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THE PROTEIN KINASE CK2 INHIBITOR TESTING BY CAPILLARY ELECTROPHORESIS

Aim. *This research aimed to enhance the methodology for efficiently evaluating CK2 inhibitors using capillary electrophoresis and validate the techniques with the CK2 inhibitor* ***FNH79****. Additionally, the study included the assessment of a novel potential inhibitor, the aurone derivative* ***BFO21****, using the optimized protocol.* ***Methods.*** *The research was conducted with the capillary electrophoresis method, and 150 mM orthophosphoric acid (pH 1.2) as a background electrolyte. Conversion of enzymatic reaction was calculated as the ratio of the phosphorylated product peak area to the total peak area of both substrate and product.* ***Results.*** *The optimal testing conditions were determined, involving 50 units of the enzyme per 50 µl of the reaction mixture, an initial peptide substrate concentration of 100 µM, and an incubation time of 40 minutes. The initial concentration of ATP was 100 µM.* ***FNH79*** *demonstrated IC50 and Ki values of 94 nM and 4.5 nM, respectively. The new aurone compound* ***BFO21*** *exhibited IC50 and Ki values of 44 nM and 2.1 nM, respectively.* ***Conclusions.*** *Under optimized testing conditions, the activity values for the* ***FNH79*** *inhibitor matched previously published results. Additionally, the activity values of the* ***BFO21*** *inhibitor revealed its significant potential as the CK2 inhibitor.*

Keywords: capillary electrophoresis, protein kinase CK2, phosphorylation, enzyme inhibition, enzyme kinetics

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Introduction

Over the last years, the inhibitors of protein kinases have become one of the most important drug classes in cancer treatment. Protein kinase CK2 plays an important role in many intracellular processes, the most important of which are cell cycle and apoptosis regulation. For many types of cancer, CK2 expression level is maintained at a consistently higher level regardless of the cell cycle stage. Hanahan and Weinberg showed that such a change in the level of expression occurs precisely at the time of oncogenic transformation [1]. The role of CK2 in oncology is the most studied, but a growing body of research indicates that it also plays a role in several serious diseases, such as multiple sclerosis, neurodegenerative diseases such as Alzheimer's and Parkinson's, atherosclerosis, thrombosis and diabetes, as well as other pathological states [2–5].

In recent years, significant efforts have been committed to the development of new CK2 inhibitors, including the ATP-competitive, allosteric, and dual inhibitors. [6–8]. According to Borad *et al.* [9], in 2016 the US Food and Drug Administration provided the protein kinase CK2 inhibitor **CX-4945** with an orphan drug status for treatment of cholangiocarcinoma. Another selective inhibitor of CK2, **CIGB-300**, is currently undergoing clinical trials [10], emphasizing the potential of this enzyme as a target for antitumor therapy.

One of the biggest problems in searching for new kinase inhibitors is the possibility of quick and effective screening. According to Gratz *et al.*, the most common are radiometric, spectroscopic and antigen-dependent studies [11]. Radiometric assays are highly sensitive and can be applied to most protein kinases. However, they involve multiple post-reaction steps and have several other disadvantages, such as special conditions for working with radioactivity and the short half-life of radioactive phosphorus isotope. In antigen-dependent assays the antibodies selectively bind to peptide sequences containing phosphorylated amino acid side chains. To date there are no commercially available phosphospecific antibodies, designed for the common CK2 substrate peptides. Most spectroscopic methods have a drawback in that they rely on a secondary enzymatic reaction that can be disrupted in the presence of test compounds. Capillary electrophoresis, in contrast, is a much more simple and affordable method that can be effectively implemented for studying CK2 enzymatic activity [11–13]. The selection of optimal testing conditions, as well as the assessment of CK2 inhibitors activities, is a promising area of research.

The aim of this study was to develop the effective techniques for testing CK2 inhibitors by capillary electrophoresis and assess the optimized techniques using the known CK2 inhibitor **FNH79**. In addition, the tasks of this research included testing a new potential inhibitor (an aurone derivative) using the optimized protocol.

Materials and methods

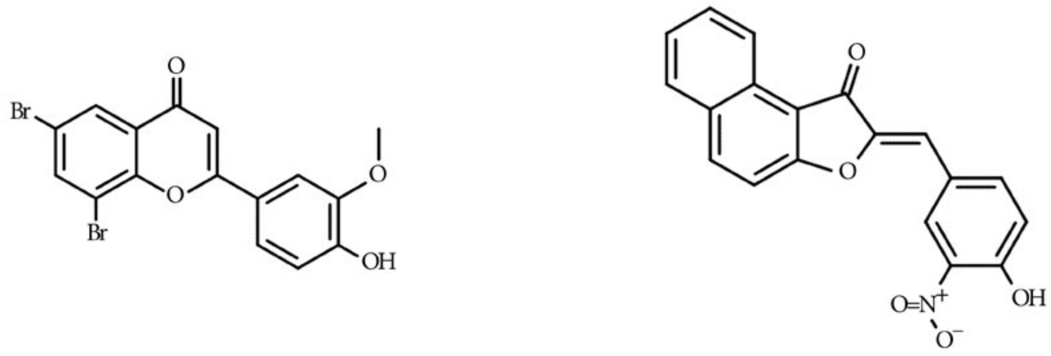
2.1. Chemicals and materials

The study was conducted using recombinant human protein kinase CK2 (New England Biolabs, USA) and peptide substrate RRRDDDSDDD (GenScript, USA). The CK2 inhibitor 6,8-Dibromo-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one **(FNH79)** was provided by OTAVA Chemicals, Ukraine. An aurone derivative (2*Z*)-2-[(4-hydroxy-3-nitro-phenyl)methylene]benzo[*e*]benzofuran-1-one (**BFO21**) was synthesized following the procedure [14] described earlier starting from the benzo[*e*]benzofuran-1-one intermediate obtained in a known manner [15].

The structural formulae of **FNH79** inhibitor and the aurone compound **BFO21** are shown in Fig. 1. A. and Fig. 1. B., respectively.

2.2. Reaction setup

The reaction mixture volume was 50 µl per sample. The reaction buffer contained 2 mM Tris, 5 mM KCl and 1 mM $MgCl_2$, pH 7.5. The starting concentration of ATP in all reactions was 100 µM. The



a *b*

Fig. 1. A. Structural formula of the CK2 inhibitor 6,8-Dibromo-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one (**FNH79**). B. Structural formula of the aurone derivative (2*Z*)-2-[(4-hydroxy-3-nitro-phenyl)methylene]benzo[*e*]benzofuran-1-one (**BFO21**)

initial concentrations of the peptide substrate and enzyme, along with the incubation time, were varied across different experiments. The incubation temperature was 30 °C. The reaction was stopped by adding 100 µl of 20 mM EDTA disodium salt.

2.3. Electrophoretic separation

All electrophoretic separations were performed on a capillary electrophoresis system Capel-105M (Lumex). Fused silica (Lumex) of a total length of 75 cm (effective length 65 cm) with inner diameter 50 µm was used. Before use, fused silica was washed for 5 minutes with 0.1 M HCl solution, then 5 min with distilled water, then 5 min with 0.1 M NaOH solution, again for 5 min with distilled water and for 5 min with background electrolyte (BGE).

Study by Gratz *et al.* suggests 2 M acetic acid (pH 1.9) as BGE for capillary electrophoresis of peptides [11]. Dawson *et al.* used 150 mM orthophosphoric acid (pH 1.2) for CE kinase assay [16]. Both BGEs were tested in this study. Before electrophoretic separation, 350 µl of deionized water was added to the samples. Samples were introduced hydrodynamically at 900 mbar*s. The electrophoresis voltage was 25 kV. Electrophoretic separation was carried out in positive polarity. Detection conducted at a wavelength of 192 nm. Instrument control, data collection and integration were performed with Elforun (Lumex) software.

2.4. Results interpretation

Accurate measurement of conversion requires a calibration curve that illustrates the relationship between peak area and concentration for the substrate. However, the construction of such a curve is time-consuming. Alternatively, the conversion can be assessed by directly comparing the peak areas of the substrate and product, since they exhibited similar absorption at the selected wavelength. In this study, conversion was calculated as the ratio of the phosphorylated product peak area to the total peak area of both substrate and product.

2.5. Selection of the optimal peptide substrate starting concentration

The reaction mixture contained 50 units of kinase per sample. The initial peptide concentration in different samples was 200, 100, 25, or 12.5 µM. The incubation time was 60 min.

2.6. Selection of the optimal incubation time

The reaction mixture contained 50 or 100 units of CK2 per sample. The initial peptide concentration

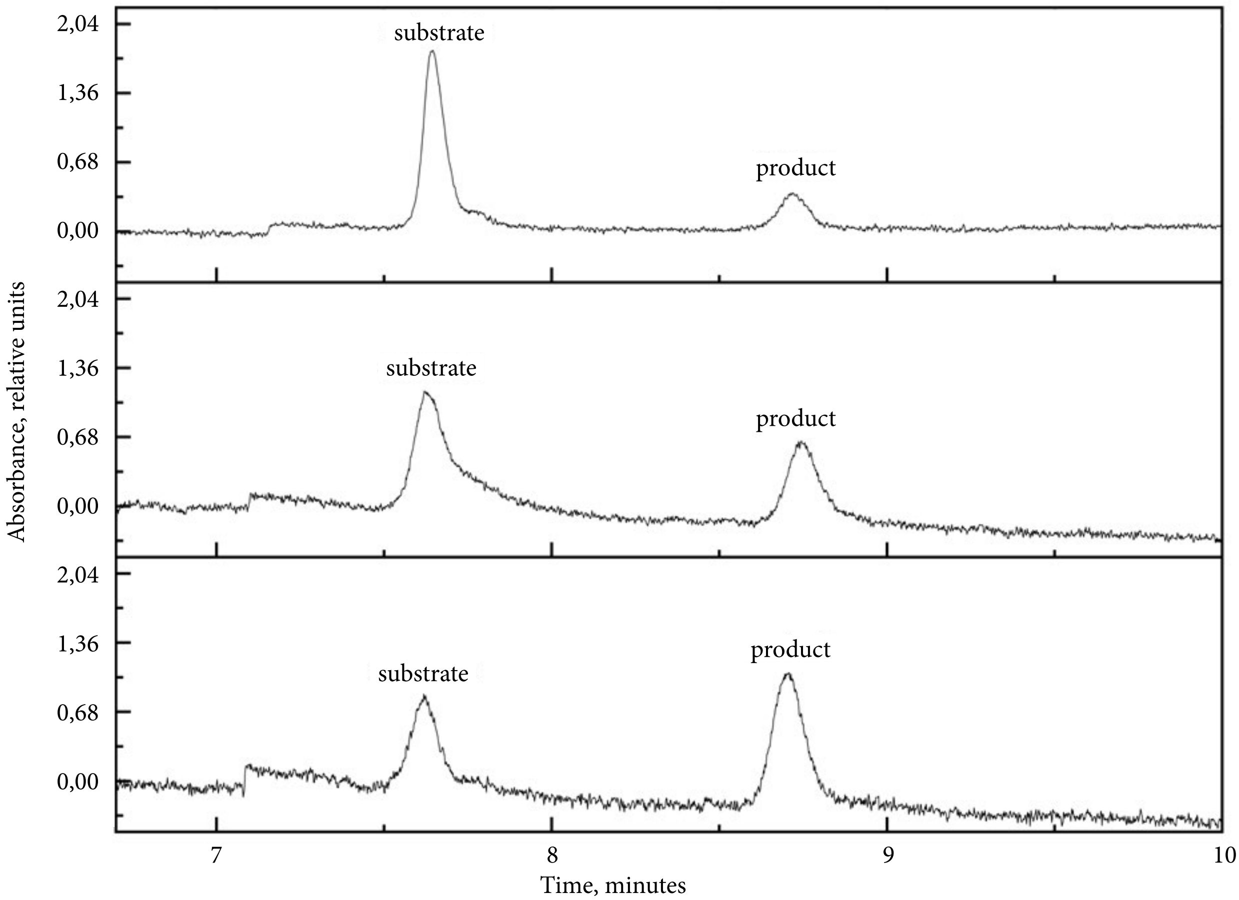


Fig. 2. Electrophoretic separation of the substrate and phosphorylated product at varying incubation time and conversion rates, 2 M acetic acid as a BGE

was 100 μM. Samples were incubated for 20, 40, 60 and 100 min.

2.7. Evaluation of the inhibition effectiveness and IC50

The reaction mixture contained 50 units of kinase per sample and 0.5 μl inhibitor solution in DMSO (final concentrations were 2 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM for **FNH79** and 5.7 μM, 2.85 μM, 1.425 μM, 0.713 μM, 0.356 μM, 0.178 μM, 0.089 μM, 0.022 μM for **BFO21**) or 0.5 μL of pure DMSO in control samples. The incubation time was 40 min. All IC_{50} studies were performed in two replicates. Mean conversion values for each concentration were used for construction of the conversion-versus-concentration curves and IC_{50} determination.

Results and discussion

3.1. Selection of electrophoretic separation conditions

Efficient electrophoretic separation was observed both using 2 M acetic acid and 150 mM orthophosphoric acid (Fig. 2 and Fig. 3, respectively).

When orthophosphoric acid was used, a higher electrophoresis current was observed (100 μA compared to 20 μA for acetic acid), as well as a larger peptide peak area. For this reason, 150 mM

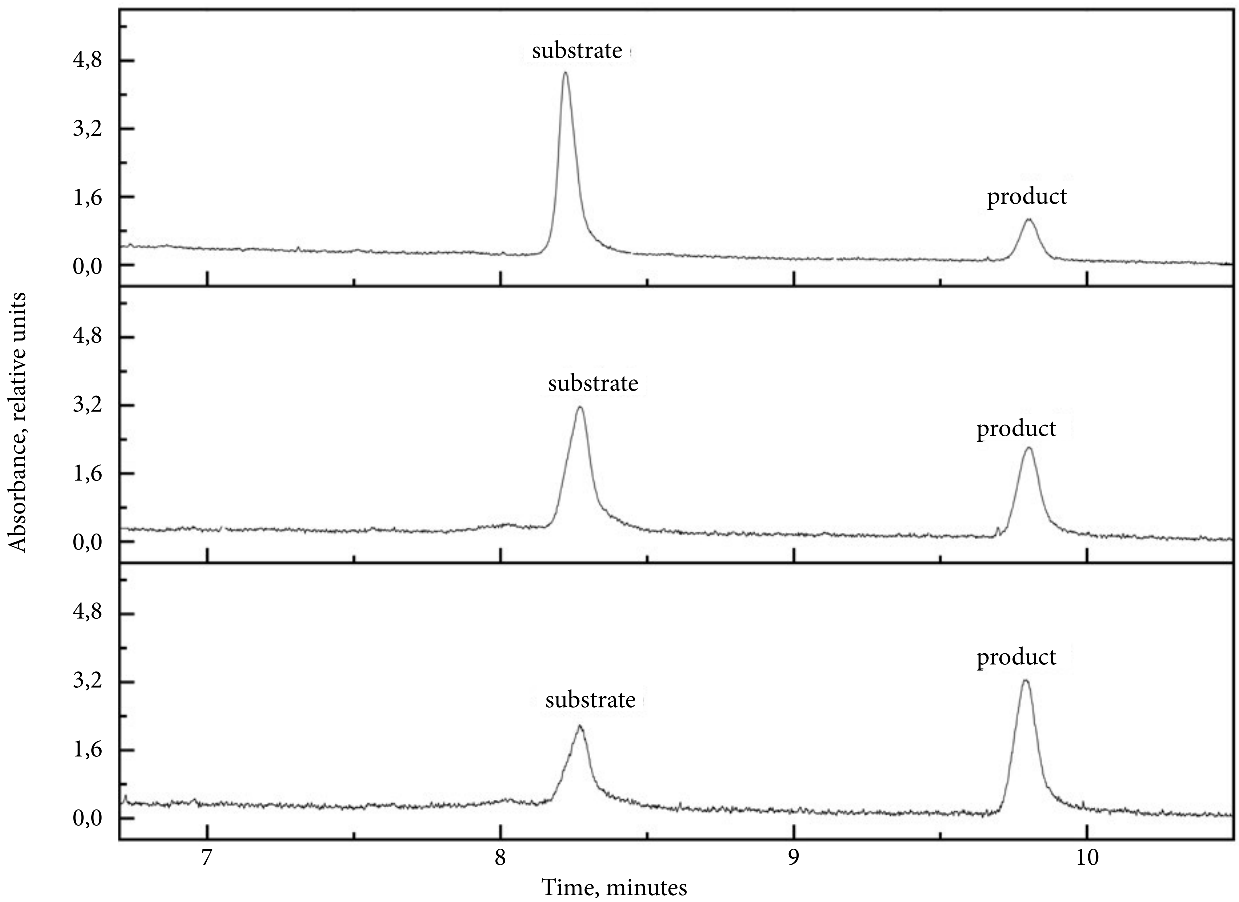


Fig. 3. Electrophoretic separation of the substrate and phosphorylated product at varying incubation time and conversion rates, 150 mM orthophosphoric acid as a BGE

orthophosphoric acid (pH 1.2) was used as a BGE in further experiments.

3.2. Determination of the optimal peptide substrate starting concentration

In order to maximize the accuracy of the measurements, it was necessary to achieve the largest possible area of the phosphorylated product. This can be achieved by increasing conversion. The conversion depends on the initial enzyme and substrate concentrations, as well as on the incubation time. In order to find the optimal starting peptide concentration, a series of experiments was conducted with different starting concentrations, in which the conversion over a fixed time was compared (Fig. 4).

As shown in Fig. 4, at an initial peptide substrate concentration of 200 μM, the conversion was only 12%. In contrast, at a starting concentration of 13 μM, the entire peptide substrate was phosphorylated within 60 minutes. Sufficiently high conversion was observed at starting concentration of 100 μM (35%).

The obtained data are consistent with the results of other studies. The initial substrate concentration of 100 μM was used by He & Yeung [12] for the CE-based protein kinase inhibitor testing, and their initial ATP concentration was 1 mM. Gratz *et al.* used a starting peptide concentration of

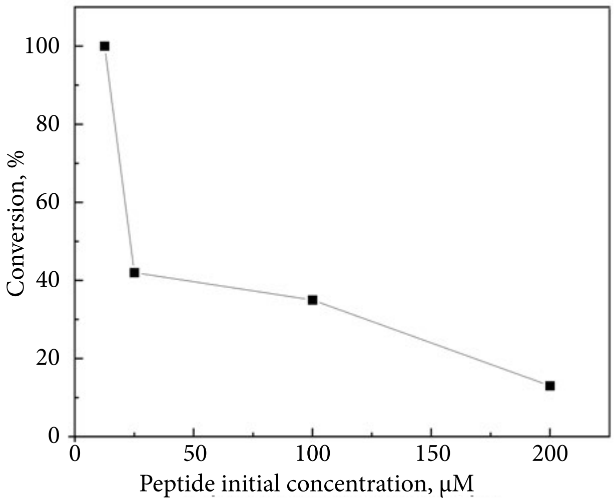


Fig. 4. The dependence curve showing the conversion as a function of the initial peptide substrate concentration

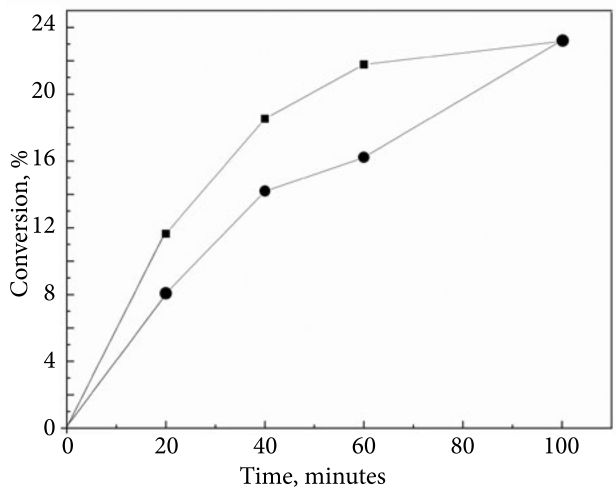


Fig. 5. The conversion versus reaction time dependence curve for 100 enzyme units (squares) and 50 enzyme units (circles) per 50 μl of reaction mixture

114 μM for the CE-based CK2 inhibitor testing, while the ATP concentration was 150 μM. Considering this data, the use of a peptide substrate in a concentration close to 100 μM is a general trend in studies on the CE-based protein kinase inhibitor testing. This initial peptide concentration was considered optimal for further experiments.

3.3. Incubation time optimization

A longer incubation time is desirable, as it allows increasing the conversion and the phosphorylated product peak area, which positively affects the accuracy of measurement results. However, the reaction rate decreases over time due to the decrease in substrate concentration, which leads to additional difficulties in determining the IC_{50} of the inhibitor. In order to optimize incubation time, a dependence curve of conversion on the reaction time was obtained (Fig. 5).

As can be seen from Fig. 5, for 40 min the conversion at 100 and 50 units of enzyme was 19% and 15%, respectively. At the same time, using 50 units of the enzyme per 50 μl of the reaction mixture linearity was observed for up to 40 minutes, in contrast to 100 units.

In general, the amount of enzyme and the incubation time in such experiments strongly depend on the enzyme activity and, accordingly, the conversion that can be achieved under these conditions. In the CE-based protein kinase inhibitor testing, the incubation time is usually in the range of 10 to 30 min [12–13], which is shorter than the optimal time in this study.This shows that the obtained conversion values turned out to be unexpectedly low for the tested time intervals. The protein kinase manufacturer states that a unit of enzyme is defined as the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to the peptide substrate (100 μM) in 1 min at 30 °C in a total reaction volume of 25 μL. Assuming a constant reaction rate, which is acceptable on a linear segment, in 40 min, 100 units of enzyme in a volume of 25 μl should theoretically catalyze the transfer of 4000 pmol of phosphate to the peptide substrate. Thus, the amount of phosphorylated product should also be 4000 pmol. Since the initial amount of peptide substrate was 8620 pmol, a conversion close to 50% was expected after this time.

In a similar research Gratz *et al.* showed that the area of the peak of the peptide substrate was sig-

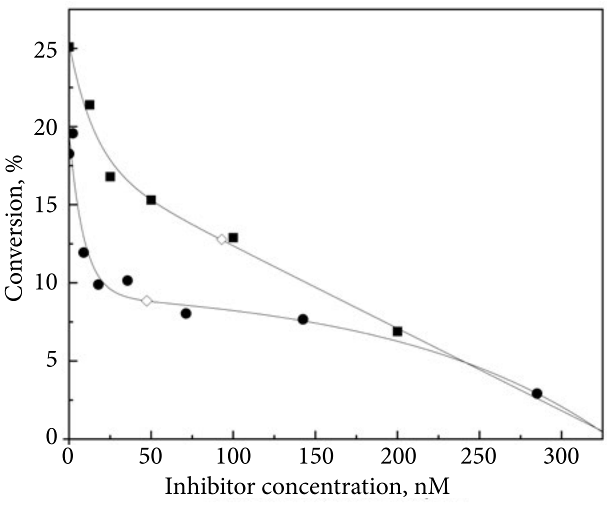


Fig. 6. The conversion versus inhibitor concentration dependence curves for FNH79 (squares) and BFO21 (circles). The IC_{50} values are shown with white marks

nificantly exceeded by the peak of the phosphorylated product already after 6 min of incubation [11]. However, in that study, the amount of enzyme was indicated in micrograms, and not in units, which makes it much more difficult to correctly compare the conditions of the current research with the results obtained by Gratz *et al.* [11]. In this study, a conversion of 15% was considered acceptable for measurements. Since it exceeded the conversion after 20 min for 100 units of protein kinase, using 50 units of enzyme per 50 μl of reaction mixture with a 40-minute incubation time was the most optimal for further tests.

3.4. Evaluation of the inhibition effectiveness and IC50

To estimate IC_{50}, a conversion-versus-concentration curve for the **FNH79** inhibitor was plotted on the basis of obtained data (Fig. 6).

The standard deviation for the obtained data was within 10% of the obtained values. The obtained IC_{50} for the **FNH79** inhibitor was 94 nM. Golub *et al.* indicate that the IC_{50} for this CK2 inhibitor was 4 nM [17]. However, the IC_{50} indicator is a rather apparent value that depends on several additional parameters, in particular substrate and co-substrate concentrations. Since these values, particularly the concentration of the ATP, differed in this study due to the specificity of the method, it is expectable that the estimated IC_{50} value differs.

The inhibition constant (K_i) is a much more universal value that can be used for direct comparison of the inhibitors tested by different methods according to the protocols that included different amounts of ATP in the reaction mixture. According to Cheng and Prusoff, K_i for competitive inhibitors can be calculated using the following equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

where [S] is the concentration of the substrate, in this case ATP, and K_m is the enzyme Michaelis constant for ATP [18].

Table 1. **Comparison of CK2 inhibitors activity**

Compound name	Testing method	[ATP] (μM)	CK2	IC_{50} (nM)	K_i (nM)	Source
BFO2	Radiometric	50	holoenzyme	3.5	0.3	[14]
Compound 3	Radiometric	90	holoenzyme	600	31.6	[22]
CX-4945	Radiometric	15	holoenzyme	1	0.3	[24]
Chrysoeriol	Radiometric	20	α-subunit	250	93.8	[20]
7h	Spectroscopic	61	α-subunit	3	0.5	[21]
GO289	Spectroscopic	3	α-subunit	7	5.6	[23]
SRPIN803-rev	Radiometric	20	α-subunit	280	105	[25]
FNH79	Radiometric	50	holoenzyme	4	0.8	[17]
FNH79	Electrophoresis	100	holoenzyme	94	4.5	This study

According to Dobrowolska *et al.* the value of K_m (ATP) is 12 μM for the alpha subunit of CK2 and 5 μM for the holoenzyme [19]. To compare the obtained results, it was necessary to analyze the data on several CK2 inhibitors, for which IC50 values were published elsewhere [14, 17, 20–25]. These values were determined using methods with varying ATP concentrations in the reaction mixture. Expected K_i values were calculated for them using the Cheng-Prusoff equation and the data of Dobrowolska *et al.* The results are shown in Table 1.

As can be seen from the table, some inhibitors with a high IC_{50} value can have low calculated K_i, for example, Compound 3, studied by Nakanishi *et al.* [22]. The obtained K_i value for the **FNH79** inhibitor is much closer to the data of Golub *et al.* despite the fact that their IC_{50} values differ significantly. Thus, the obtained data on the activity of the tested compound correspond to the previous data of Golub *et al.*, which indicates the success of the proposed protocol of CK2 inhibitors testing [17].

During the previous study from our department, conducted by Protopopov *et al.* a series of aurones with significant inhibitory activity against CK2 was discovered and synthesized [14]. As a continuation of that study, one of the newly synthesized aurones **BFO21** was tested by capillary electrophoresis. The conversion versus **BFO21** concentration dependence curve is shown in Fig. 6.

The IC_{50} value for the tested aurone was 44 nM. K_i calculated using the Cheng-Prussoff equation and the data of Dobrowolska *et al.* was 2.1 nM, which is comparable to the corresponding value calculated for the aurone **BFO2** found by Protopopov *et al.* (Table 1) [14]. The obtained IC_{50} and K_i values of **BFO21** turned out to be low, compared to the corresponding values for other known CK2 inhibitors shown in Table 1, which indicates that the tested compound is a promising inhibitor of this enzyme.

Conclusions

The efficient electrophoretic separation of the CK2 peptide substrate and its phosphorylated product by capillary electrophoresis was achieved using 150 mM orthophosphoric acid (pH 1.2) as the BGE solution. The optimal conditions for testing CK2 inhibitors by the CE method were found to be 50 units of enzyme per 50 μl of reaction mixture (with an initial peptide substrate and ATP concentrations of 100 μM) and an incubation time of 40 minutes. The obtained IC_{50} and calculated K_i values for the **FNH79** were 94 nM and 4.5 nM, respectively, which are consistent with the published data for this compound. The results may indicate the effectiveness of the proposed technique for the CK2 inhibitors testing by capillary electrophoresis. The IC_{50} and K_i values for the new aurone **BFO21** were 44 nM and 2.1 nM, respectively, which indicates the significant potential of this compound as a CK2 inhibitor.

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ТЕСТУВАННЯ ІНГІБІТОРІВ ПРОТЕЇНКІНАЗИ СК2 МЕТОДОМ КАПІЛЯРНОГО ЕЛЕКТРОФОРЕЗУ

Мета. Метою дослідження було покращення методики ефективної оцінки інгібіторів протеїнкінази СК2 за допомогою капілярного електрофорезу та її перевірка з використанням відомого інгібітора FNH79. Крім того, дослідження включало оцінку нового потенційного інгібітора, похідного аурону BFO21, з використанням оптимізованого протоколу. ***Методи.*** Дослідження проводились методом капілярного електрофорезу, фоновим електролітом слугувала 150 мМ ортофосфорна кислота (pH 1,2). Конверсію ферментативної реакції розраховували як відношення площі піку фосфорильованого продукту до загальної площі піків субстрату та продукту. ***Результати.*** Було визначено оптимальні умови тестування, що становили 50 одиниць ферменту на 50 мкл реакційної суміші із початковою концентрацію пептидного субстрату 100 мкМ та часом інкубації 40 хвилин. Стартова концентрація АТФ також становила 100 мкМ. Для FNH79 встановлені значення IC_{50} і розраховані значення K_i становили 94 нМ і 4,5 нМ відповідно. Нове похідне аурону BFO21 продемонструвало значення IC_{50} і K_i 44 нМ та 2,1 нМ відповідно. ***Висновки.*** За оптимізованих умов тестування значення активності для інгібітору FNH79 були близькі до раніше опублікованих результатів. Крім того, значення активності інгібітора BFO21 вказують на його значний потенціал в якості інгібітора СК2.

***Ключові слова*:** капілярний електрофорез, протеїнкіназа СК2, фосфорилювання, інгібування ферментів, ферментативна кінетика.