Cytotoxicity and anti-small cell lung cancer potential of 3-methoxy-5-nitrosalicylaldehyde: an analog for treatment of diabetes mellitus with considerable binding affinity to α-amylase enzyme

Hongqing Wen¹, Junyan Wang², Saad H. Alotaibi³

¹Department of PCCM, Xi’an No. 3 Hospital, the Affiliated Hospital of Northwest University, Xi’an, Shaanxi, China
²Pre-test triage, The Fourth People’s Hospital of Jinan, Jinan city, China
³Department of Chemistry, Turabah University College, Taif University, Taif, Saudi Arabia

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Abstract

Introduction: α-Amylase inhibitors are present in plants and are thought to be produced by plants to strengthen their defenses against predators. They include plant components, polyphenolic compounds and glycoproteins with enzymatic inhibitory activity.

Material and methods: In this study, the inhibitory effect of metabolic enzyme was obtained, IC₅₀: 95.14 μM. The molecular docking investigation was performed as a versatile method for the evaluation of the biological activities of 3-methoxy-5-nitrosalicylaldehyde in the presence of α-amylase. The compound exhibited a considerable binding affinity to the enzyme with a docking score of −7.676 kcal/mol.

Results: The results of the molecular docking revealed that 3-methoxy-5-nitrosalicylaldehyde is able to construct hydrophobic contacts with crucial residues of the catalytic domain of the enzyme. According to these findings, the compound has the potential to be an inhibitor of α-amylase. The MTT test was used on normal (human umbilical vein endothelial cells (HUVECs)) and small cell lung cancer (SBC-3, DMS273, and DMS114) cell lines. 3-Methoxy-5-nitrosalicylaldehyde had high cell death and anti-small cell lung cancer effects against SBC-3, DMS273, and DMS114 cell lines. Among the above cell lines, the best result of anti-small cell lung cancer properties of the molecule was obtained in the cell line DMS273.

Conclusions: The results of this study indicated the excellent anti-small cell lung cancer potential of 3-methoxy-5-nitrosalicylaldehyde in vitro conditions. After confirming the above results in the clinical trial research, this formulation may be administrated for the treatment of several types of small cell lung cancer in humans.

Key words: lung cancer, 3-methoxy-5-nitrosalicylaldehyde, α-amylase, diabetes, molecular modeling.

Introduction

Lung cancer begins when cells from structurally normal lung tissue multiply beyond need and control, forming a mass (tumor) in the lung. The mass formed here first grows in its environment, and in later stages it spreads to the surrounding tissues or to distant organs (liver, bone,
brain, etc.) through the circulation, causing damage. Lung cancer is a very common cancer. It is the cause of 12–16% of all cancers and 17–28% of cancer-related deaths. Moreover, it ranks first in cancer-related deaths in both men and women [1, 2]. Enzyme inhibition means reducing or blocking the action of an enzyme with a specific substrate, called an enzyme inhibitor, or its analogue. In modern times, besides pharmaceutical compounds, some natural compounds are marketed as enzyme inhibitors and these inhibitors exert their specific effects on enzyme inhibition in cells, bacteria, viruses and the human body [3, 4].

To control blood sugar within certain limits, diabetics avoid sugary foods and prefer starchy foods instead. High-carbohydrate diets contribute significantly to diabetes. Starch digestion is highly determinative of post-meal blood sugar level and also affects glucose metabolism. It has been suggested that hyperglycemia is an important factor leading to disruption of carbohydrate metabolism; therefore, delaying the rise in blood glucose is considered beneficial for alleviating insulin resistance and/or type 2 diabetes [5, 6]. In the gastrointestinal tract, starch is mainly digested by α-amylase in the small intestine into reducing sugars such as maltose, maltotriose and amylohextrin. The reducing sugars are then hydrolyzed by α-glucosidase to produce glucose. Therefore, amylase is the key enzyme of starch hydrolysis and regulating enzyme activity with chemical and biological components is recommended in the prevention and treatment of hyperglycemia and the resulting metabolic disorder. α-Amylase has a very important role in the treatment of diabetes [7, 8]. Inhibition of this enzyme delays glucose secretion and absorption in the small intestine. In this event that takes place in the intestine, the level of hyperglycemia is reduced by delaying carbohydrate digestion by inhibitors of enzymes [9, 10].

Nowadays, theoretical studies have become a necessary part of the laboratory evaluation of chemical compounds. Such inquiry could give a more comprehensive insight into the biological activities and experimental results [11]. Molecular docking has drawn significant attention from biologists as performing docking calculations could provide the researchers a complete point of view on the biological activities of enzyme inhibitors [12]. This information can assist the researchers to understand the mechanisms in which the compounds and biological substances would interact with each other. Various parameters will be collected from the docking study, such as binding affinity and the features of interactions.

In the current study, the properties of 3-methoxy-5-nitrosalicylaldehyde against α-amylase enzyme inhibition and molecular docking of it were investigated. Also, we decided to survey the anti-small cell lung cancer potential of 3-methoxy-5-nitrosalicylaldehyde against small cell lung cancer cell lines including SBC-3, DMS273, and DMS114.

**Material and methods**

**Materials**

Antimycotic antibiotic solution, hydrolysate, dimethyl sulfoxide (DMSO), 4-(dimethylamino) benzaldehyde, Ehrlich solution, carbazole reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco’s Modified Eagle Medium (DMED), borax-sulfuric acid mixture, and phosphate buffer solution (PBS) were all obtained from Sigma-Aldrich, USA.

**α-Amylase assay**

α-Amylase activity was determined by a method adapted from the work of Taha et al. [13]. Accordingly, α-amylase solution, phosphate buffer and starch from the given reaction components were incubated in an Eppendorf tube at 35°C for 10 min. 100 µl of dinitrosalicylic acid (DNS) was added to the reaction and the reaction mixture was boiled for 5 min to stop the reaction [14]. The reaction mixtures cooled to room temperature were diluted with 1000 µl of distilled water and absorbance measurements were performed at 540 nm. In order to determine the molar absorption coefficient, a standard maltose graph was drawn and the value was calculated as 0.0071 (µg/ml)–1 cm–1. One unit of enzyme activity was calculated as the amount of 1 µmol maltose released in 1 min under the reaction conditions [15].

**Molecular docking study**

The docking calculations were conducted to evaluate the chemical and biological activities of 3-methoxy-5-nitrosalicylaldehyde as an inhibitor for α-amylase. Acarbose was also considered to be a standard inhibitor for alpha-amylase. The structure of α-amylase (PDB ID: 1HNY) [16] at 1.8 Å resolution with the X-ray diffraction method was obtained from the Protein Data Bank (http://www.rcsb.org/pdb). The preparation of the raw structure of the enzyme was carried out using the protein preparation module of Schrödinger [17]. Two essential steps in protein preparation are the addition of hydrogens to the structure and removing unnecessary water molecules. The creation of an H-bond network was performed using the optimization step of the mentioned module. At the end of the preparation step, the structure was minimized utilizing the OPLS3e force field. The prepared structure was investigated for evaluation of the binding site of the enzyme using SiteMap of
Schrödinger [18]. In the next step, a grid box (20 × 20 × 20 Å³) was constructed around the predicted active site. The SDF forms of 3-methoxy-5-nitrosalicylaldehyde and acarbose were retrieved from the PubChem database and prepared with the LigPrep module of Schrödinger [19] to generate accurate molecular geometric and protonation states. Finally, the calculations of molecular docking were performed utilizing Glide of the Schrödinger Suite.

**Determination of anti-small cell lung cancer activities of 3-methoxy-5-nitrosalicylaldehyde**

In the present experiment, different small cell lung cancer cell lines, i.e., SBC-3, DMS273, and DMS114 cell lines, and also the human normal cell line (human umbilical vein endothelial cells (HUVECs)) were used to study the cytotoxicity and anticancer potential of small cell lung cancer towards 3-methoxy-5-nitrosalicylaldehyde using the common cytotoxicity test, i.e., MTT assay in *in vitro* conditions.

For this purpose, each cell line was placed separately in T25 flasks with a complete culture medium (including DMEM (Dulbecco’s Modified Eagle Medium), 10% complementary bovine fetal serum, and 1% penicillin-streptomycin solution) and at 37°C in the incubator, cell culture was incubated with 5% CO₂. After obtaining 80% cell density, the sample was exposed to 1% trypsin-EDTA solution and after 3 min of incubation at 37°C in a cell culture incubator with 5% CO₂ and observation of cells removed from the bottom of the plate, the sample was centrifuged at 5000 rpm for 5 min. Then, the cell suspensions after adding trypan blue dye were counted by a neobar slide and a cytotoxicity test was performed by the MTT method [20].

Initially, 10,000 cells were implanted in cell culture plates and then the cells were treated at concentrations of 1-1000 µg/ml of 3-methoxy-5-nitrosalicylaldehyde. After 24 h, 20 µl of MTT dye was added to the wells and incubated for 5 h at 37°C with 5% CO₂. DMSO was then added to the wells to dissolve the formazan crystals and the absorption rate of the wells at 570 nm was read by an ELISA reader (ELISA Teknika Oraganon reader, Netherlands) and the cell viability rate was computed by the formula below [20]: Cell viability (%) = (sample A/Control A) × 100.

After collecting data, Minitab statistical software was used for statistical analysis. Evaluation of cytotoxicity and anti-small cell lung cancer results in a completely randomized design and comparison of means was performed by the Duncan post-hoc test with a maximum error of 5%. To measure the percentage of cell survival in factorial experiments with the original design of completely randomized blocks and compare the means, the Duncan post-hoc test with a maximum error of 5% was used. The 50% cytotoxicity (IC₅₀) and 50% free radical scavenging (IC₅₀) were estimated with ED50 plus software (INER, V: 1.0). Measurements were reported as mean ± standard deviation.

**Ethics statement**

This research was approved by The Fourth People’s Hospital of Jinan animal ethical committee, Approval No. 2021-JNFH-0034.

**Results and discussion**

**α-Amylase inhibition results**

In this study, inhibition of metabolic enzyme was obtained, IC₅₀: 95.14 µM (Figure 1). Glucosidase inhibitors are highly promising in the treatment of various diseases such as diabetes, viral infections and cancer metastasis, as well as being a very effective tool for understanding the mechanism of action of glucosidases [21]. Therefore, α-amylase has been the target enzyme for the design of drug molecules suitable for the treatment of diabetes, obesity, and hyperglycemia. Generally speaking, it is known that commercially available antidiabetic drugs are α-amylase inhibitors because they reduce postprandial hyperglycemia. Liminoids purified from *Azadirachta indica* for pancreatic α-amylase inhibition have also been reported to be used as antidiabetic drugs due to their potential therapeutic effects [22]. In addition, tetra cyclic diterpenoid (also known as isosteviol) triazole derivatives obtained by acid hydrolysis of steviol glycoside extract, which is abundant in *Stevia rebaudiana*, are used as antitumor agents in the treatment of cancer. In addition to these, although it is known that disaccharides, iminosaccharides, carbasaccharides and thiosaccharides are among the inhibitors of glucosidases, there is still a need for the design and discovery of new inhibitor molecules with high specificity and efficacy [23]. Therefore, Balba et al. [24] synthesized diaryl derivatives of imidazole-thione and 1,2,4-triazole-thiol for inhibition of α-amylase

![Figure 1. IC₅₀ graph of compound against α-amylase](image-url)
and α-glucosidase and examined the inhibition potentials of these molecules. Perion et al. [25] synthesized a 1,4,5-trisubstituted 1,2,3-triazole compound and investigated its effectiveness as a glycosidase inhibitor. Anand et al. [26] demonstrated the importance of pharmacophores in the treatment of many diseases of compounds containing a triazole structure in a study they carried out, and for this reason, the synthesis of triazole glycohybrid structures and their α-glucosidase inhibition potential were examined [27]. In addition, Balan et al. [28] reported that they synthesized 2-allyl amino 4-methylsulfonyl butyric acid as a new molecule for inhibition of α-amylase and α-glucosidase, and they carried out both molecular modeling studies and enzyme kinetic studies to evaluate the inhibitory potential of the said molecule. In a previous study, molecular modeling revealed the molecular basis of the specific binding of different α-amylase inhibitors obtained from the seeds of Phaseolus vulgaris and Alpina nigra to the active site of the enzyme [29–31].

Molecular docking results

The molecular docking study as a multifaceted analytical approach was used for the investigation of 3-methoxy-5-nitrosalicylaldehyde biological activities. Figure 2 shows the docking pose of the compound in the residues of the α-amylase, and the interactions between the ligand and enzyme are presented in Figure 3. As can be seen, Gln63 has created a hydrogen bond with the ligand. NH of Gln63 of the peptide backbone has constructed this H-bond with the oxygen of 3-methoxy-5-nitrosalicylaldehyde. This oxygen atom has emerged as a hydrogen bond acceptor, which means that Gln63 is a hydrogen bond donor. Although this residue is not from the catalytic domain of the enzyme, this hydrogen bond provides a strong binding affinity for the ligand. There are eight hydrophobic contacts between the ligand and residues of α-amylase. These residues are Trp58, Trp59, Tyr62, His101, Leu165, Asp197, Asp300, and His305. Asp 197 and Asp300 are two important residues of the α-amylase active site [32]. Creating hydrophobic contacts with these two residues is essential for this compound, as it can prove the inhibitory activity of 3-methoxy-5-nitrosalicylaldehyde. The parameters obtained from the docking calculations are presented in Table I. The docking score indicates the binding affinity of the ligand to the enzyme, which is the most important parameter [33]. 3-Methoxy-5-nitrosalicylaldehyde with a docking score of –7.676 kcal/mol has shown a remarkable binding affinity to the enzyme. This value is –7.481 kcal/mol for acarbose as a standard inhibitor of α-amylase. As can be clearly seen, the docking scores have a positive correlation with the IC50 of the compounds. The binding energy between the binding partners is another important parameter. Glide ligand efficiency indicates this value. Some of the parameters are interaction related parameters, such as Glide Evdw and Glide Ecoul. The Glide Evdw and Glide Ecoul indicate Van der Waals energy and the Coulomb energy, respectively. The modified Coulomb-van der Waals interaction energy is shown with the Glide energy. The next parameter is Glide Emodel and is an indicator of the value of the interaction [34]. The first active site of the enzyme structure is presented in Figure 4. This site is the most drug-
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Table 1. Parameters obtained from the molecular docking calculations

<table>
<thead>
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<th>Parameter</th>
<th>3-Methoxy-5-nitrosalicylaldehyde</th>
<th>Acarbose</th>
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<tbody>
<tr>
<td>IC$_{50}$ [µM]</td>
<td>95.14</td>
<td>254.06</td>
</tr>
<tr>
<td>Docking score [kcal/mol]</td>
<td>–7.676</td>
<td>–7.481</td>
</tr>
<tr>
<td>Glide ligand efficiency [kcal/mol]</td>
<td>–0.548</td>
<td>–0.170</td>
</tr>
<tr>
<td>Glide Emodel [kcal/mol]</td>
<td>–56.457</td>
<td>–104.911</td>
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</table>

Figure 3. Interactions of 3-methoxy-5-nitrosalicylaldehyde and α-amylase. Green dashed lines indicate the hydrogen bonds, and semicircles show the hydrophobic contacts.

Figure 4. First predicted active site of alpha amylase. Red areas are hydrogen acceptors, blue areas are hydrogen donors, and yellow areas are hydrophobic.
gible site of the enzyme. It means that this part of the enzyme structure is able to interact with the ligands considerably. Therefore, this site can construct various contacts with inhibitor agents. The hydrogen acceptor areas are shown in red, and the blue areas are hydrogen bond donor parts of the enzyme. The hydrophilic parts of the active site are indicated with yellow color. Table II represents the residues engaged in this active site. Due to the results of the molecular docking, 3-methoxy-5-nitrosalicylaldehyde has the potential to be considered as an inhibitor of α-amylase.

Cytotoxicity and anti-small cell lung cancer effects of 3-methoxy-5-nitrosalicylaldehyde

One of the cytotoxicity test methods to measure the rate of cell death is the MTT method, which is based on the formation of formazan dye by reducing the substance dimethyl thiazole 2 and 5 diphenyltetrazolium bromide (MTT) or other tetrazolium salts [35, 36]. By breaking the MTT tetrazolium ring by mitochondrial enzymes in living cells, insoluble purple formazan crystals are formed. The formation of these crystals indicates the activity of respiratory chain enzymes and is a measure of cell viability. By measuring the amount of absorption with a spectrophotometer at specific wavelengths, the number of living cells can be determined. This test is performed according to ISO 10993-5 and its purpose is in vitro evaluation of cytotoxicity. The cytotoxicity test is performed according to the ISO10993-5 standard and in four ways: the NRU test, the CFU test, the MTT test and the XTT test. The most common method for assessing cytotoxicity is to measure cell survival by MTT [37]. The MTT method is based on the intensity of dye produced by the mitochondrial activity of cells, measured at a wavelength of 540 to 630 nm and directly proportional to the number of living cells. The increase or decrease in the number of living cells is linearly related to the activity of cell mitochondria. MTT tetrazolium dye is reduced in active (metabolically) cells. Mitochondrial dehydrogenases in living cells produce NADH and NADPH, leading to an insoluble purple precipitate called formazan. This precipitate can be dissolved by isopropanol or dimethyl sulfoxide [38]. Dead cells, on the other hand, are unable to perform this conversion due to the inactivity of their mitochondria and therefore do not show a signal. In this method, dye formation is used as a marker for the presence of living cells. In recent years, MTT testing has been the most important measurement method to evaluate the toxicity and anti-cancer effects of molecules [39].

In the current research, the cytotoxicity of 3-methoxy-5-nitrosalicylaldehyde was explored by studying its interaction with normal (HUVEC) and common small cell lung cancer cell lines, i.e., SBC-3, DMS273, and DMS114 by MTT assay for 48 h. The interactions expressed as cell viability (%) were observed at different 3-methoxy-5-nitrosalicylaldehyde concentrations (0-1000 µg/ml) with the four cell lines which are shown in Table III and Figures 5–8.

In all cases, the % cell viability is reduced with increasing 3-methoxy-5-nitrosalicylaldehyde concentrations. The IC50 values of 3-methoxy-5-nitrosalicylaldehyde against common small cell lung cancer cell lines, i.e., SBC-3, DMS273, and DMS114 cell lines were 198, 179, and 211 µg/ml, respectively (Table III and Figures 5–8).

Thus, the best cytotoxicity findings and anti-small cell lung cancer properties of the investigated molecule, 3-methoxy-5-nitrosalicylaldehyde, were observed in the case of the DMS273 cell line.

In conclusion, theoretical approaches are attractive methods for the evaluation of experimental studies. Due to the valuable information obtained from the molecular docking study, this method has gained considerable consideration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>3-Methoxy-5-nitrosalicylaldehyde [µg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 against HUVEC</td>
<td>–</td>
</tr>
<tr>
<td>IC50 against SBC-3</td>
<td>198 ±0</td>
</tr>
<tr>
<td>IC50 against DMS273</td>
<td>179 ±0</td>
</tr>
<tr>
<td>IC50 against DMS114</td>
<td>211 ±0</td>
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Conflict of interest
The authors declare no conflict of interest.

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