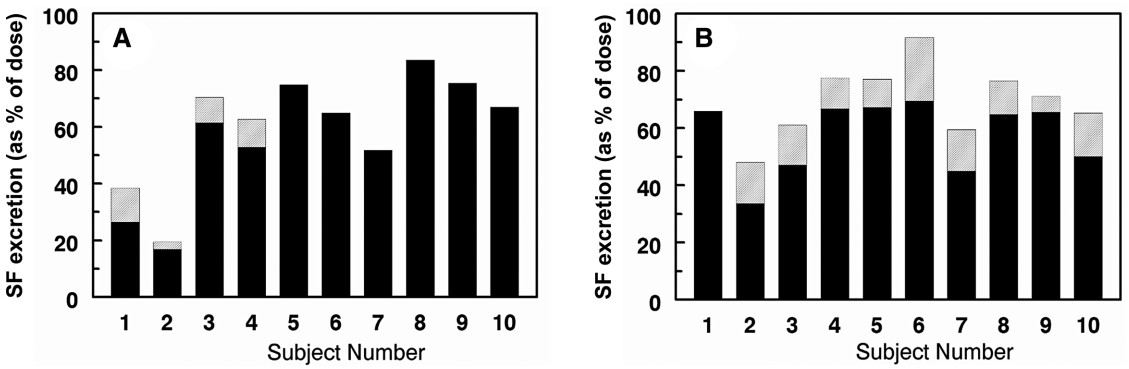

Figure 3. Bioavailability of SF-αCD and Prostaphane®. (A) Urinary excretion of SF and its metabolites following a single 200 μmol SF dose delivered orally as about 350 mg of SF-αCD powder dissolved in 25 mL water. Complete urine collections were made for the first 8 h following dose (filled bars), then the following 16 h (shaded bars), for Subjects 1–4. Subjects 5–10 collected all urine for 24 h without segregating collections (filled bars). Mean excretion for ten subjects was 62.3% of dose. (B) Urinary excretion of SF and its metabolites following a single 94.4 μmol SF dose delivered as two Prostaphane® tablets. Complete urine collections were made for the first 8 h following dose (filled bars), then the following 16 h (shaded bars), with the exception of Subject 1 who collected all urine for 24 h without segregating collections. Subject 6 was given a repeat challenge after a suitable washout period; urinary excretions were within about 11% of each other and the average values are plotted. Mean excretion for ten subjects was 71.4%. Panels A and B represent data from completely different groups of volunteers.

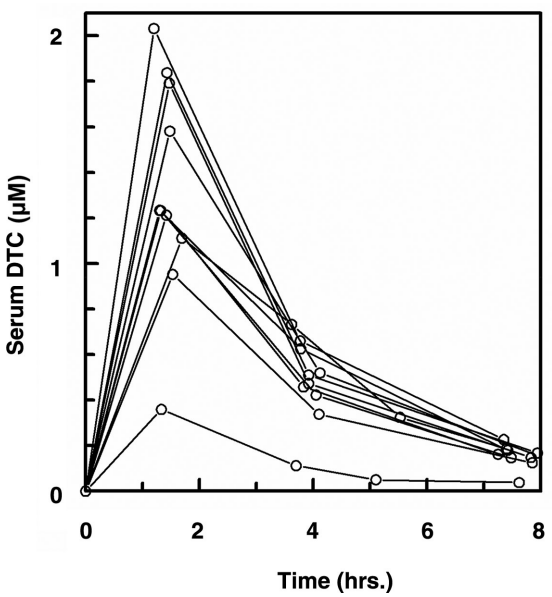

Figure 4. Serum area under the curve following a single dose of SF-αCD. SF concentrations were determined in blood taken from ten volunteers who received a single, oral dose of SF-αCD. Excretion was essentially complete by 8 h.

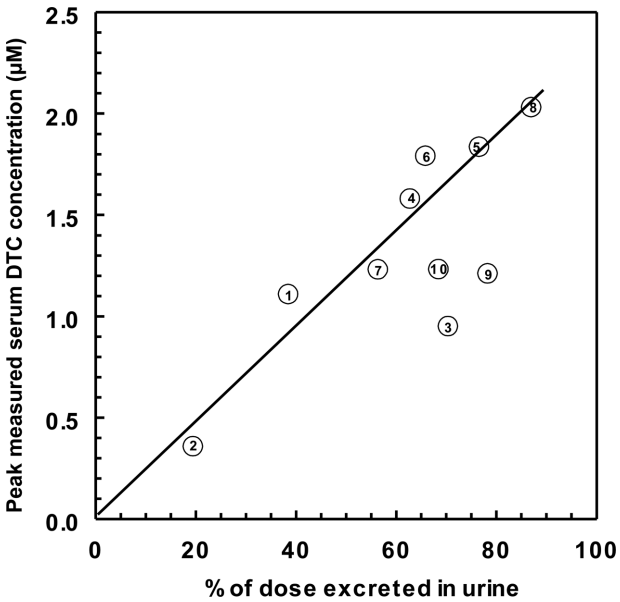

Figure 5. Comparison of serum and urinary DTC following a single dose of SF-αCD. Urinary excretion as percent of dose (from Table 1) is plotted against peak measured serum drug concentration (from Fig. 4). The two measurements are highly correlated (pwcorr or pairwise correlation is 0.757, $n = 10$).

dicalcium phosphate, microcrystalline cellulose, magnesium carbonate, diacetylated monoglycerides, silica, magnesium stearate, and stearic acid. These listed ingredients are consistent with our understanding of the way excipients are generally added to such products, but we nonetheless undertook to verify stability of the tablets in our own hands. We therefore evaluated stability of the SF concentration in these tablets when maintained at −20°C. The decline in SF content in two separate lots, shipped in boxes containing blisterpacks of tablets (Lot PO58T, expiration date 3/2014 and Lot PO50Y, expiration date 2/2017) measured over 1.5 years, equates to about 17.8% per year.

3.2.2 In vitro efficacy of Prostaphane®

The induction by SF of NQO1, the prototypical phase 2 cytoprotective enzyme, was measured in Hepa1c1c7 cells and

compared to the potency of SF delivered in Prostaphane® tablets that were homogenized and extracted in a variety of solvents (e.g. ethyl acetate, water, or a mixture of four solvents abbreviated "1:1:1:1 solvent mixture of DMSO:ACN:DMF:H_2O" consisting of equal parts of dimethyl formamide, ACN, DMSO, and water). SF is readily soluble in each of these solvents, yet they can be expected to co-extract a variety of other components of a complex supplement such as Prostaphane®. Should there have been enhanced activity over and above what could be expected from SF alone, we would have had to further examine the components of the tablets. There was none, and SF accounted for all of the activity extracted into each of these preparations (e.g. 0.22, 0.31, and 0.33 μM when ethyl acetate, water, and 1:1:1:1 solvent mixture of DMSO:ACN:DMF:H_2O, respectively, were used as extractive solvents, compared to 0.28 μM for pure SF).

3.2.3 Human study: Prostaphane® bioavailability

Ten healthy volunteers consumed a single dose of 94.4 μmol SF as Prostaphane® (two tablets). All participants' baseline excretion measured as urinary DTC level, was judged to be acceptably low (≤2 nmol DTC/mg creatinine; data not shown) to support the conclusion that they had adhered to a cruciferfree diet. DTC excretion for ten participants was measured over 24 h, divided into two collections—the first 8 h and the second 16 h. Mean excretion was 67.4 μmol or 71% of dose, range: 48–96% of dose (Fig. 3B). The participant with the highest excretion (91.2 μmol) was re-tested and her repeat score (81.6 μmol) confirmed her ranking relative to the other participants. None of the participants in this substudy had participated in the evaluation of SF-αCD bioavailability described in Section 3.1.4. All participants consumed study drug as evidenced by direct observation by the investigators. Compliance with urine collection instructions was presumed to be excellent based upon creatinine measures in all but one subject whose total 24 h urinary creatinine measurement was 481 mg (which may indicate an incomplete urine collection). Values ranged from 481 to 1886 mg, with an uncensored mean of 1238 mg, and there was no correlation between creatinine levels and DTC. Thus, bioavailability of SF from Prostaphane® was consistent with all other clinical evaluations of the bioavailability of SF from broccoli [3,29,37,56,59]. Since we have previously demonstrated the pharmacokinetics following oral (nonstabilized) SF delivery [56], we did not repeat this work with Prostaphane®. There were no adverse events and Prostaphane® was well tolerated by all participants.

4 Concluding remarks

The bioavailability of stabilized SF is identical to that of less-stable preparations made by simple extraction and freeze-drying or spray-drying of broccoli sprouts or seeds [59]. Bioavailability of oral SF is on average about seven times that of its phytochemical precursor glucoraphanin, and twice that of glucoraphanin with added myrosinase (the enzyme responsible for converting glucoraphanin to SF both in crushed plant tissue and in the human gut microbiota). We have previously reported about 70% bioavailability for SF compared to 10% for glucoraphanin on average [25,29,34,35,37,59,60]. However, there is great person-to-person variability for both SF and glucoraphanin metabolism as shown for the nonstabilized materials in those reports. For example, an examination of data from the first figure in our 2015 publication [59] (redrawn in the graphical abstract of this paper) highlights the fact that the variance in bioavailability is a much greater percentage of the mean (the standard error of the mean) for glucoraphanin than it is for SF. However, the interquantile ranges (the absolute span between the 25th and 75th percentile) are in fact much the same with glucoraphanin and SF. As we now show, there is ample variability in the bioavailability of stabilized SF: a range from 19.5 to 86.9% of dose (mean of 62.3% of dose) for SF-αCD, and a range from 48 to 96% of dose (mean of 71% of dose) for Prostaphane®. Lack of tolerance of SF-αCD in six of ten subjects, combined with its lack of dermal efficacy in our hands, suggests that this approach to stabilization may not be ideal. On the other hand, although precise details regarding the method of stabilizing the commercial supplement are not known, it clearly holds promise for further (see [51]) evaluation of its pharmacodynamic efficiency.

Our previous study did suggest that males are more efficient converters of glucoraphanin to SF, but could discern no effect of BMI, race, age, or stool frequency as a proxy for microbial metabolism [37]. The results reported herein from our small pilot studies are not powered to detect demographic effects on bioavailability.

We have highlighted the fact that availability and activity of myrosinase plays a controlling role in SF bioavailability when supplied as its precursor, glucoraphanin [59]. Dramatically reducing the intestinal bacterial populations in human volunteers completely eliminated their ability to convert glucoraphanin to SF, and this ability returned as the intestinal microbiome became re-established [3]. Given the differing magnitude of the interindividual variation in SF and glucoraphanin bioavailability, we must also consider the possibility that real differences in the innate mammalian SF metabolism, i.e. genetic, metagenetic, epigenetic, and constitutive functional differences exist which could even transcend or eclipse the variations due to differences in gut microbiota. These differences in SF bioavailability may be due to differences in gut microbial metabolism, in the levels of drug metabolizing enzymes (e.g. well-known polymorphisms of glutathione *S*-transferases that catalyze the conjugation of SF with glutathione), and in excretion kinetics. This pharmacogenetic component has become quite clear in the current study using only SF, and it should be interpreted apart from the variation in microbiome-myrosinase catalyzed glucoraphanin hydrolysis. The two effects appear to be

additive when glucoraphanin alone is supplied in food or supplements, but innate metabolic differences must not be discounted when assessing the metabolism of SF alone, delivered in supplements.

J.W.F., K.L.W., S.L.W., H.L., and P.T. conceived and designed the experiments. J.W.F., K.L.W., W.D.H., H.L., and K.K.S. performed the laboratory and animal experiments. J.W.F., K.L.W., S.L.W., and E.F. performed clinical studies. J.W.F., K.L.W., and S.L.W. analyzed the data. J.W.F. wrote the paper. All authors read and approved the final manuscript.

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5 References

[1] Fahey, J. W., Zalcmann, A. T., Talalay, P., The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 2001, *56*, 5–51. [corrigendum: *Phytochemistry* 2002, 59, 237.]

[2] Dinkova-Kostova, A. T., Kostov, R. V., Glucosinolates and isothiocyanates in health and disease. *Trends Mol. Med.* 2012, *18*, 337–347.

[3] Shapiro, T. A., Fahey J. W., Wade, K.L., Stephenson, K. K. et al., Disposition of chemoprotective glucosinolates and isothiocyanates of broccoli sprouts. *Cancer Epidemiol. Biomarkers Prev.* 2001, *10*, 501–508.

[4] Fahey, J. W., Zhang, Y., Talalay, P., Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. USA* 1997, *94*, 10367–10372.

[5] Liu, H., Talalay, P., Fahey, J. W., Biomarker-guided strategy for treatment of autism spectrum disorder (ASD). *CNS Neurol. Disord. Drug Targets* 2016, *15*, 602–613.

[6] Benedict, A. L., Knatko, E. V., Kostov, R. V., Zhang, Y., in: Juurlink, B. (Ed.), *Broccoli: Cultivation, Nutritional Properties and Effects on Health*, Nova Publishers, NY 2016, pp. 185–207.

[7] Carrasco-Pozo, C., Tan, K. N., Borges, K., Sulforaphane is anticonvulsant and improves mitochondrial function. *J. Neurochem.* 2015, *135*, 932–942.

[8] Tarozzi, A., Angeloni, C., Malaguti, M., Morroni, F. et al., Sulforaphane as a potential protective phytochemical against neurodegenerative diseases. *Oxid. Med. Cell. Longev.* 2013, art. no. 415078. doi: 10.1155/2013/415078.

[9] Fahey, J. W., Haristoy, X., Dolan, P. M., Kensler, T. W. et al., Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[*a*]pyrene-induced stomach tumors. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 7610–7615.

[10] Haristoy, X., Fahey J. W., Scholtus, I., Lozniewski, A., Evaluation of antimicrobial effect of several isothiocyanates on *Helicobacter pylori*. *Planta Med.* 2005, *71*, 326–330.

[11] Zhang, Y., Talalay, P., Cho, C.-G., Posner, G. H., A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. USA* 1992, *89*, 2399–2403.

[12] Yang, L., Palliyaguru, D. L., Kensler, T. W., Frugal chemoprevention: targeting Nrf2 with foods rich in sulforaphane. *Semin. Oncol.* 2016, *43*, 146–153.

[13] Fahey, J. W., Talalay, P., Kensler, T. W., Notes from the field: "Green" chemoprevention as frugal medicine. *Cancer Prev. Res.* 2012, *5*, 179–188.

[14] Juge, N., Mithen, R. F., Traka, M., Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell. Mol. Life Sci.* 2007, *64*, 1105–1127.

[15] Zhang, Y., Tang, L., Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. *Acta Pharmacol. Sin.* 2007, *28*, 1343–1354.

[16] Dinkova-Kostova, A. T., Talalay, P., Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol. Nutr. Food Res.* 2008, *52*, S128–S138.

[17] Talalay, P., Fahey, J. W., Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J. Nutr.* 2001, *131*, 3027S–3033S.

[18] Kensler, T. W., Wakabayashi, N., Biswal, S., Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 2007, *47*, 89–116.

[19] Kensler, T. W., Egner, P. A., Agyeman, A. S., Visvanathan, K. et al., Keap1-Nrf2 signalling: a target for cancer prevention by sulforaphane. *Top. Curr. Chem.* 2013, *329*, 163–178.

[20] Fahey, J. W., Kensler T. W., Role of dietary supplements/nutraceuticals in chemoprevention through induction of cytoprotective enzymes. *Chem. Res. Toxicol.* 2007, *20*, 572–576.

[21] Fahey, J. W., Kensler, T. W., Frugal medicine: health-span extension through green chemoprevention. *Virtual Mentor* 2013, *15*, 311–318.

[22] Franklin, S. J., Dickinson, S. E., Karlage, K. L., Bowden, G. T. et al., Stability of sulforaphane for topical formulation. *Drug Dev. Ind. Pharm.* 2014, *40*, 494–502.

[23] Jin, Y., Wang, M., Rosen, R. T., Ho, C.-T., Thermal degradation of sulforaphane in aqueous solution. *J. Agric. Food Chem.* 1999, *47*, 3121–3123.

[24] Ioannides, C., Konsue, N., A principal mechanism for the cancer chemopreventive activity of phenethyl isothiocyanate is modulation of carcinogen metabolism. *Drug Metab. Rev.* 2015, *47*, 356–373.

[25] Kensler, T. W., Chen, J.-G., Egner, P. A., Fahey, J. W. et al., Effects of glucosinolate-rich broccoli sprouts on urinary

levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo Township, Qidong, PRC. *Cancer Epidemiol. Biomarkers Prev*. 2005, *14*, 2605–2613.

[26] Dinkova-Kostova, A. T., Jenkins, S. N., Fahey, J. W., Ye, L. et al., Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane containing broccoli sprout extracts. *Cancer Lett*. 2006, *240*, 243–252.

[27] Tang, L., Zhang, Y., Jobson, H. E., Li, J. et al., Potent activation of mitochondria-mediated apoptosis and arrest in S and M phases of cancer cells by a broccoli sprout extract. *Mol. Cancer Ther.* 2006, *5*, 935–944.

[28] Tang, L., Li, G., Song, L., Zhang, Y., The principal urinary metabolites of dietary isothiocyanates, N-acetylcysteine conjugates, elicit the same anti-proliferative response as their parent compounds in human bladder cancer cells. *Anticancer Drugs* 2006, *17*, 297–305.

[29] Shapiro, T. A., Fahey, J. W., Dinkova-Kostova, A. T., Holtzclaw, W.D. et al., Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study. *Nutr. Cancer* 2006, *55*, 53–62.

[30] Dinkova-Kostova, A. T., Fahey, J. W., Wade, K. L., Jenkins, S. N. et al., Induction of the Phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts. *Cancer Epidemiol. Biomarkers Prev.* 2007, *16*, 847–851.

[31] Cornblatt, B. S., Ye, L., Dinkova-Kostova, A. T., Erb, M. et al., Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. *Carcinogenesis* 2007, *28*, 1485–1490.

[32] Talalay, P., Fahey, J. W., Healy, Z. R., Wehage, S. L. et al., Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation. *Proc. Natl. Acad. Sci. USA* 2007, *104*, 17500–17505.

[33] Munday, R., Mhawech-Fauceglia, P., Munday, C. M., Paonessa, J. D. et al., Inhibition of urinary bladder carcinogenesis by broccoli sprouts. *Cancer Res*. 2008, *68*, 1593–1600.

[34] Egner, P. A., Chen, J. G., Wang, J.B., Wu, Y. et al., Bioavailability of sulforaphane from two forms of broccoli sprout beverage: results of a short term, cross-over clinical trial in Qidong, People's Republic of China. *Cancer Prev. Res.* 2011, *4*, 384–395.

[35] Egner, P. A., Chen, J.-G., Zarth, A. T., Ng, D. K. et al., Rapid and sustainable detoxication of airborne pollutants by broccoli sprout beverage: results of a randomized clinical trial in China. *Cancer Prev. Res.* 2014, *7*, 813–823.

[36] Kensler, T. W., Ng, D., Carmella, S. G., Chen, M. et al., Modulation of the metabolism of airborne pollutants by glucoraphanin-rich and sulforaphane-rich broccoli sprout beverages in Qidong, China. *Carcinogenesis*, 2012, *33*, 101–107.

[37] Fahey, J. W., Wehage, S. L., Holtzclaw, W. D., Kensler, T. W. et al., Protection of humans by plant glucosinolates: efficiency of conversion of glucosinolates to isothiocyanates by the gastrointestinal microflora. *Cancer Prev. Res.* 2012, *5*, 603–611.

[38] Singh, K., Connors, S. L., Macklin, E. A., Smith, K. D. et al., Sulforaphane treatment of autism spectrum disorder (ASD). *Proc. Natl. Acad. Sci. USA* 2014, *111*, 15550–15555.

[39] Bierwirth, J. E., Oftedal, K. N., Civille, G. V., Fahey, J. W., Flavor misattribution: a novel approach to improving compliance and blinding in food-based clinical interventions. *NFS J*. 2015, *1*, 24–30.

[40] Bauman, J. E., Zang, Y., Sen, M., Li, C. et al., Prevention of carcinogen-induced oral cancer by sulforaphane. *Cancer Prev. Res.* 2016, *9*, 547–557.

[41] Alumkal, J. J., Slottke, R., Schwartzman, J., Cherala, G. et al., A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer. *Invest. New Drugs* 2015, *33*, 480–489.

[42] Heber, D., Li, Z., Garcia-Lloret, M., Wong, X. et al., Sulforaphane-rich broccoli sprout extract attenuates nasal allergic response to diesel exhaust particles. *Food Funct*. 2014, *5*, 35–41.

[43] Poulton, E. J., Levy, L., Lampe, J. W., Shen, D. D. et al., Sulforaphane is not an effective antagonist of the human pregnane X-receptor in vivo. *Toxicol. Appl. Pharmacol.* 2013, *266*, 122–131.

[44] Kirkwood, J. M., Singh, S. V., Lin, Y., Hahm, E. R. et al. Dose-response evaluation of biomarkers of broccoli sprout extract sulforaphane (BSE-SFN) in melanoma patients (Pts) with multiple atypical/dysplastic nevi (A/DN). *J. Clin. Oncol*. 2016, *34*(15 Suppl), e21022.

[45] Chiang, W. C. K., Pusateri, D. J., Leitz, R. E. A., Gas chromatography/mass spectrometry method for the determination of sulforaphane and sulforaphane nitrile in broccoli. *J. Agric. Food Chem.* 1998, *46*, 1018–1021.

[46] Van Eylen, D., Oey, I., Hendrickx, M., Van Loey, A., Kinetics of the stability of broccoli (*Brassica oleracea* Cv. Italica) myrosinase and isothiocyanates in broccoli juice during pressure/temperature treatments. *J. Agric. Food Chem*. 2007, *55*, 2163–2170.

[47] Dagan, I. D., Frisbee, A. R., Newsome, P. W., Baudet, M. P., Stabilized sulforaphane. US Patent No. 7,879,822 B2. Feb 1, 2011.

[48] Wu, H., Liang, H., Yuan, Q., Wang, T. et al., Preparation and stability investigation of the inclusion complex of sulforaphane with hydroxypropyl-β-cyclodextrin. *Carbohydr. Polym.* 2010, *82*, 613–617.

[49] Wu, Y., Mao, J., Mei, L., Liu, S., Kinetic studies of the thermal degradation of sulforaphane and its hydroxypropyl-β-cyclodextrin inclusion complex. *Food Res. Intl.* 2013, *53*, 529–533.

[50] Wu, Y., Zou, L., Mao, J., Huang, J. et al., Stability and encapsulation efficiency of sulforaphane microencapsulated by spray drying. *Carbohydr. Polym.* 2014, *102*, 497–503.

[51] Cipolla, B. G., Mandron, E., Marc Lefort, J., Coadou, Y. et al., Effect of sulforaphane in men with biochemical recurrence after radical prostatectomy. *Cancer Prev. Res.* 2015, *8*, 712–719.

[52] Efstathiou, T., Plu, N., Compositions containing broccoli seeds for treatment or preventing prostate cancer. U.S.

P.T.O. Application No. 14/128,874, Patent Publication No. 20140170218, (June 19, 2014).

[53] Fahey, J. W., Dinkova-Kostova, A. T., Stephenson, K. K., Talalay, P., The "Prochaska" microtiter plate bioassay for inducers of NQ01. *Methods Enzymol*. 2004, *382*, 243–225.

[54] Prochaska, H. J., Santamaria, A. B., Talalay, P., Rapid detection of inducers of enzymes that protect against carcinogens. *Proc. Natl. Acad. Sci. USA* 1992, *89*, 2394–2398.

[55] Zhang, Y., Wade, K. L., Prestera, T., Talalay, P., Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Anal. Biochem*. 1996, *239*, 160–167.

[56] Ye, L., Dinkova-Kostova, A. T., Wade, K. L., Zhang, Y. et al., Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clin. Chim. Acta* 2002, *316*, 43–53.

[57] Wade, K. L., Garrard, I. J., Fahey, J. W., Improved hydrophilic interaction chromatography method for the identification and quantification of glucosinolates. *J. Chromatogr. A* 2007, *1154*, 469–472.

[58] Troyer, J., Stephenson, K., Fahey, J., Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography. *J. Chromatogr. A* 2001, *919*, 299–304.

[59] Fahey, J. W., Holtzclaw, W. D., Wehage, S. L., Wade, K. L. et al., Sulforaphane bioavailability from glucoraphanin-rich broccoli: control by active endogenous myrosinase. *PLoS One* 2015, *10*, e0140963.

[60] Ushida, Y., Suganuma, H., Yanaka, A., Low-dose of the sulforaphane precursor glucoraphanin as a dietary supplement induces chemoprotective enzymes in humans. *Food Nutr. Sci.* 2015, *6*, 1603–1612.

