Substituted pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines – a promising class of lipoxygenase inhibitors

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The modern strategy of potential biologically active molecules search (“drug-design”) is based on several innovation approaches. The method of high throughput biological screening and method of molecular modeling deserves the most attention among such approaches. Lipoxygenase (LOX) is one of the most perspective biological target for the substituted pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines. So, molecular docking towards LOX and enzyme activating activity was investigated.

The aim: Directed search of potential inhibitors of lipoxygenases among the unknown pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines with the use of molecular docking and in vitro high throughput screening.

Materials and methods. The research of lipoxygenase activity has been conducted for a number of original pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines. Standard software was used for molecular docking and “drug-like” criteria research. Sodium letinate was used as a substrate to study soybean LOX enzyme activating activity.

Results. The results of molecular docking have shown, that substituted pyrrolo[1,2-a][1,2,4]triazolo[1,5-c]quinazolines reveal a strong affinity toward LOX. The main types of interactions with aminoacid residues of mentioned the enzyme were identified. The conducted researches showed, that the substituted pyrrolo[1,2-a][1,2,4]triazolo[2,3-c]quinazolines had the highest soybean LOX inhibition activity. Compounds with a fluorine atom and a 2-thienyl moiety in the structure revealed the highest activity inhibiting lipoxygenase by 36.33 % and 39.83 % respectively. The increased lipophilicity of triazine derivatives promotes a higher ability to inhibit soybean LOX, whereas, for triazole derivatives, which have lower molecular weight, an inverse relation is observed.

Conclusions. The research of the substituted pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines inhibition ability of soybean LOX as one of the possible mechanisms of their activity is proved and conducted. It is shown, that their lipoxygenase activity depends on lipophilicity and is defined by the availability of donor-acceptor fragments in the molecule, that is capable to form hydrogen and other types of interaction. The specified results are strong arguments for their further study as promising anti-inflammatory agents.

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The modern strategy of potential biologically active molecules search (“drug-design”) underwent significant changes and became the most important part of modern medical chemistry [1–3]. Now it is based on several innovation approaches, such as virtual screening, combinatorial chemistry, high throughput screening, molecular modeling, fragment-oriented design, optimization of the leading structure, etc. Among the above-mentioned approaches, the method of high throughput biological screening deserves the most attention. This method allows estimating activity of many compounds against a known biological target in short terms. This method always correlate with the relevant experimental data. However, it gives an understanding of the mechanism and ligand activity efficiency.

Throughout the directed search investigations of biologically active compounds among quinazoline derivatives and its condensed analogs [7–16], we have used the above-mentioned strategy. Lipoxygenase (LOX) was used as a biological target. Especially, considering that LOX part in many pathological conditions formation, such as chronic inflammations, allergy, asthma, some cancer types, cardiovascular diseases, etc [17].

**Aim**

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In vitro 1.1–1.3

\( \text{Fig. 6} \)

center_z = -3.760, size_x = 16, size_y = 16, size_z = 16 for COX-2 (3LN1); center_x = 32.978, center_y = -44.488, center_z = 53.949, size_x = 18, size_y = 16, size_z = 16 was set as following: center_x = 18.370, center_y = -52.296, center_z = 11.249, size_x = 18, size_y = 16, size_z = 16

Materials and methods

The research of lipoygenase activity has been conducted for a number of original pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)(c)quinazolines 1.1–1.3, 2.1–2.18 (Fig. 1), which were synthesized at the Department of Organic and Bioorganic Chemistry of the Zaporizhzhia state medical university (the Head of the Department, Dr hab., Professor, S. I. Kovalenko). The features of the structures of the synthesized compounds were evaluated by IR-, NMR spectroscopy, and chromatography-mass spectrometry and were discussed in detail [18].

Molecular docking. The research was conducted by flexible molecular docking, as an approach of finding molecules with affinity to a specific biological target. Macromolecules from Protein Data Bank (PDB) were used as biological targets, namely LOX (soybean) enzyme in complex with protocatechuic acid (PDB ID – 1N8Q) [19]. The choice of biological targets was due to the literature on the mechanism of anti-inflammatory drug action [17].

Ligand preparation. Substances were drawn using Marvin-Sketch 19.24 and saved in mol format [20]. After that, they were optimized by program Chem3D, using the molecular mechanical MM2 algorithm and saved as pdb-files. Molecular mechanics was used to producing more realistic geometry values for the majority of organic molecules, owing to the fact of being highly parameterized. Using AutoDockTools-1.5.6 pdb-files were converted into PDBQT, the number of active torsions was set as default [21].

Protein preparation. PDB files were downloaded from the protein data bank. Discovery Studio v 19.1.0.18287 was used to delete water molecules and ligands. Structures of proteins were saved as pdb-files [22]. In AutoDockTools-1.5.6 polar hydrogens were added and saved as PDBQT. Grid box was set as following: center_x = 18.370, center_y = -52.296, center_z = 53.949, size_x = 18, size_y = 16, size_z = 16 for COX-2 (3LN1); center_x = 32.978, center_y = -44.488, center_z = 53.949, size_x = 18, size_y = 16, size_z = 16 for COX-1 (3N8Y). Vina was used to carry docking [15]. For visualization Discovery Studio v 19.1.0.18287 was used.

Lipinski’s rule of five. Drug-like characteristics (Log P, molecular polar surface area, number of non-hydrogens, number of hydrogen bond acceptors (groups N and O), number of hydrogen bond donors (groups NH and OH) and number of rotatable bonds) were evaluated and optimized using an electronic resource [23].

Soybean LOX inhibition study in vitro. In vitro study was evaluated as it was reported previously [24,25]. To 3.880 ml of borate buffer, 40 µl 2 × 10^{-5} mol/l solution of LOX in the buffer and 40 µl of 100 uM studied compound (or nordihydroguaiaretic acid (NDGA)) solution were added. The formed mixture was shaken and incubated at ambient temperature for 5 min. After incubation, the 40 µl of 0.1 M solution of sodium linoleate was added. After 20 min. incubated at ambient temperature absorption at 234 nm was recovered. The results are calculated by the formula:

\[
\text{LOX inhibiting activity, \%} = \frac{A_{\text{control}} - A_{\text{test compound}}}{A_{\text{control}}} \times 100 \%
\]

Results

The results of molecular docking have shown, that substituted pyrrolo[1,2-a][1,2,4]triazolo[1,5-c]quinazolines (1.1–1.3) have a strong affinity for LOX (Table 1). So, their affinity is much higher, than protocatechuic acid has, a known LOX inhibitor. However, their binding energy is weaker then NDGA, which is used as the pharmacological standard. Binding energy of substituted pyrrolo[1,2-a][1,2,4]triazino[2,3-c]quinazolines (2.1–2.18) approach to NDGA value and compounds 2.6, 2.8, 2.18 binding energy exceed it.

However, high affinity to specified enzymes is not always the main factor for activity revealing. It may be due to the influence of additional factors (lipophilicity, metabolism, etc.), which are described by the «drug-like» criteria (Table 2). Analysis of “drug-like” results indicates, that the test compounds have no deviations from Lipinski’s rules (LogP ≤5; molecular weight ≤500; ability to be a proton acceptor ≤10; ability to be a proton donor ≤5; bond rotation ≤8), as

\[
\begin{align*}
1.1 & \text{ R} = \text{Me}; 1.2 & \text{ R} = \text{Ph}; & 1.3 & \text{ R} = 4-i\text{-PrC}_6\text{H}_4; \\
2.1 & \text{ R} = \text{Me}, R_1 = \text{H}; & 2.2 & \text{ R} = \text{Ph}, R_1 = \text{H}; \\
2.3 & \text{ R} = 4\text{-MePh}, R_1 = \text{H}; & 2.4 & \text{ R} = 4\text{-EtPh}, R_1 = \text{H}; \\
2.5 & \text{ R} = 4\text{-i-PrPh}, R_1 = \text{H}; & 2.6 & \text{ R} = 4\text{-i-BuPh}, R_1 = \text{H}; \\
2.7 & \text{ R} = 4\text{-EtOPh}, R_1 = \text{H}; & 2.8 & \text{ R} = \text{Ph}, R_1 = 12\text{-Me}; \\
2.9 & \text{ R} = 4\text{-MeOC}_6\text{H}_4, R_1 = 10\text{-Me}; & 2.10 & \text{ R} = \text{Ph}, R_1 = 11\text{-F}; \\
2.11 & \text{ R} = \text{Ph}, R_1 = 12\text{-F}; & 2.12 & \text{ R} = \text{Ph}, R_1 = 11,12\text{-F}; \\
2.13 & \text{ R} = \text{4-FC}_6\text{H}_4, R_1 = 11,12\text{-F}; & 2.14 & \text{ R} = \text{Ph}, R_1 = 12\text{-Cl}; \\
2.15 & \text{ R} = 4\text{-MeOC}_6\text{H}_4, R_1 = 12\text{-Cl}; & 2.16 & \text{ R} = \text{Ph}, R_1 = 12\text{-Br}; \\
2.17 & \text{ R} = 4\text{-MeOC}_6\text{H}_4, R_1 = 12\text{-Br}; & 2.18 & \text{ R} = \text{thieryl}-2, R_1 = \text{H}.
\end{align*}
\]
Table 1. The results of molecular docking and pharmacological standards

| Compd.                | R     | R<sub>i</sub> | Affinity (kcal/mol) to LOX (soybean) | The main interactions types between compounds, pharmacological standards and amino acid residues of enzymes |
|-----------------------|-------|--------------|-------------------------------------|----------------------------------------------------------------------------------------------------------|
| Proto-catechuic acid  | –     | –            | -5.0                                | HIS523<sup>a</sup>, LEU565<sup>b</sup>, HIS518<sup>b</sup>, ALA561<sup>b</sup>, LEU773<sup>b</sup>.                          |
| NDGA                  | –     | –            | -6.9                                | ASN566<sup>a</sup>, LYS276<sup>b</sup>, PHE272<sup>b</sup>, VAL26<sup>b</sup>, TYR279<sup>b</sup>.                        |
| 1.1 Me                | –     | –            | -6.8                                | LYS278<sup>b</sup>, ASN566<sup>a</sup>, LEU560<sup>b</sup>, LEU258<sup>b</sup>, ALA263<sup>b</sup>.                          |
| 1.2 Ph                | –     | –            | -6.6                                | THR445<sup>b</sup>, ARG221<sup>b</sup>, GLU573<sup>b</sup>, THR443<sup>b</sup>, ARG580<sup>a</sup>, ARG580<sup>a</sup>. |
| 1.3 4-IPrC<sub>2</sub>H<sub>5</sub> | –     | –            | -6.2                                | LYS278<sup>b</sup>, LYS278<sup>b</sup>, TYR279<sup>b</sup>.                                                              |
| 2.1 CH<sub>3</sub>    | –     | –            | -6.7                                | LYS278<sup>b</sup>, LYS278<sup>b</sup>, ASN566<sup>a</sup>, LEU560<sup>b</sup>, ALA263<sup>b</sup>.                          |
| 2.2 Ph                | –     | –            | -6.7                                | SER281<sup>b</sup>, SER564<sup>a</sup>, ARG252<sup>a</sup>, ARG252<sup>a</sup>.                                             |
| 2.3 4-MeC<sub>6</sub>H<sub>4</sub> | –     | –            | -6.5                                | ARG580<sup>a</sup>, GLU573<sup>b</sup>, GLU573<sup>b</sup>, LEU729<sup>b</sup>, PRO759<sup>b</sup>.                         |
| 2.4 4-EtC<sub>6</sub>H<sub>4</sub> | –     | –            | -6.4                                | THR445<sup>b</sup>, SER444<sup>b</sup>, SER444<sup>b</sup>, LEU729<sup>b</sup>.                                           |
| 2.5 4-IPrC<sub>2</sub>H<sub>5</sub> | –     | –            | -6.9                                | ARG580<sup>a</sup>, GLU573<sup>b</sup>, GLU573<sup>b</sup>, PRO759<sup>b</sup>, LEU729<sup>b</sup>, ARG731<sup>b</sup>. |
| 2.6 4-IBuC<sub>6</sub>H<sub>4</sub> | –     | –            | -7.0                                | TYR275<sup>b</sup>, TYR275<sup>b</sup>, ALA263<sup>b</sup>.                                                              |
| 2.7 4-EIOC<sub>6</sub>H<sub>4</sub> | –     | –            | -6.2                                | ASN556<sup>a</sup>, TYR275<sup>b</sup>, ALA263<sup>b</sup>.                                                             |
| 2.8 Ph                | 12-CH<sub>3</sub> | –          | -7.1                                | ASP255<sup>a</sup>, LYS278<sup>b</sup>, PHE272<sup>b</sup>, ALA263<sup>b</sup>.                                            |
| 2.9 4-MeOC<sub>6</sub>H<sub>4</sub> | 10-Me | –            | -5.9                                | SER281<sup>b</sup>, ARG252<sup>a</sup>, LEU563<sup>b</sup>.                                                             |
| 2.10 Ph               | 11-F  | –            | -6.6                                | LYS278<sup>b</sup>, TYR275<sup>b</sup>, ALA263<sup>b</sup>.                                                            |
| 2.11 Ph               | 12-F  | –            | -6.6                                | LEU563<sup>a</sup>, ARG252<sup>a</sup>, GLY570<sup>b</sup>, ARG252<sup>a</sup>, ARG252<sup>a</sup>, ARG252<sup>a</sup>.   |
| 2.12 Ph               | 11-F, 12-F | –        | -6.8                                | ARG252<sup>a</sup>, GLN252<sup>a</sup>, GLY570<sup>b</sup>, ASN254<sup>a</sup>, GLN282<sup>a</sup>.                     |
| 2.13 4-FPh            | 11-F, 12-F | –        | -6.8                                | PHE264<sup>a</sup>, ASN556<sup>a</sup>, LYS278<sup>b</sup>, ASP255<sup>a</sup>, ALA263<sup>b</sup>.                    |
| 2.14 Ph               | 12-Cl  | –            | -6.5                                | LEU729<sup>b</sup>, PRO759<sup>b</sup>.                                                                           |
| 2.15 4-MeOC<sub>6</sub>H<sub>4</sub> | 12-Cl | –            | -6.6                                | LYS278<sup>b</sup>, PHE272<sup>b</sup>, ALA263<sup>b</sup>.                                                           |
| 2.16 Ph               | 12-Br  | –            | -6.7                                | PHE264<sup>a</sup>, ARG252<sup>a</sup>.                                                                             |
| 2.17 4-MeOC<sub>6</sub>H<sub>4</sub> | 12-Br | –            | -5.6                                | ALA263<sup>a</sup>, ARG252<sup>a</sup>, LYS278<sup>b</sup>.                                                            |
| 2.18 thienyl-2        | –     | –            | -7.0                                | SER281<sup>b</sup>, GLY569<sup>a</sup>, GLY570<sup>b</sup>, GLY570<sup>b</sup>, HIS219<sup>a</sup>, LEU563<sup>b</sup>. |

a: hydrogen; b: hydrophobic; c: other (π-Sulfur); d: halogen; e: electrostatic.

well as the pharmacological standard “NDGA”. This was an important argument for further biological in vitro research of soybean LOX inhibition.

Conducted in vitro study of soybean LOX-inhibition activity (Table 2) showed, that among substituted pyrrolo[1,2-a] [1,2,4]triazolo[1,5-c]quinazolines highest enzyme-inhibiting activity was revealed by compound 1.1 with methyl substituent in position 2 (inhibition on 25.27 %). At the same time among substituted pyrrolo[1,2-a] [1,2,4]triazolo[2,3-c] quinazolines active were compounds 2.4–2.8, 2.13 and 2.18, that exhibited enzyme-inhibiting activity in the range of values 10.03–39.83 %. However, the activity of all obtained compounds was lower comparing to reference inhibitor NDGA.

**Discussion**

Among lipoxygenases (LOX), six isoforms are most known (LOX-5, 15-LOX, 15-LOX-2, 12-LOX, 12R-LOX and eLOX-3), which play an important role in the development of various pathological processes [19]. 5-LOX is a precursor for the synthesis of B<sub>4</sub> leukotrienes (LTB<sub>4</sub>), peptidyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub> or LTE<sub>4</sub>), and lipoxins that cause inflammatory processes. Compounds were analyzed with the use of molecular docking considering the structural similarity of LOX-5 to soybean lipooxygenase LOX (sLOX) type 1b and its role in processes of inflammation. Especially, as 1b sLOX is the molecular biological target, and the high affinity of ligands (the synthesized compounds) to lipoxygenases is one of the desirable characteristics of anti-inflammatory agents.

The visualization of complexes was conducted for evaluation of the effects of structural features of ligands on the level of binding with molecular target. The analysis of the types of main interactions with aminoacid moiety of protein was performed as well (Table 1, Fig. 2). So, visualization of the structure of NDGA with the active site to soybean LOX (Fig. 2) allows to establish, that it has hydrogen and hydrophobic interactions with the amino-acid residues:
ASN56 (2.07Å), LYS278 (3.71Å), PHE272 (5.21Å), VAL26 (5.27Å), TYR275 (5.11Å). Compound 2.18 has the highest affinity to the soybean LOX target, among the investigated ones. Visualization of this structure with the soybean LOX active site (Fig. 2) showed, that it is characterized by four hydrogen bonds with the amino acid residues: SER281 (3.16Å), GLY569 (2.69Å), GLY570 (3.40Å), GLY570 (3.45Å), hydrophobic interaction with LEU563 (5.46Å) and quite strong π-Sulfur interaction with HIS219 (4.82Å). So, an important aspect of compounds’ high affinity to soybean LOX is the presence of several hydrogen bonds, hydrophobic interactions, donor-acceptor interactions due to sulfur and fluorine lone electron pairs (Table 1).

The comparative analysis of “drug-like” results and soybean LOX inhibition has shown, that the lipoxigenase activity depends on molecule lipophilicity and availability of acceptors and donors of hydrogen bond. The last statement agreed with the data of molecular docking (Table 1, Fig. 2). So, substituted pyrrolo[1,2-a][1,2,4]triazolo[3,4-a]quinazolines with a fluorine atom (2.13) and a 2-thienyl fragment (2.18) in the molecule inhibit lipoxigenase by 36.33 % and 39.83 % respectively. The increase of lipophilicity promotes higher ability to inhibit soybean LOX (Table 2), which is speaking above derivatives 2.1–2.18. Thus, compounds 2.4–2.6 inhibit soybean LOX by 20.53–20.81 %. Whereas, for substituted pyrrolo[1,2-a][1,2,4]triazolo[1,5-c]quinazolines which have considerably smaller molecular weight inverse relation is observed. So, compound 1.1 with the indicator of lipophilicity 0.03 inhibits soybean LOX by 25.27 %. Increase in lipophilicity (compound 1.2) leads to activity decrease, and in case of compound 1.3 – its total loss.

Conclusions

The research of the substituted pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines inhibition ability of soybean LOX as one of possible mechanisms of their activity is proved and conducted. It is shown, that their lipoxigenase activity depends on lipophilicity and is defined by availability in the molecule of donor-acceptor fragments in the molecule, that are capable to form hydrogen and other types of interaction. The specified results are the strong argument for their further study as promising anti-inflammatory agents. It is planned the in vivo study of anti-inflammatory activity and toxic effects for the most active compounds.

Table 2. The value of the “drug-like” criteria and soybean LOX inhibition

| Compnd. | Log P | Molecular polar surface area, Å | Number of non-hydrogens | Molecular volume, Å³ | Number of hydrogen bond acceptors (groups N and O) | Number of hydrogen bond donors (groups NH and OH) | Number of rotatable bonds | Soybean LOX inhibition, (%) |
|---------|-------|-------------------------------|-------------------------|---------------------|-----------------------------------------------|-----------------------------------------------|--------------------------|-----------------------------|
| NDGA    | 3.48  | 80.91                         | 22                      | 392.37              | 4                                             | 5                                             | 67.19                    |
| 1.1     | 0.13  | 88.33                         | 21                      | 284.27              | 7                                             | 1                                             | 25.27                    |
| 1.2     | 2.16  | 88.33                         | 26                      | 346.35              | 7                                             | 1                                             | 3.56                     |
| 1.3     | 3.67  | 88.33                         | 29                      | 384.43              | 7                                             | 1                                             | 0.00                     |
| 2.1     | 0.02  | 105.40                        | 23                      | 312.29              | 8                                             | 1                                             | 0.00                     |
| 2.2     | 1.47  | 105.40                        | 28                      | 374.36              | 8                                             | 1                                             | 2.00                     |
| 2.3     | 1.92  | 105.40                        | 29                      | 388.38              | 8                                             | 1                                             | 3.78                     |
| 2.4     | 2.38  | 105.40                        | 30                      | 402.41              | 8                                             | 1                                             | 20.81                    |
| 2.5     | 2.98  | 105.40                        | 31                      | 416.44              | 8                                             | 1                                             | 20.63                    |
| 2.6     | 3.17  | 105.40                        | 32                      | 430.46              | 8                                             | 1                                             | 20.53                    |
| 2.7     | 1.90  | 114.63                        | 31                      | 418.41              | 9                                             | 1                                             | 15.42                    |
| 2.8     | 1.89  | 105.40                        | 29                      | 388.38              | 8                                             | 1                                             | 10.03                    |
| 2.9     | 1.92  | 114.63                        | 31                      | 418.41              | 9                                             | 1                                             | 3.00                     |
| 2.10    | 1.63  | 105.40                        | 29                      | 392.35              | 8                                             | 1                                             | 2.41                     |
| 2.11    | 1.61  | 105.40                        | 29                      | 392.35              | 8                                             | 1                                             | 2.00                     |
| 2.12    | 1.70  | 105.40                        | 30                      | 410.34              | 8                                             | 1                                             | 2.00                     |
| 2.13    | 1.86  | 105.40                        | 31                      | 428.33              | 8                                             | 1                                             | 36.33                    |
| 2.14    | 2.12  | 105.40                        | 29                      | 408.80              | 8                                             | 1                                             | 9.46                     |
| 2.15    | 2.18  | 114.63                        | 31                      | 438.83              | 9                                             | 1                                             | 1.79                     |
| 2.16    | 2.25  | 105.40                        | 29                      | 453.25              | 8                                             | 1                                             | 2.37                     |
| 2.17    | 2.31  | 114.63                        | 31                      | 483.28              | 9                                             | 1                                             | 0.00                     |
| 2.18    | 1.25  | 105.40                        | 27                      | 380.38              | 8                                             | 1                                             | 39.83                    |
Substituted pyrrolo[1,2-a][1,2,4]triazolo-(triazino-)[c]quinazolines – a promising class of lipooxygenase inhibitors

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Fig. 2. Visualization of affinity according to the docking – a: NDGA with soybean LOX; b: compound 2.5 with soybean LOX; c: compound 2.8 with soybean LOX; d: compound 2.18 with soybean LOX PLA2.
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