In Vitro Antiproliferative Activity of Isothiocyanates and Nitriles Generated by Myrosinase-Mediated Hydrolysis of Glucosinolates from Seeds of Cruciferous Vegetables

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A comparison of the effect of isothiocyanates and nitriles derived from some glucosinolates, namely, *epi*-progoitrin, sinalbin, glucotropaeolin, glucocheirolin, and glucoraphenin, on human erythroleukemic in vitro cultured cells was studied. Many studies have in fact evidenced that a consumption of vegetable containing glucosinolates could reduce the development of colorectal cancer. In the experimental conditions used, the production of isothiocyanates and nitriles from glucosinolates is almost quantitative as confirmed by HPLC or GC−MS analysis. The obtained results demonstrated that in general nitriles are considerably less potent than the corresponding isothiocyanates in inhibiting cancer cell growth. Particularly, the isothiocyanates inhibitory activity on K562 cells growth is higher in the case of products derived from *epi*-progoitrin, glucotropaeolin, glucoraphenin, and glucocheirolin; while for nitriles the higher activity in inhibiting K562 cells growth is showed by sinalbin-derived product. Considering the antiproliferative activity found for isothiocyanates and nitriles, further studies will be aimed to the possible application of glucosinolate-derived products as chemopreventive cancer agents for the reduction of colorectal cancer.

Keywords: *Enzymes and enzyme reactions; antitumor compounds; antiproliferative agents*

INTRODUCTION

The biological activity shown by glucosinolates (GLs) and their derived products, generally considered as detrimental compounds, has recently become more and more important due to their possible protective activity against intestine cancer (Lee et al., 1989; Manousos et al., 1983). GLs are characterized by a β-D-thioglycosidic group and a variable side chain, generally represented by aliphatic, aromatic, or heteroaromatic residues. These compounds are contained in various quantities and ratios in the seeds, roots, stems, and leaves of some dicotyledonous angiosperms, the most important of which are typified by Cruciferae. In the intact cells, GLs are kept separate from the endogeneous enzyme myrosinase, a β-thioglucoside glucohydrolase able to catalyze the hydrolysis of GLs. When myrosinase and GLs react together after cell disruption, they result in the production of D-glucose and a series of different compounds, i.e., isothiocyanate, thiocyanate, and nitriles, depending on the substrate and the reaction conditions used, such as pH, temperature, and substrate structure (Gil and MacLeod, 1980). In this respect, it is important to emphasize that, at neutral pH and temperature above 25 °C, the formation of isothiocyanates is definitely favored; while using a protonation mechanism and low pH, the main degradation product is represented by nitriles (Gil and MacLeod, 1980). In our previous studies, we demonstrated that GL-derived products possess interesting antiproliferative activity toward tumoral cells (Nastruzzi et al., 1996; Leoni et al., 1997). The cytotoxic mechanism of these compounds is not completely understood, although it seems that these minor dietary constituents could be important inhibitors of carcinogenesis, acting by neutralization of a wide number of carcinogens or by the suppression of proliferative activity of neoplastic cells (Wattemberg, 1982). In the first case, GLs and derived products would prevent carcinogen molecules from reaching the target site or interacting with the reactive carcinogenic molecules or activating important hepatic enzymes for the protection against several carcinogens, such as quinone reductase, glutathione *S*-transferase and UDP-glucoronosyl transferase (Zhang et al., 1992); in the second case, they may weaken the effects of genetic changes that occurred in the early stages of neoplastic transformation (Nugon-Baudon et al., 1990). In addition, other studies demonstrated that some compounds, such as isothiocyanates, are able to inhibit protein synthesis and affect carbohydrate metabolism (Leblova, 1965).

Taking into account these findings and those regarding our previous studies on the myrosinase−GL system (Leblova, 1965; Palmieri et al., 1986, 1987; Pessina et al., 1990; Visentin et al., 1992; Hochkoeppler and Palmieri, 1992; Lazzeri et al., 1993; Leoni et al., 1993), we studied the effect of some purified GLs enzymatic hydrolysis products (i.e., ITCs and nitriles) on in vitro cultured human K562 erythroleukemic cell line (Lozzio and Lozzio, 1975). This cell line is one of the best known in vitro experimental model systems mimicking erythroid differentiation when treated with suitable inducers

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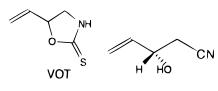
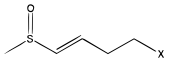
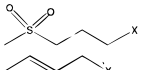
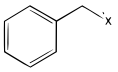
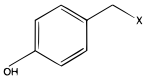
Table 1. Effect of Myrosinase-Catalyzed Hydrolysis Products of Glucosinolates on Human Erytroleukemic K562 Cells Growth[a]

Isothiocyanate and nitrile side chain	Native glucosinolate	Abbreviation GLs	activity IC_{50} nitrile (μM)	isothiocyanate (μM)
 VOT	epiprogoitrin	*e*PRO	164	32.0
	glucoraphenin	GRE	133	15.0
	glucocheirolin	GCH	43	6.0
	glucotropaeolin	GTP	420	1.5
	sinalbin	SNB	104	320

[a] X = NCS, isothiocyanate; X = CN, nitrile. Inhibitory concentration 50%, compound concentration (μM) required to cause a 50% inhibition of in vitro growth of K562 cells.

(Rutherford et al., 1979; Whaterhall et al., 1981; Gambari et al., 1984). In this view, the K562 cell line has been proposed as a very useful model system to identify the therapeutical potential of antiproliferative and differentiating compounds as well as to study the molecular mechanism(s) regulating the expression of human globin genes (Gambari et al., 1984). Studies on viability and induced differentiation of K562 cells could thus have practical applications in the search of compounds potentially useful as therapeutic agents for the treatment of different human pathologies, including neoplastic diseases and β-thalassemia.

EXPERIMENTAL PROCEDURES

Myrosinase and Glucosinolates. Myrosinase was isolated from ripe seeds of *Sinapis alba* L. The myrosinase stock solution had a specific activity of ca. 60 unit/mg of soluble protein and was stored at 4 °C in sterile distilled water until used.

GLs were isolated, purified, and characterized starting from the more convenient Crucifers species according to previously reported procedures (Palmieri et al., 1986; Thies, 1988; Visentin et al., 1992) starting from ripe seeds of some crucifers. Briefly, 50 g of freeze-dried radish roots was boiled in 50 mM imidazole−formate (pH 4.5) and methanol 3:7, v/v. The mixture was then homogenized for 20 min with a Ultraturrax TP 18/2N and left in a ice bath until the suspension decanted. After three successive extractions of the solid residues, the methanolic extracts were centrifuged at 17700*g* for 30 min using a Beckman J-21 centrifuge. The obtained supernatant was first concentrated under vacuum at 40 °C and resuspended with 50 mM imidazole−formate (pH 4.5) and then deproteinized using a solution of lead and barium acetate 50 mM. After protein precipitation, the extract was centrifuged at 17700*g* for 15 min. Each GL was then identified using NMR, mass spectrometry, and HPLC analyses of desulfoderivates (EEC, 1990) coupled with polarographic determinations of total GLs content (Iori et al., 1983).

In the present study we used the following: *epi*-progoitrin (2*S*-2-hydroxy-3-butenyl-glucosinolate) (*e*-PRO) isolated from *Crambe abissinica* cv. Belenzian, sinalbin (*p*-hydroxybenzyl-glucosinolate) (SNB) isolated from *Sinapis alba* cv. Maxi, glucotropaeolin (benzylglucosinolate) (GTP) isolated from *Lepidium sativum* L., glucocheirolin (3-methylsulfonylpropyl-glucosinolate) (GCH) isolated from *Cheirantus cheiri*, and glucoraphenin (4-methylsulfinylbutenylglucosinolate) (GRE) isolated from *Raphanus sativus* cv. Pegletta (for chemical structures, see Table 1).

Production of Isothiocyanates and Nitriles. The aromatic isothiocyanates and nitriles were obtained by Fluka (Bucs, Switzeland). The other enzymatic hydrolysis products were produced using 500 μM GLs in the presence of 50 U of myrosinase alternatively in 0.1 M phosphate buffer, pH 6.5 (ITCs), or 0.1 M acetate buffer, pH 5 (nitriles), at 37 °C (Tawfiq, 1995). The ITCs (VOT) were dried by a Buchi model 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 and filtered, and then the products were concentrated in rotary evaporator or under nitrogen stream until the solvent was completely removed.

The production of nitriles was carried out by dissolving each pure glucosinolate in 0.1 M acetate buffer at pH 5 containing 2.5 mM Fe^{2+}, 5 mM cysteine, and 50 U of myrosinase. After 3 h, the nitrile was extracted in a separator funnel with diethyl ether or methylene chloride and water (1:1, v/v). The products were then analyzed and stored under nitrogen at −20 °C in order to reduce their easy decomposition.

The obtained GL-derived products were characterized by GC−MS (isothiocyanates and nitriles derived from *epi*-progoitrin), HPLC (isothiocyanates and nitriles derived from sinalbin, glucotropaeolin, glucocheirolin, and glucoraphenin), and NMR spectroscopy (Nastruzzi et al., 1996).

Cell Culture Conditions. In the present study, human erythroleukemic K562 cells were used. Standard conditions for cell growth were α-medium (GIBCO, Grand Island, NY), 50 mg/L streptomycin, and 300 mg/L penicillin, supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Inc., McLean, VA) in 5% CO_2 at 80% humidity.

In Vitro Antiproliferative Assays. The activity of glucosinolate hydrolysis products was tested as follows. Increasing concentrations (0−500 μM) of myrosinase-catalyzed hydrolysis products obtained as previously described were poured in the cell culture medium, afterward cells were added (5×10^4 cells/1.5 cm tissue culture dish).

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

RESULTS AND DICUSSION

Production of ITCs and Nitriles Derived from GLs. The following five compounds were considered (see Table 1): *epi*-progoitrin (*e*-PRO), sinalbin (SNB), glucotropaeolin (GTP), glucocheirolin (GCH), and glucoraphenin (GRE). This choice allowed a preliminar comparison among three classes of GL-derived products, namely, aliphatic (*e*-PRO), thioaliphatic (GRE and GCH), and aromatic (SNB and GTP). The HPLC analyses of desulfoderivatives, coupled with polarographic determinations of total GLs content, showed that the

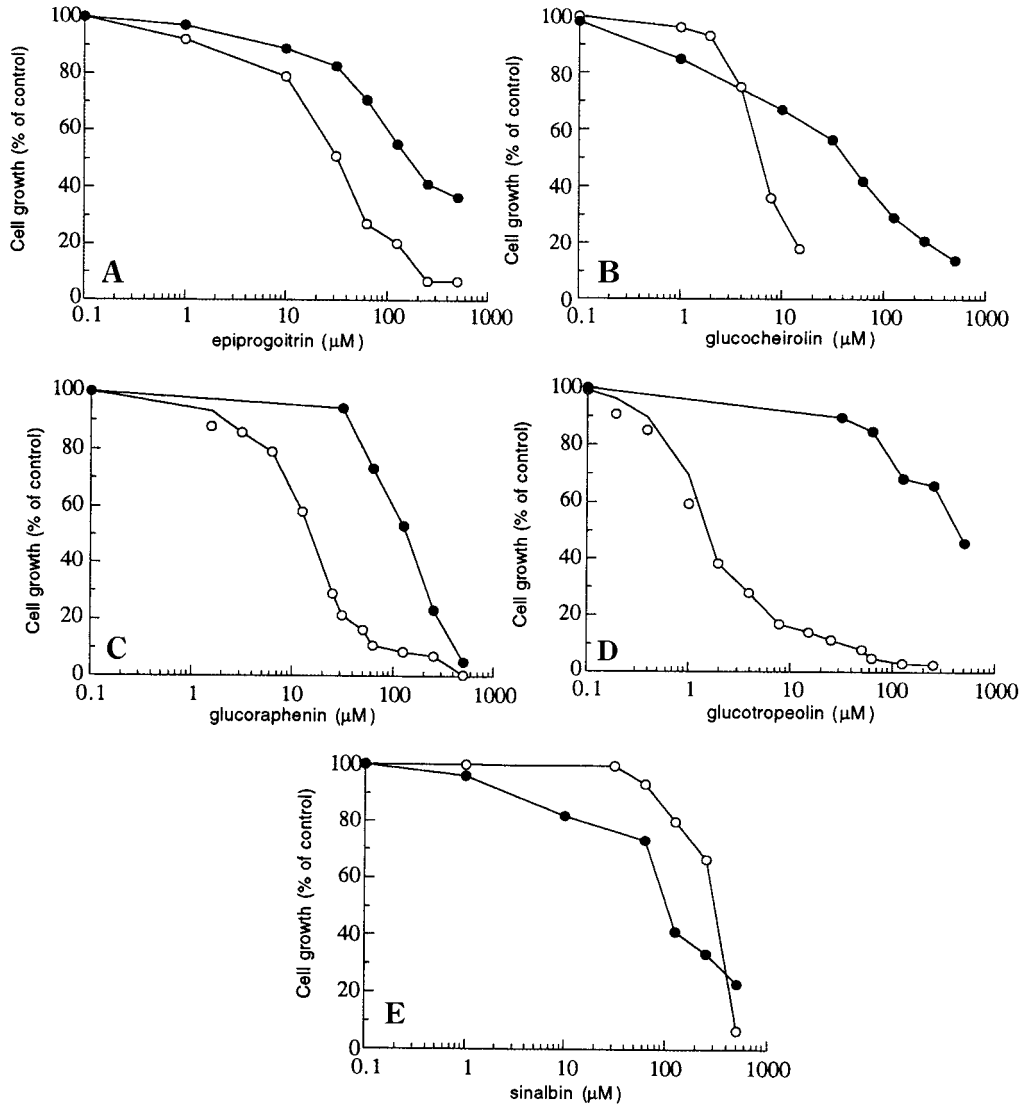


Figure 1. Differential antiproliferative activity of nitrile (●) or isothiocyanate (○) hydrolysis products on human erythroleukemic K562 cells. Data represent the average of three independent determinations: (A) epiprogoitrin, (B) glucoraphenin, (C) glucocheirolin, (D) glucotropaeolin, and (E) sinalbin.

five GLs were nearly homogeneous with an absolute purity higher than 80% (the other constituent was essentially water). Each GL was then subjected to myrosinase-catalyzed hydrolysis using different reaction parameters, such as pH and presence of cofactors (Gil and MacLeod, 1980). To this end, the myrosinase alternatively in 0.1 M phosphate buffer, pH 6.5, or 0.1 M acetate buffer, pH 5, was used. This glycoprotein isolated from *Sinapis alba* seeds is characterized by various thiol and sulfide groups together with ca. 18% of carbohydrates and consists of two identical subunits with a M_r of 71.1 kDa (Pessina et al., 1990). The reactions were performed at 37 °C. In the experimental conditions used, the GLs allowed the formation of the nitriles and isothiocyanates (ITCs) reported in Table 1 except in the case of hydroxyalkenyl GLs *epi*-progoitrin, whose hydroxy-ITC was subjected to cyclization giving the *5R*-vinyloxazolidine-2-thione (VOT) (Iori et al., 1982).

Antiproliferative Activity of ITCs and Nitriles. As reported in the Introduction, the antiproliferative activity of GLs contained in cruciferous vegetables is attributed to their enzymatic breakdown products such as isothiocyanate and/or nitriles (Wattemberg, 1982) generated after in vivo metabolization by endogenous or commensal microflora enzymes. To verify this hypothesis, the activities of a hydroxyalkenyl (e-PRO), two aromatic (SNB and GTP) and two side chain sulfur-containing (GCH and GRE) ITCs (VOT), and nitriles obtained by myrosinase catalyzed-reaction were tested on the human erythroleukemic cell line K562. In Figure 1, the antiproliferative activities are reported of increasing concentrations (up to 500 μM) of purified ITCs (VOT) and nitriles derived from GLs on human erythroleukemic K562 cells. Considering the curve trends of both GL-derived products, it is evident that, except in the case of sinalbin, the isothiocyanates are more active than the corresponding nitriles in inhibiting the growth of K562 cells. This behavior can be easily detectable considering the IC_{50} values (the concentration required to inhibit of the 50% the growth of cultured cells) of the tested ITCs and nitriles reported in Table 1. From the analysis of the IC_{50} in fact, it clearly emerges as the SNB−ITC shows the lower antiproliferative activity, while ITCs generated from GTP and GCH appear to be the most active compounds, producing a 50% cell growth inhibition at concentrations in the 1−6 μM range.

Although we considered a limited number of GLs, we

can make some preliminary considerations on the basis of the structure/activity relationship of these compounds. In our previous study we found that (a) among alkenyl-ITCs a longer side chain negatively affects the antiproliferative activity of K562 and (b) the stereochemistry of the C5 atom of the two hydroxylalkenyl-ITCs epimers, derived from PRO and *e*-PRO, does not affect the biological activity of K562 cells. Moreover, we found that antiproliferative activity appears to be correlated to the lipophilicity of the compounds rather than their nucleophilicity. The most active ITCs are, in fact, characterized by low partition coefficients independently from the group of GLs to which they belong (Nastruzzi et al., 1998).

In the present paper, the comparison within aromatic derived products gives, for ITCs and nitriles, two opposite behaviors. Particularly, for aromatic nitriles the presence of the hydroxyl group induce an increase of the antiproliferative activity, showing a IC_{50} shifting from 420 to 104 μM for GTP- and SNB-derived nitriles, respectively. Conversely, in the case of ITCs, the negative role of the hydrophilic groups bound to the aromatc ring emerges. This behavior is probably due to the formation of volatile *p*-hydroxybenzyl alcohol from SNB−ITC during myrosinase cleavage. The loss of this volatile compound thus determines a reduction of the effective quantity of ITC available for the activity.

Side chain sulfur-containing ITCs or nitriles (derived from GCH and GRE), again evidenced the negative effect of a long side chain. Nevertheless, it should be noted that the higher the oxidation state of sulfur atom present in the side chain, the higher the antiproliferative activity displayed by the glucosinolate-derived compound.

In addition, as reported in our previous paper (Leoni et al., 1997), it has to be underlined that ITCs showed a cooperative effect with suboptimal differentiating concentrations of ara-C. This finding could be of great interest since the antitumor activity exerted by ITCs in this experimental system could be also due to their ability to promote differentiation of cells.

CONCLUSIONS

The comparison between the antiproliferative activity of nitriles and isothiocyanate, reported in the present paper, clearly demonstrated that in general nitriles are considerably less potent than isothiocyanates. Considering the antiproliferative activity found for ITCs, these compounds could be considered potentially responsible for the reduction of colorectal cancer associated to diets rich in cruciferous vegetables. Further studies will be aimed to the possible application of glucosinolate-derived products as chemopreventive cancer agents.

LITERATURE CITED

EEC. Regulation 1864/90, June Enclosure VIII. *Off. J. Eur. Communities: Legis.* **1990**, *L170*, 27−34.

Gambari, R.; Del Senno, L.; Barbieri, R.; Viola, L.; Tripodi, M.; Raschellá, G.; Fantoni, A. Human leukemia K562 cells: induction of erythroid differentiation by 5-azacytidine. *Cell Differ.* **1984**, *14*, 87−97.

Gil, V.; MacLeod, A. The effect of pH on glucosinolate degradation by a thioglucoside preparation. *J. Phytochem.* **1980**, *19*, 2547−2553.

Hochkoeppler, A.; Palmieri, S. Kinetic properties of myrosinase in hydrated reverse micelles. *Biotechnol. Prog.* **1992**, *8*, 91−96.

Iori, R.; Leoni, O.; Palmieri, S. A polarographic method for the rapid determination of total glucosinolates and free glucose of cruciferous material. *Anal. Biochem.* **1983**, *134*, 195−198.

Lazzeri, L.; Tacconi, R.; Palmieri, S. In vtro activity of some glucosinolates and their reaction products toward a population of the nematode *Heterodera schachtii*. *J. Agric. Food Chem.* **1993**, *41*, 825−829.

Leblova, S. *Naturwissenschaften* **1965**, *52*, 430.

Lee, H. P.; Gourley, L.; Duffy, S. W.; Esteve, J.; Lee, J.; Day, N. E. Colorectal cancer and diet in the asian population−A case-control study among Singapore chinese. *Int. J. Cancer* **1989**, *43*, 1007−1016.

Leoni, O.; Felluga, F.; Palmieri, S. The formation of 2-hydroxybut-3-enyl cyanide from (2*S*)-2-hydroxybut-3-enyl glucosinolate using immobilized myrosinase. *Tetrahedron Lett.* **1993**, *49*, 7967−7970.

Leoni, O.; Iori, R.; Palmieri, S.; Esposito, E.; Menegatti, E.; Cortesi, R.; Nastruzzi, C. Myrosinase-generated isothiocyanate from glucosinolates: isolation, characterization and in vitro antiproliferative studies. *Bioorg. Med. Chem.* **1997**, *5*, 1799−1806.

Lozzio, C. B.; Lozzio, B. B. Human myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **1975**, *45*, 321−334.

Manousos, O.; Day, N. E.; Trichopoulos, D.; Gerevassilis, F.; Tzonou, A.; Polychronopoulou, A. Diet and colorectal cancer: a case-control study in Greece. *Int. J. Cancer* **1983**, *32*, 1−5.

Nastruzzi, C.; Cortesi, R.; Esposito, E.; Menegatti, E.; Leoni, O.; Iori, R.; Palmieri S. In vitro cyotoxic activity of some glucosinolate-derived products generated by myrosinase hydrolysis. *J. Agric. Food Chem.* **1996**, *44*, 1014−1021.

Nugon-Baudon, L.; Rabot, S.; Szylit, O.; Raibaud, P. Glucosinolates toxicity in growing rats: interactions with the hepatic detoxification system. *Xenobiotica* **1990**, *20*, 223−230.

Palmieri, S.; Iori, R.; Leoni, O. Myrosinase from *Sinapis alba* L.: A new method of purification for glucosinolate analyses. *J. Agric. Food Chem.* **1986**, *34*, 138−140.

Palmieri, S.; Iori, R.; Leoni, O. Comparison of methods for determining myrosinase activity. *J. Agric. Food Chem.* **1987**, *35*, 617−621.

Pessina, A.; Thomas, R. M.; Palmieri, S.; Luisi, P. L. An improved method for the purification of myrosinase and its physicochemical characterization. *Arch. Biochem. Biophys.* **1990**, *280*, 383−389.

Rutherford, T. R.; Clegg, J. B.; Weatherall, D. J. K562 human leukaemic cells synthesis embryonic haemoglobin in response to haemin. *Nature* **1979**, *280*, 164−165.

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

Thies, W. Isolation of sinigrin and glucotropaeolin from Cruciferous seed. *Fat Sci. Technol.* **1988**, *8*, 311−314.

Visentin, M.; Tava, A.; Iori, R.; Palmieri, S. Isolation and identification of *trans*-4(methylthio)-3-butenyl glucosinolate from radish roots *(Raphanus sativus)*. *J. Agric. Food Chem.* **1992**, *40*, 1687−1691.

Wattemberg, L. W. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.* **1982**, 2085 S.

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

Zhang, G. Y.; Talalay, P.; Cho, C.; Posner, G. H. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2399−2403.

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