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The selective cytotoxicity of the alkenyl glucosinolate hydrolysis products and their presence in *Brassica* vegetables.

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ABSTRACT

Cruciferous vegetable consumption correlates with reduced risk of cancer. This chemopreventative activity may involve glucosinolates and their hydrolysis products. Glucosinolate-derived isothiocyanates have been studied for their toxicity and chemopreventative properties, but other hydrolysis products (epithionitriles and nitriles) have not been thoroughly examined. We report that these hydrolysis products differ in their cytotoxicity to human cells, with toxicity most strongly associated with isothiocyanates rather than epithionitriles and nitriles. We explored mechanisms of this differential cytotoxicity by examining the role of oxidative metabolism, oxidative stress, mitochondrial permeability, reduced glutathione levels, cell cycle arrest and apoptosis. 2-Propenylisothiocyanate and 3-butenylisothiocyanate both inhibited cytochome P450 1A (CYP1A) enzyme activity in CYP expressing MCL-5 cells at high cytotoxic doses. Incubation of MCL-5 cells with non-cytotoxic doses of 2-propenylisothiocyanate for 24 h resulted in a dose-dependent inhibition of ethoxyresorufin *O*-deethylase, yet failed to affect CYP1A1 mRNA expression indicating interference with enzyme activity rather than inhibition of transcription. Increased reactive oxygen species (ROS) production was observed only for 2-propenylisothiocyanate treatment. 2-Propenylisothiocyanate treatment lowered reduced glutathione levels whereas no changes were noted with 3,4-epithiobutylnitrile. Cell cycle analysis showed that 2-propenylisothiocyanate induced a G2/M block whereas other hydrolysis products showed only marginal effects. We found that 2-propenylisothiocyanate and 3-butenylisothiocyanate induced cell death predominantly via necrosis whereas, 3,4-epithiobutylnitrile promoted both necrosis and apoptosis. Thus the activity of glucosinolate hydrolysis products includes cytotoxicity that is compound-class specific and may contribute to their putative chemoprotection properties.

1. Introduction

Epidemiological studies have shown that dietary cruciferous vegetables may reduce the risk of cancer development and this protective effect is attributed in part to glucosinolate degradation products. Clinical studies have reported that higher intakes of cruciferous vegetables may reduce the risk of lung, colorectal, breast and prostate cancers and major chronic diseases (Willett, 2000; Feskanich et al., 2000; Voorrips et al., 2000; Gupta et al., 2014; Zhao et al., 2001).

When *Brassica* plant tissue is disrupted by chopping or chewing, plant myrosinase comes into contact with glucosinolates, causing cleavage of the thioglucoside linkage producing an unstable thiohydroximate *O*-sulfonate that rearranges to yield hydrolysis products such as isothiocyanate (ITC), nitrile and epithionitrile (ETN) (Fig. 1). The aglycone most frequently undergoes a Lossen rearrangement to produce ITC (Bones and Rossiter, 1996, 2006;

Abbreviations: CYP, cytochrome P450; ROS, reactive oxygen species; ITC, isothiocyanate; ETN, epithionitrile; ESP, epithiospecifer protein; AhR, arylhydrocarbon receptor; 2,3-PROP-ITC, 2-propenylisothiocyanate; 3,4-BUT-NIT, 3,4-butenylnitrile; 3,4-ETBUT-NIT, 3,4-epithiobutylnitrile; 3,4-BUT-ITC, 3-butenylisothiocyanate; 4,5-PENT-NIT, 4-pentenylnitrile; 4,5-ETPENT-NIT, 4,5-epithiopentylnitrile; carboxy-H2DCFDA, 6-carboxy-2′7′-dichlorodihydrofluorescein diacetate; PBS, phosphate buffered saline; DMSO, dimethyl sulphoxide; Rho123, rhodamine 123; EROD, ethoxyresorufin-*O*-deethylase; OPA, *O*-phthaldialdehyde; GSH, reduced glutathione.

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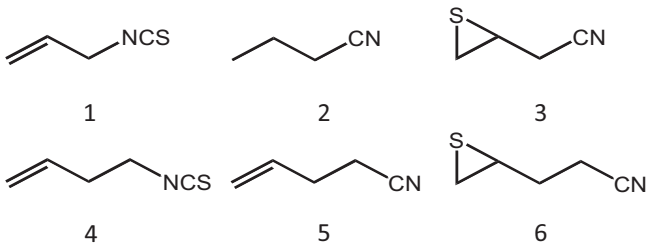

Fig. 1. Structures of the glucosinolate hydrolysis products used in this study, (1) 2,3-propenylisothiocyanate (2,3-PROP-ITC); (2) 3,4-butenylnitrile (3,4-BUT-NIT); (3) 3,4-epithiobutylnitrile (3,4-ETBUT-NIT); (4) 3-butenylisothiocyanate (3,4-BUT-ITC); (5) 4-pentenylnitrile (4,5-PENT-NIT); (6) 4,5-epithiopentylnitrile (4,5-ETPENT-NIT).

Hanschen et al., 2014). If the glucosinolate side chain contains a double bond (alkene) in the chemical structure, in the presence of epithiospecifer protein (ESP) and ferrous ions, the thiohydroximate rearranges to produce an ETN and nitrile (Bones and Rossiter, 2006). ESP is more sensitive to thermal processing than myrosinase and short periods of steaming can alter degradation profiles to increase ITCs with a marked reduction in nitriles and ETNs (Sarikamis et al., 2006). Thus ETNs and nitriles are more likely to be formed in raw vegetables (Abd Kadir, 2013; Kyung et al., 1995) such as in salads where for example cabbage is used. Commonly *Brassica* vegetables are boiled to the extent where myrosinases are deactivated. In this case the intestinal microflora can metabolise glucosinolates to give ITCs and nitriles (Fahey et al., 2012; Luang-In et al., 2014; Saha et al., 2012). While a great deal of data exists for anti cancer properties of ITCs, sulforaphane in particular (Hanschen et al., 2014; Nakamura and Miyoshi, 2010), there is far less information on other types of hydrolysis products such as ETNs and nitriles. In the seventies and eighties there was concern that ETNs being similar in structure to epoxides *i.e.* a three membered ringed heterocyle with sulphur replacing oxygen, might have similar toxicities. Few studies have revealed any negative aspects of sulforaphane although recently it has been shown that nucleotide excision repair is impaired (Piberger et al., 2014). Ring strain and the electrophilic nature of the carbon adjacent to the sulphur atom enables easy ring opening reactions with nucleophiles such as glutathione and DNA components resulting in alkylation (Druckrey et al., 1970; Luthy and Benn, 1980). Studies at this time showed that ETNs were toxic in rats but at relatively high doses compared to those that might be taken in the human diet (Brocker et al., 1984; Luthy et al., 1980; Nishie and Daxenbichler, 1980). Other work suggests that ETNs might be mutagenic while also slightly inhibitory to mutagenicity caused by benzpyrene (Uda et al., 1992). More recently the potential benefits of ETNs have been explored where it has been shown that 3,4-epithiobutylnitrile was the most potent inducer of cytoprotective enzymes of the ETNs tested (Kelleher et al., 2009).

Isothiocyanates are cancer chemopreventive in several animal models; proposed mechanisms include modulation of xenobiotic-metabolising enzymes by inhibition of cytochrome P450 enzymes (CYPs) (Smith and Yang, 2000), inducing phase II detoxifying enzymes such as glutathione *S*-transferases (GST) and NAD[P]H: quinone acceptor oxidoreductase 1 (NQ01), activating NF-E2 related factor 2 (NrF2) and the arylhydrocarbon receptor (AhR) (Hayes et al., 2008). Studies on structure-activity relationship *in vivo* and *in vitro* have demonstrated that the length of the alkyl chain of arylalkyl ITC also plays a role in the inhibition of CYP enzymes and increases their chemopreventive efficacy (Hayes et al., 2008; Munday et al., 2008; Zhang and Talalay, 1994). Isothiocyanates such as phenethylisothiocyanate and 4-methylsulfinylbutylisothiocyanate (sulforaphane) have been shown to be capable of inducing cell cycle arrest and cell death in cancer cells such as human prostate cancer cell lines (Hayes et al., 2008; Singh et al., 2004); bladder cancer cells (UM-UC-3) (Abbaoui et al., 2012); and human leukaemia cells (HL-60) (Xu and Thornalley, 2000).

For this study we have selected the potential hydrolysis products (Fig. 1) of two glucosinolates 2-propenyl- and 3-butenylglucosinolate which are found in *Brassica* vegetables. We have used the MCL-5 human lymphoblastoid cell line that has been engineered to express CYPs 1A1, 1A2, 2E1, 2A6, 3A4 (Crespi, 1991). The cHol cell line is identical to the MCL-5 line, but does not express the transfected CYP genes. The two cell lines, differing only in the metabolic competency, facilitate study of the role of CYP enzymes in xenobiotic oxidation, formation of reactive oxygenated intermediates (ROI), depletion of reduced glutathione, cellular damage, apoptosis and mutagenicity (Crespi, 1991). Here we have examined the involvement of oxidative stress and glutathione depletion leading to cell death induced by the glucosinolate hydrolysis products and show that cytotoxicity is dependent on the nature of the hydrolysis product.

2. Material and methods

2.1. Chemicals

RPMI 1640, L-glutamine, penicillin, streptomycin and hygromycin B were purchased from Invitrogen Corporation (Paisley, Scotland, UK). AlamarBlue® reagent and histidinol were purchased from Sigma–Aldrich Company (Poole, England, UK). All other chemicals unless stated in the text were obtained from Sigma–Aldrich Company. 2-Propenylisothiocyanate and 3-butenylnitrile were purchased from Sigma–Aldrich and fractionally distilled. The purity of compounds was assessed using GC–MS (Hewlett Packard 6890 GC linked to a 5973 MSD). Analysis was carried out on a Rtx®-200MS (Crossbend® trifluoropropylpolysiloxane) (30 m × 0.25 mm) 0.25 μm film thickness. The GC was programmed at an initial temperature of 50 °C (5 min) and to a final temperature of 270 °C (linear gradient, 25 min) and held for 5 min.

2.1.1. 3-Butenylisothiocyanate (3,4-BUT-ITC)

3,4-BUT-ITC was synthesised according to the procedures of Ettlinger and Hodgkins (1955). The product was purified by distillation (80 °C, 30 mmHg) and the structure confirmed by GC–MS and ^{1}H NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.5358 (q, 2H, H-4, J = 2.44), 3.6527 (t, 2H, H-3, J = 6.60), 5.2828 (m, 2H, H-1), 5.8880 (ddt, 1H, H-2, J = 17.04, 10.2, 6.76). MS (EI) m/z (%): 72 (100), 113 (M^+, 70), 55 (19), 85 (10), 114 (1, 5).

2.1.2. 3,4-Epithiobutylnitrile (3,4-ETBUT-NIT) and 4,5-epithiopentylnitrile (4,5-ETPENT-NIT)

Starting from the bromides (2-propenylbromide and 3-butenylbromide), 3,4-ETBUT-NIT and 4,5-ETPENT-NIT were synthesised according to the procedures described by Luethy et al. (1980). The resulting ETNs were purified by column chromatography on florisil. Florisil (8 g) was washed with pentane and the reaction product (approx 40 mg) dissolved in ether and applied to the column. The product was eluted (5 mL fractions) using a sequential mixture of pentane and ether in the ratio of 4:1 (10 mL), 3:1 (15 mL), 2:1 (15 mL) and finally 1:1 (20 mL). The elution of the ETNs was monitored by GC–MS. The ETNs eluted in the 3:1 pentane: ether fraction and were evaporated in a gentle stream of nitrogen gas to give approximately 25 mg of pure ETNs. The purity was confirmed by GC–MS and ^{1}H NMR spectroscopy. 4-ETBUT-NIT: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.4280 (dd, 1H, H-1, J = 5.04, 1.64), 2.7013 (dd, 1H, H-1, J = 6.12, 1.60), 2.8974 (dq, 2H, *H*-3, J = 12.96, 5.64), 3.1817 (m, 1H). MS (EI) m/z (%): 99(M^+, 100), 72 (26), 98 (8), 71 (7), 70 (5), 59 (3), 58 (3); 4,5-ETPENT-NIT: δ 1.7623 (m, 1H), 2.3608 (dd,