Journal of Research in Pharmacy

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Pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their derivatives as 15-LOX inhibitors: Design, *in vitro* studies and QSAR-analysis

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Received: 17 March 2021 / Revised: 22 June 2021/ Accepted: 28 June 2021

ABSTRACT Present manuscript is devoted to the search of 15-LOX inhibiting agents among pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines using *in silico* and *in vitro* methods. Molecular docking method was used for calculation of affinity and evaluation of protein ligand interactions features. Colorimetric *in vitro* assay was used for estimation of LOX-15-inhibiting activity of synthesized compounds. QSAR-analysis was used for formation of the models applicable for prediction of properties of not yet synthesized inhibitors of 15-LOX. It was shown that some of the studied compounds reveal LOX-inhibiting activity that was comparable or higher than activity of the reference compound – Nordihydroguaiaretic acid. The conducted molecular docking study allowed to elucidate the affinity towards the enzyme. The visualization of docking study results allowed to establish and to discuss the features of ligand – 15-LOX interactions. The correlations between structure, LOX-inhibiting activity, calculated affinity and lipophilicity were considered as well. The performed QSAR-analysis resulted the five parametric linear model that like docking study results are valuable for further search of promising 15-LOX-inhibitors. Pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines were identified as 15-LOX inhibitors. The reliable correlation between 15-LOX inhibiting activity of the synthesized compounds, their lipophilicity and calculated affinity was not observed. Visualization of molecular docking results and formed QSAR-models may be used as theoretical basis for novel LOX-inhibitors design.

KEYWORDS: Pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines; 15-lipoxygenase; molecular docking; inhibitors; SAR-, QSAR-analysis.

1. INTRODUCTION

Lipoxygenase (LOX) is a family of structurally related non-heme iron-containing enzymes that attach molecular oxygen to polyunsaturated fatty acids with *cis*-1,4-pentadiene [1, 2]. Mammalian lipoxygenases are divided into 5-, 12- and 15-LOX. That is depending on the position in which oxygen connects to arachidonic acid. The primary products of this reaction are 5-, 12- and 15-hydroperoxyeicosatetraenoic acids (HPETE), which are reduced to the corresponding hydroxy eicosatetraenoic acids (HETE) or converted to various other types of eicosanoids, such as leukotrienes. The latter, in turn, are mediators of inflammatory processes, anaphylactic reactions. Also, they regulate smooth muscle contraction, immune responses, which are important for the development of certain types of pathologies [3]. Until now, most researches were focused on the role of 5-LOX enzymes, which has ultimately led to the development of drug under trade name Zyflo®. This drug is an approved 5-LOX inhibitor and is intended to relieve chronic asthma symptoms [4-6].

Recent studies have also found an important role of 12/15-LOX in diseases that are accompanied by inflammatory processes [7-10]. In particular, ALOX12 gene polymorphism has been shown to be associated with cancer proliferation and metastasis, neurological disorders, atherosclerosis, epilepsy, hypertension, obesity and bone mineral density [7-9]. 15-LOX also initiates and/or promotes the progress of atherosclerosis, and its metabolites induce apoptosis in colon cancer cells [8]. In addition, the cytotoxic activity of 12/15-LOX is increased in neurons and endothelial cells during stroke [9]. Given the significant role of 12/15-LOX in the

How to cite this article: Krasovska N, Stavytskyi V, Nosulenko I. Kholodniak S, Antypenko O, Voskoboinik O, Kovalenko S. Pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their derivatives as 15-LOX inhibitors: Design, *in vitro* studies and QSAR-analysis. J Res Pharm. 2021; 25(5): 540-548.

https://dx.doi.org/10.29228/jrp.44

ISSN: 2630-6344

progression of many diseases, the discovery of their inhibitors may lead to the development of new therapeutic agents for the treatment of cancer, neurological, cardiovascular and other diseases [10-13]. Moreover, the search for 15-LOX inhibitors is conducted among various classes of natural and synthetic compounds, namely catechols, flavonoids, imidazole and benzimidazole derivatives, indole, purine, indolizine, pyridine benzothiazine, etc. [12-14]. Most of these compounds in their structure contain alkyl, cycloalkyl moieties, saturated heterocycles and functional groups that provide high lipophilicity of the molecule.

Previous studies have shown that substituted pyrrolo[1,2-*a*][1,2,4]triazolo(triazino-)[*c*]quinazolines are inhibitors of soybean LOX [15, 16]. It is interesting to study the effect of the original tetracyclic systems on the corresponding enzyme to determine promising areas of biological (antitumor, neuro- and immunotropic) activity. That is especially true, considering that known 15-LOX inhibitors haven't been used in medical practice [7, 9].

Therefore, the aim of this work is to find 15-LOX inhibitors among pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines, to discuss the structure-activity relationship and to select promising compounds for further directions of biological research.

2. RESULTS

The general methods for the synthesis of the target pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their substituted (**2.1-2.11**, **3.1-3.3**, **4.1-4.25**) are presented in Figure 1. The structures of the studied compounds are presented in Figure 1 and Table 1. The synthesis of compounds **2.1-2.9**, **3.1-3.3** and **4.1-4.20** was carried out by the interaction of substituted 2-(azolyl-(azinyl-)anilines (**1.1-1.23**) with ketocarboxylic acids and their esters (4-oxopentanoic, 4-oxo-4-phenylbutanoic, 2-oxopentanedioic, 4-oxoheptanedioic) in acetic acid [15-18]. Amides **2.10**, **2.11**, **4.21-4.25** were synthesized *in situ* by aminolysis of *N*-acylimidazolides [15].

At the first stage of research, the combinatorial library of pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their substituted derivatives was analyzed using molecular docking and drug-like criteria. According to the results of calculations, most of the studied compounds had a high affinity to 15-LOX (Table 1) and met the criteria of "drug-like". Compounds whose affinity was significantly lower than the NDGA reference inhibitor were excluded from further study.

In vitro biological studies have shown, that pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their substituted (**2**, **3**, **4**) in most cases were able to inhibit 15-LOX (Figure 2, 3). Thus, among 2-R_2-4*a*-R_1-5,6-dihydropyrrolo[1,2-*a*][1,2,4]triazolo[1,5-*c*]quinazoline-7(4*aH*)-ones (**2**) high enzyme-inhibiting activity (58.1-68.19%) was characteristic for compounds **2.3**, **2.5**, **2.6** and **2.9**, which exceeded the standard NDGA inhibitor (57.43%, Figure 2). With respect to substituted pyrrolo[1,2-*a*]tetrazolo[1,5-*c*]quinazolines (**3**), the high 15-LOX inhibitory activity was characteristic only for compound **3.1** (64.86%, Figure 2).

3. DISCUSSION

SAR analysis showed that the level of 15-LOX-inhibiting activity was determined by both the lipophilicity of the heterocycles themselves and the polarity of the substituents (Table 1, Figure 2, 3). Thus, for a series of substituted pyrrolo[1,2-*a*][1,2,4]triazolo[1,5-*c*]quinazolines (**2**) the most favorable for the manifestation of activity was the combination in the structure of polar and non-polar substituents at the 2nd and 4a positions: phenyl and carboxyethyl (**2.3**), carboxyl and 4-*i*-propylphenyl (**2.5**), carboxyl and carboxyethyl (**2.6**), ethylcarboxyl and carboxyethyl (**2.9**) groups. Whereas amides **2.10** and **2.11** with a non-polar substituent at the 2nd position (4-FC_6H_4) showed low ability to inhibit 15-LOX (Figure 2). SAR analysis of substituted pyrrolo[1,2-*a*]tetrazolo[1,5-*c*]quinazolines (**3**) series showed that the introduction of polar substituents (Me (**3.1**) or $(CH_2)_2COOH$ (**3.3**)) at the 4a position resulted a higher inhibitory effect compared to compound **3.2** with a non-polar phenyl substituent (Figure 2). Substituted pyrrolo[1,2-*a*][1,2,4]triazino[2,3-*c*]quinazolines (**4**), according to the structure-activity relationship, show high activity in the presence of both polar (compounds **4.10**) and non-polar (**4.3**, **4.19**, **4.20**) substituents or substituents with moderate polarity (**4.8**, **4.13**, **4.15-4.17**). Thus, the presence of 4-*i*-propylphenyl and phenyl (**4.3**), methyl and ethylcarboxyl (**4.10**), phenyl and carboxyethyl (**4.13**) heterocycle at the 3rd and 5a positions was optimal for the activity manifestation. It should also be noted, that the level of activity was positively affected by the presence of two fluorine atoms at the 11th and 12th positions (Figure 3). Amides **4.21-4.25**, as well as the above compounds **2.10** and **2.11**, showed little ability to inhibit 15-LOX (Figure 3). SAR analysis showed that the ability to inhibit 15-LOX was determined by the nature of the heterocyclic moiety, the structure of the substituents and their relative position. Thus, carboxyl and carboxyethyl groups, mainly in the angular position of the heterocycle, turned out to be substitutes that were "critical" for the enzyme-inhibiting activity manifestation.

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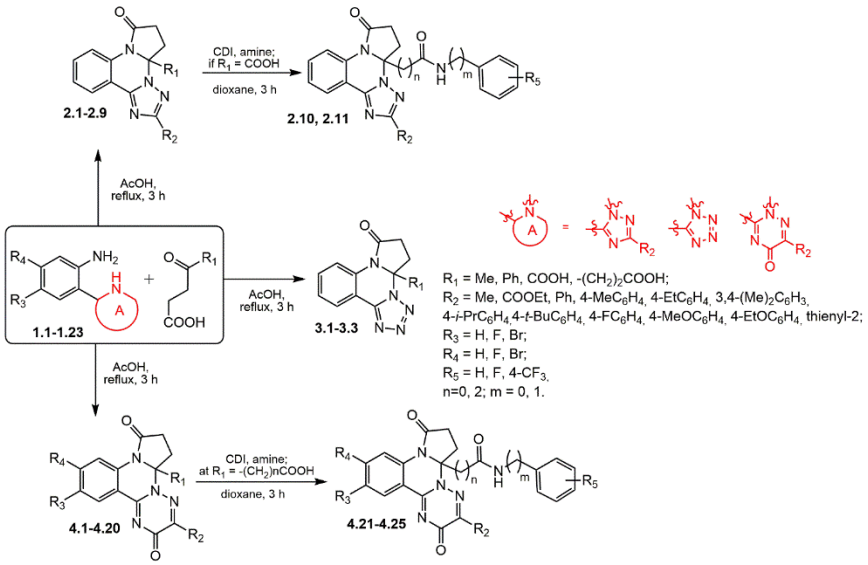

Figure 1. Approaches to the synthesis of pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their substituted derivatives.

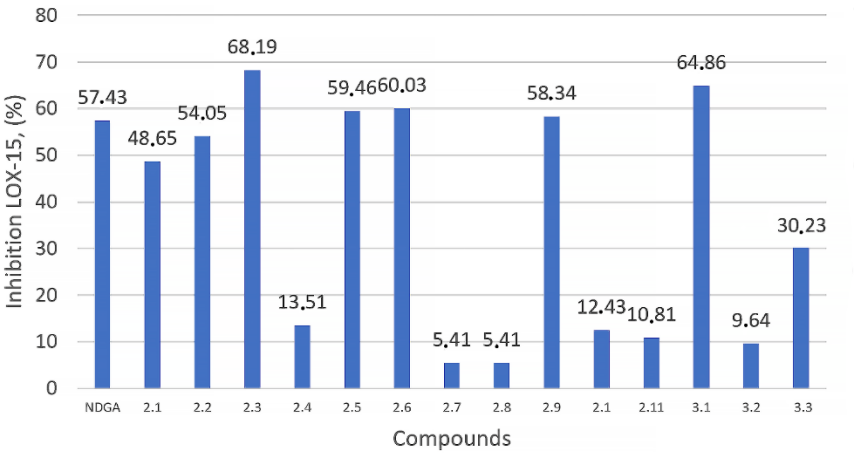

Figure 2. 15-LOX inhibition (%) of pyrrolo[1,2-*a*][1,2,4]triazolo-(tetrazolo-)[1,5-*c*]quinazolines and their substituted.

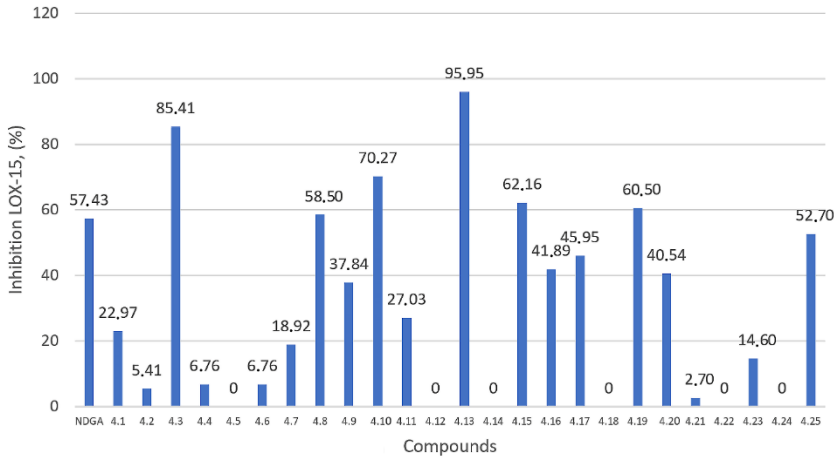

Figure 3. 15-LOX-inhibiting activity (%) of pyrrolo[1,2-*a*][1,2,4]triazino[2,3-*c*]quinazolines and their substituted.

https://dx.doi.org/10.29228/jrp.44
J Res Pharm 2021; 25(5): 540-548

Table 1. The structure and molecular docking results of the studied compounds.

Compounds	R_1	R_2	R_3	R_4	R_5	n	m	Affinity (kcal/mol) to 15-LOX (4NRE)	logP
NDGA[a]	–	–	–	–	–	–	–	-8.6	3.48
2.1	Me	4-*i*-PrC_6H_4	–	–	–	–	–	-9.7	4.80
2.2	Ph	4-*i*-PrC_6H_4	–	–	–	–	–	-9.2	6.02
2.3	Ph	COOEt	–	–	–	–	–	-8.5	2.70
2.4	COOH	Me	–	–	–	–	–	-7.9	0.13
2.5	COOH	4-*i*-PrC_6H_4	–	–	–	–	–	-8.7	3.67
2.6	COOH	COOEt	–	–	–	–	–	-9.7	0.90
2.7	$(CH_2)_2$COOH	Me	–	–	–	–	–	-8.2	0.67
2.8	$(CH_2)_2$COOH	Ph	–	–	–	–	–	-10.1	2.70
2.9	$(CH_2)_2$COOH	COOEt	–	–	–	–	–	-8.5	0.35
2.10	–	4-FC_6H_4	–	–	4-MeO	0	1	-9.7	3.94
2.11	–	4-FC_6H_4	–	–	4-MeO	1	2	-12.2	3.64
3.1	Me	–	–	–	–	–	–	-9.0	1.03
3.2	Ph	–	–	–	–	–	–	-10.3	2.25
3.3	$(CH_2)_2$COOH	–	–	–	–	–	–	-7.5	0.44
4.1	Me	Me	H	H	–	–	–	-8.2	1.15
4.2	Me	4-FC_6H_4	H	F	–	–	–	-9.0	2.90
4.3	Ph	4-*i*-PrC_6H_4	H	H	–	–	–	-9.0	5.33
4.4	COOH	Me	H	H	–	–	–	-8.6	0.02
4.5	COOH	Ph	F	H	–	–	–	-8.4	1.61
4.6	COOH	4-*t*-BuC_6H_4	H	H	–	–	–	-8.9	3.17
4.7	COOH	4-FC_6H_4	F	H	–	–	–	-9.8	1.77
4.8	COOH	C_6H_4OMe-*p*	Br	H	–	–	–	-9.2	2.31
4.9	COOH	thienyl-2	H	H	–	–	–	-9.2	1.25
4.10	$(CH_2)_2$COOH	Me	H	H	–	–	–	-8.5	0.67
4.11	$(CH_2)_2$COOH	Ph	F	H	–	–	–	-8.7	2.15
4.12	$(CH_2)_2$COOH	Ph	H	F	–	–	–	-9.0	2.31
4.13	$(CH_2)_2$COOH	Ph	F	F	–	–	–	-9.6	2.24
4.14	$(CH_2)_2$COOH	4-FC_6H_4	F	H	–	–	–	-9.7	2.17
4.15	$(CH_2)_2$COOH	4-FC_6H_4	H	F	–	–	–	-9.1	2.31
4.16	$(CH_2)_2$COOH	4-FC_6H_4	F	F	–	–	–	-9.7	2.40
4.17	$(CH_2)_2$COOEt	Me	H	H	–	–	–	-8.1	1.24
4.18	$(CH_2)_2$COOEt	Ph	F	H	–	–	–	-9.3	2.83
4.19	$(CH_2)_2$COOEt	Ph	F	F	–	–	–	-8.8	2.92
4.20	$(CH_2)_2$COOEt	4-FC_6H_4	F	F	–	–	–	-9.8	3.09
4.21	–	Me	H	H	H	2	0	-11.4	2.12
4.22	–	Me	H	H	4-F	2	1	-11.6	1.98
4.23	–	Me	H	H	4-CF_3	2	1	-10.6	2.71
4.24	–	Ph	H	H	4-F	0	1	-8.8	2.89
4.25	–	Ph	H	H	4-F	2	1	-9.2	3.73

[a] Nordihydroguaiaretic acid.

The highest 15-LOX-inhibiting activity among substituted pyrrolo[1,2-*a*][1,2,4]triazino[2,3-*c*]quinazolines (**4**) was characteristic for compounds **4.3**, **4.8**, **4.10**, **4.13**, **4.15** and **4.19**. These compounds inhibit the enzyme by 58.5-95.0%, that is higher than the standard NDGA inhibitor (Figure 3).

An attempt to find a reliable relationship between the values of LOX-inhibitory activity, calculated affinity and lipophilicity was unsuccessful. Given the above, the results of molecular docking were used exclusively to detail the main types of interactions of the most active inhibitors with amino acid residues of the enzyme to establish promising directions for the construction of agents with 15-LOX-inhibitory activity.

Analysis of visualization data showed that an effective inhibitor **4.10** (3-(3-methyl-2,8-dioxo-7,8-dihydro-2*H*-pyrrolo[1,2-*a*][1,2,4]triazino[2,3-*c*]quinazoline-5*a*(6*H*)-yl) propanoic acid) has a similar arrangement in the 15-LOX pocket as NDGA (Figure 4). This arrangement in the hydrophilic region and the docking of compound **4.10** with the active center of 15-LOX was provided by four conventional hydrogen bond with ASP:625 (1.94 Å), TRP:109 (2.76 Å), ARG:404 (2.30 Å), HIS:627 (4.77 Å) and salt bridge with ARG: 407 (2.30 Å), HIS:627 (2.30 Å). It is important that the carboxyethyl moiety of the 5a position, the CO group of the 2nd position and N-4 of the triazine ring take part in the formation of these bonds (Figure 4c). The docking of the most potent inhibitor **4.13**, in which the methyl group of the 3rd position was replaced by a phenyl substituent and additionally introduced to the 11th and 12th positions fluorine, showed a slightly different position-inverted orientation, compared to **4.10** (Figure 4d). It is obvious, that the introduction of hydrophobic

substituents resulted the interaction with the corresponding part of the enzyme, namely the appearance of π-π interactions between two fluorines with ASP:602 (3.34 Å and 3.92 Å) and the phenyl group with LEU:415 (4.57 Å), LEU:420 (4.60 Å), ALA:606 (4.16 Å) and LEU:610 (5.33 Å). In this case, an additional hydrogen bond between carboxyethyl fragment and GLY:189 (3.01 Å) of enzyme was observed. It is important, that the change in orientation for the docking also corresponded to the observed increase in inhibition for **4.13** compared to **4.10** (Figure 3). Compound **4.3** has a similar arrangement in the hydrophobic part of the enzyme, with phenyl and 4-*i*-propylphenyl substituents at the 5a and 3rd positions of the heterocycle, respectively (Figure 4b). These substituents were found to have a significant number of π-π interactions with amino acids, as well as two hydrogen bonds of CO groups of the 2nd and 8th positions with ALA:606 (2.69 Å) and ARG:429 (2.90 Å). This compound also exhibited high 15-LOX inhibitory activity.

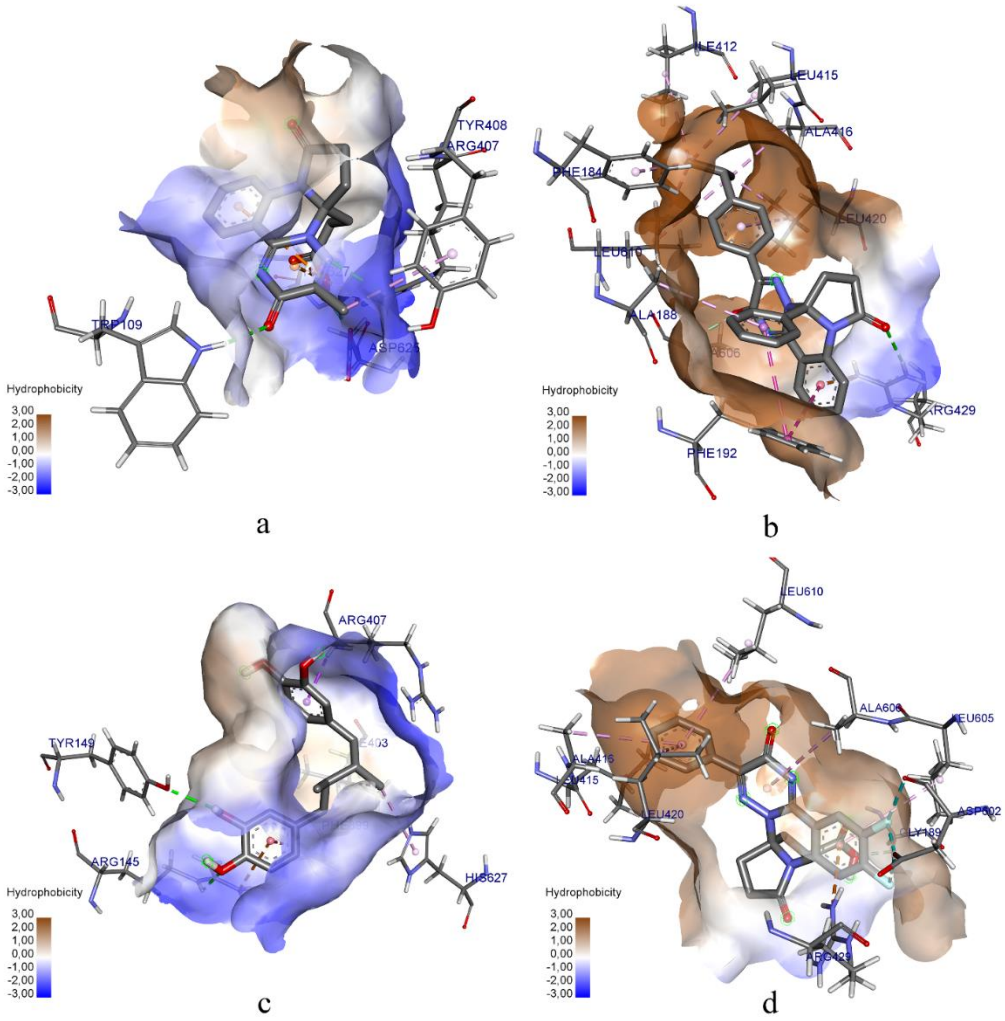

Figure 4. Visualization of affinity according to the docking a) NDGA with 15-LOX, b) compound **4.3** with 15-LOX; c) compound **4.10** with 15-LOX; d) compound **4.13** with 15-LOX.

Visualization of molecular docking results showed that the presence of both polar and nonpolar binding sites in the ligand molecules with the appropriate relative position is critical for the interaction with the molecular target. It should be noted that the data obtained during the molecular docking visualization fully correspond with the results of SAR analysis.

3.1 QSAR analysis

QSAR-analysis was performed to improve approaches of the development of highly effective 15-LOX inhibitors. As a result of the research, a five parametric linear QSAR model was obtained, for which the following statistical parameters were determined for equation 1: square correlation coefficient (R^2), adjusted squared correlation coefficient (R^2adj), Fisher's criterion (F), standard error (S), root mean square error, (RMSE), square correlation coefficient for leave-one-out cross-validation (Q^2LOO):

$$\text{Inhibition 15-LOX (\%)} = -0.551(\pm0.2381) \times \mathbf{G(N..F)} + 1218.5949(\pm759.3291) \times \mathbf{R1v+} + 44.6509(\pm15.7633) \times$$

$$\mathbf{C\text{-}003} + 66.4643(\pm17.7294) \times \mathbf{B09[O\text{-}F]} + 25.6504\ (\pm9.5018) \times \mathbf{F08[O\text{-}O]} + -65.6379(\pm48.3197)$$

$$R^2 = 0.8171,\ R^2adj = 0.7833,\ F = 24.1291,\ S = 13.2807,\ RMSE = 32.7656,\ Q^2LOO = 0.7510. \qquad \text{(Eq. 1)}$$

The descriptors included in this model belong to the following groups: 2D Atom Pairs (B09[O-F], F08[O-O]), 3D Atom Pairs (G(N..F)), Atom-centered fragments (C-003) and GETAWAY descriptors (R1v+). According to the presented equation, increasing of the descriptor G(N..F) leads to increase of 15-LOX inhibition. Conversely, increasing of the descriptor R1v+ value leads to decrease of 15-LOX inhibition. The values of the descriptors C-003, B09[O-F], F08[O-O] for the substances used to build the QSAR equation are 0, 1 or 2. Accordingly, compounds with 0 or 1 for these three descriptors have the best percentage of 15-LOX inhibition. The research results are presented in the graph (Figure 5). It also should be noted, that for the test set of structures there is a good coincidence of the experimental and predicted values.

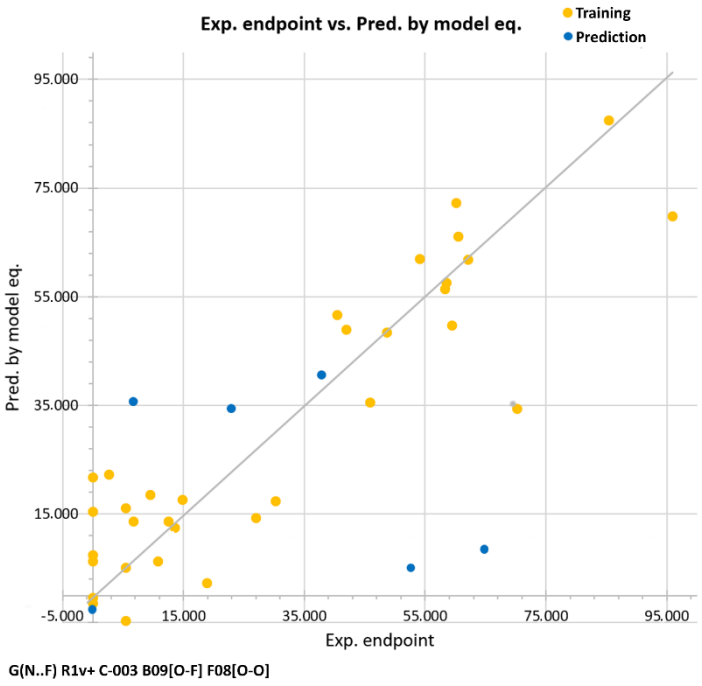

Figure 5. QSAR model validation.

4. CONCLUSION

Substituted pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines were identified as 15-LOX inhibitors and interesting objects for further studies aimed to the search of pharmacologically valued compounds. The presence of carboxy-group or carboxyethyl-group in the molecules of studied compounds was essential for 15-LOX inhibiting activity manifestation. The reliable correlation between 15-LOX inhibiting activity of the synthesized compounds, their lipophilicity and calculated affinity was not found. However, visualization of molecular docking results allowed to establish nature of the ligand – enzyme interaction and may be used as theoretical basis for novel LOX-inhibitors design.

5. MATERIALS AND METHODS

Pyrrolo[1,2-*a*]azolo-(azino-)[*c*]quinazolines and their substituted were selected to study the ability to inhibit 15-LOX. The synthesis of the tested compounds was performed as described previously [15-18]. The structures of the analyzed compounds are presented in Figure 1 and Table 1.

5.1. Molecular docking

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 human 15-LOX with a substrate mimic (PDB ID - 4NRE) [19]. The choice of biological targets was due to the literature about the mechanism of anti-inflammatory drugs activity [20]. The following software was used for molecular docking procedure and its visualization: MarvinSketch 20.20.0 [21], AutoDockTools-1.5.6, Vina [22], Discovery Studio v19.1.0.18287 [23]. Grid box was set as following: center_x = 1.722, center_y = -48.472, center_z = -23.583, size_x = 25, size_y = 25, size_z = 25 for 15-LOX (4NRE). More detailed procedure of protein and ligand preparation can be found in our previous work [17].

5.2 LOX inhibition activity

The evaluation of 15-LOX – inhibiting activity. The study of 15-LOX – inhibiting activity was conducted using Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical, Item No. 760700). In the cuvette (standard 96 cuvette plate) 90 µl of 15-LOX solution and 10 µl of 1.1 mM solution of studied compound in DMSO were mixed. The formed mixture was incubated at room temperature for 5 minutes. Then 10 µl of 1 mM solution of potassium linoleate was added and sample was placed on a shaker for 10 minutes. Then 100 µl of chromogen was added and sample was shaken for 5 minutes. After that absorbance at 495 nm was measured using a plate reader Sirio-S (Seac, Italy). NDGA was used as a reference compound. The LOX-inhibiting activity was calculated using equation 2.

$$LOX - inhibiting\ activity\ (\%) = \frac{(A\ control - A\ compound)}{A\ control} * 100\% \qquad \text{Eq. 2}$$

5.3 Lipinski's rule of five.

Drug-like characteristics were evaluated and optimized using an electronic resource [24].

5.4 QSAR and statistical analysis.

Optimized files, that were obtained in the stage of ligand preparation for docking were also used for calculations of descriptors for QSAR. The following software was used: Dragon [25, 26], MOPAC2012 [27]. The combination of the genetic algorithm and multiple linear regression analysis technique was applied to obtain the best descriptors among 1439 calculated overall, and to construct QSAR models using the QSARINS 2.2.4 [28]. The division into training and prediction sets was performed at a ratio of 80 to 20 percent relatively. Namely, 33 compounds were used for training set and 6 for prediction set. More detailed procedure of QSAR modeling was written at our previous study [29].

Acknowledgements: The work was performed with the technical support of Enamine Ltd (Kyiv, Ukraine). The work was carried out on the budgetary theme of the Ministry of Health of Ukraine «Purposeful search for anti-inflammatory agents among condensed and spiro-condensed quinazoline derivatives» (problem «Pharmacy», № state registration 0118U004370; period of study 2018-2020).

Author contributions: Concept – S.Ko.; Design – O.V.; Supervision – S.Ko.; Resources – N.K., O.A., Materials – V.S., S.K.; Data Collection and/or Processing – V.S., I.N., N.K.; Analysis and/or Interpretation – O.A., V.S.; Literature Search – N.K.; Writing – N.K.; Critical Reviews – N.K., V.S., I.N., S.K., O.A., O.V., S.Ko.

Conflict of interest statement: The authors declare that they have no conflict of interest.

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