Design, Synthesis and Biological Evaluation of Potential Novel Zinc Binders Targeting Human Glyoxalase-I; A Validated Target for Cancer Treatment

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ABSTRACT

The concept of using glyoxalase-I (Glo-I) enzyme inhibitors as anticancer agents is now becoming well-recognized due to the important role the enzyme plays in the detoxification of cytotoxic aldehydes such as methylglyoxal (MG) to harmless substances. In this study, a series of potential Glo-I inhibitor candidates possessing a zinc binding group were synthesized based on structure-based design using Discovery Studio (DS) 3.5 from Biovia®. LibDock protocol was used to dock the compounds onto the active site of Glo-I. N-substituted aminobenzamide scaffolds were chosen, and 14 compounds were synthesized, fully characterized and tested in vitro against the target enzyme. A strong positive correlation was noticed between in silico and experimental data. The top ranked compound (compound 14), which was also the most active experimentally, showed 75.9% inhibition at a 50 µM concentration.

Keywords: Computer Aided Drug Design; Glyoxalase-I; Zinc Binding Group; Anticancer, N-substituted aminobenzamide.

1. INTRODUCTION

Cancer may be simply defined as an uncontrolled somatic cell division subverting and sabotaging normal tissues, with the ability to spread into different parts or even different systems (i.e. blood and lymph systems) of the body.¹

Glyoxalase-I enzyme has gained remarkable attention as a target for developing new anticancer agents. The glyoxalase system consists of two enzymes, Glo-I (lactoylglutathione methylglyoxal lyase; EC 4.4.1.5) and Glo-II (hydroxyacylglutathione hydrolase, EC 3.1.2.6). Glo-II complements Glo-I in the detoxification of α-oxoaldehydes, mainly methylglyoxal (MG). Glo-I catalyses the irreversible isomerization of hemithioacetal, which is formed by the non-enzymatic reaction of MG with glutathione (GSH), into S-D-lactoylglutathione (SLG). SLG serves as a substrate for the Glo-II enzyme, which hydrolyses it to the final harmless product, D-lactate, thus, regenerating glutathione (Fig. 1).²
The glyoxalase system exists in both normal and tumour cells. However, since cancerous cells are highly active, they have about 200 times higher glycolytic rates than normal cells and need to be detoxified more often; therefore, the expression and activity of this system in tumour cells is much higher. Inhibiting Glo-I would stop the detoxification process, leading to accumulation of MG and/or SLG to toxic levels, finally causing cell apoptosis.

Structurally, Glo-I is considered a part of the vicinal-oxygen chelate superfamily, embracing 4β strands and 1α helix in βαβββ motif. It is a homodimeric enzyme with 183 amino acids present in each monomer. The active site is located at the dimer interface and contains one zinc ion (Zn$^{2+}$) (Fig. 2A and 2B) that shows important role in stabilizing the enediolate intermediate by lowering the proton transfer free activation energy.

Great efforts have been put into designing Glo-I inhibitors. Almost all of them have been derivatives of glutathione, mimicking the substrate of Glo-I. This started with Vince et al., who synthesized S-substituted alkyl...
glutathione compounds with p-bromobenzylglutathione, one of the most active compounds; other inhibitors included the tight-binding transition-state inhibitor and bidentate the Zn-chelating peptidic inhibitors. Takasawa et al. revealed that there are natural inhibitors of Glo-I existing in flavonoids due to the presence of planar C-4 ketone and C-5 hydroxyl groups in their structures. On the other hand, Liu et al. have exploited the polyphenol derivatives of curcumin, finding that the enol tautomers of curcumin are strong inhibitors by use of different molecular modelling and molecular dynamics techniques.

Structure-based design was first deduced by our research group focused on studying the active site of Glo-I enzyme. The study identified three vital features for an optimum mode of interaction of the inhibitor at the active site: a zinc ion acting as a cofactor and having a structural and catalytic role in the isomerization of the reactive and toxic aldehydes; a hydrophobic pocket located deep inside the active site; and a highly positive polarized area at the entrance of the active site surrounded by Arg122, Arg37, Lys150 and Lys13. Recently, we have been able to identify potential Glo-I inhibitors by utilizing a customized 3D pharmacophore dedicated to extracting ketol groups (as zinc chelators) from commercial databases (Aldrich).

In this study, 14 compounds were designed in silico using Discovery Studio software (DS 3.5). LibDock algorithm was used to predict the activity of the proposed compounds before the compounds were synthesized and evaluated in vitro against Glo-I.

Results and Discussion

1. In silico drug design

The design of novel inhibitors targeting the Glo-I enzyme was based on computer-aided drug design (CADD) approaches. Prospective inhibitors were then synthesized and biologically evaluated.

Preparation of the proposed inhibitors:

The proposed compounds were designed based on the three main features present at the Glo-I active site. Amides and sulphonamides were suggested as a zinc-binding functional group. These functionalities may form coordinate bonds with Zn\(^{2+}\) ions, and their inclusion in our proposed inhibitors was expected to enhance the binding affinity, resulting in good enzyme inhibition. Additionally, the hydrophobic pocket was fitted with many aromatic moieties that matched in size and electronic density. Moreover, the positively ionized entrance of the active site was complemented with many negatively ionized functional groups. The proposed inhibitors were sketched using ChemBioDraw Ultra 11.0 software and were imported into DS in order to be converted into their corresponding 3D structures, be assigned proper bond orders, and generate accessible tautomer and ionization states prior to docking.

Preparation of the Glo-I enzyme:

As previously mentioned, the most important features of the active site of Glo-I are the zinc atom at its heart and an adjacent hydrophobic pocket, in addition to a highly positively polarized area at the entrance of the active site. The crystal structure of Glo-I was prepared as will be mentioned in the Experimental section. The sphere was chosen to be 11Å\(^2\) covering the active site of the enzyme that would later be used in the docking study.

2. Molecular docking and scoring

In order to have a perception of how the proposed inhibitors could fit in the active site of Glo-I, and to obtain an estimation of their binding affinities, they were docked into the active site of the enzyme and scored. Molecular docking of the proposed inhibitors was performed using LibDock. LibDock uses the physicochemical properties of the ligands to match the ligand atom to the protein hotspots. As the hydrophobic groups at the Glo-I active site are important for the activity of the compounds, the docking protocol embedded in LibDock was chosen for this study. In the docking run, the Glo-I GIP complex was used to define the binding site. The LibDock scores ranged from 92.2 to 113.2, with compound 14 being ranked the highest, indicating that it could have excellent affinity for the target enzyme (Table 3).
3. Synthesis

A series of 16 compounds were synthesized. The novel compounds were characterized by proton (1H) and carbon (13C) NMR spectroscopy, melting points, FT-IR spectroscopy and mass spectroscopy. The previously synthesized compounds were characterized only by proton (1H) NMR spectroscopy and by measuring their melting points, which was compared to the literature previously reported.

Scheme 1: General workflow for the synthesis of 4-bromoanilino derivatives

General schemes for the synthesis of proposed inhibitors:

The proposed inhibitors were synthesized as described in Scheme 1. The process was started by reacting 4-bromoaniline with 4-nitrobenzoyl chloride in an acyl substitution reaction. The product was then reduced using ferrous sulfate and iron with an ethanol/water solvent mixture in a 3:1 ratio,11 and the resulting amine was coupled with acid chlorides, sulfonyl chlorides and anhydrides.

Synthesis of 4-amino-N-phenylbenzamide:

P-bromoaniline was coupled with p-nitrobenzoyl chloride. Tetrahydrofuran (THF) was used as a solvent, and the reaction took about 9 hours to complete. The product was then precipitated by pouring it into acidic water, which was then filtered to be dried in the oven.12 Then, the nitro product was reduced by using 1 equivalent of ferrous sulfate and 5 equivalents of iron. The reaction was performed under reflux for 24 hours. The amine was dissolved in an ethanol/water mixture as the product had low solubility in water alone (Scheme 2).11 This reduction method is considered to be selective for the nitro group, and it yielded no by-products as the workup consisted of filtering the product while hot, decreasing the possibility for the product to precipitate in the presence of iron. The filtrate was evaporated in vacuo, resulting in the final silver-colored product (compound 2 in Scheme 2).

Scheme 2: Synthesis of 4-nitro-N-phenylbenzamide (compound 1) and 4-amino-N-phenylbenzamide (compound 2)
Reaction of 4-amino-N-phenylbenzamide with acid chlorides:

The 4-amino-N-phenylbenzamide compound (compound 2) was dissolved in THF with the presence of pyridine at zero temperature to be reacted with eight different acid chlorides, which were added in small portions. The workup consisted of pouring the reaction mixture on an ice-cold HCl/water mixture to remove the pyridine, then letting the mixture stand until the product precipitated (Scheme 3). This was followed by filtration to collect the N-substituted amide product.13

![Scheme 3](image)

**Scheme 3:** The coupling of different acid chlorides with 4-amino-N-phenylbenzamide (compound 2), forming compounds 3 to 10 “(Procedure F)”

Reaction of 4-amino-N-phenylbenzamide with cyclic anhydrides:

Synthesis of the compounds in this group was accomplished by stirring the free amine 4-amino-N-phenylbenzamide (compound 2) with two cyclic anhydrides at room temperature overnight using THF as a solvent; the addition of heat was needed as the reaction was not moving forward. Various solvents and conditions have been reported in the literature for coupling of amine with anhydrides, such as THF,13 toluene,14 acetonitrile,15 and others, at room temperature and reflux. In our research, THF was chosen (Scheme 4).

![Scheme 4](image)

**Scheme 4:** The coupling of different anhydrides with 4-amino-N-phenylbenzamide (compound 2), forming compounds 11 and 12 “(Procedure G)”

Reaction of 4-amino-N-phenylbenzamide with sulfonyl chlorides:

The compound 4-amino-N-(4-bromophenyl) benzamide (compound 2) was dissolved in THF, pyridine was added as a base, then sulfonyl chloride was slowly added. Since the reaction resulted in too many by-
products, heat was added, and the reaction refluxed overnight. The product was washed in acidic ice-cold water, and the precipitate was filtered and dried. Low yields were observed (Scheme 5).

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{Br} \\
\text{O} & \quad \text{Pyridine} \quad \text{THF, } 0^\circ\text{C} \\
\text{O} & \quad \text{R} \\
\text{S} & \quad \text{Br}
\end{align*}
\]

Scheme 5: The coupling of different sulfonyl chlorides with 4-amino-N-phenylbenzamide (compound 2), forming compounds 13 to 16 “(Procedure H)”

4. *In vitro* enzyme assay

In order to evaluate the biological activities of the synthesized compounds against human Glo-I enzyme, an *in vitro* Glo-I enzyme inhibition assay was performed using recombinant human Glo-I (rhGlo-I) enzyme in compliance with the protocol provided by the manufacturer (R&D Systems® Corporation). The percent of inhibition of the synthesized compounds was determined as will be described in the Experimental section.

In this study, Myricetin was used as a positive control as it is a potent inhibitor with an inhibition percentage of 93% at a 50 µM concentration. The negative control was the substrate MG, with an inhibition percentage of 0%. The activities of the synthesized compounds were assessed by measuring their percent of inhibition relative to the negative control (Tables 1 to 3). The percent of inhibition showed that the most potent inhibitor of Glo-I was compound 14 with 75.9% inhibition at 50 µM.

Table 1. The percent inhibition of the 4-amino-N-phenylbenzamide acid chloride derived compounds against Glo-I enzyme

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>R group</th>
<th>Inhibition (%)</th>
<th>LibDock Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>C8H8O2</td>
<td>52 ±2.5</td>
<td>101.31</td>
</tr>
<tr>
<td>4</td>
<td>C8H7O</td>
<td>40.7 ±7.3</td>
<td>98.08</td>
</tr>
<tr>
<td>5</td>
<td>C7H4NO3</td>
<td>53.3 ±2.1</td>
<td>106.81</td>
</tr>
<tr>
<td>6</td>
<td>C8H7O</td>
<td>47.1 ±9.2</td>
<td>103.38</td>
</tr>
<tr>
<td>7</td>
<td>C7H4ClO</td>
<td>42.3 ±4.2</td>
<td>97.52</td>
</tr>
<tr>
<td>8</td>
<td>C7H4BrO</td>
<td>54.4 ±1.7</td>
<td>100.66</td>
</tr>
<tr>
<td>9</td>
<td>C5H7O3</td>
<td>43.9 ±5.5</td>
<td>99.947</td>
</tr>
<tr>
<td>10</td>
<td>C8H6ClO</td>
<td>54.2 ±3.9</td>
<td>106.43</td>
</tr>
</tbody>
</table>
Table 2. The percent inhibition of the 4-amino-N-phenylbenzamide-anhydrides derived compounds against Glo-I enzyme

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>X-Y</th>
<th>Inhibition (%)</th>
<th>LibDock Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>C2H4</td>
<td>37.4±9.1</td>
<td>100.44</td>
</tr>
<tr>
<td>12</td>
<td>C2H2</td>
<td>10.33±4.7</td>
<td>92.25</td>
</tr>
</tbody>
</table>

Table 3. The percent inhibition of the 4-amino-N-phenylbenzamide-sulfonyl chloride derived compounds against Glo-I enzyme

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>R group</th>
<th>Inhibition (%)</th>
<th>LibDock Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>C4H3O2S2</td>
<td>53.8±8.4</td>
<td>99.67</td>
</tr>
<tr>
<td>14</td>
<td>C8H7O3S</td>
<td>75.9±2.5</td>
<td>113.22</td>
</tr>
<tr>
<td>15</td>
<td>CH3O2S</td>
<td>39.4±6.7</td>
<td>94.41</td>
</tr>
<tr>
<td>16</td>
<td>C7H7O3S</td>
<td>56.5±1.4</td>
<td>108.85</td>
</tr>
</tbody>
</table>

The results of the Glo-1 inhibitory activity and the docking score (Tables 1 to 3) were analysed to test whether there was an evident correlation between the two. The Pearson product-moment correlation coefficient was calculated and found to be 0.8652, which indicates a strong positive correlation between the two sets of data. Compound 14, which was the highest in the docking ranking (113.22), was also the most active experimentally (75.9% inhibition at 50 µM). On the other hand, compound 12 ranked at the bottom of the docking study (92.25) and showed the least inhibitory activity (11% inhibition at 50 µM).

5. Structure–activity relationship (SAR)

Compound 14 had the high inhibitory activity, which may be attributable to the presence of a sulphonyl group on one hand that could coordinate in a bidentate mode with the zinc atom; while the presence of a ketone group on the other hand at para position possibly acts as a hydrogen bond acceptor, with arginine and/or lysine amino acids at the mouth of the active site.

As the hydrophobic interaction is an important feature in the design of the Glo-I inhibitor, compound 15 showed the least activity compared to the remaining sulphonyl chloride derivatives (compounds 13, 14, and 16). This may be explained by the fact that compound 15 has a small methyl group, compared to the aromatic rings on the rest of the compounds.

Compounds 6 and 10 had inhibitory activities of 47.1 and 54.8, respectively. The increase in inhibitory activity
of the later could be due to the extra chlorine atom at the para position of the phenyl ring that is possibly from a favourable steric interaction at the active site of Glo-I.

Compounds that are coupled to cyclic anhydrides were expected to yield a high percentage of inhibition; however, unfortunately, they did not. Possible explanations may be obtained by inspecting their 2D interactions with the enzyme. There were considerable variabilities in the orientation of the actual entrance of the compound into the active site; it may be assumed that the compound did not chelate to the zinc or make any relevant interactions with the enzyme (Fig. 3).

Figure 3: (A) A 2D diagram illustrating the binding mode of compound 12, showing the lack of any bond to the zinc; (B) Surface representation of the active site bound to compound 12

Experimental
1. In silico drug design

Computational materials:

The crystal structure of the target enzyme was retrieved from the Protein Data Bank (www.rcsb.org/pdb/home/home.do). Proposed inhibitors were sketched using ChemBioDraw Ultra 11.0. The in silico design was conducted using Discovery Studio 3.5 from Biovia®. Analyses of biological data and correlations between the in silico results and the in vitro ones were performed using GraphPad Prism 6. High-quality images were generated using DS 3.5. All protocol runs were performed on an Intel (R) Core (TM) 2 Duo CPU machine at 3.00 GHz with 2 GB DDR RAM.

Computational methods:

Preparation of the proposed inhibitors

The proposed inhibitors were sketched using ChemDrawUltra 11.0 and imported into DS to be subsequently converted into the corresponding 3D structures. The prepare ligand protocol within DS was utilized to generate the 3D structures, assign proper bond orders, and generate accessible tautomers and ionization states prior to virtual screening. The default parameters were used.

Preparation of the Glo-I enzyme

As Glo-I was our target, before using its crystal structure in any modelling process, it was checked using the protein report tool in DS for problems related to alternate conformations, missing loops or incomplete residues. Then, the crystal structure was cleaned using the clean protein protocol in DS to fix such issues. After having the Glo-I model checked and cleaned, the active site was defined to be used in the docking step. The definition of the active site took into consideration the
space ligands needed in the docking process. The sphere that was generated to define the active site was within 11 Å of the zinc atom.

2. Molecular docking and scoring
Molecular docking was performed using docking algorithms available in DS, namely, LibDock. LibDock is a high throughput docking algorithm that positions catalyst-generated ligand conformations in the protein active site based on polar and apolar interaction sites (hotspots).

3. Chemistry
Basic Equipment and Methods:
Reaction progress was followed using thin-layer chromatography (TLC) on silica-plated (Merck silica gel-60 F254 precoated plates) aluminium sheets and visualized under UV lamp light (UV245 Spain). Dynamic 1H-NMR and 13C-NMR experiments were performed on Bruker Avance Ultrashield spectrometer (Switzerland) at 400 MHz in DMSO-d6, CDCl3. Chemical shifts were stated in parts per million (ppm), and multiplicity indicated as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Coupling constants (J) were quoted in hertz (Hz), with the specific deuterated solvent residual peaks as reference for each of the compounds; for example, DMSO (δH 2.5, 3.3) and CDCl3 (δH 7.26). Infrared (IR) spectra were recorded to decipher the chemical signature of materials, specifically the bonds between atoms, on KBr pellets using Shimadzu IR Affinity-1 FTIR (JP) with the capillary heater at 350°C and sheath gas pressure at 45 psi (USA). The Biosystems-MDS SCIEX API 3200 apparatus was used in positive mode at 5.0 to 5.5 kV for ESI-LC/MS/MS. The Stuart Scientific Melting Point SMP1 apparatus was used in the melting point determination with degrees Celsius (°C) as the unit. The inhibitory activity was performed in vitro using the human recombinant Glo-I (rhGlo-I), coli-derived Ala2-Met184, with an N-terminal Met and 6-His tag (R&D Systems® Corporation, USA) using double-beam UV-Vis spectrophotometers (Biotech Engineering Management Co. Ltd., UK).

Synthesis

Synthesis of N-(4-bromophenyl)-4-nitrobenzamide (compound 1):
An amount of 3g of p-bromo aniline was dissolved in THF, after which 1 equivalent of pyridine (1.41 ml) was added, and 1 equivalent of 4-nitrobenzoyl chloride (3.24 g) was added slowly at zero temperature. Stirred overnight to be washed in acidic ice-cold water, the product was then filtered to yield compound 1 (4.28 g, 76.4% yield) as light green crystals. 1H NMR (400MHz): δ (DMSO-d6): 7.57 (2H, d, Ar-H, J = 8 Hz); 7.76 (2H, d, Ar-H, J = 8 Hz); 8.17 (2H, d, Ar-H, J = 8 Hz); 8.37 (2H, d, Ar-H, J = 8 Hz); 10.68 (1H, s, CO-NH). Melting point (experimental): 249 to 251°C; (published): 247°C.16

Synthesis of 4-amino-N-(4-bromo-phenyl)-benzamide (compound 2):
An amount of 5 g of N-(4-bromophenyl)-4-nitrobenzamide was dissolved in an ethanol/water mixture at a 3:1 ratio. The reaction allowed to boil, then 1 equivalent of ferrous sulfate and 5 equivalents of iron added and refluxed overnight. It was then filtrated while hot and evaporated in vacuo to yield a beige product (compound 2) (3.5g, 77.7% yield). 1H NMR (400MHz): δ (DMSO-d6): 5.73 (2H, s, CO-NH2); 6.57 (2H, d, Ar-H, J=8 Hz); 7.44 (2H, d, Ar-H, J = 8 Hz); 7.68 (4H, m, Ar-H); 9.83 (1H, s, CO-NH). Melting point (experimental): 207 to 208°C.

General procedure for reacting 4-amino-N-phenylbenzamide with acid chlorides (Procedure F for compounds 3 to 10):
One equivalent of 4-amino-N-(4-bromophenyl) benzamide (compound 2) was dissolved in 50 mL of THF, after which 1 equivalent of pyridine was added, and 1 equivalent of acid chloride was added slowly at zero temperature. The reaction mixture was then poured over water (200 mL) and left for a few minutes. The precipitated product was filtered and dried. Recrystallization was later carried out using different solvents. N-(4-bromophenyl)-4-(4-methoxybenzamido)benzamide (compound 3):
Reacting compound 2 with 4-methoxybenzoyl chloride yielded compound 3 (0.62 g, 24.12% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 3.84 (3H, s, OCH$_3$); 7.07 (2H, d, Ar-H, J = 8 Hz); 7.52 (2H, d, Ar-H, J = 8 Hz); 7.76 (2H, d, Ar-H, J = 8 Hz); 7.92 (6H, m, Ar-H); 10.25 (1H, s, CO-NH); 10.34 (1H, s, CO-NH). Melting point (experimental): 338 to 340°C.

**N-(4-bromophenyl)-4-(4-methylbenzamido)benzamide (compound 4):**

Reacting compound 2 with p-Toluoyl chloride yielded compound 4 (0.21 g, 8.57% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 3.54 (3H, s, Ar-CH$_3$), 7.07 (2H, d, Ar-H, J = 8 Hz); 7.52 (2H, d, Ar-H, J = 8 Hz); 7.76 (2H, d, Ar-H, J = 8 Hz); 7.95 (6H, m, Ar-H); 10.26 (1H, s, CO-NH); 10.34 (1H, s, CO-NH). Melting point (experimental): 344 °C.

**N-(4-bromophenyl)-4-(4-nitrobenzamido)benzamide (compound 5):**

Reacting compound 2 with 4-nitrobenzoyl chloride yielded compound 5 (0.66 g, 25% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 7.52 (2H, d, Ar-H, J=8 Hz); 7.76(4H, d, Ar-H, J = 8 Hz); 7.9 (4H, m, Ar-H); 8.2 (2H, d, Ar-H, J = 8 Hz); 8.38 (2H, d, Ar-H, J = 4 Hz); 10.30 (1H, s, CO-NH); 10.82 (1H, s, CO-NH); $^{13}$C-NMR (DMSO-d$_6$): δ 116.66, 121.12, 123.66, 125.02, 130.03, 130.77, 131.31, 132.85, 140.1, 141.71, 143.27, 150.75, 165.67; IR (KBr) cm$^{-1}$: 3331.07, 1666.50, 1517.98, 1315.43. Melting point (experimental): 349 to 349.6 °C. MS (EI) m/z: 443.5 (M+).

**N-(4-bromophenyl)-4-(2-phenylacetamido)benzamide (compound 6):**

Reacting compound 2 with phenyl acetyl chloride yielded compound 6 (0.65 g, 26.5% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 3.68 (2H, s, CH$_2$-Ar); 7.25 (2H, m, Ar-H); 7.33 (3H, m, Ar-H); 7.51 (2H, d, Ar-H, J = 8 Hz); 7.73(4H, d, Ar-H, J = 8 Hz); 7.92 (2H, d, Ar-H, J = 8 Hz); 10.23 (1H, s, CO-NH); 10.45 (1H, s, CO-NH). Melting point (experimental): 308 to 311°C.

**N-(4-(4-bromophenylcarbamoyl)phenyl)-2-chlorobenzamide (compound 7):**

Reacting compound 2 with 2-chlorobenzoyl chloride yielded compound 7 (0.92 g, 71.8% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 7.74 (1H, m, Ar-H); 7.54 (3H, d, Ar-H, J = 8 Hz); 7.61 (2H, m, Ar-H); 7.77 (2H, d, Ar-H, J = 8 Hz); 7.87 (2H, d, Ar-H, J = 8 Hz); 7.99 (2H, d, Ar-H, J = 8 Hz); 10.28 (1H, s, CO-NH); 10.80 (1H, s, CO-NH). Melting point (experimental): 269 to 271°C.

**3-bromo-N-(4-(4-bromophenylcarbamoyl)phenyl)benzamide (compound 8):**

Reacting compound 2 with 3-bromobenzoyl chloride yielded compound 8 (0.71 g, 25% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 7.44 (1H, t, Ar-H, J=8 Hz); 7.56 (4H, m, Ar-H); 7.76 (3H, m, Ar-H); 8.76 (2H, d, Ar-H, J = 8 Hz); 7.99 (2H, d, Ar-H, J = 8 Hz); 10.28 (1H, s, CO-NH); 10.79 (1H, s, CO-NH). $^{13}$C-NMR (DMSO-d$_6$): δ1115.08, 118.71, 118.82, 122.1, 127.58, 128.56, 128.77, 129.48, 131.28, 132.64, 138.52, 138.67, 141.85, 164.8. IR (KBr) cm$^{-1}$: 334.92, 1670, 1593.2. Melting point (experimental): 289 to 290°C. MS (EI) m/z: 475.36 (M+H).

**Methyl 4-(4-(4-bromophenylcarbamoyl)phenylamino)-4-oxobutanoate (compound 9):**

Reacting compound 2 with methyl-4-chloro-4-oxobutyrate yielded compound 9 (0.41 g, 16.8% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 2.6 (4H, m, CH$_2$CH$_2$); 3.59 (3H, s, O-CH$_3$); 7.51 (2H, d, Ar-H, J = 8 Hz); 7.73 (4H, m, Ar-H); 7.92 (2H, d, Ar-H, J = 8 Hz); 10.22 (1H, s, CO-NH); 10.28 (1H, s, CO-NH). $^{13}$C-NMR (DMSO-d$_6$): δ 13.72, 62.40, 115.12, 119.69, 122.05, 128.44, 130.38, 131.28, 138.47, 140.38, 155.66, 160.25, 164.79; IR (KBr) cm$^{-1}$: 3323.35, 1753.29, 1646.14, 1521.84, 1184.29. Melting point (experimental): 250 to 252°C. MS (EI) m/z: 407.2 (M+2).

**N-(4-(4-bromophenylcarbamoyl)phenyl)-2-chlorobenzamide (compound 10):**

Reacting compound 2 with 4-chlorophenyl acetyl chloride yielded compound 10 (0.44 g, 29.3% yield) as white crystals. $^1$H NMR (400MHz): δ (DMSO-d$_6$): 7.74 (1H, m, Ar-H); 7.54 (3H, d, Ar-H, J = 8 Hz); 7.76 (2H, d, Ar-H, J = 8 Hz); 7.87 (2H, d, Ar-H, J = 8 Hz); 7.99 (2H, d, Ar-H, J = 8 Hz); 10.28 (1H, s, CO-NH); 10.80 (1H, s, CO-NH). Melting point (experimental): 269 to 271°C.
orange crystals. ¹H NMR (400MHz): δ (DMSO-d6): 3.67 (2H, s, CH2); 7.35 (4H, m, Ar-H); 7.49 (2H, m, Ar-H); 7.72 (4H, m, Ar-H); 7.90 (2H, m, Ar-H); 10.20 (1H, s, CO-NH); 10.44 (1H, s, CO-NH). Melting point: (experimental) 304-305°C.

General procedure for reacting 4-amino-N-phenylbenzamide with cyclic anhydrides (Procedure G for compounds 11 and 12):
One equivalent of 4-amino-N-(4-bromophenyl)benzamide (compound 2) was dissolved in 50 mL of THF, after which 1 equivalent of anhydride was added to reflux for a couple of hours. The product was then washed in acidic ice-cold water, and the precipitate was filtered and dried to be recrystallized using different solvents.

4-(4-(4-bromophenylcarbamoyl)phenylamino)-4-oxobutanoic acid (compound 11)
Reacting compound 2 with succinic anhydride yielded compound 11 (0.67 g, 29.1% yield) as white crystals. ¹H NMR (400MHz): δ (DMSO-d6): 2.54 (2H, m, CH2CH2, J= 8 Hz); 2.60 (2H, t, CH2CH2, J = 8 Hz); 7.51 (2H, d, Ar-H, J = 8Hz); 7.73 (4H, d, Ar-H, J = 8 Hz); 7.91 (2H, d, Ar-H, J = 8 Hz); 10.21 (1H, s, CO-NH); 10.25 (1H, s, CO-NH); 12.15 (1H, s, OH). ¹³C-NMR (DMSO-d6): δ 28.64, 31.12, 115.09, 118.07, 122.16, 128.6, 131.34, 138.65, 142.34, 164.94, 170.58, 173.73; IR (KBr) cm⁻¹: 3323.35, 2924.09, 1670.3, 1516.05, 1492.90. Melting point (experimental): 284 to 287°C. MS (EI) m/z: 390 (M+1).

4-(4-(4-bromophenylcarbamoyl)phenylamino)-4-oxobut-2-enoic acid (compound 12):
Reacting compound 2 with maleic anhydride yielded compound 12 (0.5 g, 21.7% yield) as beige crystals. ¹H NMR (400MHz): δ (DMSO-d6): 6.34 (1H, d, CHCH, J=12Hz); 6.49 (1H, d, CHCH, J = 12 Hz); 7.51 (2H, d, Ar-H, J = 8 Hz); 7.74 (4H, m, Ar-H); 7.94 (2H, d, Ar-H, J = 8 Hz); 10.26 (1H, s, CO-NH); 10.66 (1H, s, CO-NH); 12.88 (1H, s, OH). ¹³C-NMR (DMSO-d6): δ 113.1, 115.72, 119.2, 122.75, 129.24, 129.94, 131.07, 131.92, 139.21, 142.32, 164.08, 165.47, 167.49; IR (KBr) cm⁻¹: 3350.35, 1645.28, 1591.27, 1533.41, 1489.05, 1317.38. Melting point (experimental): 249 to 251°C. MS (EI) m/z: 389.39 (M+).

General procedure for reacting 4-amino-N-phenylbenzamide with sulfonyl chlorides (Procedure H for compounds 13 to 16):
One equivalent of 4-amino-N-(4-bromophenyl)benzamide (QKH18) (compound 2) was dissolved in 50 mL of THF, after which 1 equivalent of pyridine was added, 1 equivalent of sulfonyl chloride was added and refluxed, and the product was washed in acidic ice-cold water. The precipitate was then filtered and dried to be recrystallized with different appropriate solvents.

N-(4-bromophenyl)-4-(thiophene-2-sulfonamido)benzamide (compound 13)
Reacting compound 2 with 2-thiophenesulfonyl chloride yielded compound 13 (0.61 g, 32.46% yield) as white crystals. ¹H NMR (400MHz): δ (DMSO-d6): 7.15 (1H, t, Ar-S-H, J = 4 Hz); 7.29 (2H, d, Ar-H, J = 8 Hz), 7.52 (2H, d, Ar-H, J = 8 Hz); 7.66 (1H, d, Ar-H, J = 4 Hz); 7.73 (2H, d, Ar-H, J = 8 Hz) ; 7.88 (2H, d, Ar-H, J = 8 Hz) ; 7.94 (1H, d, Ar-H, J = 4 Hz); 10.62 (1H, s, CO-NH); 10.89 (1H, s, CO-NH). Melting point (experimental): 249 to 251°C.

4-(4-acetylphenylsulfonamido)-N-(4-bromophenyl)benzamide (compound 14):
Reacting compound 2 with 4-acetylbenzenesulfonyl chloride yielded compound 14 (0.52 g, 18.3% yield) as white crystals. ¹H NMR (400MHz): δ (DMSO-d6): 7.15 (1H, t, Ar-S-H, J = 4 Hz); 7.29 (2H, d, Ar-H, J = 8 Hz), 7.52 (2H, d, Ar-H, J = 8 Hz); 7.66 (1H, d, Ar-H, J = 4 Hz); 7.73 (2H, d, Ar-H, J = 8 Hz) ; 7.88 (2H, d, Ar-H, J = 8 Hz) ; 7.94 (1H, d, Ar-H, J = 4 Hz); 10.62 (1H, s, CO-NH); 10.89 (1H, s, CO-NH). Melting point (experimental): 195 to 198°C.

N-(4-bromophenyl)-4-(methylsulfonamido)benzamide (compound 15):
Reacting compound 2 with methanesulfonyl chloride yielded compound 15 (0.06 g, 2.7% yield) as white crystals. ¹H NMR (400MHz): δ (DMSO-d6): 2.6 (3H, s, OCH3); 7.26 (2H, d, Ar-H, J = 8 Hz); 7.51 (2H, d, Ar-H, J = 8 Hz); 7.71 (2H, d, Ar-H, J = 8 Hz); 7.87 (2H, d, Ar-H, J = 8 Hz); 7.97 (2H, d, Ar-H, J = 8 Hz); 8.12 (2H, d, Ar-H, J = 8 Hz); 10.23 (1H, s, CO-NH); 10.93 (1H, s, CO-NH). Melting point (experimental): 195 to 198°C.
J = 8 Hz); 7.50 (2H, d, Ar-H, J = 8 Hz); 7.74 (3H, m, Ar-H); 7.93 (1H, t, Ar-H, J = 8 Hz); 9.98 (1H, s, CO-NH); 10.24 (1H, s, CO-NH). Melting point (experimental): 230°C.

N-(4-Bromo-phenyl)-4-(4-methoxy-benzenesulfonylamino)-benzamide (compound 16):
Reacting compound 2 with 4-methoxy-benzenesulfonyl chloride yielded compound 16 (2.27 g, 82.2% yield) as beige crystals. $^1$H NMR (400MHz): $\delta$ (DMSO-d6): 3.52 (3H, s, CH$_3$); 7.08 (2H, d, Ar-H, J = 8 Hz); 7.22(2H, d, Ar-H, J = 8 Hz); 7.50 (2H, d, Ar-H, J = 8 Hz); 7.67 (6H, m, Ar-H); 10.20 (1H, s, CO-NH); 10.62 (1H, s, CO-NH). Melting point (experimental): 234 to 237°C.

In vivo enzyme assay:
The in vitro inhibitory activity of the synthesized compounds against the target enzyme was assessed using the human recombinant Glo-I (rhGlo-I) according to the procedure provided by R&D Systems ® Corporation. The synthesized compounds were dissolved in DMSO at a concentration of 10mM. First, the assay buffer was prepared using a solution of 0.1M sodium phosphate dibasic and 0.1M sodium phosphate monobasic solution to a pH of 7.2. The substrate mixture was prepared freshly using a mixture of 100 mM of reduced glutathione and 100 mM of methylglyoxal solutions. The synthesized compounds were mixed with the assay buffer, substrate solution mixture and the Glo-I enzyme in a cuvette at a concentration of 50 $\mu$M, then screened at $\lambda_{\text{max}}$=240 nm for 200 seconds at 25°C.

Conclusion
In this study, a series of 14 potential Glo-I inhibitors possessing an N-substituted aminobenzamide scaffold have been designed based on structure-based drug design technique. Molecular docking of the proposed inhibitors was performed using LibDock docking protocol within DS. Those compounds were synthesized, and their biological activities were tested in vitro against the target enzyme (Glo-I). An excellent agreement was noticed between in silico and experimental data. Compound 14 showed 75.9% inhibition at a 50 $\mu$M concentration, which makes it a promising candidate for the development of novel Glo inhibitors and therefore potential new treatments for cancer.

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ملخص

أدى ظهور مرض السرطان في جميع أنحاء العالم وفي جميع المجتمعات إلى حث العلماء في هذا المجال لمقاومة انتشاره. إن مبدأ استخدام مثبتات لازيم الجليوكساليز-1 قد أصبح مرسما بشكل حاد للدور المهم الذي يقوم به الازيم في عملية إزالة السمية للأملاح. كجزء من دراسة صغيرة مبنية على الأدبيdetects، فن استخدم هذه الدراسة عملاً لتحديد الزنك المستهدفة للكائنات المضادة للسرطان فيwavصل لهذه الكائنات. يتم فحص فعالية هذه الكائنات باستخدام طريقة مخصصة لفحص مثبتات الأزم وعابرة. وان الكائن 14 هو الأكثر فعالية بنسبة 75.9%. مصطلحات: أزم الجليوكساليز، مجموعة رابطة الزنك، مضاد السرطان، مشقات الأمينوبينازاميد.