https://doi.org/10.7124/bc.000B3C
UDC 577.15.08

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2,4-DIANILINOPYRIMIDINE DERIVATIVES AS SUBMICROMOLAR CK2 INHIBITORS

Aim. *This study aims to identify and characterize new 2,4-dianilinopyrimidine derivatives as inhibitors of protein kinase CK2, a key enzyme implicated in numerous pathological processes.* ***Methods.*** *A series of newly synthesized 2,4-dianilinopyrimidine derivatives was evaluated using a luminescence-based assay to determine the inhibitory activity against CK2. Luminescence based structure-activity relationship analysis and molecular docking studies were performed to elucidate key interactions within the ATP-binding site.* ***Results.*** *Several compounds demonstrated submicromolar inhibitory activity against CK2 (IC_{50}). SAR analysis and docking revealed that the position of the carboxylic group on the aniline ring is critical for activity. Compounds bearing an ortho-carboxyl group were active, whereas meta substitution led to a loss of activity, consistent with interactions between the carboxyl group and Lys68. Additionally, the presence of polar substituents on the second aniline ring contributed to activity, likely through interactions with the hinge region residues, including Val116, Asn117, and Asn118.* ***Conclusions.*** *The 2,4-dianilinopyrimidine scaffold represents a promising platform for the development of potent CK2 inhibitors, with key structural features identified for further optimization.*

Keywords: *enzyme inhibition, bis anilinopyrimidine, molecular docking, protein kinase, luminescence.*

Introduction

Protein kinase CK2 is a well-established regulatory enzyme involved in the control of cell proliferation and apoptosis. Aberrant CK2 activity has been associated with a wide range of pathological conditions, including viral diseases [1—2], cancers [3—9], notably cholangiocarcinoma [10], as well as other disorders [11—16]. Given the potential of this enzyme as a therapeutic target, a large number

Citation: Mahdysiuk M.V., Volynets G.P., Tarnavskiy S.S., Bdzhola V.G., Lukashov S.S., Marchenko S.V., Antonenko S.V., Syniugin A.R., Yarmoluk S.M. (2026) 2,4-Dianilinopyrimidine derivatives as submicromolar CK2 inhibitors. *Biopolymers & Cell*, 2(42), 121—128. https://doi.org/10.7124/bc.000B3C

of CK2-directed compounds have been developed to date. These compounds are predominantly small molecules acting as ATP-competitive inhibitors. Among them, several major classes can be distinguished, including polyhalogenated benzimidazole and benzotriazole derivatives, anthraquinone and benzoquinone derivatives, pyrazolo[1,5-a]pyrimidine derivatives, 2-aminothiazole derivatives, indeno[1,2-b]indole derivatives, flavonoid and coumarin derivatives, carboxylic acid derivatives, and others [17].

The pyrimidine-based scaffolds have emerged as particularly promising CK2 inhibitors due to their tunable selectivity profiles. Structural modification of the clinically evaluated inhibitor CX-4945, in which the pyridine was replaced by a pyrimidine ring, has been shown to enhance both selectivity and inhibitory activity [18]. Accordingly, pyrimidine derivatives incorporating polar anchoring groups, such as carboxyl, continue to represent an active and productive direction in CK2 inhibitor development [19—20]. In this context, 2,4-dianilinopyrimidines constitute a versatile scaffold with demonstrated activity across several human protein kinases [21—22].

The aim of this study was to evaluate the inhibitory activity of 48 newly synthesized 2,4-dianilinopyrimidine derivatives against CK2 and to predict their binding modes using molecular docking.

Materials and Methods

Compound synthesis

All reagents were purchased from Merck KGaA company (Germany). The solvents were purchased from Macrochim (Ukraine). Melting points were measured on a Kofler melting point-device and are uncorrected. Thin layer chromatography was performed on DC-Alufolien Kieselgel 60 F254 plates (Merck, Germany) in $CHCl_3$: MeOH, 19 : 1. 1H NMR spectra were recorded in DMSO-d6 on Varian Gemini-2000 instrument (400 MHz, Varian, USA) using tetramethylsilane as an internal standard; Chromato-mass-spectrometric analysis (LC-MS) was performed on Agilent 1100LC/MSD SL instrument (Agilent Technologies, USA) equipped with Zorbax SB-C18 Rapid Resolution HT Cartridge (2.1 × 30 mm, 1.8 μm) using a 0—100% gradient (2 min) of CH3CN in 0.1% formic acid.

All of the compounds were synthesized using well-known methods. The synthesis of 2,4-dianilinopyrimidine derivatives was carried out according to the general well-known procedure [23]. The intermediate product IIa-m was obtained in a single step by a substitution reaction between 2,4-dichloropyrimidine I and the corresponding aniline, among which aminobenzoic acids were also used (Fig. 1). The reaction was regioselective at position C4 and could be easily carried out without the need for purification. Then, the 2-chloro group of

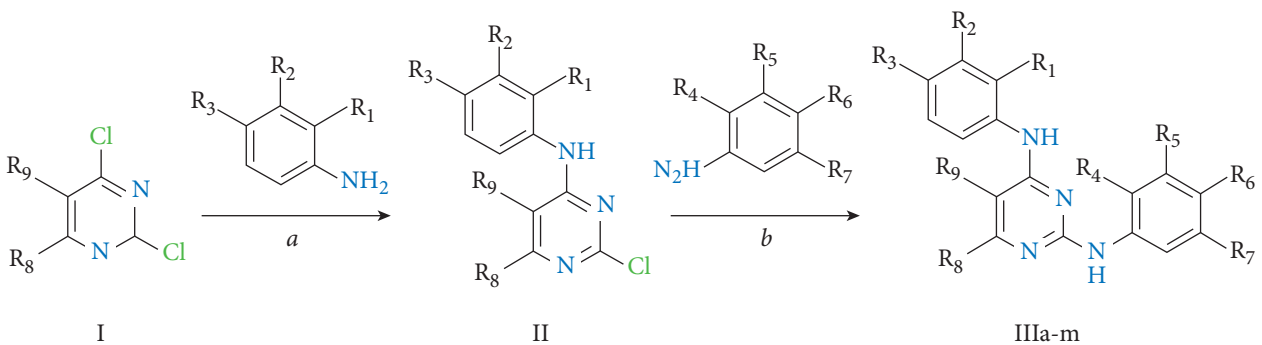
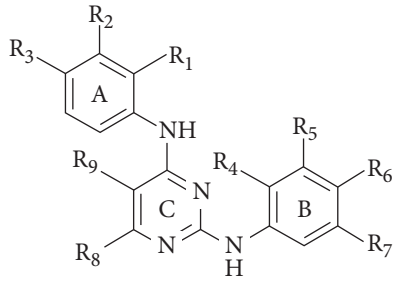


Fig. 1. Basic scheme for the synthesis of 2,4-dianilinopyrimidine derivatives. Reagents and conditions: *a* — anilines, cat. conc HCl, EtOH/H_2O, 50 °C (1 h), then rt (overnight), 86—91%; *b* — anilines, DMF, 130 °C, 40 min, 60—80%

Chemical structure of 2,4-dianilinopyrimidine derivatives and their inhibitory activity toward human protein kinase CK2



№	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	IC_{50}, μM
1*	H	H	H	H	H	COOH	H	H	H	0,22 ± 0,10
2	H	H	H	H	COOH	H	H	H	H	Inactive
3*	H	H	H	COOH	H	H	H	H	H	Inactive
4	H	H	OH	H	H	CH_2COOH	H	H	F	Inactive
5	H	H	OH	H	COOH	H	H	H	F	0,8 ± 0,05
6	H	H	COOH	H	H	H	H	H	H	>1
7	H	H	COOH	H	H	H	H	H	F	>1
8	H	H	COOH	H	H	OCH_3	H	H	H	>1
9*	H	H	COOH	H	H	COOH	H	H	H	0,7 ± 0,07
10	H	H	COOH	H	H		H	H	H	>1
11	H	H	COOH	H	H	CF_3	H	H	H	0,75 ± 0,04
12	H	H	COOH	H	F	H	H	H	H	>1
13	H	H	COOH	H	COOH	H	H	H	H	Inactive
14	H	H	COOH	H	COOH	H	H	H	F	Inactive
15	H	H	COOH	H	COOH	OH	H	H	F	Inactive
16	H	H	COOH	H	CH_3	H	CH_3	H	H	Inactive
17	H	H	COOH	COOH	H	H	H	H	H	Inactive
18	H	COOH	H	H	H	H	H	H	H	Inactive
19	H	COOH	H	H	H	OCH_3	H	H	H	Inactive
20	H	COOH	H	H	H	COOH	H	H	H	>1
21	H	COOH	H	H	H		H	H	H	Inactive
22	H	COOH	H	H	H	CF_3	H	H	H	Inactive
23	H	COOH	H	H	OH	H	H	H	H	Inactive
24	H	COOH	H	H	COOH	H	H	H	H	Inactive
25	H	COOH	H	H	F	H	H	H	H	Inactive
26	H	COOH	H	H	CH_3	H	CH_3	H	H	Inactive
27	H	COOH	H	OCH_3	H	H	OCH_3	H	H	Inactive
28	H	COOH	H	COOH	H	H	H	H	H	Inactive
29	H	COOH	OH	H	H	H	H	H	H	Inactive

End of the table

№	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	IC_{50}, μM
30	H	COOH	OH	H	H	COOH	H	H	H	Inactive
31	H	COOH	OH	H	H	CH_2COOH	H	H	H	Inactive
32	H	COOH	OH	H	COOH	H	H	H	H	0,78 ± 0,08
33	H	COOH	OH	CH_3	H	CH_3	H	H	H	Inactive
34	COOH	H	H	H	H	H	H	H	H	0,51 ± 0,16
35*	COOH	H	H	H	H	H	H	H	F	>1
36	COOH	H	H	H	H	OCH_3	H	CH_3	H	0,78 ± 0,08
37*	COOH	H	H	H	H	COOH	H	CH_3	H	>1
38	COOH	H	H	H	H		H	H	H	0,74 ± 0,06
39	COOH	H	H	H	H		H	CH_3	H	>1
40*	COOH	H	H	H	OH	H	H	H	H	0,7 ± 0,10
41	COOH	H	H	H	OH	H	H	CH_3	H	0,67 ± 0,10
42	COOH	H	H	H	F	H	H	CH_3	H	0,64 ± 0,07
43*	COOH	H	H	H	COOH	H	H	H	H	0,84 ± 0,04
44	COOH	H	H	H	COOH	H	H	CH_3	H	>1
45	COOH	H	H	OCH_3	H	H	OCH_3	CH_3	H	0,21 ± 0,05
46	COOH	H	H	OCH_3	H	H	OCH_3	H	H	0,84 ± 0,10
47*	COOH	H	H	COOH	H	H	H	CH_3	H	0,67 ± 0,10
48	COOH	H	H	H	H	OCH_3	H	H	H	0,61 ± 0,15

* Novel compound

compounds IIa-m was substituted with the corresponding aniline, forming compounds IIIa-m.

Activity determination

The study was performed using the recombinant human protein kinase CK2α subunit expressed from plasmid pZW6 (Addgene, USA; catalog no. 27086). The luminescence-based kinase assays were carried out according to the well known protocols [24] using the Kinase-Glo® assay kit (Promega, USA; catalog no. V6711). The concentrations of ATP and peptide substrate (RRRDDDSDDD) were 10 μM and 200 μM, respectively. Inhibitory activity was calculated using standard methods [24]. IC_{50} values were determined by testing inhibitors over a concentration range of 0.01—1 μM. The data plotting and IC_{50} determination were performed using OriginLab 2021 software.

Molecular Docking

Molecular docking studies were conducted with MzDOCK software [25]. The crystal structure of CK2 co-crystallized with adenylyl imidodiphosphate (PDB ID: 3NSZ) [26] was used for the simulations. For the protein preparation all ions, water and ligand molecules were removed from the PDB-file and Kollman charges were added. The forcefield MMFF94 was used for the ligand preparation. Nine ligand conformations characterized by distinct scoring function values were generated. The conformation with the lowest scoring function value was regarded as the most favorable.

Results and Discussion

Compound activity and SAR

A total of 48 2,4-dianilinopyrimidine derivatives were synthesized and evaluated for CK2 inhibitory activity. All compounds were initially screened at a concentration of 1 μM. The compounds exhibiting inhibition rates below 25% were marked as inactive. For compounds showing inhibition rate above 50%, IC_{50} values were determined (Table).

The position of the carboxyl on the A-ring was identified as the most critical structural determinant of inhibitory activity. According to published data, this functional group plays a key role in binding to essential residues within the ATP-binding site, including Lys68, Asp175, and Trp176 [17, 26—28]. Consistent with this mechanism, nearly all compounds bearing the carboxyl in the meta position (R2) were inactive, whereas several active derivatives contained the carboxyl in the para position (R1). Notably, all tested compounds with an ortho-substituted carboxyl (R3) exhibited inhibitory activity. Active compounds were also observed among the derivatives bearing carboxyl in the meta or para positions on the B-ring (R5 and R6), suggesting the possibility of an alternative binding mode for this subset of compounds.

Within ortho-substituted carboxyl (R3) active derivatives, the compounds featuring polar substituents at positions R5 or R6 were particularly prominent. This behavior may be attributed to additional interactions with the hinge region, potentially involving residue Asn118, as previously reported for pyrazolo[1,5-a]pyrimidine derivatives [17, 28]. Moderately nonpolar substituents at positions R4 and R8 were also associated with enhanced activity, likely reflecting strengthened hydrophobic interactions in this region of the binding pocket.

Binding mode

Molecular docking was performed to propose the binding modes of 2,4-dianilinopyrimidine derivatives within the ATP-binding site of CK2. The overall predicted binding mode is shown in Fig. 2. The 2,4-dianilinopyrimidine core engages in multiple hydrophobic interactions with residues of the ATP-binding pocket. The pyrimidine ring interacts with Val66, Met153, and Ile174, resembling the interaction pattern observed for the C-ring of aurones and flavones rather than that of pyrazolo[1,5-a]pyrimidine derivatives [24, 29—30]. The A-ring forms

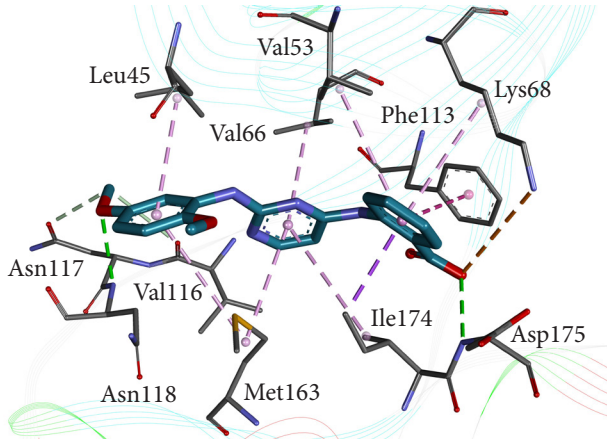


Fig. 2. The complex of compound 46 with the CK2 ATP-binding site obtained through molecular docking. Hydrogen bonds are indicated in green, hydrophobic interactions in purple, electrostatic interactions in yellow and C–H•••O interactions in white

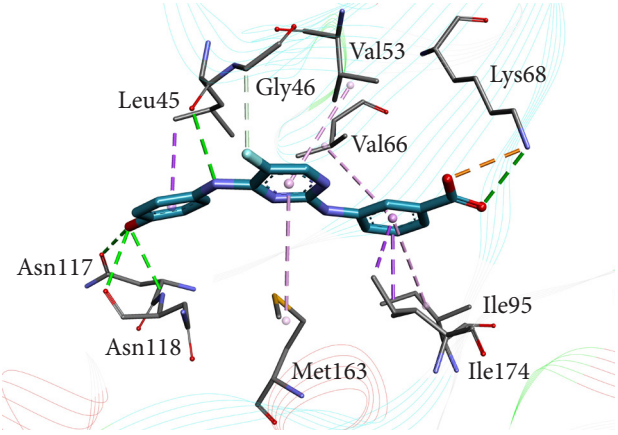


Fig. 3. The complex of compound 5 with the CK2 ATP-binding site obtained through molecular docking. Hydrogen bonds are indicated in green, hydrophobic interactions in purple, electrostatic interactions in yellow and C–H•••O interactions in white

hydrophobic contacts with Val53, Phe113, Ile174, and Lys68, whereas the B-ring interacts with Leu45 and Met153, further supporting similarity between the binding mode of 2,4-dianilinopyrimidine derivatives and that reported for flavonoid derivatives [17, 24, 29—30].

The carboxyl on the A-ring, whose position is critical for inhibitory activity, forms electrostatic and hydrogen-bond interactions with Lys68 and Asp175, in agreement with published data [17, 26—28]. Hydrogen and C–H•••O interactions between substituents of the A-ring and the hinge region were also observed, particularly involving Asn117, Asn118, and Val116. These interactions may explain the importance of these substituents for inhibitory activity. Similar contacts have been reported for polar substituents on the corresponding ring in both pyrazolo[1,5-a]pyrimidine and flavonoid derivatives [17, 24, 28—30].

The proposed binding mode for compounds bearing the carboxyl on the opposite side of the molecule, on the B-ring, is presented in Fig. 3. Owing to the substantial molecular symmetry, this orientation is generally similar to that of other derivatives, maintaining key hydrophobic contacts and hydrogen-bond/electrostatic interactions of the carboxyl with Lys68. In this case, interactions with the hinge region are instead mediated by the hydroxyl group on the A-ring. Overall, the proposed binding modes are consistent with published data and provide a structural rationale for the observed structure-activity relationships of the 2,4-dianilinopyrimidine derivatives.

Conclusions

Among the 48 tested 2,4-dianilinopyrimidine derivatives evaluated against CK2, submicromolar IC_{50} values were confirmed for 16 compounds, indicating the high potential of this scaffold. The ortho position of the carboxyl group on the aniline ring was found to be more favorable than the meta position, consistent with its interaction with residue Lys68. The presence of a polar substituent on the opposite aniline ring was also advantageous, likely enabling interactions with the hinge region, particularly involving Val116, Asn118, and Asn117. These structural features should be considered in the further design of CK2 inhibitors based on the 2,4-dianilinopyrimidine scaffold.

Funding statement. This work was supported by Rational design of protein kinase inhibitors as potential antiviral drugs project (0122U201823).

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Received: 09.04.2026
Accepted: 16.06.2026
Published: 25.06.2026

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ПОХІДНІ 2,4-ДІАНІЛІНО-ПІРИМІДИНУ
ЯК СУБМІКРОМОЛЯРНІ ІНГІБІТОРИ CK2

Мета. Це дослідження спрямоване на ідентифікацію та характеристику нових похідних 2,4-діанілінопіримідину як інгібіторів протеїнкінази CK2 — ключового ферменту, залученого до численних патологічних процесів. ***Методи.*** Серію новосинтезованих похідних 2,4-діанілінопіримідину було досліджено за допомогою люмінесцентного аналізу для визначення інгібувальної активності щодо CK2. Проведено аналіз взаємозв'язку «структура-активність» на основі люмінесцентних даних, а також молекулярне докінг-моделювання для з'ясування ключових взаємодій у сайті зв'язування АТФ. ***Результати.*** Кілька сполук продемонстрували субмікромолярну інгібувальну активність щодо CK2 (IC_{50}). Аналіз «структура-активність» та докінг показали, що положення карбоксильної групи в аніліновому кільці є критичним для активності. Сполуки з орто-карбоксильною групою були активними, тоді як мета-заміщення призводило до втрати активності, що узгоджується із взаємодіями між карбоксильною групою та Lys68. Крім того, наявність полярних замісників у другому аніліновому кільці сприяла активності, ймовірно, через взаємодії з амінокислотними залишками «шарнірної» ділянки, включаючи Val116, Asn117 та Asn118. ***Висновки.*** Молекула 2,4-діанілінопіримідину є перспективною платформою для розробки інгібіторів CK2, причому визначено ключові структурні особливості для подальшої оптимізації.

***Ключові слова*:** інгібування ферментів, біс-анілінопіримідин, молекулярний докінг, протеїнкіназа, люмінесценція.