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Myrosinase-Generated Isothiocyanate from Glucosinolates: Isolation, Characterization and In Vitro Antiproliferative Studies

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Abstract—Epidemiological and pharmacological studies have shown that colorectal cancer development could be reduced by consuming vegetables that contain glucosinolates. In view of this the effect of some glucosinolates and their isothiocyanate (ITC)-derived products on in vitro cell growth was studied. We report the isolation and characterization of ITCs derived from glucosinolates by using HPLC, GC-MS, and NMR techniques. The in vitro activity of ITCs on human erythroleukemic K562 cells has been investigated by using two alternative approaches: the in situ and pre-mix methods. No differences in antiproliferative activity were found comparing the effect of ITCs produced either of these methods. In the experimental conditions used, the production of ITCs from glucosinolates is almost quantitative as confirmed by HPLC or GC-MS analysis. The ITCs' inhibitory activity on K562 cells growth is particularly evident in the cases of ITCs derived from sinigrin, progoitrin, *epi*-progoitrin, glucotropaeolin and glucocheirolin. Finally, the antiproliferative activity of the ITCs obtained from glucoraphenin, taken as an example, was determined on other tumor cell lines with a different origin and hystotype. Considering the antiproliferative activity found for ITCs these compounds could be considered potentially responsible for the reduction of colorectal cancer associated with diets rich in cruciferous vegetables. Further studies will be aimed at the possible application of glucosinolate-derived products as chemopreventive cancer agents. © 1997 Elsevier Science Ltd.

Introduction

Recently it has been reported that a diet rich in cruciferous vegetable (e.g., cabbage, broccoli, and cauliflower) could reduce the risk of humans developing cancer. Several studies have evidenced a correlation between consumption of cruciferous vegetables containing glucosinolate (GLs) and a decreased incidence of colorectal cancer both in animals and in humans.[1–3] In particular, this cancer chemoprevention appears to be related to a series of products derived by the myrosinase cleavage of GLs.[4]

GLs are thioglycosidic compounds found in various quantities and ratios in the seeds, roots, stems, and leaves of 11 different families of dicotyledonous angiosperms, the most important of which are Cruciferae. GLs constitute a rather homogeneous class of at least 100 molecules with a common functional group (β-D-thioglucoside) and a variable side-chain (*R*) represented by aliphatic, aromatic, and heteroaromatic residues (see Fig. 1).

In the intact cells GLs are kept separate from the endogeneous enzyme myrosinase, a β-thioglucoside glucohydrolase (EC 3.2.3.1), that catalyzes the hydrolysis of GLs resulting in the production of D-glucose and a series of compounds, isothiocyanate, thiocyanate, and nitriles, depending on the substrate and the reaction conditions used (see Fig. 1).[4,5]

Myrosinase, isolated from *Sinapis alba* seeds, is a glycoprotein containing various thiol and sulfide groups together with ca. 18% carbohydrates, mainly hexose. This myrosinase consists of two identical subunits of 71.1 kD and shows a pI of 5.1.[5]

It is worth noting that over the past few decades only the antinutritional effects of GLs and their aglucon derivatives have been emphasized. This is because of their correlation with endemic hypothyroidism and hepatotoxicity, both in humans and animals, while much less has been written on the potentially advantageous aspects to human health.[4]

Taking into account these considerations and our previous results,[6] and in order to better elucidate the role of GLs on protection from carcinogenesis and tumor progression, we have focused our attention on the possible relationship between the molecular structure of some GLs and their derived products effect on in vitro cultured tumor cell lines.

In particular this paper describes: (a) the isolation and characterization of isothiocyanate-derived products (ITCs) from nine GLs—sinigrin, gluconapin, progoitrin, *epi*-progoitrin, sinalbin, glucotropaeolin, glucoerucin, glucocheirolin, and glucoraphenin, (b) their in vitro antiproliferative activity by using two alternative

Key words: glucosinolates, isothiocyanates, myrosinase, phytochemicals, antiproliferative activity.

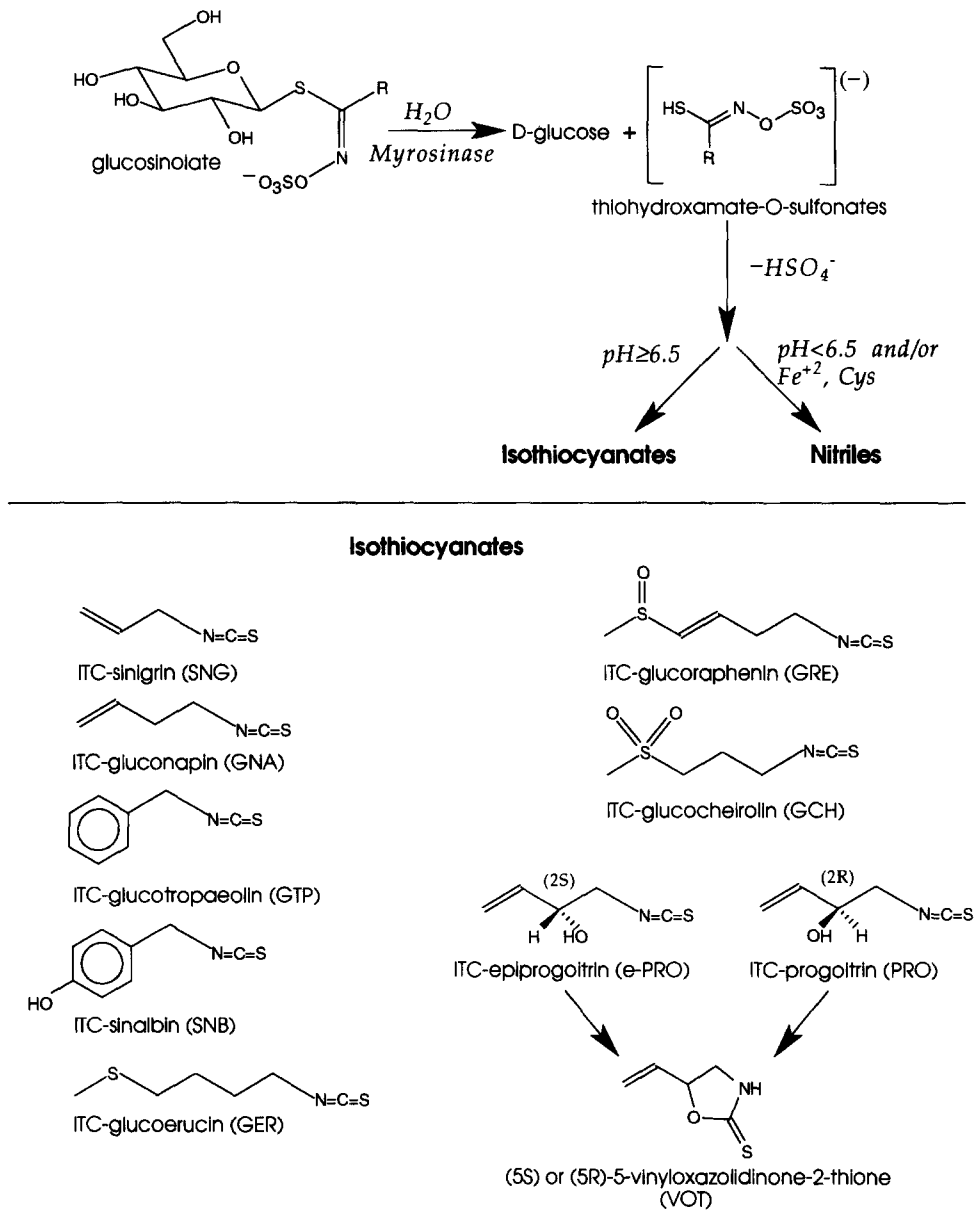

Figure 1. Scheme of the myrosinase catalyzed hydrolysis and chemical structure of glucosinolates.

approaches; the in situ and the pre-mix methods (see the Experimental section), and (c) some preliminary considerations on the structure–activity relationship of these compounds.

Results and Discussion

Production and characterization of ITCs derived from GLs

In the present study a possible relationship between the molecular structure and antiproliferative activity of a number of ITCs derived from cruciferous vegetables contained GLs has been described. In particular the following compounds were considered (see Table 1): two alkenyl compounds—sinigrin (SNG) and gluconapin (GNA); two hydroxyalkenyl compounds—progoitrin (PRO) and *epi*-progoitrin (*e*-PRO); two aromatic compounds—sinalbin (SNB) and glucotropaeolin (GTP), and three side-chain sulfur-containing GLs—glucoerucin (GER), glucocheirolin (GCH) and glucoraphenin (GRE). By using this set of compounds, differences in activity were evaluated within and between the four groups. In addition, because of the side-chain diversity of the tested compounds, some structure–activity relationships can be drawn. The GLs used for the in vitro studies were purified according to the method proposed by Thies[7] with some important modifications,[8] starting from ripe seeds of some crucifers. The HPLC analyses of desulfo derivatives,[9] coupled with polarographic determinations of total GLs content,[10] showed that the nine GLs were nearly homogeneous with an absolute purity higher than 80% (the other constituent was essentially water). Each GL was identified using HPLC and NMR spectrometry.

Myrosinase-catalyzed enzymatic hydrolysis of GLs can lead to the formation of different products as a function

Table 1. Effect of myrosinase-catalyzed hydrolysis products of glucosinolates on human erytroleukemic K562 cells growth

			Physicochemical parameters			
			Compound hydrophobicity			
Isothiocyanate side-chain	Native glucosinolate	Abbreviation GLs	w/o partition coefficient	HPLC ret. time	^{1}H NMR (R-CH_2-NCS) ppm	Activity IC_{50} (μM)[a] 'in situ'[b]
2-Propenyl-	Sinigrin	SNG	0.015	9.915	4.16	<0.1
3-Butenyl-	Gluconapin	GNA	0.628	2.101	3.58	24.0
2*R*-2-Hydroxy-3-butenyl-	Progoitrin	PRO	ND	ND	3.53[c]	55.0[c]
2*S*-2-Hydroxy-3-butenyl-	*e*-Progoitrin	*e*-PRO	0.568[c]	2.165[c]	3.53[c]	42.0[c]
p-Hydroxy-benzyl-	Sinalbin	SNB	0.468	1.606	4.45	320.0
Benzyl-	Glucotropaeolin	GTP	0.006	12.313	4.66	<0.1
4-Methyl-thio-butyl-	Glucoerucin	GER	0.012	12.455	3.57	2.5
3-Methyl-sulfonyl-propyl	Glucocheirolin	GCH	0.580	4.064	3.79	6.0
4-Methyl-sulfinyl-butenyl-	Glucoraphenin	GRE	0.405	3.868	3.71	15.0

[a]Inhibitory concentration 50%: Compound concentration (μM) required to cause a 50% inhibition of in vitro growth of K562 cells.
[b]The myrosinase catalyzed hydrolysis of the indicated glucosinolate was performed following the in situ method as reported in the Experimental section.
[c]These data have to be ascribed to (*R*) or (*S*) VOT.
ND = not determined.

of the reaction parameters, such as pH, temperature, and presence of cofactors (see Fig. 1).[4,5] For instance, at neutral pH and a temperature >25 °C, the formation of ITCs or vinyl-oxazolidine-2-thione (VOT), in the case of progoitrins,[11] is definitely favored. It has to be underlined that in the case of hydroxyalkenyl GLs progoitrin and *epi*-progoitrin, the final enzymatic hydrolysis product is represented by 5*S* and 5*R* VOTs, respectively. These products are derived from the hydroxy-ITC cyclization process described elsewhere.[11] In experimental conditions typical of cell culture (pH 7.4; 37 °C), the formation of ITCs (VOTs) is thus quantitative, as confirmed by GC-MS.[12]

The structure of the obtained ITCs (VOTs) was confirmed by ^{1}H NMR spectroscopy. Figure 2 shows the ^{1}H NMR spectra determined at 200 MHz using $CDCl_3$ or deuterated acetone as solvents. SNG (Fig. 2A): ppm 4.16 (d, 2H, CH_2-NCS), ppm 5.36 (m, 2H, CH_2=), ppm 5.79 (m, 1H, CH=); GNA (Fig. 2B): ppm 2.47 (dd, 2H, -CH_2-), ppm 3.58 (t, 2H, CH_2-NCS), ppm 5.23 (m, 2H, CH_2=), ppm 5.78 (m, 1H, CH=); SNB (Fig. 2C): ppm 4.45 (s, 2H, CH_2-NCS), ppm 6.79 (m, 2H, aromatic), ppm 7.19 (m, 2H, aromatic), ppm 8.27 (s, 1H, -OH); GTP (Fig. 2D): ppm 4.66 (s, 2H, CH_2-), ppm 7.34 (m, 5H, aromatic); GCH (Fig. 2E): ppm 2.25 (m, 2H, -CH_2-) ppm 2.99 (s, 3H, CH_3-SO_2), ppm 3.18 (t, 2H, CH_2-SO_2), ppm 3.79 (t, 2H, CH_2-NCS); GER (Fig. 2F): ppm 1.79 (m, 4H, -CH_2-CH_2-), ppm 2.11 (s, 3H, CH_3-S), ppm 2.54 (t, 2H, CH_2-S), ppm 3.57 (t, 2H, CH_2-NCS) and GRE (Fig. 2G) ppm 2.68 (m, 5H, -CH_2-, CH_3-SO), ppm 3.71 (t, 2H, CH_2-NCS), ppm 6.5 (m, 2H, CH=CH).

Antiproliferative activity of ITCs

The antiproliferative activity of GLs contained in cruciferous vegetables has been tentatively attributed to enzymatic breakdown products such as isothiocyanate and nitriles.[13] These products are generated after in vivo metabolization of GLs by endogenous or commensal microflora enzymes having a myrosinase or a myrosinase-like activity. In order to verify this hypothesis we tested the activity of ITCs derived from GLs by using two different approaches. In the first, the in situ method, the production of ITCs (VOTs) by myrosinase-catalyzed reaction was performed in a cell culture medium directly in contact with cells. In the second approach, the pre-mix method, ITCs were produced before addition to the cells. The two approaches allowed us to evaluate: (a) possible differences in antiproliferative activity of ITCs, (b) the stability of myrosinase in cell culture medium, and, eventually, (c) the antiproliferative activity of native GLs.

The nine selected GLs were tested on in vitro cultured erythroleukemic K562 cells by using both the in-situ and pre-mix methods. As an example, Figure 3 shows the activity of ITC derived from glucoraphenin (GRE-ITC). The GRE-ITC was used in the concentration range between 0.1 and 500 μM. In addition, the activity of native GRE and myrosinase is reported. From the analysis of the antiproliferative activity of all the tested GLs the following general conclusion can be drawn.

(a) Myrosinase and native GLs had no effect on tumor cell growth when used up to 36 U/mL and 500 μM, respectively. This result appears particularly important because it confirms the low antiproliferative activity of native GLs reported by other authors[4,14] and because a suitable experimental control is available.

(b) The inhibitory curves obtained by using both the in situ and the pre-mix methods displayed similar trends even if, excepted for SNB, a slightly higher activity can be observed for the in situ method. This is more evident

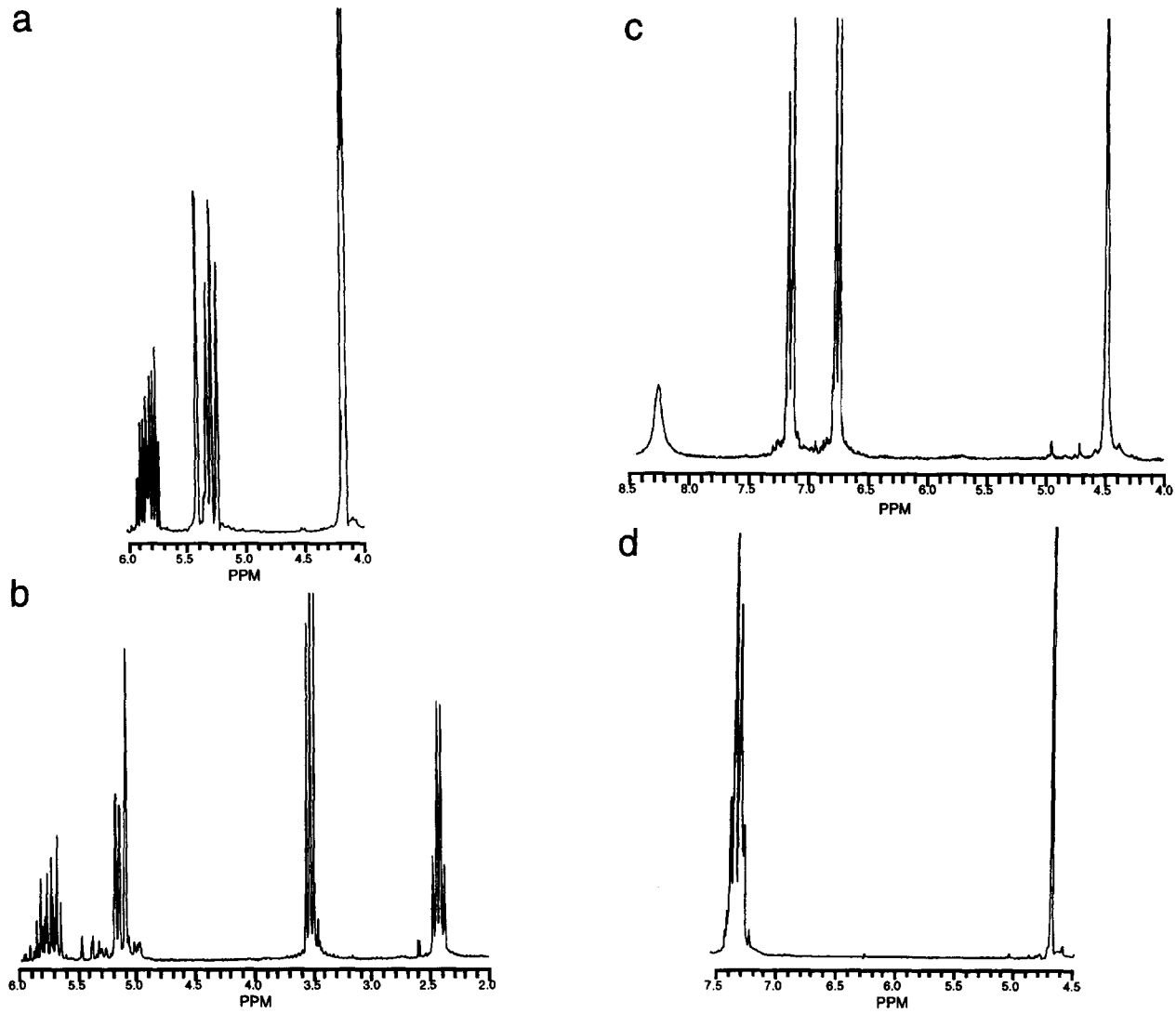

Figure 2. ^{1}H NMR spectra of ITCs derived from SNG (2-propenyl-glucosinolate) (panel A); GNA (3-butenyl-glucosinolate) (panel B); SNB (*p*-hydroxy-benzyl-glucosinolate) (panel C); GTP (benzyl-glucosinolate) (panel D); GCH (3-methyl-sulfonyl-propyl-glucosinolate) (panel E); GER (4-methyl-thio-butyl-glucosinolate) (panel F); GRE (4-methylsulfinyl-butenyl-glucosinolate) (panel G). See chemical structure in Fig. 1.

in the case of SNG, PRO, *e*-PRO, GTP, and GCH (data not shown). The last point demonstrates that myrosinase remains stable in tissue culture.

The IC_{50} values (the concentration inhibiting the growth of 50% of cultured cells) of the tested ITCs are reported in Table 1. From the analysis of the IC_{50} it is evident that the SNB-ITC shows the lower antiproliferative activity, on the contrary, ITCs generated from SNG and GTP appear to be the most active compounds, producing a 50% cell growth inhibition at concentrations in the 100 nM range.

Structure–activity relationship

Figure 4 shows the in vitro activity of the nine ITCs obtained by the in situ method grouped as function of their side-chain chemical structure. For instance, in panel A includes the alkenyl, panel B the hydroxyalkenyl, panel C the aromatic, and finally panel D the side-chain sulfur-containing ITCs. Although the limited number of GLs tested does not allow a definitive evaluation of the structure–activity relationship, it is possible to draw some preliminary considerations.

The comparison within alkenyl-ITCs suggests that a higher alkenyl side-chain length negatively affects the antiproliferative activity, as demonstrated by the differences in the IC_{50} values (see Table 1).

Concerning hydroxylalkenyl-ITCs, it is evident that the activity of the two epimers 5*S* or 5*R* VOTs (derived from PRO and *e*-PRO) is almost superimposable (see Fig. 4, panel B). This indicates that the stereochemistry of the group at the C-5 atom does not affect the biological activity of these compounds.

Data relative to aromatic-ITCs (derived from SNB and GTP), differing only for the *p*-hydroxyl group, suggest the negative role of the hydrophilic groups (see panel C). The SNB-ITC shows the lowest antiproliferative

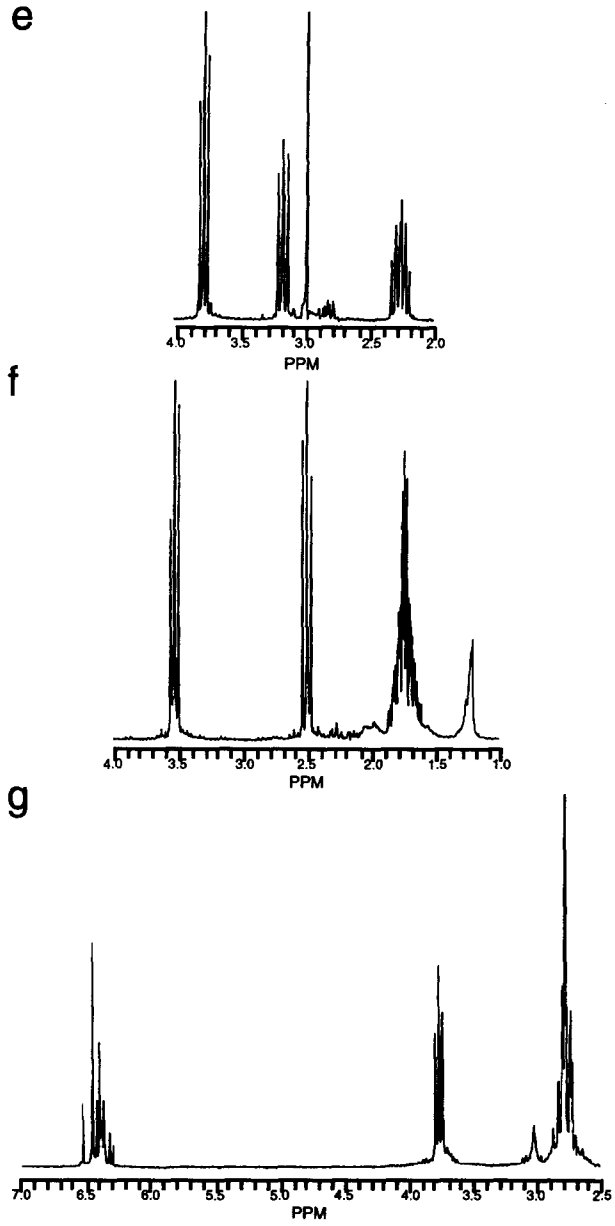

Figure 2. *continued.*

activity. IC_{50} is, in fact, 320 μM, while the IC_{50} of its unhydroxylated counterpart (GTP-ITC) is 0.1 μM. In addition SNB-ITC was found to be unstable as probed by GC-MS[12] leading to the formation of *p*-hydroxybenzyl alcohol.

Finally, side-chain sulfur-containing ITCs (derived from GER, GCH, and GRE), show intermediate antiproliferative activity. In particular, within this group, GER-ITC shows the highest activity (IC_{50} = 2.5 μM). These differences in antiproliferative activity could be attributed to the oxidation state of the sulfur atom present in their side-chain.

In this regard, sulforaphane (the ITC derived from glucoraphanin), which contains the same sulfinyl group contained in the molecule of glucoraphenin, has been indicated by Zhang et al.[15] to be the most potent inducer of some detoxification enzymes such as quinone

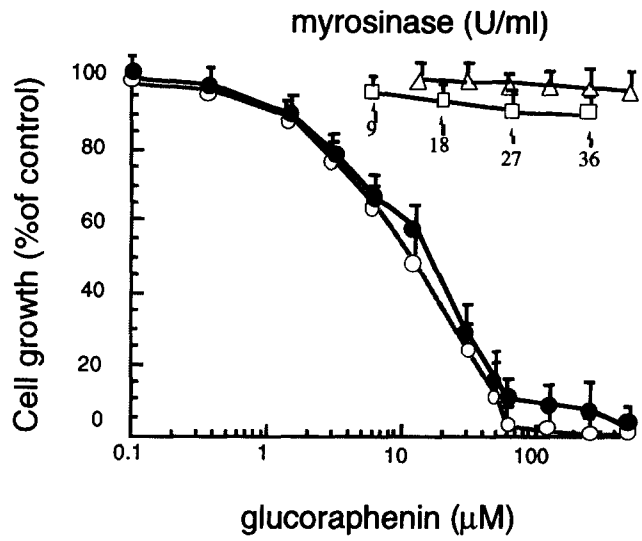

Figure 3. Effect of myrosinase (□), GRE (△) and GRE-ITC on in vitro cultured human erythroleukemic K562 cells in the concentration range comprised between 0.1 and 500 μM. Experiments were conducted either following the in situ (○) or pre-mix methods (●) (see Experimental). Arrows indicate the myrosinase concentration espressed in U/mL. Data represent the average of four independent determinations ± SD.

reductase and glutathione-*S*-transferase, in growing murine hepatoma cells. In the same paper, the authors report that other ITCs containing sulfone groups, although less potent, show similar activity in term of enzyme induction and chemoprotection.

In order to find a rationale for the differences in antiproliferative activity shown by the tested ITCs we determined some physicochemical properties possibly related to biological activity for all the compounds. Table 1 gives the water/oil partition coefficient and the chemical shift of the methylene group adjacent to the isothiocyanate group. In particular, the partition coefficient, being a measure of molecular lipophilicity or hydrophobicity, assumes particular importance in this case. In fact, due to the wide distribution in nature of lipids, which are also important costituents of biological membranes, it is not surprising that the water/oil partition coefficient is, in this case, a parameter of practical interest, providing information on the ability of ITCs to pass through cell membranes. On the other hand, chemical shift value could be taken as an indication of the nucleophilicity of the compounds and thus of their reactivity with critical target sites (i.e., enzyme systems). By the analysis of the results in Table 1 it is clearly evident that antiproliferative activity appears to be correlated to the lipophilicity rather than the nucleophilicity of the compounds. In fact, it is evident that the most active ITCs are characterized by low partition coefficients independently from the group of GLs to which they belong. For instance, GNA-ITC and GER-ITC display very similar chemical shift values, while their activity is quite different. This difference in activity fits very well with their respective water/oil partition coefficient. Aromatic ITCs again show very similar chemical shifts (4.45 and 4.66 ppm, respectively), while their partition coefficients are 0.468 and

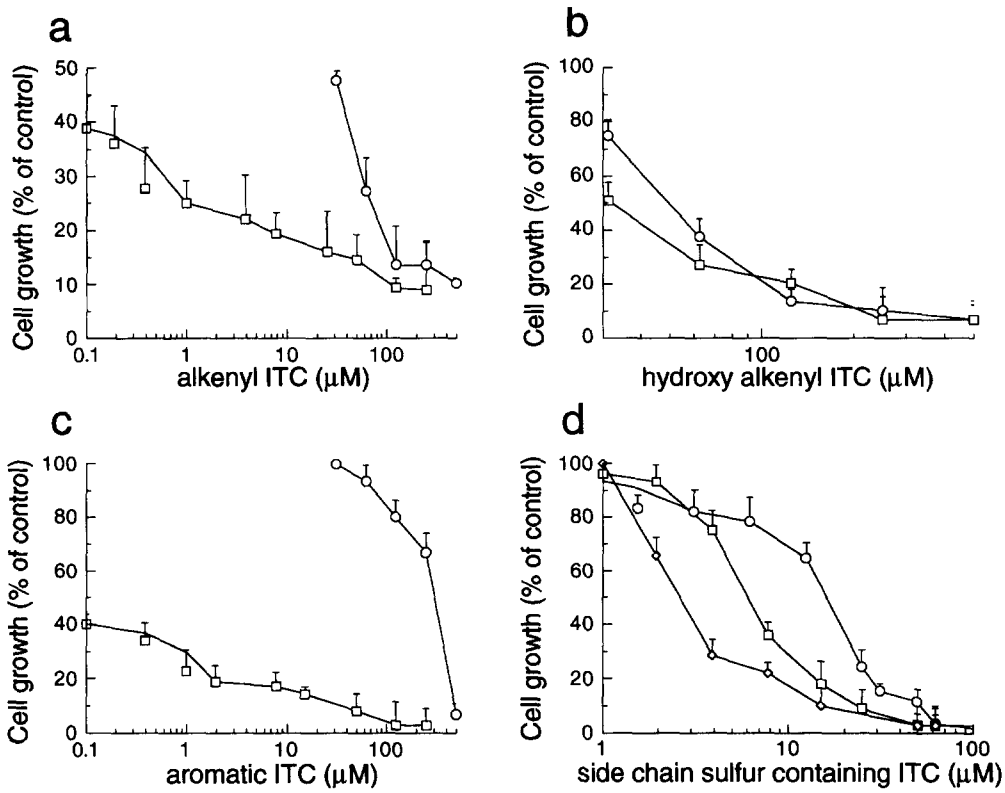

Figure 4. Antiproliferative activity on K562 cells of alkenyl (panel A): GNA-ITC (○), SNG-ITC (□); hydroxy-alkenyl (panel B): PRO-ITC (○), *e*-PRO-ITC (□); aromatic (panel C): SNB-ITC (○), GTP-ITC (□); and side-chain sulfur-containing ITCs (panel D): GRE-ITC (○), GER-ITC (◇), GCH-ITC (□). Results were obtained by the in situ method (see Experimental). Data represent the average of four independent determinations ± SD.

0.006. This difference in partition coefficient values appears to be well correlated with the antiproliferative activity having IC_{50} values obtained by using either the in situ (320 vs 0.1 μM) (see Table 1) or the pre-mix method (100 vs 1.5 μM) (data not shown).

Finally, in order to determine how general the antiproliferative activity of ITCs is, 4-methyl-sulfinyl-butenyl-isotiocyanate, taken as an example, was tested on six different tumor cell lines: human erythroleukemic K562 cells, murine eythroleukemic FL cells, human T-lymphoid Jurkat cells, human cervix carcinoma HeLa cells, H9-38 cells, and H3-T1-1 cells. The result of this experiment is shown in Figure 5. Data indicate that when employed at concentrations ranging from 0.1 to 50 μM the inhibitory curves obtained are almost superimposable for all cell lines tested.

Effects of GRE-ITC on erythroid differentiation of K562 cells

In order to determine the possible toxic effects we evaluated the activity of GRE-ITC (taken as an example) on the activation of differentiated functions. In particular, the effect of GRE-ITC on ara-C-mediated erythroid induction of K562 cells was studied. The results of these experiments, shown in Figure 6, demontrated that not only does GRE-ITC not interfere with the erythroid induction of K562 cells but it has a cooperative effect with ara-C when this drug is used at suboptimal concentrations. This latest finding could be of great interest since the antitumor activity exerted by ITCs in this experimental system could also be due to their ability to promote differentiation of cells.

Conclusions

The biological activity of ITCs derived from enzymatic hydrolysis of GLs described here is particularly important. Our results reconsider the minor constituents contained in cruciferous vegetables that were generally considered as detrimental compounds.

These compounds could be responsible for the protective activity of a cruciferous-rich diet against intestinal cancer. The mechanism by which the protective effect is developed is not yet completely clear, although there is evidence that some isothiocyanates are able to induce phase II enzyme activities[16] and affect the carbohydrate metabolism.[17] The activity of these compounds was ascribed to the chemical reactivity of the isothiocyanate group which can easily react with proteins.[18]

Finally, in light of our results, we would like to underline that diets particularly rich in glucosinolate-containing vegetables or a possible introduction of GL-enriched foods is to be carefully considered. In fact, the actual level of knowledge about the relationship between ingestion of specific levels of ITCs and cancer prevention is not yet completely elucidated. Never-

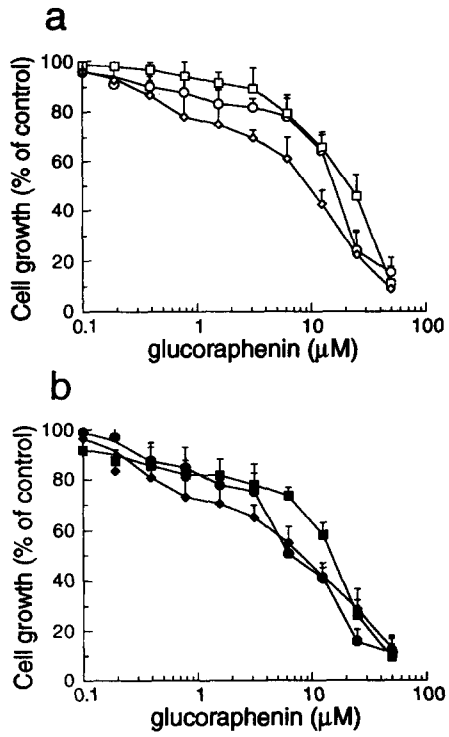

Figure 5. Antiproliferative activity of GRE-ITC assayed by the in situ procedure (see the Experimental section), on different in vitro cultured cell lines. Panel A: human erythroleukemic K562 cells (○), murine eythroleukemic FL cells (□), human T-lymphoid Jurkat cells (◇). Panel B: human cervix carcinoma HeLa cells (○), H9-38 cells (■) and H3-T1-1 cells (♦). Data represent the average of three independent determinations ± SD.

theless, it could be of great interest to achieve cancer chemoprevention in humans through an alternative strategy employing phytochemicals. These non-nutritive substances indeed represent a new possibility for both cancer prevention and treatment.

Experimental

Myrosinase

Myrosinase was isolated from ripe seeds of white mustard (*Sinapis alba* L.) by concanavalin A affinity chromatography and chromatofocusing as reported elsewhere.[19,20] The myrosinase stock solution used in the present study had a specific activity of ca. 60 units/mg of soluble protein, measured with sinigrin as the substrate and assayed as described by Palmieri et al.[20] Myrosinase solution was stored at 4 °C in sterile distilled water until used.

Glucosinolates

The GLs used were isolated from different cruciferous seeds according to a previously reported procedure.[7,8] In the present study we considered the following GLs: sinigrin (2-propenyl-glucosinolate) (SNG) isolated from *Brassica carinata*, gluconapin (3-butenyl-glucosinolate) (GNA) isolated from *Brassica rapa* cv. Silla, progoitrin

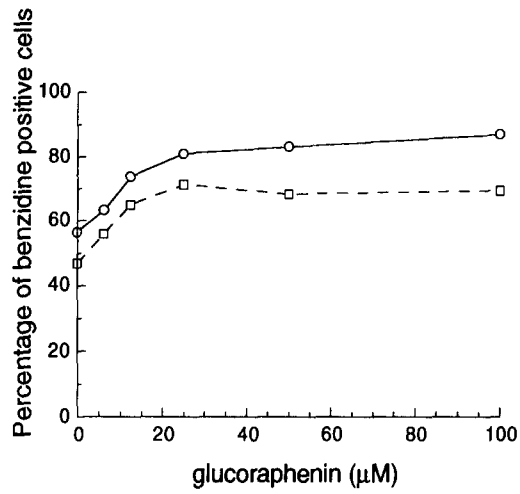

Figure 6. Effects of GRE-ITC on erythroid differentiation of human erythroleukemic K562 cells. K562 cells were induced to erythroid differentiation with 250 nM (□) and 500 nM (○) ara-C. Determinations were performed after 6 days of cell culture.

(2*R*-2-hydroxy-3-butenyl-glucosinolate) (PRO) isolated from *Brassica napus* cv. Jet Neuf, *epi*-progoitrin (2*S*-2-hydroxy-3-butenyl-glucosinolate) (*e*-PRO) isolated from *Crambe abissinica* cv. Belenzian, sinalbin (*p*-hydroxy-benzyl-glucosinolate) (SNB) isolated from *Sinapis alba* cv. Maxi, glucotropaeolin (benzyl-glucosinolate) (GTP) isolated from *Lepidium sativum* L., glucoerucin (4-methyl-thio-butyl-glucosinolate) (GER) isolated from *Eruca sativa* M., glucocheirolin (3-methyl-sulfonyl-propyl-glucosinolate) (GCH) isolated from *Cheirantus cheiri*, glucoraphenin (4-methyl-sulfinyl-butenyl-glucosinolate) (GRE) isolated from *Raphanus sativus* cv. Pegletta (chemical structure given in Fig. 1).

Production of ITCs

The enzymatic hydrolysis products were produced using free or immobilized myrosinase in 0.1 M phosphate buffer pH 6.5 at 37 °C.[19] The reaction products, isothiocyanates (ITCs), and vinyl-oxazolidine-2-thiones (VOTs) were dried by a Buchi RE 121 rotary evaporator at 50–60 °C under vacuum and then solubilized in CH_2Cl_2. The solution was dried using anhydrous Na_2SO_4, filtered and then the ITCs were concentrated in a rotary evaporator or under a stream of nitrogen until the solvent was completely removed. Finally, the ITCs were characterized by GC-MS and/or HPLC, and NMR spectroscopy.

ITCs analyses

GC-MS analyses of ITCs were performed on a Hewlett Packard GCD G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5. The flow rate of the carrier gas (He) was 1 ml/min and the sample (1 μL of CH_2Cl_2 solution) was injected in the splitless mode. Column temperature was 40 °C at the start and 220 °C at the end with a rate of 10 °C/min. Injector and detector were at 250 and 280 °C, respectively. Mass spectrum of each

ITC was scanned in the range 10–425 *m/z*. In some cases peak identification was done by comparing with spectra reported in the literature.[12] The column was eluted for 22 min using a linear gradient of acetonitrile (20–75%) at 40 °C with a flow of 2 mL/min. The chromatographic fraction were detected at 200 and 210 nm. Partition coefficients were determined as previously described.[6]

Spectroscopy

Nuclear magnetic resonance (^{1}H NMR) spectra of ITCs were recorded on a Bruker AC-200 spectrometer after solubilization in $CDCl_3$, except in the case of SNB-ITC, where acetone was used as solvent. Spectra were run at 200 MHz. Peak positions are given in parts per millions down field from tetramethylsilane as an internal standard.

Cell line and culture conditions

Cell lines used in the present study were human erythroleukemic K562 cells, murine erythroleukemic FL cells, human T-lymphoid Jurkat cells, human cervix carcinoma HeLa cells, human T-lymphoid H9 cells and HeLa transfected with a LTR-HIV-1-CAT plasmid H3-T1-1 cells. Standard conditions for cell growth were α-medium (GIBCO, Grand Island, NY, U.S.A.), 50 mg/L streptomycin, 300 mg/L penicillin, supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Inc., McLean, VA, U.S.A.) in 5% CO_2, 80% humidity.

In vitro antiproliferative assays

The activity of glucosinolate hydrolysis products was tested using two different approaches as described below.

The in situ method. Myrosinase was directly added to the complete cell culture medium containing increasing concentrations of GLs (up to 500 μM). Cells were subsequently added (5×10^4 cells/1.5 cm tissue culture dish).

The pre-mix method. In this case myrosinase-catalyzed hydrolysis of glucosinolates was previously carried out in phosphate buffer pH 7.4 at 37 °C. After 30-min incubation, the time needed in order to obtain the complete hydrolysis of GLs, the buffer solution was diluted with an equal volume of 2 × cell culture medium to which cells were subsequently added.

Cell growth determinations were performed by an electronic counting with a ZF Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.). Assays were carried out in triplicate and usually counts differed by <5%.

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Hemoglobin determination

K562 cells containing heme or hemoglobin were detected by specific reaction with a benzidine/hydrogen peroxide solution as reported elsewhere.[21]

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References

1. Graham, S.; Dayal, H.; Swanson, M.; Mittleman, A.; Wilkinson, G. *J. Natl. Cancer Inst.* **1978**, *61*, 709.

2. Manousos, O.; Day, N. E.; Trichopoulos, D.; Gerevassilis, F.; Tzonou, A.; Polychronopoulou, A. *Int. J. Cancer* **1983**, *32*, 1.

3. Lee, H. P.; Gourley, L.; Duffy, S. W.; Esteve, J.; Lee, J.; Day, N. E. *Int. J. Cancer* **1989**, *43*, 1007.

4. Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. *CRC Crit. Rev. Food Sci.* **1983**, *18*, 123.

5. Pessina, A.; Thomas, R. M.; Palmieri, S.; Luisi, P. L. *Arch. Biochem. Biophys.* **1990**, *280*, 383.

6. Nastruzzi, C.; Cortesi, R.; Esposito, E.; Menegatti, E.; Leoni, O.; Iori, R.; Palmieri, S. *J. Agric. Food Chem.* **1996**, *44*, 1014.

7. Thies, W. *Fat Sci. Technol.* **1988**, *8*, 311.

8. Visentin, M.; Tava, A.; Iori, R.; Palmieri, S. *J. Agric. Food Chem.* **1992**, *40*, 1687.

9. *Official Journal of European Community*. Regulation (EEC) 1864/90, June Enclosure VIII, J.O. n. L170, **1990**, 27–34.

10. Iori, R.; Leoni, O.; Palmieri, S. *Anal. Biochem.* **1983**, *134*, 195.

11. Leoni, O.; Marot, C.; Rollin, P.; Palmieri, S. *Tetrahedron: Asymmetry* **1994**, *5*, 1157.

12. Spencer, G. S.; Daxenbichler, M. E. *J. Agric. Food Chem.* **1980**, *31*, 359.

13. Wattemberg, L. W. *Cancer Res.* **1982**, 2085 S.

14. Bell, J. M. *J. Anim. Sci.* **1984**, *58*, 996.

15. Zhang, G. Y.; Talalay, P.; Cho, C.; Posner, G. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2399.

16. Talalay, P. In *Cancer Chemoprevention*. Wattenberg, L.; Lipkin, M.; Boone, C. W.; Kelloff, G. J., Eds.; CRC Press: Boca Raton, 1992; pp 469–478.

17. Tawfiq, N. *Carcinogenesis* **1995**, *16*, 1191.

18. Kawarishi, S.; Kaneko, T. *J. Agric. Food Chem.* **1987**, *35*, 85.

19. Palmieri, S.; Iori, R.; Leoni, O. *J. Agric. Food Chem.* **1986**, *34*, 138.

20. Palmieri, S.; Leoni, O.; Iori, R. *Anal. Biochem.* **1982**, *123*, 320.

21. Wheatherall, D. J.; Clegg, J. B. In *The Thalassemia Syndromes*. Wheatherall D. J.; Clegg J. B., Eds.; Blackwell Scientific: Oxford, 1981, pp 744–780.