Abstract: In this paper, the synthesis, characterization, and biological evaluation of the novel tetrahydropyrimidines—THPMs are described. THPMs are well-known for wide pharmacological activities such as antimicrobial, anticancer, antiviral, etc. This research includes obtained results of in vitro antimicrobial, anticancer, and α-glucosidase inhibitory activities of the eleven novel THPMs. An antibiotic assessment was done against five bacteria (two Gram-positive and three Gram-negative) and five fungi by determining the minimal inhibitory concentration (MIC), using the broth tube dilution method. The most active antibacterial compounds were 4a, 4b, and 4d, while the best antifungal activity was shown by 4e, 4f, and 4k. The lowest MIC value (0.20 mg/mL) was measured for 4e, 4f, and 4k against the Trichophyton mentagrophytes. Moreover, examining the α-glucosidase inhibitory activity revealed the compound 4g as the one with the best activity. The cytotoxic activity was performed on the tumor cell lines (HeLa, K562, and MDA-MB-231) and normal cells (MRC-5). The best antitumor activity was shown by compounds 4b and 4k against HeLa cell lines. The influence on cell cycle and mechanism of action of the most active compounds were examined too. Compound 4b had good antibacterial and anticancer activities, while 4k showed promising antifungal and anticancer activities.

Keywords: Biginelli reaction; tetrahydropyrimidine; antimicrobial; anticancer; apoptosis

1. Introduction

Pietro Giacomo Biginelli (1860–1937) was an Italian chemist who discovered a one-pot multicomponent reaction. He invented the procedure that would subsequently be named Biginelli synthesis in his honor [1,2]. Despite being the first multicomponent reaction, the scientific community dismisses the Biginelli reaction as outmoded. Nevertheless, since the finding of monastrol as a kinesin-5 inhibitor [3], as well as its anticancer activity, Biginelli chemistry has expanded [4–6]. Huge numbers of tetrahydropyrimidines—THPMs (former name 3,4-dihydropyrimidine-2(1H)-(thi)ones-DHPMs) have been synthesized using the Biginelli reaction to date. THPMs derivatives have a wide range of biological effects, including anti-inflammatory, antiviral, anticancer, calcium channel inhibition, antibacterial, antifungal, and antioxidant [7,8]. Significant anti-inflammatory activity of the Biginelli thioxo-hybrids was denoted towards various inflammatory diseases [9–11]. Although
compounds delivered from a Biginelli reaction could possess broad spectra of antiviral activities, among them the most important were those that had inhibitory potential against HIV [12–14]. However, it was shown that thio-Biginelli adducts are significantly better calcium channel inhibitors compared to appropriate oxo- and aza-hybrids. In that sense, scientists have found that the (R)- was more potent than the (S)-enantiomer, which implies that the configuration at the C4 stereocenter plays a key role in this kind of activity [15,16]. Some Biginelli compounds bearing a pyrazole scaffold exhibited good-to-high potency against Mycobacterium tuberculosis [17,18]. In addition, the presence of 1,3-dihydro-2H-indol-2-one core in the structure of oxo- and thioxo-Biginelli’s analogs also displayed good antibacterial activities against Bacillus subtilis, E. coli, and S. aureus [19]. Indeed, the most useful aza-nucleophile in Biginelli’s chemistry is urea or N-methylurea. However, over the years were shown as quite hard to apply thiourea or thiourea-based nucleophiles in Biginelli’s chemistry [20–25]. This fact lies predominantly in its behavior and huge sensitivity towards strong acids and bases, even the Lewis acid/base salt type. In the above-mentioned conditions with heating, it is very hard to apply thioureas in the Biginelli reaction with success. Furthermore, efficient synthesis of Biginelli’s hybrids containing thiourea-based nucleophiles was and is quite challenging. Considering this, our goal was to synthesize a small library of novel THPMs derivatives containing N-methylthiourea functionality. Specific objectives were an investigation of their potential for dual biological activities against various microbes and cancer cell lines.

2. Materials and Methods

2.1. Chemistry

All solvents and substrates (acetyl, benzoyl, and p-toluoyl chloride, methyl acetocetate, N-methylthiourea, vanillin, 5-bromovanillin, 5-iodovanillin, 5-nitrovanillin) were purchased from Sigma. Vanillic derivatives 1d–k were prepared following the procedures described previously in the literature [26]. We determined melting points (Mp) on a Mel-Temp apparatus. For recording IR spectra, we used a Perkin–Elmer Spectrum One FT-IR spectrometer on KBr pellet. A Varian Gemini 200 MHz NMR spectrometer (1H at 200 and 13C at 50 MHz) was utilized for NMR characterization of compounds 4a–k. The compounds were dissolved in DMSO-d6. 1H and 13C NMR spectra are presented in Supplementary Materials (see Figures S1–S22).

Experimental Procedure for Synthesis of 4a–k

A total of 6 mmol of N-methylthiourea and 2 mmol of aldehyde were dissolved in the mixture of dioxane/CHCl3 (4/1, v/v) in a 25 mL round-bottom flask. Ten minutes later, 3 drops of HCl (36.5%) were added at room temperature. In all cases, yellow color appeared. Then, two hours later, 6 mmol of methyl acetocetate and 10 mol% of 2-amino-1-(4-nitrophenyl)-1,3-propanediol (ANP) were loaded. After one day, the solvent was evaporated. Crude solid had been dissolved in ethanol/water mixture (2/1, v/v). The product was then put in the fridge; after 24 h in those conditions, a precipitate appeared. The formed powder was filtered, and washed with acetone and water. Recrystallization from acetone or ethanol/water was done to reach the desired product with a good purity grade (>95%).

2.2. Sample Preparation for MS Analysis

Having in mind that the experiment was not quantitative in nature, care was taken to introduce about 10 ng of the compound into the MS system; a quantity that will provide adequate response without saturating the detector of the instrument. Solid samples were weighted on an analytical balance into the 10 mL volumetric flask up to cca. 10 mg and dissolved in HPLC-grade acetonitrile, solutions were vigorously shaken and subsequently sonicated for 10 min at 40 °C in order to dissolve completely. Further dilution to the final concentration of cca. 10 ng/μL (in acetonitrile) was prepared directly into the autosampler vials.
Confirmation of theoretical masses was carried out on a Shimadzu (Kyoto, Japan) LC-MS/MS system consisting of two LC-40D × 3 pumps, DGU-405 degassing unit, CTO-40S column oven, SIL-40C × 3 autosampler, and LCMS-8050cl mass spectrometer with ESI interface. The flow injection analysis (FIA) technique was used for direct introduction of the sample into the inlet system avoiding chromatographic separation. Stainless steel tubing (4 m × 0.1 mm ID) was attached instead of the column to the LC pumps/autosampler and mass spectrometer providing a retention time of about 25 s. The mobile phase was 0.1% formic acid in acetonitrile, flow rate was set to 0.2 mL/min.

Positive ESI was used for all compounds with the following parameters: nebulizing gas flow 3 L/min; drying gas flow 10 L/min; heating gas flow 10 L/min; interface (capillary) voltage 4.5 kV; interface temperature 350 °C, desolvation temperature 600 °C; desolvation line temperature 250 °C; heat block temperature 300 °C.

The mass spectrometer operated in Q3 scan mode (unit resolution), the scanning range was set from 200 to 800, the scanning speed was 600 u/s.

All materials and methods used for biological examination (antibacterial, antifungal, anticancer, and α-glucosidase inhibitory activities) and crystal structure determination are presented in the Supplementary Materials, File S1.

3. Results and Discussion

3.1. Chemistry

At the beginning of our research, we were seeking the best reaction conditions to synthesize tetrahydropyrimidine 4a under a one-pot manner by applying various acid-based catalysts. For this part of the investigation, methanol, n-butanol, and dioxane were utilized as solvents. Under classical Biginelli conditions with hydrochloric acid as a catalyst, the desired product was isolated in a lower yield than expected in all applied solvents. When amidosulfonic acid was used as a catalyst, 4a was obtained in the yield of 35–50% in the mentioned solvents under reflux. Among the applied solvents, the reaction in dioxane gave the best yield of 4a. However, reactants 1a, 2, and 3 were not fully converted into the expected product. At room temperature, the yield was lower. We decided to apply another strategy preparing an imine intermediate that would allow better production of 4a. First, we mixed the aldehyde with N-methylthiourea, and 3 drops of 36.5% HCl at room temperature in the mixture of dioxane and CHCl₃. Based on TLC, one hour later, we added active methylene 3, and substituted 2-amino-1,3-propanediol. The targeted product 4a was obtained in 63% yield. The reaction without 2-amino-1,3-propanediol only produced imine intermediate. Going forward, different substituted aldehydes were employed to broaden the substrate scope (Scheme 1). The usage of thioureas and their derivatives is quite challenging in Biginelli chemistry, and there are a couple of described procedures in the literature [27,28]; in this research, we performed a reaction at room temperature in a specific mixture of solvents with good yields.

The structures of the isolated Biginelli hybrids (4a–k) were confirmed by IR, NMR, and ESI-MS spectroscopy. The compound 4j was suitable for crystallographic analysis. Benzylic proton appeared as a doublet in the range 5.10–5.40 ppm. Thioamide proton and carbon were detected at around 10 ppm, and 178 ppm, respectively. In the ESI-MS spectrum of 4a, a molecular ion was found at 401 [M]+ and 403 [M + 2]+ m/z.
Scheme 1. Substrate scope in the synthesis of tetrahydropyrimidines 4a–k. Reaction conditions: (a) aldehyde and N-methylthiourea, dioxane/CHCl₃, room temperature, (b) methyl acetoacetate, HCl, 2-amino-1-(4-nitrophenyl)-1,3-propanediol (ANP), room temperature.

3.2. X-ray Crystallography

Figure 1 shows a perspective view of the molecular structure of methyl 4-(4'-benzoyloxy-3'-methoxyphenyl)-1,2,3,4-tetrahydro-1,6-dimethyl-2-thioxopyrimidine-5-carboxylate (4j). A list of selected torsion angles, bond angles, and bond lengths is tabulated in Table 1.

The asymmetric unit of compound 4j consists of two moieties: one methyl 4-(4'-benzoyloxy-3'-methoxyphenyl)-1,2,3,4-tetrahydro-1,6-dimethyl-2-thioxopyrimidine-5-carboxylate molecule and half of 1,4-dioxane molecule. The conformation of 4j is best defined by the torsion angles $\angle C11$–O4–C8–C7 = 103.7 (2)$^\circ$, $\angle C11$–O4–C8–C9 = −80.8 (3)$^\circ$, $\angle C10$–C5–C4–C3 = 81.9 (2)$^\circ$, and $\angle C6$–C5–C4–C3 = −97.22 (18)$^\circ$ (Table 1). Two phenyl moieties are in the nearly gauche position, with the angle between the mean planes of both phenyl rings of 69.63 (10)$^\circ$. On the other hand, both phenyl rings are nearly perpendicular with the pyrimidine moiety, whereas the angle between the least-squares planes through N1/C1/N2 and C5/C6/C7/C8/C9/C10 atoms is 88.67 (19)$^\circ$, and through N1/C1/N2 and C12/C13/C14/C15/C16/C17 atoms, it is 82.8 (2)$^\circ$.

Figure 1. MERCURY [29] drawing of the molecular structure of compound 4j with labeled non-H atoms. Displacement ellipsoids are shown at 30% probability. Symmetry code: (i) $-x$, $-y+1$, $-z+2$. 
Table 1. Selected geometrical parameters for methyl 4-(4'-benzoyloxy-3'-methoxyphenyl)-1,2,3,4-tetrahydro-1,6-dimethyl-2-thioxopyrimidine-5-carboxylate (4j).

| Bond Lengths [Å]          |          |          |          |
|---------------------------|----------|----------|----------|
| O1—C20                    | 1.203 (3)| N1—C1   | 1.339 (2) |
| O2—C20                    | 1.334 (3)| N1—C4   | 1.453 (2) |
| O2—C21                    | 1.442 (2)| N2—C2   | 1.373 (3) |
| O3—C7                     | 1.354 (2)| N2—C2   | 1.410 (3) |
| O3—C22                    | 1.424 (3)| N2—C18  | 1.472 (3) |
| O4—C11                    | 1.354 (2)| C3—C2   | 1.336 (3) |
| O4—C8                     | 1.402 (2)| C4—C3   | 1.517 (2) |
| O5—C11                    | 1.196 (3)| S1—C1   | 1.6733 (19) |
| O6—C24                    | 1.423 (3)| O6—C23  | 1.425 (3) |

| Bond angles [°]            |          |          |          |
|---------------------------|----------|----------|----------|
| C11—O4—C8                 | 116.71 (15) | C1—N1—C4 | 122.60 (14) |
| C24—O6—C23                | 110.08 (16) | N1—C1—N2 | 115.90 (16) |
| C20—O2—C21                | 116.98 (17) | N1—C1—S1 | 120.25 (14) |

| Torsion angles [°]         |          |          |          |
|---------------------------|----------|----------|----------|
| C4—N1—C1—N2               | −23.7 (2) | C2—C3—C20—O1 | −19.9 (3) |
| N1—C4—C3—C2               | −31.1 (2) | C4—C3—C20—O1 | 157.6 (2) |
| C2—N2—C1—N1               | −10.4 (2) | C2—C3—C20—O2 | 162.28 (16) |
| N1—C4—C3—C20              | 151.29 (15) | C4—C3—C20—O2 | −20.2 (2) |
| C18—N2—C1—S1              | −8.3 (3) | O3—C7—C8—O4 | −3.0 (3) |
| C2—N2—C1—S1               | 20.2 (3) | C11—O4—C8—C9 | −80.8 (3) |
| C1—N2—C2—C3               | 169.09 (13) | C11—O4—C8—C7 | 103.7 (2) |
| C5—C4—C3—C2               | 92.44 (18) | C17—C12—C11—O5 | 9.3 (3) |

Conformation of molecular structure of 4j is stabilized by N—H⋯O hydrogen bond and C—H⋯O contacts (Table 2 and Figure 2).

Table 2. Intramolecular and intermolecular contact parameters in the crystal structure of methyl 4-(4'-benzoyloxy-3'-methoxyphenyl)-1,2,3,4-tetrahydro-1,6-dimethyl-2-thioxopyrimidine-5-carboxylate (4j).

| D—H       | D—H       | H⋯A       | D⋯A       | D—H⋯A       |
|-----------|-----------|-----------|-----------|-------------|
| N1—H1⋯O6  | 0.86      | 2.23      | 2.897 (2) | 134.9       |
| C19—H19B⋯O1 | 0.93    | 2.95      | 3.809 (2) | 154.0       |
| C9—H9⋯S1   | 0.96      | 2.61      | 3.363 (3) | 135.7       |
| C21—H21C⋯O5 | 0.96    | 2.61      | 3.363 (3) | 135.7       |
| C22—H22C⋯O6 | 0.96    | 2.62      | 3.407 (3) | 139.5       |

Symmetry codes: 1 x, y + 1, z; 2 x, y, z; 3 x, y + 1, z, z.

Molecular arrangement in the crystal packing of 4j is governed by a network of C—H⋯S and C—H⋯O contacts (Table 2) in a head-to-tail manner, whereas C—H⋯S form a path of interactions in the [010] direction, forming a thread, as seen in Figure 2. Moreover, the molecules are linked by weak C—H⋯O interactions with C21 and C22 methyl atoms as donors and ester atom O5 (x−1, y, z) and dioxane atom O6 (x+1, y, z) as acceptors in these linkages in the [100] direction (Figure 3).
As shown in the tables, the tested substances manifested relatively strong antimicrobial efficiency. They inhibited growth of all the used microorganisms, except for Proteus mirabilis (MIC value was 0.20 mg/mL). The strongest antibacterial effect was demonstrated by compound 4h, while compound 4j demonstrated the best antifungal effect. The most susceptible microorganism towards all components was Trichophyton mentagrophytes.

### 3.3. Biology

#### 3.3.1. Antimicrobial Activity

The obtained MICs of the studied compounds are summarized in Tables 3 and 4. As shown in the tables, the tested substances manifested relatively strong antimicrobial efficiency. They inhibited growth of all the used microorganisms, except 4e which acted selectively (this compound had no inhibitory effect on the Proteus mirabilis). The measured MICs for the tested components against the used bacteria and fungi ranged from 0.20 to 3.25 mg/mL. The maximum antimicrobial activity showed 4e, 4f, and 4k against the Trichophyton mentagrophytes (MIC value was 0.20 mg/mL). The strongest antibacterial effect was found in 4b and 4d, while 4f and 4k demonstrated the best antifungal effect. The most susceptible microorganism towards all components was Trichophyton mentagrophytes.
Table 3. The antibacterial activity of the tested compounds.

| Tested Compounds | Staphylococcus aureus | Bacillus subtilis | Klebsiella oxytoca | Proteus mirabilis | Escherichia coli |
|------------------|-----------------------|------------------|-------------------|------------------|-----------------|
| 4a               | 1.62                  | 3.25             | 1.62              | 0.81             | 1.62            |
| 4b               | 1.62                  | 3.25             | 1.62              | 0.81             | 1.62            |
| 4c               | 1.62                  | 3.25             | 1.62              | 1.62             | 1.62            |
| 4d               | 0.81                  | 3.25             | 1.62              | 0.81             | 1.62            |
| 4e               | 3.25                  | 3.25             | 3.25              | ND               | 1.62            |
| 4f               | 3.25                  | 3.25             | 3.25              | 1.62             | 3.25            |
| 4g               | 3.25                  | 3.25             | 1.62              | 0.81             | 1.62            |
| 4h               | 3.25                  | 3.25             | 3.25              | 3.25             | 3.25            |
| 4i               | 3.25                  | 3.25             | 1.62              | 1.62             | 1.62            |
| 4j               | 3.25                  | 3.25             | 3.25              | 1.62             | 3.25            |
| 4k               | 3.25                  | 3.25             | 3.25              | 3.25             | 3.25            |
| Streptomycin     | 0.031                 | 0.016            | 0.008             | 0.062            | 0.062           |

ND—not detected.

Table 4. The antifungal activity of the tested compounds.

| Tested Compounds | Trichophyton mentagrophytes | Mucor mucedo | Penicillium italicum | Aspergillus flavus | Aspergillus niger |
|------------------|-----------------------------|--------------|----------------------|-------------------|------------------|
| 4a               | 0.40                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4b               | 0.40                        | 1.62         | 1.62                 | 1.62              | 0.81             |
| 4c               | 0.40                        | 1.62         | 1.62                 | 0.40              | 0.81             |
| 4d               | 0.40                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4e               | 0.20                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4f               | 0.20                        | 1.62         | 1.62                 | 1.62              | 0.81             |
| 4g               | 0.40                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4h               | 0.81                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4i               | 0.81                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4j               | 0.40                        | 1.62         | 1.62                 | 1.62              | 1.62             |
| 4k               | 0.20                        | 1.62         | 1.62                 | 1.62              | 0.81             |
| Fluconazole      | 0.25                        | 1            | 1                    | 1                 | 0.5              |

3.3.2. Cytotoxic Activity of the Compounds

The cytotoxic activity of eleven novel compounds was examined against HeLa, K562, MDA-MB-231, and MRC-5. The tested compounds exerted concentration-dependent cytotoxic effects on cell lines. Among the tested compounds, 4k and 4b showed the strongest cytotoxicity against HeLa cells with IC_{50} values of 43.63 µM and 52.59 µM, as presented in Table 5. Compounds 4j and 4a exerted lower cytotoxic effects on HeLa cells with IC_{50} values of 78.11 µM and 97.40 µM when compared with the cytotoxicity of the two most active compounds. Compared to compound 4j, 4k has a methyl group in para position of benzoyl fragment; so, probably this structural difference is responsible for better activity and selectivity of compound 4k on HeLa (43.63 ± 1.49 µM) and K562 (39.11 ± 2.90 µM). In addition, the difference between 4a and 4b in position C5’ (4a has bromine, and 4b iodine) provides significantly stronger activity of 4b on HeLa cell lines. The compounds 4e, 4f, 4g, 4h, and 4i exhibited cytotoxic activity against HeLa cells with IC_{50} values ranging from 120.85 µM-197.49 µM, while compounds 4c and 4d showed very low cytotoxicity at concentrations up to 200 µM. K562 cells were the most sensitive to the cytotoxic activity of compound 4k with an IC_{50} value of 39.11 µM.
The four tested compounds 4a, 4b, 4h, and 4j showed moderate cytotoxic activity against K562 cells (IC_{50} values in the range from 67.97 µM–79.94 µM). The observed intensities of cytotoxic activity against K562 cells for the other six examined compounds were in the range from 99.36 µM to 180.21 µM, presented as IC_{50} values. Compound 4k was the most active against MDA-MB-231 cells (IC_{50} value of 74.12 µM). Cytotoxicity of compounds 4a, 4b, 4f, 4h, and 4j on MDA-MB-231 cells was weaker in comparison with activity of 4k (determined IC_{50} values were in the range from 114.02 µM–161.29 µM). Compounds 4c, 4d, 4e, 4g, and 4i had very low cytotoxic activity, with IC_{50} values higher than 200 µM. Each of the eleven examined compounds showed cytotoxic effects on normal fibroblasts MRC-5 (determined IC_{50} values ranging from 77.82 µM–196.08 µM).

Regarding selectivity in the cytotoxic activity, compounds 4a, 4b, 4j, and 4k exerted higher intensity of cytotoxic activity against HeLa and K562 cells when compared with activity against normal MRC-5 cells. In addition, compound 4a showed higher cytotoxicity on MDA-MB-231 cells in comparison with cytotoxicity on MRC-5 cells. It is noteworthy that compound 4k exerted the strongest cytotoxic activity against all tested cancer cell lines. This compound showed good selectivity in the cytotoxic action with selectivity coefficients 2 and 2.23 for HeLa and K562 cells, respectively. The selectivity in the cytotoxic activity against HeLa cells, when compared with activity against MRC-5 cells, was observed for compound 4b (selectivity coefficient 2.13). Compounds 4k and 4b, which showed the strongest selective cytotoxic activity against HeLa cells, were chosen for further analysis of mechanisms of cytotoxic activity.

### 3.3.3. Effects of the Compounds 4b and 4k on Cell Cycle

The treatment of HeLa cells for 24 h with IC_{50} and 2IC_{50} concentrations of compounds 4b and 4k induced an increase in the percentage of dead cells within subG1 phase of the cell cycle when compared with that percentage in the control, untreated cell sample, as it can be seen in Figure 4. In addition, both tested compounds applied at IC_{50} and 2IC_{50} concentrations triggered a pronounced increase in the percentage of HeLa cells in the G1 phase. The observed changes in the amounts of treated cells within subG1 and G1 phases of the cell cycle were accompanied with decreased percentages of cells within the S and G2/M phases. The effect on the cell-cycle phase distribution in HeLa cell samples was concentration-dependent for compound 4b, while compound 4k showed similar effects on HeLa cells at both tested concentrations. These results indicate that cytotoxic effects of compounds 4b and 4k against HeLa cells could be attributed to an increase in the percentage of cells in the subG1 phase and G1 cell cycle arrest.

### Table 5. Cytotoxic activity of compounds.

| Tested Compounds | HeLa       | K562       | MDA-MB-231 | MRC-5      |
|------------------|------------|------------|------------|------------|
| 4a               | 97.40 ± 5.40 | 78.98 ± 7.49 | 144.50 ± 8.29 | 186.92 ± 4.66 |
| 4b               | 52.59 ± 4.45 | 76.83 ± 5.01 | 115.65 ± 8.71 | 111.87 ± 10.85 |
| 4c               | >200        | 180.21 ± 9.15 | >200       | 195.83 ± 5.89 |
| 4d               | >200        | 164.66 ± 8.50 | >200       | 192.71 ± 10.32 |
| 4e               | 197.22 ± 3.93 | 149.08 ± 5.74 | >200       | 127.98 ± 3.20 |
| 4f               | 135.34 ± 9.32 | 122.91 ± 8.52 | 161.29 ± 9.69 | 196.08 ± 5.54 |
| 4g               | 197.49 ± 3.55 | 152.68 ± 2.58 | >200       | 193.22 ± 9.59 |
| 4h               | 120.85 ± 9.96 | 79.94 ± 6.73 | 114.02 ± 12.86 | 77.82 ± 2.57 |
| 4i               | 169.06 ± 4.12 | 99.36 ± 9.38 | >200       | 152.34 ± 10.15 |
| 4j               | 78.11 ± 5.78 | 67.97 ± 6.53 | 122.61 ± 2.19 | 104.17 ± 8.61 |
| 4k               | 43.63 ± 1.49 | 39.11 ± 2.90 | 74.12 ± 1.25 | 87.23 ± 7.31 |
| cisPt            | 4.91 ± 0.74 | 6.89 ± 0.21 | 14.74 ± 0.36 | 9.35 ± 1.29 |

The results are presented as average ± standard deviation of three independent experiments performed in triplicate.
The proapoptotic effect of compound 4b was to some extent stronger when comparing with the effect of compound 4k. This result is in accordance with the higher amount of subG1 cells detected in the HeLa cell sample exposed to 2IC50 concentration of compound 4b in comparison with the amount of subG1 cells in cells exposed to compound 4k.

3.3.4. Morphological Evaluation of HeLa Cell Death Mode Induced by the Compounds 4b and 4k

The photomicrographs of control HeLa cells and HeLa cells incubated for 24 h with 2IC50 concentrations of the compounds 4b and 4k and stained with a mixture of acridine orange/ethidium bromide are presented in Figure 5. Green-stained rounded cells with condensed chromatin and shrunk nuclei were observed in HeLa cell samples treated with compound 4b, pointing to the ability of this compound to induce apoptotic cell death. Orange-red-stained shrunken cells with condensed chromatin in later stages of apoptosis were detected as well. Compound 4k also showed the ability to activate apoptosis in HeLa cells after 24 h treatment, as demonstrated by rounded green cells with condensed nuclei. The proapoptotic effect of compound 4b was to some extent stronger when comparing with effect of compound 4k.

3.3.5. Inhibitory Effects of Compounds on α-Glucosidase Enzymatic Activity

The tested compounds showed the ability to inhibit α-glucosidase enzymatic activity, with the exception of compounds 4c and 4j, as can be seen in Table 6. The obtained IC50 values were in the range from 191.80 µM to 1121.91 µM. Among the tested compounds, the compound 4g exerted the best α-glucosidase inhibitory activity with an IC50 value of 191.80 µM. Its activity was stronger than the activity of the anti-diabetic drug acarbose with an IC50 value of 304.21 µM. The compounds 4a and 4b showed inhibitory activities similar to acarbose. The inhibitory effects of the other examined compounds on α-glucosidase enzymatic activity were weaker when compared with the effect of acarbose.
Table 6. α-glucosidase inhibitory activity of the compounds.

| Compounds | IC$_{50}$ [µM] |
|-----------|----------------|
| 4a        | 312.91 ± 7.73  |
| 4b        | 291.77 ± 6.67  |
| 4c        | ND             |
| 4d        | 1121.91 ± 20.59 |
| 4e        | 674.81 ± 5.65  |
| 4f        | 767.91 ± 5.91  |
| 4g        | 191.80 ± 5.95  |
| 4h        | 932.75 ± 25.16 |
| 4i        | 418.02 ± 5.95  |
| 4j        | ND             |
| 4k        | 1083.41 ± 54.54|
| acarbose  | 304.21 ± 14.62 |

The results are presented as average ± standard deviation of two experiments. ND—non-determined.

4. Conclusions

Eleven new THPM derivatives were synthesized under mild conditions, while good yields were obtained. For the reaction, methyl acetoacetate, vanillic aldehydes, and N-methylthiourea were used. The obtained compounds were characterized by IR, NMR, ES-MS, while molecular structure of compound 4j has been determined by single-crystal X-ray diffraction analysis. Moreover, their biological activities—antimicrobial, anticancer, and α-glucosidase inhibitory potential—were investigated. The antimicrobial activities of the tested compounds were evaluated against five strains of bacteria and fungi. Compounds 4a, 4b, and 4d have the most effect on bacteria, while the compounds 4e, 4f, and 4k showed the best antifungal activity. Generally, all tested compounds have very good antimicrobial activities which make them worthy of potential pharmacological usage.

The cytotoxic activity was examined against HeLa, K562, MDA-MB-231 cells, and normal human lung fibroblasts MRC-5. The compounds 4k and 4b presented the strongest cytotoxicity against HeLa cells with IC$_{50}$ values of 43.63 µM and 52.59 µM. In addition, the effects of the 4k and 4b on the cell cycle were examined. Cytotoxic effects of 4k and 4b against HeLa cells could be attributed to an increase in the percentage of cells in the subG1 phase and G1 cell cycle arrest. Furthermore, the investigation of the α-glucosidase inhibitory activity revealed compound 4g as the one with the best activity among all the tested ones. It possesses stronger α-glucosidase inhibitory activity than the anti-diabetic drug acarbose. Further, compounds 4a, 4b, and 4i showed similar activity as acarbose.

Compound 4b had good antibacterial and anticancer activities, compound 4k showed promising antifungal and anticancer activities, while compound 4a has very good α-glucosidase inhibitory activity and antibacterial activity, and compound 4b possesses similar activity as acarbose, and has good anticancer activity. Therefore, we confirmed the potential of some THPMs to be effective agents for more diseases at the same time. All the results have shown the importance of this type of compound as one with the most diverse, and interesting biological activities. The mentioned facts make THPMs excellent candidates for further investigations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pharmaceutics14102254/s1, Supplementary Materials File S1: Materials and methods for biological examination and crystal structure determination; Figures S1–S22: NMR spectra; Table S1: Crystallographic data and refinement parameters for 4j; Supplementary Materials File S2: MS spectra of 4a–k. Refs. [30–40] are cited in Supplementary Materials File S1.
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