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Cytotoxic and Antioxidant Activity of 4-Methylthio-3-butenyl Isothiocyanate from *Raphanus sativus* L. (Kaiware Daikon) Sprouts

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There is high current interest in the chemopreventive potential of *Brassica* vegetables (cruciferae), particularly due to their content in glucosinolates (GL), which upon myrosinase hydrolysis release the corresponding isythiocyanates (ITC). Some ITCs, such as sulforaphane (SFN) from broccoli (*Brassica oleacea italica*), have been found to possess anticancer activity through induction of apoptosis in selected cell lines, as well as indirect antioxidant activity through induction of phase II detoxifying enzymes. Japanese daikon (*Raphanus sativus* L.) is possibly the vegetable with the highest per capita consumption within the Brassicaceae family. Thanks to a recently improved gram scale production process, it was possible to prepare sufficient amounts of the GL glucoraphasatin (GRH) as well as the corresponding ITC 4-methylthio-3-butenyl isothiocyanate (GRH-ITC) from its sprouts. This paper reports a study on the cytotoxic and apoptotic activities of GRH-ITC compared with the oxidized counterpart 4-methylsulfinyl-3-butenyl isothiocyanate (GRE-ITC) on three human colon carcinoma cell lines (LoVo, HCT-116, and HT-29) together with a detailed kinetic investigation of the direct antioxidant/radical scavenging ability of GRH and GRH-ITC. Both GRH-ITC and GRE-ITC reduced cell proliferation in a dose-dependent manner and induced apoptosis in the three cancer cell lines. The compounds significantly ($p < 0.05$) increased Bax and decreased Bcl2 protein expression, as well as producing caspase-9 and PARP-1 cleavage after 3 days of exposure in the three cancer cell lines. GRH-ITC treatment was shown to have no toxicity with regard to normal human lymphocytes ($-15 \pm 5\%$) in comparison with SFN (complete growth inhibition). GRH and GRH-ITC were able to quench the 2,2-diphenyl-1-picrylhydrazyl radical, with second-order rate constants of 14.0 ± 2.8 and 43.1 ± 9.5 M^{-1} s^{-1}, respectively (at 298 K in methanol), whereas the corresponding value measured here for the reference antioxidant α-tocopherol was 425 ± 40 M^{-1} s^{-1}. GRH reacted with H_2O_2 and *tert*-butyl hydroperoxide in water (pH 7.4) at 37 °C, with rate constants of $1.9 \pm 0.3 \times 10^{-2}$ and $9.5 \pm 0.3 \times 10^{-4}$ M^{-1} s^{-1} (paralleling recently developed synthetic antioxidants) being quantitatively (>97%) converted to GRE. It is demonstrated that GRH-ITC has interesting antioxidant/radical scavenging properties, associated with a selective cytotoxic/apoptotic activity toward three human colon carcinoma cell lines, and very limited toxicity on normal human T-lymphocytes.

KEYWORDS: ***Raphanus sativus*; isothiocyanate; glucosinolate; antioxidant; apoptosis; cancer**

INTRODUCTION

Colon cancer is the second leading cause of cancer-related mortality in Western populations, and its pathogenesis and chemoprevention activity are currently areas of intense investigation (*1*). Diets rich in vegetables are often associated with a lower risk of colon cancer. In particular, *Brassica* vegetables contain a variety of health-promoting chemicals, among them vitamins C and E, carotenoids, polyphenols and, especially, glucosinolates. This, associated with the ready availability and high per capita consumption of such vegetables (10–15% of total vegetable intake in most of the world), highlights their potential as healthy food. The interest of the scientific community toward *Brassica* (cruciferous) vegetables was boosted by the earthshaking report by Zhang et al. that consumption of cruciferous vegetables, particularly broccoli (*Brassica oleracea*

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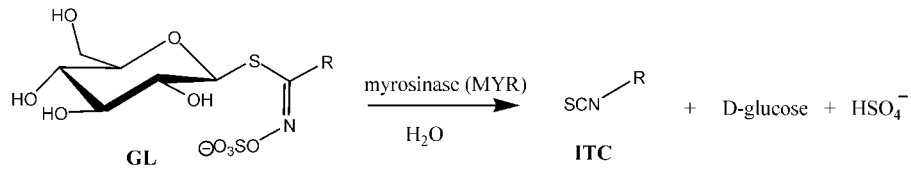
Scheme 1



Side chain (R)	*Chemical name*	***GL**s trivial name*	***ITC**s trivial name*
$CH_3S(O)CH_2CH_2CH_2CH_2$-	4-methylsulfinylbutyl-	Glucoraphanin (GRA)	Sulforaphane (SFN)
$CH_3SCH_2CH_2CH_2CH_2$-	4-methylthiobutyl-	Glucoerucin (GER)	Erucin (ERN)
$CH_3S(O)CH{=}CHCH_2CH_2$-	4-methylsulfinyl-3-butenyl-	Glucoraphenin (GRE)	Sulforaphene (GRE-ITC)
$CH_3SCH{=}CHCH_2CH_2$-	4-methylthio-3-butenyl-	Glucoraphasatin (GRH)	Raphasatin (GRH-ITC)

italica), reduces the risk of developing cancer, which was attributed in particular to sulforaphane (SFN) (*2*), an isothiocyanate released by myrosinase hydrolysis of the precursor glucosinolate glucoraphanin (GRA), on chewing, cutting or chopping the raw vegetable (**Scheme 1**). Subsequent research has concentrated on SFN, showing its beneficial properties in greater detail: for instance, it eliminated intracellular *Helicobacter pylori* in a human epithelial cell line and demonstrated anticancer activity in the animal, blocking benzo[a]pyrene-evoked forestomach tumors in mice (*3*). SFN has also been attributed antioxidant properties due to the induction of phase-II enzymes (*4*). Although the main focus has been on broccoli and sulforaphane, anticancer and health promoting properties appear to be a common feature of several species of the Brassicaceae family (*5*), and isothiocyanates (ITCs), or their precursors glucosinolates (GLs), are widely distributed (*6*). We have recently shown that 4-(methylthio)butyl isothiocyanate (erucin) and its bioprecursor glucoerucin from rocket (*Eruca sativa*, Mill.) seeds and sprouts have even more interesting antioxidant properties: unlike sulforaphane, they also possess direct antioxidant behavior, being efficient scavengers of hydrogen peroxide and organic hydroperoxides; furthermore, upon reaction with these reactive oxygen species (ROS), erucin oxidizes to sulforaphane itself, thereby sharing its beneficial detoxifying properties (*7*). One of us has also shown that erucin has selective pro-apoptotic activity against human leukemia cells (*8*). More recently we have focused our interest on Japanese daikon (*Raphanus sativus* L.) both because it is possibly the vegetable with the highest per capita consumption within the Brassicaceae family (*9*), and because of its content in the unsaturated homologue of glucoerucin: 4-(methylthio)-3-butenyl glucosinolate (glucoraphasatin, GRH) (*10*). GRH has interesting redox properties: in previous investigations it was able to quench hydrogen peroxide being oxidized to 4-methylsulfinyl-3-butenyl glucosinolate (glucoraphenin, GRE), and possessed a Trolox equivalent antioxidant capacity (TEAC) value of 0.13 mM (*10*). GRH and its oxidation product GRE have recently been shown to possess a wide and complex pattern of phase I and phase II enzymes inducing activity in vivo, providing controversial arguments for their chemopreventive role (*11*). This prompted further research toward the understanding of the health-promoting potential and mechanisms of the GLs (or ITCs) contained in daikon sprouts.

Here we report the results we obtained on the cytotoxic activity of the 4-methylthio-3-butenyl isothiocyanate (GRH-ITC), released from GRH, compared with the oxidized counterpart 4-methylsulfynil-3-butenyl isothiocyanate (GRE-ITC), toward three human colon carcinoma cell lines LoVo, HCT-116, HT-29. The toxicity of GRH-ITC compared to GRE-ITC and SFN was also investigated in normal human T-lymphocytes. These results were completed by a detailed kinetic investigation on the reactivity of GRH with hydrogen peroxide and organic hydroperoxides under pseudophysiological conditions, as well as on the reactivity of GRH and the corresponding ITC with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), a persistent isoelectronic mimic of peroxyl radicals.

MATERIALS AND METHODS

Chemicals. All solvents and chemicals, except when noted, were of the highest grade commercially available (Fluka-Aldrich-Sigma) and were used without purification. DPPH$^{\bullet}$ was purchased from Aldrich and stored at 5 °C; fresh solutions in methanol were prepared immediately prior to use. (*R*,*R*,*R*)-α-Tocopherol (Aldrich) was purified by column chromatography on silica gel as previously described (*12*). Styrene (Aldrich, 99+%) was distilled under reduced pressure and percolated through activated basic alumina and dry silica. AAPH and AMVN were available from previous studies and stored at −20 °C; AMVN was recrystallized from hexane prior to use.

Preparation of Myrosinase, Sinigrin, GRH, GRE, GRA, and Derived ITCs. GLs were purified in two sequential steps, by anion exchange and size exclusion chromatography, according to the previously reported method (*7*). GRH and GRE were purified from *R. sativus* L. sprouts and seeds, respectively (*10*). GRA was isolated from *B. oleracea* L. (var. *acephala* subvar. *laciniata*) seeds supplied by Suba & Unico (Longiano, FC, Italy). Sinigrin was obtained from *Brassica carinata* L. seeds (Selection ISCI 7; CRA-ISCI). Individual GLs were characterized by ^{1}H and ^{13}C NMR spectrometry and the purity was assayed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method (*13*), proving to be about 99% based on peak area value and more than 95% on weight basis due to their high hygroscopic properties. The enzyme myrosinase was isolated from seeds of *Sinapis*

alba L. according to a reported method (*14*) with some modifications. The stock solution used in the present study had a specific activity of ~60 units/mg of soluble protein and was kept at 4 °C after dilution in sterile distilled H_2O at ~30 U/mL. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 μmol/min of sinigrin at pH 6.5 and 37 °C. GLs were dissolved in H_2O at a concentration of 10 mM and kept at −20 °C. The relative ITCs were produced via myrosinase catalyzed hydrolysis that was performed by an in situ method (*15*). In every treatment 5 μL of myrosinase was added to the medium containing increasing concentration of GLs to which cells were added.

Preparation of Reference Compound GRH-ITC. GRH-ITC was produced via myrosinase catalyzed hydrolysis of pure GRH, performed in 0.1 M phosphate buffer pH 6.5 at 37 °C. The total conversion of GL into ITC was confirmed by HPLC analysis of the desulfo derivative (*13*), which allowed us to monitor the reduction until there was a complete disappearance of GRH in the reaction mixture. GRH-ITC was purified by EXtrelut (Merck) NT20 column extraction. The mixture was applied to the dry column filled with granular EXtrelut support NT. Elution was carried out using dichloromethane that extracted GRH-ITC from aqueous phase into organic phase. The solutions containing GRH-ITC were concentrated to a few milliliters in a rotary evaporator at 45 °C, and finally the solvent was completely removed under a stream of nitrogen. The GRH-ITC structure was ascertained by GC-MS spectrometry using a Hewlett-Packard GCD G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5 (*16*): EI+ (70 EV), *m/z* 163, $M^{\bullet+}$ (<0.1%), 161 (~3%), 159 (30%), 87 (100%), 72 (20%), 45 (42%).

Cell Cultures. The human cell lines were maintained in RPMI (Roswell Park Memorial Insitute, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Cambrex, Belgium), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin and grown at 37 °C in a humidified air with 5% CO_2.

The human tumor cell lines, HT-29, LoVo, and HCT-116, were obtained from Interlab Cell Line Collections (Genoa, Italy). We generated the ITCs in situ by adding myrosinase to the respective glucosinolate precursor. The concentration of the solvent (water) in the highest dose of drugs did not affect cell proliferation of the cell lines.

MTT Assay. The effect of ITCs on cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the reduction of the number of metabolically active cells, and the results were expressed as percentage of the controls. Briefly, 3×10^3 cells/well were seeded into 96-well plates and treated with several concentrations of GRH-ITC and GRE-ITC for 3 and 6 days, after which 0.5 mg/mL of MTT (Sigma, St. Louis, MO) were added to each well and incubated for 4 h at 37 °C. Following the incubation, a solution containing 10% sodium dodecyl sulfate (SDS) and 0.01 M HCl was added. After at least 18 h at 37 °C, the absorbance of each well was measured in a microplate reader (Bio-Rad, Hercules, CA) at 570 nm. The results were expressed as percentage of the controls (*17*).

Sulforhodamine (SRB) Assay. To confirm data obtained by the MTT assay, we have evaluated the amount of cells in samples by the SRB assay. The SRB assay is based on the ability of SRB dye to bind protein basic aminoacid residues. The amount of dye incorporated by the cells indicate the number of cells. Cells were plated in 96-well plates (100 μL/well) and treated as with the MTT assay. At the end of the treatment, the cell culture medium was eliminated and only RPMI (50 μL/well) was added. The cells were fixed using 25 μl/well of 50% aqueous trichloroacetic acid (TCA) for 1 h at 4 °C and then rinsed with water several times and 50 μL/well of sulforhodamine B solution (0.4%) was added for 30 min. After being rinsed with 1% acetic acid and solubilized in 10 mM Tris for 5 min, the absorbance of each well was measured in a microplate reader (Bio-Rad) at 570 nm. The results were expressed as percentage of the controls (*18*).

Cytotoxity Test on T-Cells. T lymphocytes from blood samples of several donators (men and women) were isolated by density gradient centrifugation and erythrocyte resetting. T-Cells (8×10^4 cells/mL) were cultured in RPMI 1640 containing 10% FBS and 20 μg/mL phytohemagglutinin P (Difco Laboratories, Detroit, MI) (*19*). Viability was determined using MTT assay after treatment with GRH-ITC, GRE-ITC, and SFN.

Annexin V Apoptosis Detection Assay. Experiments were performed on cells seeded in 6-well plates with slides, at a density of 8×10^4 cells/well, treated for 1 day with GRH-ITC and GRE-ITC. Samples were prepared using 0.5 μg of annexin V FITC (Bender MedSystems, Vienna, Austria) for 15 min in the dark. After incubation, the cells were fixed with paraformaldehyde at 4 °C for 30 min and marked with 10 μL (1 μg/mL) of propidium iodide per 100 μL of assay buffer. After washing with PBS and water, samples were observed under fluorescence microscope using a dual filter set for FITC and rhodamine (*20*).

Western Blot Analysis. To determine Bax, Bcl2, caspase-9, and poly(ADP-ribose) polymerase (PARP) levels, cells were plated and treated with GRH-ITC and GRE-ITC at different concentrations. The cells were detached and were collected by centrifugation at 300*g* for 10 min, and pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 μM Na_3VO_4) and sonicated on ice, in the presence of protease inhibitors. Protein concentration was determined according to the method of Lowry (*21*).

Cell lysates (50 μg of protein per lane) were size fractioned in 10% SDS−polyacrylamide gel prior to transfer to Hybond TM-C Extra membranes (GE Healthcare, Buckinghamshire, U.K.) by standard protocols. Membranes were blocked for 2 h with 5% milk in transfer buffer saline (Tris-HCl, 2.42%, NaCl, 8%, Tween 20, 0,1%, pH 7.4) (TBS) at room temperature. The membranes were incubated overnight at 4 °C with antibodies diluted 1:300: anti-Bax (Calbiochem, Darmstadt, Germany), anti-Bcl2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anticaspase-9 (Sigma) and anti-PARP (Santa Cruz Biotechnology Inc.) dissolved in TBS-milk 5%. The membranes were washed twice with TBS-milk 5% and were incubated for 1 h with peroxidase-conjugated antibody. The antirabbit peroxidase conjugated antibody was diluted 1:1000 with 5% milk in TBS Tween 1%. The proteins were detected by luminol (GE Healthcare).

Bands were quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). The amount of protein in each lane was the same, as confirmed by Actin (Sigma).

Statistical Analysis. Data were expressed as mean ± SE. Differences were analyzed by Student's *t* test and considered statically significant at $p < 0.05$ between the control and experimental samples.

Kinetic Measurements with HOOH and ROOH. GRH [(1–5) × 10^{-4} M] was incubated with hydrogen peroxide [(1–5) × 10^{-2} M] or *tert*-butyl hydroperoxide (0.01–0.2 M) in water containing 0.1 mM phosphate buffer (pH 7.4) at 323.0 ± 0.1 K, using a thermostatted water bath equipped with a sealed magnetic microstirrer. Aliquots of the reaction mixture were sampled at time intervals and analyzed by ESI-MS in a Micromass ZMD ESI-MS spectrometer, with direct injection in the electrospray source through a syringe pump (Harvard Apparatus Pump II) at a flow rate of 15 μL/min. The most appropriate instrumental setting were determined in a preliminary set of experiments: ESI type, negative ions; desolvation gas (N_2), 280 L/h; cone flow (skimmer), 40; desolvation temperature, 150 °C; capillary voltage, 3.0 kV; cone voltage, 40 V; hexapole extractor 3.0 V; RF lens, 0.3 V. GRE was the only relevant product detected resulting from the oxidation of GRH. Sinigrin was used as the internal standard since preliminary experiments had shown it was not affected by our experimental conditions. Calibration curves were then obtained both for GRH and for GRE, using authentic samples, under identical instrumental settings from the peak areas of signals at *m/z* 418 and 434 respectively, normalized against the signal at *m/z* 358 due to sinigrin. Measurements were repeated at least with three different starting concentrations of peroxide and, for each concentration, experiments were triplicate.

Kinetic Measurements with DPPH$^\bullet$ Radical. The kinetics of the reaction with DPPH$^\bullet$ radicals was investigated for GRH [(5–15) × 10^{-5} M), and the corresponding ITC, 4-(methylthio)-3-butenyl isothiocyanate [(1–10) × 10^{-4} M]. α-Tocopherol [(5–15) × 10^{-5} M] was also tested as a reference antioxidant. A diluted stock solution of DPPH$^\bullet$ radical [(0.5–3) × 10^{-5} M] in methanol was rapidly mixed (in 1:1 ratio) with a solution containing at least a 10-fold excess of the antioxidant dissolved in methanol (ITC and α-tocopherol) or in methanol/water

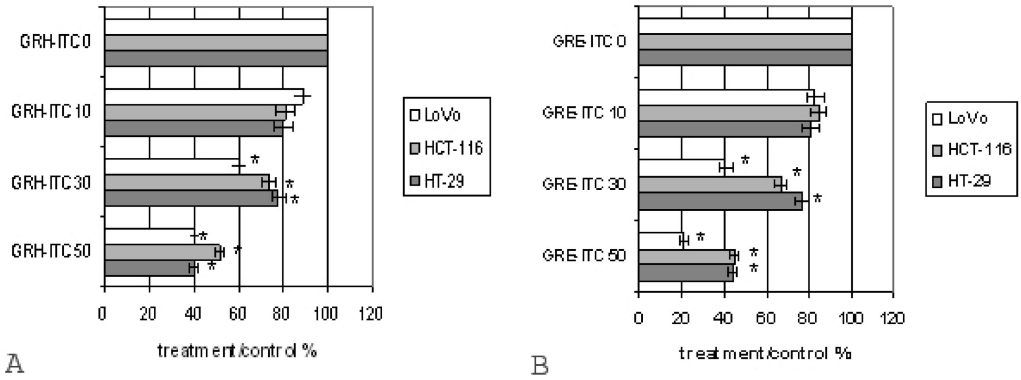


Figure 1. Effect after 6 days of different doses (0, 10, 30, and 50 μM) of GRH-ITC (**A**) and GRE-ITC (**B**) on LoVo, HCT-116, and HT-29 cell growth. Each bar represents the mean (±SE) of six replicate cultures from three independent experiments. *, $p < 0.05$ (Student's t test).

99:1 (GRH), at 298 ± 2 K using a stopped-flow apparatus (HI-TECH SFA-20 Rapid Kinetic Accessory) with the mixing chamber sitting in the optical path of a Jasco V-550 spectrophotometer. The reaction was monitored by following the time-course of DPPH$^{\bullet}$ absorbance at λ_{max} = 517 nm (ϵ = 12400 M^{-1} cm^{-1}). Good pseudo-first-order decays were obtained that afforded pseudo-first-order rate constants k_1 by analyzing the upper one-third of the decay according to eq 1.

$$\ln[\mathrm{DPPH}]_t - \ln[\mathrm{DPPH}]_0 = A - k_1 t \quad (R^2 > 0.98) \qquad (1)$$

Second-order rate constants k_2 were obtained by performing the reaction at different starting concentrations of the antioxidant and plotting pseudo-first-order rate constants k_1 versus the concentration of the antioxidant (eq 2).

$$k_2 = k_1[\mathrm{antiox}] \quad (R^2 > 0.97) \qquad (2)$$

Autoxidation Experiments in Homogeneous Solution. Autoxidation experiments were performed as previously described (*22–24*), in a two-channel oxygen uptake apparatus, based on a Validyne DP 15 differential pressure transducer, that has already been described elsewhere (*25*). The entire apparatus was immersed in a thermostatted bath that ensured a constant temperature within ± 0.1 °C. In a typical experiment, an air-saturated chlorobenzene solution of styrene containing the antioxidant mixture [(0.25–5.0) × 10^{-4} M] was equilibrated with the reference solution containing only an excess of α-tocopherol [(0.1–1.0) × 10^{-2} M] in the same solvent at 30 °C. After equilibration, a concentrated chlorobenzene solution of AMVN [final concentration of (0.5–5.0) × 10^{-2} M] was injected in both the reference and sample flasks and the oxygen consumption in the sample was measured, after calibration of the apparatus, from the differential pressure recorded with time between the two channels. Initiation rates, R_i, were determined for each condition in preliminary experiments by the inhibitor method using α-tocopherol as the reference antioxidant: $R_i = 2[\alpha\text{-tocopherol}]/\tau$. Blank experiments were performed by following the autoxidation of styrene in the absence of any antioxidant, while reference experiments were performed using α-tocopherol (1.0 × 10^{-5} M) as the standard antioxidant.

Autoxidation Experiments in SDS Micelles. Autoxidation experiments in the presence and in the absence of antioxidants were carried out by monitoring the oxygen concentration with a miniaturized Clark-type electrode (Instech, Plymouth Meeting, PA) provided with an automatic data recorder (World Precision Instruments, Sarasota, FL). Experimental procedures were similar to those previously reported in other heterogeneous media (*26*). The measurement chamber (internal volume of 0.6 mL) was kept at constant temperature by circulating water and was protected from room light to avoid initiator photodecomposition. After thermal equilibration of the oxidizable substrate (styrene or methyl linoleate dispersed in sodium dodecyl sulfate) at 37 °C, the appropriate amount of AAPH (final concentration of 1.7 × 10^{-2} M) was injected into the cell at the beginning of data collection. After a few minutes, a concentrated ethanol or water/ethanol solution of the antioxidant was added to obtain a final concentration of (0.1–5.0) × 10^{-4} M.

RESULTS

Growth Inhibition. The effects of GRH-ITC and GRE-ITC on cell growth in three colon cancer cell lines (LoVo, HCT-116, and HT-29) were measured by MTT and sulforhodamine assay. Both ITCs reduced cell proliferation in a dose-dependent manner, as shown in **Figure 1**.

Interestingly, whereas our results show that ITCs derived from Japanese daikon sprouts and seeds are potent inhibitors of colon cancer cell growth, neither GRH nor GRE alone (i.e., in the absence of myrosinase) showed growth inhibitory activity. Also, treatment with myrosinase alone had no effect on cell growth (data not shown). All the results obtained upon cell treatment with GL + myrosinase must therefore be attributed to the effect of the corresponding ITC.

Apoptotic Activity. Apoptosis (programmed cell death) is a natural form of cell death, which can be induced by an "intrinsic" mitochondria-mediated pathway. This pathway leads to activation of caspases (like caspase-9) which are responsible for the execution of cell death by cleaving cellular substrates. During apoptosis, the DNA repair enzyme PARP (116 kDa) is cleaved by caspases into 89 and 24 kDa fragments. PARP cleavage represents a hallmark of late apoptosis (*27–29*).

To evaluate whether ITC induction of growth inhibition was accompanied by apoptosis, annexinV/propidium iodide staining analysis was performed. FITC labeled annexin V binds the newly exposed phosphatidylserine (PS) at the outer membrane leaflet (*30*). As demonstrated by annexin V analysis, both ITCs displayed apoptotic activity in the three lines tested; furthermore, apoptotic activity induced by GRH-ITC was higher than that induced by GRE-ITC in HT-29 cells: treatment with 50 μM GRH-ITC or GRE-ITC increased the percentage of apoptosis, respectively, to 34 and 24 (**Figure 2**).

Bcl2 and Bax Expression. Proteins of the Bcl-2 family, which comprise pro-apoptotic (e.g., Bax) as well as antiapoptotic (e.g., Bcl-2) members, are major regulators of the intrinsic mitochondria-mediated pathway. They form homo- and hetero-oligomers, which act directly at the outer mitochondrial membrane. The ratio of pro- to antiapoptotic oligomers has been suggested to play an important role in determining commitment to cell death (*31, 32*). Indeed, Bcl2 overexpression has been demonstrated in many cancer cells where its down-regulation is indicative of apoptosis. Instead, Bax overexpression allows apoptosis.

We therefore evaluated the effect of GRH-ITC and GRE-ITC on Bcl2 and Bax protein expression (**Figure 3**). As demonstrated by Western blot analysis, both isothiocyanates caused a slight down-regulation of Bcl2 protein and an up-regulation of Bax in all colon cancer cells tested.

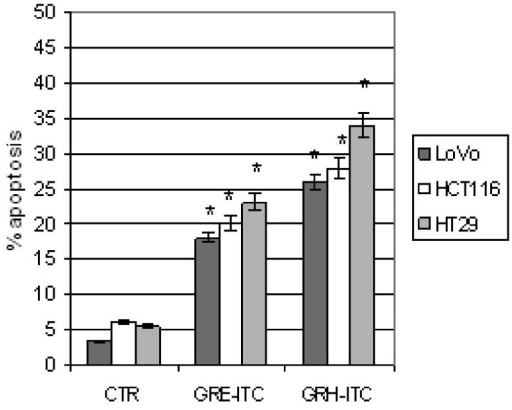


Figure 2. Apoptosis induction by 50 μM GRH-ITC and GRE-ITC in HT-29, LoVo, and HCT-116 cells after 24 h of exposure, as assessed by annexin V apoptosis assay. Apoptosis was evaluated by counting FITC labeled cells in five random fields at least and expressed as percentage of total cells. CTR, untreated cells. Each bar represents the mean (±SE) of four experiments. *, $p < 0.05$ (Student's *t* test).

Isothiocyanate Activation of Caspase-9 and Poly(ADP-ribose) Polymerase-1 (PARP-1). Activation of caspase-9 and PARP-1 is most important for execution of the apoptotic process. As shown in **Figure 4A**, we found that GRH-ITC and GRE-ITC at the dose of 50 μM induced a strong cleavage of the 47 kDa proenzyme caspase-9 into its active 35 kDa form, which was observed starting from 3 days of treatment onward in HT-29 cell line. We also observed (**Figure 4B**) the PARP-1 cleavage, seen as a typical 89 kDa band, which was almost complete after 3 days of treatment in the HT-29 cell line.

These results were confirmed also in LoVo and HCT-116 colon cancer cell lines (data not shown).

Lymphocyte Cell Viability. Normal lymphocytes can undergo cell death induced by drugs as a side-effect of chemotherapy aimed at malignant cells. To assess the cytotoxic effect of GRH-ITC and GRE-ITC purified from sprouts and seeds of *R. sativus* L. in comparison with SFN, normal T-lymphocytes were exposed to equimolar doses for 6 days. The effects of GRH-ITC, GRE-ITC, and SFN on lymphocytes are shown in **Figure 5**. Interestingly, SFN caused a complete growth inhibition at 50 μM, whereas GRH-ITC showed only 15(±5)% inhibition.

Hydrogen Peroxide and Alkyl Hydroperoxides Scavenging Activity. Incubation of GRH dissolved in water with a 4-fold excess of H_2O_2 at room temperature for 6 h resulted in complete disappearance of the starting material as judged by HPLC-MS analysis of the reaction mixture. The compound was completely (>97% by HPLC-MS) converted into a product giving, in ESI-MS spectrometry, a pseudomolecular ion peak ($[M - H]^-$) at *m*/*z* 434 in negative ionization mode, that is, at an *m*/*z* value higher by 16 amu than that recorded for the starting glucosinolate GL (*m*/*z* 418). The reaction product was identified as GRE by comparison with a reference sample. Similarly the reaction of the corresponding GRH-ITC, in methanol with an excess H_2O_2 yielded the corresponding sulfinyl (EI 70 EV, $M^{\bullet+}$ *m*/*z* 175) with 93% conversion, measured by GC-MS analysis of the reaction mixture. Clearly, similarly to previous observations with structurally related erucine and glucoerucine (*7*) and in line with a preliminary investigation on GRH (*10*), with both starting materials (the GL or the ITC) regioselective oxidation of the methylthio moiety occurs to quantitatively yield the corresponding methyl sulfoxide, while the subsequent oxidation to methyl sulfone was not observed under our experimental conditions.

The reaction of GRH with hydrogen peroxide and *tert*-butyl hydroperoxide in water was investigated at 37 °C, under pseudo-first-order conditions by incubating the potassium salt of the glucosinolate [(1–5) × 10^{-4} M] with a 100-fold excess of the peroxide. For convenience the reaction was followed at time-intervals by direct injection of the reaction mixture in the ESI-MS spectrometer through a syringe pump. The reaction course was monitored from the disappearance of the signal at *m*/*z* 418 ($[M - H]^-$ ion of GRH) and appearance of the signal at *m*/*z* 434 ($[M - H]^-$ of GRE) in negative ionization mode. Preliminary studies allowed adjustment of the optimal instrumental setting for direct analysis of the reaction mixture under conditions in which the two pseudomolecular ions were the only signals due to the glucosinolates. Sinigrin (*m*/*z* 358, $[M - H]^-$ ion) was used as the internal standard. Calibration of the spectrometer response with authentic samples was performed using the integrated signal peak. As can be seen from **Figure 6**, decay of GRH and growth of GRE, monitored through the time evolutions of signals at *m*/*z* 418 and 434, respectively, afforded nice first-order complementary kinetic traces, that is, the rate of disappearance of the signal due to GRH was almost identical to the rate of formation of GRE. Because decay traces usually showed lower scattering of the mass signal, for convenience they were chosen to calculate the second-order rate constants. Measurements were always performed under pseudo-first-order conditions with various amounts of starting GL and peroxide. The results are collected in **Table 1**.

Reactivity with DPPH$^\bullet$ Radical. The reactivity of GRH and the corresponding ITC with the persistent DPPH$^\bullet$ radical was studied spectrophotometrically from the disappearance of its signal at 517 nm, under pseudo-first-order conditions, as shown in **Figure 7**. Analysis of the kinetic traces (vide supra) afforded the second-order rate constants reported in **Table 2**, together with the value recorded for α-tocopherol under identical experimental conditions. Both compounds showed good reactivity toward DPPH$^\bullet$, although the rate of reaction was approximately 1 order of magnitude lower than that of α-tocopherol. Interestingly, the ITC was consistently about 3 times more reactive than the corresponding glucosinolate.

Autoxidation Experiments. No chain-breaking antioxidant activity was observed either with 4-methylthio-3-butenylisothiocyanate in the autoxidation of styrene in homogeneous (chlorobenzene) solution or with this ITC or GRH in the autoxidation of styrene or methyl linoleate in SDS/water micellar solution. Under very similar experimental conditions neat inhibition of the autoxidation was instead always observed with the reference antioxidant α-tocopherol (data not shown).

DISCUSSION

Both GRH and the corresponding ITC are effective scavengers of hydrogen peroxide and organic hydroperoxides, thereby possessing a direct preventive antioxidant activity, similarly to the saturated analogues glucoerucin and erucin (*7*). Because such a reaction has been shown to proceed regiospecifically on the methylthio function, no difference in reactivity is expected between the glucosinolate and the isothiocyanate; for convenience, we therefore measured the kinetics of reaction of the glucosinolate. With respect to glucoerucin (GER), GRH exhibits slightly lower reactivity toward peroxides (see **Table 1**), conceivably due to the electron withdrawing activity of the C=C group on the methylthio function; however, it still compares favorably with synthetic antioxidants such as phenylaminoethyl sulfide and phenylaminoethyl selenide, for instance, the rate constants of which with hydrogen peroxide at 37 °C have been

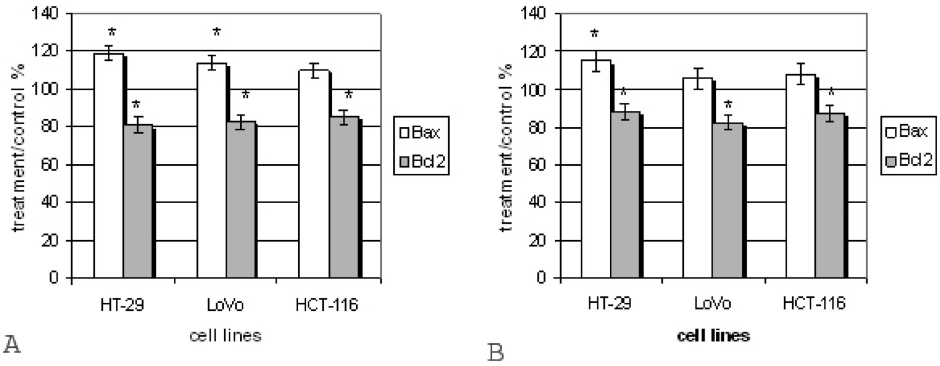


Figure 3. Western blot analysis for Bcl2 and Bax protein level in HT-29, LoVo, and HCT-116 cells, after 3 days of exposure to 50 μM GRH-ITC (**A**) or 50 μM GRE-ITC (**B**). Densitometric data are expressed as percentage of treated samples with respect to the control. Each bar represents the mean ($\pm$SE) of three independent experiments. $*$, $p < 0.05$ (Student's t test).

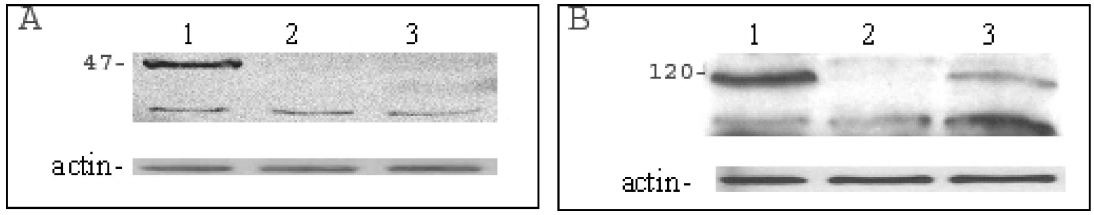


Figure 4. Western blot analysis for caspase-9 (**A**) and PARP (**B**) protein in HT-29 cells, after 3 days of exposure to 50 μM GRH-ITC (lanes 2) or 50 μM GRE-ITC (lanes 3). Lanes 1, untreated cells. We show representative blots from one of three experiments.

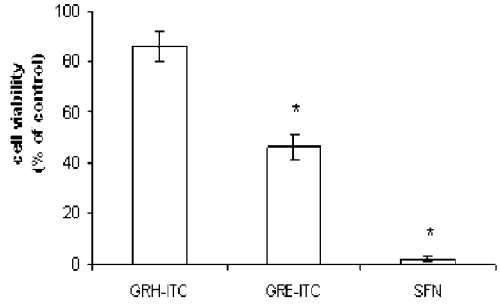


Figure 5. Effect after 6 days of 50 μM of GRH-ITC, GRE-ITC, and SFN on lymphocyte cell viability as assessed by MTT assay. $*$, $p < 0.05$ (Student's t test).

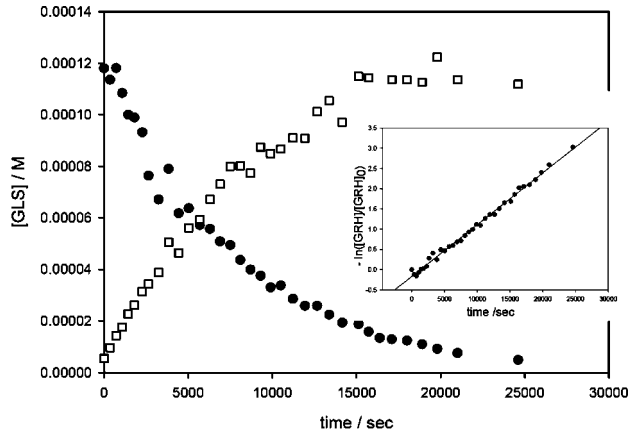


Figure 6. Time evolution of the concentration of GRH (solid circles) and GRE (open squares), measured by ESI-MS analysis of the reaction mixture of GRH (1.2×10^{-4} M) and *tert*-butyl hydroperoxide (0.155 M) in water (containing phosphate buffer, pH 7.4) at 310 K. (Inset) First-order plot of the decay of GRH concentration.

reported as 4.2×10^{-4} and 4.7×10^{-2} M^{-1} s^{-1}, respectively (*35*). In line with previous observations with erucin and GER (*7*), neither GRH nor the corresponding ITC possesses chain-breaking antioxidant activity. Indeed, they were not able to stop or retard methyl linoleate or styrene autoxidation, forcedly initiated under controlled conditions. A similar lack of chain-breaking antioxidant behavior was observed both in homogeneous organic solution (chlorobenzene) and in water/SDS micelles, while under identical experimental settings the reference antioxidant α-tocopherol always showed neat inhibition of the autoxidation. As previously discussed (*7*), this might be attributed either to the lack of reactivity of GRH or GRH-ITC with peroxyl radicals, which we consider to be unlikely, or more conceivably to the fact that the reaction product is still able to propagate the autoxidation chain. To further investigate the homolytic reactivity of GRH and GRH-ITC, we studied the kinetics of reaction with the persistent radical DPPH$^{\bullet}$, which has a strong absorption at 517 nm and is suited for stopped-flow kinetics with a UV–vis spectrometer. Furthermore, the DPPH$^{\bullet}$ radical is electronically related to peroxyl radicals and gives similar reactions with common antioxidants such as phenols, although the actual reactivity is decreased by about 3 orders of magnitude (*36*).

Table 1. Second-Order Rate Constants (k_2, $\pm$ Standard Deviation) for the Reaction of GRH with *tert*-Butyl Hydroperoxide and Hydrogen Peroxide in Water at 310 K, Compared to Structurally Related GER

	k_2 (M^{-1} s^{-1})	
glucosinolate	*t*-BuOOH	H_2O_2
GRH	$(9.5 \pm 0.3) \times 10^{-4}$	$(1.9 \pm 0.3) \times 10^{-2}$
GER[a]	$(4.5 \pm 0.2) \times 10^{-3}$	$(6.9 \pm 0.1) \times 10^{-2}$

[a] Data from ref (*7*).

As can be seen from **Table 2**, both GRH and GRH-ITC gave relatively fast reactions with DPPH$^{\bullet}$ radicals, being able to quench this persistent radical species with a rate constant about ten times higher that that reported for common phenolic antioxidants like 2,6-di-*tert*-butyl-4-methoxyphenol (BHA) and 2,6-di-*tert*-butyl-4-methyphenol (BHT), and about 4 orders of magnitude higher than that of phenol itself. The reactivity of α-tocopherol with DPPH$^{\bullet}$ was measured for comparison under

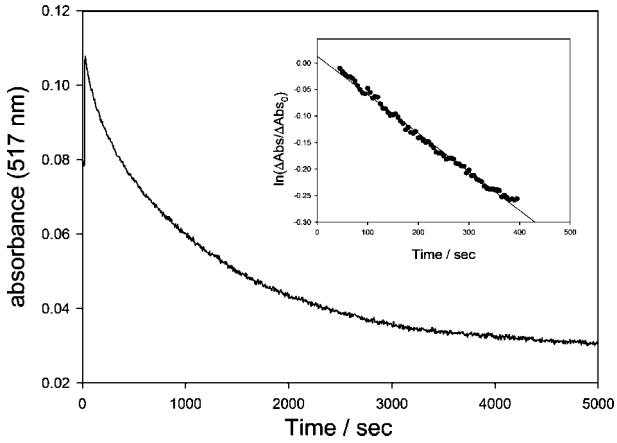


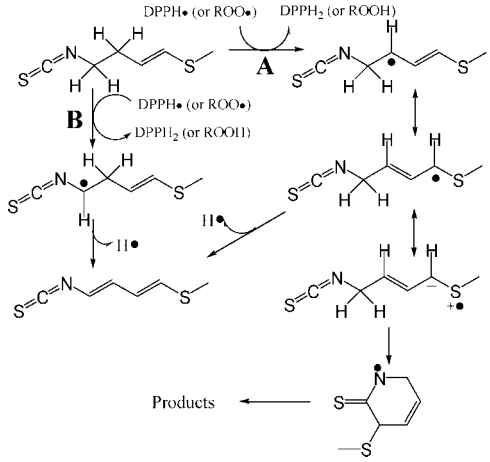
Figure 7. Typical kinetic trace recorded at 298 K for the decay of DPPH• radical (1.0×10^{-5} M) in methanol upon quick mixing with GRH (1.0×10^{-4} M) and its fist-order plot (inset).

Table 2. Absolute Second-Order Rate Constant at 298 K for the Reaction of DPPH• Radical with GRH, the Corresponding ITC, and α-Tocopherol

substrate	k_2 (M^{-1} s^{-1})	solvent
GRH	14.0 ± 2.8	methanol/water 99:1
GRH-ITC	43.1 ± 9.5	methanol
α-tocopherol[a]	425 ± 40	methanol
BHT[b]	0.86	ethanol
BHA[b]	3.9	ethanol
phenol[c]	2.9×10^{-3}	*t*-BuOH

[a] Some reference values from the literature are $k_2 = 390$ M^{-1} s^{-1} in MeOH (*33*) and $k_2 = 570$ M^{-1} s^{-1} in *t*-BuOH (*12*). [b] From ref *34*. [c] From ref *12*.

Scheme 2



identical experimental settings and is in excellent agreement with literature data (*12, 33*). Hence GRH-ITC has a reactivity toward DPPH• radicals (and conceivably toward peroxyl radicals) intermediate between α-tocopherol and BHA; this high reactivity could be attributed to hydrogen atom abstraction either in the alylic position (see **Scheme 2**, route A) or in alpha to the NCS group (route B) or both. The relevance of route B can be inferred from the 3-fold higher reactivity of the ITC as compared to the glucosinolate. In this contest, the observed lack of chain-breaking antioxidant activity of GRH and GRH-ITC is conceivably due to the reaction, in oxygen-rich environments, of the carbon-centered radical, formed upon H-abstraction by peroxyl radical, with molecular oxygen to generate a chain-propagating peroxyl radical. According to the concentration of oxygen, this reaction could become faster than the subsequent H-atom abstraction (by ROO• or DPPH•) or cyclization (route A) illustrated in **Scheme 2**. In this regard GRH-ITC might behave in biological systems similarly to retinol (vitamin A) or β-carotene, that is, move from antioxidant to pro-oxidant behavior according to the local partial pressure of oxygen and to the availability of other antioxidants in the same compartment (*37*).

It is intriguing, but speculative, to envisage a link between the controversial antioxidant behavior of GRH and GRH-ITC and their cytotoxic/apoptotic activity, particularly in comparison with other compounds of the same class (vide infra).

The data presented herein demonstrate that GRH-ITC and its oxidation product GRE-ITC have a strong antiproliferative effect. This effect is accompanied by a pro-apoptotic action. During apoptosis, the loss of phospholipid asymmetry occurs, which results in exposure of PS residues at the outer plasma membrane, that may serve as a trigger for phagocytosis. The exposure of PS has been demonstrated to occur early, during the *effector phase* of apoptosis, probably downstream of changes in the mitochondria, and precedes the loss of plasma membrane integrity and DNA fragmentation. Treatment with GRH-ITC and GRE-ITC induces a PS exposure, still detectable after 24 h, as demonstrated by annexin V test. We have also shown that GRH-ITC and GRE-ITC induce apoptosis in human colon cancer with an up-regulation of Bax and down-regulation of Bcl2. Overexpression of the antiapoptotic protein Bcl2, reported in a wide variety of cancer cells, is associated with a diminished apoptotic response. Indeed Bcl2 blocks apoptosis by inhibiting the release of mitochondrial proteins and PS exposure, while Bax is an important pro-apoptotic protein (*32*). In all of the colon cancer cells tested, 50 μM treatment with GRH-ITC or GRE-ITC activated caspase-9 and PARP cleavage, suggesting that apoptosis might be initiated through an internal mitochondrial pathway. These results are in line with other investigations on the apoptotic effects of ITC mediated caspase-9 (*38, 39*).

The finding that the effect of GRH-ITC on apoptosis was higher than that recorded with GRE-ITC in all cancer cell lines tested is very interesting. The behavior of GRH-ITC on normal lymphocytes was also worthy of note, as it differed significantly from what we recorded with the two sulfinyl-functionalized ITC (GRE-ITC and SFN), possibly indicating a role of such moiety on the toxicity of the compound.

This parallels the previous finding, by some of us, that the corresponding glucosinolates, GRH and GRE, had a different behavior in the induction of phase I and phase II enzymes, despite the fact that the oxidation status of the sulfur is the only structural difference (*11*). This was tentatively attributed to the direct antioxidant/radical scavenging activity of GRH but not of GRE. Although the link between these phenomena, e.g. antioxidant activity versus enzyme induction, or antioxidant activity versus apoptotic effect, is far from being understood, we note that a similar, but actually greater, difference in behavior has become apparent for the structurally related redox couple erucin/sulforaphane. Unlike SFN, erucin was found to possess direct antioxidant activity (*7*); in a recent investigation erucin was found to induce apoptosis selectively in Jurkat T-leukemia cells, but not on normal human T-lymphocytes (*8*), whereas the data presented here confirmed that sulforaphane does not share this selectivity. At this conjunction it is of significance that glucoraphanin, the bioprecursor of SFN, was found to have neat pro-oxidant and DNA-damaging activity in rat liver (*40, 41*).

Accumulating evidence suggests that ROS and hydrogen peroxide play an important role in cancer development, and experimental data show that cancer cells produce high amounts of hydrogen peroxide (*42*). On the other hand, there is evidence

that hydrogen peroxide can selectively induce apoptosis in cancer cells and that the activity of several anticancer compounds involves hydrogen peroxide, suggesting that cancer cells are more susceptible to hydrogen peroxide-induced cell death than normal cells (*42*). Although not completely understood, this dual role of hydrogen peroxide (and ROS) moving between cell signaling and cytotoxicity, could be one key to achieve selective chemoprevention. Possibly the fine balancing of the "direct" antioxidant/radical scavenging activity combined with the "indirect" antioxidant activity (e.g., by the induction of phase II enzymes) and pro-oxidant activity (e.g., by the induction of phase I enzymes) is related to selectivity in cytotoxic/apoptotic activity of the thio-functionalized GRH-ITC.

In conclusion, chemoprevention is defined as the use of natural or synthetic agents that reverse, inhibit, or prevent the development of cancer. Effective and safe chemoprevention requires the use of nontoxic agents that inhibit specific molecular steps in the carcinogenic pathway. We have demonstrated that GRH-ITC has interesting antioxidant/radical scavenging properties, associated with a selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines, and very limited toxicity on human T-lymphocytes. Particularly when compared to other structurally related ITCs such as SFN, GRH-ITC obtained from *R. sativus* L. (kaiware daikon) sprouts can be regarded as a promising chemopreventive agent.

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