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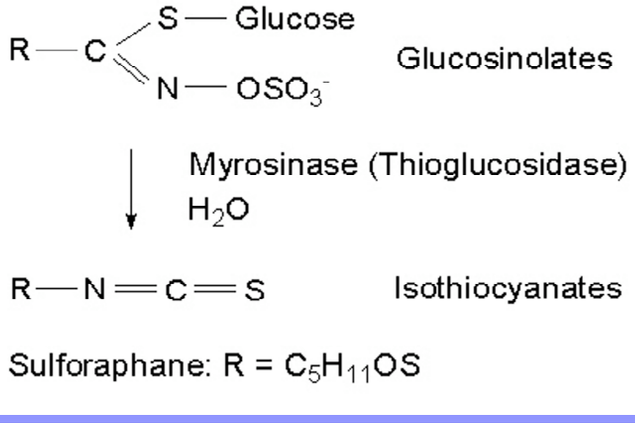

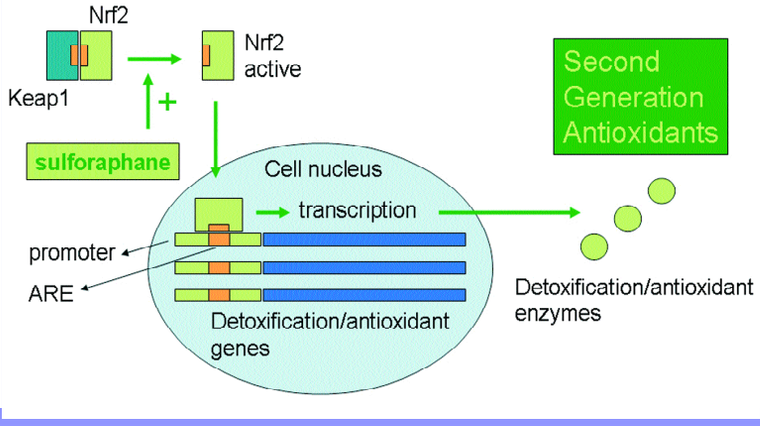

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D. Schmid, C. Schürch, S. Müller, F. Zülli:

Second Generation Antioxidants from Cress Sprouts

D. Schmid, C. Schürch, S. Müller, F. Zülli*

Second Generation Antioxidants from Cress Sprouts

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Summary

Cress sprouts were used as the source material for the development of a cosmetic ingredient rich in isothiocyanates. The scientifically well established efficacy of isothiocyanate phytonutrients to detoxify environmental pollutants and to neutralize reactive oxygen species, could be reproduced in an *in vitro* skin model. The ingredient was found in a preliminary study to stimulate the cells' inherent detoxification system. In a second study, skin cells pre-treated with the cress sprout ingredient proved to be more resistant to toxic oxidants.

The Vegetables of the Mustard Family

Mom was right: »Eat your vegetables, they are good for you!« Vegetables are high in vitamins, minerals and fibers and low in calories, fat, cholesterol and sodium. They have been linked to many health benefits, including lowered risk for certain cancers, stroke, heart disease, and high blood pressure. Vegetables in the mustard (*Brassicaceae*) family have extra health value. They have been proved in numerous studies to help lower the risk of colon, stomach, lung and prostate cancer. Isothiocyanates, a class of plant chemicals that are characteristic of the *Brassicaceae* family, have been identified as the anti-cancer actives. Also belonging to this family are many salad greens, leafy foods and root crops present in our diet. Well known members include cabbage, broccoli, cauliflower, kale, rapeseed, mustard, radishes, horse radish, water and garden cress. The family *Brassicaceae* was formerly named *Cruciferae*, because they all have four petals in a cross-like arrangement.

Sprouts are the Richest in Nutrients

Sprouts have naturally occurring levels of nutrients higher than any whole food. Plant seeds contain the embryo and stored food reserves. When under favourable conditions the seeds begin to germinate, the food reserves are mobilized. The fats are transformed into free fatty acids, starch into maltose and proteins into free amino acids. At this stage, some other very important nutrients start coming up in the growing seed, such as vitamins, enzymes and phytonutrients. The latter include substance families like flavonoids, saponins, monoterpenes, phytosterols and isothiocyanates. Members of these families are shown to have a beneficial effect on health or an active role in the amelioration of disease. Sprouts that are a few days old have the highest concentration of phytonutrients per calorie of any food.

Isothiocyanates: The Phytonutrients in Brassicaceae

The publicity on sprouts was especially driven by the research on the isothiocyanates of broccoli sprouts done at the Johns Hopkins University. The concentra-

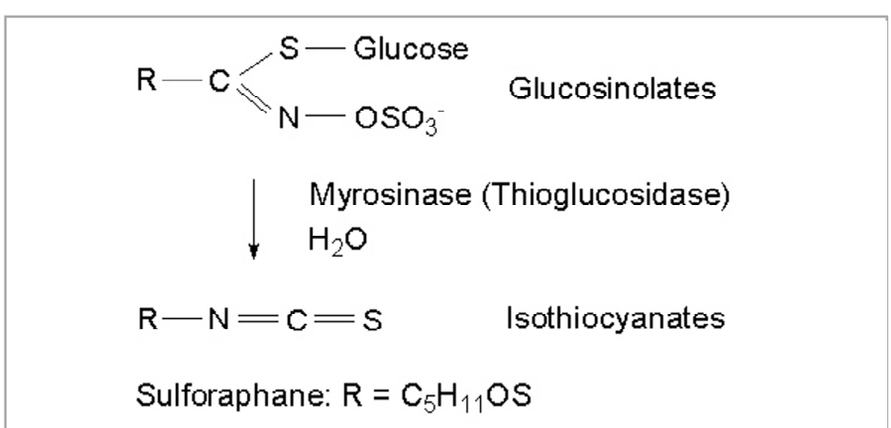

Fig. 1 Formation of isothiocyanates and chemical formula of sulforaphane

tion of the anti-cancer isothiocyanate active was found to be 20 to 50 times higher in 3-day-old sprouts than in mature broccoli (1). Isothiocyanates are sulphur-containing compounds that are present in the living plant as glucose-derivatives, called glucosinolates **(Fig. 1)**. When the vegetables are chewed, the plant cells are broken and the enzyme myrosinase is liberated that hydrolyses the glucosinolates into isothiocyanates. In the past years, a lot of research was done on the mechanism of the anti-cancer activity of the isothiocyanate sulforaphane. It was shown to act as an indirect antioxidant by reducing the activity of phase I enzymes and by enhancing the activity of phase II enzymes (2). These enzymes are part of two main types of metabolism that deal with the elimination of xenobiotics (e.g. environmental pollutants), drugs and carcinogens **(Fig. 2)**. In general, phase I enzymes catalyze reactions that increase the reactivity of hydrophobic compounds, preparing them for reactions catalyzed by phase II enzymes. The latter generally increase water solubility and promote the elimination of the compound from the body. Nowadays, antioxidant enzymes are also regarded as part of the phase II metabolism. Cytochrome P450 (CYP) enzymes are responsible for most phase I reactions. Reactive oxygen species are likely products of CYP enzymes. Some procarcinogens require biotransformation by CYP enzymes in order to become active carcinogens (electrophilic compounds) that are capable of binding DNA and inducing mutations. In several clinical studies isothiocyanates were found to inhibit CYP-dependent activation of procarcinogens. Phase 2 enzymes, such as glutathione transferase and NADPH: quinone reductases, detoxify electrophilic carcinogens, which if left unchecked can lead to mutations in DNA and so to cancer. Isothiocyanates have a potent capacity for inducing phase 2 enzymes, one of our body's important natural defense mechanisms. **Fig. 3** shows the detoxification of quinones by the phase II enzyme NADPH: quinone reductase. Quinones are environmental pollutants present in cigarette smoke and diesel exhaust. Cycling by 1-electron reduction leads to the formation of the dangerous semi-

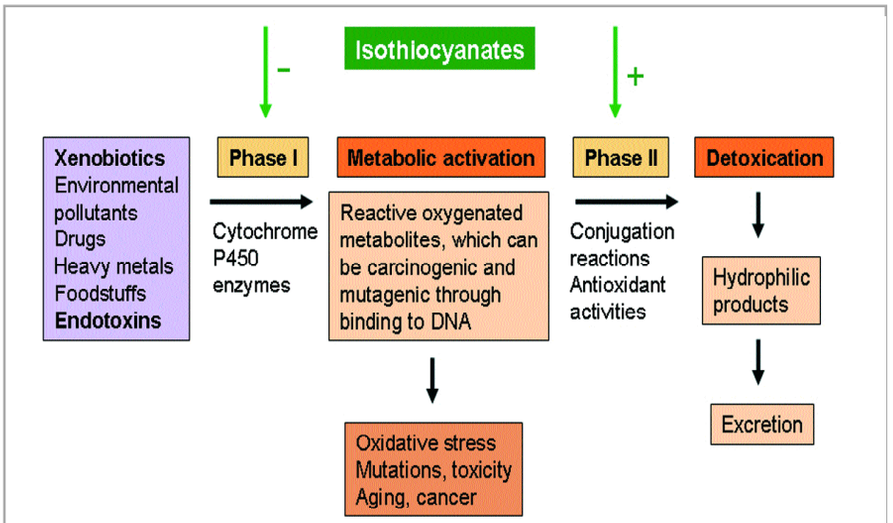

Fig. 2 Classical scheme of phase I and phase II xenobiotic-metabolizing enzymes

quinone and reactive oxygen species that exert toxic and mutagenic effects. The cycle can be interrupted by the 2-electron reduction to hydroquinone, catalyzed by NADPH: quinone reductase. The genes of classical phase II enzymes and antioxidant enzymes contain a specific sequence of DNA called an antioxidant response element (ARE). Isothiocyanates have been found to increase the transcription of genes containing a promoter with an ARE sequence. The exact mechanism is shown in **Fig. 4**. The transcription factor Nrf2 is the critical regulator of ARE-dependent transcription. Under basal conditions, Nrf2 is largely bound in the cytoplasm to Keap1. As a heterodimer, Nrf2 is inactive. But disruption of the Nrf2-Keap1 complex leads to nuclear translocation of Nrf2, binding to the ARE sequence and expression of detoxification and antioxidant enzymes. The isothiocyanates are speculated to activate this pathway either by specific interaction with Keap1 and thereby liberating Nrf2 (3) or by induction of Nrf2 expression and suppression of that of Keap1 (4).

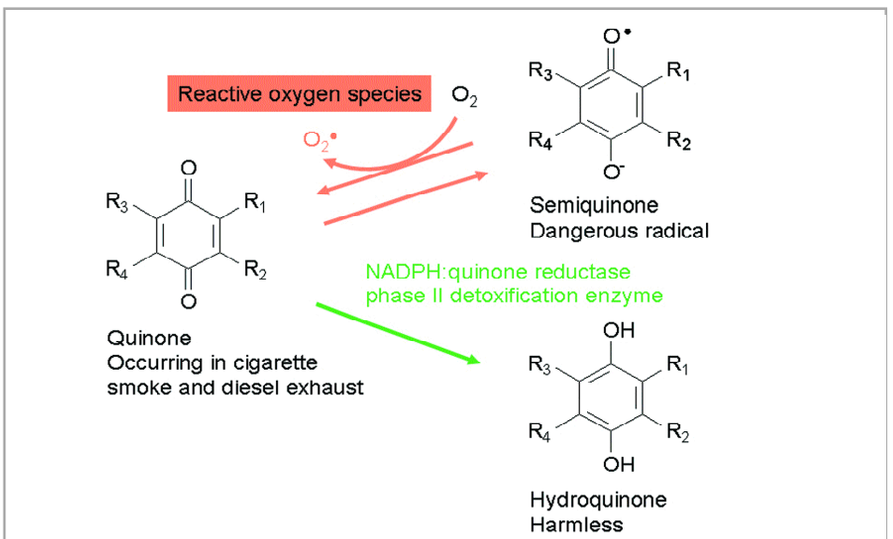

Fig. 3 Neutralization of quinone pollutants by the phase II enzyme NADPH:quinone reductase

Detoxophane: A Cosmetic Ingredient Based on Garden Cress Sprouts

Garden cress displays a spicy aroma and a refreshing, peppery-pungent taste. Like the other members of the Brassicaceae family, garden cress owes its aroma to isothiocyanates. Garden cress is suitable for hydroponic cultivation and typically harvested just a week after germination. 4 to 5 day old garden cress sprouts were used as the raw material to produce a cosmetic ingredient. The composition of the ingredient is the following (INCI): Lepidium Sativum Sprout Extract, Glycerin, Lecithin, Phenoxyethanol and Aqua. For a better skin uptake, the actives of Detoxophane are incorporated into liposomes. In the sprouts, sulforaphane is present as glycoside, called glucoraphanin. For analysis of the sulforaphane content, the sprout extract was first treated with myrosinase to hydrolyze the glycosides and the resulting sulforaphane was measured by HPLC after cyclocondensation with 1,2-benzenedithiol (5). The percentage of sulforaphane in the ingredient was standardized at 0.005 to 0.012%.

Detoxophane: Repression of Phase I Enzymes / Induction of Phase II Enzymes

The capacity of Detoxophane to modulate the expression of phase I and II enzymes was analyzed *in vitro* using normal human epidermal keratinocytes. The method of real-time polymerase chain reaction (PCR) was used to measure the expression of selected genes. As a typical representative of phase I enzymes the cytochrome P450 isoenzyme 2E1 (CYP2E1) was chosen. CYP2E1 metabolizes many xenobiotics into nucleophilic, reactive species. Well known substrates are the drug paracetamol, organic solvents such as acetone, acetonitrile, chloroform and carcinogenic environmental pollutants such as vinyl chloride. A couple of antioxidant enzymes were chosen as representatives of phase II enzymes. NADPH: quinone reductase 1 (NQO1) is a major anticarcinogenic enzyme with a principal role in transformation of quinones into stable hydroquinones. Heme oxygenase 1 (HO-1) is induced after exposure to oxidative stress, such as UV irradiation or hyperoxia, indicating its role in cellular defence. Thioredoxin reductase 1 (TrxR1) works together with NADPH to control the redox balance of the cell. Glutathione peroxidase (GPX1) enzyme has a major role in the reduction of lipid peroxides and of free hydrogen peroxide. The keratinocytes were grown in standard growth medium to 80% confluence. Then the cells were incubated for 24 hours with 0.05 or 0.2% Detoxophane. After incubation, the cells were harvested and total RNA was extracted. Compared to the untreated control, the unfavourable phase I enzyme CYP2E1 was found to be clearly less expressed after incubation with both concentrations of Detoxophane **(Table 1)**. The antioxidant enzyme NQO1 was moderately stimulated at 0.05% and strongly stimulated at 0.2% Detoxophane. HO-1 and TrxR1 were both stimulated strongly even at the lower Detoxophane concentration. The enzyme GPX1 did not respond to Detoxophane in this trial.

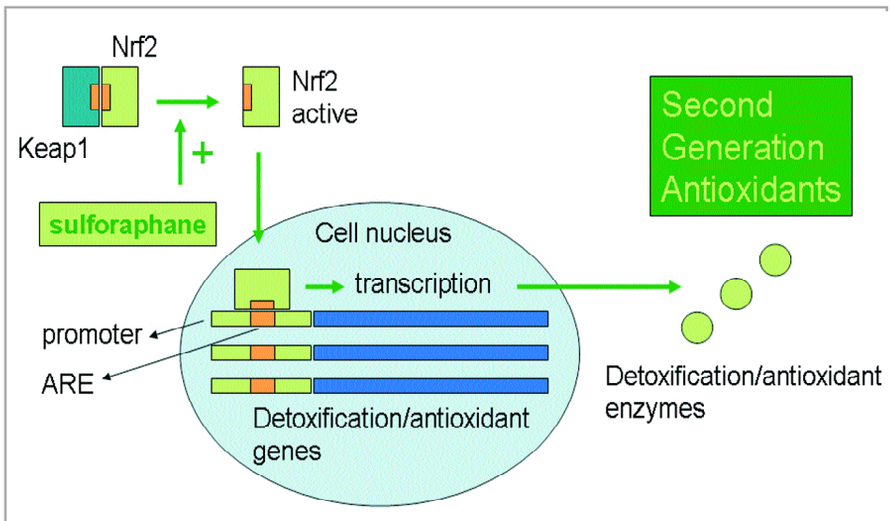

Fig. 4 Activation of expression of genes with the antioxidant response element (ARE)

Detoxophane: Protection against Oxidative Stressors

Detoxophane was found to positively modulate the expression of phase I and phase II enzymes. As consequence, cells in culture pretreated with Detoxophane should be more resistant against toxic chemicals. This protective effect of Detoxophane was analyzed *in vitro* using normal human epidermal keratinocytes. The chemical stressors used were tert-butyl hydroperoxide (t-BH) and 4-Hydroxynonenal (HNE). T-BH is a strong oxidiz-

Concentration of Detoxophane (%)	0.05	0.2
	Expression (%) to untreated	
Phase I enzyme Cytochrome P450 isoenzyme 2E1	60	62
Phase II enzymes NADPH:quinone reductase 1	175	314
Heme oxygenase 1	312	4282
Thioredoxin reductase 1	284	2416

Table 1 Effect of Detoxophane on expression of phase I and phase II enzymes

ing organic peroxide. HNE is a reactive, cytotoxic aldehyde that is released during the oxidation of unsaturated fatty acids. HNE is a key mediator in oxidative stress-induced apoptosis. The keratinocytes were precultured in standard growth medium. Then the cells were pretreated for 24 hours with 0.01 or 0.05% Detoxophane. After this pre-treatment, the culture supernatant was removed and standard growth medium with t-BH or HNE was added. After 4 hours' incubation, the cell viability was measured by MTT assay. Cell viability was analyzed again 20 hours after incubation with the stressors. Detoxophane at 0.05% exerted an impressive protection against t-BH **(Fig. 5)**. The IC_{50} value, indicating the concentration of the stressor inducing 50% mortality, increased from 258 µM in the control without Detoxophane to 646 µM in the culture pre-treated with Detoxophane. The protective effect persisted even after 20 hours after incubation with t-BH with an IC_{50} value of the control of 146 µM shifted to 236 µM with Detoxophane. Even at 0.01 % there was a slight protection by Detoxophane against t-BH, but only when measured just after the oxidative stress period (IC_{50} of control 253 µM; IC_{50} with Detoxophane 317 µM). Protection of keratinocytes against HNE was found only with the higher Detoxophane concentration **(Fig. 6)**. Detoxophane led to an increase of the IC_{50} value from 76 µM (control) to 90 µM. Also this effect persisted even 20 hours after the incubation with HNE.

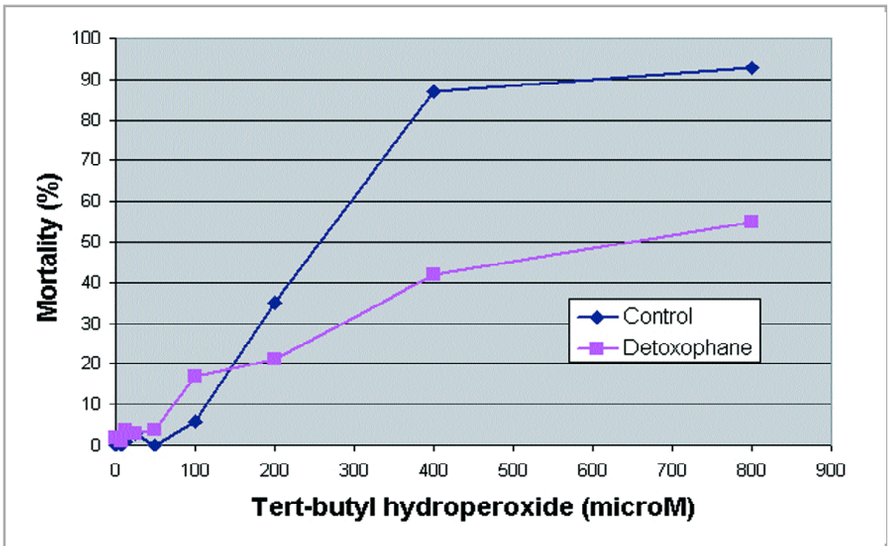

Fig. 5 Detoxophane protects human keratinocytes against tert-butyl hydroperoxide

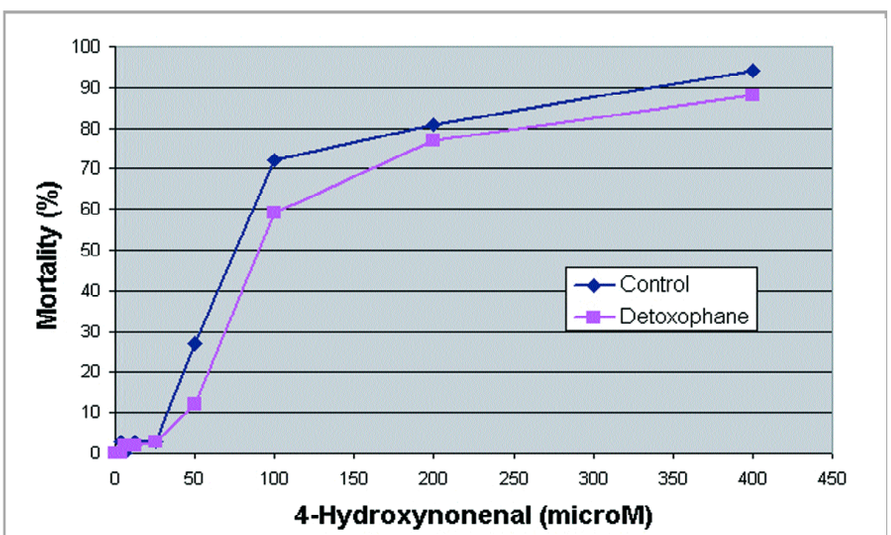

Fig. 6 Detoxophane protects human keratinocytes against 4-Hydroxynonenal

Conclusion

Environmental stressors are the cause of premature skin aging. Beside ultraviolet radiation, the most prominent stressors are xenobiotic pollutants but also drugs and certain stimulants. Isothiocyanate phytonutrients help to eliminate these stressors. By inhibition of phase I enzymes they reduce the oxidative transformation of precursor compounds into toxic intermediates. As inducer of phase II enzymes they promote the elimination of toxic intermediates by excretion and the enzymatic neutralization of oxidants. Isothiocyanates are therefore much more than just single antioxidants. They represent the next generation of antioxidants. Topically applied isothiocyanates will protect skin cells against DNA damage and thus prevent apoptosis (cell death), the typical mechanisms leading to premature skin aging.

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* *Authors' addresses:*

Dr. Daniel Schmid, Dr. Cornelia Schürch,
Sabrina Müller, Dr. Fred Zülli
Mibelle AG Biochemistry
Bolimattstrasse 1
5033 Buchs
Switzerland
Email: Daniel.Schmid@Mibelle.ch