Research Results in Pharmacology

Research Results in Pharmacology 10(2): 119–133
UDC: 547.785.51; 615.212.7
DOI 10.18413/rrpharmacology.10.465

Research Article

Synthesis and pharmacological activity of various organic and inorganic salts of phenyl derivatives of imidazobenzimidazole

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Academic editor: Oleg Gudyrev ♦ **Received** 20 February 2024 ♦ **Accepted** 25 April 2024 ♦ **Published** 30 June 2024

Citation: Zhukovskaya ON, Eliseeva NV, Vassiliev PM, Pridvorov GV, Grechko OYu, Morkovnik AS, Spasov AA (2024) Synthesis and pharmacological activity of various organic and inorganic salts of phenyl derivatives of imidazobenzimidazole. Research Results in Pharmacology 10(2): 119-133. https://doi.org/10.18413/rrpharmacology.10.465

Abstract

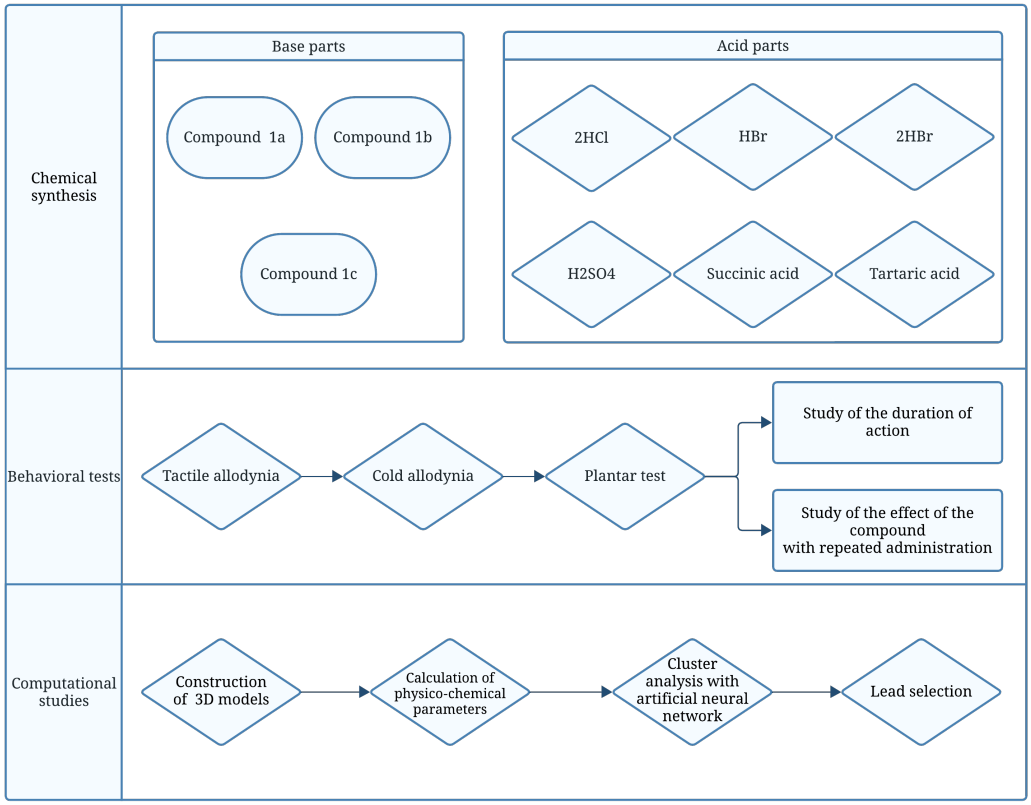
Introduction: The creation of new opioid analgesics that are devoid of the main undesirable effects – euphoria, respiratory depression, tolerance – is an important task for the treatment of pain syndrome. Eighteen salts of 9-pyrrolidinoethyl-(1a), 9-piperidinoethyl-(1b), and 9-morpholinoethyl-2-(4-fluorophenyl)benzo[d]imidazo[1,2-a]imidazole (1c) were synthesized and tested for kappa agonist and analgesic activity.

Material and Methods: Specific analgesic activity studies were conducted using models of nociceptive pain in the "Hot/Cold-plate" and "Plantar Test" tests, and in neuropathic pain against the background of sciatic nerve ligation. The relationship between the physicochemical and pharmacological properties of the compounds was studied *in silico* using quantum chemistry methods and artificial neural network technology.

Results: The most active compound identified was 4-(2-(2-(4-fluorophenyl)-benzo[d]imidazo[1,2-a]imidazol-9-yl)ethyl)morpholine dihydrochloride (1c.2HCl). EC_{50}, acute toxicity, and conditional therapeutic index values were calculated, which were 38.8 times superior to the kappa agonist U50488. It was established that compound 1c.2HCl exhibits a dose-dependent analgesic effect in the "Hot/Cold-plate" test, which is six times superior to that of butorphanol, maintaining a statistically significant effect for six hours in the "Plantar test" and without causing the formation of tolerance with a ten-day administration. In models of neuropathic pain syndrome, 1c.2HCl with a fourteen-day administration significantly reduced tactile and cold allodynia by three and 1.6 times, respectively, and was not inferior in activity to gabapentin. The kappa-opioidergic mechanism of the antinociceptive action of 1c.2HCl compound was confirmed. Quantum chemistry methods and artificial neural network technologies have shown that the high level of kappa-opioid agonist activity of hydrochlorides, in contrast to other salts, results in lower total energy production; therefore, there is a significant increase in energy during the formation of a salt supramolecular complex.

Conclusion: The most active compound was identified – 1c.2HCl, for which kappa-opioid activity *in vitro* and analgesic properties were revealed in various *in vivo* models. It has been shown that the compound does not cause the formation of tolerance and is not toxic.

Graphical abstract



Keywords

artificial neural networks, imidazobenzimidazole derivatives, kappa-opioid agonists, nociceptive and neuropathic pain, opioid analgesics, quantum chemical modeling

Introduction

Severe pain constitutes a significant component of numerous diseases and pathological conditions, sometimes substantially worsening the clinical prognosis for patients. In modern medical practice, various opioid and non-opioid analgesics are available for severe pain. However, in certain clinical scenarios, such as major trauma or heart attacks, the use of potent analgesics from the first class is necessary due to a high level of pain. Thus, opioids like morphine are considered being the most effective painkillers used in clinical settings (Stein 2020; Paul et al. 2021). Nevertheless, their clinical use is significantly limited due to severe side effects. Recently, the escalating medical utilization and abuse of opioids have led to a remarkable increase in deaths primarily associated with their irrational usage, resulting in what is called “a 21st-century opioid crisis”. This crisis has prompted the search for safer and more effective analgesics, including opioids. All clinically approved opioid analgesics are mu-opioid receptor agonists. This mechanism of action leads to their severe toxicity such as the development of tolerance and drug-dependence, constipation, and, most importantly, respiratory depression (Pasternak et al. 2020). Additionally, access to these analgesics remains challenging due to the current system of stringent government control of their usage, which also affects medical professionals (Kaminskaya 2022).

The development of new highly potent opioid analgesics without serious toxicity that can be observed in mu-opioid receptor agonists is potentially better to explore among ligands of the kappa subtype of opioid receptors. The rationale is that they do not initiate severe toxicity such as respiratory depression, pose lesser risks in case of overdose, and importantly, they do not trigger the reward pathway, indicating they lack narcotic potency (Edinoff et al. 2021).

Analysis of the structures of the known ligands of kappa-opioid receptors showed that many of them are derivatives of nitrogen-containing heterocycles, in particular, unfused and fused benzimidazoles (Lifanova et al. 2023). Examples of type 1 benzimidazole agonists include phenothiazine benzimidazole RP-61127 and benzimidazole SR-14136 (Cahil et al. 2021).

The development of highly selective kappa agonists is based on scaffolds that are bioisosteric with benzimidazole. One such scaffold, benzithiazoline, is found in ligands like 2-arylbenzthiazoline SA14867. The development of kappa-opioid receptor agonists within the condensed benzimidazole series was facilitated by an analysis of databases containing pharmacophores specific to the activity under consideration obtained *in silico*. This analysis enabled the identification of certain structural types of benzimidazoles containing a 2-aminobenzimidazole scaffold (Sasmal et al. 2015) as highly selective agonists of the kappa opioid receptor. Original *in vitro* studies of several structural types, particularly those containing a 2-aminobenzimidazole scaffold integrated into tricyclic systems were conducted at the Research Institute of Physical and Organic Chemistry of the Southern Federal University (located in Rostov-on-Don, Russia). They were conducted in collaboration with the Department of Pharmacology and Bioinformatics at Volgograd State Medical University. These studies aimed to identify several compounds exhibiting high selectivity towards kappa-opioid receptors (Spasov et al. 2018, Kalitin et al. 2023), along with a pronounced and naloxone-reversible analgesic and anticonvulsant effects *in vivo* (Kalitin et al. 2017, 2018; Vasil'ev et al. 2017; Mohammed et al. 2023). A standard model of behavioral nociceptive reactions in the 'tail flick' test (Spasov et al. 2021) was used. This led to further exploration and investigation of compounds exhibiting a kappa receptor profile of pharmacological activity among benzimidazole derivatives.

The objective of this study was to synthesize salts with various acids, both inorganic and organic, of a subset demonstrating such activity and basic properties. Specifically, the study focused on 2-(4-fluorophenyl)benzo[d]imidazo[1,2-a]imidazoles with N9-substituents of 2-dialkylaminoethyl types, wherein the amino group is integrated into cyclic systems of morpholine, piperidine, and pyrrolidine. Additionally, the study aimed to assess the influence of the nature of the acid salts incorporated in their composition on their kappa-agonist and specific analgesic activity.

In aqueous solutions, salts of organic compounds, as previously assumed, form ion pairs, contact or solvate-separated. Later studies established that in solution these salts exist in the form of supramolecular complexes, stabilized by electrostatic and exchange interactions, in which the formal charges of the ions are significantly reduced, primarily due to the donation of electron density from the anion to the cation (Steed and Atwood 2022). If complexes with bimolecular stoichiometry exhibit sufficient stability within a living organism, it is likely that they may interact with appropriate receptors as a unified entity. Therefore, potentially modulating pharmacological activity to a varying extent was compared to the ligand bases. This modulation may be attributed in part to the significant influence of the electrostatic factor on the ligand's affinity for the receptor and its activation efficiency (Lin et al. 2022). When compared to fluoride and chloride ions, larger anions can also pose steric hindrances for the entry of supramolecular complexes formed with their involvement into the specific binding site's space. Alternatively, in the absence of such hindrances, they may stabilize the resultant guest-host structure through polycentric electrostatic interactions, which may include deprotonated carboxyl groups.

Given this, the following working hypothesis was formulated: the kappa agonist and analgesic activities of the studied salts should be determined by the stability of the corresponding supramolecular complexes, their sizes, and the influence of acidic residues on the interaction affinity with the kappa receptor.

To test this hypothesis, neural network models to examine the dependencies of kappa-agonist and analgesic activities on the quantum-chemical and physicochemical parameters of the salts under investigation were developed. Subsequently, based on the established models, we identified molecular descriptors conducive to the manifestation of these activities.

Materials and Methods

Experimental animals

The studies were carried out on mature male rats weighing 200-230 g, male mice weighing 20-25 g, and male Chinchilla rabbits weighing 2.5-3.5 kg. They were obtained from Rappolovo Laboratory Animal Nursery of the Russian Academy of Medical Sciences (Saint-Petersburg, Russia). Before the start of the experiment, the animals were subjected to an adaptive quarantine for 14 days in the vivarium of the Department of Pharmacology and Bioinformatics of Volgograd State Medical University, followed by a visual assessment of the condition and culling of individuals with deviations. The animals had 24-hours access to feeders and drinkers ad libitum, and were kept in standardized vivarium conditions (Resolution No. 51 of August 29, 2014 "On Approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the design, equipment and maintenance of experimental biological clinics (vivariums)", Directive of the European Parliament and the Council of the European Union 2010/63/EU of September 22, 2010 "On the protection of animals used for scientific purposes"). Animals were kept in groups of 5 individuals, with an adjustable combined light regime (12/12 h) and temperature 20-22°C. 12 hours before the experiment, the animals were deprived of food with free access to water. The experiments were approved by the Regional Research Ethics Committee of the Volgograd region (Registration number IRB 00005839 IORG 0004900 (OHRP), Minuters No. 2077-2018 dated October 30, 2018).

Chemical synthesis

The synthesis of 9H-benzo[d]imidazo[1,2-a]imidazole salts 1a-c·nHY (n=1,2) containing a para-fluorophenyl group directly linked to position 2 of imidazobenzimidazole system is presented in Figure 1.

In addition to the heterocyclic backbone and para-fluorophenyl group, these compounds contain N-formacophore groups linked to the N^9 nitrogen atom. The synthesis of salts 1·nHY included the stages of quaternization of 1-dialkylaminoethyl-2-aminobenzimidazoles 3a-c with 2-bromo-1-(4-fluorophenyl)ethanone-1 and imidazole-type cyclization of the resulting hydrobromides of 2-iminobenzimidazolines 2a-c.

The best results were obtained under the low temperature (not exceeding 40°C). Under these conditions, monohydrobromides3a-c·HBr are formed in almost quantitative yield and can be used for the

cyclization stage without additional purification. Subsequent thermal cyclization of these hydrobromide leads to the tricyclic base forms 1a-c, from which the majority of final salts are then prepared. In this case, monohydrobromides 1a-c with HBr were obtained directly as products of thermal recyclization, and the corresponding dibromides were obtained as products by treating the monobromides in hot saturated alcohol or aqueous alcohol solutions with 47% HBr, adjusting the pH to 1. After 1-2 hours, the precipitate of dihydrobromide is filtered off. Sulfates, succinates and tartrates are obtained by treating solutions of bases 1a-c with sulfuric, succinic or tartaric acids, respectively.

IR spectra (ν/cm^{-1}) of the compounds obtained were recorded on a Varian Excalibur 3100 FT-IR spectrophotometer (Varian, USA), using the method of attenuated total reflection in powder; ^{1}H NMR spectra were recorded on Varian Unity-300 (Varian, USA) spectrometer in DMSO-d6. Chemical shifts for ^{1}H are given relative to the signals of residual protons of a deuterated solvent (δ2.49). Melting points were measured on a Fisher-Johns Melting Point Apparatus (Fisher Scientific, USA). Elemental analysis was carried out using a classical method (Gelman et al. 1987).

Reaction progress and purity of synthesized compounds were monitored by TLC (plates with Al_2O_3 III degree of activity, eluent $CHCl_3$, visualization with iodine vapors in a moist chamber).

General procedure for synthesizing of 2-amino-3-[(2-(4-fluorophenyl)-4-yl)-2-oxoethyl]-1-R-1H-benzimidazolium bromides 2a-c

To a hot solution of the corresponding amine 3 (3 mmol) in acetone at room temperature of 2-bromo-1-(4-fluorophenyl)ethan-1-one (3 mmol) was added. The mixture was stirred until it dissolved, heated to the beginning of precipitation of the quaternary salt, and after that it was kept in a hot water bath (40-45°C) for 30 minutes or 6-8 hours at 20ºC. The quaternary salt was filtered off and washed thoroughly with acetone. The resulting chromatographically pure salts were dried in air and used in the next step without further purification. The structure of salts 2a-c was confirmed by their transformation into corresponding benzo[d]imidazo[1,2-a]imidazoles, as well as by spectroscopic data.

2-Amino-3-(2-(4-fluorophenyl)-2-oxoethyl)-1-(2-(pyrrolidin-1-yl)ethyl)-1H-benzimidazolium bromide (2a)

Yield 96 %, m.p. 179–180 °C (dec.). IR spectrum, ν/cm^{-1}: 3210, 3247 (NH_2), 1688 (C=O). Found (%): C 56.38; H 5.52; Br 17.75; F 4.40; N 12.64. $C_{21}H_{24}BrFN_4O$. Calculated (%): C 56.38; H 5.41; Br 17.86; F 4.25; N 12.52. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.64-1.68 (m, 4H, (C-CH_2)$_2$-C); 2.54-2.80 (m, 4H, N(CH_2)$_2$, cyclic); 2.86-3.07 (m, 2H, CH_2-pyrrolidinyl); 4.31-4.55 (m, 2H, NCH_2CH_2-pyrrolydinyl); 6.03 (s, 2H, CH_2CO); 7.15-7.67 (m, 6H, H_{Ar}); 8.12-8,28 (m, 2 H, H_{Ar}); 9.0 (c, 2H, N^+H_2).

2-Amino-3-(2-(4-fluorophenyl)-2-oxoethyl)-1-(2-(piperidin-1-yl)ethyl)-1H-benzimidazolium bromide (2b)

Yield 92 %, m.p. 185–186°C. IR spectrum, n/cm^{-1}: 3210, 3247 (NH_2), 1688 (C=O). Found (%): C 57.38; H 5.75; Br 17.25; F 4.18; N 12.32. $C_{22}H_{26}BrFN4O$. Calculated (%): C 57.27; H 5.68; Br 17.32; F 4.18; N 12.14. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.31-1.60 (m, 6H, C-(CH_2)$_3$-C); 2.60-2.78 (m, 4H, CH_2NCH_2,); 2.93-3.36 (m, 2H, CH_2N⊖)$_2$piperidin); 4.22-4.43 (m, 2H, NCH_2); 5.98 (s, 2H, CH_2CO); 7.20-7.64 (m, 6H, H_{Ar}); 8.11-8.27 (m, 2 H, H_{Ar}); 9.05 (c, 2H, N^+H_2).

2-Amino-3-(2-(4-fluorophenyl)-2-oxoethyl)-1-(2-(morpholine-1-yl)ethyl)-1H-benzimidazolium bromide (2c)

Yield 97%, m.p. 206-207°C. IR spectrum, ν/cm^{-1}: 3215, 3245 (NH_2), 1685 (C=O). Found (%): C 54.40; H 5.18; Br 17.15; F 4.08; N 12.22. $C_{22}H_{26}BrFN_4O$. Calculated (%): 54.44; H 5.22; Br 17.24; F 4.10; N 12.09. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm, J (Hz): 2.42-2.61 (m, 4H, N(CH_2)$_2$ cyclic); 2.71 (t, 2H, CH_2N, J=6.8); 3.50 (br. t., 4H, CH_2OCH_2); 4.42 (t, 2 H, N-CH_2, J=6.7); 6.08 (c, 2H, CH_2CO), 7.17-7.44(m, 6H, H_{Ar}); 7.53 (d, 2H, $H^{4,7}$, J=7.2); 8.20 (dd, 2H, $H^{5,6}$, J=9.5, J=8.5); 9.11 (br.s., 2H, N^+H_2)

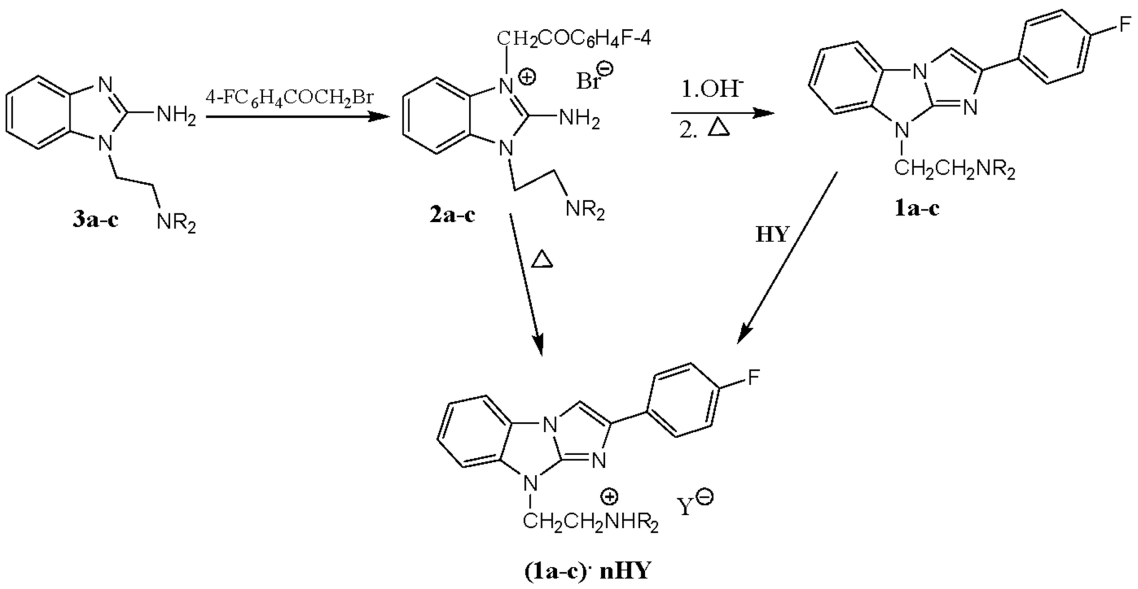


Figure 1. Synthesis of 2-(4-fluorophenyl)-9-(2-dialkylaminoethylbenzo[d]imidazo[1,2α]imidazole salts. HY: HBr, HCl, H_2SO_4, $(CH_2COOH)_2$, $[CH(OH)COOH]_2$, n=1,2.

The General procedure for synthesizing of 2-(4-fluorophenyl)-9R-benzo[d]imidazo[1,2-a]imidazole monohydrobromides 1a-c·HBr

Suspension of quaternary salt 2a-c (9 mmol) in 80 mL of water was refluxed until completion of the reaction (2-4 hours, control - TLC). In this case, the initial salt gradually dissolves and a salt of final imidazobenzimidazole 1 is formed instead. After completion of the reaction, the reaction mixture was cooled, the precipitate of hydrobromide filtered off, washed with cold water and dried in air. Then chromatographically pure salt was recrystallized from ethyl alcohol.

2-(4-Fluorophenyl)-9-(2-(pyrrolidin-1-yl)ethyl)-benzo[d]imidazo[1,2-a]imidazole hydrobromide (1a·HBr)

Yield 94, m.p. 226°C (EtOH) (dec.). Found (%): C 58.68; H 5.21; Br 18.68; F 4.35; N 13.08. $C_{21}H_{21}FN_4HBr$. Calculated (%): C 58.75; H 5.16; Br 18.61; F 4.43; N 13.05. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm, J (Hz): 2.27 (m, 4H, C-$(CH_2)_2$-C); 3.43 (m, 2H, NCH_2), 3.74-3.78 (m, 4H, $N(CH_2)_2$, cyclic), 5.20 (t, 2H, N^9CH_2, J=6.0), 7.32-7.93 (m, 6H, H_{Ar}), 8.05-8.31 (m, 2H, $H^{5,8}$), 8.51 (s, 1H, H^3), 12.03 (br. s, 1H, N^+H).

2-(4-Fluorophenyl)-9-(2-(piperidin-1-yl)ethyl)-benzo[d]imidazo[1,2-a]imidazole hydrobromide (1b·HBr)

Yield 98 %, m.p. 245°C (EtOH) (dec.). Found (%): C 59.68; H5.40; Br 18.07; F4.25; N 12.60. $C_{22}H_{23}FN_4$. HBr. Calculated (%): C 59.60; H 5.41, Br 18.01; F 4.29; N 12.61. ^{1}H NMR spectrum (300 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.16-1.96 (m, 6H, C-$(CH_2)_3$-C, cyclic); 2.33-3.11 (m, 6H, $N(CH_2)_2$, cyclic + NCH_2); 4.12 (t, 2H, N^9-CH_2, J=5.9); 7.19-8.20 (m, 9H, H_{Ar}); 11,84 (br.s, 1H, N^+H).

4-(2-(2-(4-Fluorophenyl)-benzo[d]imidazo[1,2-a]imidazole-9-yl)ethyl)morpholine hydrobromide (1c·HBr)

Yield 99 %, m.p. 273-275°C (EtOH-H2O). Found (%): 56.60; H 5.06; Br 17.90; F 4.18; N 12.64. C21H21FN4O · HBr. Calculated (%): C 56.64; H.4.98; Br 17.94; F 4.27; N 12.58. 1H NMR spectrum (300 MHz, DMSO-d6), δ, ppm, J (Hz): 3.28 (br.s., 4H, N(CH2)2, cyclic); 3.72-4.04 (m, 6H, NCH2+O(CH2)2); 5.03 (s, 2H, N9-CH2); 7.14-7.67 (m, 6H, HAr); 7.82 (d, 1H, H5, J=7.8); 8.12 (s, 1H, H3).

General procedure for synthesizing of dihydrobromides 1a-c·2HBr

Hot saturated alcoholic or hydroalcoholic solution of corresponding monohydrobromide (5 mmol) was acidified with conc. HBr to pH 1-2. After 1 hour dihydrobromide that precipitated during cooling is filtered off, washed thoroughly with acetone and dried at 105-110°C.

Dihydrobromide (1a.2HBr). Yield 89%, m.p. 295°C dec.(EtOH). Found (%): C 49.38; H 5.00; Br 31.26; F 3.76; N 11.00. C21H21FN4 2HBr. Calculated (%): C 49.43; H 4.54; Br 31.33; F 3.72; N 10.98.

Dihydrobromide (1b.2HBr). Yield 88 %, m.p. 298-300°C dec. (EtOH). Found (%): C 50.46; H 4.77; Br 30.41; F 3.65; N 10.71. C22H23FN4 · 2HBr. Calculated (%): C 50.40; H 4.81; Br 30.48; F 3.62; N 10.69.

Dihydrobromide (1c.2HBr). Yield 92 %, m.p. 280-281°C dec. (EtOH). Found (%): C 47.98; H 4.37; Br 30,30; F 3,58; N 10,71. C21H21FN4O · 2HBr. Calculated (%): C 47.93; H 4.41; Br 30.37; F 3.61; N 10.65.

General procedure for synthesizing of 2-(4-fluorophenyl)-9-dialkylaminoethyl-benzo[d]imidazo[1,2-a]imidazole dihydrochlorides 1a-c·2HCl

A mixture of corresponding 2-amino-1-dialkylaminoethylbenzimidazole 3 (4 mmol), 2-chloro-1-(4-fluorophenyl)etan-1-one (4 mmol) and 15 mL of dry acetonitrile was boiled until completion of the quaternization reaction (~8-10 hours, control – TLC). Then solvent was evaporated on a rotary evaporator and the residue, 2-amino-3-[(2-(4-fluorophenyl)-4-yl)-2-oxoethyl]-1-dialkylaminoethyl-1H-benzimidazolium chloride, cyclized in 100 mL of boiling water, monitoring the progress of the reaction using TLC. At the end of the reaction, two-thirds of the water was distilled off; the remaining mass was acidified with conc. HC land leaves for a day in the refrigerator. Precipitation of dihydrochloride was filtered off, squeezed thoroughly on the filter, washed with ice water and acetone.

2-(4-Fluorophenyl)-9-(2-(pyrrolidin-1-yl)ethyl)-benzo[d]imidazo[1,2-a]imidazole dihydrochloride (1a·2HCl)

Yield 90%, m. p. 257-258°C dec. (i-PrOH). Found (%): C 59.79; H 5.60; Cl 16.78; F 4.44; N 13.39. $C_{21}H_{21}FN_4$·2HCl. Calculated (%): C 59.86; H 5.50, Cl 16.83; F 4.51; N 13.30.

2-(4-Fluorophenyl)-9-(2-(piperidin-1-yl)ethyl)-benzo[d]imidazo[1,2-a]imidazole dihydrochloride (1b·2HCl)

Yield 91%, m. p. 260-262°C (EtOH). Found (%): C 60.73; H 5.73; Cl 16.37; F 4.33; N 12.84.$C_{22}H_{23}FN_4$·2HCl. Calculated (%): C 60.69; H 5.79; Cl 16.29; F 4.36; N 12.87.

4-(2-(2-(4-Fluorophenyl)-benzo[d]imidazo[1,2-a]imidazole-9-yl)ethyl)morfoline dihydrochloride (1c·2HCl)

Yield 89 %, m. p. 278-280°C dec. (70% EtOH). Found (%): C 57.62; H 5.37; Cl 16.12; F 4.41; N 12.88. $C_{21}H_{21}FN_4O$·2HCl. Calculated (%): C 57.67; H 5.30; Cl 16.21; F 4.34; N 12.81.

General procedures for the synthesis of bases of 2-(4-fluorophenyl)-9-dialkylaminoethyl-benzo[d]imidazo[1,2-a]imidazoles (1a-c)

A. Quaternary salt 2 (10 mmol) was cyclized in boiling water (2-4 hours, control TLC). Then the reaction was cooled, made alkaline by addition of a solution of NH_4OH or N_2CO_3 and base 1a extracted with chloroform. The extract passed through a layer of Al_2O_3 (eluent $CHCl_3$). Upon evaporation of $CHCl_3$ from chromatographically pure snow – white crystals were obtained from the eluate, which could be used for synthesis of various salts without additional purification.

B. Hydrobromide 1·HBr was treated with a 22% solution of NH_4OH for 0.5-1 hour. After that, the precipitate of base form had been filtered off, washed with water, dried first in air and then in a desiccator over KOH.

2-(4-Fluorophenyl)-9-(2-(pyrrolidin-1-ylethyl)benzo[d]imidazo[1,2-a]imidazole (1a)

Yield 87.5%, m.p. 98-99°C. Found (%): C 72.32; H 6.12; F 5.52; N 16.04. $C_{21}H_{21}FN_4$. Calculated (%): C 72.39; H 6.08; F 5.45; N 16.08.

2-(4-Fluorophenyl)-9-(2-(piperidin-1-yl)ethyl)-9H-benzo[d]imidazo[1,2-a]imidazole (1b)

Yield 91 %, m.p. 93-94°C. Found (%): C 72.96; H 6.35; F 5.18; N 15.49. $C_{22}H_{23}FN_4$. Calculated (%): C 72.90; H 6.40; F 5.24; N 15.46.

4-(2-(2-(4-Fluorophenyl)-9H-benzo[d]imidazo[1,2-a]imidazole-9-yl)ethyl)morpholine (1c)

Yield 93,5%), m.p. 91-92°C. Found (%): C 69.28; H 5.77; F 5.14. $C_{21}H_{21}FN_4O$. Calculated (%): C 69.21; H 5.81; F 5.21; N 15.37.

Synthesis of sulfates (1a-c·H_2SO_4)

A solution of 2 mmol of corresponding base 1 in acetone was acidified by solution of H_2SO_4 in acetone to pH 1. The precipitate that forms was filtered off after 1 hour, washed with acetone until the washing solvent is neutral, dried at 105-110°C.

Sulfate (1a· H_2SO_4). Yield 99 %, m.p. 273-275°C (dec. EtOH). Found, %: C 56.42; H 5.25; F 4.30; N 12.48; S 7.22. $C_{21}H_{21}FN_4 \cdot H_2SO_4$. Calculated, %: C 56.49; H 5.19; F 4.25; N 12.55; S 7.18.

Sulfate (1b· H_2SO_4). Yield 95%, m.p. 260-262°C (dec. EtOH). Found, %: C 57.44; H 5.40; F 4.18; N 12.10; S 7.03. $C_{22}H_{23}FN_4 \cdot H_2SO_4$.

Sulfate (1c· H_2SO_4). Yield 96%, m.p. 274-276°C (dec. EtOH). Found, %: C 54.49; H 5.08; F 4.05; N 12.18; S 6.87. $C_{21}H_{21}FN_4O \cdot H_2SO_4$. Calculated, %: C 54.54; H 5.01; F 4.11; N 12.11; S 6.93.

Synthesis of succinates (1a-c·$C_4H_6O_4$)

The mixture of equimolar amounts of the corresponding base 1 (2 mmol) and succinic acid (2 mmol) with hot acetone was boiled for 30 minutes and then left to stand at room temperature. After 2-3 hours, the precipitate that forms is filtered off, washed with acetone and dry first in air and then at 105°C.

Succinate (1a$C_4H_6O_4$). Yield 85%, m.p. 167-169°C (dec. EtOH). Found, %: C 64.42; H 5.77; F 4.14; N 11.96. $C_{21}H_{21}FN_4 \cdot C_4H_6O_4$. Calculated, %: C 64.37; H 5.83; F 4.17; N 12.01.

Succinate (1b $C_4H_6O_4$). Yield 88%, m.p. 171-172°C (dec. EtOH). Found, %: C 65.07; H 6.02; F 3.88; N 11.70. $C_{21}H_{23}FN_4 \cdot C_4H_6O_4$. Calculated, %: C 64.99; H 6.08; F 3.95; N 11.66.

Succinate (1c $C_4H_6O_4$). Yield 81%, m.p. 154-155°C (dec. EtOH). Found, %: C 62.18; H 5.70; F 3.88; N 11.67. $C_{21}H_{21}FN_4O \cdot C_4H_6O_4$. Calculated, %: C 62.23; H 5.64; F 3.94; N 11.61.

Synthesis of tartrates (1a-c$C_4H_6O_6$)

To a solution of 2 mmol of corresponding base 1 in acetone, prepared by heating, boiling acetone solution of an equimolar amount of tartaric acid (2 mmol) is added. The solution is boiled for 10 minutes, cooled and after 1 hour precipitate of the corresponding tartrate was filtered, washed with acetone, dried first in air and then at 80-90°C.

Tartrate (1a$C_4H_6O_6$). Yield 86.5%, m. p. 176-177°C (dec. EtOH). Found, %: C 60.18; H 5.52; F 3.74; N 11.30. $C_{21}H_{21}FN_4 \cdot C_4H_6O_6$. Calculated, %: C 60.23; H 5.46; F 3.81; N 11.24.

Tartrate (1b$C_4H_6O_6$). Yield 80.5%, m.p. 187-188°C (dec. EtOH). Found, %: C 61.00; H 5.65; F 3.67; N 11.00. $C_{22}H_{23}FN_4$ $C_4H_6O_6$. Calculated, %: C 60.93; H 5.70; F 3.71; N 10.93.

Tartrate (1c$C_4H_6O_6$). Yield 85.6%, m.p. 141-143°C (dec. EtOH). Found, %: C 52.28; H 5.34; F 3.75; N10.80. $C_{21}H_{21}FN_4O \cdot C_4H_6O_6$. Calculated, %: C 58.36; H 5.29; F 3.69; N 10.89.

Experimental design

The impact of salts derived from 9-substituted derivatives of 2-(4-fluorophenyl)benzo[d]imidazo[1,2-a]imidazole on kappa-opioid receptors was investigated *in vitro* using a platelet activation model employing small-angle light scattering in a saline medium (Tris-HCl buffer, pH 7.4) with a laser particle size analyzer "LASKA-1K" (TMK-Engineering, Russia) at a concentration of test salts of 1×10^{-4} M. The selective kappa agonist U-50488 (Sigma, USA) was used as the reference drug. The degree of kappa-agonist activity of the substances under study was assessed by the change in the light transmission signal compared to the control (Δ%) (Spasov et al. 2011). The specificity of the kappa-opioid action of the investigated substances was evaluated under conditions of a 2-minute preincubation with the selective kappa antagonist norbinaltorphimine (norBNI) at a final concentration of 1×10^{-4} M (Sigma, USA).

Considering that the analgesic activity of these salts may depend not only on the nature of the corresponding bases but also on the molecules integrated into the acid salts, we assessed the thresholds of the nociceptive reaction in the electrical stimulation test of the tail root ("tail-flick") using an ELS-device 2 (Russia), operating in the mode of generating rectangular pulses with a frequency of 100 Hz and a duration of 10 ms (Yam et al. 2020).

The threshold magnitude for the pain was characterized by the voltage, measured in volts, at which the tail-flick reflex occurred. Test substances and the reference drug, butorphanol tartrate (Moscow Pharmaceutical Factory, Russia), were administered intraperitoneally at doses of 0.01, 0.1, and 1 mg/kg (at a rate of 1 mL of aqueous solution per 1 kg of body weight). Control animals received an equivalent volume of distilled water. To determine the time dependence of the effect, electrical stimulation was conducted at intervals of 20, 60, 120, 180, 240, 300, 360, and 420 minutes post-compound administration. Test results were evaluated using the method developed by Abboud et al. (2021).

The acute daily toxicity of the studied compounds was studied on white outbred male mice weighing 18-22 g with intraperitoneal administration. The LD_{50} value was calculated using the Prozorovsky method (Prozorovsky et al. 1978). The therapeutic indices for the test substances and the reference drug were calculated as the ratio of LD_{50} to the concentration of the substance corresponding to the EC_{50} value.

For the most active salt, pharmacological activity was additionally studied at different levels of the nociceptive reaction: spinal, supraspinal, in a model of neuropathic pain. As a result, the risk of developing tolerance was determined.

For the assessment of nociceptive pain, the Plantar Test apparatus (UgoBasile S.r.l., Varese, Italy) operating at a radiation power of 40% was used. Analgesic activity evaluation was based on the extension of the latency period of the nociceptive response to a painful stimulus (infrared radiation), observed as licking or withdrawal of the paw exposed to the radiation, indicating the substance's spinal action level. Five minutes prior to testing, animals were introduced to the new environment within the apparatus, followed by the measurement of the baseline reaction time. Subsequently, animals received an intraperitoneal injection of the test substance at a dose of 10 mg/kg, and testing was repeated after 30 minutes. To assess the compound's

duration of action, the experiment was repeated every 30 minutes for 3.5 hours. For each measurement, the average duration of the latency period along with ± standard error of the mean were calculated. To investigate the development rate of substance tolerance, the experiment was conducted daily for 11 days (Di Cesare Mannelli et al. 2015).

Mean latency periods were calculated for each group ± standard error of the mean for each day. Measurements were taken twice: before compound administration and 30 minutes afterward.

The assessment of thermal nociceptive pain at the supraspinal level was conducted using a "Hot/Cold-plate" apparatus (UgoBasile, Italy, 2016) – featuring a thermostatically controlled, electrically heated surface encircled by a transparent cylinder with a diameter of 15 cm, maintained at 55°C. Temperature sensitivity was determined by observing motor reactions of distress during thermal stimulation, specifically licking of the hind paw pads. Test substances and the reference drug butorphanol tartrate were administered intraperitoneally within the dosage range of 0.001-10 mg/kg. To confirm the kappa-opioid specificity of action, tests were conducted with the kappa-opioid receptor antagonist norBNI (10 mg/kg subcutaneously, administered 20 minutes prior to the test substance). To minimize the risk of thermal damage to the paws, the maximum experimental duration did not exceed 60 seconds. If an animal showed no activity during the 60-second interval, the latency reaction time was recorded as 60 seconds, a key parameter for calculating the MPE (maximum possible effect).

The calculation was carried out according to the formula (1):

$$MVE = \frac{LP_e - LP_c}{60 - LP_c} \times 100\% \quad (1),$$

where LPe – latent period of nociceptive reaction in the experimental group; LPc - latent period of nociceptive reaction in the control group; 60 – maximum exposure time.

The criterion for assessing the analgesic effect was defined as a statistically significant increase in the latency period of the reaction following the administration of the test product compared to the control group.

Neurogenic pain syndrome was modeled using ligation of the sciatic nerve by applying 3 ligatures around the sciatic nerve (Sadler et al. 2022). Chloral hydrate 400 mg/kg intraperitoneally was used as anesthesia for surgical procedures. The animals were divided into 5 groups:

Group 1 – "falsely operated" (n=6) – the animals were isolated from the sciatic nerve without ligating it, and a solvent was administered at a dose of 0.1 mL/100 g of weight;

Group 2 – "neuropathy" (n=6) – animals with right-sided ligation of the sciatic nerve were injected with a solvent – distilled water;

Group 3 – "neuropathy + gabapentin" (n=6) – animals with right-sided ligation of the sciatic nerve were administered gabapentin (10 mg/kg);

Group 4 – "neuropathy + compound" (n=6) – animals with right-sided ligation of the sciatic nerve were injected with the studied compound (1 mg/kg);

Group 5 – "neuropathy + morphine" (n=6) – animals with right-sided ligation of the sciatic nerve were injected with morphine (1 mg/kg).

The studied compounds and solvent (distilled water) were administered intraperitoneally twice a day, starting from the day of surgery and for the next 14 days.

The experiments were conducted using the following methods recommended for the study of neuropathic pain (Sadler et al. 2022):

1. Tactile allodynia test;
2. Thermal hyperalgesia test ("Hot/Cold-plate").

Tactile allodynia in rats was evaluated by measuring the pressure at which the animals withdrew their right hind paw, thereby minimizing the influence of escalating stimuli. VonFrey hairs, comprising 20 monofilaments of nylon threads with varying diameters affixed to plastic handles, were applied to the animal's right hind paw from below (operated). The experiment commenced with the monofilament marked – 4.31. If there was no response after five touches, the next monofilament with greater force was sequentially administered. The test results were measured upon reaching the filament labeled 5.18 or after four tests following the initial positive response. The 50% paw withdrawal threshold was determined by incrementally increasing and decreasing the stimulus strength using the "UP-AND-DOWN" method (Sadler et al. 2022).

The 50% pain threshold was calculated using the formula (2):

$$A(50\%) = \frac{10^{(Xf + K\delta)}}{10000} \quad (2),$$

where Xf – the value of the final Frey hair (in log units); K – standard coefficient value; δ – average difference between stimuli (in log units).

Thermal (cold) allodynia was examined using a Hot/Cold-plate apparatus (UgoBasile, Italy) with the plate cooled to 5°C. This test considered the duration throughout the entire observation period (2 minutes) that the animals endured the cold temperatures, with all limbs resting on the cooled surface (Modi et al. 2023).

The criterion for the analgesic effect was considered to be a statistically significant decrease in the time of holding the limb on weight under the influence of the test substances in comparison with the control group.

The statistical significance of the differences was calculated based on two-way analysis of variance (2 way ANOVA). Graphing and mathematical calculations were performed using the GraphPadPrism 8 (GraphPad Software, Inc., USA) and Microsoft Office Excel 2007 (Microsoft Corporation, USA) software package.

Design of computational studies

For the calculations, 2D structures of the 18 synthesized salts were utilized, considering the effects of protonation/deprotonation of the corresponding molecular fragments. The computational studies encompassed several stages:

1. Construction of optimized 3D models of the studied compounds employing molecular mechanics and quantum chemistry methods.
2. Calculation of energetic and physico-chemical parameters of the studied molecules using quantum chemistry methods.
3. Nonparametric Spearman correlation analysis and identification of significant molecular parameters for subsequent neural network modeling.
4. Utilization of artificial neural network technology

to construct models illustrating the dependence of kappa-agonist and analgesic activities on significant molecular parameters of the studied compounds.

5. Sensitivity analysis of neural network models to identify the most crucial variables influencing the kappa-agonist and analgesic activity of the substances under study.

6. Cluster analysis of activity indicators.

7. Determination of the influence of the most important variables on the high kappa-agonist and analgesic activity of the analyzed salts.

The construction of optimized 3D models of connections was carried out in three stages.

Initially, for each structure, a step-by-step conformational analysis was conducted using molecular mechanics methods via the Chem3D program in the MM2 force field, considering conformationally mobile bonds, and minimizing the steric energy of the molecule at each step. Subsequently, the geometry of the resulting 3D models was optimized using the AM1 semi-empirical quantum chemical method via the HyperChem program. At the final stage, the constructed conformation underwent further optimization using the semi-empirical quantum chemical method PM3, also utilizing the HyperChem program.

The construction of optimized 3D models was performed for all 18 investigational salts, as well as for three structures of bases and five acids constituting the studied salts, aimed at subsequent calculation of the stabilization energies of the corresponding supramolecular complexes.

Calculation of energetic and physical-chemical parameters of compounds. At the level of the PM3 method, using the constructed optimized 3D models of molecules in the HyperChem program, the following characteristics of the structures were calculated:

· E_{tot} – the total energy of formation of the entire molecule, eV;

· ΔE_{tot1} – energy of formation of salt 1.nHY (n=1.2) taking into account the energy of the acid residue (the difference between two total energies of formation: the molecule under study and the starting base), eV;

· ΔE_{tot2} – energy of salt formation without taking into account the energy of the acid residue (the difference between the three total energies of formation: the molecule under study, the initial base and the initial acidic residue), eV;

· E_{HOMO} – energy of the highest occupied molecular orbital, eV;

· E_{LUMO} – energy of the lowest unoccupied molecular orbital, eV;

· ΔE_{HL} – energy difference between the highest occupied and lowest unoccupied molecular orbitals, eV;

· LogP – logarithm of the distribution coefficient, arb. units;

· V – the volume of the molecule, $Å^3$;

· S – surface area of the molecule, $Å^2$;

· μ – dipole moment, D;

· MR – molecular refraction, $Å^3$.

Nonparametric Spearman correlation analysis

Conducted using the Statistica program. Spearman's correlation coefficient R_{Sp} reflects the degree of monotonic interdependence of two variables (continuous, but not necessarily linear). However, the relationship between two variables may not always be monotonic; it may even be discrete. Therefore, in this study, a molecular parameter was deemed significant if the Spearman correlation coefficient RSp reflected a discernible trend in the relationship between kappa opioid agonist or analgesic activity measures and any molecular characteristic, corresponding to a significance level of $p<0.2$. For kappa-opioid agonist activity, seven indicators were found to be significant: E_{tot}, ΔE_{tot1}, ΔE_{tot2}, E_{HOMO}, ΔE_{HL}, V, MR; for analgesic activity, four indicators were significant: E_{tot}, ΔE_{tot1}, ΔE_{tot2}, μ.

Neural network modeling

Conducted using the Statistica program. In accordance with Kolmogorov's theorem (Ismayilova and Ismailov, 2024), a two-layer artificial neural network (ANN) can approximate dependencies of any complexity. Therefore, in this study, a two-layer MLP k-m-l(f1, f2) perceptron was selected as the ANN architecture, where *k* represents the number of input neurons (in this case, parameters of connection structure significant for the analyzed activity); *m* denotes the number of hidden neurons, varied by the program during the learning process (ranging from 3 to 12); *l* indicates the number of output neurons, set to one for regression models (in this case, representing the predicted activity); f1 and f2 represent activation functions (Identity, Logistic, Tanh, Exponential) for the hidden and output layers, respectively. The program conducts pairwise search of these functions during the training process.

Regression neural network models were calculated separately for kappa agonist and analgesic activities. In each cycle, 4000 neural networks were trained, from which the program automatically selected the top 1000. Upon completing training for a specific activity, the best model was manually chosen from the 1000 top neural networks based on overall training and testing accuracy metrics. Model accuracy was evaluated using a random rolling control method, where the test sample was generated using a random number generator. The accuracy of constructed regression dependencies was assessed using correlation coefficients R calculated on the training, testing, and combined samples.

Sensitivity analysis of neural network models was conducted using the Statistica program. Calculated values of neuronal sensitivity were normalized by their sum, and highly sensitive neurons were identified based on the relative values. These neurons reflected the most crucial molecular characteristics influencing the kappa-agonist and analgesic activities of the substances under study.

Cluster analysis of activity indicators was performed using the Statistica program employing the k-means method. This involved dividing the values of each of the two activities into two clusters and evaluating the statistical reliability of the clustering outcomes.

Determining the influence of variables on high activity levelsImidazo[1,2-a]benzimidazole salt

Conducted using the Statistica program and the Excel data analysis package. The normality of distribution for the four most crucial molecular characteristics, identified during the sensitivity analysis of neural networks, was assessed using the Shapiro-Wilk test. Subsequently, employing the Student's t-test, the statistical significance of differences between the two mean values of compound parameters within clusters exhibiting high and low activities was computed.

Results and Discussions

In a rat model of central analgesia, all 18 salts of the studied compounds exhibited varying levels of analgesic activity, as evidenced by an increase in the area under the pain threshold-time curve compared to control values (Table 1). The calculation principle is based on the assumption that the curve of nociceptive response threshold follows a logarithmic dependence. Consequently, the level of analgesic activity of substances can be evaluated based on the extent of increase in the area under the 'nociceptive threshold-time' curve, calculated using the trapezoidal rule.

Table 1. Effect of salts of the studied compounds on platelet activation and on the pain threshold in the tail-flick test

Imidazo[1,2-a]benzimidazole salt	Kappa agonist activity (delta % platelet activation 10^{-4})	Analgesic activity Area under the curve for 6 hours of observation
Control group	-	145.2
1a.2HCl	110±6.5*	262.0
1a·HBr	10.9±1.4	396.6
1a·2HBr	33.4±2.3*	448.3
1a·H_2SO_4	10.5±1.6	121.7
1a, succinate	10.4±1.8	217.5
1a, tartrate	11.5±2.1	312.0
1b·2HCl	123±4.8*	316.8
1b·HBr	Insoluble	465.0
1b·2HBr	Insoluble	343.9
1b·H_2SO_4	Insoluble	329.4
1b, succinate	Insoluble	252.0
1b, tartrate	Insoluble	402.2
1c·2HCl	146±7.1*	329.4
1c·HBr	10.9±1.4	362.9
1c·2HBr	27±3.1*	330.2
1c·H_2SO_4	16.2±1.8	304.9
1c, succinate	Insoluble	575.7
1c, tartrate	10.5±1.6	253.8
U-50488	23.4 ±2.4*	-
Butorphanol tartrate	25.6±1.0*	351.0

The dihydrobromide 1a.2HBr exhibited the highest analgesic activity. However, compounds C4H6O4 and hydrobromide1b.HBr either could not be adequately studied due to insufficient solubility or were found to be inferior to the hydrochlorides of 2-(4-fluorophenyl)substituted benzo[d]imidazo[1,2-a]imidazoles 1a-c in *in vitro* platelet activation models. These hydrochlorides, within the concentration range of 10-1000 µM, induced a dose-dependent, norBNI-reversible platelet activation, surpassing U-50.488 surpassing U-50.488 in kappa-opioid agonistic effect by an average of 3-4 times ($p<0.05$) (Table 2). Their relative safety index was even higher, at 23, 37.6, and 38.8 times, respectively (Table 3).

Table 2. Dose-dependent activation of platelets caused by the administration of imidazo benzimidazole dihydrochloride 1a-c and the reference drug U50.488

Factor	1a.2HCl	1b.2HCl	1c.2HCl	U50,488
pEC50(-logM)	1.63 (2.46-1.10)	1.67 (2.32-1.20)	1.59 (1.9-1.32)	2.17 (2.86-1.64)
EC50(mM)	42.66 (288.40-11.80)	46.80 (208.90-16.00)	38.90 (79.40-20.90)	147.91 (724.40-43.70)

Table 3. Indicators of kappa-opioid agonist activity (EC_{50}), acute toxicity (LD_{50}) and conditional therapeutic index determined for dihydrochloride 1a-c

Compound	EC50, mM	EC50, mg/kg	LD50, mg/kg	Conditional therapeutic index (LD50/EC50)
1a.2HCl	42.66 (288.40-11.80)	0.018	192.7	9635.0
1b.2HCl	46.80 (208.90-16.00)	0.021	328.9	15662.0
1c.2HCl	38.90 (79.40-20.90)	0.017	274.3	16135.0
U50,488	147.91 (724.40-43.70)	0.060	25.0*	416.7

***Note*:** * – LD_{50} U50,488 according to Kuzeff et al. (2004).

4-(2-(2-(4-Fluorophenyl)-benzo[d]imidazo[1,2-a]imidazole-9-yl)ethyl)morpholine dihydrochloride (1c.2HCl) demonstrated the highest analgesic and kappa-opioid agonist activity in micromolar concentrations, significantly surpassing that of the reference drug U-50,488. It also demonstrated advantages in terms of efficacy and breadth of therapeutic action.

Upon examining the duration of the compound's effect in the Plantar Test, it was observed that a pronounced effect manifested after 30 minutes, gradually intensifying over the subsequent 90 minutes. The maximum antinociceptive effect was noted between 90 to 150 minutes, followed by a steady decline until the end of the recording period. The observed effect maintained statistical significance compared to the control group during the entire observation period. These results aligned

with findings reported for morphine (Gholami et al. 2015), which similarly retained effectiveness during the same period of time (Fig. 2).

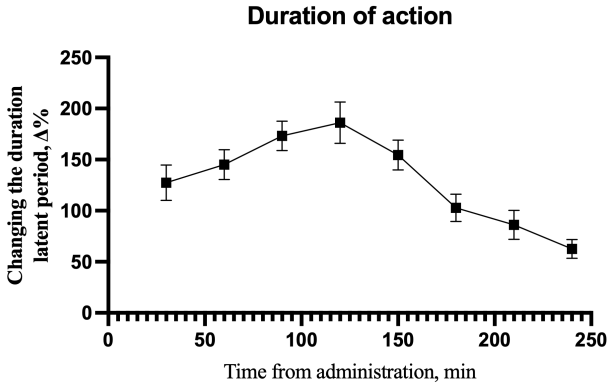


Figure 2. Study of the duration of action of 1c.2HCl (10 mg/kg, intraperitoneally) in the plantar test on rats. ***Note*:** All data are reliable in relation to control (Two-way ANOVA, $p<0.05$).

A study on the effects of repeated administration of the substance over 10 days revealed no significant alteration in the effect over time. The latent period of the reaction was statistically significant, in absolute and relative values (Fig. 3).

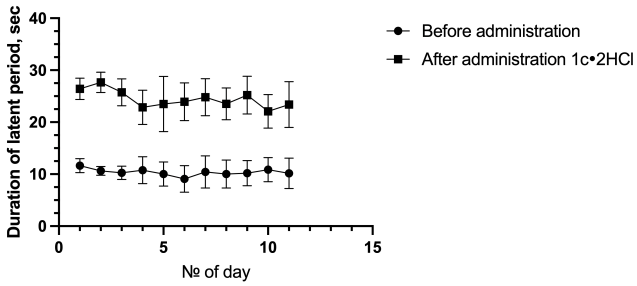


Figure 3. The effect of the compound 1c.2HCl (10 mg/kg, intraperitoneally) on the extension of the latent period of withdrawal in the plantar test on rats with repeated administration. ***Note*:** All data are reliable in relation to control (Two-way ANOVA, $p<0.05$).

Data for the reference drug were extracted from the original publication Di Cesare Mannelli et al. (2015). Starting from day 6, statistically significant differences were noted between the parameters of the test substance and the reference drug due to the development of tolerance in laboratory animals. Analysis of changes in the duration of the latent period during the administration of morphine at a dosage of 10 mg/kg revealed a sharp decrease in its effectiveness by day 6. Considering the continued prolongation of the studied parameter during the administration of 1c.2HCl, it can be concluded that this compound does not induce tolerance development for at least 11 days (Fig. 4).

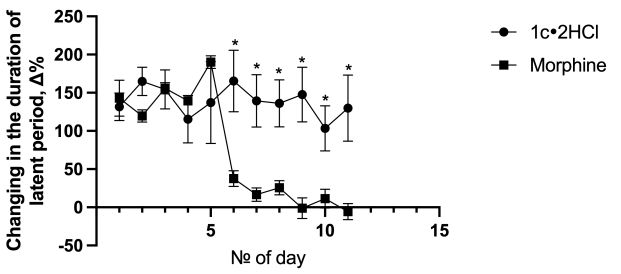


Figure 4. Comparison of the relative change in withdrawal latency in the plantar test on rats during repeated administration of 1c.2HCl (10 mg/kg, intraperitoneally) and morphine (10 mg/kg, intraperitoneally). ***Note*:** * – data are reliable in relation to control (Two-way ANOVA, $p<0.05$).

”Hot/Cold-plate” model characterized the central mechanism of antinociceptive action at the supraspinal level. Dihydrochloride (1c.2HCl) surpassed the ED_{50} of butorphanol by more than 6 times. Following preliminary subcutaneous administration of norbinaltorphimine, a kappa-opioid receptor blocker (nor-BNI), the analgesic activity of the most active compound 1c.2HCl and the reference drug, butorphanol, administered at ED_{80} doses, decreased statistically significantly by 2.5 and 1.6 fold, respectively.

Currently, neuropathic pain is gaining interest among physicians due to its growing prevalence and the challenges associated with achieving therapeutic efficacy compared to other clinical forms of chronic pain (Palekhov and Vvedenskaya 2020). Unlike nociceptive pain, neuropathic pain does not correlate with the nature and severity of injuries and is often linked with comorbid conditions such as dyssomnia, anxiety disorders, depression, etc. These factors can worsen the disease progression and exacerbate its impact on the patient’s quality of life, ability to work, and social engagement, thus contributing significantly to socio-economic losses (Cohen et al. 2021). Classic opioids, which are the first choice drugs in the treatment of nociceptive pain, such as morphine (Serrano et al. 2021), are not effective enough in the treatment of neuropathic pain (Martínez-Navarro et al. 2019). In recent years, the primary focus in the scientific research has been devoted to investigation of selective kappa opioid receptor agonists as potential agents for the treatment of such pain (Li et al. 2023). Therefore, one of the stages of an additional pharmacodynamic study of the compound 1c.2HCl and its salts as a substance with a confirmed kappa-opioid-mediated mechanism of analgesic action was to study its effect on neurogenic pain syndrome.

The gold standard for the treatment of all neuropathic pain syndromes is anticonvulsants. The most effective drug is gabapentin. This is the reason why it was chosen as the main comparator drug when assessing the effect on neuropathic pain syndrome. When modeling neurogenic pain syndrome in the control group of “sham-operated animals,” no signs of the development of tactile allodynia were observed. In animals of the “neuropathy” group, initial signs of allodynia were recorded on day 7 and on day 14. The value of the 50% pain response threshold was 2.1±0.4 g (Fig. 5).

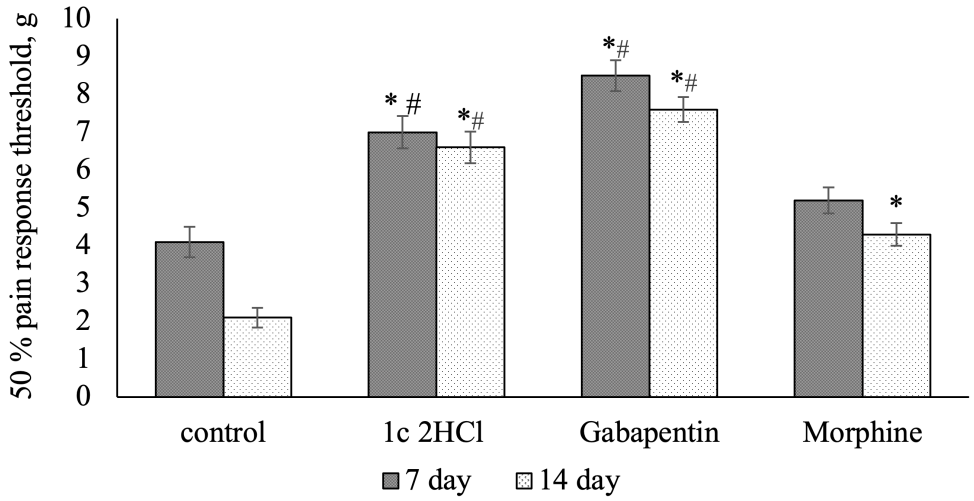


Figure 5. Effect of compound 1c.2HCl (1 mg/kg intraperitoneally), gabapentin (10 mg/kg intraperitoneally) and morphine (1 mg/kg intraperitoneally) with 14-day administration on the severity of tactile allodynia. ***Note*:** * – data are reliable in relation to control, $p<0.05$; # – data are reliable in relation to morphine, $p<0.05$.

The studied compound, imidazobenzimidazole dihydrochloride 1c, administered for 14 days at a dose of 1 mg/kg, led to a statistically significant increase in pain thresholds on both the 7th and 14th days of the study, exceeding the indicators of the control group by 1.8 and 3 times, respectively. No statistically significant differences were observed between the test compound 1c.2HCl and the reference drug gabapentin, even at a 10-fold increase in the dose of 1c.2HCl. Morphine hydrochloride exhibited lower activity compared to the test compound and gabapentin by 1.3 and 1.7 times, respectively, confirming literature data on its limited effectiveness in treating neuropathic pain.

During the assessment of thermal allodynia, intact animals maintained contact with cold temperatures throughout the observation period, resting all limbs on the cooled floor. However, with the onset of neuropathy, the duration of limb contact with the cold plate significantly decreased, reaching 32.3±2.2 seconds by the 14th day of the experiment (Fig. 6). The dihydrochloride of compound 1c, along with gabapentin, notably reduced the duration of limb contact with the weight by 1.6 and 2.1 times, respectively. Morphine did not lead to a statistically significant increase in the duration of contact of the injured limb with the cold plate.

Employing the methodologies of molecular mechanics and quantum chemistry, we constructed optimized 3D models for the 18 studied salts, three original base structures, and five acid structures that compose these salts. Subsequently, utilizing the constructed 3D models, the semi-empirical quantum chemical method PM3 was employed to compute 11 energetic and physicochemical parameters of the salts under investigation, as listed in Table 4.

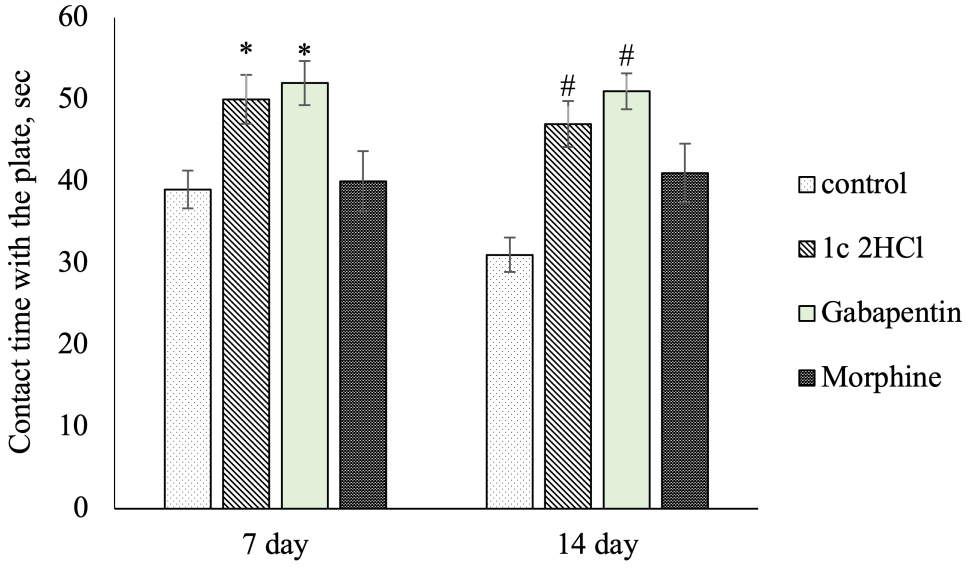


Figure 6. Effect of compound 1c.2HCl (1 mg/kg intraperitoneally), gabapentin (10 mg/kg intraperitoneally) and morphine (1 mg/kg intraperitoneally) with 14-day administration on the severity of cold allodynia (t=50°C). ***Note*:** * – data are reliable in relation to control on day 7 $p<0.05$; # – data are reliable in relation to control on day 14 $p<0.05$

Nonparametric correlation analysis showed that 7 variables were significant for kappa-agonist activity: E_{tot}, ΔE_{tot1}, ΔE_{tot2}, E_{HOMO}, ΔE_{HL}, V, MR; for analgesic activity – 4 indicators: E_{tot}, ΔE_{tot1}, ΔE_{tot2}, μ (Table 4).

Table 4. Nonparametric Spearman correlation coefficients of kappa-opioid agonistic and analgesic activities and physicochemical parameters of salts of the studied compounds

Indicator	Kappa-opioid agonistic activity	Analgesic activity
Etot[1]	**0.344**	**0.345**
ΔEtot1[2]	**0.389**	**0.317**
ΔEtot2[3]	**-0.512**	**-0.352**
E_{HOMO}[4]	**-0.354**	0.172
E_{LUMO}[5]	-0.041	0.113
ΔE_{HL}[6]	**-0.577**	-0.138
LogP[7]	-0.001	0.278
V[8]	**-0.317**	-0.234
S[9]	-0.083	-0.293
μ[10]	-0.262	**-0.410**
MR[11]	**-0.333**	0.167

Note: [1]Total energy of formation. [2] Energy of salt formation taking into account the energy of the acid residue. [3] Energy of salt formation without taking into account the energy of the acid residue. [4] Energy of the highest occupied molecular orbital.[5] Energy of the lowest unoccupied molecular orbital. [6] Energy difference between the highest occupied and lowest unoccupied molecular orbitals. [7] Logarithm of the distribution coefficient. [8] Volume of the molecule. [9] Surface area of the molecule. [10] Dipole moment. [11] Molecular refraction.

The values of the Spearman correlation coefficient for significant parameters ($p<0.2$) selected as input variables (neurons) for neural network modeling are highlighted in bold.

In the process of neural network modeling, using significant molecular characteristics from Table 3 as input neurons, about 10 thousand neural networks were trained. The two best neural networks were found. Their characteristics are given in Table 5.

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Table 5. Accuracy indicators of the best neural networks describing the dependence of kappa-opioid agonistic and analgesic activities of salts of the studied compounds on the parameters of their chemical structure

Activity	Number[1]	Architectory[2]	RTrain[3]	RTest[4]	RGen[5]
Kappa-opioid agonistic	275	MLP 7-6-1 Exp Ident	0.99992 $p<5\cdot10^{-7}$	0.99997 $p<5\cdot10^{-7}$	0.99989 $p<5\cdot10^{-7}$
Analgesic	827	MLP 4-7-1 Tanh Ident	0.99819 $p<5\cdot10^{-7}$	0.94448 $p<5\cdot10^{-7}$	0.89923 $p<5\cdot10^{-7}$

Note: [1] Number of the best neural networks. [2]Architecture of the best neural network. [3]Correlation coefficient for the training sample. [4]Correlation coefficient for the test sample. [5]Correlation coefficient for the combined sample.

Sensitivity analysis of neural network models demonstrated that the most important three molecular characteristics for the formation of k-opioid agonistic activity of the studied salts are: E_{tot}, ΔE_{tot2} and E_{HOMO}. For the formation of analgesic activity the most important molecular characteristics are E_{tot} and ΔE_{tot1} (Table 6).

All these significant parameters reflect the characteristics of the formation of salt supramolecular complexes, i.e. reflect their stability. Steric characteristics do not have a significant effect on the level of activity.

Table 6. Sensitivity indicators of neurons from the best neural networks describing the kappa-agonist and analgesic activities dependencies of salts from the studied compounds on the parameters of their chemical structure

Indicator[1]	Kappa-opioid agonistic activity		Analgesic activity		General[4]
	Base[2]	Rel[3]	Base[2]	Rel[3]	
Etot	**12351**	**0.262**	**21.05**	**0.315**	**0.577**
ΔEtot1	1837	0.039	**24.11**	**0.360**	**0.399**
ΔEtot[2]	**6585**	**0.140**	10.50	0.157	**0.297**
E_{HOMO}	**22351**	**0.475**	—	—	**0.475**
ΔE_{HL}	1337	0.028	—	—	0.028
V	1412	0.030	—	—	0.030
μ	—	—	11.28	0.168	0.168
MR	1214	0.026	—	—	0.026

Note: [1]Parameter designations correspond to table 3. [2]Initial values, conditional units. [3]Relative values reduced to the sum of the initial values. [4]Sum of two relative values for k-agonist and analgesic activities. The highest sensitivity values are highlighted in bold.

The results of cluster analysis of k-opioid agonistic and analgesic activities, and their significant parameters identified as a result of sensitivity analysis of neural networks are shown in Table 7.

The cluster of high k-opioid agonist activity included all three hydrochlorides, type 1.HCl; salts of other acids form a cluster of low k-opioid agonist activity.

The cluster of high analgesic activity included five salts – 1c.C4H6O4, 1b.HBr, 1a.2HBr, 1b.C4H6O6 and 1a.HBr; the remaining salts form a cluster of low analgesic activity.

The statistical significant differences in the means in the two clusters in the case of k-agonist activity for two variables were identified: E_{tot} (total energy of salt formation) and ΔE_{tot2} (energy of salt formation excluding the energy of the acid residue).

Consequently, the high kappa-opioid agonist activity of the analyzed compounds is evidently attributed to the lower total energy of salt formation, coupled with a greater increase in energy during the formation of the salt supramolecular complex.

The significantly higher kappa-opioid agonist activity of hydrochlorides may be elucidated by the higher mobility of the chloride ion in the salt supramolecular complex, albeit to a lesser extent compared to other salts, stabilized by exchange interactions. This facilitates the adaptation of the molecule to the binding site of the kappa-opioid receptor.

Table 7. Indicators of cluster analysis of kappa-opioid agonistic, analgesic activities and the important molecular parameters of the salts of the studied compounds

Indicator	Act[1]	Etot[2]	ΔEtot1[2]	ΔEtot2[2]	E_{HOMO}[2]
Kappa-opioid agonistic activity[3]					
M_1[5]	**126.3**	**-108695**	—	**-13.82**	-8.8
M_2[6]	**13.4**	**-123639**	—	**8.82**	-8.7
p(W)[7]	0.0000	0.2084	—	0.0824	0.1396
p(t)[8]	**0.0036**	**0.0033**	—	**0.0173**	0.3927
Analgesic activity[4]					
M_1[5]	**457.6**	-118250	-25584	—	—
M_2[6]	**287.4**	-122263	-28667	—	—
p(W)[7]	0.7976	0.2084	0.0234	—	—
p(t)[8]	**0.0014**	0.3610	0.3846	—	—

Note: [1]Activity indicator. [2]Parameter designations correspond to Table 3. [3]Activity indicator – percentage of platelet activation at a concentration of 10^{-4} M. [4]Activity indicator - area under the curve for 6 hours of observation. [5]Arithmetic mean for cluster 1 of highly active compounds. [6]Arithmetic mean for cluster 2 of non-highly active compounds. [7]Probability of normal distribution according to the Shapiro-Wilk test. [8]Probability of differences in mean values using the Student's test. Statistically significant differences in means in two clusters are highlighted in bold.

Other salt residues are characterized by the presence of several relatively mobile p- and d-orbitals. They engage in exchange interactions with the π-orbitals of the aromatic system of the organic base. It significantly enhances the strength of the salt supramolecular complex during interaction with the site of the kappa-opioid receptor; the salt residue impedes the compound from forming the optimal biological conformation for receptor activation. This is likely to happen due to competitive binding with any other amino acid residues of this protein that are incongruent with the formation of the optimal biological conformation.

Regarding analgesic activity, no statistical significance was observed for differences in means in the two clusters for either of the two analyzed variables. However, the high reliability of the constructed neural network model (Table 4) indicates that the relationship between the analgesic activity of the studied salts and the energy parameters of their formation still exists, but not as prominently as in the case of kappa-opioid agonistic activity.

Conclusion

A pharmacological investigation of various salts of 2-(4-fluorophenyl)imidazo[1,2-a]benzimidazoles containing 2-dialkylaminoethyl type substituents at position 9 revealed that 9-morpholinoethyl-2-(4-fluorophenyl)imidazo[1,2-a]benzimidazole hydrochloride is the most promising among these salts. It exhibits high kappa-opioid agonistic and antinociceptive activity, identified by the code 1c.2HCl. In nociceptive experimental tests, it demonstrated a pronounced dose-dependent analgesic effect *in vivo*. It was comparable to reference drugs such as butorphanol and morphine, and superior to butorphanol by nearly 6 times in the model of central algesia at the supraspinal level. Unlike the classic opioid analgesic morphine, the test compound did not demonstrate the rapid formation of tolerance and also exhibited activity in a neuropathic pain model, where it demonstrated slightly lower activity than gabapentin at a ten-fold dose relative to 1c.2HCl. The antinociceptive activity of the test salt was determined to be due to stimulation of kappa-opioid receptors. It was confirmed by tests with opioid antagonists both *in vitro* and *in vivo*. Furthermore, upon intraperitoneal administration, the duration of action was consistent with other classical opioid analgesics, rendering this compound a promising subject for further investigation.

Utilizing artificial neural network technology, two statistically significant ($p<5\cdot10\text{-}7$) dependencies of kappa-opioid agonistic and analgesic activities on the quantum-chemical and physico-chemical parameters of the studied salts were determined. Subsequent analysis of neural network sensitivity and cluster analysis indicated that the high level of kappa-agonist activity of the studied compounds can be attributed to the lower total energy of formation of the entire salt molecule (E_{tot}), in connection with a greater increase in energy during the formation of the salt supramolecular complex (ΔE_{tot2}). The significantly higher kappa-opioid agonist activity of hydrochlorides may be attributed to the higher mobility of the chloride ion in the salt supramolecular complex to a smaller extent compared to other salts, stabilized by exchange interactions and facilitating the molecule's adaptation to the binding site of the kappa-opioid receptor. However, in the case of analgesic activity, no such obvious pattern was observed. It was established that their steric characteristics do not affect the level of kappa-opioid agonistic and analgesic activities of the analyzed salts significantly.

Conflict of interests

The authors have declared that no competing interests exist.

Funding

The chemical section was financially supported by the Ministry of Science and Higher Education of the Russian Federation (state task in the field of scientific activity, Southern Federal University, grant FENW-2023-0011).

Acknowledgments

This work was carried out using the equipment of the Common Use Center of the Southern Federal University, Rostov-on-Don, Russia.

Data availability

All of the data that support the findings of this study are available in the main text.

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