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Evaluation of *in vitro* activity and SAR study of the novel hetarylamino-3-aryl-1*H*-indazole derivatives as inhibitors of protein kinase CK2

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Aim. To identify novel protein kinase CK2 inhibitors among the 5-hetarylamino-1H-indazoles. **Methods.** Biochemical testing was carried out with the aid of γ-^{32}P ATP *in vitro* kinase assay. Molecular docking via the Autodock 4.2.6 program package was executed, rescoring of docking results was performed using DrugScore scoring function. **Results.** Among the 17 studied 5-amino-3-arylindazole derivatives 11 inhibitors of protein kinase CK2 with IC$_{50}$ in nanomolar range were identified. The most active compound has IC$_{50}$ = 2 nM. SAR study and additional molecular modeling of these compounds allowed us to select prospective substituents for construction of novel compounds with improved activity and physicochemical properties. **Conclusions.** As a result of this work 11 nanomolar protein kinase CK2 inhibitors were developed and the binding modes of these compounds with the ATP-acceptor site were proposed using molecular docking methods. The physicochemical properties and SAR of substituents of studied compounds were analyzed and 6 novel compounds were designed for further development as protein kinase CK2 inhibitors. Summarizing, 5-heterylamino-1H-indazoles are a good basis for further CK2 inhibitors development.

K e y w o r d s: protein kinase CK2 inhibitor, *in vitro* kinase assay, molecular docking, indazole, pyrazolo[3,4-d]pyrimidine.

Introduction

Serine/threonine protein kinase CK2 was discovered among the first ones in 1954 [1]. Despite a long history of CK2 investigations, a number of its functions are not completely determined. At the same time CK2 has unique properties that differ it from the majority of protein kinases [2]. In contrast to the common protein kinases CK2 cannot be turned off by the mechanisms such as phosphorylation or dephosphorylation, second messenger binding and reversible association with regulatory subunits, but can be only "modulated" by means of the mechanisms, which are still not completely understood [3]. Unlike the most of other oncogenic protein kinases, the malignant behavior of CK2 is not caused by deregulation of the kinase activity at a genetic level. CK2 has the unique ability to use GTP as well as ATP as the phosphate donors [4]. The ATP binding pocket of CK2 is smaller than that of the other protein kinases, that is why the ATP competitive inhibitors with higher selectivity for CK2 could be developed. CK2 has high pleiotropicity (CK2 phosphorylates more than 500 proteins) [5] – most of the substrates have been found to be transcriptional factors (60), effectors of DNA/RNA structure (50), signaling proteins (more than 80), a limited number of metabolic enzymes and even over 40 viruses use CK2 to phosphorylate essential proteins in their life cycle [6]. Experimental results clearly showed that CK2 is involved in regulation of transcription, translation, cell proliferation, survival and apoptosis [7]. Overexpression and overactivity of CK2 are associated with the development of neurodegenerative, inflammatory, cardio-vascular diseases, virus infections, and all the cancers having been examined [8, 9]. Therefore, small-molecular inhibitors of CK2 would be important compounds for the development of clinical agents.

In the previous work we have described chemical synthesis of the novel 5-hetarylamino-1*H*-indazole derivatives as prominent inhibitors of human protein kinase CK2 [10]. Here we are representing measurements of their *in vitro* activity and SAR study as well as molecular docking simulation.

Materials and Methods

Molecular docking

Flexible docking was conducted using the program Autodock 4.2.6 [11]. The 3D structure of human protein kinase CK2 was obtained from the Brookhaven Protein Data Bank (PDB ID: 3NSZ and 4UBA) [12, 13].

Water molecules, ions, and ligand were removed from the PDB file of the receptor. Ligands were prepared by Vega ZZ (command line) [14] and MGL Tools 1.5.6 [11]. To carry out a calculation with the aid of the Autodock program, the incoming formats of receptor and ligands data were converted into PDBQT-format with Vega ZZ in the AUTODOCK force field. This format contains the coordinates of the atoms and partial charges. Hydrogen atoms were removed from nonpolar adenine parameters: translation step – 2 Å, quaternion step – 50, torsion step – 50. Torsional degrees of freedom and coefficient were 2 and 0.274, respectively. Cluster tolerance – 2 Å. External grid energy – 1,000, max initial energy – 0,

max number of retries – 10,000. Number of individuals in the population – 300, the maximum number of energy evaluations – 850,000, the maximum number of generations – 27,000, number of top individuals, which survived to the next generation – 1, rate of gene mutation – 0.02, rate of crossover – 0.8, mode of crossover – arithmetic. Alpha parameter of Cauchy distribution was 0, Beta parameter Cauchy distribution – 1. The number of iterations of the Lamarckian genetic algorithm was 50 for each ligand.

Visual analysis of the best-scored complexes was performed using Discovery Studio Visualizer 4.0 [Discovery Studio Visualizer: http://accelrys.com/].

LogP calculation

LogP values were calculated using Marvin Sketch 19.12 (https://www.chemaxon.com).

Biological evaluation

Compounds were tested using *in vitro* kinase assay [15]. Each test was carried out in triplicate in 30 μL reaction volume, which contains 6 μg of peptide substrate RRRDDDSDDD (New England Biolabs); 10 units of recombinant human CK2 holoenzyme (New England Biolabs); 50 μM ATP and 0.05–0.1 μCi γ-labeled ^{32}P ATP; CK2 buffer (20 mM Tris-HCl, pH 7.5; 50 mM KCl; 10 mM $MgCl_2$) and inhibitor in varying concentrations. The incubation time was 20 min at 30 °C. The reaction was stopped by adding an equal volume of 10 % orthophosphoric acid and the reaction mixture was loaded onto 20-mm discs of phosphocellulose paper (Whatman). Disks were washed three times with 1 % orthophosphoric acid solution, air-dried at room temperature

and counted by the Cherenkov method in a beta-counter (LKB). As a negative control, an equal volume of dimethyl sulfoxide (DMSO) was added to the reaction mixture. Inhibition percentage was calculated as the ratio of substrate-incorporated radioactivity in the presence of inhibitor to the radioactivity incorporated in control reactions, i.e., in the absence of inhibitor. Serial dilutions of inhibitor stock solution were used to determine its IC_{50} concentration. The IC_{50} values represent means of triplicate experiments with SEM never exceeding 15 %.

Results and Discussion

Earlier we have found new promising CK2 inhibitors with IC_{50} values in a range 0.007–0.1 μM in *in vitro* assay with radiolabeled ATP [15] among the 4-substituted quinazoline derivatives of 5-amino-3-arylindazoles 1–3 [20]. We had left 3-(3,4-dichlorophenyl) substituent of the most active compound 1 unmodified and have focused on the variation of heterocyclic residue at the 5-position of indazole moiety [10].

A complex of compound 1 with a CK2 ATP-binding site is shown in Figure 1. An indazole heterocyclic moiety is involved in the hydrophobic interactions with amino acid residues Val66, Ile95, Phe113, Val116, Met163 and Ile174 in the adenine-binding region. Also, the indazole moiety forms a hydrogen bond with Val116 amino acid residue of the CK2 hinge region. The 3,4-dichlorophenyl substituent is directed towards the exit of ATP-binding pocket. It forms hydrophobic interaction with Leu45 and Met163. The (2-trifluoromethyl) quinazoline moiety lies inside the hydrophobic pocket I. It has hydrophobic interaction with
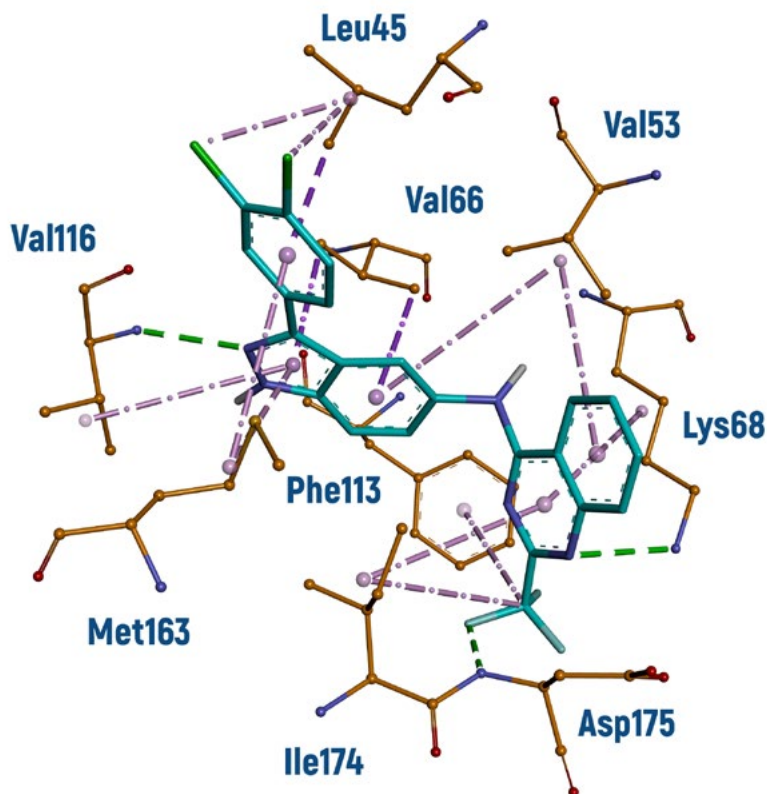
Fig. 1. Molecular modelling of the complex of compound **1** with human protein kinase CK2. Hydrogen bonds are indicated as green dashed lines and hydrophobic interactions – as magenta dot-dashed lines.

Phe113, Val53, Lys68 and Ile174. Additionally, 2-trifluoromethylquinazoline forms hydrogen bonds with Lys68 and Asp175.

Chemical structures, IC_{50}, calculated LogP and LipE (CLogP and CLipE) values of the 14 new heterocyclic derivatives of substituted 5-amino-3-(3,4-dichlorophenyl)indazole are represented in the Table 1. In general, 5-amino-3-aryl indazole derivatives show high inhibitory activity toward CK2 but mostly have had extremely high lipophilicity (LogP>5). In this case, to estimate the efficiency of studied compounds as CK2 inhibitors, it was used a special composite parameter that linked inhibitory activity and lipophilicity parameter. This parameter is lipophilic efficiency or LipE [17].

LipE is the most "correct" [18] composed parameter, and it is determined using the following formula: $CLipE = pIC_{50} - ClogP$. The structure and IC_{50} values of the most effective studied inhibitors are shown in Table 2.

According to *in vitro* tests, 11 compounds (including **1–3** having been synthesized earlier) have submicromolar IC_{50} values ranging from 0.002 μM (**4**) to 0.8 μM (**6**). It turns out that negative steric influence of the bulky substituents at 2nd position of quinazoline heterocycle on the compounds inhibitory activity is not as crucial as that of the substituents at the 6th position. Small methyl group at the 6th quinazoline's position of compound **8** causes decrease of IC_{50} by two orders of magnitude

Table 1. **Chemical structure, IC$_{50}$, CLogP and CLipE of 5-amino-3-arylindazole derivatives.**
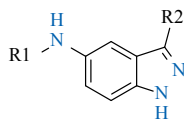
Com-pound	R$_1$	R$_2$	IC$_{50}$, μM	CLogP	CLipE	Com-pound	R$_1$	R$_2$	IC$_{50}$, μM	CLogP	CLipE
1			0.007	7.5	0.5	**9**			8	6.7	-1.6
2			0.016	6.2	2.3	**10**			0.5	7.1	-0.8
3			0.1	6	1	**11**			>30	6.1	<-1.6
4			0.002	6	2.7	**12**			>30	7	<-2.5
5			0.015	7.6	0.2	**13**			3.5	5.9	-0.4
6			0.8	8.3	-2.2	**14**			0.33	5.4	1.1
7			0.18	8.9	-2.1	**15**			0.8	4.6	1.5
8			0.5	6.5	-0.2	**16**			0.004	5.4	3
						17			2.5	4.9	0.7

compared to the **4** with unsubstituted quinazoline ring. This decrease is even more than the decrease caused by the bulky 2-chlorophenyl residue of compound **7**. On the other hand, 2nd position substituents having restricted dimensions cause almost no negative effect on compound activity – the IC$_{50}$ of **1** and **5** with 2-trifluoromethyl and 2-isopropyl group is not far from the IC$_{50}$ of **4**. The 4-phenylpyrimidine moiety of compound **9** in comparison to the quinazoline ring system of **4** probably have no enough hydrophobic interactions in ATP-binding site of CK-2, as a result, the IC$_{50}$ of **9** is more than one thousand times higher than the IC$_{50}$ of **4**. However, the most surprisingly poor results in this testing were shown by quinoline derivatives **11** and **12** as well as thienopyrimidine thieno[2,3-d]pyrimidine derivative **13**. **11** has almost the same geometry as **4** but shows no activity towards CK2. This might be a result of a combination of the lower basicity of quinoline heterocycle compared to quinazoline one and lower water solubility of **11**. Difficulties with water solubility also may be a reason of significant variation in IC$_{50}$ of more polar and hydrophilic pyrazolo[3,4-d] pyrimidine derivatives **14–17** which, according to IC$_{50}$ compound **16** in 0.002 µM, can be active enough.

Regarging the lipophilicity – the analysis of FDA approved protein kinase inhibitors showed that average LipE value is 4.99 with a range from 2 (vandetanib) to 8.5 (tofacitinib) [19]. Our best compounds **2**, **4** and **16** have values of CLipE 2.3, 2.7 and 3, respectively (Table 1). It is significantly lower than CLipE values of known promising CK2 inhibitors BFO13, FLC26, FNH79 and CX-4945–4.94, 3.85, 4.48 and 4.84, respectively. Examined

5-amino-3-arylindazole derivatives have strong inhibitory potential toward CK2, but their physicochemical properties should be thoroughly optimized. In this case, the new 5-amino-3-arylindazole derivatives with the same binding mode and activities but with better CLipE values should be constructed.
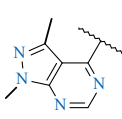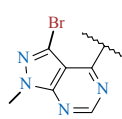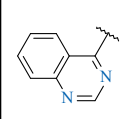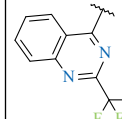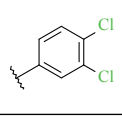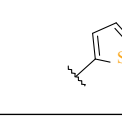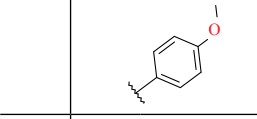
The CLogP values of the most effective substituents R$_1$ and R$_2$ were calculated to determine the direction of optimization (see Figure 2).

R$_1$ substituent [3-(trifluoromethyl)-1-quinazolyl] and R$_2$ substituent 3,4-dichlorophenyl had the highest CLogP values – 2.6 and 3.6, respectively. These substituents were not taken into account for the design of the new 5-amino-3-arylindazoles.

Based on the SAR study, the results of molecular docking and calculated LogP values of each substituent, six new 5-amino-3-arylindazole derivatives were designed as possible effective human protein kinase CK2 inhibitors.

All new six compounds comply with Lipinski's rules. The structure and CLogP values of these compounds are represented in Figure 3.

The protein part of a crystal structure of the protein kinase CK2 complex with inhibi-

R$_1$			
CLogP = 0.1	CLogP = 1.2	CLogP = 1.6	CLogP = 3.6
R$_2$			
CLogP = 2.6	CLogP = 1.2		CLogP = 1.4

Fig. 2. Structure and CLogP values of R$_1$ and R$_2$ substituents
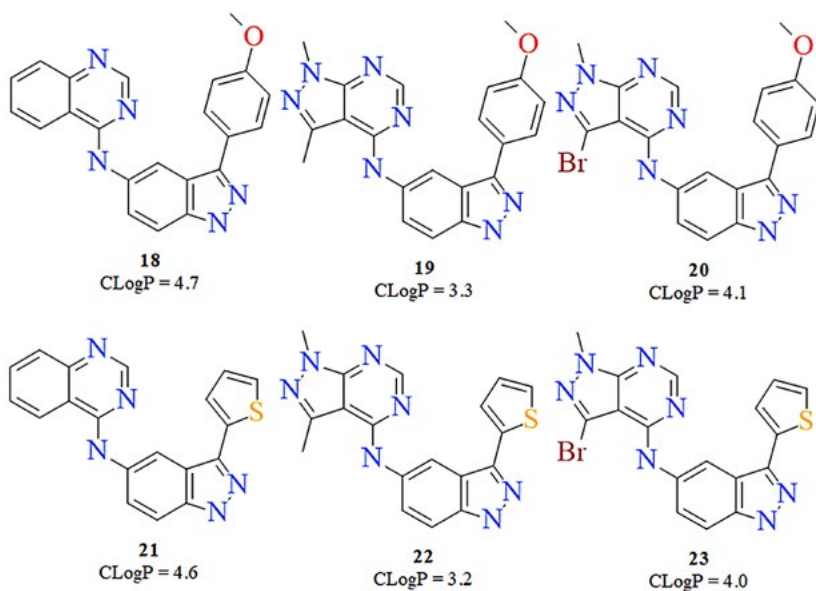
**Fig. 3.** Structure and CLogP values of new designed 5-amino-3-arylindazole derivatives

Table 2. **Drug score values, molecular weight and the ratio of drug score value to molecular weight and numbers of heavy atoms of compounds.**

Compound	CLogP	Number of heavy atoms	MW	DrugScore	DrugScore/ Number of heavy atoms	DrugScore/MW
FLC26	3.9 (CLipE = 3.85)	23	440	-96 (IC$_{50}$ = 0.009 μM)	-4.2	-0.22
BFO13	3.5 (CLipE = 4.94)	22	362	-86.2 (IC$_{50}$ = 0.0036 μM)	-3.9	-0.24
CX-4945	4.20 (CLipE = 4.5)	25	350	-102 (IC$_{50}$ = 0.002 μM)	-4.1	-0.29
1	7.5 (CLipE = 0.5)	32	571	-115.1 (IC$_{50}$ = 0.01 μM)	-3.6	-0.2
2	6.2 (CLipE = 2.3)	32	435	-93.7 (IC$_{50}$ = 0.003 μM)	-2.9	-0.22
4	6 (CLipE = 2.7)	28	495	-98.5 (IC$_{50}$ = 0.002 μM)	-3.5	-0.2
16	5.4 (CLipE = 3)	29	578	-92.2 (IC$_{50}$ = 0.004 μM)	-3.2	-0.16
18	4.7	28	456	-105.6	-3.8	-0.23
19	3.3	29	385	-105.3	-3.6	-0.27
20	4.1	29	539	-116.6	-4	-0.22
21	4.6	25	424	-79.4	-3.2	-0.19
22	3.2	26	442	-103.1	-4	-0.23
23	4	26	507	-96.3	-3.7	-0.19
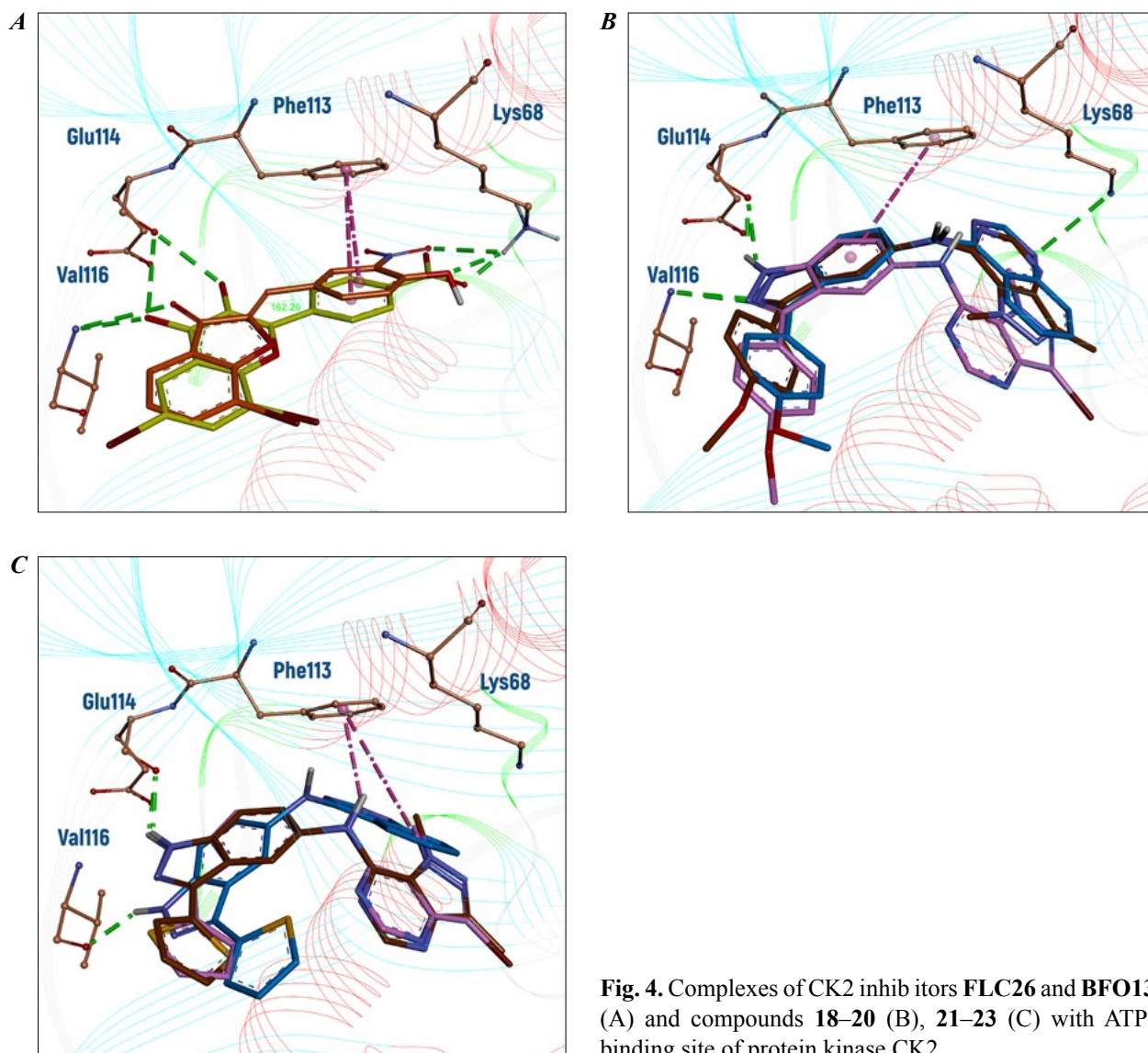
Fig. 4. Complexes of CK2 inhibitors **FLC26** and **BFO13** (A) and compounds **18–20** (B), **21–23** (C) with ATP-binding site of protein kinase CK2.

tor FLC26 (PDBID: 4UBA) was chosen as the target for docking studies and following comparison of docking scores of compounds **18–23** with known CK2 inhibitors **FLC26**, **BFO13**, **CX-4945**, **1**, **2**, **4** and **16**. Score values were calculated using a knowledge-based scoring function Drug Score [20] (see Table 2).

To avoid the influence of different molecular weights on the results of scoring function, the drug score values were approximated to molecular weights and numbers of heavy atoms of compounds. It is required for more detailed differentiation of its possible inhibitory activity.

Molecular docking showed that binding modes of compounds **18**–**23** (see Figure 4) are similar to the binding mode of FLC26 proved by crystallography. R_1 substituents are oriented to the hydrophobic pocket I and R_2 – to the exit of the ATP-binding pocket. Indazole heterocycle is located in an adenine-binding pocket and forms hydrogen bonds with hinge amino acid residues.

Among known inhibitors the compounds **1**, **CX-4945**, compound **4**, and **FLC26** had the best Drug Score scoring function values (−115.1, −102, −98.8, −96 respectively), **FLC26**, **CX-4945**, **BFO13** and compounds **1** – DrugScore/Number of heavy atoms coefficient (−4.2, −4.1, −3.9, −3.6, respectively) and **CX-4945**, **BFO13**, **FLC26** and compounds **2** – DrugScore/Molecular weight coefficient (0.29, −0.24, −0.22, −0.22, respectively). Among designed compounds, compounds **20**, **18** and **19** had the best Drug Score scoring function values (-116.6, 105.6 and 105.3, respectively), compounds **20**, **22** and **18** – DrugScore/ Number of heavy atoms coefficient (-4, −4 and −3.8), compounds **19**, **22** and **18** – DrugScore/Molecular weight coefficient (−0.27, −0.23 and –0.23). Taking into account CLogP, Drug Score scoring function, DrugScore/Number of heavy atoms and DrugScore/Molecular weight values, the best compounds for further development are **22** and **19**. We anticipate that designed 5-amino-3-arylindazole derivatives will have lipophilic efficiency comparable with the best known CK2 inhibitors.

Conclusions

This research has demonstrated that 5-hetaryl-amino-3-arylindazoles have good potential as CK2 inhibitors. Eleven compounds with sub-micromolar inhibitory activity were identified (compounds **4**, **16** and **1** have shown IC_{50} values 2, 4 and 7 nM, respectively). The binding mode of these compounds with CK2 was proposed using molecular docking methods. It demonstrates hydrogen bonds with amino-acids of the hinge region and numerous hydrophobic interactions which provide high activity of 5-amino-3-arylindazoles. SAR and docking studies allowed us to suggest lipophilic efficiency optimization methods and to design compounds with good probability to inhibit protein kinase CK2 for further development.

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Визначення активності in vitro та аналіз співвідношення структура-активність нових гетероциклічних похідних 5-аміно-3-арил-1*H*-індазолу як інгібіторів протеїнкінази CK2

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Мета. Пошук нових інгібіторів протеїнкінази CK2 серед 5-гетариламіно-1*H*-індазолів. **Методи.** Біохімічне тестування проводили *in vitro* за допомогою радіоактивно міченого γ-^{32}Р АТФ. Для молекулярного докінгу застосовували програмний пакет Autodock 4.2, рескоринг результатів докінгу виконували з використанням фукції скорингу DrugScore. **Результати.** Серед 17-ти похідних 5-аміно-3-ариліндазолу що було досліджено було знайдено 11 інгібіторів протеїнкінази CK2 з IC_{50} в наномолярному діапазоні. Найбільш активна сполука мала $IC_{50} = 2$ нМ. Дослідження співвідношення структура-активність та додатковий молекулярний докінг цих сполук дозволив обрати перспективні модифікації для одержання нових сполук з кращими фізико-хімічними характеристиками. **Висновки.** В результаті проведеної роботи було отримано 11 нових наномолярних інгібіторів протеїнкінази CK2. Спираючись на результати молекулярного докінгу було запропоновано модель зв'язування цих молекул з АТФ-зв'язувальним сайтом кінази. На підставі аналізу фізико-хімічних характеристик та співвідношення структура-активність для замісників індазольного ядра досліджених сполук було запропоновано структури 6-ти нових похідних для подальшої розробки інгібіторів протеїнкінази CK2. Загалом 5-гетариламіно-1*H*-

індазоли виявились вдалою основою для побудови нових інгібіторів протеїнкінази CK2.

К л ю ч о в і   с л о в а: інгібітор протеїнкінази CK2, тестування кіназної активності *in vitro*, молекулярний докінг, індазол, піразоло[3,4-d]піримідин.

Определение активности in vitro и анализ соотношения структура-активность новых гетероциклических производных 5-амино-3-арил-1*H*-индазола в качестве ингибиторов протеинкиназы CK2

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Цель. Поиск новых ингибиторов протеинкиназы CK2 среди 5-гетериламино-1*H*-индазолов. **Методы.** Биохимическое тестирование проводили *in vitro* при помощи радиоактивно меченого γ-^{32}P АТФ. Для молекулярного докинга применяли программный пакет Autodock 4.2, рескоринг результатов докинга проводили с использованием фукции скоринга DrugScore. **Результаты.** Среди 17-ти исследованных производных 5-амино-3-арилиндазола было обнаружено 11 ингибиторов протеинкиназы CK2 с IC$_{50}$ в наномолярном диапазоне. У наиболее активного соединения IC$_{50}$ = 2 нМ. Исследование соотношения структура-активность и дополнительный молекулярный докинг этих соединений позволил выбрать перспективные модификации для получения новых соединений с лучшими физико-химическими характеристиками. **Выводы.** В результате проведённой работы было получено 11 новых наномолярных ингибиторов протеинкиназы CK2. Опираясь на результаты молекулярного докинга была предложена модель связывания этих молекул с АТФ-связывающим сайтом киназы. На основе анализа физико-химических характеристик и соотношения структура-активность для заместителей индазольного ядра исследованных соединений были предложены структуры 6-ти новых производных для дальнейшей разработки ингибиторов протеинкиназы CK2. Вцелом 5-гетериламино-1*H*-индазолы оказались удачной основой для синтеза новых ингибиторов протеинкиназы CK2.

К л ю ч е в ы е   с л о в а: ингибитор протеинкиназы CK2, тестирование киназной активности *in vitro*, молекулярний докинг, индазол, пиразоло[3,4-d]пиримидин.