STUDIES ON THE ANTIMYCOBACTERIAL CLASS ACTION OF A NOVEL COMPOUND OF THE THIADIAZOLE CLASS, 2-(PROPYL-THIO)-5H-[1,3,4]-THIADIAZOLE[2,3-B]-QUINAZOLINE-5-ONE

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Abstract

The aim of the current study was the antimicrobial screening of a newly synthesised thia diazolic compound, 2-(propylthio)-5H-[1,3,4]-thiadiazolo[2,3-b]-quinazoline-5-one. The study was conducted for the qualitative and quantitative assessment of the antimicrobial activity, as well as its influence on the adherence capacity inhibition of certain microbial strains. Antimicrobial activity has been approached by means of instrumental techniques, such as flow cytometry. The results of this study have demonstrated the effect of the newly synthesised compound against Gram-negative bacteria through the permeabilization of the bacterial cell wall and against Gram-positive bacteria through the inhibition of the efflux pumps activity.

Rezumat

Scopul prezentului studiu a fost screening-ul antimicron al unui compus tiadiazolic nou sintetizat, 2-(propiltio)-5H-[1,3,4]-tiadiazolo[2,3-b]-chinazolin-5-onă. Studiul a fost efectuat pentru a evalua calitativ și cantitativ activitatea antimicrobiană, precum și de a inhiba capacitatea de aderență a anumitor tulpiți microbieni. S-au folosit tehnici instrumentale pentru evaluarea activității antimicrobiene, precum citometria în flux. Rezultatele acestui studiu au demonstrat faptul că noul compus sintezat acționează prin permeabilizarea peretelui celular bacterian asupra bacteriilor Gram-negativ și prin inhibiția pompelor de eflux asupra bacteriilor Gram pozitive.

Keywords: tuberculosis, thiadiazoles, antimicrobial action, flow cytometry

Introduction

Tuberculosis is one of the most dangerous infectious diseases, annually resulting in the death of 1.5 million people worldwide [6]. The estimated prevalence of all tuberculosis forms in the Republic of Moldova and in Romania is among the highest in the European Union and in countries of the Eastern Partnership, amounting to 234.1 and 144.6 cases per 100,000 inhabitants, respectively [3, 6, 14]. World Health Organization (WHO) data [6] show high Mycobacterium tuberculosis resistance to the action of antituberculosis medication. Based on these data, it is imperative to develop novel compound classes with a higher antituberculous potential [1].
A study performed by Macaev et al. [10] reported the synthesis of novel compounds from the 5-aryl-2-thio-1,3,4-oxidiazoles class, as well as correlations between their chemical structure and antimycobacterial activity [2, 8]. Between 2005 and 2016, Macaev and collaborators published their results on the synthesis and activity against Mycobacterium tuberculosis, obtained for the compound 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one. From the series of newly synthesised compounds, this molecule has been selected to establish the antituberculosis activity because, by QSAR approaches, it has shown a potential biological action [12]. The determination of the antituberculosis activity has been performed by the nitrate reductase (NRA) method [5, 11]. The results of these studies revealed intense antituberculous activity against Mycobacterium tuberculosis strain H37Rv (ATCC 27294), expressed in inhibition percentage of 100% for a minimum inhibitory concentration (MIC) of 6.25 mg/mL, in comparison with rifampicine [5, 12]. The aim of the current study was to extend Macaev and collaborators research on the antimicrobial screening of a newly synthesised thia diazolic compound, 2-(propylthio)-5H-[1,3,4]-thiadiazole [2,3-b]-quinazoline-5-one.

The study was conducted for the qualitative and quantitative determination of antimicrobial activity, as well as its influence on the adherence capacity inhibition of certain microbial strains. Instrumental techniques such as flow cytometry were employed for the evaluation of the antimicrobial activity.

Materials and Methods

**Qualitative assessment of the antimicrobial activity — the adapted diffusion method**

The microbial strains selected for the assessment of the antimicrobial activity of the new compound have been: *E. faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10231). Bacterial 1.5 x 10^8 colony forming units (CFU)/mL suspensions have been prepared, using McFarland 0.5 nephelometric standard, from each 24 h - microbial culture developed on solid medium (agar A). The bacterial inoculum was seeded on Müller-Hinton solid medium, in the groove. The 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one compound was then added in the middle of the plated spot. The plate was incubated for 24 h at 37°C. Results have been estimated after 24 h, by assessment of the striatal microbial growth off/around the powder set out on the culture medium.

**Quantitative assessment of the antimicrobial activity - the microdilution method**

The quantitative determination of the antimicrobial activity has been conducted by the binary serial microdilution method, in Müller-Hinton liquid medium, in 96 well plates, concentrations of 0.5 - 256 μg/mL. 1.5 x 10^5 CFU/mL microbial suspensions have been prepared and the wells were inoculated at a 10% (v/v) rate. Controls have been used, both positive - microbial growth controls and negative controls - blank dilutions of the test compound. The plates have been incubated at 37°C, for 24 hours. Determination of the minimum inhibitory concentration (MIC) has been performed macroscopically, by assessing the smallest concentration for which no microbial growth and broth turbidity were observed as well as by spectrophotometrical evaluation at 620 nm.

**The influence on the inert substrate adherence capacity of the tested microbial strains**

The influence over the inert substrate adherence capacity has been quantified after conducting the protocol for the quantitative analysis of the antimicrobial effect. This was achieved using the microtitration method, by assessing the adhered biomass, after fixation with methanol and crystal violet staining [4, 7]. The plate with 96 wells was cleared of content (microbial cultures, controls), washed 3 times in sterile physiological water (SPW), and fixed with cold methanol (150 μL methanol volume to each well), for 5 min. The plate was cleared, dried, and stained with a 0.1% crystal violet solution, for 15 min, washed in SPW. After drying, the adhered biological material was resuspended in an acid solution.

**The bacterial efflux pump activity assay**

Assessment of the 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b] quinazoline-5-one compound effect on the bacterial efflux pump activity has been performed by flow cytometry methods: the determination of cell viability by propidium iodide marking and evaluation of the bacterial efflux pumps activity by ethidium bromide marking. Data have been acquired using a FACS Calibur cytometer. The analysis software of a BD CellQuest Pro v.4.0.2 cytometer has been used for data statistical analysis.

Flow cytometry is an analytical method for the simultaneous measurement of the physical and molecular characteristics of the various cells and at the same time for cell sorting. The method is based on the measurement of light scatter as compared to laser incident radiation - *forward scatter* (FSC) at low (2º - 10º) angles, correlated with cell size, and *side scatter* (SSC) at 90º angles, coupled with cellular complexity and cytoplasmic granularity –
as well as measurement of fluorescence parameters, on passing of a linear laminar cell flow by the laser beam at a right angle. Fluorochromes are chemicals able to absorb light at a certain wavelength and to emit light at a higher wavelength (with lower energy). Propidium iodide and ethidium bromide are DNA specific fluorochromes, intercalating in the DNA double-helical structure and causing an increase of the fluorescence [15].

Propidium iodide (3,8-diamino-5-diethylmethyl-amino-propyl-6-phenyl-phenanthridinium diiodide) is an intercalating fluorochrome, with an absorption spectrum in the 300 - 650 nm wavelength range and an emission spectrum in the 550 - 724 nm wavelength range. It is used to determine cell viability, because live cells are impermeable to this dye due to membrane integrity.

Ethidium bromide (2,7-diamino-9-phenyl-10-ethylphenanthridinium bromide) is a fluorochrome, with an absorption spectrum in the 400 - 625 nm wavelength range and an emission spectrum in the 525 - 750 nm wavelength range. Due to its ability to emit weak fluorescence outside cells and strong fluorescence by intracellular accumulation, it is used for the detection and the quantification of bacterial efflux pump activity. Fluorescence intensity is thus a measure of cell content in ethidium bromide and a net ratio between ethidium bromide influx, due to bacterial cell wall permeabilization, and its extracellular elimination, due to efflux pump activity [15].

100 mL of MIC/2 wells were collected in Eppendorf tubes for each microbial strain. Next, the tubes were centrifuged at 13,000 rpm for 3 min and the supernatant was further disposed of the tubes. The sediment was next washed with 200 mL SPW. The tubes were again centrifuged at 13,000 rpm for 3 min. The supernatant was again disposed of the tubes and the sediment was marked with either 10 mL propidium iodide to give a final 5 mg/mL concentration, or with 10 mL ethidium bromide for a final 10 mg/mL concentration. Then, the tubes were incubated at 4°C, in the dark, for 10 min. Marked samples were diluted with 100 mL filtered SPW and submitted to flow cytometry.

Positive controls for viable cells were prepared from 1.5 x 10^8 CFU/mL microbial cell suspensions marked with propidium iodide or ethidium bromide. Negative controls were prepared by heat treatment of 1.5 x 10^8 CFU/mL bacterial suspensions (QBD1 Granthermoblock, 100°C, for 30 min). Samples were marked with propidium iodide or ethidium bromide.

Results and Discussion

Qualitative assessment of the antimicrobial activity – the adapted diffusion method

The qualitative assessment of the antimicrobial activity has revealed microbial growth for all strains under study in the bacterial culture traced on solid medium. This may be correlated with the absence of diffusion for the test substance in the culture medium which does not allow for a gradient of its concentration in the environment and neither the assessment of the antimicrobial activity by the diffusion method (Figure 1).

![Figure 1](image1.png)

Qualitative testing of the antimicrobial activity of the 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one compound, the adapted diffusion method – tested strains: 1 - P. aeruginosa (ATCC 27853), 2 - E. coli (ATCC 25922), 3 - E. faecalis (ATCC 29212), 4 - B. subtilis (ATCC 6633), 5 - C. albicans (ATCC 10231), 6 - S. aureus (ATCC 6538)

The method could be appreciated as semiquantitative for antibiotics, but not for other compounds, because of the substance diffusion through the culture medium. However, even if no antimicrobial effect could be observed in the qualitative testing step, the protocol was followed. Quantitative assessment of the antimicrobial activity – the microdilution method

This study has allowed the evaluation of the antimicrobial activity range of the new compound by quantitative assessment. Dimethyl sulfoxide (DMSO), the solvent used for the solubilisation of 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one compound, did not exhibit antimicrobial activity over the investigated strains. MIC values for strains under study are within the 640 – 20 μg/mL range, and the most sensitive are the fungal strain (C. albicans (ATCC 10231)) and the E. faecalis strain (ATCC 29212). The studied substance displays maximum antimicrobial activity against the Gram-positive and the fungal strains (Figure 2).
The extended study particularly attempted to confirm the valuable antifungal activity of the compound. A wide spectrum of the antimicrobial activity has been demonstrated, but conclusions concerning the specificity of the antimicrobial activity might be possible after testing more strains belonging to the same species.

Figure 2.
The influence of the compound 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one to the MIC microorganisms studied

The influence on the inert substrate adherence capacity of the tested microbial strains
The inert substrate adhesion capacity of certain microbial strains is an important milestone in establishing an infectious process induced thereof, because it is determined by certain adhesins produced by bacterial cells. The adhesion capacity of bacterial strains tested may be highlighted by interactions of bacterial cells with the cell substrate. Results of this study showed that the tested compound exhibited antiadherence activity against all strains at MIC/2 concentrations harvested from the wells with halved minimum inhibitory concentrations.

The assay is a colorimetric method that could be appreciated by visual inspection or spectrophotometrical reading. The results have been estimated qualitatively. The importance of the study is that the subinhibitory concentrations of the compound could lead to the decrease of the pathogenic effect for all the tested strains. Therefore, the results could be a premise for assessing the antipathogenic effect of the tested compound.

The bacterial efflux pump activity assay
The alternative marking of samples with two fluorochromes at halved minimum inhibitory concentrations (MIC) allowed the assessment of its mechanism against microbial strains, i.e. permeabilization of cell coatings or inhibition of microbial efflux pumps. This latter mechanism is of particular interest since it may cause conversion of resistant microbial phenotypes by targeting efflux pumps activity by means of an inhibitor, which should restore the microorganism responsiveness to antibiotics. Ideally, the inhibitor potentiates antibiotic activity over-drug-multi resistant microorganisms and therefore reduces the minimum inhibitory concentration of the tested antibiotic [9]. Phenyl-arginyl-β-naphthylamide (PaβN) has been the first inhibitor identified in Pseudomonas strains displaying MexAB-OprM type pumps. This peptidomimetic compound shows a competitive inhibition mechanism, but its toxic effects do not allow its use for clinical applications [13]. Figures 3 and 4 are diagrams of the average fluorescence for sample alternative marking.

Figure 3.
Average fluorescence intensity (MFI) for ethidium bromide (EB) labelled samples

Figure 4.
Average fluorescence intensity (MFI) for propidium iodide (PI) labelled samples
Typical aspects of histograms obtained for sample labelled with propidium iodide - histograms 1 and 2 (figure 5), have revealed permeabilization of cellular coatings for P. aeruginosa (Figure 5a.) and E. coli (Figure 5b.), as evidenced by the increase of samples fluorescence compared to the viable cells control (filled grey peak).

Typical aspects of the histograms obtained for the samples labelled with ethidium bromide, c, d, e and f (Figure 6), revealed increased MFI for the studied Gram-positive bacteria (E. faecalis, B. subtilis, S. aureus) and fungi (C. albicans), in comparison with the viable cells control (filled grey peak). This particular result could be correlated with the efflux pump activity inhibition.

**Conclusions**

The qualitative evaluation of data regarding the antimicrobial activity (by the adjusted diffusion method) has shown microbial growth for all studied strains.

The quantitative assessment of the antimicrobial activity (by the microdilution method) has shown MIC values for the studied strains within the range of 640 - 20 mg/mL. C. albicans (ATCC 10231) and the E. faecalis (ATCC 29212) were the most sensitive.

Thus, it may be appreciated that the newly synthesised 2-(propylthio)-5H-[1,3,4]thiadiazole-[2,3-b]-quinazoline-5-one compound has maximum antimicrobial activity against Gram-positive and the fungal strains, and also a large spectrum of antimicrobial activity.

By correlating quantitative data with qualitative aspects of histograms obtained by fluorochrome
marking, it may be concluded that 2-(propylthio)-5H-[1,3,4]-thiadiazole[2,3-b]-quinazoline-5-one acts in line with the affinity for Gram staining of the studied strains. It acts against Gram-negative bacteria by cell coating permeabilization and against Gram-positive bacteria by efflux pumps inhibition. However, the results could be a premise for assessing the antipathogenic effect of the tested compound, as a new and valuable approach against microbial development.

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