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Identification of novel protein kinase CK2 inhibitors among indazole derivatives

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Aim. To synthesize the novel purine bioisosteres — indazole derivatives and evaluate inhibitory activity of these compounds towards CK2 in the *in vitro* system. **Methods.** Chemical synthesis, ^{1}H and ^{13}C NMR spectroscopy, LC-MS method, determination of residual enzyme activity using ATP consumption tests with a luciferase Kinase-Glo® luminescent kinase assay. **Results.** Known synthetic methods of indazole chemistry were originally applied to the synthesis of 3-aryl-indazole-7-carboxylic acids. Conditions of cross-coupling of 3-bromo-indazole derivatives with arylboronic acids were substantially improved. 3-aryl-indazole 5- and 7-carboxylic acids have shown IC_{50} in a range 3.1–6.5 µM in luciferase luminescent kinase assay. **Conclusions.** The synthesis of 3-aryl-indazole-7-carboxylic acids has been developed. Novel inhibitors of the protein kinase CK2 among indazole derivatives have been identified among the 3-aryl-indazole 5- and 7-carboxylic acids. It has been shown the crucial impact of carboxyl group on the inhibitory activity of studied compounds.

K e y w o r d s: Indazole, chemical synthesis, luciferase luminescent kinase assay, protein kinase inhibitor, protein kinase CK2.

Introduction

Casein kinase 2 (CK2) is a serine/threonine protein kinase usually consisting of two catalytic subunits (α) and/or (α') and two regulatory (β) subunits. This enzyme stands out among the most protein kinases by its high pleiotropicity. CK2 is involved in various biological processes — it has hundreds of substrates for phosphorylation [1] and can use both ATP and GTP as a phosphate source [2]. For a long time, this ubiquity was considered as an obstacle to use CK2 in the drug development until discovering the fact that cancer cells are much more dependent on CK2 than normal cells [3]. It has turned CK2 into an important player in overcoming drug resistance, albeit with some limitations [4]. Abnormally high

levels of CK2 occur during the various neuro-degenerative, inflammatory, cardio-vascular diseases, virus infections and all the cancers having been examined [5, 6]. This increased CK2 level having no genetic background often reduces the treatment efficiency, so until the reasons are not clarified the only possible way of attenuating CK2 activity is therapeutic use of the potent and selective CK2 inhibitors [7].

A number of CK2 inhibitors belonging to different chemical classes have been published [8–11] and one of them — silmitasertib (CX-4945) — is undergoing clinical trials now supporting the notion that CK2 is recognized as a druggable protein kinase. Therefore, the discovery of small-molecular CK2 inhibitors is a significant area of medicinal chemistry research.

Earlier, we found a number of chemical classes of CK2 inhibitors using the computer-aided drug design approach. Here we are representing the CK2 inhibitors identified among the indazole derivatives. Purine bioisosteres are a common target in search for new binders of the ATP-binding site as protein kinase inhibitors [7]. The indazole carboxylic acid derivatives drew our attention for two reasons: the first — an indazole ring is a bioisostere of an adenine ring, the second — a carboxyl group is an essential part of several indazole-containing CK2 inhibitors, such as 4,5,6,7-tetrabromo-1H-indazole and 3,4,5,6,7-pentabromo-1H-indazole derivatives [8], benzo[g]indazole derivatives [9] and a series of 4,6-disubstituted pyrazine derivatives including inhibitor CC04820. The silmitasertib (CX-4945) molecule also contains a carboxyl group involved in electrostatic interaction with the Lys68 of CK2 ATP-binding site. Thus, this work aims at the synthesis of indazole-carboxylic acids and related derivatives and evaluation of their inhibitory activity against CK2 in the *in vitro* system.

Materials and Methods

Starting materials and solvents were purchased from commercial suppliers and used without further purification. NMR spectra were recorded on a Varian VXR 400 instrument at 400 MHz for ^{1}H NMR and 101 MHz for ^{13}C NMR spectra. Chemical shifts are described as parts per million (δ) downfield from an internal standard of tetramethylsilane, and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), td (triple doublet), t (triplet), q (quartet), quintet (quintet) or m (overlapped multiplets). HPLC-MS analysis was performed using the Agilent 1100 LC/MSD SL separations module and Mass Quad G1956B mass detector with electrospray ionization (+ve or −ve ion mode as indicated), and HPLC was performed using a Zorbax SB-C18, Rapid Resolution HT cartridge, 4.6 mm × 30 mm, 1.8 µm i.d. column (Agilent P/N 823975-902) at a temperature of 40 °C with gradient elution of 0–100 % CH_3CN (with 1 mL/L HCOOH)/ H_2O (with 1 mL/L HCOOH) at a flow rate of 3 mL/min and a run time of 2.8 min. Compounds were detected at 215 nm using a diode array G1315B detector. All tested compounds gave $\geq$ 95 % purity as determined by these methods.

3-Phenyl-1H-indazole-7-carboxylic acid hydrochloride (**8**). *Procedure A*: The mixture of 1.00 g (2.71 mmol) of 1-tert-butyl-7-ethyl 3-bromoindazole-1,7-dicarboxylate **6**, 0.35 g (2.88 mmol) of phenylboronic acid, 0.50 g (4.72 mmol) of sodium carbonate, 0.03 g (0.136 mmol) of palladium diacetate, 0.10 g

(0.38 mmol) of triphenylphosphine, 15 ml of ethanol and 5 ml of water was refluxed under stirring in Ar atmosphere for 16 hours. After evaporation of solvents under reduced pressure the residue was diluted with 20 ml of dichloromethane and 10 ml of water, dark insoluble precipitate was filtered off and two layers were separated. Chromatographic purification of the residue after evaporation of the dichloromethane layer mixed with the insoluble precipitate gave 0.11g (15 %) of ethyl 3-phenyl-1H-indazole-7-carboxylate **7**; ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.16 (br s, 1H), 8.28 (d, J = 7.7 Hz, 1H), 8.04 (d, J = 7.2 Hz, 1H), 7.96 (d, J = 7.6 Hz, 2H), 7.50 (d, J = 7.3 Hz, 2H), 7.39 (t, J = 7.8 Hz, 1H), 7.28 (t, J = 7.6 Hz, 1H), 4.50 (q, J = 6.7 Hz, 2H), 1.45 (t, J = 6.9 Hz, 3H). Acidification of the aqueous layer gave 0.32 g (43 %) of precipitate of 3-phenyl-1H-indazole-7-carboxylic acid hydrochloride **8** as light grey powder m.p. 158°C ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.01 (br s, 1H), 8.23 (d, J = 8.1 Hz, 1H), 8.00 (d, J = 7.2 Hz, 1H), 7.94 (d, J = 7.4 Hz, 2H), 7.47 (t, J = 7.3 Hz, 2H), 7.36 (t, J = 7.3 Hz, 1H), 7.25 (t, J = 7.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.1, 144.4, 140.2, 133.9, 129.1, 129.0, 128.0, 127.5, 126.3, 122.4, 120.6, 114.5. LC-MS (ESI) t_r = 0.886, m/z 239.0 ([M+H]⁺).

Procedure B: The mixture of 1.00 g (2.71 mmol) of 1-tert-butyl-7-ethyl 3-bromoindazole-1,7-dicarboxylate **6**, 0.35 g (2.88 mmol) of phenylboronic acid, 0.50 g (4.72 mmol) of sodium carbonate, 0.03 g (0.136 mmol) of palladium diacetate, 0.075 g (0.135 mmol) of dppf, 15 ml of ethanol and 5 ml of water was refluxed under stirring in Ar atmosphere for 16 hours. Then 0.45 g (8 mmol) of potassium hydroxide were added and the

mixture was refluxed for one more hour. After evaporation of solvents under reduced pressure the residue was diluted with 20 ml of water, treated with charcoal and filtered. Acidification of the mother liquid gave 0.62 g (83 %) of 3-phenyl-1H-indazole-7-carboxylic acid hydrochloride **8** as a light grey precipitate.

Procedure C: The mixture of 1.00 g (2.71 mmol) of 1-tert-butyl-7-ethyl 3-bromoindazole-1,7-dicarboxylate **6**, 0.35 g (2.88 mmol) of phenylboronic acid, 0.80 g (7.14 mmol) of potassium *tret*-butoxide, 0.035 g (0.133 mmol) of Pd(dba)$_2$, 0.085 g (0.137 mmol) of BINAP, 10 ml of toluene was refluxed under stirring in Ar atmosphere for 16 hours. Solvent was evaporated under reduced pressure, the residue was diluted with 20 ml of water, then 0.45 g (8 mmol) of potassium hydroxide were added and the mixture was refluxed for one hour. After discoloration with charcoal and filtration the mother liquid was acidified giving 0.13 g (48 %) of 3-bromo-1H-*indazole*-7-carboxylic acid hydrochloride **15** as a light brown precipitate. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.48 (s, 1H), 8.06 (d, J = 7.2 Hz, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H).

3-Pyridin-3-yl-1H-indazole-5-carbonitrile (**9**). As beige powder m.p. 108°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.78 (s, 1H), 9.16 (s, 1H), 8.57 (d, J = 6.8 Hz, 2H), 8.34 (d, J = 7.9 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.61 (d, J = 8.6 Hz, 1H), 7.47 (dd, J = 7.2, 4.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.3, 148.1, 142.8, 142.4, 134.5, 129.0, 128.4, 127.5, 124.0, 120.3, 119.7, 112.5, 104.5. LC-MS (ESI) t_r = 0.663, m/z 221.0 ([M+H]+).

3-pyridin-3-yl-1H-indazole-5-carboxylic acid dihydrochloride (**10**). As beige powder m.p. 123°C. ^{1}H NMR (400 MHz, DMSO-d_6)

δ 13.60 (br s, 1H), 12.50 (br s, 1H), 9.14 (br s, 1H), 8.62 (s, 2H), 8.30 (d, J = 7.0 Hz, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.4 Hz, 1H), 7.53 (br s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.7, 149.1, 147.9, 143.5, 142.4, 134.3, 129.4, 127.1, 124.2, 123.4, 120.2, 110.8. LC-MS (ESI) t_r = 0.565, m/z 240.0 ([M+H]+).

3-(4-Methoxy-phenyl)-1H-indazole-5-carboxylic acid (**11**). As white powder m.p. 246ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.94 (br s, 2H), 8.61 (s, 1H), 7.93 (d, J = 8.6 Hz, 1H), 7.87 (d, J = 8.2 Hz, 2H), 7.54 (d, J = 8.7 Hz, 1H), 7.05 (d, J = 8.2 Hz, 2H), 3.83 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.8, 159.5, 145.1, 143.6, 128.4, 126.8, 126.2, 124.0, 123.8, 120.2, 114.5, 110.3, 55.3. LC-MS (ESI) t_r = 0.794, m/z 269.0 ([M+H]+).

3-(3-Fluoro-phenyl)-1H-indazole-5-carboxylic acid (**12**). As beige powder m.p. 86 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.44 (br s, 1H), 12.67 (br s, 1H), 8.63 (s, 1H), 7.95 (d, J = 8.2 Hz, 1H), 7.79 (d, J = 7.7 Hz, 1H), 7.67 (d, J = 10.0 Hz, 1H), 7.58 (d, J = 8.9 Hz, 1H), 7.53 (t, J = 6.4 Hz, 1H), 7.14 (t d, J = 8.24, 1.93 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.9, 164.2 (d, J = 245.1 Hz), 144.0, 143.8, 136.1 (d, J = 7.5f Hz), 131.0 (d, J = 7.8 Hz), 127.1, 124.5, 123.7, 123.1, 120.2, 115.0 (d, J = 20.0 Hz), 113.9 (d, J = 23.0 Hz), 110.8. LC-MS (ESI) t_r = 0.821, m/z 257.0 ([M+H]+).

3-(4-Fluoro-phenyl)-1H-indazol-5-ylamine (**13**). As light brown powder m.p. 98ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.72 (br s, 1H), 7.92 (br s, 2H), 7.28 (d, J = 8.7 Hz, 1H), 7.20 (t, J = 8.3 Hz, 2H), 7.13 (s, 1H), 6.84 (d, J = 8.3 Hz, 1H), 4.60 (br s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.8 (d, J = 244.3 Hz), 142.3, 140.6, 136.8, 131.5, 128.2 (d, J = 6.7 Hz), 121.2, 118.4, 115.5 (d, J = 21.2 Hz), 111.0, 101.6. LC-MS (ESI) t_r = 0.613, m/z 228.0 ([M+H]+).

3-(4-Fluoro-phenyl)-1H-indazole-5-carbonitrile hydrochloride (**14**). As white powder m.p. 135ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.74 (br s, 1H), 8.53 (s, 1H), 7.94 (d, J = 8.2 Hz, 1H), 7.92 (d, J = 1.1 Hz, 1H), 7.70 (d, J = 8.7 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 7.48 (t, J = 7.8 Hz, 1H), 7.37 (d, J = 7.1 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 142.7 (d, J = 244.3 Hz), 134.9, 134.3, 130.6, 128.1, 127.3, 126.8, 125.7, 120.0, 119.5, 112.4, 104.3. LC-MS (ESI) t_r = 1.002, m/z 238.0 ([M+H]+).

The enzyme activity of protein kinase CK2 was determined using ATP consumption tests. Each reaction mixture contained 3 μl of buffer solution (20 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl$_2$), 3 μl of peptide substrate RRRDDDSDDD (2 mg/ml), 0.02 μl (10 units) of recombinant human CK2α catalytic subunit obtained by us [10], 13 μl of H$_2$O and 1 μl of inhibitor 300 μM stock solution in dimethyl-sulfoxide (DMSO).

The reaction was started with addition of 10 μl of 3^x ATP solution (30 μM ATP, 30 mM MgCl$_2$, 15 mM MOPS pH 7.2) to each micro-plate well. The microplate was put on "TERMIT" incubator and incubated at 31 °C for 20 min.

The reaction was stopped by adding 30 μl of luciferase mix (Kinase-Glo® Luminescent Kinase Assay, Promega) to each microplate well. Luciferase suppresses ATP consumption by kinase and starts the luciferase reaction. The luminescence was counted with Victor reader using Dual Luciferase assay protocol. The heating option on "Victor" was switched on 21 °C.

Results

In order to provide enough hydrophobic interactions to keep binding in the pocket of ATP-binding site we have focused on a synthesis of indazole-carboxylic acids carrying the aryl-moiety at the 3-rd position of the indazole ring.

The synthesis of 3-aryl-indazole-5-carboxylic acid derivatives as the inhibitors of various protein kinases was reported earlier [11–14]. At the same time only few instances of synthesis of indazole-7-carboxylic acids and no examples of synthesis of 3-aryl-substituted indazole-7-carboxylic acids have been reported. Here we are representing a method of synthesis of 3-phenyl-indazole-7-carboxylic acid that can be easily applied to the synthesis of any 3-aryl-substituted indazole-7-carboxylic acid. We have used synthetic methods known for indazole derivatives with other substitution. The indazole ring assembly starts from the 3-methyl-anthranilic acid **1** ester (Fig. 1). Bromination or iodination of the indazole-7-carboxylic acid ester **2** leads to the 3-halogeno-substituted intermediates **3** [15] that could possibly be converted into the 3-aryl-indazole-7-carboxylic acid **4** esters under the Suzuki-Miyaura cross-coupling conditions. This convenient synthetic route may involve a wide range of aryl- and heteroaryl-boronic

acids, however, it requires additional steps of protection/deprotection of NH on the indazole ring. The halogen on the 3-rd position of the indazole ring shows no reactivity unless it's NH is covered with a protective group, moreover, NH-arylation with the arylboronic acids takes place under the same conditions as cross-coupling.

Moving along this synthetic route (Scheme 1) we have obtained the ethyl indazole-7-carboxylate **2** in moderate yield by the nitrosylation of the ethyl 3-methylanthranilate **1** with *tret*-butylnitrite in acetic acid. After the bromination and the N-Boc protection, this intermediate was used in the Suzuki-Miyaura cross-coupling with phenylboronic acid. Several attempts were unsuccessful. The cross-coupling product was not formed when BINAP-Pd(0), tBuOK in toluene were used. However, the ethyl 3-phenyl-indazole-7-carboxylate appeared in the reaction mixture when the reaction ran in aqueous ethanol media in presence of $Pd(PPh_3)_4$ and Na_2CO_3 as a base. The replacement of $Pd(PPh_3)_4$ with $Pd(OAc)_2$/dppf enhanced the yield of the process. Noteworthy, under the conditions of mentioned reaction the indazole moiety completely lost its N-Boc protection. Additionally, a significant amount — up to half the ethyl es-
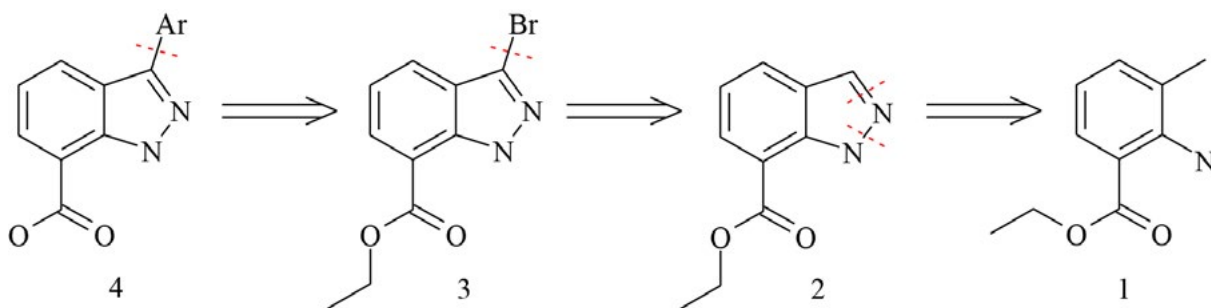

Fig. 1. Preparation of the 3-aryl-indazole-7-carboxylic acids from an indazole-7-carboxylic acid ester.
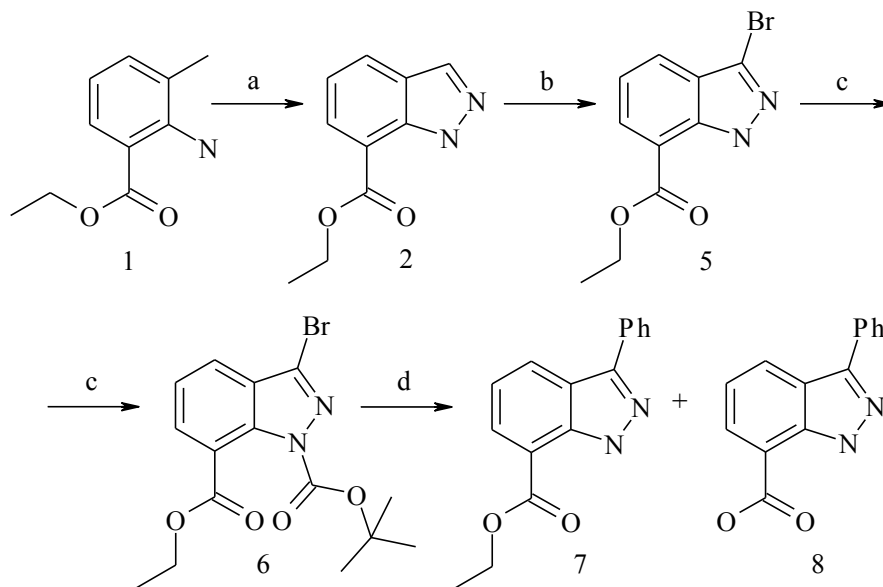
Fig. 2. Conditions: a: AcOH, *t*BuONO, 10°C ÷ r.t., 12h; b: NBS, DMF, r.t., 8h; c: Boc₂O, dioxane, reflux, 1h; d: PhB(OH)₂, Na₂CO₃, Pd(OAc)₂, dppf, EtOH, H₂O, reflux, overnight.

ter — underwent hydrolysis, so, it turned out reasonable to complete the hydrolysis by adding KOH and refluxing for one more hour before the isolation of the final product. The isolation of carboxylic acid also has an advantage — the rest of a catalyst and most impurities can be easily removed from the basic aqueous solution with further precipitation of the 3-phenyl-indazole-7-carboxylic acid after acidification (Fig. 2).

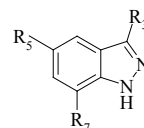5-substituted 3-aryl-1H-indazoles **9–14** were synthesized following known procedures.

Discussion

We have tested the compounds **8–14** for inhibitory activity toward human CK2α catalytic subunit using Kinase-Glo® Luminescent Kinase Assay. The chemical structure of substituents and IC$_{50}$ values are presented in Table 1.

Several important structural features of indazole derivatives can be identified from a

qualitative analysis of their activity toward CK2. As can be seen from Table 1, CK2 inhibitory activity of the studied derivatives

Table 1. **Structure and inhibitory activity towards CK2 of indazole derivatives.**

Cpd	R$_3$	R$_5$	R$_7$	IC$_{50}$, µM
8	phenyl	H	COOH	5.2
9	pyridyl	CN	H	>10
10	pyridyl	COOH	H	3.4
11	methoxyphenyl	COOH	H	5.1
12	fluorophenyl	COOH	H	6.5
13	fluorophenyl	NH$_2$	H	>10
14	fluorophenyl	CN	H	>10

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strongly depends on the presence of a carboxyl group in their structures regardless of the position in phenyl moiety. Compound **8** with a carboxyl group at the 7-th position of the indazole ring shows the same level of inhibition of CK2 as compounds **10–12** with a carboxyl group at the 5-th position. In our opinion[, the] electrostatic interaction between negatively charged carboxylate and side chain residues of the ATP-binding pocket of CK2 plays an important role in the affinity of compounds **8** and **10–12**. Compounds **9** and **14** having neutral nitrile group (which meanwhile can act as a hydrogen bond acceptor in the same way as carboxylate) demonstrate no inhibitory activity unlike the corresponding carboxylic acid derivatives. Compound **13** with the slightly basic hydrogen bond donor amino group also shows no inhibitory activity. The structure of the aromatic R_3 substituents in a range of the studied compounds seems not to be important. A slight advantage of the pyridine moiety over phenyl rings among other reasons may be a result of the contribution of higher polarity pyridine ring in the water solubility of the compound.

Conclusion

The synthesis of 3-aryl-indazole-7-carboxylic acid has been developed. Novel inhibitors of the protein kinase CK2 among indazole derivatives have been identified. The activity of the studied compounds strongly depends on the presence of carboxyl group as substituent R_5 or R_7. The identified indazole derivatives have shown promising inhibitory activity toward CK2, although they require further chemical optimization and biological investigations.

Conflict of Interest

None

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Ідентифікація нових інгібіторів протеїнкінази CK2 серед похідних індазолу

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Мета. Синтез нових біоізостерів пурину — похідних індазолу та перевірка інгібувальної активності отриманих сполук щодо протеїнкінази CK2 в системі *in vitro*. **Методи.** Хімічний синтез, спектроскопія ^1H та ^{13}C ЯМР, метод ВЕРХ з мас-детектором. Визначення залишкової ензимної активності за витратою АТФ і люмінесценцією люциферазної реакції в системі Kinase-Glo®. **Результати.** Відомі синтетичні методи хімії індазолу було вперше застосовано для синтезу 3-арил-індазол-7-карбонових кислот. Було суттєво вдосконалено умови крос-сполучення похідних 3-бром-індазолу з арилбороновими кислотами. 3-арил-індазол 5- та 7-карбонові кислоти продемонстрували в люциферазному кіназному тесті IC_{50} в межах 3.1–6.5 μM. **Висновки.** Було розроблено синтез 3-арил-індазол-7-карбонових кислот. Серед похідних 3-арил-індазол 5- та 7-карбонових кислот було знайдено нові інгібітори протеїнкінази CK2. Було продемонстровано вирішальний вплив карбоксильної групи на інгібувальну активність досліджених сполук.

К л ю ч о в і с л о в а: Індазол, хімічний синтез, люмінесцентний люциферазний тест, інгібітор протеїнкінази, протеїнкіназа CK2.

Идентификация новых ингибиторов протеинкиназы CK2 среди производных индазола

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Цель. Синтез новых биоизостеров пурина –производных индазола и проверка ингибирующей активности полученных соединений относительно протеинкиназы CK2 в системе *in vitro*. **Методы.** Химический синтез, спектроскопия ЯМР на ядрах ^1H и ^{13}C, метод ВЭЖХ с масс-детектором. Определение остаточной энзимной активности по расходу АТФ и люминесценции люциферазной реакции в системе Kinase-Glo®. **Результаты.** Известные синтетические методы химии индазола были впервые применены для синтеза 3-арил-индазол-7-карбоновых кислот. Были существенно усовершенствованы условия кросс-сочетания производных 3-бром-индазола с арилборными кислотами. 3-арил-индазол 5- и 7-карбоновые кислоты продемонстрировали в люциферазном киназном тесте IC_{50} в пределах 3.1–6.5 μM. **Выводы.** Был разработан синтез 3-арил-индазол-7-карбоновых кислот. Среди производных 3-арил-индазол 5- и 7-карбоновых кислот были обнаружены новые ингибиторы протеинкиназы CK2. Было продемонстрировано решающее влияние карбоксильной группы на ингибирующую активность исследованных соединений.

К л ю ч е в ы е с л о в а: Индазол, химический синтез, люминесцентный люциферазный тест, ингибитор протеинкиназы, протеинкиназа CK2.