Inhibition of rat liver cytochrome P450 isozymes by isothiocyanates and their conjugates: a structure–activity relationship study

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A series of arylalkyl and alkyl isothiocyanates, and their glutathione, cysteine, and N-acetylcysteine conjugates were used to study their inhibitory activity toward the dealkylation of ethoxyresorufin (EROD), pentoxyresorufin (PROD), and methoxyresorufin (MROD) in liver microsomes obtained from the 3-methylcholanthrene or phenobarbital-treated rats. These reactions are predominantly mediated by cytochrome P450 (P450) isozymes 1A1 and 1A2, 2B1 and 1A2, respectively. All isothiocyanates inhibited PROD more readily than EROD. Increases in the alkyl chain length of arylalkyl isothiocyanates to Cα resulted in an increased inhibitory potency in these assays; at longer alkyl chain lengths (Cα-Cβ) the inhibitory potency declined. The IC50s for phenethyl isothiocyanate (PEITC) were 47, 46 and 1.8 μM for EROD, MROD and PROD, respectively. Substitution of an additional phenyl group on PEITC also increased the inhibitory potency; the IC50s for 1,2-diphenylethyl isothiocyanate (1,2-DPEITC) and 2,2-diphenylethyl isothiocyanate (2,2-DPEITC) were 0.9 and 0.26 μM for EROD, and 0.045 and 0.13 μM for PROD, respectively. The relative inhibitory potency of PEITC and its conjugates was N-acetylcysteine-PEITC (PEITC-NAC) < glutathione-PEITC (PEITC-GSH) < cysteine-PEITC (PEITC-CYS) < PEITC. The observations that the parent isothiocyanates were more potent inhibitors than the conjugates suggest that dissociation of the conjugate is required for activity. Naturally occurring alkyl isothiocyanates, sulforaphane (SFO) and allyl isothiocyanate (AITC), were very weak inhibitors in the assays. These results suggest the potential of isothiocyanates as structural probes for studying P450 features of isothiocyanates required for inhibition of P450 isozymes and the role of their conjugates as inhibitors. The purpose of present studies was to elucidate structure–activity relationships for inhibition of 2B1, 1A1 and 1A2 activity, in vitro inhibition of 2B1, 1A1 and 1A2 in rat liver microsomes by isothiocyanates and their glutathione, cysteine conjugates, and N-acetylcysteine conjugates. Conjugates of isothiocyanates were studied because they are the major metabolites of parent isothiocyanates in vivo (2,16–18). Basic information was generated regarding the structural features of isothiocyanates required for inhibition of P450 isozymes and the role of their conjugates as inhibitors. The dealkylation assays of PROD, EROD and MROD were used to assess inhibition of 2B1, 1A1 and 1A2 activity, respectively.

Materials and methods

Chemicals

AITC, benzyl isothiocyanate (BITC) and PEITC of the highest purity available and tris(hydroxymethyl)methyl isothiocyanate with purity >99.9% were purchased from Aldrich Chemical Co. (Milwaukee, WI); 1,2-diphenylethyl isothiocyanate (1,2-DPEITC), 2,2-diphenylethyl isothiocyanate (2,2-DPEITC), 2-hexyl isothiocyanate (2-HITC), 1-hexyl isothiocyanate (HITC) and tert-octyl isothiocyanates and their conjugates were studied because they are the major metabolites of parent isothiocyanates in vivo (2,16–18). Basic information was generated regarding the structural features of isothiocyanates required for inhibition of P450 isozymes and the role of their conjugates as inhibitors. The dealkylation assays of PROD, EROD and MROD were used to assess inhibition of 2B1, 1A1 and 1A2 activity, respectively.

Introduction

Isothiocyanates are widely distributed as thioglycoside conjugates, i.e. glucosinolates, in cruciferous vegetables such as watercress, horseradish, cabbage, cauliflower, Brussels sprouts and radishes; the isothiocyanates are released upon hydrolysis of glucosinolates by the enzyme myrosinase (1,2). Isothiocyanates have been reported to inhibit tumorigenesis by environmental carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines (3–6).

Synthetic and naturally occurring isothiocyanates vary considerably with regard to their chemopreventive potency and target tissue, which in rats includes lung, mammary gland, esophagus, liver, small intestine, colon and bladder (7). Studies with a series of arylalkyl isothiocyanates used as inhibitors of lung tumorigenesis by the tobacco-specific nitrosamine (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) in A/J mice have demonstrated that increasing alkyl chain length up to six carbons increased the chemopreventive potency (4,8,9). Phenethyl isothiocyanate (PEITC) and its homologue 6-phenylethyl isothiocyanate (PHITC) were also potent inhibitors against NNK induced lung tumorigenesis in the F344 rat (10). The isothiocyanate group has been demonstrated to be essential for chemopreventive activity (11), and the potency has been correlated with low reactivity and high lipophilicity of isothiocyanates (12).

Studies of mechanisms have shown that isothiocyanates, when administered in vivo, are inhibitors of isozymes that metabolize carcinogens such as NNK in lung, nasal mucosa, and liver of rats and mice [13]. Isothiocyanates are also inducers of certain phase II enzymes such as β-nicotinamide adenine dinucleotide, reduced, tetrasodium salt (NAD(P)H):quinone oxidoreductase and glutathione S-transferase (14). Specific P450 isozymes identified that are inhibited by isothiocyanates include 2B1, 2A1, 1A1, 1A2 and 2E1 (15). The purpose of present studies was to elucidate structure–activity relationships for in vitro inhibition of 2B1, 1A1 and 1A2 in rat liver microsomes by isothiocyanates and their glutathione, cysteine conjugates and N-acetylcysteine conjugates. Conjugates of isothiocyanates were studied because they are the major metabolites of parent isothiocyanates in vivo (2,16–18). Basic information was generated regarding the structural features of isothiocyanates required for inhibition of P450 isozymes and the role of their conjugates as inhibitors. The dealkylation assays of PROD, EROD and MROD were used to assess inhibition of 2B1, 1A1 and 1A2, and 1A2 activity, respectively.

Abbreviations: AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; PBITC, 4-phenethyl isothiocyanate; PDITC, 10-phenyldeceyl isothiocyanate; PEITC, phenethyl isothiocyanate; PHTIC, 6-phenylethyl isothiocyanate; POITC, 8-phenylethyl isothiocyanate; 1,2-DPEITC, 1,2-diphenylethyl isothiocyanate; 2,2-DPEITC, 2,2-diphenylethyl isothiocyanate; HITC, 1-hexyl isothiocyanate; 2-HITC, 2-hexyl isothiocyanate; r-OITC, tertiary octyl isothiocyanate; SFO, (+)-1-isothiocyanate-4-methylsulfinylbutane (sulforaphane); P450 1A1 etc., cytochrome P450 1A1 etc.; GSH, glutathione conjugate; -CYS, cysteine conjugate; -NAC, N-acetylcysteine conjugate; NADPH, β-nicotinamide adenine dinucleotide, reduced, tetrasodium salt, 3-MC, 3-methylcholanthrene; NNK, (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone; PB, phenobarbital sodium.

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Table I. Activity of rat liver microsomal preparations

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Inhibitor</th>
<th>Activity, pmol/min/mg protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (saline)</td>
<td>-</td>
<td>EROD (1A1, 1A2) 164 ± 3, PROD (2B1) 73 ± 8, MROD (1A2) 49 ± 4</td>
</tr>
<tr>
<td>3-Methylcholanthrene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>12,664 ± 341, 88 ± 7, 634 ± 42</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>0.1 µM 7,8-benzo[alpha]fluorone</td>
<td>199 ± 15</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>1.0 µM 7,8-benzo[alpha]fluorone</td>
<td>62&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbital&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>416 ± 13, 4568 ± 32, 48 ± 8</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1.0 µM metapyrone</td>
<td>1368&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>10 µM metapyrone</td>
<td>177&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*All values with standard deviations: three determinations.
<sup>b</sup>20 mg/kg in corn oil, daily for 3 days, i.p.
<sup>c</sup>75 mg/kg in saline, daily for 3 days, i.p.
<sup>d</sup>One determination.

Fig. 1. Structures of isothiocyanates.

Cyanate (l-OTC) were purchased from Trans World Chemicals, Inc. (Rockville, MD). (−)-1-isothiocyanate-4-methylsulfinylbutane (sulforaphane) (SFO) was custom synthesized by LKT Laboratories, Inc. (St. Paul, MN). Cysteine, N-acetylcyesteine, glutathione conjugate (GSH), NADPH, 7-pentoxy-3H-phenoxazin-3-one (resorufin methyl ether), 7-hydroxy-3H-phenoxazin-3-one (resorufin), 7-ethoxy-3H-phenoxazin-3-one (resorufin pentyl ether), 7-ethoxy-3H-phenoxazin-3-one (resorufin ethyl ether), 7,8-benzoflavone, phenobarbital sodium (PB), 3-methylcholanthrene (3-MC) and rhodamine B were obtained from Sigma Chemical Co. (St. Louis, MO); 7-methoxy-3H-phenoxazin-3-one (resorufin methyl ether) was ordered from Molecular Probes, Inc. (Eugene, OR).

The 4-phenylbutyl isothiocyanate (PBITC), 6-phenylhexyl isothiocyanate (PHPTC), 8-phenyloctyl isothiocyanate (POITC) and 10-phenyldecyl isothiocyanate (PDITC) were previously synthesized and characterized in our laboratory (8,12). GSH conjugates of allyl isothiocyanate (AITC), BITC, PEITC, PBITC, PHPTC and 2,2-DPEITC were synthesized using published procedures and characterized previously (18,19). The purity of chemicals was established by reverse phase HPLC. The structures of the isothiocyanates used in this study are shown in Figure 1.

Treatment of animals

Male F344 rats, 230–241 g, purchased from Taconic Farms (Germantown, NY), were housed two per cage and fed NIH-07 diet (Dyes, Bethlehem, PA); rats were acclimated 1 week prior to treatment. Four rats were treated with 75 mg/kg PB in 5 ml/kg 0.9% saline, i.p., once per day for three consecutive days. Four other rats were administered 3-MC, 20 mg/kg in 5 ml/kg corn oil i.p. for 3 days. Four additional rats were treated with 5 ml/kg 0.9% saline, by gavage, as uninduced controls, and killed 20 h later by CO₂ asphyxiation. Twenty-four hours after the final dose of PB or 3-MC, the rats were killed with CO₂.

Enzyme assay studies

Liver microsomes were prepared according to a previously described method (20). Preliminary studies to delineate the time course of inhibition of PROD by PEITC, in the absence and presence of NADPH, were carried out in a manner similar to that reported earlier (21). Stock solutions of isothiocyanates and their conjugates in DMSO were prepared immediately before assays, and 10 µl of inhibitor was used in each 2 ml assay. Five to eight concentrations were used, each in triplicate; the inhibitor concentrations ranged from 0 to 100 µM to define isozyme activity versus inhibitor concentration curves for each compound and to determine the IC₅₀.

PROD activity was assayed using a fluorescence method (20,22). The reaction mixture consisted of 20 µg PB-induced rat liver microsomal protein in 1.96 ml 0.1 M Tris buffer pH 7.8, 2.5 mM MgCl₂ pre-incubated at 37°C. Exactly 1 min after addition of the inhibitor, 10 µl 2 mM resorufin pentyl ether in DMSO was added (final concentration 10 µM); the reaction mixture was immediately transferred to a quartz cuvette that was placed in a Perkin-Elmer model 650-40 fluorescence spectrophotometer maintained at 37°C. The baseline fluorescence was monitored (excitation wavelength: 230 nm; emission wavelength: 285 nm) until the reaction was initiated at 3 min by addition of 250 µM NADPH in 10 µl H₂O; the slope of the fluorescence increase was used to calculate enzyme activity. For quantitation purposes the reaction product resorufin, 0.1 µmol in 2 ml 0.1 M Tris buffer pH 7.8, was prepared. Rhodamine B in ethylene glycol, freshly diluted to 8 ng/ml with 0.1 M Tris buffer pH 7.8, was utilized as a secondary standard (23).

EROD activity was assayed in an identical manner as PROD, except that 10 µg microsomal protein from 3-MC-induced rat liver was used, and 10 µl 0.34 mM resorufin ethyl ether in DMSO (final concentration 1.7 µM) was used as substrate. MROD activity was assayed as above using 40 µg microsomal protein from 3-MC-induced rat liver and 10 µl 1.0 mM resorufin methyl ether in DMSO (assay final concentration 5 µM) as substrate (24). The IC₅₀s were determined graphically using curves of mean isozyme activity vs. initial inhibitor concentration.

Results and discussion

The inhibition of EROD by 0.1 and 1.0 µM 7,8-benzo[alpha]fluorone and inhibition of PROD by 1.0 and 10 µM metapyrone were first demonstrated to confirm the assays employed (Table I). The inhibition of PROD by PEITC was time dependent (Figure 2A). A sharp decrease in enzyme activity was seen immediately after adding PEITC. When the assay was initiated at varying times (0–30 min) after addition of the inhibitor, a steady reduction in enzyme activity was noted. If 250 µmol NADPH were added to the assay mixture prior to addition of substrate, an even faster reduction in PROD activity was observed (Figure 2B), suggesting that, in addition to direct inhibition...
Inhibition of rat liver cytochrome P450 isozymes

Fig. 2. Time course of inhibition of PROD by PEITC. Assay mix (20 μg microsomes from PB-induced rat liver, 2.5 mM Mg²⁺, 2 μM PEITC) pre-incubated for various times prior to initiation of enzyme assay (see Materials and methods). (A) Pre-incubation of assay mix with substrate (10 μM resorufin pentyether) prior to addition of NADPH. (B) Pre-incubation of assay mix with 250 μM NADPH prior to addition of substrate. Time points studied were 15 s, 3 min, 5 min, 10 min and 20 min. Zero time indicates enzyme activity in absence of 2 μM PEITC.

by PEITC, microsomal metabolism of PEITC forms an unidentified metabolite with inhibitory activity, possibly related to isocyanate formation (25). A slight time-dependent reduction in enzyme activity was also observed (~1.3%/min) when microsomes were incubated in buffer without inhibitor (data not shown). On the basis of these results, a 3-min pre-incubation period in the absence of NADPH was used as a standard condition for all inhibition assays.

The IC₅₀'s for 21 compounds were determined in the EROD and PROD assays (Table II). The MROD assay, which is specific for P450 1A2, was conducted to differentiate the relative inhibitory potency of isothiocyanates and their conjugates for P450 1A1 and P450 1A2. The activity of MROD in 3-MC induced microsomes was ~5% of the activity for EROD (Table I) and relative effects of inhibitors on the two isozymes could not be readily detected. IC₅₀'s for the four compounds tested in the MROD assay were quite similar to the IC₅₀'s in the EROD assay (Table II); hence, further MROD assays were not conducted.

Many of the isothiocyanates tested were also tested as corresponding GSH conjugates. The conjugates of PEITC, PEITC-GSH, PEITC-cysteine conjugate (CYS), and PEITC-N-acetylcysteine conjugate (NAC) were considerably less potent as inhibitors of both PROD and EROD when compared with PEITC itself. Conjugates of isothiocyanates are, however, generally less toxic and are readily absorbed upon oral administration; hence, conjugates may be the preferred forms for isothiocyanates as chemopreventive agents (26). Conjugates are dissociated in solution to free isothiocyanates and the respective thiol; the free isothiocyanate rather than the conjugate itself may be the actual inhibitor (11,20,27,28). The IC₅₀ value for the cysteine conjugate of PEITC most closely approached the value for the IC₅₀ of PEITC using both PROD and EROD. Of the three conjugates of PEITC, PEITC-CYS is the most readily dissociated at pH 7.4 (29).

### Table II. Inhibition of rat liver microsomal P-450 isozymes by isothiocyanates and conjugates

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀, μM</th>
<th>PROD</th>
<th>EROD</th>
<th>MROD</th>
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<tr>
<td>AITC</td>
<td>78</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>AITC-GSH</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
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<tr>
<td>SFO</td>
<td>68</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>BITC</td>
<td>5.0</td>
<td>54</td>
<td></td>
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<tr>
<td>BITC-GSH</td>
<td>19.2</td>
<td>62</td>
<td></td>
<td></td>
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<tr>
<td>PEITC</td>
<td>1.8</td>
<td>47</td>
<td></td>
<td>46.0</td>
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<tr>
<td>PEITC-GSH</td>
<td>13.45</td>
<td>&gt;100</td>
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<td></td>
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<tr>
<td>PEITC-CYS</td>
<td>4.8</td>
<td>53</td>
<td></td>
<td></td>
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<tr>
<td>PEITC-NAC</td>
<td>30</td>
<td>&gt;100</td>
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<tr>
<td>PBITC</td>
<td>0.17</td>
<td>17.5</td>
<td>14.3</td>
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<tr>
<td>PBITC-GSH</td>
<td>0.025</td>
<td>7.0</td>
<td>5.8</td>
<td></td>
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<tr>
<td>PHITC</td>
<td>0.085</td>
<td>55</td>
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<tr>
<td>PHITC-GSH</td>
<td>0.052</td>
<td>&gt;100</td>
<td></td>
<td></td>
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<tr>
<td>PDITC</td>
<td>0.17</td>
<td>&gt;100</td>
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<tr>
<td>1,2-DPEITC</td>
<td>0.045</td>
<td>0.9</td>
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<tr>
<td>2,2-DPEITC</td>
<td>0.13</td>
<td>0.26</td>
<td>0.75</td>
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<tr>
<td>2,2-DPEITC-GSH</td>
<td>0.8</td>
<td>2.8</td>
<td></td>
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<tr>
<td>HITC</td>
<td>0.75</td>
<td>45</td>
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<tr>
<td>2-HITC</td>
<td>4.4</td>
<td>83</td>
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<tr>
<td>I-OITC</td>
<td>0.06</td>
<td>58</td>
<td></td>
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</table>

*Microsomes from phenobarbital-treated rats.

bMicrosomes from 3-methylcholanthrene-treated rats.

cPoor solubility of POITC and PDITC at concentrations above 20 μM.
A clear correlation of the inhibitory potency for both PROD and EROD with chain length of the arylalkyl isothiocyanates was observed. The pattern extended from C₁ (BITC) up to C₆ (PHITC), and then the inhibitory potency declined with further increase of chain length (C₈–C₁₀) (Figure 3). In another study, inhibition of P450 2E1-catalysed N-nitrosodimethylamine demethylation by a series of primary alcohols increased with increasing alkyl chain length up to C₆–C₇, and then decreased with greater alkyl chain length; a maximum binding for secondary alcohols occurred at C₆ (30). It was proposed that the substrate access channel of the active site on the P450 2E1 isozyme permitted binding by the hydrophobic alkyl chain and allowed optimum binding for compounds with six or seven alkyl carbons. Increased inhibition of ethylmorphine demethylation (P450 2C12) by alkylbenzenes with increasing length of the hydrocarbon chain (C₆–C₈) has also been reported (31). An increase in potency with longer alkyl chain length has been previously reported when arylalkyl isothiocyanates (C₁–C₆) were assayed as competitive inhibitors of the metabolism of NNK using microsomes prepared from A/J mouse lung, F₃₄₄ rat lung and rat nasal mucosa (13).

The phenyl ring does not appear to be essential for inhibition of cytochrome P450 isozymes, since t-OITC, HITC and 2-HITC, which do not contain the phenyl ring, were relatively strong inhibitors of both PROD and EROD. Naturally occurring isothiocyanates AITC and SFO, however, were not strong inhibitors. 1,2-DPEITC, 2,2-DPEITC and 2,2-DPEITC-GSH, which possess an additional phenyl ring on the PEITC structure, were much stronger inhibitors than PEITC and PEITC-GSH, respectively, for both PROD and EROD. The potency of 1,2-DPEITC, 2,2-DPEITC and 2,2-DPEITC-GSH as inhibitors for EROD was among the highest for all the isothiocyanates examined in this study, suggesting that the active site on P450 1A1 is shaped to harbor compounds with multiple phenyl groups. The high substrate specificity of P450 1A1 for polycyclic aromatic hydrocarbons, halogenated polycyclics, and planar molecules supports such a model (32).

Previous tumor bioassays have shown that the arylalkyl isothiocyanates are potent inhibitors of NNK-induced lung tumorigenesis in the A/J mouse; the potency increased with increasing chain length up to C₆ (4,8,12). Furthermore, an increase in the alkyl chain length to C₈ or C₁₀ as in POITC and PDITC did not significantly improve the inhibitory activity as compared to its C₆ homologue PHITC (12). Introducing a phenyl ring to PEITC as in 1,2-DPEITC and 2,2-DPEITC enhanced its potency to that similar to PHITC. Both naturally occurring alkyl isothiocyanates, AITC and sulforaphane (F.-L.Chung and D.Jiao, unpublished), showed no inhibitory activity against NNK-induced lung tumorigenesis (12). The close agreement of inhibition toward PROD with tumor inhibitory activity supports the theory that P450 2B1 plays an important role in activation of NNK in the A/J mouse, and suggests that P450 2B1 is a key target for the chemopreventive action of isothiocyanates (15). These results also suggest the potential of the PROD assay as a convenient method for prescreening isothiocyanates as inhibitors of NNK-induced lung tumorigenesis.

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References


