Saudi Journal of Biological Sciences (2016) 23, 34–38

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Modulation of the antibiotic activity against multidrug resistant strains of 4-(phenylsulfonyl) morpholine

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Received 17 October 2014; revised 29 January 2015; accepted 1 February 2015
Available online 10 February 2015

KEYWORDS

4-(Phenylsulfonyl) morpholine;
Antimicrobial agent;
Modulation;
Microbial resistance

Abstract The compound 4-(Phenylsulfonyl) morpholine belongs to the class of sulfonamides, which are widely used in the treatment of a large number of diseases caused by microorganisms. This compound has a morpholine group, which is also known for its antimicrobial properties. The aim of the present study was to investigate the antimicrobial and modulating activity of 4-(Phenylsulfonyl) morpholine against standard and multi-resistant strains of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and strains of the fungi *Candida albicans*, *C. tropicalis* and *C. krusei*. Antimicrobial activity was assessed based on the minimum inhibitory concentration (MIC) using the microdilution method. MIC was $\geqslant$1024 μg/mL for all microorganisms. Regarding modulating activity, the most representative effect occurred with the combination of 4-(Phenylsulfonyl) morpholine at a concentration of 128 μg/mL (MIC 1/8) and amikacin against *P. aeruginosa* 03, with a reduction in MIC from 312.5 to 39.06 μg/mL.

1. Introduction

Sulfonamides are substances with structures correlative to that of *p*-aminobenzoic acid. As competitive antagonists, sulfonamides impede their use by bacteria in the synthesis of folic acid, thereby affecting microorganisms that need to synthesize their own folic acid. As mammals do not synthesize folic acid, sulfonamides do not affect their metabolism (Alaburda et al., 2007). The importance of the sulfonamide nucleus is well

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2015.02.001
1319-562X

established in pharmaceutical chemistry. New synthesized 5-substituted amino pyrazole sulfates in an attempt to find a therapeutic alternative for combating infection (Borges et al., 2004). A number of sulfonamides, especially those derived from *p*-aminobenzenesulfonamide, have structural variations that enhance their efficacy to obtain a greater action spectrum and increase their solubility in biologic systems (Coutinho et al., 2008a).

Morpholine derivatives constitute a new antifungal chemical group not correlated with other currently available medications with antifungal activity. These derivatives inhibit the biosynthesis of sterol by blocking two successive enzymatic processes: (1) inhibiting the biotransformation of lanosterol into zymosterol by blocking the enzyme C-14 sterol reductase and; (2) inhibiting the synthesis of ergosterol from the biotransformation of fecosterol into episterol by blocking the enzyme C-8 sterol isomerase; these enzymes are different from those inhibited by allylamines or azoles (Kerkenaar, 1987; Polak, 1988). The advantage in preparing morpholine derivatives resides in the fact that these compounds provide chlorhydrates that are soluble in water for pharmacological assays (Pinto et al., 2013). Different sulfonyl-hydrazones obtained from sulfonyl chloride exhibit anti-neoplasm, antibacterial, antinociceptive and other pharmacological activities against several and different targets (Oliveira, 2012). Synthetic substances have demonstrated efficacious antimicrobial action against resistant microorganisms. The determination of synergism or antagonism between antimicrobial agents is important to understanding the action mechanisms of these substances as well as resistance mechanisms. Moreover, *in vivo* and *in vitro* analyses are performed for the detection of therapeutic potential with the aim of finding alternative pathogen control methods (Catão et al., 2010).

The aim of the present study was to investigate the antimicrobial and modulating activity of 4-(Phenylsulfonyl) morpholine against standard and multi-resistant strains of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and strains of the fungi *Candida albicans*, *Candida. tropicalis* and *Candida. krusei*.

2. Materials and methods

2.1. Synthesis

The precursor's benzenesulfonyl chloride and morpholine were purchased commercially from Sigma–Aldrich (St. Louis, USA). The precursors were used without further purification. The title compound 4-(Phenylsulfonyl) morpholine was prepared by reaction of 1 equivalent of benzenesulfonyl chloride (1 mmol) and morpholine (2 mmol) in methanolic mixture of pyridine (5 mL) at low temperature (~0 °C) under stirring by 2 h as a previously described procedure (Buchmann and Schalinatus, 1962). The material thus formed was filtered and washed with methanol solution then dried. The resulting material was recrystallized by using heating methanol solution. The crystals were formed by slow solvent evaporation at room temperature. The complete structure elucidation was confirmed by NMR ^{1}H and ^{13}C spectroscopy analysis by comparison with literature data (Modarresi-Alam et al., 2009). The NMR spectra in $CDCl_3$, were recorded in Varian-Mercury 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer, using tetramethylsilane (TMS) as internal standard. Analytical Data: ^{1}H RMN ($CDCl_3$, 300 MHz): t (4H; 3.01 ppm); t (4H; 3.74 ppm); t (2H, J = 7.56 Hz); t (1H; 7.64 ppm); d (2H; 7.77 ppm) ^{13}C RMN ($CDCl_3$, 75 MHz): δ = 46; 66.10; 127.85; 129.15; 133.09; 135.13. The synthesis is described by the scheme present in Fig. 1.

2.2. Microorganisms

The following strains were used: *E. coli* ATCC10536 and *E. coli* EC 27; *S. aureus* ATCC25923 and *S. aureus* SA358; *P. aeruginosa* ATCC15442 and *P. aeruginosa* PA; *C. albicans* ATCC40006; *C. krusei* ATCC6258 and *C. tropicalis* ATCC13803. Table 1 displays the resistance profile of the microorganisms. All strains were maintained on heart infusion agar (HIA, Difco Laboratories Ltd., San Diego, USA). Prior to the assays, the strains were cultivated in Brain-Heart Infusion broth (BHI, Difco Laboratories Ltd., San Diego, USA) for 18 h at 37 °C.

2.3. Antimicrobial activity and antibiotic modulating activity

The minimum inhibitory concentration (MIC) of all microorganisms was determined in broth microdilution assays (CLSI, 2005) using an inoculum of 100 μL of each strain suspended in BHI broth at a concentration of 10^5 colony forming units/mL in 96-well microtitration plates, with dilutions in ½ series. An aliquot of 100 μL of 4-(Phenylsulfonyl) morpholine was added to each well. The final concentrations of the substance ranged from 512 to 8 μg/mL. The standard antibiotics (amikacin, gentamicin and neomycin) and antifungals (amphotericin B, benzoilmetronidazol, mebendazole and nystatin) were assayed at concentrations ranging from 512 to 8 μg/mL and were used as controls. The plates were incubated at 35 °C for 24 h, after which the readings were performed with the aid of resazurin. The MICs were recorded as the least concentration necessary to the growth inhibition. For the assessment of the substance as a modulator of antibiotic and antifungal action, the MIC of antibiotics and antifungals was evaluated in the presence and absence of the substance in sterile microplates. The antibiotics and antifungals were analyzed at concentrations ranging from 512 to 0.5 μg/mL. All antibiotics tested were obtained from Sigma–Aldrich (St. Louis, USA).

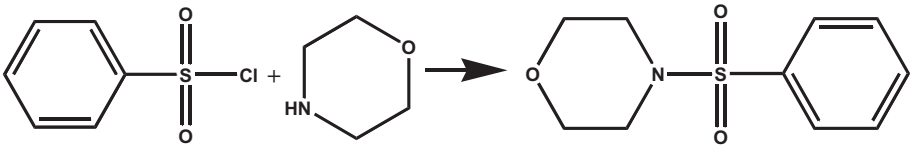

Figure 1 Synthesis of the compound 4-(Phenylsulfonyl) morpholine ($C_{10}H_{13}NO_3S$).

Table 1 Bacterial strains origin and resistance profile.

Bacteria	Origin	Resistance profile
Escherichia coli 27	Surgical wound	AZT, AMI, AMP, CFR, KAN, CAZ, CCL, CF, CIP, CHL, IMI, SXT, TET, TOB
Escherichia coli ATCC10536	–	–
Staphylococcus aureus 358	Surgical wound	AMI, BUT, KAN, GEN, NEO, NET, OXA, PARA, SIS, TOB
Staphylococcus aureus ATCC25923	–	–
Pseudomonas aeruginosa 03	Uroculture	AMI, CIP, CPM, CAZ, IMI, LEV, MEM, PTZ
P. aeruginosa ATCC 15442	–	–
Candida albicans ATCC 40006	–	–
Candida krusei ATCC 6258	–	–
Candida tropicalis ATCC 13803	–	–

AZT – Aztreonam; Amp – Ampicillin; AMI – Amikacin; CFR – Cefadroxil; KAN – Kanamycin; CAZ – Ceftazidime; CIP – Ciprofloxacin; CCL – Cefaclor; CF – Cephalothin; CHL – Chloramphenicol; IMI – Imipenem; SXT – Sulfamethoxazole and Trimethoprim; TET – Tetracycline; TOB – Tobramycin; BUT – Butirosin; GEN – Gentamicin; NEO-Neomycin; NET – Netilmicin; OXA – Oxacillin; PARA – Paromomycin; SIS – Sisomicin; CPM – Cefepime; CAZ – Ceftazidime; PTZ – Piperacillin-tazobactam; LEV – Levofloxacin; MEM – Meropenem.

4-(Phenylsulfonyl) morpholine was mixed in 10% BHI broth at sub-inhibitory concentrations, which were determined during the MIC evaluation tests. For the modulation test, the concentration of the extract solution was reduced eight fold (MIC/8). The preparation of the antibiotic solutions was performed with the addition of sterile distilled water at double concentration (1024 μg/mL) in relation to the initially defined concentration and volumes of 100 μL serially diluted (proportion: 1:1) in 10% BHI broth. Each well with 100 μL of the culture medium contained the diluted bacterial suspension (proportion: 1:10). The controls for the modulation assays were the same as those used in the MIC assays (Coutinho et al., 2008b). The plates were incubated at 35 °C for 24 h, after which the reading was performed with the aid of resazurin. The antimicrobial assays were carried out in triplicate, with mean values used for the analysis.

3. Results and discussion

The MIC of the compound was > 512 μg/mL for all bacterial (*E. coli*, *S. aureus* and *P. aeruginosa*) and fungal (*C. albicans*, *C. krusei* and *C. tropicalis*) strains, demonstrating a lack of clinical significance within the limits established by the protocol (Sriram et al., 2010). Moreover, a pilot test was performed using DMSO alone and no antimicrobial or modulating activity was found, indicating a lack of toxicity.

Table 2 displays the effect of 4-(Phenylsulfonyl) morpholine on aminoglycoside activity, demonstrating modulation of the activity of amikacin and gentamicin, with a reduction in MIC against the Gram-negative strains. The most representative effect was obtained with the combination of 4-(Phenylsulfonyl) morpholine at a concentration of 128 μg/mL (MIC 1/8) and amikacin against PA 03, with a reduction in the MIC from 312.5 to 39.06 μg/mL. In contrast, the substance demonstrated no capacity to modulate the action of aminoglycosides against strains of *S. aureus*. These findings are in agreement with data reported in the literature testing synthetic products against bacteria for the reduction of microbial resistance (Sriram et al., 2010; Ravat et al., 2009; Zhanel et al., 2009).

The synergism found in the modulation of antibiotic activity against *E. coli* and *P. aeruginosa* may be explained by the fact that 4-(Phenylsulfonyl) morpholine is derived from sulfonamides. This class of antibiotics is represented in therapy by sulfamethoxazole, the action mechanism of which consists of the blocking of the enzyme dihydropteroate synthase in bacteria. Sulfonamides are bacteriostatic agents that act as antimetabolites of *p*-aminobenzoic acid, which is the substrate for bacterial dihydropteroate synthase, impeding the formation of the dihydropteroate and, consequently, the formation of N^5,N^{10}-methylenetetrahydrofolate (Halland et al., 2014).

The morpholine group may also have affected the antimicrobial activity against *E. coli* and *P. aeruginosa*, as a study involving the combination of morpholine and dimorpholine demonstrated efficacy against bacteria and yeasts. The same study reports a significant decrease in fungal contamination (99.9%) and the degree of efficacy against this microorganism was maintained for a period of 28 days (Takahashi, 2012). In contrast, no significant results were achieved with regard to the strains of *Candida* tested.

The lack of modulating activity in neomycin against all strains may be explained by the difference in the structure of this aminoglycoside, which is a hydrophilic molecule composed of an aminocyclitol ring linked to one or more amino sugar through a glycosidic bond. In most of these compounds

Table 2 MIC values (128 μg/mL) of aminoglycosides in the absence and presence of the compound 4-(Phenylsulfonyl) morpholine in multiresistant strains.

Antibiotic	EC27		SA358		PA03	
	MIC	MIC combined	MIC	MIC combined	MIC	MIC combined
Amikacin	78.125	19.53	39.06	39.06	312.5	39.06
Gentamicin	39.06	9.76	4.88	4.88	39.06	9.76
Neomycin	156.25	156.25	156.25	156.25	312.5	312.5

EC27 – *Escherichia coli*; SA358 – *Staphylococcus aureus*; PA03 – *Pseudomonas aeruginosa*; MIC – Minimum inhibitory concentration.

with clinical usefulness, the aminocyclitol group is 2-deoxystreptamine, which can be substituted in the 4 and 5 or 4 and 6 position (Magnet and Blanchad, 2005), thereby influencing the solubility, polarity and absorption of the drug.

Due to absorption to the intercellular space, a toxic effect is common to all aminoglycosides, except spectinomycin. Renal toxicity causes nerve damage, being the oto/neurotoxicity the main toxic effect of aminoglycosides (Vallejo et al., 2001; Oliveira et al., 2006).

Thus, the combination of compounds with aminoglycosides may be an option to minimize undesirable effects when used for the treatment of infection by *E. coli* and *P. auruginosa*, since such combinations have a synergic effect, with a considerable reduction in the MIC of these drugs, allowing the reduction of the essential dose necessary for treatment success (Figueredo et al., 2013).

The lack of a significant effect against *S. aureus* may be explained by the fact that this microorganism has a number of virulence mechanisms and a high degree of versatility regarding pathogenic strategies, which enhances its resistance to antibiotics (Coutinho et al., 2008a; Tavares, 2000). Epidemiological studies evaluating the resistance of *S. aureus* report mean resistance rates of 63.25%, 76.5%, 56% and 71.25% to ciprofloxacin, clindamycin, tetracycline and trimethoprim–sulfamethoxazole, respectively (Almeida et al., 2007).

4-(Phenylsulfonyl) morpholine exhibited no significant modulating effect on the antifungal agents amphotericin B, benzoilmetronidazol, mebendazole and nystatin against *C. albicans*, *C. tropicalis* or *C. krusei*. The findings are in agreement with previous study demonstrating fungal resistance (Silva, 2009). This resistance may occur due to the presence of chitin in the cell wall of these microorganisms, which serves as structural support (Merzendorfer and Zimoch, 2003). As chitin is insoluble in most solvents, it hinders the entrance of the antifungal agent (Mathur and Narang, 1990).

4. Conclusion

The MIC of 4-(Phenylsulfonyl) morpholine was >512 μg/mL for all bacterial and fungal strains tested, demonstrating a lack of antimicrobial activity. However, when used as a modulating agent of amikacin and gentamicin, the synthetic substance tested lowered the MICs of these aminoglycosides against Gram-negative strains. This finding demonstrates that 4-(Phenylsulfonyl) morpholine has antibacterial and antibiotic modulatory activities, being this synthetic compound an interesting weapon against drug resistant bacteria. However, more data, mainly toxicological assays must be performed before this use.

Conflict of interest

The authors declare to have no conflict of interest regarding the contents of this article.

Acknowledgement

The authors acknowledge the support of the Brazilian research agencies FUNCAP and CNPq.

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