Journal Pre-proofs

Exploration of piperazine-derived thioureas as antibacterial and anti-inflammatory agents. *In vitro* evaluation against clinical isolates of colistin-resistant *Acinetobacter baumannii*

Sarah Mazzotta, Tania Cebrero-Cangueiro, Luca Frattaruolo, Margarita Vega-Holm, Marta Carretero-Ledesma, Javier Sánchez-Céspedes, Anna Rita Cappello, Francesca Aiello, Jerónimo Pachón, José Manuel Vega-Pérez, Fernando Iglesias-Guerra, María Eugenia Pachón-Ibá ñez

PII: S0960-894X(20)30522-9
DOI: https://doi.org/10.1016/j.bmcl.2020.127411
Reference: BMCL 127411

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 25 April 2020
Revised Date: 6 July 2020
Accepted Date: 12 July 2020

Please cite this article as: Mazzotta, S., Cebrero-Cangueiro, T., Frattaruolo, L., Vega-Holm, M., Carretero-Ledesma, M., Sánchez-Céspedes, J., Rita Cappello, A., Aiello, F., Pachón, J., Manuel Vega-Pérez, J., Iglesias-Guerra, F., Eugenia Pachón-Ibá ñez, M., Exploration of piperazine-derived thioureas as antibacterial and anti-inflammatory agents. *In vitro* evaluation against clinical isolates of colistin-resistant *Acinetobacter baumannii*, *Bioorganic & Medicinal Chemistry Letters* (2020), doi: https://doi.org/10.1016/j.bmcl.2020.127411

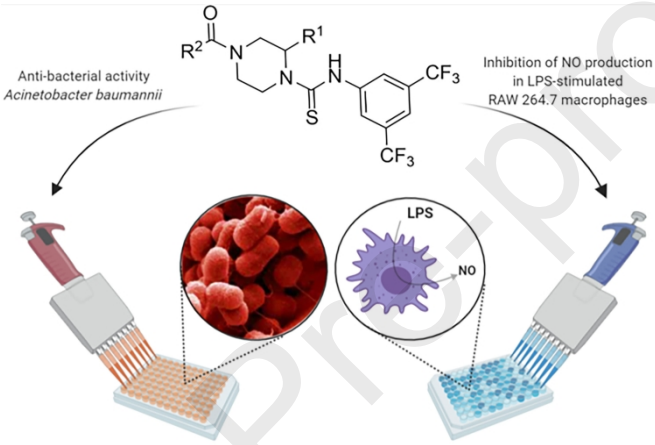
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Exploration of piperazine-derived thioureas as antibacterial and anti-inflammatory agents. *In vitro* evaluation against colistin-resistant *Acinetobacter baumannii*

Leave this area blank for abstract info.

Sarah Mazzotta, Tania Cebrero-Cangueiro, Luca Frattaruolo, Margarita Vega-Holm, Marta Carretero-Ledesma, Javier Sánchez-Céspedes, Anna Rita Cappello, Francesca Aiello, Jerónimo Pachón, José Manuel Vega-Pérez, Fernando Iglesias-Guerra, Maria Eugenia Pachón-Ibáñez



ELSEVIER

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Exploration of piperazine-derived thioureas as antibacterial and anti-inflammatory agents. *In vitro* evaluation against clinical isolates of colistin-resistant *Acinetobacter baumannii*.

Sarah Mazzotta[a,d], Tania Cebrero-Cangueiro[b,c], Luca Frattaruolo[d], Margarita Vega-Holm[a*], Marta Carretero-Ledesma[b,c], Javier Sánchez-Céspedes[b,c], Anna Rita Cappello[d], Francesca Aiello[d], Jerónimo Pachón[c,e], José Manuel Vega-Pérez[a*], Fernando Iglesias-Guerra[a], María Eugenia Pachón-Ibáñez[b,c]

[a]*Department of Organic and Medicinal Chemistry, Faculty of Pharmacy, University of Seville E-41071, Seville, Spain*
[b]*Clinical Unit of Infectious Diseases, Microbiology, and Preventive Medicine, University Hospital Virgen del Rocío, CSIC, University of Seville. Seville, Spain*
[c]*Institute of Biomedicine of Seville. University Hospital Virgen del Rocío, CSIC, University of Seville, Seville, Spain*
[d]*Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Arcavacata di Rende (CS) Italy*
[e]*Department of Medicine. University of Seville. Seville, Spain*

S.M., T.C.C contributed equally to this work (joint first authors)

ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Piperazine thiourea derivatives
Antibacterial agents
Multidrug-resistant
Acinetobacter baumannii
Anti-inflammatory

ABSTRACT

A. baumannii is one of the most important multidrug-resistant microorganisms in hospital units. It is resistant to many classes of antibiotics and the development of new therapeutic strategies is necessary. The aim of this study was to evaluate the antibacterial activity of a set of piperazine-derived thioureas against 13 clinical strains of colistin-resistant *A. baumannii*. Six derivatives were identified to inhibit bacterial growth of 46% of the *A. baumannii* strains at low micromolar concentrations (Minimum Inhibitory Concentration from 1.56 to 6.25 μM). A common structural feature in most active compounds was the presence of a 3,5-bis-trifluoromethyl phenyl ring at the thiourea function. In addition, the ability of the compounds to inhibit production of nitric oxide (NO) was examined in RAW 264.7 murine macrophages, highlighting the potential of piperazine-derived thioureas as promising scaffolds for the design of new combined anti-bacterial/anti-inflammatory agents.

Multidrug resistance (MDR) in bacteria is an emerging problem for public health all over the world. The inappropriate overuse of antibiotics for the treatment of infectious diseases contributes to the insensitivity of bacteria strains to these drugs.[1]

Acinetobacter baumannii is a gram-negative bacillus (GNB) of great clinical relevance for being a MDR and extensive drug antimicrobial resistant bacterium (XDR), also carbapenems-resistant (CRAb). It is an important healthcare-associated microorganism that causes a high number of nosocomial infections in hospitalized patients of intensive care units.[2,3] Many nosocomial infections caused by it involve organ systems containing high levels of fluids, such as blood stream infections (BSI), septicemia and pneumonia, with high mortality rates.[4] During the years, the antimicrobial resistance of different species of *A. baumannii* has increased dramatically. Colistin (COL) is one of the last therapeutic options for the treatment of these infections, though resistance to polymyxins has been also reported.[5,6] There is therefore a need to find novel active compounds with growth inhibition properties against *A. baumannii* strains.[1,7]

Many nitrogen heterocycles derivatives with potential antimicrobial activity against *A. baumannii* have been described recently. Synthetic sets of pyrazole derivatives were prepared and evaluated against *A. baumannii.* (**1**, Figure 1) Compounds with halogen substituents on the *N*-aryl moiety as fluorine, chlorine or bromine are the most active (lowest MIC value 1.56 μg/mL).[8,9] Also, novel 2-(pyrrolidin-1-yl)-thiazole derived compounds (**2**, Figure 1)[10] and 1,2,5-oxadiazoles were identified as new potential anti-Acinetobacter agents, reaching modest activity (lowest MIC 0.5 mM) in the case of oxadiazole derivatives.[11]

In the last few years, many compounds with piperazine core have been assessed as new potential antibiotics. Compounds having 4-aminoquinoline moiety and 1,3,5- triazine core linked through a piperazine ring have been prepared (**3**, Figure 1) and have shown activity against GNB (MIC from 3.12 to 6.25 μg/mL). A thiourea function was employed to connect the piperazine ring with the other moieties.[12] In other studies, the piperazine ring is part of molecular hybrids. Recently, the synthesis of new cumarine-piperazine derivatives with good antimicrobial and anti-inflammatory properties has been described.[13] Moreover, some conjugates of adamantane and

pipe

reported as growth inhibitors of gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, (MIC from 0.25 to 8 μg/mL). The most active compounds presented a 3,5-bis-trifluoromethyl group on the phenyl ring (**4**, Figure 1).[14] At present, we have found one study that reports piperazine-derived compounds as potential antimicrobial agents including anti-A. *baumannii* activity.[15]

In this letter we report the biological evaluation of a set of 39 4-N-acyl-1-phenylaminothiocarbonyl-2-substituted piperazine derivatives (34 of them previously described as new anti-adenovirus compounds,[16] and 5 described here) against 13 colistin-resistant *A. baumannii* clinical strains. We decided to develop this preliminary screening based on the presence of those privileged structures (the piperazine ring and the thiourea function) of the general backbone of these molecules, that are present in reported antimicrobial agents.[17] As shown in Figure 1 the general scaffold consists on a piperazine ring as central core with a phenylaminothiocarbonyl group at nitrogen 1 and different acyl groups at nitrogen 4 (compounds **5-43**).

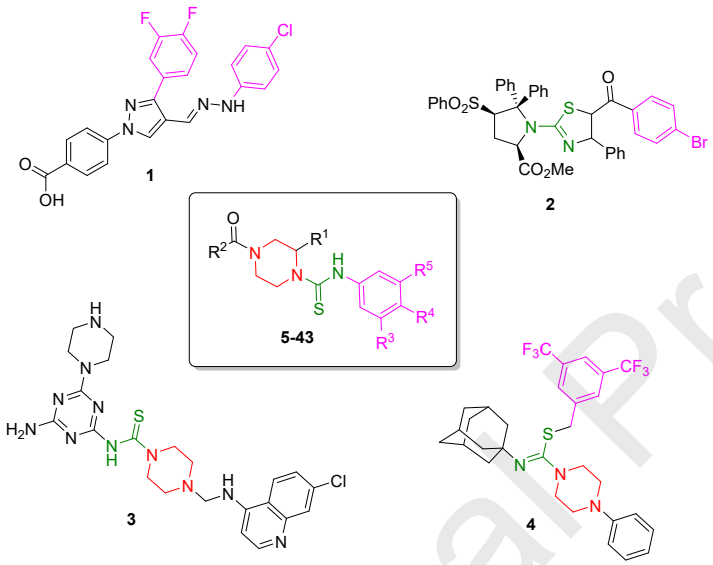
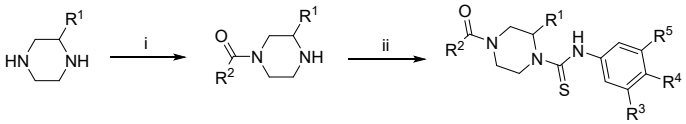


Figure 1. Some reported nitrogen heterocyclic compounds with antibacterial activity (**1-4**) and general structure of piperazine-derived anti-Acinetobacter agents (**5-43**).

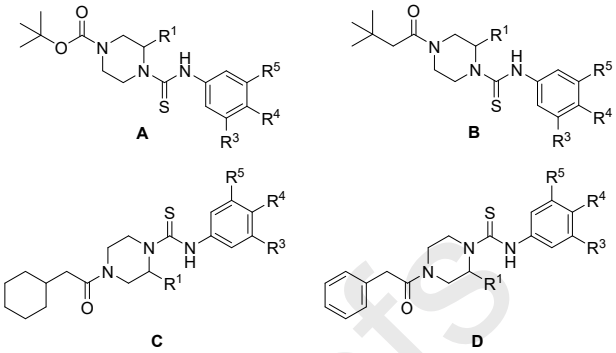
The synthetic methodology was a brief and high yielded route, previously described, that consists of two reactions.[16] The first step involved a chemoselective *N*-acylation reaction of commercial 2-substituted piperazines and provided the introduction of an amide or urethane functions at the less hindered nitrogen. From these monoacyl derivatives the thiourea group was introduced at the other nitrogen by the reaction with the appropriate phenylisothiocyanate (Scheme 1).[18] Spectral (NMR, mass spectrometry) and analytical data of the new synthesized compounds (**39-43**)[19] were in full agreement with the proposed structures (Supplementary Information)



i: starting material 1 eq, acyl halyde or anhydride 1 eq, piridine 1.5 eq, dichloromethane
ii: monoamide 1 eq, isothiocyanate 1.2 eq, dichloromethane

Scheme 1. Synthetic route for the piperazine-derived thioureas.

Table 1. 4-Acyl-1-phenylaminothiocarbonyl-2-substituted-piperazine derivatives **5-43**.



Comp.	Structure	R^1	R^3	R^4	R^5
5	A	CH_3	H	NO_2	H
6	A	CH_3	H	Cl	H
7	A	CH_3	H	CN	H
8	A	CH_3	H	F	H
9	A	CH_3	H	CF_3	H
10	A	CH_3	H	OCH_3	H
11	A	CH_3	H	CH_3	H
12	A	CH_3	CF_3	H	CF_3
13	B	CH_3	H	NO_2	H
14	B	CH_3	H	Cl	H
15	B	CH_3	H	CN	H
16	B	CH_3	H	F	H
17	B	CH_3	H	CF_3	H
18	B	CH_3	H	OCH_3	H
19	B	CH_3	H	CH_3	H
20	B	CH_3	CF_3	H	CF_3
21	C	CH_3	H	NO_2	H
22	C	CH_3	H	Cl	H
23	C	CH_3	H	CN	H
24	C	CH_3	H	F	H
25	C	CH_3	H	CF_3	H
26	C	CH_3	H	OCH_3	H
27	C	CH_3	H	CH_3	H
28	C	CH_3	CF_3	H	CF_3
29	D	CH_3	H	NO_2	H
30	D	CH_3	H	Cl	H

31	D	CH_3	H	CN	H
32	D	CH_3	H	F	H
33	D	CH_3	H	CF_3	H
34	D	CH_3	H	OCH_3	H
35	D	CH_3	H	CH_3	H
36	D	CH_3	CF_3	H	CF_3
37	B	Ph	CF_3	H	CF_3
38	D	Ph	CF_3	H	CF_3
39[a]	A	Ph	CF_3	H	CF_3
40[a]	C	Ph	CF_3	H	CF_3
41[a]	A	H	CF_3	H	CF_3
42[a]	B	(*R*)-CH_3	CF_3	H	CF_3
43[a]	B	(S)-CH_3	CF_3	H	CF_3

[a]New compounds

The antibacterial activity of these piperazine derivatives was evaluated through a growth inhibition screening, against 13 clinical colistin-resistant *A. baumannii (Ab*) strains from an outbreak in 2002 in the University Hospital *Virgen del Rocío* of Seville.[20]

Briefly, the inhibition activity at 50 μM of each compound was carried out in 96-well plate to identify which compounds produced growth inhibition of $5x10^5$ Unit Forming Colonies (UFC/ml) of the tested strains. Compounds were found to inhibit the bacterial growth of 46% of strains.[21] It is important to note that our active compounds showed growth inhibition activity against Ab 11, Ab 14, Ab 17, Ab 21R, Ab 22P and Ab 24 (Supplementary Information, Table S10). Then, the Minimal Inhibitory Concentration (MIC) was determined for those compounds that showed growth inhibition in the previous assay by the standard broth microdilution method.[22] MIC values of COL were also determined. (Supplementary Information, Table S12). Table 2 shows those compounds with MIC values ≤ 12.5 μM.

Table 2. MIC values (μM) of the selected piperazine-derived thioureas and COL against colistin-resistant *A. baumannii* clinical strains and CC50 values (μM).

Comp	MIC (μM) *Acinetobacter baumannii*						CC_{50} (μM)[a]
	11	14	17	21R	22P	24	
12	-	3.12	-	-	-	-	112.4
20	-	50	-	6.25	6.25	6.25	73.2
21	-	-	-	25	12.5	-	148.1
22	-	50	-	12.5	12.5	25	200.0
25	-	12.5	-	25	6.25	3.12	142.2
28	-	50	-	3.12	1.56	25	82.0
36	6.25	3.12	3.12	3.12	-	6.25	129.7
37	3.12	-	-	3.12	3.12	3.12	91.8
38	-	-	3.12	-	-	-	104.3
40	3.12	-	-	3.12	6.25	3.12	124.6
41	12.5	6.25	6.25	6.25	12.5	-	108.6
42	-	-	-	6.25	12.5	6.25	162.1
43	-	-	-	6.25	6.25	12.5	73.5
COL	14	56	> 111	1772	3545	7	-

[a] Cytotoxic concentration 50%.

Most of the monosubstituted derivatives at *para* position of the phenyl ring, having electron-releasing groups (R^3 = OCH_3, CH_3) or electron withdrawing groups (R^3 = NO_2, Cl, CN, F, CF_3) exhibited MIC values from 25 to 50 μM (Supplementary Information, Table S13). Only compound **25** (2-cyclohexylacetyl derivative) with R^3 = CF_3 showed 6.25 and 3.12 μM against the Ab22R and Ab24 strains, respectively.

The most active compounds (lowest values of MIC) were analogues **20**, **28** and **36**-**38**. The presence of a 3,5-bis-trifluoromethyl phenyl ring at the thiourea function seems to be crucial to improve the *in vitro* activity (reduced MIC values, from 1.56 to 6.25 μM). Depending on the acyl group at N-4, they were active against different strains. Urethane derivative **12** was active only against Ab 14 with low MIC (3.12 μM) while amide derivatives (**20**, **28** and **36**) were very active against, mainly, three different strains, Ab 21R, Ab 22P and Ab 24. **28** (2-cyclohexylacetyl derivative) gave the lowest MIC (1.56 μM against Ab 22P) and **36** (2-phenylacetyl derivative) gave also low MIC values (3.25 μM and 6.25 μM) with a broad spectrum of activity (five strains). From the methyl pipeperazine-derived thioureas collection, compounds **12**, **28** and **36** were selected.

Keeping this substitution pattern at the phenyl ring, the 2-methyl piperazine central core was exchanged with 2-phenylpiperazine to give analogues **37**-**40**. We focused not only on trying to reduce MIC values but also on increasing the number of strains that could be affected by these compounds. They were active against the same strains than their methyl analogues. Regarding the acyl group at N-4, 3,3-dimethylbutanoyl derivative **37** and 2-cyclohexylacetyl derivative **40** achieved lower MIC values (3.12 μM) than their 2-methyl piperazine analogues against most of the strains. However, compound **38**, 2-phenylacetyl derivative, did not improve the activity of **36**.

Compound **41** (with an unsubstituted piperazine core) was prepared as an achiral analogue of **12** (2-methylpiperazine core) and **39** (2-phenylpiperazine core). It showed low MIC against five strains, increasing the spectrum of activity compared to its analogues.

We have also prepared both pure enantiomers of the racemic compound **20** (**42** and **43**). They were both active against the same strains and showed almost similar MIC values than **20** (Table 2).

As it was one of the of the broadest spectrum derivatives, including the two most colistin-resistant strains (Ab 21R, Ab 22P), compound **41** was chosen to develop bactericidal studies against them by time kill curves. We tested it at different conditions: 1xMIC (6.25 μM), 2xMIC (12.5 μM), and 4xMIC (25 μM) for each strain. We found that compound **41** showed bactericidal effect at 4xMIC after 4 hours against Ab21R. Furthermore, it reached a reduction close to 3-log_{10} after 4 hours at 2xMIC, compared to the initial inoculum, against Ab21R (Figure 2). No bactericidal activity was observed at any condition against Ab22P strain (Figure 3).

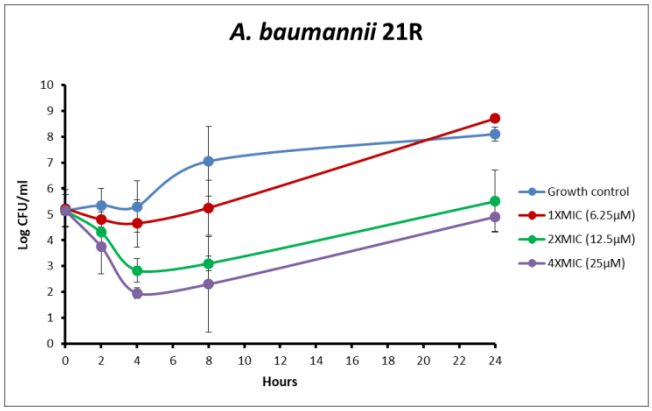


Figure 2. Bactericidal activity of compound **41** against *A. baumannii* 21R.

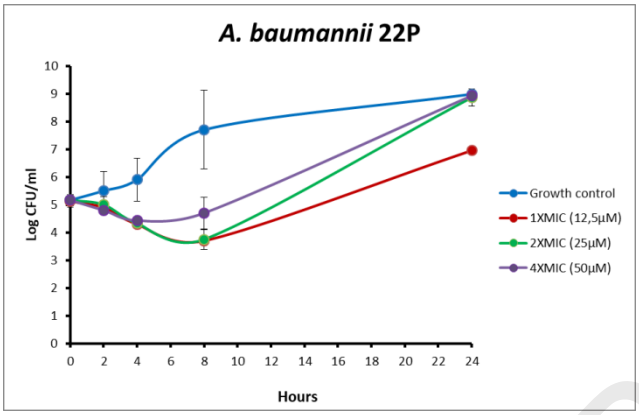


Figure 3. Bactericidal activity of compound **41** against *A. baumannii* 22P.

Considering the drawbacks of COL and other potential active antibiotics in monotherapy, combination therapy has been raised as an interesting strategy to overcome these limitations. The synergistic activity of **41** in combination with standard antibiotic COL against Ab 21R and Ab 22P was also investigated.[23] The outcomes were determined by calculation of Fractional Inhibitory Concentration (FIC) Index values and presented in Table 3. Compound **41** and COL displayed FICI values of 0.5 and 0.37 against Ab 21R and Ab 22P, which indicated a synergistic interaction. It was noted that the compound markedly reduced the MIC value of COL by fourfold against both strains.

Table 3. Determination of FIC values and interaction effects of compound **41** and COL against two colistin-resistant *A. baumannii* clinical strains.

Strain	Agent	MIC (μM)		FIC[a]	
		Alone	Combination	FIC Individual	FIC Index
Ab 21R	Comp. **41**	6.25	0.78	0.12	0.5 (S)
	COL	1178	443	0.38	
Ab 22P	Comp. **41**	12.5	1.56	0.12	0.37 (S)
	COL	3545	886	0.25	

[a]The FICI was interpreted as follow: synergism=FICI≤0.5; indifferent> 0.5 to ≤4; antagonism=FICI > 4. The experiments were performed in triplicate.

it is important to study if they can selectively kill microorganisms without being significantly toxic to host cells. All active compounds were tested towards A549 cell line at several concentrations (Supporting Information) and their CC_{50} values were also calculated (Supplementary Information, Table S13). Selectivity index (SI) of selected compounds (**12**, **28**, **36**, **37**, **40** and **41**) was determined (Supplementary Information, Table S13). The results showed that these compounds had relatively high safety index, with values in the range of 8.69-52.56, being most of them > 20, suggesting high selective toxicity towards bacterial cells over host ones.[24-26]

Microbial infections are always associated with the triggering of inflammatory processes, necessary for the body to fight the cause of the damage and stimulate the healing process.[27] However, if the inflammatory process becomes chronic it can lead to severe tissue damage that can be associated with a loss of the functionality of the affected tissue, providing greater harm than benefit.[28] In recent years, numerous *in vitro* and *in vivo* studies highlighted the unexplored anti-inflammatory potential of molecules having a piperazine moiety.[13,29-32] This action seems to be due to the ability of these molecules to act, in macrophages, as antagonists of histamine receptors ,[29,33,34] involved in the modulation of different inflammatory mediators, such as NO, pro-inflammatory cytokines and TNF-α.[35,36] Furthermore, several studies have highlighted the remarkable versatility of piperazine-scaffold, whose anti-inflammatory activity often coexists with the anti-bacterial one.[37-39]

This scenario prompted us strongly to evaluate the anti-inflammatory potential of the piperazine-derived thioureas subject of this study, in order to reveal their possible double effect that would find a useful use in the clinical practice. The anti-inflammatory potential was assessed by monitoring the ability of different compounds to inhibit, in the macrophage line RAW 264.7, the production of nitric oxide (NO), one of the main mediators of phlogistic processes.[40]

An initial screening was performed by treating macrophages with all the different molecules at a concentration of 50 μM (Supplementary Information, Table S14). Surprisingly, the compounds that showed, in this first phase, an inhibitory capacity on the production of NO were all characterized by possessing two trifluoromethyl substituents (**12**, **20**, **28**, **36**, **37**, **38**, **39**, **40**, **41**), which previously turned out to be the best antimicrobial agents. Trifluoromethyl group, indeed, is well known moiety in pharmaceutical chemistry, with peculiar chemical and physiological stability and able to enhance the anti-inflammatory potential of bioactive compounds.[41,42] Among our results, an exception is represented by the compounds **21**, **23**, **30**, **33**, differently substituted on the phenyl ring, characterized by a weak or absent antibacterial activity against *A. baumannii*, but which responded positively to the anti-inflammatory screening. Subsequently, the active compounds were tested at lower concentrations (1 and 10 μM), in order to highlight reductions in NO production at concentrations that are closest to the previously identified MICs (Figure 4A). Highest reduction of NO production (percentage of inhibition ≥ 80%) was observed with 2-phenylpiperazine derivatives **37** (3,3-dimethylbutanoyl derivative), **39** (*tert*-butoxycarbonyl derivative) and **40** (2-cyclohexylacetyl derivative), while the inhibition observed with their methyl analogues (**20**, **12** and **28** respectively) was significantly lower (highest value observed 40%). The phenyl substituent on piperazine ring seems to improve the anti-inflammatory profile.

production could be by macrophages death, the effect on the cell viability of our compounds was also evaluated (Supporting information). In Figure 4B it is shown these results expressed as percentage of cell viability versus control. Compounds **39** and **40** were the only ones with cytotoxic effects on the macrophage line under examination.

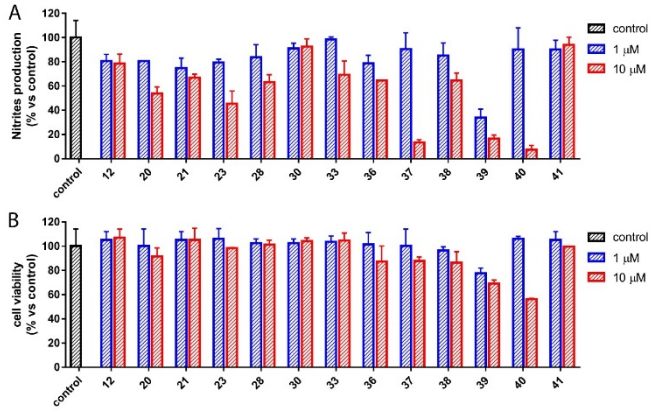


Figure 4. Anti-inflammatory activity of Piperazine-derived thioureas. Nitrites production (A) and cell viability (B) assessments after treatment of LPS-stimulated RAW 264.7 cell line with different concentration of compounds for 24 hours.

In summary, we have evaluated the antibacterial activity of a collection of 39 piperazine-derived thioureas. All active compounds showed growth inhibition against 46% of the tested colistin-resistant *A. baumannii* clinical strains. The SAR analysis has indicated that those compounds with a 3,5-bis-trifluoromethyl phenyl ring at the thiourea function (**12, 28, 36, 37, 40** and **41**) were the most effective. Their potent activity, no cytotoxicity, anti-inflammatory activity and easy synthetic accessibility allow us to consider them as potentially strong candidates for development of new anti-Acinetobacter agents. The optimization process will be focused on improving their potency as both antibacterial and anti-inflammatory agents. They potentially could be used as adjuvants in combination therapy in order to restore the efficacy of current employed antibiotics in the clinical setting.

Declaration of Competing Interest

The authors declare no competing financial interest or personal relationships that could have appeared to influence the work reported in this paper. S.M, TCC, M.V.H, J.S.C., J.P., J.M.V.P., F.I.G., M. V.H. and M.E.P.I. are co-inventors of the European patent EP16382073.1 (Name of the Invention: Piperazine derivatives as antiviral agents with increased therapeutic activity; year of application: 2016).

Acknowledgments

This work has been supported by Ministerio de Economía y Competitividad, Plan Estatal 2013-2016 Retos-Proyectos I+D+i (CTQ2016-78580-C2-2-R). MVH also thanks Ministerio de Economía y Competitividad, Plan Estatal 2013-2016 Excelencia I+D+i (CTQ2016-78703-P). This work has been also supported by Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0009)—co-financed by European Operative program Intelligent Growth 2014-2020.). T.C.C. is supported by the V Plan Propio of the University of Seville with a postdoctoral contract as research personnel in training. JSC is a researcher belonging to the program "Nicolás Monardes" (C-0059-2018), Servicio Andaluz de Salud, Junta de Andalucía, Spain. MEPI is a researcher belonging to the program "Nicolás Monardes" (C1-0038-2019), Servicio Andaluz de Salud, Junta de Andalucía, Spain. S.M, F.A, L.F and A.R.C. thank Dipartimento di Farmacia e Scienze della Salute e della Nutrizione-Dipartimento di Eccellenza MIUR L 232/2016". Authors also thank CITIUS (Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla) for its important contribution in recording NMR and Mass spectra.

References and notes

1. Tanwar, J.; Das, S.; Fatima, Z.; Hameed, S. *Interdiscip. Perspect. Infect. Dis*. **2014:** 541340.
2. Lee, H.; Lee, H. *Infect. Chemother*. **2016**, *48*, 174-180.
3. Cano, M. E.; Domínguez, M. A.; Ezpeleta, C.; Padilla, B.; Ramírez de Arellano, E.; Martínez-Martínez, L. *Enferm. Infecc. Microbiol. Clin*. **2008**, *26*, 220-229.
4. Mody, L.; Gibson, K. E.; Horcher, A.; Prenovost, K.; McNamara, S. E.; Foxman, B.; Kaye, K. S.; Bradley, S. *Infect. Control Hosp. Epidemiol*. **2015**, *36*, 1155-1162.
5. Pogue, J. M.; Cohen, D. A.; Marchaim, D. *Clin. Infect. Dis*. **2015**, *60*, 1304–1307.
6. Hua, X.; Liu, L.; Fang, Y.; Shi, Q.; Li, X.; Chen, Q.; Shi, K.; Jiang, Y.; Zhou, H.; Yu, Y. *Front. Cell. Infect. Microbiol.* **2017**, doi: 10.3389/fcimb.2017.00045.
7. Asif, M.; Alvi1, I. A.; Rehman, S. U. *Infect. Drug Resist.* **2018**, *11*, 1249-1260.
8. Zakeyaha, A. A.; Whitta, J.; Dukea, C.; Gilmoreb, D. F.; Meekerc, D. G.; Smeltzerc, M. S.; Alama, M. A. *Bioorg. Med. Chem. Lett.* **2018**, *28*, 2914–2919
9. Allison, D.; Delancey, E.; Ramey, H.; Williams, C.; Alsharif, Z. A.; Al-khattabi, H.; Ontko, A.; Gilmore, D.; Alam, M. A. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 387-392. .
10. Nural, Y.; Gemili, M.; Ulger, M.; Sari, H.; c, De Coen, L. M.; Ertan Sahin, E. *Bioorg. Med. Chem. Lett.* **2018**, *28*, 942-946.
11. Christoff, R. M.; Murray, G. L.; Kostoulias, X. P.; Peleg, A. Y.; Abbott, B. M. *Bioorg. Med. Chem. Lett* **2017**, *25*, 6267–6272.
12. Verma, A.; Prateek, P.; Thakur, A.; Shukla, P. K. *Int. J. Chemtech Res.* **2016**, *9*, 261-269.
13. Koparde, S.; Hosamani, K. M.; Kulkarni, V.; Joshi, S. D. *Chem. Dat. Collect.* **2018**, *15/16*, 197-206.
14. Al-Wahaibi, L. H.; Hassan, H. M.; Abo-Kamar, A. M.; Ghabbour, H. A.; El-Emam, A. A. *Molecules* **2017**, *22*, 710.
15. Patila, M.; Poyilb, A.N.; Joshic, S.D.: Patild, S.A.; Patila, S.A.; Bugarine, A. *Bioorganic Chemistry* **2019**, *92*, 103217
16. Mazzotta, S.; Marrugal Lorenzo, J. A.; Vega-Holm, M.; Serna-Gallego, A.; Álvarez-Vidal, J.; Berastegui-Cabrera, J.; Pérez del Palacio, J.; Díaz, C.; Aiello, F.; Pachón, J.; Iglesias-Guerra, F.; Vega-Pérez, J. M.; Sánchez-Céspedes, J. *Eur. J. Med. Chem.* **2020**, *185*, 111840.
17. Vega-Pérez, J. M.; Periñán, I.; Argandoña, M.; Vega-Holm, M.; Palo-Nieto, C.; Burgos-Morón, E.; López-Lázaro, M.; Vargas, C.; Nieto, J. J.; Iglesias-Guerra, F. *Eur. J. Med. Chem.* **2012**, *58*, 591-612.
18. Synthesis of the thiourea derivatives **(39-43).** To a solution of the monoacyl derivative (0.75 mmol) in dry dichloromethane (10 mL) was added the corresponding isothiocyanate (0.9 mmol). The reaction mixture was stirred at room temperature until TLC showed that all the starting material had reacted, than was evaporated to dryness. Compounds were purified by flash chromatography on silica gel using the appropriate eluent.
19. Spectra data of the representative compound **41**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.81 (s, 1H), 8.11 (s, 2H), 7.77 (s, 1H), 3.97 (t, J = 5.1 Hz, 4H), 3.48 (t, J = 5.1 Hz, 4H), 1.44 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 180.8, 153.8, 143.1, 130.1, 129.8, 129.6, 129.3, 129.2, 124.4, 124.3, 122.2, 116.6, 79.2, 47.8, 42.8, 28.0. HRMS (*m/z*): calcd. for $C_{18}H_{21}F_6N_3O_2SNa$ 480.1151 $[M+Na]^+$; found 480.1143. Anal. calcd for $C_{18}H_{21}F_6N_3O_2S$: C, 47.26; H, 4.63; N, 9,19. Found: C, 46.95; H, 4.77; N, 9.17.
20. Valencia, R.; Arroyo, L. A.; Conde, M.; Aldana, J. M.; Torres, M. J.; Fernández-Cuenca, F.; Garnacho-Montero, J.; Cisneros, J. M.;

2009, *50*, 257-265.
21. The activity screening studies were performed as follows: All compounds were screened at 50 µM in 96-well plate to identify which produced inhibition of growth against 13 colistin-resistant *A. baumannii* clinical strains. For each strain, approximately $1x10^5$ CFU/mL bacteria in Müller Hinton Broth (MHB) were dispensed into the plates containing 50µM of each piperazine derivate. Plates were incubated at 37º C with humidity for 18-24 hours. The compounds were dissolved in DMSO, which was used as negative control. As a positive control, $1x10^5$ CFU/mL bacteria in broth, without compound was incubated in the same conditions. Growth was indicated by turbidity, a single large dot, or a filamentous network of growth. The experiments were performed in triplicate and were read independently by two observers.
22. Minimal inhibitory concentration (MIC) was determined by the broth microdilution method (cation-adjusted Mueller-Hinton broth; Becton Dickinson, Cockeysville, Md.), in accordance with the CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).The compounds for which inhibition of the bacterial growth was observed in the screening studies were tested (6clinical strains). A range of piperazine derivates concentrations from 50µM to 0.195µM was tested. MICs were read as the lowest concentrations of piperazine derivate which inhibited visible growth after incubation for 24 h. The experiments were performed in triplicate and were read independently by two observers.
23. Xu, J.; Pachón-Ibañez, M. E.; Cebrero-Cangueiro, T.; Chen, H.; Sánchez-Céspedes, J.; Zhou, J. *Bioorg. Med. Chem. Lett.* **2019**, *29*, 1399-1402.
24. Lyu, Y.; Yang, Y.; Lyu, X.; Dong, N.; Shan, A. *Sci. Rep.* **2016**, *6*, 27258 https://doi.org/10.1038/srep27258, Article number: 27258.
25. Cherdtrakulkiat, R.; Boonpangrak, S.; Sinthupoom, N.; Prachayasittikul, S.; Ruchirawat, S.; Prachayasittikul, V. *Biochem. Biophys. Reports* **2016**, *6*, 135-141.
26. Ong, Z. Y.; Gao, S. J.; Yang, Y. Y. *Adv. Funct. Mater.* **2013**, *23*, 3682–3692.
27. Carullo, G.; Cappello, A. R.; Frattaruolo, L.; Badolato, M.; Armentano, B.; Aiello, F. *Future Med. Chem.* **2017**, *9*, 79-93.
28. Furman, D.; Campisi, J.; Verdin, E.; Carrera-Bastos, P.; Targ, S.; Franceschi, C.; Miller, A. H. *Nat. Med.* **2019**, *25*, 1822-1832.
29. Corrêa, M. F.; Varela, M. T.; Balbino, A. M.; Torrecilhas, A. C.; Landgraf, R. G.; Troncone, L. R. P.; Fernandes, J. P. S. *Chem. Biol. Drug Des.* **2017**, *90*, 317-322.
30. Li, J.; Yin, Y.; Wang, L.; Liang, P.; Li, M.; Liu, X.; Wu, L.; Yang, H. *Molecules* **2016**, *21*(11), 1544.
31. Silva, D. P.; Florentino, I. F.; Oliveira, L. P.; Lino, R. C.; Galdino, P. M.; Menegatti, R.; Costa, E. A. (2015). *Pharmacology Biochemistry and Behavior* **2015**, *137*, 86-92.
32. Ma, Y.; Zheng, X.; Gao, H.; Wan, C.; Rao, G.; & Mao, Z. *Molecules* **2016**, *21*(12), 1684.
33. Corrêa, M. F.; Barbosa, Á. J.; Teixeira, L. B.; Duarte, D. A.; Simões, S. C.; Parreiras-e-Silva, L. T.; Balbino, A.M.; Landgraf, R.G.; Bouvier, M.; Costa-Neto, C.M.; Fernandes J.P.S. *Frontiers in Pharmacology* **2017**, *8*, 825.
34. Ahmadi, A.; Khalili, M.; Chavrogh, S.; Nahri-Niknafs, B. *Iranian journal of pharmaceutical research: IJPR* **2012**, *11*(4),1027.
35. Chen, X. F.; Zhang, Z.; Dou, X.; Li, J. J.; Zhang, W.; Yu, Y. Y.; Yu, B. *Cellular and Molecular Biology* **2016**, *62*(1), 84-89
36. Thangam, E. B.; Jemima, E. A.; Singh, H.; Baig, M. S.; Khan, M.; Mathias, C. B.; Church, M. K.; Saluja, R. *Frontiers in immunology* **2018**, *9*, 1873.
37. Shaquiquzzaman, M.; Verma, G.; Marella, A.; Akhter, M.; Akhtar, W.; Khan, M. F.; Tasneem, S.; Alam, M. M. *European Journal of Medicinal Chemistry* **2015**, *102*, 487-529.
38. Mohan, N. R.; Sreenivasa, S.; Manojkumar, K. E.; Rao, T.; Thippeswamy, B. S.; Suchetan, P. A. *Journal of the Brazilian Chemical Society* **2014**, *25*(6), 1012-1020.
Yale, R. H.; Kamhole, V. M. (2012). *Bioorganic & medicinal chemistry letters* **2012**, *22*(20), 6385-6390.
40. Inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Briefly, RAW 264.7 cells were seeded at a density of 2 × 105 cells/well in 24 well plates and incubated overnight in complete medium. Media were aspirated and replaced with fresh DMEM, then, cells were simultaneously treated for 24 h with LPS (1 µg/mL) and different concentrations of extracts or isolated compounds. DMSO was used as a vehicle control. After treatment end, the presence of nitrite (a stable oxidized product of NO) was measured in cell culture media by using the Griess assay, and results are expressed as percentage of nitrites production versus control (untreated LPS-stimulated cells).
41. Yale, H. L. *J. Med. Chem*. **1958**, *1*, 121-133.
42. Perri, F.; Frattaruolo, L.; Haworth, I.; Brindisi, M.; El-magboub, A.; Ferrario, A.; Gomer, C.; Aiello, F.; Adams, J. D. *Heliyon* **2019**, 5, e01366.

Supplementary Material

Full description of biological assays, chemical characterization and copies of NMR spectra of piperazine derivatives **39-43**, growth Inhibition screening data, MIC values and NO production screening for compounds **5-43**; Selectivity Index for the six selected compounds (**12, 28, 36, 37, 40** and **41**) can be found in the Supplementary Material.

Declaration of interests

☐X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

As corresponding author I sign this declaration in the name of all authors of this manuscript
Margarita Vega Holm

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights for: Exploration of piperazine-derived thioureas as antibacterial and anti-inflammatory agents. *In vitro* evaluation against

cli

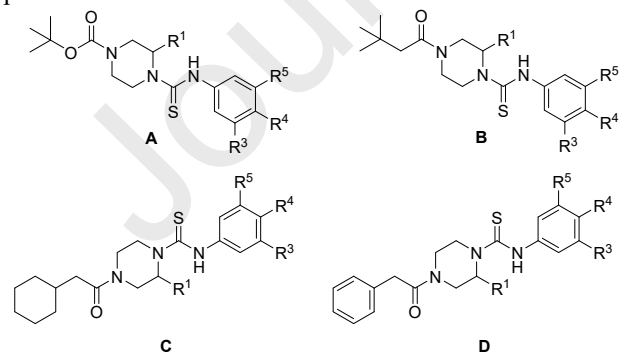
baumannii.

S. Mazzotta, T.Cebrero-Cangueiro, L. Frattaruolo, M. Vega-Holm*, M. Carretero-Ledesma, J. Sánchez-Céspedes, A.R. Cappello, F. Aiello, J. Pachón, J.M. Vega-Pérez*, F. Iglesias-Guerra, M.E. Pachón-Ibáñez

E-mail: mvegaholm@us.es
E-mail: vega@us.es

- A set of 39 piperazine-derived thioureas was evaluated against colistin-resistant *Acinetobacter baumannii* clinical strains.
- Six derivatives inhibited bacterial growth of 46% of the *A. baumannii* strains at low micromolar concentrations.
- Bactericidal activity (time kill curve) was tested for compound **41** against two colistin-resistant *A. baumannii* strains.
- Compound **41** was also tested for synergistic combination with colistin against the same two clinical strains.
- Their potential anti-inflammatory activity was assessed by their ability to inhibit the production of nitric oxide

Table 1. 4-Acyl-1-phenylaminothiocarbonyl-2-substituted-piperazine derivatives **5-43**.



Comp.	Structure	R^1	R^3	R^4	R^5
5	A	CH_3	H	NO_2	H
6	A	CH_3	H	Cl	H
7	A	CH_3	H	CN	H
8	A	CH_3	H	F	H
9	A	CH_3	H	CF_3	H
10	A	CH_3	H	OCH_3	H
11	A	CH_3	H	CH_3	H
12	A	CH_3	CF_3	H	CF_3
13	B	CH_3	H	NO_2	H
14	B	CH_3	H	Cl	H
15	B	CH_3	H	CN	H
16	B	CH_3	H	F	H
17	B	CH_3	H	CF_3	H
18	B	CH_3	H	OCH_3	H
19	B	CH_3	H	CH_3	H
20	B	CH_3	CF_3	H	CF_3
21	C	CH_3	H	NO_2	H
22	C	CH_3	H	Cl	H
23	C	CH_3	H	CN	H
24	C	CH_3	H	F	H
25	C	CH_3	H	CF_3	H
26	C	CH_3	H	OCH_3	H
27	C	CH_3	H	CH_3	H
28	C	CH_3	CF_3	H	CF_3
29	D	CH_3	H	NO_2	H
30	D	CH_3	H	Cl	H
31	D	CH_3	H	CN	H
32	D	CH_3	H	F	H
33	D	CH_3	H	CF_3	H
34	D	CH_3	H	OCH_3	H
35	D	CH_3	H	CH_3	H
36	D	CH_3	CF_3	H	CF_3
37	B	Ph	CF_3	H	CF_3
38	D	Ph	CF_3	H	CF_3
39[a]	A	Ph	CF_3	H	CF_3
40[a]	C	Ph	CF_3	H	CF_3
41[a]	A	H	CF_3	H	CF_3
42[a]	B	(*R*)-CH_3	CF_3	H	CF_3
43[a]	B	(S)-CH_3	CF_3	H	CF_3

[a]New compounds.

Table 2. MIC values (μM) of the selected piperazine-derived thioureas and COL against colistin-resistant *A. baumannii* clinical strains and CC50 values (μM).

Comp	MIC (μM) *Acinetobacter baumannii*						CC_{50} (μM)[a]
	11	14	17	21R	22P	24	
12	-	3.12	-	-	-	-	112.4
20	-	50	-	6.25	6.25	6.25	73.2
21	-	-	-	25	12.5	-	148.1
22	-	50	-	12.5	12.5	25	200.0
25	-	12.5	-	25	6.25	3.12	142.2
28	-	50	-	3.12	1.56	25	82.0
36	6.25	3.12	3.12	3.12	-	6.25	129.7
37	3.12	-	-	3.12	3.12	3.12	91.8
38	-	-	3.12	-	-	-	104.3
39	-	12.5	6.25	-	-	-	127
40	3.12	-	-	3.12	6.25	3.12	124.6
41	12.5	6.25	6.25	6.25	12.5	-	108.6
42	-	-	-	6.25	12.5	6.25	162.1
43	-	-	-	6.25	6.25	12.5	73.5
COL	14	56	> 111	1772	3545	7	-

[a] Cytotoxic concentration 50%.

Table 3. Determination of FIC values and interaction effects of compound **41** and COL against two colistin-resistant *A. baumannii* clinical strains.

Strain	Agent	MIC (μM)		FIC[a]	
		Alone	Combination	FIC Individual	FIC Index
Ab 21R	Comp. **41**	6.25	0.78	0.12	0.5 (S)
	COL	1178	443	0.38	
Ab 22P	Comp. **41**	12.5	1.56	0.12	0.37 (S)
	COL	3545	886	0.25	

[a]The FICI was interpreted as follow: synergism=FICI≤0.5; indifferent> 0.5 to ≤4; antagonism=FICI > 4. The experiments were performed in triplicate

Hours

ii: monoamide 1 eq, isothiocyanate 1.2 eq, dichloromethane