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New benzamides with potential pharmacological activity

PHD THESIS ABSTRACT

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Introduction

The development of new therapeutic agents, active not only against planktonic microorganisms but also on biofilms, is one of the fundamental challenges in medicinal chemistry [1, 2]. The facile synthesis of benzamides has allowed the production of numerous derivatives, many of which have been evaluated for the antimicrobial activity.

Given these considerations and to continue the efforts in the development of new antimicrobial agents, the present work describes two research studies, which have as a common starting point the strategy to design and synthesize new bioactive compounds, containing simultaneously two pharmacophores, namely an *N*-acyl-thiourea fragment and a heterocyclic ring (thiazole, benzothiazole, pyridine or pyrimidine).

I. General part

1. Studies on combating resistance phenomena by developing new molecules

The increased resistance, established in most classes of antimicrobial agents, is recognized as a global health challenge [3, 4] and a worldwide public health problem [5]. Bacterial pathogenesis focused on acute infections has been debated in numerous scientific studies [6-8]. Alongside these issues, recent research studies has focused attention on chronic conditions caused by continuously evolving bacteria, displayed as mucilaginous aggregates, commonly known as biofilms [9].

Thiourea derivatives have been reported in the literature for their multiple biological activities, including antibacterial [10], antifungal [11], anticancer [12], and antioxidant [13] properties. Thiourea derivatives are also versatile intermediates in the synthesis of heterocyclic compounds such as 1,3-thiazoles, pyrimidines, 1,2,4-triazines, and 1,3-quinazoline [14].

Numerous studies have reported the potential of the *N*-acyl-thiourea moiety as a valuable component in the discovery of new anti-infective candidates [15].

2. Computational tools used in the development of new chemical compounds

The docking study is described as a computational method in which the favorable interaction between two molecules is predicted, where one entity is called the receptor and contains affinity sites, which provide and create binding sites for the second species - the ligand [16].

As thiourea derivatives are the subject of the current Ph.D. project, the proven affinities of thiourea derivatives towards the evaluated microbial strains have been sustained with data from the literature. The N-H and C=S groups in the thiourea fragment act as active binding sites between the newly studied ligands and the protein receptor on the surface of the microorganism [17]. The presence of multiple reactive binding sites (C=S, N-H, C=O) potentiates the biological activity of the designed molecules by enhancing the interaction possibilities [18, 19]. This is further supported by the fact that the binding sites between a compound and the receptor surface of microorganisms increase with the increasing number of thiourea moieties [20].

3. Research hypothesis and general objectives

The present research comprises the *in silico* studies, synthesis, antimicrobial, antibiofilm and antioxidant evaluation for two series of *N*-acyl-thiourea derivatives. Each of the two series of compounds was designed starting from a common core structure of the series.

In the first series of compounds, 15 derivatives of 2-((4-ethylphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide were obtained.

The second series contains 7 compounds, which share the molecular skeleton of 2-((4-methoxyphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide.

The objective of the work is to address topical research directions, starting from the development of novel analogues of structures that have proven to be effective.

4. General research methodology

For each set of compounds, the *in silico* studies were conducted using two computational tools: software programs (I) *Spartan 14* and (II) *CLC Drug Discovery Workbench 2.4*.

For the quantitative evaluation of the antimicrobial activity of the tested compounds, the standard broth microdilution method was applied using the following test microorganisms: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Minimum inhibitory concentrations (MICs) were determined by culturing different broth dilutions on Muller-Hinton agar plates.

The anti-biofilm activity of the compounds was evaluated using the microtiter plate test and crystal violet assay.

Total antioxidant activity (AAT) was evaluated using the DPPH assay. A UV-Vis UVD-3500 spectrophotometer was used for this purpose.

For cytotoxicity assays, I used ileocecal colorectal adenocarcinoma cells HCT-8 (ATCC CCL-244), colorectal adenocarcinoma cells HT29 (ATCC HTB-38), hepatocellular carcinoma cells HepG2 (ATCC HB-8065) and cervical carcinoma cells HeLa (ATCC CCL-2). Cell viability after treatment with the novel *N*-acyl-thiourea derivatives was assessed using fluorescein diacetate staining (FDA).

Caspase-1 activated by the new *N*-acyl-thiourea derivatives could be detected by the Caspase-Glo® 1 Inflammasome Assay (Promega, Madison, WI, USA).

The hemolysis test was performed according to Lourenço et al [21].

Flow cytometry cell cycle analysis was performed after treatment of HCT 8 cells with *N*-acyl-thiourea derivatives by propidium iodide reaction.

For the quantitative determination and separation of the compounds that stood out from each series, two analytical methods were developed and optimized by using the high-performance liquid chromatography technique. The Waters Alliance HPLC system, consisting of the following modules, was used for the determination: 2695 + 2998 separation module, 998 PDA detector, and PC equipped with *Empower PDA Software*.

Four representative compounds of the first series were loaded into three types of matrices (MCM-41 mesoporous silica, FDU-12 mesoporous silica and magnetite). The resulting nanomaterials were analyzed by Brunauer-Emmett-Teller analysis, FT-IR spectroscopy, thermogravimetric analysis and differential scanning calorimetry, and X-ray diffraction.

5. Contribution to the design, synthesis, characterization, separation and quantification of novel 2-((4-ethylphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide derivatives with antimicrobial and antioxidant potential

The study has aimed to design, synthesize, characterize and develop a method for the separation and quantification of novel 2-((4-ethylphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide derivatives incorporating a thiazole or pyridine nucleus.

In this chapter, 15 compounds have been evaluated by determining a series of molecular descriptors (e.g., area, volume, polarizability, log P, polar surface area) interconnected to elucidate

and predict their behaviour in the human body, based on their chemical structure. As the central theme is to obtain derivatives with antimicrobial activity, I initially performed molecular docking studies to predict the antimicrobial potential towards selected target proteins. Analyzing the docking results, correlated with the interaction group of co-crystallized natural ligands of *Staphylococcus aureus* and *Escherichia coli* strains, the studied molecules took appropriate conformations in the active site of the receptor protein, forming hydrogen bonds with the rest of the amino acids in the selected protein. The established conformation was then quantified in a docking score, describing the highest fit of the studied molecule in the active site of the receptor.

The *in silico* results encouraged the initiative to synthesize the 2-((4-ethylphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide derivatives. Thus, the new compounds were obtained by treating 2-((4-ethylphenoxy)methyl)benzoyl isothiocyanate with a heterocyclic amine. The isothiocyanate was obtained *in situ* by the reaction of 2-((4-ethylphenoxy)methyl)benzoic acid chloride with ammonium thiocyanate. The acid chloride was prepared by refluxing 2-((4-ethylphenoxy)methyl)benzoic acid with thionyl chloride in anhydrous 1,2-dichloroethane medium. This acid was obtained by treating the corresponding potassium salt with a mineral acid, salt that in turn was obtained from phthalide and *p*-ethylphenol.

The resulting compounds were characterized by determining their melting points and assessing their solubility in different solvents, and their structure was confirmed by ^{1}H-NMR, ^{13}C-NMR, infrared spectroscopy and mass spectrometry (APCI+).

The *in vitro* antimicrobial activity of the new molecules has been evaluated on bacterial strains, which have developed resistance to currently available antibiotics and have a high capacity for biofilm growth [22]. Specifically, four compounds, 2-((4-ethylphenoxy)methyl)-*N*-(thiazol-2-yl-carbamothioyl)benzamide (**1a**), 2-((4-ethylphenoxy)methyl)-*N*-((5-chloropyridin-2-yl)carbamothioyl)benzamide (**1g**), 2-((4-ethylphenoxy)methyl)-*N*-((2-chloropyridin-3-yl)carbamothioyl)benzamide (**1h**) and 2-((4-ethylphenoxy)methyl)-*N*-((3,5-dibromopyridin-2-yl)carbamothioyl)benzamide (**1o**) were the most potent inhibitors of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 strains at minimum inhibitory concentrations of 625 µg/ mL. In the bacterial adherence test, compound **1e** showed the lowest biofilm inhibitory concentration of *Escherichia coli* ATCC 25922 (MBIC = 312 µg/ mL).

Subjected to the DPPH assay, 13 compounds in Series I showed antioxidant activity. Of the series, the compound 2-((4-ethylphenoxy)methyl)-*N*-((2-chloropyridin-4-yl)carbamothioyl)benzamide (**1i**) had the highest free radical scavenging capacity at 87%, followed by **1a** (44%), compared to ascorbic acid, used as the reference antioxidant.

The compounds were subjected to cytotoxicity assay performed on HCT-8 adenocarcinoma cells. The results of flow cytometry analysis raise the likelihood of G0/G1 phase block after prolonged contact time with *N*-acyl-thiourea derivatives.

Following cell monitoring using the Incucyte automated system, no difference in toxicity levels was observed in the cell lines tested for Series I. By determining the level of hemolysis induced when treating erythrocytes with *N*-acyl-thiourea derivatives of Series I, the compounds tested were found to be non-hemolytic.

For isomeric chlorinated compounds, I developed a separation method, using reversed-phase high-performance liquid chromatography (RP-HPLC). By evaluating the validation parameters, according to ICH guidelines [23], the method was found to be suitable for the intended purpose, i.e., separation and quantification of the analyzed isomers.

6. Contribution to the design, synthesis, characterization and quantification of novel 2-((4-methoxyphenoxy)methyl)-*N*-(heteroaryl-carbamothioyl)benzamide derivatives with antimicrobial and antioxidant potential

Similar to the steps taken in the evaluation of the first series, I have resorted to characterizing new compounds of the 2-((4-methoxyphenoxy)methyl)-*N*-(heteroaryl-carbamotioyl)benzamide series by *in silico* approaches. Molecular descriptors described the nature of the compounds and their ability to exert a bioactive effect. Through molecular docking studies, it was possible to predict the binding affinities between the studied molecules and the receptors of the bacterial strains *Staphylococcus aureus* and *Escherichia coli*. Consequently, *in silico* data provided a means to explore the in vitro affinity of the designed molecules towards selected proteins.

Synthesis of the *N*-acyl-thiourea derivatives in this series, containing thiazole, benzo[d]thiazole, pyridine or pyrimidine moieties in the structure, was performed similarly to the Series I study, i.e. condensation of 2-(4-methoxyphenoxymethyl)benzoic acid chloride with

ammonium thiocyanate in anhydrous acetone, followed by reaction of the resulting isothiocyanate with a heterocyclic amine. Since the synthetic yield of the compound *N*-(benzo[d]thiazol-2-yl-carbamotioyl)-2-((4-methoxyphenoxy)methyl)benzamide (**1b**) was low, I conducted a study to optimize its synthesis. For this purpose, I used a phase transfer catalyst, tetra-*n*-butylammonium bromide (TBAB). The yield was improved to 76% compared to the 41% yield of the reaction carried out without the catalyst.

The structures of the synthesized compounds were proved by Fourier transform infrared spectrometry (FT-IR), nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (FT-ICR).

The compounds were investigated biologically *in vitro* by testing antimicrobial, antibiofilm and antioxidant capacities.

The antimicrobial activity of the Series II compounds was examined on selected standard bacterial strains. The tested compounds showed low antimicrobial activity against planktonic cells, with MIC values of > 5000 - 1250 µg/ mL compared to the control (ciprofloxacin). In terms of antibiofilm effects, the results showed MBIC values between > 5000 and 625 µg/ mL. Compounds **1b** and 2-((4-methoxyphenoxy)methyl)-*N*-((6-methylpyridin-2-yl)carbamothioyl)benzamide (**1d**) showed the best antibiofilm activity against *Escherichia coli* strain ATCC 25922 at MIC values of 625 µg/ mL.

The highest antioxidant capacity was recorded for compound **1d** (~ 43%) compared to sodium ascorbate, used as the reference antioxidant.

The compounds were tested for cytotoxicity and the determination was performed on HCT-8 adenocarcinoma cells. After 48 hours of treatment, HCT-8 cells were harvested to examine the effects on cell cycle phases by flow cytometry. The results indicated that *N*-acyl-thiourea derivatives blocked the G0/G1 phase of the tested cells and caused a comparable decrease in the S phase. The results are consistent and statistically significant.

Following the results of the experimental analyses and highlighting the most promising compound of the series, I developed a quantitative analysis method using the RP-HPLC technique. The validation of the analytical procedure was also carried out in this case according to the ICH Q2 (R1) guidelines. The statistical data calculated from the chromatographic records demonstrated that the analytical method is suitable for the quantitative determination of compound **1d** and can be used in routine analyses.

7. Conclusions and personal contributions

The two research studies focus on the design, synthesis, characterization, separation and quantification of novel 2-((4-ethylphenoxy)methyl)-*N*-(heteroaryl-carbamotioyl)benzamide and 2-((4-methoxyphenoxy)methyl)-*N*-(heteroaryl-carbamotioyl)benzamide derivatives, not reported in the literature, with antimicrobial and antioxidant potential.

The research methodology was based on the evaluation of new chemical structures by *in silico* approaches. Obtaining the two series of compounds includes several optimized and reproducible synthesis steps. The resulting compounds were characterized by determining melting points and assessing solubility in different solvents, and their structure was confirmed by ^{1}H-NMR, ^{13}C-NMR spectra, infrared spectroscopy and mass spectrometry.

Following *in vitro* testing of biological action, the compounds showed antimicrobial, antibiofilm, and antioxidant activities.

The originality of the work is represented by the presence of two biologically active pharmacophores, namely the *N*-acyl-thiourea moiety and the heterocyclic core in the same molecule. The design (*in silico*), the synthesis process and the yield optimization method consist of original aspects.

Personal contributions also include characterization of new compounds, spectral analysis and high-performance liquid chromatography. The validated separation and quantification methods are of originality character and are the subject of my own contribution in the field of HPLC developments.

The loading of MCM-41 mesoporous silica, FDU-12 mesoporous silica and magnetite matrices with *N*-acyl-thiourea derivatives and the characterization of the resulting nanomaterials are also the own contribution to the research on this type of nanostructures and their potential utility in improving antimicrobial activity.

Deepening the relationships between the chemical structure and the biological activity of the new compounds, analyzing the mechanisms of action for the derivatives that have shown antimicrobial, antibiofilm and antioxidant activities, completing the bioactive profile of the new *N*-acyl-thiourea derivatives, as well as evaluating the antimicrobial and antibiofilm activity of the nanoparticles loaded with the studied derivatives, open new perspectives and may be the subject of future research.

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2. **Roman R**, Pintilie L, Nuță D, Avram S, Buiu C, Sogor C, Limban C. In Silico Prediction, Characterization and Molecular Docking Studies on New Benzamide Derivatives. *Processes*, *11*, 479, 2023. https://doi.org/10.3390/pr11020479

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(Article from the chapter *6. Contribution to the design, synthesis, characterization and quantification of novel 2-((4-methoxyphenoxy)methyl)-N-(heteroaryl-carbamothioyl)benzamide derivatives with antimicrobial and antioxidant potential, 6.3.2. Synthesis of novel compounds 1a - 1g, 6.3.3. Physico-chemical characterization of new compounds 1a - 1g, 6.3.4. Evaluation of the biological activity of series II compounds, 6.3.5. Analytical method for quantitative determination by RP-HPLC, 6.4. Discussion*)