



Published in final edited form as:

J Appl Microbiol. 2013 October ; 115(4): 943–954. doi:10.1111/jam.12288.

Growth-inhibitory activity of natural and synthetic isothiocyanates against representative human microbial pathogens

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Abstract

Aims—The aim of this study was to test the growth inhibition activity of isothiocyanates (ITC), defense compounds of plants, against common human microbial pathogens.

Methods and Results—In this study we have tested the growth inhibitory activity of a diverse collection of new and previously known representative ITC of various structural classes against pathogenic bacteria, fungi and molds by a serial dilution method. Generally, the compounds were more active against Gram-positive bacteria and fungi exhibiting species-specific bacteriostatic or bactericidal effect. The most active compounds inhibited the growth of both drug-susceptible and multi drug resistant (MDR) pathogens at micromolar concentrations. In the case of *Mycobacterium tuberculosis* some compounds were more active against MDR, rather than against susceptible strains. The average anti-microbial activity for some of new derivatives was significantly higher than previously reported for the most active ITC compounds. The structure-activity relationship (SAR) established for various classes of ITC with *Bacillus cereus* (model organism for *B. anthracis*) followed a distinct pattern, thereby enabling prediction of new more efficient inhibitors. Remarkably, tested bacteria failed to develop resistance to ITC. While effectively inhibiting microbial growth, ITCs displayed moderate toxicity towards eukaryotic cells.

Conclusions—High antimicrobial activity coupled with moderate toxicity grants further thorough studies of the ITC compounds aimed at elucidation of their cellular targets and inhibitory mechanism.

Significance and impact of the study—This systematic study identified new ITC compounds highly active against common human microbial pathogens at the concentrations comparable with those for currently used antimicrobial drugs (e.g. rifampicin, fluconazole). Tested representative pathogens do not develop resistance to the inhibitors. These properties justify further evaluation of ITC compounds as potential antimicrobial agents for medicinal use and for industrial applications.

Keywords

Isothiocyanate; natural; synthetic; bacteria; fungi; growth; inhibition; bacteriostatic; bactericidal

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Supporting information includes: i) the analytical data on ITC compounds; ii) the diagrams for proliferation rate of *B. cereus* in the presence of various concentrations of ITC 37, and iii) the dependence of ITC 37 growth inhibitory potency against *B. cereus* on the compound pre-incubation time in LB culture medium.

INTRODUCTION

Despite availability of numerous diverse collections of synthetic products and high throughput approaches for their biological testing, natural compounds remain a major source for antimicrobial drugs development. These compounds are especially valuable, since they have undergone natural selection overtime. In our study we concentrated on isothiocyanates (ITC), which are secondary metabolites, used by certain plants for the defense against microbial pathogens (Drobnica et al. 1957; Fenwick, Heaney and Mullin 1983; Delaquis and Mazza 1995; Osborn 1996; Fahey, Zalcmann and Talalay 2001; Tierens et al. 2001). ITCs are stored in the plant cells as inactive precursors, glucosinolates, which can be converted to active compounds by the enzyme myrosinase, normally separated from ITC precursors by a cellular compartment. Destruction of the compartment upon plant damage (which greatly increases the incidence of infection) brings the enzyme in contact with the pro-drug, causing glucose release from the substrate followed by an intramolecular reaction of the liberated aglicone yielding ITC (Fahey, Zalcmann and Talalay (2001) and references therein)). ITC compounds subsequently kill opportunistic pathogenic bacteria, or fungi that enter a plant through the damaged site (Osborn 1996; Mewis 2006). These defense compounds are typical for the family *Cruciferae* and have been known for a long time for their antimicrobial, nematocidal, and allelopathic activities (Fahey, Zalcmann and Talalay (2001) and references therein). Some of ITC-precursors-enriched plants have been used in folk medicine (Swart, Van Dyk and Malan 2002), while synthetic compound, 4-bromophenyl ITC constitutes an active ingredient of a formulation for dermatomycoses treatment (Drobnica et al. 1967).

ITCs have gained recognition following the discovery of their anti-cancer chemoprotective properties (Zhang and Talalay 1994; Michaud et al. 1999; Hecht 2000; Talalay and Fahey 2001; Conaway et al 2002) and selective apoptotic activity against major types of cancer cells (Zhang et al 2003; Srivastava et al 2003; Xiao et al 2003). Many representatives of cruciferous plants naturally enriched with ITC precursors (broccoli, cabbage, watercress, Brussels sprouts, etc.), are widely consumed by humans. Epidemiological studies established reliable correlation between the dietary intake of ITCs and decreased risk of cancer (Michaud et al. 1999; Lam et al. 2009; Herr and Buchier 2010). Remarkably, many ITCs possess both anticancer and antimicrobial activity. Thus ITC sulforaphan has been suggested as a means for treatment of stomach ulcer caused by *Helicobacter pylori* and for prophylactics of stomach cancer (Fahey et al. 2002).

High antimicrobial activity of many ITCs revealed in previous studies merits search for more active representatives of this class and thorough investigation of their action mechanism in order to use the compounds to combat infections. The studies of ITCs as potential anti-microbial agents are yet at the starting stage (Johns et al. 1982; Chmel et al. 1958; Drobnica et al 1968; Tajima et al 1998; Tajima, Kimoto and Taketo 2001; Fahey et al 2002; Tajima, Kimoto and Taketo 2003; Johansson, Pavia and Chiao 2008; Jung et al 2010, Dufour et al 2012; Conrad et al 2013). Very limited set of ITC have been used in these studies. To this end, in the present research we have tested a diverse collection of known and newly synthesized ITC compounds against common classes of human pathogens including a variety of clinical strains of bacteria, yeasts and fungi.

MATERIALS AND METHODS

All chemicals were from Sigma, Aldrich, Acros Organics, Fluka or TCI America. All solvents used in the synthesis were anhydrous. The structures of tested ITC compounds are presented in Table 1. The synthetic protocols for each compound is indicated in Table 1 and described below. The compounds identity was confirmed by UV, NMR, and mass spectroscopy (see supplemental materials). Gas chromatography with MS detection was

performed on 6890GC/5973MS chromatograph (Hewlett Packard). Thin layer chromatography (TLC) was performed on Sigma-Aldrich TLC plates, column chromatography (20 × 2.5 cm) – on Kieselgel 60 (Fluka). UV spectra were recorded on UV160U spectrophotometer (Shimadzu).

Synthesis of ITC

Protocol I (Dyson and George 1937)—Solution of 1 mmol of amino compound in 2 ml of chloroform was added dropwise to the mixture of 1.1 mmol of thiophosgene and 3 ml of chloroform under rigorous agitation in the ice bath. In the case of diamino compounds the amount of thiophosgene was doubled. After 5 min anhydrous triethylamine (two molar equivalents per each amino group) in 2 ml of chloroform was added dropwise under continuous agitation. The mixture was brought to room temperature and supplemented with water (40 ml). After partitioning organic phase was collected, washed by another 40 ml of water and evaporated in *vacuo*. The products were purified by silicagel column chromatography using hexane/acetone mixture as eluent. Fractions, containing the product were detected by UV light, combined and evaporated under reduced pressure. The products were collected and stored at –20 °C.

Protocol II—One mmol of amino compound was dissolved in 0.5 ml of methylene chloride and added dropwise to the solution of 1.1 mmol of thiocarbonyldiimidazole in 2 ml of the same solvent, followed by addition of 2 eq (to each amino group) of trifluoroacetic acid. After 2 h incubation at 20 °C 2.2 eq of triethylamine was added, the mixture extracted twice with 3 ml of water, organic phase collected, dried over anhydrous Na₂SO₄, evaporated under reduced pressure and the product purified described above.

Protocol III—Solution of 1 mmol of ITC **5** in 0.2 ml of trimethylphosphate was treated with 1.1 molar excess of POCl₃ at room temperature for 1h followed by addition of 0.1 ml of water and rigorous agitation for 20 min. The product was subjected to preparative TLC in acetonitrile-water (5:1) developing system and visualized using UV monitor. After elution with 50 % aqueous methanol the product was concentrated by evaporation under reduced pressure.

Microbial strains and growth conditions—All strains of bacteria and fungi (Tables 2–4) were from the collection of Public Health Research Institute Tuberculosis Center, UMDNJ. Growth conditions for microorganisms are specified below. Components for growth media preparation were purchased from Difco and Sigma.

Determination of MIC—Consecutive two-fold dilutions of ITCs were prepared in liquid growth media (specified below) in 96 well plates and immediately mixed with equal volume of a microorganism suspension (~2 × 10⁴ CFU ml⁻¹ as determined by counting the colonies after plating of an aliquot of the bacterial suspension with known turbidity at 600 nm). Visible microbial growth was examined after 24 h incubation at 37 °C. In the case of mycobacteria the growth was determined after 3 weeks incubation at 37 °C. In another version the MIC was determined by plating of the bacteria suspension on agar containing a testing compound (Anon 2000). MICs for bacteria were determined using Muller-Hinton broth or agar; in case of mycobacteria 7H10 Middlebrook culture medium was used. MICs for fungi and yeast were determined using RPMI 1640 liquid medium.

The effect of pre-incubation of the ITC 37 in the culture medium on its growth-inhibitory activity—To determine the ITC **37** stability in culture medium at 37 °C, we incubated the compound (4 µg ml⁻¹) in Muller Hinton broth for various periods of time indicated in Fig. S3 prior to the inoculation with equal volume of *Bacillus cereus*

suspension. After incubation for 20 h at 37 °C the bacterial growth was assessed by measuring the turbidity at 600 nm using microtiter plate reader.

Determination of the growth-inhibitory mode for ITC 37—To determine the action mode for the compound on *B. cereus* or *S. aureus*, the ITC was added to the bacterial cultures in MHB medium containing 2×10^4 CFU ml⁻¹ at the concentrations indicated in Fig. 1. After incubation for 1 and 2 h at 37 °C, the number of surviving cells was determined by plating the serial dilutions of the culture medium onto ITC-free Muller Hinton agar and subsequent incubation for 24h.

Toxicity studies—The toxicity assays of the compounds were performed on human macrophages kindly provided by Dr. R. Shepard (PHRI) using cell proliferation kit (Promega). To assess the toxicity of ITC compounds, we used macrophages isolated from donor human blood and immobilized in 96-well plates employing an established protocol (<http://www.promega.com/resources/protocols/technical-bulletins/0/celltiter-96-aqueous-nonradioactive-cell-proliferation-assay-protocol/>). The assay takes advantage of the ability of life cells to generate colored compound, which was detected by light absorption.

Attempt to select spontaneous ITC-resistant mutants of *B. cereus*, *Staphylococcus aureus*, *Mycobacterium bovis*, and *Candida albicans*—

Microbial culture suspension of about $10^9 - 10^{11}$ CFU (for various species) was plated onto the Muller-Hinton agar medium containing ITC 37 at a concentration of 10 µg ml⁻¹. The plating density was $10^7 - 10^8$ CFU per cm².

RESULTS

SAR for ITC in *B. cereus* and *Escherichia coli* growth inhibition assay

First we assessed growth inhibitory activity of newly synthesized and known ITC of various classes on *B. cereus* and *E. coli* as representative of Gram-positive (G⁺) and Gram-negative (G⁻) bacterial species correspondingly. Table 1 (see also Fig. 2) shows the MIC values for tested compounds. As reference compounds in this testing we included previously described ITCs 2 and 20, possessing high antibacterial activity (Tajima 1998; Swart 2002). It is seen that with *B. cereus* ITCs with short hydrocarbon chains (ITC 8, 22, 7) displayed poor activity (MIC = 128 µg ml⁻¹). Remarkably, addition of a hydroxyl group to the terminal carbon of ITC 7 (creating ITC 18) strongly increased the potency (8-fold). In this subset of compounds variation of the spacer between the hydroxyl and ITC groups from 4 to 7 carbons (ITC 18, 5, 21, 44) did not change the ITC activity, while 8-carbon compound (45) displayed lower MIC. Adamantane-based ITC 35 displayed moderate activity (MIC = 8 µg ml⁻¹).

Bifunctional compounds containing two ITC groups in the molecule (ITC 38–42) possessed high activity (MIC 2 µg ml⁻¹) irrespectively on the linker size. Notably, the substitution of two carbons in the spacer with oxygens in ITC 42 creating ITC 43, reduced the activity. Aromatic ITC efficiently inhibit the growth of various microorganisms (Drobnica et al 1968; Tajima 1998; Swart 2002, Sofrata et al 2011). In SAR studies as reference compound for this group we have chosen benzyl-ITC (ITC 1) possessing moderate activity (MIC = 16 µg ml⁻¹). In control experiments we established a crucial role of the isothiocyano group of ITC 1 in bacterial growth inhibition. Indeed, neither of the parent chemically related derivatives - isocyanate 23 or thiocyanate 25 - displayed antibacterial activity (Table 1).

The deletion of a methylene group in ITC 1 (leading to ITC 4) dramatically decreased the activity, while insertion of an extra methylene group (producing ITC 17), or introduction of

an additional methyl substitution into the methylene group adjacent to ITC group (ITC **27**) had no effect (Table 1). The insertion of hydroxy- or methoxy groups in the *para*- position on the phenyl ring (compare ITC **1** – ITC **2**, ITC **4** – ITC **3**, and ITC **17** – ITC **20**) increased growth-inhibitory potency. Similarly to the trend seen in aliphatic compounds bifunctional aromatic ITCs possessed enhanced activity. Thus MIC for ITC **16** and **37** was 2 and 0.5 $\mu\text{g ml}^{-1}$, correspondingly.

Heterocyclic ITC compounds displayed various levels of activity. Derivatives of indole (ITC **19**), pyridine (ITC **30–32**) and imidazole (ITC **34**) showed comparable potency (MIC = 8–16 $\mu\text{g ml}^{-1}$). The growth inhibitory efficiency of pyridine derivatives strictly depended on the position of a nitrogen atom in the ring and on the size of methylene spacer between the ITC group and the ring. Thus placing a nitrogen in the *para*-position (ITC **29**) strongly decreased the activity (MIC > 128 $\mu\text{g ml}^{-1}$) compared to the reference ITC **1**, while *meta*-substitution (ITC **30**) yielded better inhibitor (MIC = 8 $\mu\text{g ml}^{-1}$). ITC derivative with a bulky side chain, fluorescein had low activity, while ITC compounds with more hydrophobic pyrene ring (ITC **33**) was exceptionally active (MIC = 0.5 $\mu\text{g ml}^{-1}$). ITC **33** was also highly potent against *Staphylococcus epidermidis*, and *methicillin-resistant Staphylococcus aureus* strains (MIC = 0.5 $\mu\text{g ml}^{-1}$) (not shown). As seen from Tab. 1, the ITC compounds were much less active against *E. coli*, a gram-negative bacterium. The most efficient was ITC **37** (MIC = 8 $\mu\text{g ml}^{-1}$). The MIC for ITC **1**, **16**, and **32** were in the range of 64–128 $\mu\text{g ml}^{-1}$, while for the rest of the compounds it was beyond the detection range (>128 $\mu\text{g ml}^{-1}$).

Activity of the representative ITCs against gram positive and gram-negative bacteria

Next we assessed growth-inhibitory potency of some representative ITC compounds with wide selection of (G^-) and (G^-) bacterial species listed in Tab. 2. For these testing we have chosen ITCs from various structural groups possessing significant growth inhibitory activity towards *B. cereus*. Notably, ITC compounds were much more active against G^+ than against G^- bacteria (Tab. 2). The most susceptible genera were *Staphylococcus*, *Streptococcus*, and *Bacillus* (MIC from <0.5 to 2 $\mu\text{g ml}^{-1}$). Bifunctional ITC **37**, **41** and **42** were highly active against G^+ strains. ITC **37** was also moderately effective against some G^- strains while ITC **42** and **43** were highly discriminative, affecting only G^+ species. Generally, the compounds synthesized in the present study were 2–32 times more active than the reference ITC **2** and **20**.

Activity against mycobacteria

Table 3 represents the results of the growth inhibition of clinical isolates of tuberculosis and non-tuberculosis mycobacteria by the same ITC compounds. The ITCs were generally more efficient against *M. bovis* and *M. tuberculosis*, than against non-tuberculosis mycobacteria. The most effective were ITC **37**, **40–41**, and **45**, inhibiting the growth of all tested clinical isolates of *M. tuberculosis* including multi drug resistant (MDR) strains, at concentrations comparable to that of common TB drug rifampicin. Again, the average efficiency of new ITCs was 2–32 times higher than that for the known ITCs.

Activity against pathogenic fungi and molds

The testing results for anti-fungal and anti-yeast activity of ITC compounds are presented in Table 4. It is seen that synthesized ITC compounds efficiently inhibited the growth of the pathogens, being generally more active compared not only to the reference ITC **2** and **20** but also in comparison to the common antifungal drug fluconazol. The most active were ITC **30**, **37**, and **41**, while ITC **33** highly effective against *B. cereus*, displayed only moderate species-specific antifungal activity.

Testing of the growth-inhibitory mode of ITCs

As previously reported, ITC compounds displayed bactericidal activity against *Helicobacter pylori* (Fahey et al 2002) and *Aggregibacter actinomycetemcomitans* (Sofrata et al 2011), while having mostly bacteriostatic effect on *E. coli* (Tajima et al 2001). Both of these bacterial species belong to G⁻ class. In the present research we investigated the growth inhibitory mechanism of one of the most potent ITC **37** on G⁺ bacterial pathogens *B. cereus* and *S. aureus* (which are both highly and equally sensitive to ITC **37**) by determining the number of the surviving cells after the pre-incubation with the ITC. In the case of *B. cereus* (Fig. 1A), increasing ITC concentration (up to 4 µg ml⁻¹) caused progressive decrease in cell viability so that no CFUs were detected after subsequent 20 h incubation on the same media without ITC. Some colonies emerged only after 4 days of plating subculturing greatly impaired fitness of the survived cells. Notably, neither increase of the drug concentration, nor incubation duration reduced the number of the residual bacterial colonies. The effect of the ITC **37** on *S. aureus* was more complex (Fig. 1B). Increasing the concentration of the drug up to 1 µg/ml⁻¹ and incubation time with the ITC from 1 to 2 h, progressively reduced the number of survived cells. However, further increase in the inhibitor's concentration restored the viability "paradoxical effect", so that at ITC concentration 64 µg ml⁻¹ the bactericidal effect completely disappeared.

As seen from Fig. S2, the action of ITC **37** was fast, so that at the concentration equal to MIC complete inhibition of the bacterial growth was observed after 30 min exposure to the compound.

Effect of ITC pre-incubation in culture medium on its growth inhibitory activity

It was suggested that ITC can decay due to reaction with culture media components (Adesida et al 1996). To examine the stability of ITC in a culture medium we investigated the effect of ITC **37** pre-incubation in the MHB medium on the ITC growth-inhibitory activity against *B. cereus*. No growth of bacteria was detected upon immediate addition of the bacterial culture to the inhibitor at concentration equal to MIC and subsequent incubation for 24 h (Fig. S3). However, progressive increase in growth rate was observed if the bacteria were added to the ITC **37** pre-incubated for various periods of time at 37 °C, indicating gradual decrease of inhibition potency and therefore, ITC decay.

Cytotoxicity of selected ITC compounds on human monocytes

The results of this testing (Fig. 3) indicate that the toxic effect for all of the ITC compounds became detectable at the concentration 16–32 µg/ml⁻¹, which is much higher than MIC values for the ITC-susceptible bacteria. Also, SAR for cytotoxicity was less sharp than for bacterial growth inhibition (compare Fig. 3 with Tables 1–4). An example could be MICs for *B. subtilis* (8, 0.5, and 8 µg/ml⁻¹ for ITC **2**, **37**, and **45** µg/ml⁻¹ respectively) and the concentrations of the same compounds reducing 2-fold the number of survived blood monocytes (32, ~22, and >32 µg/ml⁻¹).

An attempt to select ITC-resistant mutants

Developing resistance to growth inhibitors is an inherent property of most microorganisms. Usually resistance is developed through selection of bacteria that have pre-existing mutations abolishing the binding of a drug, or the enhanced expression of the efflux systems that removes a drug from the cell. The frequency of resistant mutations is specific to both the tested microorganism and a particular drug. To the best of our knowledge, no studies have been conducted so far on resistance to ITCs. We attempted to select pre-existing resistance mutants to one of the most potent ITC **37** by plating *B. cereus*, *S. aureus*, *M. bovis* and *C. albicans* suspension on agar in the presence of the ITC compound. Optimistically, the

frequency of resistance mutations was below the detection limit (less than 10^{-9} – 10^{-11}). Notably, we were able to see the resistant mutants as countable separate colonies at the same growth conditions when rifampicin was used as control drug at MIC concentrations.

DISCUSSION

SAR for ITCs in microbial growth inhibition

As follows from the testing, ITC growth-inhibitory activity was much higher against G^+ , than towards G^- bacterial species (Tables 1 and 2). SAR for *B. cereus* has identified the general structural determinants of ITC compounds that strongly affect antimicrobial activity (Table 1 and Fig. 2). They include: **i**) the size of the organic radical attached to ITC group, which is evident from the poor performance of ITC derivatives with small-size substituents (ITC **8**, **22** and **7**) compared to those of bigger size; **ii**) the presence of a methylene group adjacent to ITC group in aromatic compounds, which follows from the comparison of the activity of ITC **1** and **4**, as well as **3** and **4**. We also showed the importance of presence (in some types of compounds) of a polar uncharged group at distance 3 – 8 carbons from ITC group as follows from comparing the activities of the ITC **1** and **2**; **7** and **18**; **1** and **30**; **17** and **32**; **17** and **20**. This is also supported by high antimicrobial activity of sulforaphane (Fahey et al., 2002), which can be viewed as derivative of ITC **7** with methylsulfoxide substitution at terminal methyl group. The presence of negatively charged group strongly reduced the inhibitory activity (compare ITC **5** and **6**), most likely affecting the cellular uptake.

Bi-functional compounds with two ITC groups show more than 2-fold increase of the activity compared to corresponding mono-functional compounds (compare ITC **1** and **37**; **7** and **38**; **4** and **16**; **1** and **16**), displaying strong synergistic effect. In this class of compounds general lipophilicity of the linker seems to be a factor contributing to their activity. Indeed, the substitution of two methylene groups by polar oxygen atoms in ITC **42**, while preserving the size and geometry of the molecule, created less active ITC **43**. Notably, significant difference of ITC SAR was observed for various microorganisms. Indeed, with *B. cereus* ITC **37** was much more active than ITC **45**, but the activity of the ITCs was reversed in the case of mycobacteria (compare Table 1 and Table 3). Also, ITC **33** was highly active against *B. cereus*, while displaying poor performance with fungi and mold (compare Tables 1 and 4). Finally, ITC **30** was much more efficient against fungi and molds than against bacteria in relation to ITC **37** (which displayed comparable activity with both bacterial and fungal pathogens) as seen from Tables 1, 3, and 4. These species-specific effects can be explained by the different efficiency of ITC cellular uptake in the tested microorganisms or by different cellular target(s) for the ITCs.

Notably, in case of *Mycobacterium tuberculosis* some compounds were more active against MDR, than against drug susceptible clinical isolates. This can be explained by reduced fitness of the most of MDR strains, since resistance mutations in many cases affect normal functioning of a targeted enzyme or protein (Comas, 2011).

Establishment of SAR for ITC provides guidance for the future design of more efficient inhibitors. For example, it can be predicted that highly active compound ITC **37** can be further improved by introducing a nitrogen atom into the aromatic ring, since this modification strongly augments the general antimicrobial activity of corresponding mono-functional ITC (compound **1**). Also, further enhancement of the activity can be expected for hydroxy-ITC derivatives with longer carbon spacer.

Generally, this study yielded efficient antimicrobials active against pathogenic bacteria, fungi, and yeast. The activity of the best of new compounds was 4–32 fold higher than that for previously described ITCs.

The mechanism of ITC inhibitory activity

The actual mechanism by which ITCs inhibit the growth of microbial pathogens remains unknown. The presence of an ITC group is the most crucial factor determining the compounds activity, since related cyanate, or thiocyanate derivatives (compounds **23** and **25**, respectively) do not inhibit bacterial growth. It has been suggested that ITC group covalently cross-links to a cellular target (Tajima et al 2003). ITC compounds can target a unique protein or limited number of proteins (that possess a vital function) by modifying a group crucial for the activity of the targeted protein. As such, it can be envisioned that a group adjacent to the reactive ITC function can also contribute to inhibition through establishing additional interactions, thereby facilitating the binding of the compound in the vicinity of the targeted aminoacid residue and increasing the crosslinking efficiency. In this way the process can be considered as a case of affinity modification. This mechanism is supported by: i) deleterious effect of ITC group inactivation by pre-incubation of ITCs with sulfhydryl compounds (Adesida et al 1996; present study) or just in culture medium, on growth-inhibitory potency; ii) strong influence of the structure and size of the substituent adjacent to ITC group on antimicrobial activity of the compounds. Indeed, ITC with small carrier groups display poor activity (since they might not provide substantial additional interaction with the targets), while ITCs with larger groups are much more potent. Additional studies are required to confirm this mechanism.

The SAR for ITC compounds does not allow for simple interpretation, as it could reflect the interaction of an ITC with a target, involvement of multiple targets, the efficiency of cellular uptake, or all of the above. G^- bacteria (generally having less permeable membranes) are more resistant to ITC inhibitors than G^+ type, suggesting intracellular localization of a target. This is consistent with elimination of the growth-inhibitory activity of ITC **18** by phosphorylation at hydroxyl group leading to ITC **6**, which renders the compound less permeable due to acquisition of two negative charges.

Strong positive effect of uncharged polar groups (hydroxyls, methoxy-, or heterocyclic nitrogens) on ITC activity detected for ITC **2**, **3**, **18**, **20**, **30**, and **32**, could be explained by favorable interactions with the target. Alternatively, these groups could maintain an optimal solubility of ITC in the intracellular matrix, since highly lipophilic compounds would be retained by cellular membrane, preventing ITC diffusion inside the cell.

To test whether microbial species develop resistance to ITC we attempted to select pre-existing resistance mutants in large population of microbial species. However, none of the tested three bacterial and one fungal cell populations contained mutants resistant to ITC **37**. This could be explained either by high evolutionary conservation of the ITC binding site on the cellular target(s), or by presence of multiple targets, so that the incidence of organisms having simultaneous resistance mutations in all targets, is very low. Given high dependence of microbial resistance on various conditions (e.g. concentration of compound and microorganisms, time, species, growth phase of organisms, etc.), additional studies with various ITCs and microbial species are required to generalize the above finding. High antimicrobial activity of some ITC synthesized in this study and their moderate toxicity grants further evaluation of these compounds as potential medicinal drugs as well as candidates for industrial applications (e.g. food and wood preservation).

The next challenge in developing more active and specific ITC compounds is the identification of cellular targets in various microbial pathogens, followed by co-

crystallization of the targeted molecules with ITC compounds. These data can provide the structural information about the interactions of the ITC with the target, necessary for rational drug design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant RO1 GM-30717-21 for AM. We are grateful to Dr. Krasnoperov (NJIT) for introduction to gas chromatography and MS analysis.

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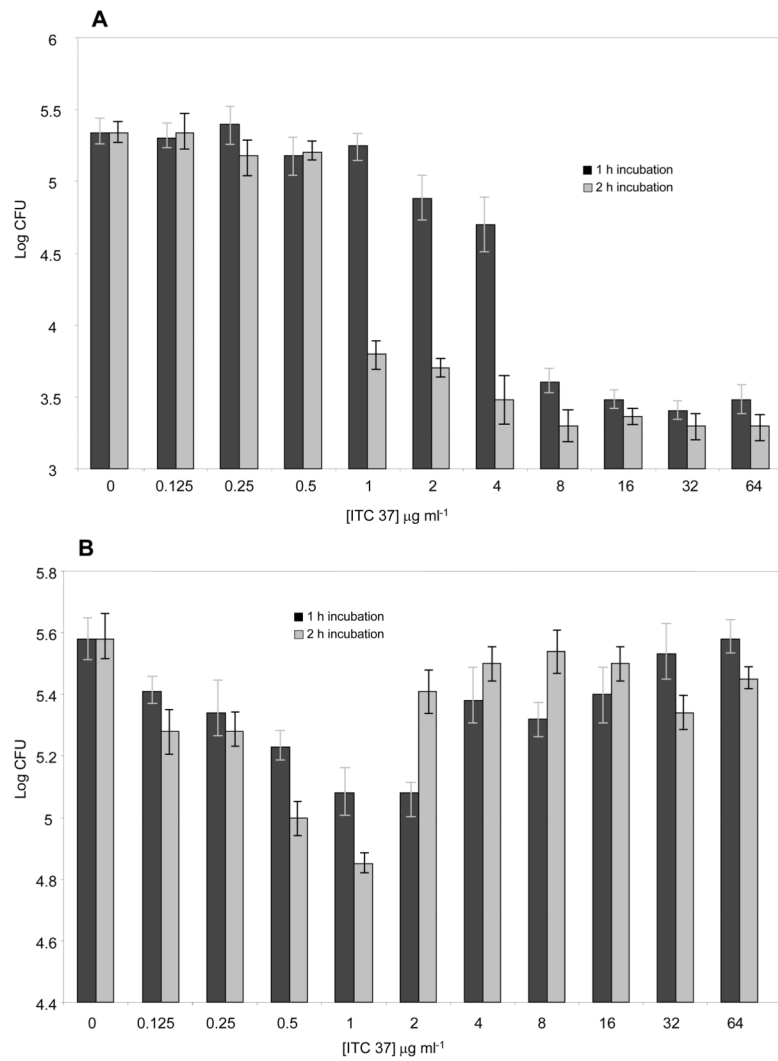


Fig. 1. Effect of incubation of *B. cereus* (A) and *S. aureus* (B) with various concentrations of ITC 37 on cell viability. Solid bars correspond to 1 h incubation, shaded bars – 2 h incubation.

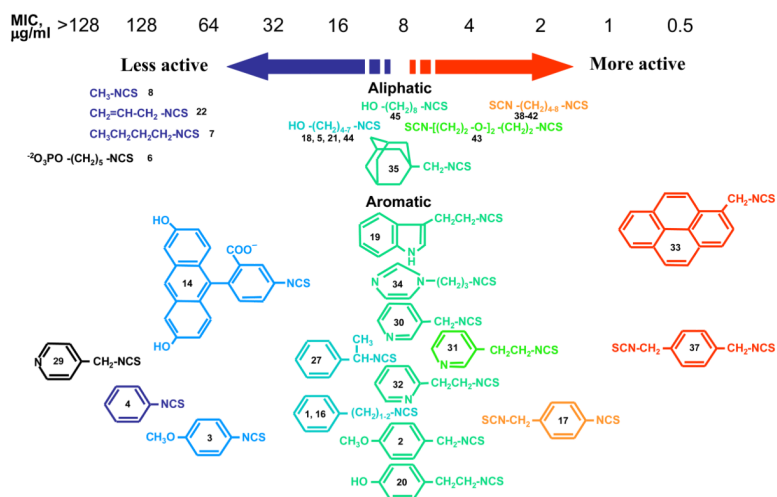


Fig. 2. Growth inhibitory activity of ITC compounds against *B. cereus*. The compounds are numbered as in Table 1. The ITCs are colored and positioned according to their growth inhibitory potency indicated on the top.

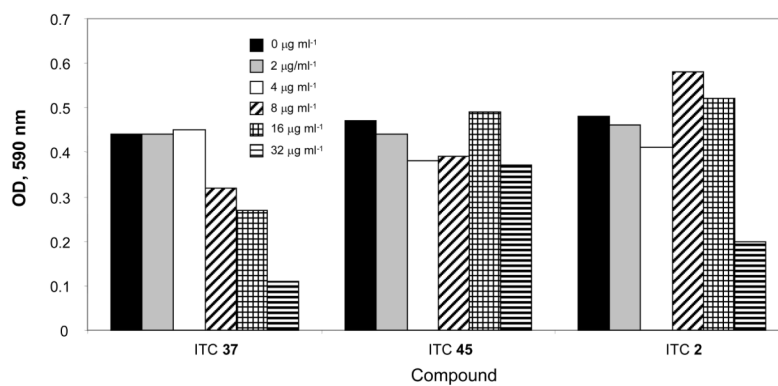





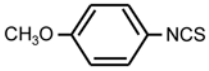
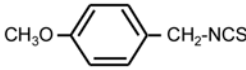
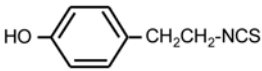
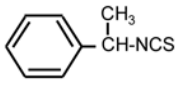
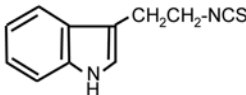
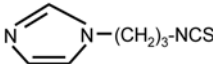
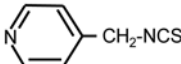
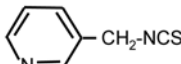
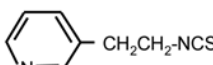
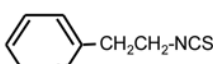
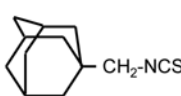
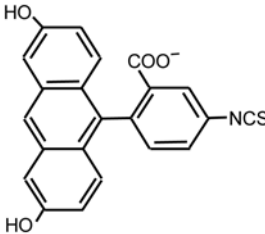


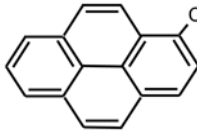
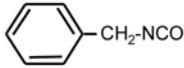
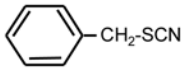
Fig. 3. Cytotoxicity of ITC on human monocytes. The cells were incubated with an ITC at various concentrations for 24 h and their viability was tested using Cell Proliferation kit (Promega). The optical density reflects the degree of cell viability.

Table 1

Structure and growth-inhibitory activity of ITC and related compounds against *B. cereus*.

#	Structure	MIC, $\mu\text{g ml}^{-1}$		Synthetic protocol
		<i>E. coli</i>	<i>B. cereus</i>	
Aliphatic ITC derivatives				
8.	CH ₃ -NCS	>128	128	*
22.	CH ₂ =CH-CH ₂ -NCS	>128	128	*
7.	CH ₃ CH ₂ CH ₂ CH ₂ -NCS	>128	128	*
Hydroxy- ITC derivatives				
18.	HO-(CH ₂) ₄ -NCS	>128	16	*
5.	HO-(CH ₂) ₅ -NCS	>128	16	*
21.	HO-(CH ₂) ₆ -NCS	>128	16	*
44.	HO-(CH ₂) ₇ -NCS	>128	16	*
45.	HO-(CH ₂) ₈ -NCS	>128	8	I
Charged ITC derivative				
6.	⁻² O ₃ PO-(CH ₂) ₅ -NCS	128	>128	III
Bifunctional ITC derivatives				
38.	SCN-(CH ₂) ₄ -NCS	>64	2	I
39.	SCN-(CH ₂) ₅ -NCS	>64	2	I
40.	SCN-(CH ₂) ₆ -NCS	>64	2	I
41.	SCN-(CH ₂) ₇ -NCS	>64	2	I
42.	SCN-(CH ₂) ₈ -NCS	>64	2	I
43.	SCN-[(CH ₂) ₂ -O] ₂ -(CH ₂) ₂ -NCS	>64	4	I
37.	SCN-CH ₂ -  -CH ₂ -NCS	8	0.5	I
16.	SCN-CH ₂ -  -NCS	64	2	I
Aromatic and heterocyclic ITC derivatives				
4.	 -NCS	>128	>128	*
1.	 -CH ₂ -NCS	64	16	*
17.	 -CH ₂ CH ₂ -NCS	>128	16	*

#	Structure	MIC, $\mu\text{g ml}^{-1}$		Synthetic protocol
		<i>E. coli</i>	<i>B. cereus</i>	
3.		>128	64	*
2.		64	8	*
20.		128	8	*
27.		>128	16	I
19.		>128	8	I
34.		>128	8	II
29.		>128	>128	II
30.		>128	8	II
31.		>128	4	II
32.		128	8	II
35.		>128	8	I
14.		>128	64	**

#	Structure	MIC, $\mu\text{g ml}^{-1}$		Synthetic protocol
		<i>E. coli</i>	<i>B. cereus</i>	
33.		>128	0.5	I
Control compounds				
23.		>128	>128	**
25.		>128	>128	**

* Known compounds

Table 2

Activity (MIC, $\mu\text{g ml}^{-1}$) of selected ITC against representative bacterial species.

Bacteria	Gram type	ITC compound							
		2	20	37	41	42	44		
<i>Acinetobacter</i> sp., BK12786	-	>64	>64	>64	32	>64	64		
<i>Acinetobacter</i> sp., BK12787	-	>64	>64	>64	32	>64	64		
<i>Enterobacter cloacii</i> BK12313	-	>64	>64	>64	>64	64	>64		
<i>Enterobacter cloacii</i> BK12315	-	>64	>64	>64	>64	64	>64		
<i>Klebsiella pneumoniae</i> BK12260	-	>64	>64	>64	>64	>64	>64		
<i>Klebsiella pneumoniae</i> BK12261	-	>64	>64	>64	>64	>64	>64		
<i>Pseudomonas aeruginosa</i> BK12347	-	>64	>64	>64	64	>64	>64		
<i>Pseudomonas aeruginosa</i> BK12348	-	>64	>64	>64	64	>64	>64		
<i>Serratia marcescens</i> BK12421	-	>64	>64	64	>64	64	>64		
<i>Serratia marcescens</i> BK12426	-	>64	>64	64	>64	64	>64		
<i>Shigella</i> sp., BK 12440	-	64	32-64	16	64	>64	64		
<i>Shigella</i> sp., BK 12441	-	32-64	32-64	16	64	>64	64		
<i>Bacillus subtilis</i> BK11590	+	32-64	16-32	4	2	2	4		
<i>Enterococcus</i> sp., BK11936	+	64	32	16	8	8	32		
<i>Enterococcus</i> sp., BK11940	+	64	32	16	8	8	32		
MRSA (<i>Staphylococcus aureus</i>) BK12060	+	32-64	32	8	4	2-4	8		
MRSA (<i>Staphylococcus aureus</i>) BK12064	+	64	32	4	4	4	16		
<i>Staphylococcus epidermidis</i> BK12798	+	32	16-32	0.5	4	2	8		
<i>Staphylococcus epidermidis</i> BK12799	+	32	16-32	0.5	1	2	8		
<i>Streptococcus</i> sp., BK12233*	+	4	2	<0.5	1-2	1-2	ND		
<i>Streptococcus</i> sp., BK12235*	+	8	4	<0.5	1-2	2	ND		

* Determined on Tryptic Soy agar medium due to the poor growth on Muller-Hinton medium

BK# indicates the number in PHRI TB Center Bacterial Strains Collection

ND – not determined

Table 3

Activity (MIC, $\mu\text{g ml}^{-1}$) of selected ITC against mycobacteria

<i>Mycobacterium</i>	Compound										
	2	20	30	37	40	41	42	45	Rif		
<i>M. intracellulare</i>	32	32	32	32	32	32	32	4	0.5		
<i>M. avium</i>	32	32	32	32	32	32	32	4	0.5		
<i>M. scrofulaceum</i>	32	32	32	16	32	32	32	8	0.5		
<i>M. cansassi</i>	32	32	32	4	8	8	4	1	0.5		
<i>M. fortuitum</i>	32	16	16	32	16	32	32	4	>4		
<i>M. tuberculosis W4</i>	ND	ND	ND	2	2	2	4	1	0.5		
<i>M. tuberculosis 210</i>	ND	ND	ND	2	2	4	4	1	0.5		
<i>M. tuberculosis W*</i>	8	4	4	1	0.5	2	4	1	>4		
<i>M. tuberculosis H37Rv</i>	32	16	32	2	2	2	4	1	0.5		
<i>M. tuberculosis CDC1561</i>	32	16	16	4	4	2	8	2	0.5		
<i>M. tuberculosis P*</i>	8	4	4	2	2	4	4	2	>4		
<i>M. tuberculosis AB</i>	ND	ND	ND	2	4	2	4	2	0.5		
<i>M. bovis BCG</i>	ND	ND	ND	2	2	2	4	0.5	0.5		

* MDR strains

ND – not determined

Table 4

Activity (MIC, $\mu\text{g ml}^{-1}$) of selected ITC on pathogenic yeast and fungi.

Fungus, strain	Compound										
	2	20	30	33	37	41	45	45	45	45	Fluconazole
<i>Candida albicans</i> 5108 ^{ndr}	2	4	0.5	>64	0.5	8	4	4	4	4	32
<i>Candida crusei</i> 6258 ^{ndr}	2	ND	0.5	>64	0.5	2	4	4	4	4	32
<i>Candida parapsilosis</i> 22019	4	ND	1	32	0.5	2	8	8	8	8	2
<i>Candida albicans</i> 90028	4	ND	2	32	0.5	8	8	8	8	8	0.25
<i>Candida albicans</i> 90030	4	ND	1	8	0.5	<1	4	4	4	4	4
<i>Candida dlabrata</i> 250 ^{ndr}	1 ^{**}	ND	2 ^{**}	64	0.125 ^{**}	<1	4 ^{**}	4 ^{**}	4 ^{**}	4 ^{**}	>64
<i>Cryptococcus neoformans</i> CNH99	<1	ND	1	4	<1	<1	<1	<1	<1	<1	4
<i>Aspergillus fumigatus</i> Rit 1	8	4	4	>64	0.5	4	8	8	8	8	>64
<i>Aspergillus fumigatus</i> Rit 11	8	4	4	>64	0.5	4	8	8	8	8	>64
<i>Aspergillus fumigatus</i> Rit 21	8	4	4	>64	0.5	4	8	8	8	8	>64

^{**} MICs after 48 hours of growth

ND – not determined