The antibacterial properties of isothiocyanates

Virginie Dufour,¹ Martin Stahl² and Christine Baysse¹

¹Equipe EA1254, Microbiologie Risques Infectieux, University of Rennes 1, F-35042 Rennes cedex, France
²Division of Gastroenterology, BC’s Children’s Hospital, Child and Family Research Institute and University of British Columbia, Vancouver, BC, Canada

Isothiocyanates (ITCs) are natural plant products generated by the enzymic hydrolysis of glucosinolates found in Brassicaceae vegetables. These natural sulfur compounds and their dithiocarbamate conjugates have been previously evaluated for their anti-cancerous properties. Their antimicrobial properties have been previously studied as well, mainly for food preservation and plant pathogen control. Recently, several revelations concerning the mode of action of ITCs in prokaryotes have emerged. This review addresses these new studies and proposes a model to summarize the current knowledge and hypotheses for the antibacterial effect of ITCs and whether they may provide the basis for the design of novel antibiotics.

Introduction

The interest in natural plant products as antimicrobials is supported by the necessity to reduce the use of conventional antibiotics in food preservation and to overcome the emergence of antibiotic resistance in bacterial pathogens. Isothiocyanates (ITCs; R–N\textsubscript{3}C\textsubscript{5}=S) are the products of the reaction of plant glucosinolates with myrosinase, an enzyme released by the disruption of plant tissues. This myrosinase–glucosinolate system is present in plants of the family Brassicaceae, such as cauliflower, broccoli, mustard and cabbage. ITCs are volatile substances that display an inhibitory effect on a variety of pathogenic microorganisms at low concentrations, making them promising antimicrobial candidates. The use of allyl ITC (AITC) is already approved in Japan for food preservation, provided it has a natural plant source (Nadarajah et al., 2005), but little information exists about the antibacterial effects of the other ITCs. ITCs can also be found in food with added condiments made from Brassicaceae vegetables, usually mustard, horseradish or wasabi (Delaquis & Sholberg, 1997). Additionally, the properties of ITCs as antibacterial, anti-fungal and anticancer compounds have increased their interest as food supplements (for reviews, see Singh & Singh, 2012; Zhang, 2012). This review intends to summarize the current knowledge about the cellular targets of ITCs, with particular focus on bacterial targets, the potential bacterial resistance to ITCs and the related research perspectives.

Sources of ITCs

Sulfur metabolism in plants is a source of natural products able to affect human health. Amongst these products, the ITCs are strongly electrophilic and are used to modify both thiols and amines. ITCs are known to have a role in protecting plants against both pest and microbial infection (Bending & Lincoln, 2000; Brown et al., 1997; Fahey et al., 2001). Glucosinolates themselves are not known to be toxic; however, the products of their degradation by the myrosinase (thioglucosidase) enzyme, which cleaves the sulfur–glucose bond, creates toxic ITCs. Myrosinase was discovered in the fungus Aspergillus sydowi (Öhtsuru et al., 1969). The first bacterial myrosinase enzyme was found in Enterobacter cloacae (Tani et al., 1974a, b). It has also been found in bacteria associated with mammal gut microflora (Fahey et al., 2012) and in crucivorous aphids (MacGibbon & Beuzenberg, 1978). In plants, myrosinase is known to be a cytosolic enzyme; however, the cellular organization of the myrosinase–glucosinolate system remains unclear. Myrosinase and glucosinolate are kept separate to prevent the production of toxic compounds in an intact plant. In Arabidopsis thaliana, high glucosinolate concentrations are found in specialized, sulfur-rich cells located between the phloem and the endoderm. Myrosinase is located in the adjacent phloem parenchyma (Koroleva et al., 2010). Upon disruption of the tissues by chopping or chewing, glucosinolate and myrosinase meet and the degradation process starts. Thioester glucosinolates are converted to reactive ITCs, as well as thiocyanates, nitrates, epithionitrides and oxazolidine 2-thione (Dinkova-Kostova & Kostov, 2012; Fahey et al., 2001) (Fig. 1).

The family Brassicaceae, which includes more than 350 genera and 3000 species, is able to synthesize glucosinolates. There are over 120 known glucosinolates (Agerbirk & Olsen, 2008).

Abbreviations: BSH, bacillithiol; ER, erucin; GSH, glutathione; GST, glutathione S-transferase; HSL, homoserine lactone; ITC, isothiocyanate; AITC, allyl ITC; BITC, benzyl ITC; PEITC, 2-phenylethyl ITC; SFN, sulforaphane.
which are both stable and water soluble. The sulfur-linked β-D-glucopyranose structure is invariant (Fig. 1), whereas side chains differ since they are derived from different amino acids (Table 1). These differences confer on the resulting ITC rather different biological properties due to their specific side chain structure. These variations depend on the speed at which ITCs penetrate the cell, and the persistence and accumulation of each ITC (Ye & Zhang, 2001; Zhang & Talalay, 1998). Table 1 presents the major ITCs that are cited in this review and the originating glucosinolates (Fahey et al., 2001; Nugon-Baudon & Rabot, 1994). Glucosinolate side chains contain a variety of chemical structures. The most intensively studied glucosinolates are the aliphatic, aromatic, heterocyclic and methylthioalkyl variants, mostly found in *Brassica*. Many ITCs, such as the z-methylsulfonylbutyl ITC, named erysolin, have also been chemically synthesized in *vitro* for several decades (Kjaer & Conti, 1954).

**Chemistry of ITCs**

The reactivity of ITCs can lead to random modifications of proteins *in vivo*, which can disturb biochemical processes. ITCs readily attack thiols and amines, but ITC activity is mainly linked to their reactivity with sulphydryl groups. The carbon atom of the ITC group (−N=C=S) is highly electrophilic and reacts with amines, thiols and hydroxyls to generate thiourea, thioicarbamates and carbamates respectively (Fig. 1). ITCs react with z-amino groups in N-terminal residues and/or amino groups of lysine through alkylation at a rate at least 1000 times less than that of thiocarbamation (Podhradsky et al., 1979). Cysteine, when highly conserved in proteins, plays a crucial role in protein structure and regulatory function owing to the ability of thiol groups to stabilize protein structure with covalent disulfide bonds, to coordinate metal ions, and to confer redox properties. Therefore, ITC-thiol conjugates may have pleiotropic effects. For example, ITCs are known to inhibit
When the target is the small thiol glutathione (GSH, \(\gamma\)-L-glutamyl-L-cysteinyl-L-glycine). The reactivity of specific amines and thiols in target proteins also depends on their \(p_K_a\) values, which are affected by surrounding amino acid residues (Podhradsky et al., 1979; Snyder et al., 1981). Not all cysteine residues are reactive toward electrophiles. The \(p_K_a\) value, itself affected by electrostatic interactions with nearby residues, directs the protonation state of cysteine and thus its reactivity toward ITCs. The hydrophobicity of the environment and steric hindrance can also modulate the reactivity of nucleophile residues (Nagahara et al., 2009). Since thiol and amino groups react in their dissociated form, higher pH values favour the interactions with ITC (Drobnica & Sturdik, 1979).

The conjugation of ITCs with thiols is reversible (Jiao et al., 1996). The decomposition rates of ITC conjugates seem to influence their efficacy, as demonstrated by their activity towards the cytochrome P450 enzyme (Conaway et al., 2001). Although the thiocarbamyl linkage is unstable, the thiol-ITC product, a dithiocarbamate, can react with adjacent amines to yield a more stable thiourea (Nakamura et al., 2009). Dithiocarbamate can also react with water without forming a covalent adduct (Hanschen et al., 2012) (Fig. 2). This feature of ITC to spontaneously bind thiols or amine groups has already been explored in order to create molecular research tools such as an ITC-fluorophore (fluorescein ITC) that react with thiol groups of proteins or amino groups of oligonucleotides for labelling experiments. Phenyl ITC and its derivatives have already been employed for protein sequencing via the Edman reaction, owing to their reactivity with the N-terminal amine (Jin et al., 1986).

Conjugation with GSH is a major metabolic route of ITC accumulation and detoxification in both rodents and humans. GSH is the most abundant non-protein thiol in many organisms, being present in almost all eukaryotes but only in a small number of prokaryotes, such as Cyano- bacteria, Proteobacteria and a few Gram-positive strains (Fahey & Sundquist, 1991; Newton et al., 1996). The thiol group gives GSH its biological activity, while the \(\gamma\)-linkage between glutamic acid and cysteine prevents the action of most proteases. Only the peptidase \(\gamma\)-glutamyl transpeptidase (GGT) is known to be able to hydrolyse GSH. GGTs are widely distributed from bacteria to mammals (Chikhi et al., 1999; Tate & Meister, 1981). Small thiols such as GSH are antioxidants and scavengers of reactive electrophiles; ITCs may impair various detoxification processes by sequestering these small thiols. ITCs in humans are excreted as S-(N-acetyl)cysteine conjugates in urine (Jiao et al., 1994). These mercapturates are the degradation products of ITC-GSH conjugates via the mercapturic acid pathway. GST enzymes are involved in the first step of this process (Kolm et al., 1995; Meyer et al., 1995). GSTs catalyse the conjugation of GSH to the electrophilic centres of potentially harmful compounds, including ITCs. The resulting conjugates are more water soluble and can be further metabolized and excreted. Mammalian GSTs are grouped in eight classes based on primary structure similarities. Prokaryotic GSTs are less well characterized (Sheehan et al., 2001).

**Antibacterial properties of ITCs**

**MIC against various pathogens**

ITCs exhibit biocidal activities against various bacterial pathogens. In 1995, Brabban and Edwards reported that

**Table 1. Structures of some glucosinolates and names of glucosinolates and related ITC**

<table>
<thead>
<tr>
<th>Side chain R*</th>
<th>Side chain name</th>
<th>Trivial name</th>
<th>Isothiocyanate</th>
</tr>
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<tbody>
<tr>
<td>CH₃-</td>
<td>Methyl</td>
<td>Glucocapparin</td>
<td>Methyl ITC</td>
</tr>
<tr>
<td>CH₃-CH₂-</td>
<td>Ethyl</td>
<td>Glucoepidin</td>
<td>Ethyl ITC</td>
</tr>
<tr>
<td>CH₃-CH₂-CH(CH₃)-</td>
<td>1-Methylpropyl</td>
<td>Glucocochlearin</td>
<td>Methylylpropyl ITC</td>
</tr>
<tr>
<td>CH₃=CH-CH₂-</td>
<td>Prop-2-enyl</td>
<td>Sinigrin</td>
<td>Allyl ITC</td>
</tr>
<tr>
<td>CH₃-S-(CH₂)₄-</td>
<td>4-Methylthiobutyl</td>
<td>Glucoerucin</td>
<td>Eru cin</td>
</tr>
<tr>
<td>CH₃-SO-(CH₂)₄-</td>
<td>3-Methylsulfinylpropyl</td>
<td>Glucoiberin</td>
<td>Iberin</td>
</tr>
<tr>
<td>CH₃-SO-(CH₂)₅-</td>
<td>3-Methylsulfinylbutyl</td>
<td>Glucoraphanin</td>
<td>Sulforaphane</td>
</tr>
<tr>
<td>C₆H₅-CH₂-</td>
<td>Benzyl</td>
<td>Glucopaeolin</td>
<td>Benzyl ITC</td>
</tr>
<tr>
<td>C₆H₅-(CH₂)₂-</td>
<td>2-Phenylethyl</td>
<td>Gluconasturtii</td>
<td>Phenyylethyl ITC</td>
</tr>
</tbody>
</table>

*for the general structure of glucosinolates, see Fig. 2.

**Fig. 2. Structure of glucosinolates.**
only the hydrolysis products of the glucosinolate sinigrin displayed an effect upon the growth of micro-organisms (Brabban & Edwards, 1995). Several years later, the volatile ITC components of two Brassicaceae plant extracts were identified for their activity against yeasts, bacteria and fungi (Hashem & Saleh, 1999). There is now ample evidence for the antimicrobial properties of ITCs (Aires et al., 2009a, b), but most reports of the suppression of bacterial growth by ITCs are still limited to MIC determination. However, MIC values are not a biological constant and are greatly influenced by the method used. Therefore the published MIC values of ITCs (some examples are displayed in Table 2) cannot be compared since they were not measured under well standardized conditions. There is no general rule that arises concerning the efficacy of ITCs toward various bacteria; however, the aromatic and/or hydroxy ITCs seem to consistently display a higher antimicrobial activity than aliphatic ones between studies (Table 2). The effect is dose-dependent, and both bacteriostatic and bactericidal effects have been described. More importantly, the inhibitory concentrations of active ITCs are in ranges equivalent to or below those of conventional antibiotics (Aires et al., 2009b). Some ITCs display a synergy with conventional antibiotics: it was demonstrated that 2-(4-hydroxyphenyl)ethyl ITC displayed antimicrobial synergism with aminoglycosides such as streptomycin, against E. coli and Staphylococcus aureus grown in glucose-containing medium (Tajima et al., 2001). However, small changes in the concentrations of both ITC and streptomycin affect their combined action from synergism to suppression of antimicrobial activity. The mechanism of synergism and suppression remains unclear (Tajima et al., 2003). AITC was effective at reducing the MIC of erythromycin against Streptococcus pyogenes (Palaniappan & Holley, 2010), and both AITC and PEITC displayed a synergy with streptomycin against Gram-negative bacteria such as E. coli and Pseudomonas aeruginosa (Saavedra et al., 2010). PEITC was tested against 11 isolates of E. coli with extended β-lactamases, in combination with the aminoglycoside gentamicin. A significant additive effect between PEITC and gentamicin was observed for most of the isolates (Freitas et al., 2013). Therefore, not only do ITCs display an antimicrobial activity, but also they may work synergistically with antibiotics, potentially allowing for reduced doses achieving the same potency, but with reduced chance for the development of bacterial resistance.

Food preservation and control of soil-borne disease

Antimicrobial food packaging aims to reduce, inhibit or retard the growth of micro-organisms on food products. AITC from mustard seeds is used both as a flavour compound and as an antimicrobial. AITC is responsible for the pungent taste of mustard, horseradish and wasabi and it is sometimes added to some prepared vegetable meals to enhance the flavour. This pungent volatile compound has been used as a natural food preservative since the late 20th century in Japan (Delaquis & Mazza, 1995; Nadarajah et al., 2005). AITC vapour has been found to be more effective than liquid AITC (Shin et al., 2010), but its strong odour can limit its use in food systems. The use of AITC as a flavouring substance has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by the EFSA (European Food Safety Authority) Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC). This report concluded that there were no safety concerns from AITC consumption at the estimated levels of intake (EFSA, 2010). Moreover, evaluations have been conducted of the use of 4-hydroxybenzyl ITC, which is found in white mustard essential oil (WMEO), derived from white mustard seeds (Sinapis alba L.), and is obtained by the hydrolysis of the glucosinolate sinalbin by myrosinase. WMEO was found to reduce the number of Salmonella recovered from inoculated frozen vegetables and chicken particulates in a dose-dependent manner (David et al., 2013).

Methyl ITC has proven its ability to reduce soil-borne disease in agriculture systems. However, similar to other fumigants such as methyl bromide, it is toxic and may leave chemical residues in products. The use of glucosinolates, the precursors to ITC, was investigated in search of alternative processes (Rosa & Rodriguez, 1999). The role of glucosinolates and their breakdown products in the selection of the natural rhizosphere community was evaluated in Arabidopsis thaliana (Bressan et al., 2009) using a transgenic Ara. thaliana that overexpressed p-hydroxybenzyl glucosinolate (gene from sorghum). The metabolites can be released via root exudation, as shown in Brassica rapus (Choesin & Boerner, 1991) and in mustard (Schreiner & Koide, 1993). The authors hypothesized that glucosinolates and their hydrolysis products diffused from the roots into the rhizosphere, where they are degraded by extracellular myrosinases as described by Borek et al. (1996). In the rhizosphere of the transgenic plant, the proteobacteria and fungal community were significantly affected, with significant differences detected with the heavy DNA profiles for α-Proteobacteria, γ-Proteobacteria and fungal communities in rhizospheric soil (Bressan et al., 2009). Modification of the glucosinolate content of the plant could be an alternative to the use of pesticides, although no ITCs were experimentally detected because of their low concentrations and rapid turnover.

Arabidopsis mutants with a reduced aliphatic glucosinolate content [reduction of 60-fold of 4-methylsulfinylbutyl glucosinolate, the precursor of sulforaphane (SFN)] showed an increased susceptibility to Fusarium oxysporum, whereas the resistance toward other pathogens, such as Alternaria brassicicola, Erwinia carotovora, Pseudomonas syringae and Botrytis cinerea, was unchanged compared with isogenic WT plants (Tierens et al., 2001).

Mode of action of ITCs on prokaryotic cells

Effect on membranes

The antimicrobial mechanisms of ITCs are not well understood. The ability of ITCs to bind thiol groups of proteins...
or thiol peptides such as GSH was largely demonstrated in vitro, whereas the physiological responses of bacteria to ITC challenges have been poorly investigated.

Lin and collaborators have compared the responses of E. coli O157 : H7, Salmonella Montevideo and Listeria monocytogenes to AITC, with those to conventional antibiotics with known targets, such as polymixin B, streptomycin and penicillin, which affect the cell membrane, ribosomes and the cell wall, respectively. AITC displayed bactericidal activity at all growth stages, and when used as a vapour, seemed to provoke the leakage of cellular metabolites, similar to the effect of polymixin B (Lin et al., 2000). However, the exact mechanism behind these effects was not found. The hypothesis that AITC damages the cell wall was countered by further studies of Ahn and collaborators, who found that when L. monocytogenes was treated with AITC, neither leakage of ATP nor damage to the cell wall was observed (Ahn et al., 2001). Nevertheless, the treatment induced altered internal structures as observed by electron microscopy. In a similar way, in the presence of essential oil from Salvadora persica, which contains 98 % benzyl ITC (BITC), or pure BITC, small protrusions were observed by electron microscopy in Aggregatibacter actinomycetemcomitans HK1519 after only 2 min exposure. These protrusions increased with the incubation time until a loss of membrane integrity occurred (Sofrata et al., 2011). The authors hypothesized that BITC, having both lipophilic and electrophilic properties, might penetrate through the outer bacterial membrane and hamper the ability of the bacterium to maintain its membrane potential, similar to the effect observed with cationic peptides (Sofrata et al., 2011).

### Inhibition of enzymic or regulatory (quorum sensing, QS) activities

AITC was reported to inhibit specific enzymic activity in E. coli, interfering with the function of both thioredoxin reductase and acetate kinase (Luciano & Holley, 2009). The enzymic inhibition was monitored in vitro, using purified enzymes. In Helicobacter pylori (a gastric pathogen), SFN inhibits urease activity, which generates ammonia and thus neutralizes gastric acidity and promotes inflammation (Fahey et al., 2013). Recently, a number of sulfur-containing compounds have been employed as a new class of QS inhibitors, including ajoene from garlic extract (with disulfide and sulfanyl groups) (Jakobsen et al., 2012b) and sulfur-containing molecules that were found to inhibit the AI-2 QS system of Vibrio harveyi (Li et al., 2008; Peng et al., 2009). Similarly, ITCs have also been found to display QS-inhibiting activity.

The first whole transcriptomic assay on a bacterium with ITC was carried out by Jakobsen and collaborators. In this study, 69 common food products and plant extracts were analysed for their ability to inhibit the QS system of Ps. aeruginosa. Ps. aeruginosa produces two N-acylhomoserine lactones (N-acetyl-HSLs) as QS signalling molecules: 3-oxo-C12-HSL, recognized by the receptor LasR, and C4-HSL, the ligand of the receptor RhlR (Jakobsen et al., 2012a). The QS regulons of both RhlR and LasR have been identified (Jimenez et al., 2012). Amongst the 69 products tested, horseradish (Armoracia rusticana) extract displayed the better inhibitory activity, based on the repression of both the lasB-gfp and rhlA-gfp transcriptional fusions. Horseradish belongs to the family Brassicaceae and the main component of horseradish oil is allyl ITC (AITC) (78–94.4 %) (Park et al., 2006; Yu et al., 2001). A recently improved HPLC method identified AITC and PEITC as the two main horseradish ITCs, whereas iberin and SFN were at a concentration below the detection limit. Iberin and SFN were, however, detected in both cauliflower and cabbage (Wilson et al., 2012). Iberin (3-methylsulfinylpropyl ITC) is an analogue of SFN, differing only in a shorter lateral chain (Table 1). Both ITCs possess a sulfanyl lateral group. Interestingly, a sulfanyl lateral group is also present in the QS-inhibiting compound ajoene. Despite being a minor ITC, iberin was purified and identified as the QS-inhibiting compound within the different horseradish extract fractions tested by HPLC. Pure iberin was used to confirm the QS-inhibiting activity and to perform the transcriptomic analysis at several subinhibitory concentrations that did impact growth or cell viability. The expression of 64 Ps. aeruginosa genes was downregulated by sublethal concentrations of pure iberin, including 49 QS-regulated genes. These data confirmed an antagonist interaction between the QS system and iberin in Ps. aeruginosa, although not all QS-regulated genes were affected at the transcriptional level. It is interesting to note that the subinhibitory concentrations of iberin did not decrease the expression of the las reporter, whereas the rhl-lux reporter transcription was decreased.

SFN and its deoxy analogue erucin (ER) were also tested for their ability to inhibit the QS system in Ps. aeruginosa (Ganin et al., 2013). ER is a precursor of SFN but does not contain the sulfanyl group found in SFN, iberin and ajone (Table 1). The ITC ER is formed both from the enzymic hydrolysis of glucoerucin (Table 1), a glucosinolate found at high levels in rocket species of the family Brassicaceae (Eruca sativa), and from the in vivo reduction of SFN (Kassahun et al., 1997), derived from broccoli and the action of myrosinase on glucoraphanin from Brassica oleracea. ER possesses anticancer properties by the modulation of phase I, II and III detoxification, the induction of apoptosis and cell cycle arrest, the induction of oxidative stress and the regulation of androgen receptor pathways (for review see Melchini & Traka, 2010). The QS-inhibiting ability of ER and SFN was assayed using a reporter strain of Ps. aeruginosa containing luminescent transcriptional fusions activated by either 3-oxo-C12-HSL or C4-HSL, the two signalling molecules of Ps. aeruginosa, via their binding to their specific intracellular receptors, LasR and RhlR respectively. ER and SFN efficiently competed with 3-oxo-C12-HSL to inhibit in a concentration-dependent manner the activation of LasR-specific reporter fusions, whereas no antagonism of C4-HSL upon the RhlR specific reporter system was observed. The
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<th>Main result</th>
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<tbody>
<tr>
<td>Phenethyl</td>
<td>Intestinal bacteria: <em>Bifidobacterium bifidum</em> ATCC 29521, <em>Bif. breve</em> ATCC 15700, <em>Clostridium difficile</em> ATCC 9689, <em>Cl. perfringens</em> ATCC 13124, <em>E. coli</em> ATCC 11775, <em>Lactobacillus acidophilus</em> ATCC 4356, <em>Lb. casei</em> ATCC 27216</td>
<td>Paper disc agar diffusion method: 0.5–5 mg ITC per disc; EG agar medium</td>
<td>Diameter inhibition zone</td>
<td>Phenethyl, benzyl, benzoyl ITC only inhibited harmful <em>Cl. difficile, Cl. perfringens, E. coli</em></td>
<td>Kim &amp; Lee (2009)</td>
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<td>(extracted from seed of <em>Sinapis alba</em> L., white mustard), acetyl, allyl, benzyl, butyl, ethyl, methyl</td>
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<td>Allyl, benzyl, 2-phenylethyl, SFN</td>
<td>Human faecal material isolates: <em>Enterococcus faecalis</em>, <em>Staphylococcus aureus</em>, <em>Acinetobacter baumanii</em>, <em>Citrobacter freundii</em>, <em>Enterobacter asburiae</em>, <em>E. cloacae</em>, <em>E. hormaechei</em>, <em>E. coli</em> (two strains), <em>Hafnia alvei</em>, <em>Klebsiella oxytoca</em>, <em>K. pneumoniae</em>, <em>Morganella morganii</em>, <em>Proteus mirabilis</em>, <em>Pseudomonas aeruginosa</em>, <em>Salmonella typhi</em>, <em>Stenotrophomonas maltophilia</em></td>
<td>Paper disc agar diffusion method: 15 μl of 0.015, 0.15, 0.75 or 3 μmol ITC per disc; Mueller–Hinton agar</td>
<td>Diameter inhibition zone; percentage of relative inhibition zone diameter with control antibiotics</td>
<td>SFN, BITC: highest antimicrobial activities against Gram+ and Gram−; PEITC: high inhibitory activity against Gram+</td>
<td>Aires <em>et al.</em> (2009b)</td>
</tr>
<tr>
<td>3-Butenyl, 4-pentenyl, 2-phenylethyl, benzyl</td>
<td><em>Bacillus cereus</em>, <em>Bac. subtilis</em>, <em>Listeria monocytogenes</em>, <em>Staph. aureus</em>, <em>Aeromonas hydrophila</em>, <em>Ps. aeruginosa</em>, <em>Salmonella choleraesuis</em>, <em>Salm. enterica</em>, <em>Serratia marcescens</em>, <em>Shigella sonnei</em>, <em>Vibrio parahaemolyticus</em></td>
<td>Paper disc agar diffusion method: 1 μl per disc ITC at 0.05–2 μl ml⁻¹; 2-layer plate nutrient agar</td>
<td>Diameter inhibition zone</td>
<td>2-Phenylethyl and benzyl ITC (aromatic): higher antibacterial activities than 3-butenyl and 4-pentenyl ITCs (aliphatic)</td>
<td>Jang <em>et al.</em> (2010)</td>
</tr>
<tr>
<td>Essential oil of <em>Salvadora persica</em> root containing 73.8 % of benzyl ITC</td>
<td>Oral periodontal Gram− pathogens: <em>Aggregibacter actinomycetemcomitans</em> HK 1519, <em>Porphyromonas gingivalis</em> ATCC 33277, <em>Oral Gram + pathogen: Streptococcus mutans</em> (CCUG 27624)</td>
<td>Viable colony counting; bacteria incubated 90 min with 0.1–1 % (v/v) essential oil in species-specific medium (BHI, MRS, TSB or LB); spreading onto same agar medium</td>
<td>C.f.u. determination</td>
<td>BITC-rich essential oil of <em>Salva. persica</em>: dose-dependent high antibacterial activity against Gram− bacteria; no or limited growth inhibition for Gram+ bacteria</td>
<td>Sofrata <em>et al.</em> (2011)</td>
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Table 2. cont.

<table>
<thead>
<tr>
<th>ITC</th>
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<th>Methods</th>
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<tr>
<td>SFN</td>
<td>Three reference strains (26695, J99, ATCC 43504), 45 clinical isolates of <em>Helicobacter pylori</em></td>
<td>Agar dilution method: NCCLS* procedures; Time-to-kill assay: <em>Brucella</em> broth, incubation with SFN at 0.25, 0.5, 1, 5 x MIC, 0, 2, 4, 6, 8, 24 h at 37 °C, microaerophilic conditions; c.f.u. determination</td>
<td>MIC defined as lowest concn each compound that resulted in no visible growth after 3 days incubation at 37 °C under microaerophilic conditions; bacterial activity defined as &gt;1000-fold reduction in viable c.f.u.</td>
<td>MIC 0.06–8 μg ml⁻¹ (mean 2.5 μg ml⁻¹, median 2 μg ml⁻¹); MIC90 4 μg ml⁻¹; for strain LBN20 1:1000-fold reduction within 24 h exposure to 5 x MIC SFN</td>
<td>Fahey et al. (2002)</td>
</tr>
<tr>
<td>2-Phenylethyl</td>
<td>Food-poisoning bacterial strains: <em>V. parahaemolyticus</em> KCCM 11965, <em>Salmonella choleraesuis</em> KCCM 40050, <em>Staph. aureus</em> KCCM 40935, <em>Bac. cereus</em> KCCM 41034</td>
<td>Paper disc agar diffusion method: 30 μl per disc ITC at 100, 200, 1000, 2000 μg ml⁻¹; 2-layer plate nutrient agar; liquid culture in nutrient broth plus ITC; OD600 at 12 h intervals for 72 h</td>
<td>Diameter inhibition zone; MIC defined as lowest concn at which growth of strains cannot be detected by measurement of absorbance</td>
<td>Strong activity (i.z. &gt;20 mm) against <em>V. parahaemolyticus</em> and <em>Staph. aureus</em>; clear activity (i.z. 10–15 mm) against <em>Bac. cereus</em> at 1000 μg l⁻¹; MIC 500 μg ml⁻¹ for <em>V. parahaemolyticus</em>, MIC &gt;2000 μg ml⁻¹ for other strains</td>
<td>Hong &amp; Kim (2008)</td>
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<td>Several synthetic hydroxyphenyl ITCs</td>
<td><em>Staph. aureus</em> IFO12732; <em>E. coli</em> C</td>
<td>Twofold agar dilution method: ITC in nutrient agar at 1–250 μg ml⁻¹; bacteria streaked onto plates and incubated 37 °C 48 h; plates rinsed and washing transferred into nutrient agar; incubated 37 °C 48 h</td>
<td>MIC, minimum concn at which no colony observed; MBC, minimal concn at which no regrowth observed</td>
<td>2-(4-hydroxyphenyl)ethyl ITC: higher antimicrobial activity, MIC 15.6 μg ml⁻¹, MBC 62.5 and 31.3 μg ml⁻¹ for <em>Staph. aureus</em> and <em>E. coli</em>, respectively</td>
<td>Tajima et al. (1998)</td>
</tr>
<tr>
<td>Allyl, benzyl</td>
<td><em>Campylobacter jejuni</em> reference strains NCTC 11168 and 81-17 plus 24 Ca. <em>jejuni</em> strains isolated from chicken faeces, human infections (blood, faeces), contaminated processed meats</td>
<td>Agar dilution method: 10-fold serial dilutions ITC in Mueller–Hinton agar; liquid culture in Mueller–Hinton broth plus ITC; final concns 10, 5, 2.5, 1.25 μg ml⁻¹ for AITC, 1.25, 0.625, 0.312, 0.156 μg ml⁻¹ for BITC; OD600 before and after 18 h microaerobic incubation 37 °C; c.f.u. determination</td>
<td>MIC defined as lowest concn that inhibited any visible growth of a 10⁻⁵–5 x 10⁻⁹ c.f.u. spot after 48 h at 37 °C in microaerobic conditions; MBC defined as lowest concn ITC that kills 99.9 % bacteria (i.e. 3 log reduction) after 18 h incubation</td>
<td>MIC AITC 50–200 μg ml⁻¹, MIC BITC 2.5–5 μg ml⁻¹; identical MIC and MBC values for both ITCs</td>
<td>Dufour et al. (2012)</td>
</tr>
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</table>

*NCCLS, National Committee for Clinical Laboratory Standards.
†MIC₉₀, MIC at which growth of 90 % of strains is inhibited.
‡i.z., Diameter of inhibition zone.
authors postulated that ER and SFN were inactivating 3-oxo-C₁₂-HSL binding to LasR by the formation of a complex with the nucleophilic cysteine residue of the binding pocket. However, the authors failed to isolate an ITC-LasR dithiocarbamate conjugate. Nevertheless, in this case, they ruled out the implication of the sulfoxide moiety in the QS-inhibitory activity, although the QS-inhibiting ability of SFN and ER on the Las QS system showed slight differences in efficacy (Ganin et al., 2013). A recent study has confirmed the QS-inhibitory activity of three ITCs (AITC, PEITC and BITC), using the CviIR QS system in Chromobacterium violaceum (Borges et al., 2014). Each ITC reduced violacein (a QS-induced pigment) production in Ch. violaceum but at growth-inhibitory levels, with BITC being the most potent inhibitor.

Effect of ITCs on respiratory enzymes

The study carried out by Jakobsen et al. found that ITC molecules altered physiological pathways other than just the QS system in bacteria. At sublethal concentrations in Ps. aeruginosa, iberin activated the expression of the respiratory genes cyoA and cyoB, encoding the cytochrome O ubiquinol oxidase (bo₃ oxidase), whereas the gene PA4133, encoding an uncharacterized subunit I of a putative cytochrome c oxidase cbb₃ type, was downregulated (Jakobsen et al., 2012a). Ps. aeruginosa possesses five described terminal oxidases for aerobic respiration, with the cbb₃ type being O₂ high-affinity oxidases (including CIO, cbb₃-1 and cbb₃-2 oxidases). They can support microaerobic growth with as little as 2% oxygen and are probably involved in energy conservation under low O₂ concentrations (Kawakami et al., 2010). ITCs seem to affect normal O₂ respiration while activating alternative respiratory pathways to maintain energy levels. The effect of ITCs on respiration was previously described in yeast by Kojima & Ogawa (1971), for which oxygen uptake was diminished by several ITCs (AITC, methyl ITC, PITC and thymol).

Induction of heat-shock and oxidative stress responses

In Ps. aeruginosa, groEL and dnaK, encoding heat-shock proteins (Hsp), were induced by iberin (Jakobsen et al., 2012a). Both genes encode folding chaperones that rely on ATP-driven conformational changes to mediate refolding or unfolding of their protein targets (Lund, 2001). However, in this study, the expression levels of groES, dnaJ and grpE, encoding the associated factors of these two chaperones, were not significantly changed in iberin-treated Ps. aeruginosa cells. Overexpression of GroEL/GroES or DnaK/DnaJ prevented protein aggregation in E. coli (Gragerov et al., 1992). Interestingly, similar differential expression of heat-shock proteins was observed in ITC-treated eukaryotic cells. In Caco-2, HeLa and rat colon carcinoma cells, SFN induced a heat-shock response, mainly resulting in an overexpression of Hsp27, a small heat-shock protein, usually induced by a variety of stresses, and displaying several functions such as redox homeostasis and chaperone activity. The overexpression of Hsp27 by SFN was mediated by activation of the heat-shock transcription factor Hsf1, but the activation mechanism remains unknown (Gan et al., 2010). How ITC impacts on the heat-shock response is variable according to the cell lines and experimental conditions used. In pancreatic cancer cells, SFN blocked the interaction of Hsp90 with its co-chaperone P50 (Li et al., 2011) and in breast cancer cell lines, SFN downregulated the expression of Hsf1 and of several Hsps, inducing a cascade that led to increased apoptosis (Sarkar et al., 2012). Although there is a link between ITCs and the heat-shock response, the effect seems to depend on the conditions used.

We have recently performed a whole transcriptomic analysis of Campylobacter jejuni treated with subinhibitory concentrations of BITC (Dufour et al., 2013). We had previously determined the MIC and bactericidal action of BITC on several Ca. jejuni strains, showing that BITC was the most efficient ITC at killing Ca. jejuni, relative to AITC and ethyl ITC (Dufour et al., 2012). From this transcriptomic study, it clearly appeared that BITC elicited a heat-shock-like response in Ca. jejuni, with the genes clpB, dnaK, grpE, groEL, groES, cbpA and hrcA all being upregulated in response to BITC exposure. ClpB functions to solubilize and reactivated aggregated proteins via an ATP-driven process, coupled with the action of DnaK-DnaJ-GrpE activity. CbpA is a DnaJ homologue and a DnaK co-chaperone (Chae et al., 2004). HrcA is a transcriptional repressor that can fold into an active conformation only in non-heat-shock-treated cells since it requires the assistance of the chaperones GroEL/ES to fold properly. Indeed, in cells undergoing heat shock, the available GroEL/ES chaperone levels are decreased by the accumulation of unfolded proteins (Holmes et al., 2010; Lund, 2001). All of these upregulated heat-shock-related proteins are...
ATP-dependent. It is interesting to note that this upregulation is correlated with an increase in ATP content in BITC-treated Ca. jejuni cells, along with an overexpression of genes from the TCA cycle and respiratory pathways such as nitrate reductase, lactate oxidase, hydrogenases and fumarate reductase (Dufour et al., 2013). In E. coli, an aerobic organism for which O2 is the preferential electron acceptor, treatment with 2-(4-hydroxyphenyl)ethyl ITC decreased the cellular content of ATP (Tajima et al., 1998). The two O2-dependent respiratory chains of E. coli may be sensitive to ITC while, in the microaerophile Ca. jejuni, the upregulation of O2-independent pathways for fumarate and nitrate respiration may overcome the inhibition of O2 respiration by ITCs. We have also demonstrated that subinhibitory concentrations of BITC reduced O2 consumption by Ca. jejuni and promoted protein aggregation in treated Ca. jejuni cells (Dufour et al., 2013). This effect may contribute to the antimicrobial action of ITC in bacterial cells (Fig. 3). BITC also induces an oxidative stress response in Ca. jejuni, with the upregulation of genes encoding superoxide dismutase (SodB), ruberythrin-like proteins (Rrc) (Yamasaki et al., 2004), and a sulfite oxidase involved in nitrogen-reactive species detoxification (Cj0379c) (Hitchcock et al., 2010). These data confirmed that ITC disrupted the redox homeostasis of the cells, as previously described in eukaryotic cells.

**Induction of a stringent response**

Nowicki et al. (2014) have reported the efficacy of PEITC in inhibiting the growth of enterohaemorrhagic E. coli (EHEC) as well as Shiga toxin production. The authors correlated these effects to the induction of a stringent response. PEITC provoked a strong inhibition of RNA synthesis, except in relA and relA spoT mutants deficient in ppGpp synthase and therefore unable to induce a stringent response. Moreover, PEITC caused an accumulation of ppGpp and its precursor, pppGpp. The authors proposed that the induction of the stringent response was due to the depletion of cellular amino acids, through their interaction with PEITC. Indeed, supplementation of glycine or arginine in the growth medium, thereby increasing the pool of free amino acids at inhibitory concentrations of ITC, was able to restore growth of bacterial cultures treated with PEITC and increased the MIC of PEITC for E. coli. This MIC-increasing effect was also observed by supplementing lysine, methionine, phenylalanine, serine and threonine (Nowicki et al., 2014).

![Hypothetical model for the antimicrobial effects of ITC and resistance mechanisms.](http://mic.sgmjournals.org/237)
Mechanisms of resistance to ITCs

Resistance to SFN was investigated in the plant pathogen Ps. syringae by Fan et al. (2011). They identified a group of genes (the sax genes, for ‘Survival in Arabidopsis eXtract’) responsible for the resistance of virulent host-associated Ps. syringae to SFN produced by Arabidopsis. When introduced into E. coli, the first three genes of the operon, saxABC, conferred the ability to overcome the plant defences and therefore to grow on Arabidopsis extracts. How the sax genes mediate resistance of virulent Ps. syringae is unclear, as are the mechanisms of homologous genes known to be present in various pathogens of ITC-producing plants. The sax operon is composed of seven genes, amongst which only three are required for ITC resistance. The product of saxA is related to class B β-lactamase, although it does not confer resistance to conventional β-lactam antibiotics. SaxB is an isochorismatase, and SaxC is a transcriptional regulator of the AraC/XylS family. Only the absence of saxA and/or saxC totally abolished the bacterial resistance to ITC-containing extracts. saxA-like genes are found only in the genome of pathogenic strains of Ps. syringae. Further investigations are required to understand the implication of a β-lactamase-like enzyme in the resistance to ITC. Recently, Chan and collaborators showed that AITC was not mutagenic for E. coli, since it did not activate the DNA repair gene recA. As a result, the authors postulated that the risk of evolving antimicrobial resistance to AITC by mutagenesis was reduced (Chan et al., 2013). A transcriptomic analysis of the response of Ps. aeruginosa to iberin revealed some potential resistance mechanisms. Amongst the 51 upregulated genes, the authors pointed out the genes mexEF-oprN, encoding a well characterized efflux pump in Ps. aeruginosa. Originally identified as being linked to fluoroquinolone resistance, MexEF-OprN mediates resistance to a variety of antimicrobials, including trimethoprim and chloramphenicol (Köhler et al., 1997). Recently, it was demonstrated that MexEF-OprN was induced by nitrosative stress, while not being directly involved in nitric oxide resistance (Petar et al., 2011). Interestingly, the genes are under the control of the regulator MexT (LysR-type regulator), which was recently found to be a redox-responsive regulator controlling disulfide stress provoked by diamides, which deplete cellular GSH in a fashion similar to ITC (Fargier et al., 2012). The authors postulated that this efflux pump may be involved in the efflux of electrophilic compounds; therefore it is tempting to postulate that MexEF-OprN may also be involved in the efflux of iberin.

In E. coli O157:H7, the complete deletion of the two-component system BaeSR increased sensitivity to AITC via a currently undefined mechanism (Cordeiro et al., 2014). BaeSR has been previously linked to the ability of E. coli to resist exposure to Acacia mearnsii (black wattle) tannin extract (Zoetendal et al., 2008), as well as to the induction of the multidrug resistance cluster mdtABCD (Baranova & Nikaido, 2002).

In a further study on the effects of ITC on Ps. aeruginosa, a gene encoding a GST was identified as being upregulated by iberin (Jakobsen et al., 2012a). The genome of Ps. aeruginosa PA01 encodes 10 putative GSTs (www.pseudomonas.com), meaning that, while the upregulation of only one GST gene by iberin suggests that Ps. aeruginosa does use the GST-GSH detoxification system for iberin, these GSTs have specific substrates, of which only one may apply to iberin exposure. Additionally, GST has also been reported as a detoxification enzyme for ITC in the cyanobacterium Synechococcus elongatus PCC 6301 (Wittkeius & Stenberg, 2007). As mentioned previously, GSTs are also part of a common route of ITC detoxification in eukaryotes (Kolm et al., 1995), and expression of GST was found to be induced by BITC in the pathogenic fungus Alt. brassicicola (Sellam et al., 2006). GSH is not, however, present in all bacterial species. In low-GC Gram-positive bacteria, such as Bacillus and Staphylococcus, bacillithiol (BSH), a glycoside formed between L-cysteinyl-D-glucosamine and malic acid, is the major low molecular mass thiol, along with coenzyme A and cysteine (Helmann, 2011). The thiol group of BSH functions as a nucleophile, reacting with electrophilic molecules. A recent study has demonstrated the implication of BSH in the detoxification of toxins and thiol-reactive compounds such as rifamycin in Staph. aureus, via the formation of BSH conjugates and the generation of mercapturic acid (Newton et al., 2012). So far, the detoxification processes for electrophilic molecules in bacteria lacking both BSH and GSH are unknown.

Given that several bacteria, such as Ca. jejuni, possess neither GST nor GSH biosynthesis pathways, how do these bacteria mediate the detoxification of ITC conjugates? The first step in the process needs to be a thiol exchange mechanism to separate the dithiocarbamate conjugates, as demonstrated for the GSH/GST system. The conjugation of electrophilic ITC with GSH is a means to decrease the intracellular levels of both free ITC and the protein-ITC conjugates. Interestingly, the transcriptomic analysis of Ca. jejuni exposed to BITC showed the upregulation of the thioredoxin gene trxA (Dufour et al., 2013). The thioredoxin/thioredoxin reductase systems are often redundant with the GSH/glutaredoxin/glutathione reductase system or can replace it (Toledano et al., 2007). These systems are responsible for maintaining a reducing environment and for the regulation of thiol-based enzymic activity. All thioredoxins have a three-dimensional structure comprising a central core of four β-strands, surrounded by three α-helices (Martin, 1995). The active site contains two redox-active cysteines. The pKₐ of the N-terminal cysteine is lower than the pKₐ of a free cysteine, which enables thioredoxin to attack the disulfide in proteins as a nucleophile (Holmgren, 1985; Roos et al., 2009). Because of their low redox potential (−270 to −330 mV in E. coli) (Aslund et al., 1997; Krause & Holmgren, 1991), thioredoxins are efficient thiol-disulfide reductants. In bacteria, thioredoxins serve as enzyme reductants, hydrogen donors to ribonucleotide reductases (DNA synthesis) and phosphoadenosine phosphosulfate reductases (sulfur assimilation), and methionine sulfoxide reductases (protein repair). They
provide reducing equivalents to peroxiredoxins, involved in peroxide detoxification (for review see Zeller & Klug, 2006). So far, their role in the resistance to ITC via their possible thiol exchange ability, the reduction of diithiocarbamate conjugates or ITC depletion has not been experimentally evaluated. However, it is interesting to notice that, in eukaryotic cells, treatment with ITCs often resulted in over-expression of thioredoxin reductase or thioredoxin-encoding genes, suggesting that these proteins have a role in the defence against ITCs. In human colon carcinoma Caco-2 cells, iberin triggered the overexpression of the thioredoxin reductase-1 gene (Jakubiková et al., 2006), and a similar result was obtained in human hepatoma HepG2 cells cultured with SFN (Zhang et al., 2003). Along with the thioredoxin reductase genes, SFN also induced the expression of thioredoxin in both HepG2 and Caco-2 cells (Bacon et al., 2007). In mice, both intraperitoneal and oral SFN induced increased production of thioredoxin protein in retinal tissues, reducing light damage to retinol (Tanito et al., 2005). Thioredoxin may have an essential role in maintaining reduced thiol groups as a means to scavenge electrophilic ITC. In eukaryotic cells treated with 14C-radiolabelled PEITC or SFN, only a small number of proteins were found to contain radioactive ITC conjugates (Mi et al., 2008, 2011), suggesting that either ITC binding in vivo was selective or, as previously reported, ITC conjugates dissociate to reach an equilibrium with free ITCs. The proportion and identification of prokaryotic ITC target proteins in vivo have not as of yet been investigated.

**Conclusion and perspectives**

Fig. 3 is a hypothetical model of the different ITC activities in prokaryotes based on experiments carried out in different species: (1) ITCs may accumulate in bacteria as GSH, small thiols like BSH or thioredoxin diithiocarbamate conjugates, and attack the active centre of enzymes (such as receptors of QS signalling molecules), or affect protein conformation via exchange processes (2) and binding to thiol or amine groups. These interactions impact specialized enzymatic activities, respiration, metabolism and transcription of genes. Moreover, conjugation of ITCs with the thiol groups of proteins may promote the aggregation of misfolded proteins and thus the activation of heat-shock responses, while the conjugation with amino acids may induce a stringent response (3). The binding of ITCs by thioredoxin, and of small thiols such as GSH or BSH, may affect oxidative stress responses by impairing peroxiredoxin regeneration (reduction) and cell redox homeostasis (4). The conjugation of ITCs to GSH (via the enzyme GST), BSH or thioredoxin may be a first step in the detoxification of ITCs. The product of the *sax* gene homologues, found in SFN-resistant *Ps. syringae* (Fan et al., 2011), may also be involved in ITC detoxification (5). Efflux systems (such as MexEF-OprN of *Ps. aerugi-nosa*) may promote the extrusion of ITCs from the cell (6). These hypotheses are based mainly on gene expression assays and are awaiting experimental confirmation. The use of ITCs as antimicrobials is often impaired by their high volatility and strong odour. In the absence of myrosinase, such as when plants are cooked and myrosinase is heat-inactivated, animals can efficiently convert glucosinolates to ITCs through the action of myrosinase derived from the microbiota of the gastrointestinal tract (Fahey et al., 2012). In this case, the dietary intake of Brassicaceae vegetables is sufficient to obtain beneficial effects on health. However, for the purpose of treating food for preservation or to fight against infectious diseases, processes including a combination of glucosinolates and myrosinase are not as easy to implement. Several packaging systems are being evaluated to reduce the detrimental effect of ITC on food appearance and taste. So far there is no report of the antimicrobial effect of ITC conjugates; however, in eukaryotic cells, GSH-ITC and cysteine-ITC diithiocarbamates, formed from AITC and BITC, displayed a similar biological activity to their parent ITCs in inhibiting the growth of epithelial cancer cells. Other studies demonstrated that ITC conjugates could act as prodrugs, with similar efficacy to the accumulated ITCs in cells treated with GS-ITC conjugates. This result demonstrates that diithiocarbamates are potent ITC donors in eukaryotic cells (Zhang, 2000). Since GSH-ITC and other ITC conjugates are reversible complexes (Hwang & Jeffery, 2005), they may be able to release free ITC inside the cells, and thus not deplete endogenous GSH or other thiols. However, the inhibitory efficacy of these conjugates may vary significantly compared with free ITC. Indeed, some bacteria overcome the inhibitory effect of free ITC via their conjugation to GSH, but an excess of GSH-ITC conjugates may be a sufficient source of ITC for endogenous thiol-exchange processes. Conaway et al. (2005) have demonstrated that thiol conjugates of ITCs in an aqueous medium exist in equilibrium with the free form, and that free ITCs, rather than conjugates, exert enzyme-inhibitory activity (tested *in vitro* on cytochrome P450). They have also shown that N-acetyl-cysteine conjugates are more stable than GSH and cysteine conjugates and postulated that increasing the number of alkyl carbons should increase the stability of ITC-thiol conjugates. Further investigations will be required to determine the effect of thiol conjugates and related chemical factors on bacteria.

In summary, ITCs are natural plant products with interesting antimicrobial and anticancerous properties. Further studies are needed to better understand their mode of action and reveal potential synergic activity with antibiotics.

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