Glycosylation of small molecule-based drugs can dramatically improve the biological activities of the parent scaffold. In the current study, S-glycosides and N-glycosides of polyfunctionalized pyridine-biphenyl system tethered with benzotriazole moiety were designed and synthesized. S-Glycosides of pyridine-2-thione derivatives 5a–h and N-glycosides of pyridine-2-one derivatives 9a,b were synthesized by a facile, convenient, and high-yielding procedure. The epimers glucose and galactose, acetylated or deacetylated, were used to form the glycone part. The structures of these compounds were confirmed by microanalysis and spectroscopic data (IR, 1H–NMR, and 13C-NMR). The anticancer activities of the target compounds, in comparison with standard cisplatin, were assessed by MTT assay against MCF7 cell line. Compounds 4f, 4g, 5f, and 5h exhibited the highest cytotoxic effect on MCF7. The anticancer effect of these four compounds induced the apoptosis as evident by the up-regulated expression of the apoptotic genes Bax and p53 and down-regulated expression of the anti-apoptotic gene BCl2. S-Glycoside derivatives are more active than N-glycosides. Moreover, the nontoxic doses of the tested compounds were evaluated in MA104, FRHK4, BGM, Hep2, and Vero cells. Compounds 4a–d and 5a–d were also evaluated for their antiviral effect against HSV-1, HAV, and rotavirus Wa strain. The compounds’ results showed less, moderated, and high antiviral activities. The docking study for these compounds with MDM2 revealed that deacetylated galactose is important for binding with the receptor as it facilitates the formation of hydrogen bond in the receptor. Rapid overlay of chemical structures analysis was employed to understand the compounds’ similarity on the basis of their shape structure using the Tanimoto scores.


**INTRODUCTION**

Breast cancer is considered as the first common leading cause of cancer deaths, representing 14.7% of all cancer cases in women in the recent years [1]. The formation of glycoside bond with heterocyclic compounds is still in the mind of the majority of chemists and drug design strategists [2]. These compounds containing glycoside bond have played a vital role as established cancer chemotherapeutic agents [3–16]. For example, N-nucleoside of pyridine-2-one and S-nucleoside of pyridine-2-thione derivatives are applied in the treatment of metastatic breast cancer, hairy cell leukemia, lung carcinoma cell line, liver carcinoma cell line, and brain carcinoma cell line [14–19]. In this context, dihydropyridine derivatives with glycoside bonds were identified as a strong P-glycoprotein antagonist with significant cytotoxic activity against human colon carcinoma cells (Fig. 1) [20].

Furthermore, some of the glycosides of pyridine-2-thione and pyridine-2-one derivatives illustrated antiviral activities against HIV-1 [21–23]. The biphenyl system is an important pharmacophore that is incorporated in different bioactive compounds especially in cancer therapy [24,25].
Several benzotriazole and 1,2,4-triazole derivatives represented an interesting class of heterocyclic compounds [26]. Numerous benzotriazoles, including 4-(1H-benzo-1,2,3-triazol-1-ylmethyl-oxy)-3-methoxybenzaldehyde, showed high effectiveness against human breast carcinoma cells (MCF7) [27]. Additionally, pyridine-2-(1H)-ones and pyridine-2-(1H)-thione derivatives showed the following: anticancer effect against MCF7, ovarian adenocarcinoma cells (SK-OV-3), and blood cancer cells (CCRF-CEM) [28,29]; antiviral activities [30] against human rhinoviruses [31,32] and HIV-1 [21–23,33]; anti-inflamatory activity [34]; eukaryotic elongation factor-2 kinase inhibitor activity [35]; antidiabetic effects [36]; potent antiviral effects [30]; and antimicrobial effect [37].

In view of the aforementioned observations, we report here the synthesis, characterization, and glycosylation of new 2-oxopyridine/2-thioxopyridine derivatives. The aglycone part is a biphenyl system (ring A and ring B) linked by benzotriazole moiety. The glycone part originated from the two epimer monosaccharides, glucose and galactose, acetylated or deacetylated, and the effect of N-glycoside or S-glycoside in activity relationship was examined (Fig. 2). Their anticancer activities against breast cancer (MCF7) including apoptosis studies were evaluated. The cytotoxicity of these compounds against the normal cells and their antiviral activities were also determined. Docking studies and shape similarity studies were also investigated.

RESULTS AND DISCUSSION

Chemistry. The key intermediates for the synthesis of cyclic S-glycosides are shown in Scheme 1. Treatment of pyridine-2-thione derivatives 1a–d [38] with aq. KOH in acetone furnished the corresponding potassium salts of 2-thioxopyridines (2a–d), which in turn were treated with 2,3,4,6-tetra-O-acetyl-α-d-glucopyranosyl bromide (3a) or 2,3,4,6-tetra-O-acetyl-α-d-galactopyranosyl bromide (3b) to afford the S-glycosylated [15,39–47] compounds 4a–h in good to high yields (50–99%) (Scheme 1). The structures of the S-glycoside 4a–h were confirmed by elemental analysis and spectral data (IR, 1H-NMR, 13C-NMR). The 1H-NMR (400 MHz, DMSO-d6) spectrum of compound 4a, as an example, showed the anomeric proton of the glucose moiety as a doublet at δ 6.02 ppm.
with a coupling constant $J_{1',2'} = 10.00$ Hz indicating $\beta$-configuration of the anomeric center. The other protons of the glucopyranose ring resonated at $\delta = 4.11$–$5.32$ ppm, while the four acetoxy groups appeared as four singlets at $\delta = 1.07$–$1.66$ ppm. The $^{13}$C-NMR (400 MHz, DMSO-$d_6$) revealed the absence of the thione carbon atom at about $179.40$ ppm and a resonance of $\text{–N=\text{C\text{\textendash}}}\text{S\textendash}$carbon atom (C-2) at $\delta = 163.30$ ppm, which indicated the chemical shift of the corresponding carbon atom (Fig. 3).

Removal of the acetyl groups from the glycone moiety of 4a–h with saturated solution of NH$_3$/MeOH at room temperature furnished the corresponding free glycosides 5a–h (Scheme 1). The structures of 5a–h were confirmed on the basis of their spectroscopic data. The $^1$H-NMR (400 MHz, DMSO-$d_6$) spectrum of compound 5a, as an example, showed the disappearance of four acetyl signals and the presence of a doublet at $\delta = 5.56$ with $J_{1',2'} = 10.00$ Hz, corresponding to the $1'$-H, indicating a $\beta$-configuration. C-2 of 5a that resonated at $\delta = 166.06$ ppm established the $\text{S\textendash}glycosylation.

Then, our attention was directed to synthesize some of $N$-glycoside derivatives in order to examine the effect of glycoside linkage on activity. Treatment of compound 6 with $\text{K}_2\text{CO}_3$ or NaH in dimethylformamide (DMF) followed by the addition of 2,3,4,6-tetra-$O$-acetyl-$\alpha$-$D$-glucopyranosyl bromide (3a) or 2,3,4,6-tetra-$O$-acetyl-$\alpha$-$D$-galactopyranosyl bromide (3b) afforded the $N$-glycosylated nucleosides 8a,b in good yields (50–60%) (Scheme 2). The suggested reaction mechanism was through the formation of the sodium/potassium salts of 2-oxopyridines (7) (Scheme 2).

The structures of the $N$-glycoside 8a,b were confirmed by elemental analysis and spectral data (IR, $^1$H-NMR, $^{13}$C-NMR). The IR spectrum of compound 8a, as an example, showed frocked bands at 3417–3336 cm$^{-1}$ according to $\text{NH}_2$, additionally cyano, carbonyl, and ester groups at 2211, 1739, and 1629 cm$^{-1}$, respectively. The $^1$H-NMR (400 MHz, DMSO-$d_6$) spectrum of compound 8a showed a very broad band at $\delta = 3.81$ ppm according to $\text{NH}_2$ and absence of signal at 10.27 according to NH. The anomeric proton of the glucose moiety as a doublet at $\delta = 6.27$ ppm with a coupling constant $J_{1',2'} = 8.40$ Hz indicated $\beta$-configuration of the anomeric center. The other protons of the glucopyranose ring resonated at $\delta = 4.04$–$5.31$ ppm, while the four acetoxy groups appeared as four singlets at $\delta = 1.93$–$2.13$ ppm. The $^{13}$C-NMR (400 MHz, DMSO-$d_6$)
revealed the presence of the oxo carbon atom at about 165.20 ppm and a resonance of $-\text{N} = \text{C} = \text{O}$ carbon atom (C-2) at $\delta = 161.02$ ppm, establishing the N-glycosylation.

**Anticancer activity against MCF7.** *In vitro anticancer screening.* The cytotoxicity of the newly synthesized compounds was evaluated in vitro against MCF7 cells by MTT assay [49]. The results of novel compounds, compared with the reference cisplatin, showed IC$_{50}$ values, as shown in Table 1. The results showed that compounds 4f, 4g, 5f, and 5h have moderate activity against MCF7 cells with IC$_{50}$ = 30.63, 24.39, 27.24, and 20.49, respectively. Considering the glycone part, it was observed that compounds that contain galactose moiety were more active than others that contain the glucose part, and this also stratifies on their free acetyl of moieties: compound 4f against 4b, compound 4g against 4c, compound 5f against 5b, and compound 5h against 5d. Regarding the aglycone part, compounds that contain R$_1$ = CN, R$_2$ = NH$_2$ or OH are the least active. We hope that the synthesized compounds serve as lead chemical entities for further modification to render them clinically useful drug agents. N-Glycosides are less active than are S-glycosides.

**Apoptosis study.** To check whether the cytotoxic effect of compounds 4f, 4g, 5f, and 5h occurred through induction of apoptosis in MCF7 cells, changes in the gene expression of apoptotic genes *Bax* and *p53* and the anti-apoptotic gene *BCl2* were detected by quantitative polymerase chain reaction (qPCR) before and after the addition of these compounds. The addition of these compounds resulted in a significant ($P \leq 0.05$) increase in the expression of the *Bax* and *p53* genes in MCF7 cells, as compared with vehicle- [dimethyl sulfoxide (DMSO)] treated MCF7 cells, with the highest expression in 5h followed by 4g, then 5f, and finally 4f (Fig. 4). However, the *BCl2* expression was significantly decreased in MCF7 cells treated by the four compounds, in the order 5h $>$ 4g $>$ 5f $>$ 4f, than in vehicle-treated MCF7 cells (Fig. 4).

**Antiviral activity.** The study used three types of virus, namely, rotavirus Wa, HAV HM175, and herpes simplex virus type 1. The study commenced with the examination

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>56.66</td>
</tr>
<tr>
<td>4a</td>
<td>88</td>
</tr>
<tr>
<td>4b</td>
<td>51</td>
</tr>
<tr>
<td>4c</td>
<td>100</td>
</tr>
<tr>
<td>4d</td>
<td>59</td>
</tr>
<tr>
<td>4e</td>
<td>62</td>
</tr>
<tr>
<td>4f</td>
<td>39</td>
</tr>
<tr>
<td>4g</td>
<td>32</td>
</tr>
<tr>
<td>4h</td>
<td>74</td>
</tr>
<tr>
<td>5a</td>
<td>93</td>
</tr>
<tr>
<td>5b</td>
<td>130</td>
</tr>
<tr>
<td>5c</td>
<td>100</td>
</tr>
<tr>
<td>5d</td>
<td>51</td>
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<td>5e</td>
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</tr>
<tr>
<td>5f</td>
<td>44</td>
</tr>
<tr>
<td>5g</td>
<td>71</td>
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<tr>
<td>5h</td>
<td>32</td>
</tr>
<tr>
<td>8a</td>
<td>88</td>
</tr>
<tr>
<td>8b</td>
<td>60</td>
</tr>
<tr>
<td>9a</td>
<td>170</td>
</tr>
<tr>
<td>9b</td>
<td>180</td>
</tr>
</tbody>
</table>
of the nontoxic dose for these compounds against MA104, Hep2, Vero, BGM, and FRHK cells as shown in Table 2. Most of compounds exhibited a nontoxic dose at 100 μg/mL.

The antiviral activities for selected compounds rotavirus Wa, HAV HM175, and herpes simplex are illustrated in Table 3. Statistical analyses for the percent inhibition of viral titers achieved by each of the title compounds’ nontoxic doses against each of the study viruses were conducted. In our study, only percent inhibition values of viral titers above 50% were considered significant [50]. Compound 5a was more active than other derivatives against rotavirus Wa and herpes simplex virus type 1 strains by 63.3% and 80%, respectively, than are acyclovir as standard antiviral drug (IC50% 13.5 μg/mL). Compounds 4b, 5b, and 5c were moderate and similar in activity against rotavirus Wa with range 50–56%. Compounds 4a, 4b, and 5b exhibited good activity against herpes simplex virus type 1 strain with range 60–66.5%. In general, compounds 5a, 4b, and 5b were higher in activity against all three types of virus, while compounds 4c and 5d showed an activity less than that of others, as shown in Table 3.

In general, the deacetylated glycosides are more reactive than acetylated analogs. The deacetylated analogs are more reactive while in considering the aglycone part, the compounds contain R1 = nitrile or acetyl functionality and R2 = OH, or NH2 in the pyridine part is more reactive.

**Molecular docking study and structure-activity relationship.** P53 is an endogenous protein that acts as a tumor suppressor and stops cancer cells from growing and multiplying. The overexpression of MDM2 has been observed in a wide range of tumor types. MDM2 interacts with P53, and so there is a suppression effect of P53, and apoptosis is avoided [51–54]. As our compounds induce apoptosis and increase P53, the goal was directed to examine the docking of these compounds with MDM2 and to examine their activity to inhibit P53–MDM2 interactions.

In this regard, a library of target compounds was energy minimized using MMFF94 force field calculations. The catalytic domain of MDM2 (PDB code 5law) [55] was prepared for docking using OpenEye. OpenEye Omega application was used to generate different conformations...
of each ligand. Docking was conducted using FRED, and the data were visualized by the Veda application. This software package generates consensus scoring, a filtering process, to obtain virtual binding affinity; the lower the consensus score, the better the binding affinity of the ligand toward the receptor.

The most active compound 5h binds with the specific receptor of MDM (ID: 5law) with the best consensus score 1 and forms hydrogen bonding (HB) through its OH of C-5 of galactose moiety with Tyr 100 AA and through OH of C-4 with both Gly 24 AA and Ala 21 AA. The triazole ring forms HB with Gly 58 AA (Fig. 5A). The pyridine and the aryl ring from biphenyl system are adopted perpendicularly in the receptor through the formation of hydrophobic–hydrophobic interactions. Compound 5f docks with consensus score 5 and overlays completely with 5h but with the formation of one HB through its triazole ring with Gly 58 AA (Fig. 5B). However, compound 4g showed consensuses score 23 and docked with different pose and mode through the formation of HB with Ala 21 AA. Acetylated galactose (4g) forms hydrophobic–hydrophobic interaction with the receptor (Fig. 5C). In order to understand the effect of galactose and glucose moieties, the docking pose and mode for compound 4f against 4b were compared, compound 4g against 4c, compound 5f against 5b, and compound 5h against 5d. It was clear that the axial position of epimeric hydroxyl in galactose moiety switches the molecule to form HB interactions.

**Rapid overlay of chemical structures (ROCS) analysis and structure–activity relationship.** The final compounds behave in special manners, as the epimeric isomers are not near each other in activity (compound 4g versus 4c, compound 4f versus 4b; and compound 5h versus 4d, compound 5f versus 4b) and also variations of substituent on pyridine ring play an important role in activity. To

![Figure 5.](image-url)

(A) Visual representation of 5h docked with 5law showing hydrophobic–hydrophobic interaction through its biphenyl system and HB interaction through glucose and benzotriazole parts as shown by Vida. (B) Visual representation of 5h overlay 5f docked with 5law as shown by Vida. (C) Visual representation of 4g docked with 5law showing hydrophobic–hydrophobic interaction through biphenyl system and acetylated glucose and forming HB interaction through benzotriazole parts as shown by Vida. [Color figure can be viewed at wileyonlinelibrary.com]
gain insight about structure–activity relationship and to understand the compounds’ activity, rapid overlay of chemical structures (ROCS) was employed [56–58]. ROCS are a shape-based superposition method and is used to perceive similarity between molecules based on their three-dimensional shape. Shape similarity is as a fundamental descriptor in drug design. ROCS alignment requires (a) query molecules, and the queries here are the most active compounds in both acetylated (4g) and deacetylated (5h) sugar and (b) the database molecules of our final compounds.

The quality of alignment between database and query was calculated using the Tanimoto combo. Tanimoto combo is the summation of shape Tanimoto and colour Tanimoto. Shape Tanimoto represents the shared volume and mismatch volume and has a scale from 0 to 1. Colour Tanimoto (also with scale from 0 to 1) is reflective of the degree of matching or mismatching of light chemical features in three dimensions. From ROCS model (shape and color), query volume showed many points of acceptors, donors, and rings. The quality of alignment, using ROCS, between compounds 4g and 5h (queries) (Fig. 6A and 6C, respectively) and database molecules (final compounds) was calculated using Tanimoto combo (Table 4). Compound 4f overlays completely within the query volume shape (Fig. 6). Similarly, compound 5f overlays with query 5h (Fig. 6D). Based on the ROCS data, shape Tanimoto data revealed good correlation with biological activities. For example, compounds 4f and 4b exhibited high shape Tanimoto score using 4g as query and compounds 5d and 5f using 5h as query.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tanimoto combo</th>
<th>Shape Tanimoto</th>
<th>Color Tanimoto</th>
</tr>
</thead>
<tbody>
<tr>
<td>5h</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4g</td>
<td>1.999</td>
<td>1</td>
<td>0.999</td>
</tr>
<tr>
<td>4e</td>
<td>1.981</td>
<td>0.997</td>
<td>0.9840</td>
</tr>
<tr>
<td>5e</td>
<td>1.818</td>
<td>0.929</td>
<td>0.888</td>
</tr>
<tr>
<td>5g</td>
<td>1.784</td>
<td>0.923</td>
<td>0.862</td>
</tr>
<tr>
<td>5d</td>
<td>1.769</td>
<td>0.979</td>
<td>0.7900</td>
</tr>
<tr>
<td>4c</td>
<td>1.7320</td>
<td>0.9090</td>
<td>0.8230</td>
</tr>
<tr>
<td>4f</td>
<td>1.724</td>
<td>0.949</td>
<td>0.775</td>
</tr>
<tr>
<td>5f</td>
<td>1.714</td>
<td>0.9200</td>
<td>0.794</td>
</tr>
<tr>
<td>5a</td>
<td>1.675</td>
<td>0.921</td>
<td>0.754</td>
</tr>
<tr>
<td>4a</td>
<td>1.620</td>
<td>0.8750</td>
<td>0.7460</td>
</tr>
<tr>
<td>4d</td>
<td>1.5690</td>
<td>0.8580</td>
<td>0.711</td>
</tr>
<tr>
<td>5c</td>
<td>1.563</td>
<td>0.896</td>
<td>0.667</td>
</tr>
<tr>
<td>5b</td>
<td>1.5490</td>
<td>0.8700</td>
<td>0.6790</td>
</tr>
<tr>
<td>5h</td>
<td>1.209</td>
<td>0.818</td>
<td>0.3910</td>
</tr>
<tr>
<td>4h</td>
<td>0.880</td>
<td>0.519</td>
<td>0.361</td>
</tr>
</tbody>
</table>

Table 4. Tanimoto scales for compounds 4a–g and 5a–h.

Figure 6. (A) Representation of shape and color atoms of 4g by vROCS application. (B) Overlay and alignment 4f on 4g shape. (C) Representation or shape and color of 5h by vROCS application. (D) Visual representation of 5f with 5h by vROCS. [Color figure can be viewed at wileyonlinelibrary.com]
Structure–activity relationship studies revealed the following features: (1) S-glycoside pyridines are more active than N-glycosides probably owing to strength and rigidity of N-glycosides; (2) compounds contain deacetylated sugar moiety are more reactive than acetylated derivatives. This hypothesis is clear because the alcoholic hydroxyl groups form HB with the receptor amino acids; (3) the galactosyl compounds are more reactive than glucosyl analogs because the epimeric OH forms HB when it located axially; compounds that contain glucosides of acetyl galactose are more active than those that contain acetylated glucose; (4) the pyridine ring perpendicular to the methoxyphenyl ring allows this biphenyl system to from hydrophobic–hydrophobic interaction.

CONCLUSION

A biphenyl system from polyfunctionalized pyridine tethered with benzotriazole moiety was synthesized using a very simple method. This system linked to glycoside formation with glucose and galactose epimers. The glycoside side chain was either thioglycosides or N-glycosides. S-Glycosides are more reactive than N-glycosides analogs. Compounds 4f, 4g, 5f, and 5h were the most active derivatives against MCF7. These compounds induced apoptosis in MCF7 cells, changing the expression of apoptotic genes Bax and p53 and the anti-apoptotic gene BCl2. The docking study explained for us the kind of interaction with MDM2, as these compounds increase the level of P53. ROCS analysis was employed to understand compounds’ similarity based on their shape structure using Tanimoto scores. Also, in case of antiviral activity, the deacetylated sugars are more active, which indicates the importance of lipophilicity.

EXPERIMENTAL

All the melting points are uncorrected. It taken in open glass capillaries using melting point 3 samples apparatus, Rumo, model 4000. All samples were carried out by thin-layer chromatography (TLC), which was performed on an EM silica gel 60 F254 sheet. The spot was detected by UV lamp. IR spectra (KBr) were measured on Thermo Fisher Scientific (Waltham, MA) spectrometer in the Faculty of Science, Mansoura University; microanalysis was performed in the Regional Center for Mycology and Biotechnology at Al-Azhar University. 1H-NMR and 13C-NMR spectra were measured on a variation GEM 400 MHz in DMSO in the Faculty of Science, Kafrelsheikh University. Tetramethylsilane as internal standard and chemical shifts are expressed as δ ppm. The antiviral activity of all synthesized compounds was carried out at National Research Centre, Cairo, Egypt.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>CCTGTCACCAAAAGGTGGCCGAACCT</td>
<td>CCACCCCTGCTTGGATGCATCCAGCC</td>
</tr>
<tr>
<td>BCl2</td>
<td>AGGAAAGTGAACATTGTGGTAC</td>
<td>GCTGAGCTACCAAGGACCAGGC</td>
</tr>
<tr>
<td>p53</td>
<td>TAAACGTTTCGACTGAGGGGGGC</td>
<td>AGGACAGGGCAACAAACCGCAC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CACCAACTGGGACGCAT</td>
<td>ACACGCTTTGAATGACAG</td>
</tr>
</tbody>
</table>

Table 5

Primers used in qPCR.
C$_{36}$H$_{33}$N$_3$O$_{14}$S.5H$_2$O (767.74): C, 54.63; H, 4.55; N, 12.74. Found: C, 54.68; H, 4.13; N, 12.39.

5-(Methylcarbonyl)-3-cyano-6-hydroxy-4-(4-(1H-benzof[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl-2-(2',3',4',6'-tetra-O-acetyl-b-D-glucopyranosylthiophioryl) (4b). This compound was obtained as white solid (methanol), yield 685 mg (99%), reaction time 2 h, mp 177°C; IR (KBr, cm$^{-1}$): 3434 (OH), 2216 (CN), 1751 (CO$_{Ac}$), 1629 (CO$_{Ac}$). 1H-NMR (400 MHz, DMSO-$d_6$, ppm): 1.96, 2.01, 2.03, 2.08 (12H, 4s, 4CH$_3$), 3.36 (3H, s, COCH$_3$), 3.40 (1H, s, br, OH), 3.70 (3H, s, OCH$_2$), 4.12 (1H, m, H-6'), 4.23 (1H, d, $J = 10.0$ Hz, H-5'), 5.08 (1H, t, $J = 9.8$ Hz, H-4'), 5.17 (1H, t, $J = 9.8$, H-2'), 5.33 (1H, t, $J = 9.2$ Hz, H-3'), 6.01 (1H, d, $J_{1,2'} = 10.4$ Hz, H-1'), 6.76 (2H, s, OCH$_2$), 6.80–8.12 (7H, m, Ar-H); 13C-NMR (100 MHz, DMSO-$d_6$, ppm): 20.76, 20.81, 20.96, 29.48 (SCH$_3$), 56.35 (OCH$_2$), 75.38 (OCH$_2$), 61.96 (C6'H'), 68.79 (C5'), 68.79 (C4'), 73.93 (C3'), 75.85 (C2'), 80.56 (C1'), 117.70 (CN), 87.57, 94.65, 111.4, 113.75, 115.49, 119.81, 121.78, 125.06, 128.74, 129.31, 130.47, 133.36, 145.81, 147.19, 150.19, 158.62 (C-Ar), 160.04 (CO$_{Ac}$), 163.29 (C2'-C), 167.88, 169.65, 170.11, 170.47 (4Ac); Calcd. for C$_{37}$H$_{37}$N$_5$O$_{14}$S.5H$_2$O (816.78): C, 54.35; H, 4.65; N, 8.57. Found: C, 54.38; H, 3.79; N, 8.42.

6-Amino-3,5-dicyano-4-(4-(1H-benzof[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl-2-(2',3',4',6'-tetra-O-acetyl-b-D-galactopyranosylthiophioryl) (4e). This compound was obtained as white solid (methanol), yield 538 mg (83%), reaction time 7 h, mp 140–145°C; IR (KBr, cm$^{-1}$): 3328 (NH$_2$), 2214 (CN), 1749 (CO$_{Ac}$). 1H-NMR (400 MHz, DMSO-$d_6$, ppm): 1.92, 1.99, 2.06, 2.16 (12H, 4s, 4CH$_3$), 3.36 (2H, br, s, NH$_2$), 3.71 (3H, s, OCH$_3$), 3.96 (1H, m, H-6'), 4.15 (1H, m, H-5'), 4.42 (1H, t, $J = 6.0$ Hz, H-4'), 5.22 (1H, d, $J = 6.4$, H-2'), 5.44 (1H, m, H-3'), 6.02 (1H, d, $J_{1,2'} = 11.2$ Hz, H-1'), 6.80 (2H, s, OCH$_2$), 7.09–8.12 (7H, m, Ar-H); 13C-NMR (100 MHz, DMSO-$d_6$, ppm): 20.79 20.86, 20.89, 20.94 (4CH$_3$), 56.35 (OCH$_3$), 75.38 (OCH$_2$), 61.61 (C6'), 66.38 (C5'), 67.91 (C4'), 71.84 (C3'), 74.98 (C2'), 81.00 (C1'), 117.69 (CN), 87.66, 94.78, 111.14, 113.75, 115.48, 119.81, 121.79, 125.07, 128.75, 129.31, 133.36, 145.81, 147.19, 150.19, 158.63, 160.04 (Ar-C), 163.23 (C2'-C), 170.05, 170.01, 170.33, 170.47 (4Ac); Calcd. for C$_{35}$H$_{33}$N$_3$O$_{14}$S (759.74): C, 55.33; H, 4.38; N, 12.91. Found: C, 54.68; H, 4.19; N, 12.39.

5-(Methylcarbonyl)-3-cyano-6-methyl-4-(4-(1H-benzof[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl-2-(2',3',4',6'-tetra-O-acetyl-b-D-galactopyranosylthiophioryl) (4f). This compound was obtained as white solid (methanol), yield 685 mg (99%), reaction time 14 h, mp 215–220°C; IR (KBr, cm$^{-1}$): 3327 (br, OH), 2215 (CN), 1749 (CO$_{Ac}$), 1652 (CO$_{Ac}$). 1H-NMR (400 MHz, DMSO-$d_6$, ppm): 1.87, 2.06, 2.16 (12H, 4s, 4CH$_3$), 3.34 (4H, s, OCH$_2$), 3.71 (3H, s, OCH$_3$), 3.96 (1H, m, H-6'), 4.14 (1H, m, H-5'), 4.41 (1H, t, $J = 6.0$, H-4'), 5.22 (1H, d, $J = 6.0$ Hz, H-2'), 5.44 (1H, s, OCH$_2$), 6.03 (1H, d, $J = 6.0$ Hz, H-3'), 6.80 (2H, s, OCH$_2$), 7.12–8.13 (7H, m, Ar-H); 13C-NMR (100 MHz, DMSO-$d_6$, ppm): 20.79, 20.86, 20.88, 20.94 (5CH$_3$), 56.35 (CH$_3$O), 75.37 (OCH$_2$), 61.60 (C6'), 66.37 (C5'), 67.90 (C4'), 71.84 (C3'), 74.98 (C2'), 81.00 (C1'), 117.69 (CN), 87.66, 94.78, 111.14, 113.75, 115.48, 115.52, 119.82, 121.79, 125.06, 128.74, 129.30, 133.36, 145.81, 147.18, 150.19, 158.64 (Ar-C), 160.04 (CO$_{Ac}$), 163.22
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5. (Ethyloxyacarbonyl)-3-cyano-6-hydroxy-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranosyl-thio)]pyridines (4h).

This compound was obtained as white solid (methanol), yield 689 mg (99%), reaction time 6 h, mp 198–200°C; IR (KBr, cm⁻¹): 3436 cm⁻¹ (OH), 2215 cm⁻¹ (CN), 1749 (CO₃), 1628 (CO₂Et); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 1.98, 2.06, 2.16 (12H, 4s, 4CH₃), 1.06 (3H, t, J = 6.8 Hz, CH₂CH₂CH₃), 1.65 (2H, q, J = 6.9 Hz, CH₂CH₂), 3.35 (1H, s, br. OH), 3.71 (3H, s, OCH₃), 3.96 (1H, m, H-6'), 4.13 (1H, m, H-5'), 4.41 (1H, t, J = 6.2 Hz, H-4'), 5.22 (1H, d, J₁,₂ = 6.8 Hz, H-2'), 5.44 (1H, s, H-3'), 6.03 (1H, d, J = 10.4 Hz, H-1'), 6.80 (2H, s, OCH₂), 7.11–8.13 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 20.79, 20.86, 20.88, 20.94 (4CH₃), 22.57 (CH₂CH₂), 65.19 (OCH₃), 75.38 (OCH₂), 64.01 (C₆), 66.39 (C₅'), 67.91 (C₄'), 71.79 (C₃'), 75.56 (C'₂), 80.92 (C'₁), 117.72 (CN), 86.74, 94.75, 111.09, 113.71, 115.50, 119.79, 121.78, 125.10, 128.78, 129.32, 133.33, 147.58, 147.15, 149.48, 150.21, 158.70 (Ar-C), 163.27 (C-2), 170.10, 170.04, 170.38, 170.52 (4Ac); Calcd. for C₃₅H₃₂N₆O₁₂S

149.48, 150.21, 158.60 (Ar-C), 163.27 (C-2), 170.04, 170.00, 170.32, 170.47 (4Ac); Calcd. for C₉₃H₃₂N₆O₁₂S (777.75): C, 55.59; H, 4.54; N, 9.00. Found: C, 56.28; H, 4.06; N, 9.38.

6-Amino-3,5-dicyano-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2(β-glucopyranosylthio)pyridine (5a).

This compound was obtained as white solid (methanol), yield 434 mg (75%), reaction time 1 h, mp 177–180°C; IR (KBr, cm⁻¹): 3420 (OH), 3347 (NH₂), 2214 (CN); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 3.27–3.65 (5H, m, H-6', H-5', H-4', H-3', H-2'), 3.36 (2H, br. s, NH₂), 3.71 (3H, s, OCH₃), 3.98 (1H, s, 2'-OH), 4.42 (1H, s, 3'-OH), 5.09 (1H, d, J = 4.5 Hz, 4'-OH), 5.21 (1H, d, J₁,₂ = 10.0 Hz, H-1'), 6.80 (2H, s, OCH₂), 7.10–8.13 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 56.35 (OCH₃), 75.41 (OCH₃), 61.12 (C-6'), 69.91 (C-5'), 72.00 (C-4'), 79.05 (C-3'), 81.48 (C-2'), 83.79 (C-1'), 117.72 (CN), 86.77, 94.46, 111.15, 113.75, 114.85, 115.91, 121.75, 125.72, 128.75, 129.56, 133.35, 145.81, 147.08, 150.19, 158.27, 160.02 (C-Ar), 166.06 (C-2); Calcd. for C₂₇H₂₇N₇O₇S₂H₂O (627.6): C, 51.62; H, 4.30; N, 15.61. Found: C, 51.69; H, 4.48; N, 15.24.

5-(Methylcarbonyl)-3-cyano-6-hydroxy-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2(β-glucopyranosylthio)pyridine (5b).

This compound was obtained as white solid (ethanol), yield 479 mg (77%), reaction time 18 h, mp 190–195°C; IR (KBr, cm⁻¹): 3489 (OH), 3382 (OH), 2193 (CN), 1615 (CO₃), 1749 (CO₃), 1628 (CO₂Et); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 3.10–3.69 (5H, m, H-6', H-5', H-4', H-3', H-2'), 3.40 (3H, s, OCH₃), 3.69 (3H, s, OCH₃), 3.98 (1H, br, s, 2'-OH), 4.19 (1H, br, s, 3'-OH), 4.41 (1H, t, J = 7.6 Hz, 4'-OH), 5.45 (1H, br, s, 6'-OH), 6.95 (1H, d, J₁,₂ = 8 Hz, H-1'), 6.75 (2H, s, OCH₂), 6.87 (1H, s, br. OH), 7.09–8.12 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 22.96 (CH₃), 56.32 (OCH₃), 75.43 (OCH₃), 61.71 (C-6'), 63.72 (C-5'), 70.09 (C-4'), 70.79 (C-3'), 75.59 (C-2'), 83.75 (C-1'), 117.71 (CN), 83.92, 111.15, 113.69, 115.58, 116.02, 119.80, 121.73, 125.07, 128.75, 129.75, 133.35, 145.81, 147.01, 150.14, 160.54, 161.51 (Ar-C), 161.65 (CO₃), 166.25 (C-2); Calcd. for C₂₉H₂₇N₉O₇S (609.61): C, 55.17; H, 4.46; N, 11.49. Found: C, 55.26; H, 4.11; N, 11.87.

5.6-Dicyano-6-hydroxy-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2(β-glucopyranosylthio)pyridine (5c).

This compound was obtained as white solid (methanol), yield 336 mg (55%), reaction time 19 h, mp 148°C; IR (KBr, cm⁻¹): 3415 (OH), 2212 (CN); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 3.06–3.17 (5H, m, H-6', H-5', H-4', H-3', H-2'), 3.71 (3H, s, OCH₃), 3.06 (1H, br. s, OH), 3.98 (1H, br. s, 2'-OH), 4.56 (1H, br. s, 3'-OH), 4.33 (1H, br. s, 4'-OH), 5.05 (1H, br. s, 6'-OH), 5.23 (1H, d, J₁,₂ = 12 Hz, H-1'), 6.69 (2H, s,
5-((Ethoxycarbonyl)-6-hydroxy-4-((4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2-(β-galactopyranosylthio)pyridine (5d). This compound was obtained as white solid (methanol), yield 512 mg (80%), reaction time 19 h, mp 190°C; IR (KBr, cm⁻¹): 3428 (OH), 2214 (CN), 1630 (CO₂Et); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 1.18 (3H, t, J = 6.0 Hz, CH₂CH₃), 1.53 (2H, q, J = 6.0 Hz, CH₂CH₂), 3.08–3.45 (5H, m, H-6', H-5', H-3', H-2'), 2.95 (1H, s, OH), 3.71 (3H, s, OCH₃), 4.04 (1H, br. s, 2'-OH), 4.29 (1H, br. s, 3'-OH), 4.62 (1H, br. s, 4'-OH), 4.97 (1H, br. s, 6'-OH), 5.10 (1H, d, J₁₁₂ = 6.8 Hz, H-1'), 6.25 (2H, s, OCH₂), 6.74–8.06 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 22.49 (CH₂CH₃), 56.31 (OCH₃), 75.18 (OCH₂CH₃), 75.47 (OCH₃), 61.41 (C-6'), 70.62 (C-5'), 72.63 (C-4'), 73.43 (C-3'), 77.02 (C-2'), 83.65 (C-1'), 117.79 (CN), 44.21, 97.13, 104.15, 111.02, 113.57, 116.61, 119.72, 121.64, 125.19, 128.86, 129.72, 132.27, 145.71, 146.89, 150.14, 160.42 (Ar-C), 161.59 (COOEt), 166.24 (C-2').

6-Amino-3,5-dicyano-4-((4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2-(β-galactopyranosylthio)pyridine (5e). This compound was obtained as white solid (methanol), yield 544 mg (85%), reaction time 8 h, mp 210°C; IR (KBr, cm⁻¹): 3446 (OH), 2213 (CN), 1642 (CO₂Et); ¹H-NMR (DMSO-d₆, 400 MHz): 1.22 (3H, t, J = 7.2 Hz, CH₂CH₃), 1.62 (2H, q, J = 6.7 Hz, CH₂CH₂), 3.01–3.60 (5H, m, H-6, H-5, H-4, H-3, H-2'), 3.62 (1H, s, OH), 3.71 (3H, s, OCH₃), 3.97 (1H, s, 2'-OH), 4.60 (1H, m, 3'-OH), 4.77 (1H, m, 4'-OH), 5.11 (1H, m, 6'-OH), 5.56 (1H, d, J₁₁₂ = 10.4 Hz, H-1'), 6.71 (2H, s, OCH₂), 7.00–8.12 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 22.74 (CH₂CH₃), 56.34 (OCH₃), 75.08 (OCH₂CH₃), 75.44 (OCH₃), 60.66 (C₆'), 63.27 (C₅'), 68.75 (C₃'), 69.43 (C₂'), 80.07 (C₁'), 117.71 (CN), 83.92, 91.84, 111.14, 113.69, 159.11, 189.90, 121.66, 125.07, 128.76, 129.56, 133.35, 145.80, 147.01, 150.20, 160.54, 161.51 (Ar-C), 161.65 (CO₂Ac), 166.25 (C-2).

5-(Ethoxycarbonyl)-3-cyano-6-hydroxy-4-((4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-2-(β-galactopyranosylthio)pyridine (5f). This compound was obtained as white solid (ethanol), yield 444 mg (75%), reaction time 10 h, mp 130°C; IR (KBr, cm⁻¹): 3421 (OH), 2212 (CN); ¹H-NMR (400 MHz, DMSO-d₆, δ, ppm): 2.99–3.31 (5H, br, m, H-6', H-5', H-4', H-3', H-2'), 3.81 (3H, s, OCH₃), 4.24 (1H, br. s, 2'-OH), 4.33 (1H, br. s, 3'-OH), 5.01 (1H, br. s, 4'-OH), 5.69 (1H, br. s, 6'-OH), 5.90 (1H, d, J₁₁₂ = 8 Hz, H-1'), 6.73 (2H, s, OCH₂), 7.05–8.08 (8H, m, Ar-H, OH); ¹³C-NMR (100 MHz, DMSO-d₆, δ, ppm): 56.25 (OCH₃), 75.41 (OCH₃), 60.69 (C₆'), 60.98 (C₅'), 68.75 (C₄'), 75.03 (C₇'), 79.59 (C₂'), 83.87 (C₁'), 117.83 (CN), 83.87, 110.96, 113.54, 115.66, 116.03, 119.71, 121.64, 125.25, 128.91, 129.80, 133.25, 145.67, 146.86, 150.20, 160.47, 161.64 (Ar-C), 166.29 (C-2').

General procedure for the synthesis of the protected nucleosides of pyridine-2-ones 8a,b. 6-Amino-3,5-dicyano-4-((4-(1H-benzo[d][1,2,3]triazol-1-yl)methoxy)-3-methoxyphenyl)-1-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosylthio)pyridine (8a). To a mixture of compound 6 [38] (1 mmol, 413 mg) in aq. K₂CO₃ (138 mg, 1 mmol) in 6 mL of distilled water was added a solution of 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl bromide (3a) (1.1 mmol, 452.1 mg) in (10 mL) DMF. The reaction mixture was stirred at room temperature, and the reaction progress was monitored with TLC every 1 h. The reaction mixture was evaporated under reduced pressure at 40°C, and the residue washed with distilled water to remove KBr, dried, and recrystallized from the suitable solvent to give the pure product 8a. White solid, methanol, yield 316 mg (50%), reaction time 30 h, mp 120–123°C; IR (KBr, cm⁻¹): 3417 (NH₂), 2212 (CN), 1629 (CO₂Ac), 1739 (CO₂pyridinone); ¹H-NMR (400 MHz,
6-Amino-3,5-dicyano-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl) methyl)-3-methoxyphenyl)-1-(2,3',4'-tetra-O-acetyl-β-D-galactopyranosyl)pyridine (9a). This compound was obtained as white solid (methanol), reaction time 25 h, mp 195°C, yield 512 mg (85%); IR (KBr, cm⁻¹): 3431 (OH), 3431 (NH2), 2207 (CN), 1664 (COpyridine); ¹H-NMR (400 MHz, DMSO-d₆, δ ppm): 3.34–3.39 (5H, m, H-6, H-5, H-4, H-3, H-2), 4.00 (2H, br. s, NH₂), 3.71 (3H, s, OCH₃), 4.75 (1H, m, 2'-OH), 4.87 (1H, m, 3'-OH), 5.00 (1H, m, 4'-OH), 5.34 (1H, m, 6'-OH), 5.98 (1H, d, J = 8.4 Hz, H-1'), 6.84 (2H, s, OCH₂), 7.39–8.13 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ ppm): 56.15 (OCH₃), 60.11 (C₆'), 62.71 (C₅'), 74.80 (C₄'), 74.90 (C₃'), 75.44 (OCH₃), 77.99 (C₂'), 82.23 (C₁'), 117.01 (CN), 109.90, 111.17, 114.77, 119.77, 119.90, 124.98, 125.21, 125.77, 128.64, 128.87, 133.27, 145.80, 149.69, 150.81, 154.95, 150.71 (Ar-C), 162.56 (C₂); Calcd. for C₃₂H₂₅N₅O₈; 2H₂O (611.53): C, 52.98; H, 4.47; N, 16.02. Found: C, 52.69; H, 4.48; N, 16.34.

6-Amino-3,5-dicyano-4-(4-(1H-benzo[d][1,2,3]triazol-1-yl) methyl)-3-methoxyphenyl)-1-(β-D-galactopyranosyl)pyridine (9b). This compound was obtained as white solid (methanol), reaction time 15 h, mp 140°C, yield 500 mg (83%); IR (KBr, cm⁻¹): 3433 (OH), 3658 (NH₂), 2209 (CN), 1634 (COpyridine); ¹H-NMR (400 MHz, DMSO-d₆, δ ppm): 3.69–3.82 (5H, m, H-6, H-5, H-4, H-3, H-2), 3.41 (2H, s, NH₂), 3.71 (3H, s, OCH₃), 4.01 (1H, br. s, 2'-OH), 4.06 (1H, br. s, 3'-OH), 4.75 (1H, br. s, 4'-OH), 5.73 (1H, m, 6'-OH), 6.33 (1H, d, J₁',₂' = 8 Hz, H-1'), 6.75 (2H, s, OCH₂), 6.95–8.12 (7H, m, Ar-H); ¹³C-NMR (100 MHz, DMSO-d₆, δ ppm): 20.72, 20.76, 20.83, 20.84 (4CH₃), 56.24 (OCH₃), 75.48 (OCH₂), 62.17 (C-6'), 66.76 (C-5'), 68.92 (C-4'), 71.34 (C-3'), 72.90 (C-2'), 80.89 (C-1'), 117.68 (CN), 116.87 (CN), 89.50, 94.25, 111.14, 113.76, 116.25, 119.78, 121.65, 125.02, 128.76, 130.51, 133.36, 145.80, 146.90, 150.07, 157.96, 161.95 (Ar-C), 163.19 (C-2'), 170.15, 170.05, 170.27, 170.58 (4Ac).

ANTICANCER ACTIVITY

In vitro cytotoxicity screening. Cell line propagation. Mammary gland breast cancer cell line (MCF7) was obtained from the American Type Culture Collection (Manassas, VA). The reagents Roswell Park Memorial Institute (RPMI)-1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and fetal bovine serum were obtained from Gibco (New York, NY). The different cell lines mentioned earlier were used to determine the inhibitory effects of the tested compounds on cell growth using the MTT assay [49]. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells.
MTT assay. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics were added 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO₂ incubator. The cells were seeded in a 96-well plate at a density of 1.0 × 10⁴ cells/well at 37°C for 48 h under 5% CO₂. After incubation, the cells were treated with different concentrations of compounds and incubated for 24 h; 20 μL of MTT solution at 5 mg/mL was added and incubated for 4 h. DMSO in volume of 100 μL was added into each well to dissolve the formed purple formazan. The colorimetric assay was measured and recorded at absorbance of 570 nm using a plate reader (StatFax-2100, Awareness Technology, Inc., Palm City, FL). Cytotoxic activities of the target compounds were expressed as IC₅₀ (the concentrations of compounds required to produce 50% inhibition of cell growth). IC₅₀ values were calculated using sigmoidal concentration response curve fitting models by the sigmoidal curve. The results reported are means of at least three separate experiments. Significant differences were analyzed using one-way analysis of variance (ANOVA) wherein the differences were considered to be significant at P < 0.05.

Quantitative real-time PCR analysis. Total RNA was isolated from MCF7 cells using RNeasy Mini kit according to the manufacturer’s protocol and as previously described [59]. After determination of RNA purity and concentration, cDNA was synthesized from 4 μg of total RNA (per sample) using Quant script reverse transcriptase. The isolated cDNA was amplified using 2× Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer’s protocol (Thermo Fisher Scientific, #K0221) and gene-specific primers (Table 5). Reaction volume and qPCR thermal conditions were as previously described [60]. The reference gene, β-actin, was used to calculate fold change in target gene expression. At the end of the last cycle, temperature was increased from 60°C to 95°C to produce a melt curve. The relative change in the last cycle, temperature was increased from 60°C to 95°C to produce a melt curve. The relative change in expression was represented as fold change using quantity critical threshold (Ct) and 2⁻ΔΔCt method [61].

Statistical analysis. Statistical analysis was performed using ANOVA with Tukey-Kramer multiple comparisons test as a post-ANOVA test. Significant differences among means were estimated at P < 0.05. The results were expressed as mean ± standard error of the mean. Values were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

ANTIVIRAL ACTIVITY

Cytotoxicity testing. The nontoxic doses of the test compounds were estimated as previously described by Simões et al. [62] and Meh dizadeh et al. [63]. Briefly, after being dissolved in DMSO, 1 mL of each sample was treated with 24 μL of a 100× antibiotic–antimycotic mixture. Twofold dilutions were prepared with 100 μL of the original dissolved samples, and 100 μL of each dilution was inoculated in MA104 (rhesus monkey kidney cell line used to detect rotavirus), Hep2 (human epithelial type 2 cells, considered to originate from a human laryngeal carcinoma; it has been used for virus and tumorigenicity studies), African green monkey kidney cell line (used to detect a wide variety of viruses), and FRHK4 (fetal rhesus kidney–4) cell lines obtained from the Holding Company for Biological Products & Vaccines, VACSERA, Egypt. Cytotoxicity was assessed by cell morphology evaluation with an inverted light microscope, and cell viability was assessed using a trypan blue dye exclusion method [64].

Measurement of rotavirus Wa, −, and herpes simplex virus type 1 titers using plaque assays. Nontoxic dilutions (100 μL) were mixed with 100 μL of different titers of rotavirus Wa, HAV HM175, and herpes simplex virus type 1 (1 × 10⁵, 1 × 10⁶, and 1 × 10⁷, respectively). The infectivity of the rotavirus stocks was activated with 10 μg/mL trypsin for 30 min at 37°C. The mixture was incubated for 30 min at 37°C. The inoculation of (100 μL) 10-fold dilutions of treated and untreated rotavirus Wa, HAV HM175, and herpes simplex virus type 1 was performed separately in MA104, FRHK4, BGM cell, Hep2 and Vero cell lines in 12-well plates. After 1 h of incubation for adsorption at 37°C in a 5% CO₂–water vapor atmosphere without constant rocking, the plates were rocked intermittently to keep the cells from drying. After adsorption, 1 mL of 2× media (Dulbecco’s modified Eagle medium; Gibco-BRL, DMEM) and 1 mL of 1% agarose were added to each well, and the plates were incubated at 37°C in a 5% CO₂–water vapor atmosphere. After the appropriate incubation period, the cells were stained with 0.4% crystal violet after formalin fixation, and the number of plaques was counted. The viral titers were then calculated and expressed as plaque-forming units per milliliter [65].

Docking studies. Docking studies were performed using the OpenEye molecular modelling software (OpenEye Scientific Software) [66]. A library of synthesized compounds was energy minimized using the MMFF94 force field, which was followed by the generation of multiconformers using the Omega application. The entire energy-minimized library was docked with the prepared catalytic domain of MDM2 (PDB code 5 law) [55] using the FRED application to generate a profile of the ligand–receptor complex. The Vida application can be employed as a visualization tool to show the potential binding interactions of the ligands with the receptor of interest.
ROCS study. The basic method to represent shape and color features in ROCS is by using ROCS application OpenEye Scientific Software. Most active compounds were selected as query molecules. Compound library was adopted as the database file. Both query and database files were energy minimized by Omega applications OpenEye Scientific Software. ROCS runs were employed by personal PC in very fast vROCS interface. vROCS was employed to run and analyze/visualize the results. ROCS application searched the database with the query molecules with similar shape and colors. Compound conformers were scored on the basis of the Gaussian overlap to the query, and the best scoring parameters are Tanimoto combo scores (shape + color); the highest score is the best matched with query compound.

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REFERENCES AND NOTES