RESEARCH ARTICLE

Repeated oral administration modulates the pharmacokinetic behavior of the chemopreventive agent phenethyl isothiocyanate in rats

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The principal objective of this study was to evaluate whether repeated oral administration influences the pharmacokinetic behavior of the chemopreventive agent phenethyl isothiocyanate (PEITC) in rat. Animals were treated orally with 0.5, 1.0 and 5.0 mg/kg of the isothiocyanate for 4 days, and plasma levels at various times post-administration were determined by LC/MS after the first and last day. To determine absolute bioavailability, a group of animals was treated with a single (0.5 mg/kg) intravenous dose of PEITC. Following single oral dose administration, PEITC was rapidly absorbed, peak plasma concentrations being attained within the hour, and achieved an absolute bioavailability of 77%, but displayed dose-dependent pharmacokinetics, with bioavailability decreasing and clearance increasing moderately with dose; C_{max} values did not rise proportionately to the dose and volume of distribution increased. At the higher doses of 1.0 and 5.0 mg/kg, repeated administration led to higher PEITC plasma C_{max} concentrations and decreased plasma clearance of the isothiocyanate leading to enhanced bioavailability.

Keywords:
Chemoprevention / Isothiocyanates / Pharmacokinetics / Phenethyl isothiocyanate

1 Introduction

Strong epidemiological evidence has repeatedly linked consumption of cruciferous vegetables to lower cancer risk at a number of sites [1, 2]. Indeed, these vegetables can act as sources of anticarcinogenic chemicals; one such class of chemicals are the isothiocyanates, which are present in these vegetables in the form of glucosinolates, but are released following exposure to the enzyme myrosinase (β-thioglucoside glucohydrolase) that comes into contact with these compounds during the harvesting, chopping and mastication processes. The generation of isothiocyanates from their glucosinolate precursors is further facilitated by microbial myrosinase activity in the human intestine [3].

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Abbreviation: PEITC, phenethyl isothiocyanate

The anticarcinogenic activity of isothiocyanates appears to involve a number of distinct mechanisms. At the initiation stage of carcinogenesis, substantial experimental evidence has been published indicating the potential of these phytochemicals to perturb the enzyme systems involved in the metabolism of carcinogenic chemicals leading to reduced availability of their reactive metabolites that are responsible for their genotoxicity [4–6]. This is achieved through two different mechanisms; the first, and more important, involves stimulation of enzymes, such as glutathione S-transferase and quinone reductase that are involved in the detoxication of reactive intermediates, in tissues such as liver and lung and, to a lesser extent, by inhibiting cytochrome P450 activity leading to their decreased generation, thus protecting DNA from chemical insult [7–10]. At the post-initiation level, isothiocyanates may exert their chemopreventive action by inducing apoptosis, suppressing cellular proliferation and inhibiting histone deacetylases [11–13].

One such isothiocyanate is phenethyl isothiocyanate (PEITC), which is encountered in watercress, its most

important source, as the glucosinolate gluconasturtiin. In animal studies, PEITC antagonized effectively the carcinogenicity of chemicals in many tissues. It protected against carcinogenesis induced by nitroso compounds, azoxymethane and polycyclic aromatic hydrocarbons in many tissues [14–18]. Moreover, exposure to PEITC led to a decrease in DNA-adduct levels in the liver, colon and prostate of rats treated with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine [19]. Commensurate with these findings, PEITC was found to modulate carcinogen-metabolizing enzymes in a tissue-specific manner. For example, quinone reductase was markedly up-regulated in the liver but was refractive in the lung [5, 20, 21].

The principal objective of the present study was to evaluate the effect of repeated administration of dietary PEITC on its plasma levels and pharmacokinetic behavior in comparison with single-dose administration. Appreciation of the pharmacokinetic behavior of PEITC following intake of low doses would facilitate the extrapolation of *in vitro* findings to the *in vivo* situation, and enable science-based advice on the optimal consumption of these vegetables to be given to the consumer.

2 Materials and methods

PEITC [*CAS* 2257-09-02] and 2 M ammonia in isopropanol were purchased from Sigma (Poole, Dorset, UK). To serve as an internal standard, 1,1′, 2,2′-tetra-deutero-PEITC was chemically synthesized. To a solution of 200 mg (1.6 mmol) 1,1′, 2,2′-tetra-deutero phenylethylamine in 5 mL chloroform at 0°C, 5 mL 0.1 M NaOH solution was added followed by 184 mg (1.6 mmol) thiophosgene. The solution was stirred at 0°C for 20 min and the phases separated. The aqueous phase was extracted with chloroform (2 × 5 mL) and the combined organic phases dried over $MgSO_4$, filtered and the solvent removed in vacuum. The resulting oil was further purified by Kugelrohr distillation in vacuum to give 1,1′, 2,2′-tetra-deutero-PEITC as a yellow oil (201 mg, 70%). IR (liquid) [cm^{-1}] 2950, 2872 (CH), 2187, 2136, 2107, 2049 (NCS and CD); ^{1}H-NMR (500 MHz, $CDCl_3$) [ppm] 7.31(2H, t, $J = 8.1$ Hz, ArH), 7.25 (1H, t, $J = 8.0$ Hz, ArH), 7.19 (2H, d, $J = 8.1$ Hz, ArH); ^{2}H-NMR (76.5 MHz, $CDCl_3$) [ppm] 3.7 (s, br), 2.95 (s, br), ^{13}C-NMR (125 MHz, $CDCl_3$) [ppm] 136.9, 128.7, 127.1, 46.5 (q), 26.7 (q); MS (ESI, positive ion mode, $CHCl_3$/MeOH 10:1) 168 (M + H), 190 (M + Na).

Male Wistar albino rats (250 ± 10 g) were obtained from B&K Universal (Hull, East Yorkshire, UK). The animals were housed at 22 ± 2°C and 30–40% relative humidity in an alternating 12-h light:dark cycle with light onset at 07.00 h. Rats, four per group, received single daily administrations of PEITC, dissolved in 0.1% DMSO, by gastric intubation, at three dose levels, namely 0.5, 1.0 and 5.0 mg/kg for 4 days. Following the first and last intake of PEITC, blood samples (150 μL) were withdrawn from the rat tail at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 h following intake, and placed into lithium-heparinized centrifuge tubes. A sample was also obtained 24 h after administration as well as at 0 time, *i.e.* before administration of the isothiocyanate. Finally, to determine absolute bioavailability, one group of animals was treated through the tail vein with a single intravenous dose of PEITC (0.5 mg/kg), dissolved in 15% hydroxypropyl-β-cyclodextrin [22], and blood samples were withdrawn at the same time points post-administration.

2.1 Sample preparation

The extraction and ammonia derivatization procedures to generate the phenethyl thiourea were performed essentially as described by Ji and Morris [22]. Aliquots of the rat plasma (60 μL) were spiked with the deuterated PEITC (final concentration 1.5 μM). Plasma was extracted twice with heptane (200 μL), and to the combined extracts was added 2 M ammonia in isopropanol (1 mL). Following a 24-h incubation in a shaking water bath at room temperature, the mixture was dried down under a stream of nitrogen at room temperature and re-suspended in 60 μL of the HPLC mobile phase (40% ACN in water containing 0.1% formic acid); 10 μL was injected for analysis.

2.2 Determination of PEITC in rat plasma

Plasma concentrations of PEITC in the rat plasma were determined by LC-MS following conversion to phenethyl thiourea. Separation of the thiourea was achieved by HPLC employing a C18 reverse phase column (4 μm particle size, 150 × 2.1 mm) supplied by Phenomenex (Macclesfield, UK). Mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN), and the following gradient was adopted; 5% of solvent B increasing to 80% in 10 min, remaining at this level for 5 min, decreasing to 5% in the next 5 min; a flow rate of 0.2 mL/min was used. Under these conditions, the retention time of phenethyl thiourea and its deuterated analogue was about 15 min in a total run time of 25 min. At the PEITC retention time there were no interfering peaks from blank rat plasma extracts (results not shown).

Phenethyl thiourea was detected using a Micromass LCT^{TM} (Micromass UK, Manchester, UK) orthogonal acceleration TOF mass spectrometer (Applied Biosystems, Warrington, UK) equipped with an electrospray (ESI^+) probe, operating in the positive ionization mode. The analyte and internal standard were detected by monitoring the m/z 181 and 185 ions, respectively (Fig. 1), under the following conditions: capillary voltage, 3000 V; cone voltage, 20 V; source temperature, 100°C; desolvation temperature, 300°C; desolvation gas flow, 800 L/h, and cone gas flow of 80 L/h.

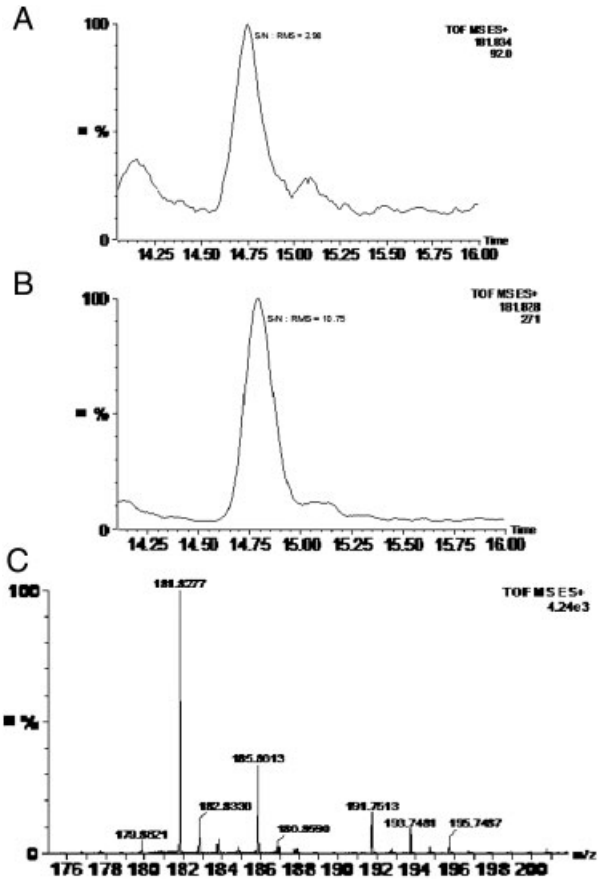

Figure 1. Determination of PEITC in rat plasma by LC/MS. (A) Representative extracted ion chromatogram of *m/z* 181 at the LOD (0.1 μM of PEITC in spiked rat plasma); (B) representative extracted ion chromatogram of *m/z* 181 at the limit of quantification (0.5 μM PEITC in spiked rat plasma); (C) mass spectrum under peak at 14.75 min; *m/z* 181, phenethyl thiourea (5 μM); *m/z* 185, deuterated PEITC derivative serving as an internal standard (1.5 μM).

2.3 Pharmacokinetic analysis

Pharmacokinetic analysis was carried out using PK solutions^{TM} software package (version 2.0, Summit Research Services, Ashland, OH, USA). Absolute bioavailability (F) was determined from the ratio of the oral to intravenous dose-normalized $AUC_{0-\infty}$ values. Apparent volume of distribution (V_d) was calculated from the equation $V_d = FD/AUC_{0-\infty}\ k_{el}$, where k_{el} is the elimination rate constant, F is the bioavailabilty and D the dose. Plasma clearance (Cl) was determined using the equation $Cl = FD/AUC_{0-\infty}$. For the calculation of the pharmacokinetic parameters, the F value for each animal was calculated from AUC_{oral}/AUC_{iv} and corrected for dose where appropriate. Finally, C_{max} and T_{max} were determined graphically from the plasma concentration *versus* time plots.

Animal data were analyzed individually and are presented as mean ± SD; statistical evaluation was performed using the Student's *t*-test, where $n = 4$.

3 Results

An LC-MS method was used for the determination of PEITC in rat plasma. No PEITC was detectable in rat plasma prior to administration of the isothiocyanate either following a single or repeated intake. LOD, defined as the lowest concentration of analyte that generates a minimum signal to noise ratio of 3, and limit of quantification, defined as the lowest concentration of analyte that gives rise to an instrument response with a minimum signal to noise ratio of 5, were 0.1 and 0.5 μM, respectively, following a 10-μL injection (Figs. 1A and B). The calibration curve, at a plasma concentration range of 0.5–15 μM showed excellent linear relationship, with an R value of 0.996. Recovery of PEITC at three plasma concentrations, 0.5, 2.5 and 7.5 μM, were 84 ± 2, 103 ± 3 and 97 ± 3%, respectively ($n = 6$). At concentrations of 0.5, 1.5 and 2.5 μM, inter-assay variation were 7.1, 4.0 and 3.4%, respectively, whereas intra-assay variation, established at the same concentrations, were 8.4, 7.6 and 4.6%, respectively ($n = 6$). Finally, accuracy was established by comparing calculated and theoretical values at four plasma concentrations (0.5, 1.0, 1.5 and 2.5 μM); calculated and theoretical values did not differ more than 15% ($n = 6$).

Figure 2 shows the time-course changes in the plasma concentrations of PEITC, plotted using a semi-logarithmic plot, following a single intravenous bolus administration to rats; the plasma profile is best described by an one-compartment pharmacokinetic model. Plasma levels declined rapidly with a half-life of 1.36 h (Table 1), and no PEITC was detectable in the 24-h samples. Similarly, following oral administration of the same dose, plasma kinetic profile was better described by a one-compartment model (Fig. 2). PEITC was rapidly absorbed, with maximal levels been attained within the hour.

The pharmacokinetic parameters of PEITC following single and repeated oral administration are shown in Table 1. The bioavailability of PEITC following single oral administration, at a dose level of 0.5 mg/kg, was 77%, but decreased with increasing dose, being only 23% at the highest dose studied of 5.0 mg/kg. The C_{max} and $AUC_{0-\infty}$ values in orally treated rats increased with dose, but not proportionately; rise in $AUC_{0-\infty}$ and C_{max} values was lower than would be anticipated. Plasma clearance values increased with dose, and similarly volume of distribution increased but statistical significance was not attained; half-life, and the absorption and elimination rate constants were unaffected by dose within the investigated range (Table 1).

Repeated oral administration of PEITC influenced the pharmacokinetic behavior of the isothiocyanate, but only at the higher two doses, namely 1.0 and 5.0 mg/kg (Fig. 2, Table 1). Although T_{max} values were unaffected, C_{max} values rose significantly, being nearly trebled at the 5.0 mg/kg dose level; biological half-life and plasma clearance values as well as volume of distribution decreased. Finally, $AUC_{0-\infty}$ increased leading to higher bioavailability.

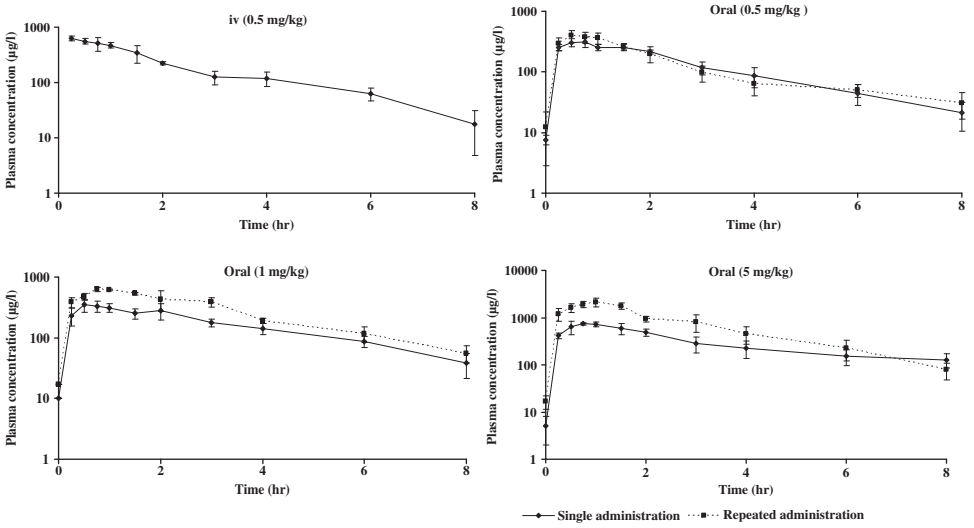

Figure 2. Plasma levels of PEITC in rats treated with single and repeated oral doses of PEITC. Rats were treated with single or repeated, for four days, oral doses, 0.5, 1.0 and 5.0 mg/kg, of PEITC, or a single intravenous dose (0.5 mg/kg). Results are presented as mean ± SD for four animals.

Table 1. Pharmacokinetic parameters of PEITC after single and repeated administration to rats

Parameter	Intravenous (0.5 mg/kg)	Oral 0.5 (mg/kg)		Oral (1.0 mg/kg)		Oral (5.0 mg/kg)	
		Single	Repeated	Single	Repeated	Single	Repeated
C_{max} (μg/L)	–	318 ± 62	404 ± 80	387 ± 73	647 ± 38**	769 ± 107^{+++}	2139 ± 444***
T_{max} (h)	–	0.73 ± 0.15	0.70 ± 0.24	0.45 ± 10^{+}	0.90 ± 0.12	0.78 ± 0.21	0.95 ± 0.10
$t_{1/2}$ (h)	1.36 ± 0.36	2.62 ± 1.37	2.55 ± 0.26	2.78 ± 0.36	1.86 ± 0.2**	3.19 ± 0.37	1.62 ± 0.44**
k_{el} (h^{-1})	0.54 ± 0.15	0.31 ± 0.12	0.27 ± 0.03	0.25 ± 0.03	0.33 ± 0.05**	0.22 ± 0.02	0.45 ± 0.12*
k_{ab} (h^{-1})	–	3.52 ± 1.58	4.26 ± 2.31	5.19 ± 2.14	2.26 ± 0.48	3.88 ± 0.40	3.02 ± 2.03
$AUC_{0-\infty}$ (μg L^{-1} h)	1346 ± 378	1033 ± 174	1117 ± 282	1646 ± 284^{++}	2311 ± 258**	2438 ± 462^{++}	5796 ± 1004***
V_d (L/kg)	0.75 ± 0.06	1.53 ± 0.81	1.50 ± 0.14	1.75 ± 0.29	1.05 ± 0.10**	2.15 ± 0.40	0.88 ± 0.28**
Cl (L h^{-1} kg^{-1})	0.40 ± 0.11	0.40 ± 0.01	0.41 ± 0.01	0.43 ± 0.02^{+}	0.40 ± 0.01*	0.46 ± 0.04^{+}	0.39 ± 0.01**
F (%)	100	77 ± 13	83 ± 21	61 ± 11	86 ± 10	23 ± 5^{++}	43 ± 7***

Results are presented as Mean ± SD for four animals. *$p<0.05$; **$p<0.01$; ***$p<0.001$ when compared with single dose administration. $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ when compared with the 0.5 mg/kg intravenous dose.

4 Discussion

PEITC is a naturally occurring isothiocyanate with chemopreventive activity, which in animal studies afforded protection against the tumourigenicity of established chemical carcinogens [14–18]. Many studies, largely conducted *in vitro*, have reported the ability of this isothiocyanate to modulate the initiation and post-initiation stages of carcinogenesis. However, for the outcome of these studies to be extrapolated to the human situation and be related to dietary levels of intake, it is desirable that the pharmacokinetic behavior of the isothiocyanate is appreciated. To our knowledge this is the first study investigating the effects of repeated intake of PEITC on plasma levels and pharmacokinetic parameters. In the present study, three dose levels were used, the lowest (0.5 mg/kg, 3 μmol/kg) representing human dietary intake, being equivalent to about 150 g of watercress, the principal source of PEITC [23].

The first studies concerned with the pharmacokinetics of PEITC employed a radiolabeled compound or measured total dithiocarbamates, so that plasma levels represent the sum of the parent compound and its metabolites [24]. Since metabolites of isothiocyanates can attain concentrations in the plasma higher than those of the parent compound [25], the plasma levels of total radioactivity or dithiocarbamates reflect mostly metabolites and can not be used to define the fate of the parent isothiocyanate. In subsequent studies, Ji *et al.* [26] determined the plasma levels of PEITC in rats using LC/MS/MS methodology that could discriminate between the parent compound and metabolites. However, in these studies only single doses were investigated, and the lowest oral dose employed was 10 μmol/kg, which is more than three times higher than the human dietary intake. In our studies, PEITC was rapidly absorbed following oral administration with peak levels attained within the hour, in agreement with previous studies [26]. PEITC achieved very good bioavailability of over 75%; it is likely that PEITC

undergoes modest first-pass metabolism as metabolites of isothiocyanates can be generated by intestinal as well as hepatic enzymes, or even possibly in the blood as it contains low concentrations of glutathione that can interact chemically with the isothiocyanate. Studies using human jejunum *in situ* have established that sulforaphane, an aliphatic isothiocyanate, is conjugated with glutathione in the enterocytes during absorption and secreted back into the lumen [27].

Similar to our studies with sulforaphane [28], dose-dependent pharmacokinetic behavior was evident even when the oral dose of PEITC was only doubled. The C_{max} and $AUC_{0-\infty}$ values in orally treated rats increased with dose, but not proportionately; rise in $AUC_{0-\infty}$ and C_{max} values was lower than would be anticipated. As a result, the bioavailability dropped markedly so that at the 5.0 mg/kg dose it was only about a third of what was observed at the 0.5 mg/kg dose. These observations imply that intake of isothiocyanate supplements containing PEITC may not be effective at raising the plasma concentrations of the compound. Since the absorption rate constant was not perturbed by the dose of PEITC, it is unlikely that absorption processes are responsible for the dose-dependent behavior. PEITC displays very high protein binding [26], and it is conceivable that at the higher doses protein-binding sites may be saturated so that the isothiocyanate remains free and can be eliminated by metabolism and excretion; the observation that the elimination rate constant and plasma clearance increase with dose would support such a mechanism. However, it has been reported that the protein binding of PEITC in rat serum remains constant over a wide range of concentrations [26]. The lowest concentration utilized in those studies, however, was 10 μM, which is considerably higher than the C_{max} levels achieved in the present study, being 2.0 and 4.7 μM at the 0.5 and 5 mg/kg doses, respectively. Since non-linear pharmacokinetics in rats has also been reported for sulforaphane, having an aliphatic substituent [28], it may be inferred that the isothiocyanate group is more likely to be responsible for this effect rather than the substituent.

A principal mechanism of the chemopreventive activity of isothiocyanates is enhanced detoxication of the genotoxic metabolites of carcinogens, and repeated intake would be required for maximal induction of enzymes like the glutathione S-transferases and quinone reductase to be achieved [5]. Indeed, suppression of the genotoxic effects of experimental carcinogens by isothiocyanates in animals was noted after their addition to the diet continuously for extended periods of time [19, 29, 30]. Consequently, it is important to understand the pharmacokinetic behavior of PEITC after repeated intake as this may differ from what occurs after single intake, *e.g.* as a result of modulation of enzyme systems involved in its metabolism. At doses of 1.0 and, in particular, 5.0 mg/kg, the pharmacokinetic profile of PEITC was altered following repeated intake; plasma levels and $AUC_{0-\infty}$ increased, leading to enhanced bioavailability. As PEITC is a mechanism-based inhibitor [5, 7, 8], the rise in plasma levels could be attributed to the decreased metabolic clearance of PEITC. Although isothiocyanates may be metabolized by cytochromes P450 [31], such metabolism is not a major route of isothiocyanate biotransformation. A plausible mechanism that may be, at least partly, responsible for the higher plasma levels is saturation of intracellular levels of PEITC on repeated exposure. This suggestion is consistent with the decrease in apparent volume of distribution with increasing dose. Isothiocyanates attain very high intracellular concentrations as a result of their interaction with glutathione and may reflect the catalytic efficiency of glutathione *S*-transferases toward the isothiocyanate [32, 33]. As the absorbed isothiocyanate conjugates with glutathione, the concentration gradient drives the further cellular uptake of the isothiocyanate, which can achieve millimolar concentrations, and is accompanied by a marked drop in glutathione levels. The glutathione and cysteinylglycine conjugates are exported through membrane transporters such as P-glycoprotein [34].

Comparison of the single dose plasma levels of PEITC with those attained by sulforaphane, in studies conducted at the same dose levels and rat strain [28], reveals important differences. The C_{max} values of PEITC are orders of magnitude higher compared with sulforaphane at the same dose levels. Most likely this reflects the fact that sulforaphane achieves higher intracellular concentrations compared with PEITC [32]. The much higher volume of distribution of sulforaphane, compared with PEITC, concords with such mechanism.

PEITC and erucin, the latter found primarily in rocket salad, are probably the most promising chemopreventive isothiocyanates as they are consumed largely uncooked, thus avoiding poor bioavailability due to loss of glucosinolates and deactivation of myrosinase that may occur during cooking. At least in the case of PEITC, repeated intake results in greater bioavailability.

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The authors have declared no conflict of interest.

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