Full Paper

Synthesis and Biological Evaluation of 2-Mercapto-1,3-benzothiazole Derivatives with Potential Antimicrobial Activity

Carlo Franchini1, Marilena Muraglia1, Filomena Corbo1, Marco Antonio Florio1, Antonia Di Mola1, Antonio Rosato1, Rosanna Matucci2, Marta Nesi2, Francoise van Bambeke3, Cesare Vitali1

1 Department of Pharmaceutical Chemistry, University of Bari, Bari, Italy
2 Department of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy
3 Department of Pharmaceutical Sciences, Cellular and Molecular Pharmacology Unit, Catholic University of Louvain, Bruxelles, Belgium

The enhancement of bacterial resistance of pathogens to currently available antibiotics constitutes a serious public health threat. So, intensive efforts are underway worldwide to develop new antimicrobial agents. To identify compounds with a potent antimicrobial profile, we designed and synthesized low molecular weight 2-mercaptobenzothiazole derivatives 2a–2l and 3a–3l. Both series were screened for in-vitro antibacterial activity against the representative panel of Gram-positive and Gram-negative bacteria strains. The biological screening identified compounds 2e and 2l as the most active ones showing an interesting antibacterial activity with MIC values of 3.12 μg/mL against Staphylococcus aureus and 25 μg/mL against Escherichia coli, respectively. The replacement of the S-H by the S-Bn moiety resulted in considerable loss of the antibacterial action of the 3a–3l series. The antibiotic action of compounds 2e and 2l was also investigated by testing their activity against some clinical isolates with different antimicrobial resistance profile. Moreover, the involvement of the NorA efflux pump in the antibacterial activity of our molecules was evaluated. Finally, in this paper, we also describe the cytotoxic activity of the most interesting compounds by MTS assay against HeLa and MRC-5 cell lines.

Keywords: Antibacterial activity / Benzothiazole / Efflux pump / MTS assay

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Introduction

The current interest in the development of new antimicrobial agents can be partially ascribed both to the increasing emergence of bacterial resistance to antibiotic therapy and to newly emerging pathogens [1, 2]. Despite advances in antibacterial therapy, many problems remain to be solved for most available antimicrobial drugs. For example, in the hospital setting, the re-emergence of Gram-negative pathogens is of major concern. In fact, the most important cases of sepsis were caused by virulent Gram-negative bacteria such as Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, and Enterobacter spp. [3, 4]. Furthermore, emerging resistance among new pathogens such as Acinetobacter baumanii, and also the appearance of multidrug resistant Gram-positive bacteria, in particular, methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococci, are causing a serious menace to public health. Therefore, the development of new and different antimicrobial drugs is a very important goal, and most of the research program efforts in this field are directed towards the design of new agents. A review of the recent literature revealed that many effective antimicrobial agents show a heterocyclic moiety within their structure [5] and, in particular, that substituted benzimidazole, benzoxazole,
and benzothiazole derivatives bring different biological properties such as chemotherapeutical, antibacterial, antifungal, and antiviral activities, with a low toxicity for the antimicrobial therapeutic use in man [6–8]. Structure-activity relationship (SAR) studies carried out on these types of heterocycles have shown that positions 2 and 6 are crucial for antibacterial activity against Gram-positive and Gram-negative bacteria strains [9].

All these observations prompted us to start a research program for the synthesis of small molecules potentially useful as antimicrobial agents. After a careful screening of various heteronuclei, we have chosen to focus our attention on benzothiazole derivatives. In particular, we synthesized 2-mercaptobenzothiazole derivatives 2a–2f and 3a–3l in order to explore the effects of substituents at positions 2 and 6 on the antibacterial activity. Especially, the main objective of our program was to investigate how the potency and selectivity against different Gram-positive (Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis) and Gram-negative (Escherichia coli, Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa) bacteria, can be modulated by the replacement of the hydrogen at the 6-position of the heterocyclic nucleus with groups that can generate electronic and electrostatic effects as well as different steric properties. In addition, we also investigated the role of lipophilicity on the antibacterial activity through the synthesis of the benzylated series 3a–3l. Although compounds of the 2a–2l series are commercially available, they have been included in our research program to screen their antimicrobial profile in order to have more data for SAR (structure-activity relationship) proposal. Because of the prohibitive price, we synthesized compounds 2a–2f ex novo in short reaction time, with very good yields and in a cheap synthetic procedure.

In the present work, we report the synthesis of two series of 2-mercaptopbenzothiazoles 2a–2f and 3a–3l and their preliminary antibacterial profile against different Gram-positive and Gram-negative bacterial strains belonging to American Type Culture Collection (ATCC). The most active compounds of the series were also studied by using clinical isolates S. aureus with different antibiotic resistance profile. Finally, the most interesting molecules were also characterized with regard to their cytotoxic effects by testing them against a human cervical cancer cell line (HeLa) and a normal human lung fibroblasts cell line (MRC-5).

Results and discussion

Chemistry

The synthetic routes of compounds 1d, 2a–2f, and 3a–3l are reported in Scheme 1. The 6-substituted-2-mercaptop-
Mercaptobenzothiazole Derivatives: Antibacterial Activity

Table 1. Antimicrobial activity results\(^a\) of 2-mercaptobenzothiazole derivatives 2a–2l (MIC in \(\mu\)g/mL).

<table>
<thead>
<tr>
<th>Compd R</th>
<th>S. a.(^b)</th>
<th>S. a.(^b)</th>
<th>S. a.(^b)</th>
<th>E. f.(^c)</th>
<th>E. f.(^c)</th>
<th>E. f.(^c)</th>
<th>B. s.(^d)</th>
<th>B. s.(^d)</th>
<th>B. s.(^d)</th>
<th>E. c.(^e)</th>
<th>E. c.(^e)</th>
<th>E. c.(^e)</th>
<th>A. b.(^f)</th>
<th>K. p.(^g)</th>
<th>P. a.(^h)</th>
</tr>
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<tbody>
<tr>
<td>2a</td>
<td>CH(CH(_3))(_2)</td>
<td>12.5</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>6538P</td>
</tr>
<tr>
<td>2b</td>
<td>Cl</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>29213</td>
</tr>
<tr>
<td>2c</td>
<td>CH(_3)</td>
<td>25</td>
<td>25</td>
<td>50</td>
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<td>100</td>
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<td>50</td>
<td>25</td>
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<td>50</td>
<td>R</td>
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<tr>
<td>2e</td>
<td>CF(_3)</td>
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<td>6.25</td>
<td>3.12</td>
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<td>100</td>
<td>50</td>
<td>50</td>
<td>100</td>
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<td>R</td>
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<td>F</td>
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<td>100</td>
<td>25</td>
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<tr>
<td>2h</td>
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<td>R</td>
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<td>R</td>
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<tr>
<td>2i</td>
<td>CH(CH(_3))(_2)</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>R</td>
<td>R</td>
<td>50</td>
<td>50</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>R</td>
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<td>R</td>
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<tr>
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<td>12.5</td>
<td>100</td>
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</tr>
<tr>
<td>NRF*</td>
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<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>2</td>
<td>0.125</td>
<td>0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>4</td>
<td>0.25</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
<td>29213</td>
</tr>
</tbody>
</table>

\(^a\) Antimicrobial activity was estimated by using NCCLS assay \([12]\).

Abbreviations: \(^b\) S. a., S. Aureus; \(^c\) E. f., E. Faecalis; \(^d\) B. s., B. Subtilis; \(^e\) B. c., B. Cereus; \(^f\) E. c., E. Coli; \(^g\) A. b., A. Baumannii; \(^h\) K. p., K. Pneumoniae; \(^i\) P. a., P. aeruginosa.

* NRF, norfloxacin; R, resistant.

Acceptable quality-control range of MICs for reference strains \([NCCLS M7-A4]: S. aureus ATCC 29213: 0.5-2; E. faecalis ATCC 29212: 2-8; P. aeruginosa ATCC 27853: 1-4."

Benzothiazole derivatives 2a–2f were prepared according to the literature procedure \([10]\). Reaction of the appropriate anilines 1a–1c, 1e, 1f, commercially available, with potassium ethyl xanthate in DMF gave the corresponding 6-substituted-1,3-benzothiazole-2-thiols 2a–2c, 2e, 2f, respectively. Compounds 2g–2l are commercially available.

The 3a–3l series was prepared by the treatment of 6-substituted-1,3-benzothiazole-2-thiols 2a–2c, 2e, 2f with benzyl bromide, K\(_2\)CO\(_3\), dissolved in a mixture of dioxane/water, providing the corresponding 2-benzylthio-6-substituted-1,3-benzothiazoles derivatives.

The starting 2-bromo-4-methoxyaniline 1d was synthesized according to a reported methodology via a bromination strategy by using tetrabuthylammonium tribromide (Bu\(_4\)NBr\(_3\)) following a synthetic procedure described in the literature \([11]\).

As it is outlined in Scheme 1, compounds 2a–2f and 3a–3l were synthesized in high yields. The structures of the obtained compounds were elucidated by spectral data.

**Biological evaluation**

The antibacterial activity of compounds 2a–2l was evaluated in vitro against an assortment of Gram-positive and Gram-negative bacteria belonging to the ATCC collection. All MIC determinations were carried out using NCCLS guidelines \([12]\). MIC values are given in \(\mu\)g/mL and were compared to MIC values for the standard antibacterial drug norfloxacin. Screening results are summarized in Table 1.

The combined data showed that compounds 2a–2l exerted inhibitory activity against the tested bacterial strains with MIC values between 3.12 and 100 \(\mu\)g/mL. The obtained results generally indicate that most of the tested molecules are more active against Gram-positive than Gram-negative bacterial strains. Among the mentioned derivatives 2a–2l, the most promising results were obtained with compounds 2e and 2l. In particular 2e was the most active derivative giving the best antibacterial activity against S. aureus with a MIC value of 3.12 \(\mu\)g/mL. On the other hand, compound 2l showed a wide antimicrobial activity toward Gram-positive such as S. aureus (MIC: 12.5 \(\mu\)g/mL) and Gram-negative such as E. coli (MIC: 25 \(\mu\)g/mL). Furthermore, it is noteworthy that the data registered in Table 1 reveal that 2a–2l derivatives generally have a significant influence on the antibacterial profile of S. aureus. In this series, compounds 2a, 2b, 2d, 2h, and 2l were found to inhibit S. aureus at a MIC
value of 12.5 μg/mL. In addition, compounds 2c, 2d, and 2g revealed moderate antimicrobial activity against Gram-negative bacteria such as E. coli strains.

With regards to the antimicrobial activity, the behaviour of the 3a—3l series was very different compared with the 2a—2l series: under the same experimental conditions it was found that derivatives having a thiobenzyl group at the 2-position of the heterocyclic nucleus did not inhibit the bacterial growth in spite of biological results previously observed on benzothiazole derivatives [9].

Due to both the small numbers of evaluated compounds and the low diversity of the involved chemical features of the series reported herein, an attempt to analyse the structure-activity relationships does not seem reasonable. Several comparisons on the results could be made. Initially, it should be noted that small structural changes at the 6-position do not significantly alter the antibacterial activity, except for compounds 2e and 2l carrying a trifluoromethyl group and a nitro group at the 6-position of the 2-mercaptop 1,3-benzothiazole, respectively. In detail, the biological results identified compound 2e as a potent and selective inhibitor of S. aureus strains (MIC: 3.12 μg/mL) and whereas compound 2l was less active than 2e against Gram-positive bacteria, it was able to inhibit the growth of Gram-negative microorganism such as E. coli (MIC: 25 μg/mL).

By evaluating the biological results, it is possible to observe that the presence of a hydrogen atom (2g) or a methyl group (2c) at the 6-position on the heterocyclic nucleus, preserves a wide spectrum of action. In addition, it is interesting to consider that an increase of steric hindrance, though an increment of lipophilicity, probably could lead to a loss of activity against Gram-negative strains, as observed for compound 2a.

Moreover, the displacement of the hydrogen atom (2g) at the 6-position with chlorine (2b) and fluorine (2f) atoms as well as with methoxy (2d) and ethoxy (2i) groups generally produced an increment in the activity against S. aureus with MIC values in the range of 12.5 to 100 μg/mL. Furthermore, the introduction of a polar group such as the aminic one (2h) led to an interesting antibacterial activity against Gram-positive strains.

Because among the mentioned derivatives a remarkable activity was registered against S. aureus strains, we focused our attention on our best molecules, 2e and 2l, to investigate their antimicrobial profile against seven clinical isolates of S. aureus belonging both to NARSA and private collections with a different antimicrobial resistance profile as detailed in the experimental section. In Table 2 the microbiological data obtained according to NCCLS protocol [12] are reported and compared with norfloxacin (NRF), vancomycin (VAN), and oxacillin (OXA) used as standard drugs. From the results reported in Table 2, it appears that compound 2e generally revealed better growth inhibitory effects against the clinical isolates than compound 2l. In particular, compound 2e was surprisingly efficacious against two vancomycin-resistant clinical isolate strains (VRS2 and NRS52) which were close to VAN in the control experiments. In detail, compound 2e displayed an interesting antibacterial activity against VRS2, a vancomycin fully resistant strain, showing a MIC value three-fold smaller than the MIC value registered for the commercially available drug VAN (MIC values: 8 μg/mL vs. 64 μg/mL, respectively). Additionally, compound 2e was active against NRS52, a vancomycin intermediate resistant strain (VISA), having a MIC value of 8 μg/mL comparable to the reference drug VAN.

To further investigate the antibacterial effects in several clinical isolates, molecules 2e and 2l were tested against two different methicillin-resistant bacteria strains (MRSA) such as N4120032 and N4112910. As shown in Table 2, both compound 2e and 2l possessed interesting antimicrobial effects against N4112910 bacteria strain in comparison to the control drugs NRF and VAN. The biological data reported in Table 2 indicate that compound 2e was more active against N4112910 rather than N4120032 when compared to OXA, showing MIC values of 8 μg/mL and 16 μg/mL, respectively. Despite this relevant data, it is worthy to note that compound 2e inhibited the growth of the clinical isolate N4120032 with a MIC value similar to the reference drug OXA. Taking into account that the last-mentioned bacteria strain belongs to the HA-MRSA family characterized by high morbidity and mortality degrees, data registered for compound 2e appear to be very significant.

Moreover, compound 2e showed a remarkable antimicrobial profile against the multi-drug resistant strain NRS100, an oxacillin/tetracycline-resistant strain, with a MIC value of 16 μg/mL in comparison to 256 μg/mL of OXA.

Table 2. Antimicrobial activity results (MIC in μg/mL) of compounds 2e and 2l against clinical isolates of S. aureus.

<table>
<thead>
<tr>
<th></th>
<th>2e</th>
<th>2l</th>
<th>NRF[a)</th>
<th>VAN[b)</th>
<th>OXAc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. a.</td>
<td>25923</td>
<td>4</td>
<td>15</td>
<td>n.e.</td>
<td>n.e.</td>
</tr>
<tr>
<td>VRS2</td>
<td>8</td>
<td>32</td>
<td>128</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>NRS52</td>
<td>8</td>
<td>16</td>
<td>256</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>N4120032</td>
<td>16</td>
<td>32</td>
<td>256</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>N4112910</td>
<td>8</td>
<td>32</td>
<td>256</td>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>NRS100</td>
<td>16</td>
<td>32</td>
<td>1</td>
<td>1</td>
<td>256</td>
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<tr>
<td>STA268</td>
<td>8</td>
<td>32</td>
<td>1</td>
<td>1</td>
<td>n.e.</td>
</tr>
</tbody>
</table>

[a) NRF, Norfloxacin.
[b) VAN, Vancomycin.
[c) OXA, Oxacillin.
Finally, the antibacterial activity exhibited by both compound 2e and 2l against STA268, a potentially lethal strain producing the Panton–Valentine Leukocidin (PVL+) [13], seems to be negligible in comparison to NRF and VAN.

Results obtained during our preliminary investigation caused us to carry out additional tests using multidrug-resistant bacteria SA-1, a modified S. aureus strain that overexpresses the NorA, the most studied efflux pump at the present time [14].

Generally, the overexpression of multidrug-resistance (MDR) efflux pumps confer clinically relevant resistance to antibiotics (e.g., fluoroquinolones), dyes, detergents, and disinfectants. In particular, the typical substrate profile of NorA includes quinolones, chloramphenicol, and several unrelated substances [15].

The literature clearly describes a detailed biological assay used to evaluate the NorA involvement in the mechanism of antibacterial multidrug resistance, by using reserpine as NorA blocker [16, 17]. In fact, this alkaloid seems to be able to inhibit multidrug transporters like NorA, increasing the intracellular concentration of fluoroquinolones, thus potentially lowering MICs.

In light of this evidence, we investigated the involvement of the NorA efflux pump in the mechanism of action of our best molecules 2e and 2l by using the biological assay reported before and detailed in the experimental section, herein. The antimicrobial results exhibited by compounds 2e and 2l against both wild-type and SA-1 bacteria strains were compared with the control drug NRF as reported in Table 3. By reading Table 3, it is clear that the standard drug NRF produced a lower SA-1 bacteria growth in the presence, rather than in absence, of reserpine (MIC values: 4 µg/mL vs. 16 µg/mL, respectively) suggesting that the latter is a good substrate for the NorA efflux pump. Herein, we also noticed that molecules 2e and 2l exhibited comparable inhibition of bacteria growth in absence so as in presence of reserpine indicating that these compounds are not involved in this active efflux system.

This interesting result suggests that both compounds 2e and 2l could be considered as candidates for new tools for the synthesis of antibacterial compounds acting on resistant bacterial strains overexpressing NorA efflux pump.

Finally, taking into account the well-known antimicrobial and anticancer activities of benzothiazole derivatives, structurally related to our 2-mercaptobenzothiazole compounds but, of course, with different chemical features, we also investigated the cytotoxic activity of the molecules with most meaningful antibacterial activity, 2e and 2l. This study was realized by using the MTS assay against HeLa and MRC-5 cell lines, following a 3-days exposure [18].

In general, the cancer cells were more sensitive to the tested agents. As shown in Fig. 1, the derivatives 2e and 2l tested at the single dose of 100 µM, did not produce a relevant change in cell viability in MRC-5 cells (maximal inhibition of cell growth never exceeded 25%), whereas their cytotoxic effect in HeLa cells was remarkable (about 80% of inhibition).

Data obtained confirm that our tested molecules can be considered moderately toxic for HeLa cells while they lack of any toxicity for normal cells like MRC-5.

### Conclusion

In summary, in the present study, we report the synthesis and the antimicrobial studies of 2-mercapto 1,3-benzothiazole derivatives. It was observed that the synthesized compounds substituted with a S-H moiety at the 2-position of the heterocyclic nucleus (2a–2l) favored the antibacterial activity especially against the Gram-positive strains. On the contrary, compounds bearing the S-Bn moiety 3a–3l at the 2-position of the benzothiazole nucleus did not show any antimicrobial profile.
Among the series 2a–2l, the most prominent and consistent antimicrobial activity was obtained with compound 2e (MIC: 3.12 μg/mL) carrying a trifluoromethyl moiety at the 6-position of the heterocycle. Compound 2l showed an appreciable broad spectrum of action against both Gram-positive and Gram-negative bacteria. Its MIC value (25 μg/mL) toward E. coli is very significant.

Interesting, cytotoxicity against the MRC-5 cells was not observed for compounds 2e and 2l. In light of the results presented in this work and taking into account that this preliminary study does not produce conclusive evidence regarding a structure-antibacterial relationship, we stopped our attention on the most promising compounds 2e and 2l as an interesting starting point for the development of a new class of antimicrobial agents. Therefore, the synthesis of novel 2-mercaptobenzothiazole derivatives aimed to optimize the chemical features involved in the antibacterial activity such as to investigate the mechanism of action is currently going on in our laboratory.

This work was accomplished thanks to the financial support of the Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR).

The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were recorded on Gallenkamp melting point apparatus (Weiss-Gallenkamp, London, UK) in open glass capillary tubes. The IR spectra were recorded on a Perkin-Elmer FT spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) and band positions are given in reciprocal centimeters (cm⁻¹). ¹H-NMR spectra were recorded on a FT Bruker Aspect 3000 spectrometer (Bruker Bioscience, USA) using CDCl₃ as the solvent, unless otherwise indicated. Chemical shifts are reported in part per million (ppm) relative to the solvent resonance: CDCl₃, δ = 7.26 (¹H-NMR). Amino-proton assignments were confirmed by D₂O exchange. J values are given in Hz. EIMS spectra were recorded with a Hewlett-Packard 6890-5973 MSD gas chromatograph | mass spectrometer at low resolution (Hewlett-Packard, Palo Alto, CA, USA). Elemental analyses were performed on a Eurovector Euro EA 3000 elemental analyzer (EuroVector, Milan, Italy). The data for C, H, and N were within ± 0.4 of the theoretical values for all final compounds. Silica gel chromatographic separations were performed by chromatography with silica gel (Kieselgel 60, 40–63 μm; Merck, Germany) packed in glass columns, using the technique described in the literature [19]. The weight of the silica gel was approximately 100-times that of the substance. The eluting solvent indicated in parentheses for each purification was determined by TLC performed on precoated silica gel on aluminum sheets (Kieselgel 60, F₂₅₄, Merck). TLC plates were visualized with UV light and/or in an iodine chamber. All chemicals, compounds 2g–2l included, were purchased from Aldrich Chemical Co. (Sigma-Aldrich, Germany) in the highest quality commercially available. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Only spectra for compounds not previously described are given.

Synthesis of 2-bromo-4-methoxyaniline 1d

Compound 1d was prepared according to the literature procedure [11] starting from 4-methoxyaniline (commercially available). Column chromatography (silica gel, eluent: Et₂O/petroleum ether, 4:6) of the reaction crude provided 2-bromo-4-methoxyaniline 1d as purple oil (37% yield). ¹H-NMR and MS spectra were in agreement with those reported in the literature.

General procedure for the synthesis of 6-substituted-1,3-benzothiazole-2-thiols 2a–2f

6-Substituted-1,3-benzothiazole-2-thiols 2a–2c, 2e, 2f were prepared from the reaction of the corresponding aniline derivatives 1a–1c, 1e, 1f commercially available, with potassium ethyl xanthate by using a literature procedure [10], while 2d was prepared using aniline derivative 1d (see above).

6-(1-Methylethyl)-1,3-benzothiazole-2-thiol 2a

Yield: 88%; m.p.: 133–135 °C (EtOAc); IR (CHCl₃): 3092, 2911, 1496, 1257, 895 cm⁻¹; ¹H-NMR (300 MHz, DMSO-d₆) δ: 13.9 (br s, 1H), 7.81 (s, 1H), 7.40 (d, J = 8.5 Hz, 1H), 7.24 (d, J = 8.8 Hz, 1H); MS (70 eV) m/z (%): 211 [M⁺ + 2] (8), 210 [M⁺ + 1] (10), 209 [M⁺] (72), 194 (100), 161 (15). Anal. calcd. for C₆H₅NOS₂: C, 41.38; H, 2.15; N, 6.92.

6-Chloro-1,3-benzothiazole-2-thiol 2b

Yield: 83%; m.p.: >245 °C (EtOAc/petroleum ether); IR (KBr): 3094, 2914, 1494, 1253, 893 cm⁻¹; ¹H-NMR was in agreement with the published data [10]. MS (70 eV) m/z (%): 203 [M⁺ + 2] (47), 202 [M⁺ + 1] (12), 201 [M⁺] (100), 166 (35). Anal. calcd. for C₆H₄ClNOS₂: C, 41.69; H, 2.00; N, 6.94; found: C, 41.38; H, 2.15; N, 6.92.

6-Methyl-1,3-benzothiazole-2-thiol 2c

Yield: 77%; m.p.: 180–181 °C (EtOAc/petroleum ether); IR (CHCl₃): 3094, 2914, 1494, 1253, 893 cm⁻¹; ¹H-NMR and MS were in agreement with the published data [10]. Anal. calcd. for C₆H₅ClNOS₂: C, 51.72; H, 4.07; N, 7.54; found: C, 51.76; H, 3.73; N, 7.48.

6-Methoxy-1,3-benzothiazole-2-thiol 2d

Yield: 90%; m.p.: 206–207 °C (EtOAc/petroleum ether); IR (KBr): 3036, 2936, 1495, 1296, 893 cm⁻¹; ¹H-NMR (300 MHz) δ 13.2–13.9 (br s, 1H), 7.26 (s, 1H), 7.02–6.97 (m, 2H), 3.82 (s, 3H); MS (70 eV) m/z (%): 199 [M⁺ + 2] (12), 198 [M⁺ + 1] (13), 197 [M⁺] (100), 182 (78), 154 (17). Anal. calcd. for C₆H₅ClNOS₂: C, 48.16; H, 3.66; N, 7.02; found: C, 48.02; H, 3.35; N, 6.85.

6-Trifluoromethyl-1,3-benzothiazole-2-thiol 2e

Yield: 99%; m.p.: >240 °C (EtOAc/petroleum ether); IR (KBr): 3106, 2931, 1487, 1262, 882 cm⁻¹; ¹H-NMR was in agreement with the published data [20]. MS (70 eV) m/z (%): 237 [M⁺ + 2] (12), 236 [M⁺ + 1] (13), 235 [M⁺] (100), 157 (10). Anal. calcd. for C₆H₅F₃NOS₂: C, 40.84; H, 1.71; N, 5.95; found: C, 41.08; H, 1.71; N, 5.86.

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6-Fluoro-1,3-benzothiazole-2-thiol 2f
Yield: 98%; m.p.: 227–228 °C (EtOAc/petroleum ether); IR (KBr): 3442, 2938, 1467, 1287 cm⁻¹; ¹H-NMR (300 MHz, DMSO-d₆): δ: 7.75 (s, 1H), 7.67 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 8.2 Hz, 2H), 7.38–7.25 (m, 4H), 4.61 (s, 2H); ¹³C-NMR (75 MHz, DMSO-d₆): δ: 161.81, 146.61, 135.69, 128.69, 124.04, 117.81, 114.86, 48.83; MS (70 eV) (m/z): 293 [M + 1] (61), 272 [M + 1] (21), 268 [M + 1] (100), 240 (17), 91 (100). Anal. calcd. for C₁₇H₁₀NS₂: C, 68.47; H, 5.84; N, 4.62.

General procedure for the synthesis of 2-(benzylthio)-6-substituted-1,3-benzothiazoles 3a–3f

The preparation of 2(benzylthio)-6-isopropyl-1,3-benzothiazole 3a can be taken as the reference for the synthesis of 2(benzylthio)-6-substituted-1,3-benzothiazoles 3a–3f. 6-Isopropyl-1,3-benzothiazole-2-thiol 2a (1.91 mmol) was distilled in dioxane (16 mL), and added to a solution of K₂CO₃ (2.87 mmol) in water (5 mL). The reaction mixture was stirred at 75 °C for 1.5 h. After the addition was completed, the resulting mixture was stirred for another 15 min. The dioxane was evaporated under reduced pressure and the residue was extracted with EtOAc and washed with 2 N NaOH (3 × 20 mL) up to pH 11. The organic phase was dried over Na₂SO₄, filtered, and evaporated to dryness. Column chromatography (silica gel, eluent: EtOAc/petroleum ether; 3:7) of the reaction crude afforded the desired product.

2-(Benzylsulphanyl)-6-(1-methylethyl)-1,3-benzothiazole 3b
Yield: 98%; m.p.: 80–81 °C (EtOAc); IR (KBr): 3058, 2950, 1438, 1237, 830 cm⁻¹; ¹H-NMR (300 MHz, DMSO-d₆): δ: 7.75 (s, 1H), 7.76 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 8.2 Hz, 2H), 7.38–7.25 (m, 4H), 4.61 (s, 2H); ¹³C-NMR (75 MHz, DMSO-d₆): δ: 161.81, 146.61, 135.69, 128.69, 124.04, 117.81, 114.86, 48.83; MS (70 eV) (m/z): 293 [M + 1] (61), 272 [M + 1] (21), 268 [M + 1] (100), 240 (17), 91 (100). Anal. calcd. for C₁₇H₁₀NS₂: C, 68.47; H, 5.84; N, 4.62.

2-(Benzylsulphanyl)-6-chloro-1,3-benzothiazole 3c
Purified by chromatography (acetone/petroleum ether; 3:7). IR, ¹H-NMR, and MS were in agreement with the published data [21]. Anal. calcd. for C₁₇H₁²NS₂: C, 66.38; H, 4.31; N, 5.44; found: C, 65.24; H, 4.40; N, 5.44.

2-(Benzylsulphanyl)-6-ethoxy-1,3-benzothiazole 3d
Yield: 91%; m.p.: 60 °C (EtOAc/petroleum ether; 3:7); ¹H-NMR (300 MHz, CDCl₃): δ: 7.37, 7.26, 7.09, 7.02 (s, 1H), 6.95 (s, 2H); ¹³C-NMR (75 MHz, CDCl₃): δ: 146.77, 135.65, 129.07, 123.08, 49.99, 49.87; MS (70 eV) (m/z): 292 [M + 1] (100), 273 [M + 1] (10), 258, 240, 210, 183 (100). Anal. calcd. for C₁₄H₁₀NS₂: C, 70.31; H, 4.32; N, 4.93.

2-(Benzylsulphanyl)-6-amino-1,3-benzothiazole 3e
Yield: 98%; m.p.: 102–104 °C (EtOAc); IR (KBr): 2925, 1566, 1437, 1308, 1235, 993 cm⁻¹; ¹H-NMR (300 MHz, DMSO-d₆): δ: 8.15–7.92 (m, 2H), 7.65 (d, J = 7.9 Hz, 1H), 7.45 (d, J = 7.65 Hz, 2H), 7.38–7.22 (m, 3H), 4.62 (s, 2H); MS (70 eV) (m/z): 327 [M + 2] (6), 326 [M + 1] (10), 299 [M + 1] (32), 292 (19), 238 (19), 210 (100). Anal. calcd. for C₁₄H₁₀FNS₂: C, 55.37; H, 3.10; N, 4.30; found: C, 55.40; H, 3.09; N, 4.32.

2-(Benzylsulphanyl)-6-trifluoromethyl-1,3-benzothiazole 3f
Yield: 98%; m.p.: 102–104 °C (EtOAc); IR (KBr): 2925, 1566, 1437, 1308, 1235, 993 cm⁻¹; ¹H-NMR (300 MHz, DMSO-d₆): δ: 8.15–7.92 (m, 2H), 7.65 (d, J = 7.9 Hz, 1H), 7.45 (d, J = 7.65 Hz, 2H), 7.38–7.22 (m, 3H), 4.62 (s, 2H); MS (70 eV) (m/z): 327 [M + 2] (6), 326 [M + 1] (10), 299 [M + 1] (32), 292 (19), 238 (19), 210 (100). Anal. calcd. for C₁₄H₁₀FNS₂: C, 55.37; H, 3.10; N, 4.30; found: C, 55.40; H, 3.09; N, 4.32.

Biology

Test organisms

Twelve bacteria strains belonging to the ATCC collection were used: Gram-positive such as Staphylococcus aureus ATCC 6538P, ATCC 25923, and ATCC 29213, Enterococcus faecalis ATCC 19433 and ATCC 29212, Bacillus subtilis ATCC 6633, Bacillus cereus

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SA-1, a NorA overexpressing strain, selected from Huang in the Chang Gung Children's Hospital of Taiwan and such as Panton–Valentine Leukocidin (PVL+) [13] isolated by Y. C. l

The in-vitro Minimal Inhibitory Concentrations (MICs, µg/mL) of the prepared compounds were determined by the broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003).

For antibacterial assays the microdilution method, encoded by CLSI, formerly NCCLS, using 96-wells plates (Microtiter1), was used [12]. Stock solutions of the tested compounds 2a – 2l, 3a – 3l, were obtained in absolute ethanol at a concentration of 800 µg/mL. Stock solutions of lower concentrations were prepared for those substances which did not dissolve well. Further two-fold serial dilutions in the test medium between 100 and 0.78 µg/mL were plated. In each well, 200 µL of these solutions was added. To be sure that the solvent had no an adverse effect on bacterial growth, a control test was carried out by using ethanol at its maximum concentration along with the medium. Norfloxacin (NRF) was used as the standard drug.

To evaluate the antimicrobial profile towards the clinical isolates, the microdilution method [12] was used. Stock solutions of the tested compounds 2e and 2l, were obtained in absolute ethanol at a concentration of 1024 µg/mL. NRF, VAN, and OXA were used as standard drugs. The antibacterial activity against SA-1 strain was registered in the presence as well as in absence of reserpine added at 10 µg/mL following the experimental procedure suggested by Brenwald et al. [16].

Cultures were grown on Petri dishes with Müller–Hinton agar (Merck, Darmstadt, Germany) for 24 h at 37 ± 1°C. A number of colonies were drawn using a sterile metal loop. They were then dissolved in MHB (Müller–Hinton broth) and were incubated for approximately 3 to 4 h. The absorbance of these cell suspensions calibrated at a wavelength of 625 nm using spectrophotometric method (Thermo Spectronic, Genesis 20), should be 0.08 to 0.10 for the 0.5 McFarland standard, corresponding approximately to 10⁶ CFU (Colony Forming Unit)/mL. The inocula were diluted to a ratio of 1:40 (100 µL of inocula in 3.9 mL of MHB) and 20 µL of this dilution were pipetted into each well. The final inoculum value was 2.5 × 10⁶ CFU/mL. A number of wells containing only inoculated broth as control growth were prepared. After incubation for 24 h at 37 ± 1°C, the last well containing no microbial growth was recorded to show the MIC, in values of µg/mL. The MIC were determined by using an antibacterial assay repeated twice in triplicates.

Inoculum preparation

Antimicrobial assay

The reagent (16% total well volume) was added to each well. Plates were incubated at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air. HeLa cells were subcultured three times weekly to maintain continuous logarithmic growth. MRC-5 cells were grown to confluence without subculturing; the medium was renewed twice a week. Confluent cells were harvested after trypsinization using Trypsin-EDTA solution 1X (Sigma–Aldrich).

Agent treatment

Cytotoxicity assay

The tested compounds were assayed against cell lines HeLa (human epithelial cervical cancer cells) and MRC-5 (normal human lung fibroblasts) for their cytotoxic effect, by using the MTS cytotoxicity assay a variant of the widely used MTT assay [18].

Cell culture

HeLa and MRC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 50 units/mL of penicillin G and 0.05 mg/mL streptomycin (Sigma–Aldrich), 2 mM glutamine (Sigma–Aldrich), 0.25 µg/mL amphotericin B (Sigma–Aldrich) in a humidified atmosphere consisting of 5% CO₂ and 95% air. HeLa cells were subcultured three times weekly to maintain continuous logarithmic growth. MRC-5 cells were grown to confluence without subculturing; the medium was renewed twice a week. Confluent cells were harvested after trypsinization using Trypsin-EDTA solution 1X (Sigma–Aldrich).

The MTS cytotoxicity assay (Cell Titer 96 Aq One Solution Cell Proliferation Assay, Promega), was used to screen the viability of the cells incubated with the test compounds. This assay measures cell viability and is based on the bioreduction of MTS tetrazolium into formazan by NADH and NADPH produced by dehydrogenase enzymes only in metabolically active, viable cells.

Compounds were prepared as 100 mM stock solutions, dissolved in DMSO, and stored at 4°C. For each cytotoxicity assay with HeLa cells, cells were seeded into 96-well microtiter plates at a density of 200 cells per well and allowed 24 h to adhere before drugs were introduced (final concentration 100 µM). For the cytotoxicity assay with MRC-5, 600 cells per well were seeded in 96-well microplates and allowed 24 h to adhere before drug addition (final concentration 100 µM). Compounds that exhibited activity in single-dose testing were further evaluated to generate dose-response curves, which were used to determine CC₅₀ (cytotoxic concentration able to destroy 50% of the initial cell amount). To perform dose-response curves, serial drug dilutions (final concentration 1 to 100 µM) were prepared in medium immediately prior to each assay. Cells were incubated for 72 h at 37°C in a humidified atmosphere and 5% CO₂.

The reagent (16% total well volume) was added to each well. Plates were incubated at 37°C until sufficient colour development had occurred (usually 3–4 h). The purple formazan product was then measured spectrophotometrically at 490 nm. The optical density (O.D.) value of each culture is a function of the amount of formazan produced and is proportional to the number of viable cells [18].

References