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Research Article

Synthesis and ophthalmic hypotensive effect of new potential benzimidazole-based Rho-kinase-2 inhibitors

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Abstract

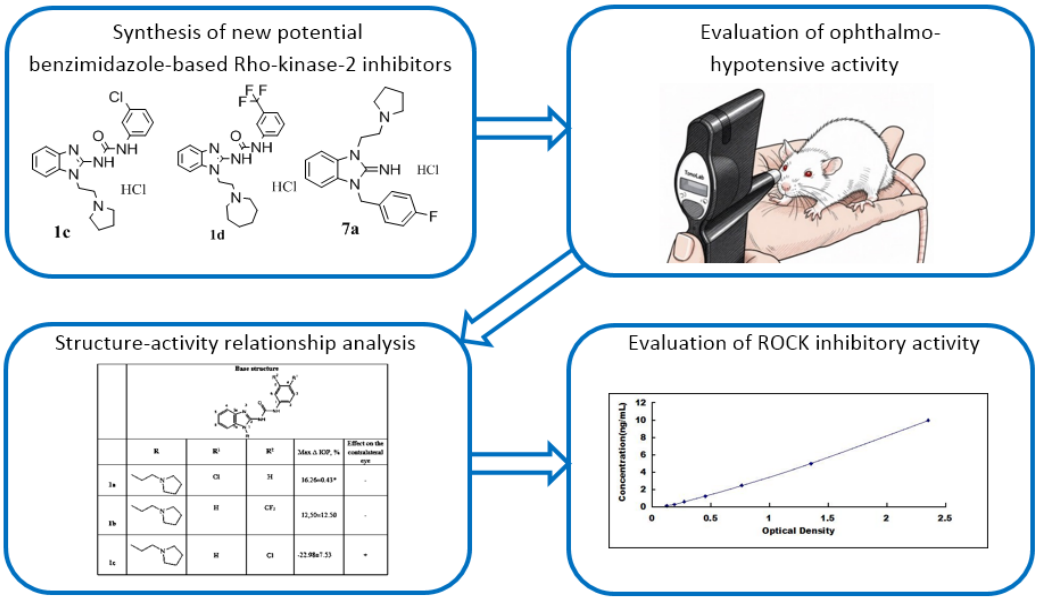
Introduction: Based on the chemical structures of known Rho-kinase-2 inhibitors, 18 benzimidazole derivatives were synthesized and studied for their ophthalmic hypotensive activity in animals with normal intraocular pressure. The dependence of the pharmacological effect on the chemical structure of the compounds was analyzed. The effect of the most active compounds on Rho-kinase-2 activity was assessed.

Materials and Methods: Ophthalmic hypotensive activity was assessed by measuring intraocular pressure with a TonoVet veterinary tonometer in 120 mongrel rats (6 in each group) before and after instillation of reference drug solutions (timolol and melatonin) and 18 test substances. The effect of the test compounds on Rho-kinase activity was assessed using an in vitro enzyme-linked immunosorbent assay spectrophotometrically.

Results: The most active compounds among the new benzimidazole derivatives after a single instillation at a concentration of 0.4% were compound 7a (1-(4-fluorobenzyl)-3-(2-(pyrrolidin-1-yl)ethyl)-1,3-dihydro-2H-benzo[d]imidazol-2-imine hydrochloride), which reduced intraocular pressure in normotensive animals by 28.21%, exceeding the effect of the reference drug timolol (26.84%), but inferior to melatonin (30.95%), and compound 1d (1-(1-(2-(azepan-1-yl)ethyl)-1H-benzo[d]imidazol-2-yl)-3-(3-trifluoromethyl)phenyl)urea hydrochloride), which reduced ophthalmotonus by 23.96%, slightly inferior to timolol. The test compounds do not affect intraocular pressure dynamics in the contralateral eye and, therefore, do not have a systemic effect, unlike the reference drugs. It was also found that compounds 7a and 1d at a concentration of 1*10-4 mol/L inhibit Rho-kinase-2 by 26.72% and 18.11%, respectively.

Conclusion: The most active compounds, 7a and 1d, were identified as Rho kinase-2 inhibitors that exhibit ocular hypotensive effects in normotensive animals in vivo.

Graphical Abstract



Keywords

glaucoma; benzimidazole; azepan; biphenyl; intraocular pressure; Rho-kinase (ROCK)

Introduction

The synthesis and study of the biological activity of compounds spans various areas of modern medicinal chemistry. The presence of a heterocyclic fragment significantly influences the biological activity of hybrid substances. For example, the benzimidazole fragment is present in the structure of many drugs with varying biological activity. Among the widely used medications, mention should be made of dabigatran (an anticoagulant, trade name Prodaxa), dibazol (an antispasmodic), omeprazole (an antiulcer drug), droperidol and pimozide (neuroleptics), astemizole (an antihistamine), and others.

Glaucoma is currently one of the leading causes of blindness or visual impairment (Movsisyan et al. 2022). The leading symptom of this disease is increased IOP, primarily caused by impaired aqueous humor (AHF) outflow, followed by the development of typical visual defects and optic nerve atrophy (Cheng et al. 2012; Cholkar et al. 2015; Jonas et al. 2018; Efimenko et al. 2024). Thus, the main goal of glaucoma therapy is to reduce ophthalmotonus, regardless of the type and stage of glaucoma (Razhko et al. 2021).

Current glaucoma treatments primarily focus on reducing intraocular pressure but do not address pressure-independent neurodegenerative mechanisms. The endogenous indoleamine, melatonin, has attracted the attention of scientists due to its potential to regulate intraocular pressure (through activation of melatonin receptors and synergism with adrenergic and enzymatic regulators), as well as its ability to correct neurodegeneration (protecting retinal ganglion cells, mitigating oxidative stress, preventing mitochondrial dysfunction, and inhibiting apoptotic and inflammatory cascades) (Babkov et al. 2024). Benzimidazole compounds are melatonin isosteres and exhibit affinity for melatonin receptors, making this class promising not only as ophthalmic hypotensive agents but also as neuroprotective agents (Marcus et al. 2018a; Marcus et al. 2018b; Turanlı et al. 2020; Taran et al. 2025).

Recently, the emergence of a new class of antihypertensive drugs, Rho-kinase-2 inhibitors, has been discussed in the literature. They have a neuroprotective effect, affect trabecular tissue, increase the outflow of intraocular fluid, and reduce intraocular pressure (Abbhi et al. 2017; Yao et al. 2018; Wu et al. 2024; Hou and Pan 2025). Among the Rho-kinase-2 inhibitors, there are representatives of several groups depending on the chemical structure, such as: derivatives of

isoquinoline, urea, indazole, aminopyrimidine, chroman-3-amine, benzimidazole, quinazolinone, indole, 7-azaindole (Cholkar et al. 2015).

Ripasudil and netarsudil are isoquinoline derivatives, and sovesudiil is a biphenyl derivative of pyridine (Fig. 1). These compounds are approved by the US Food and Drug Administration (FDA) (Tan et al. 2025).

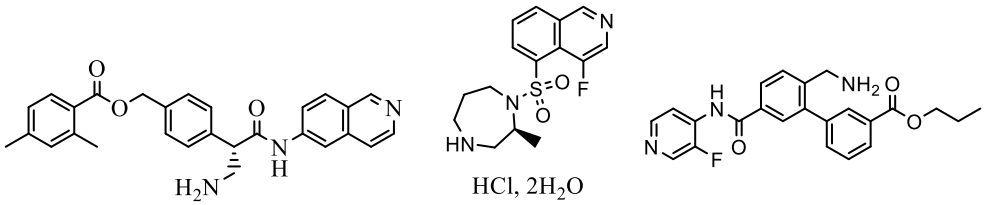


Figure 1. Chemical structures of Rho kinase-2 inhibitors

The structures of Rho-kinase-2 inhibitors containing benzimidazole as a structural subunit have been reported in (Fig. 2) (Abbhi et al. 2017; Marcus et al. 2019). In this study, using the privileged structure of benzimidazole and existing data on the chemical structure of a new class of antiglaucoma agents, potential ophthalmic hypotensive agents were synthesized that exert their effect through inhibition of Rho-kinase-2.

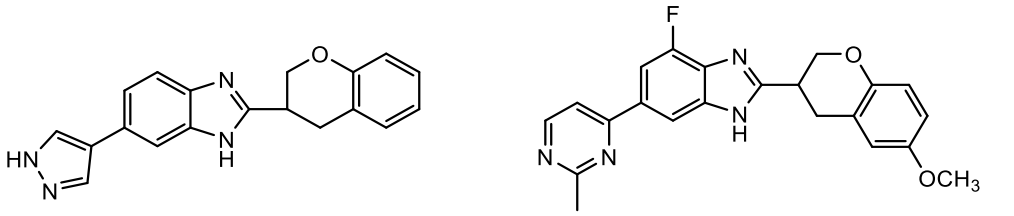


Figure 2. Chemical structures of benzimidazole-based Rho kinase-2 inhibitors.

Materials and Methods

Experimental animals

120 outbred rats took part in an experiment to study the effect on intraocular pressure.

All animal procedures in the study were conducted in accordance with generally accepted ethical standards for animal handling. Animal care complied with the rules of laboratory practice for preclinical studies in the Russian Federation (GOST 351.000.3-96 and 51000.4-96), Order No. 708n of the Ministry of Health and Social Development of the Russian Federation dated August 23, 2010, "On Approval of the Rules of Laboratory Practice", and also complied with Directive 2010/63/EU of the European Parliament and of the Council of the European Union dated September 22, 2010, on the protection of animals used for scientific purposes. The experiments were approved by the Biomedical Ethics Committee of Volgograd State Medical University (IRB 00005839 IORG 0004900, OHRP, Certificate No. 2021/056 dated June 15, 2021). All sections of this study comply with the ARRIVE Guidelines for Reporting Animal Studies. Animals were maintained in the vivarium of Volgograd State Medical University, Ministry of Health of the Russian Federation, at a temperature of 24°C and a relative humidity of 60% under a natural light cycle with free access to food and water.

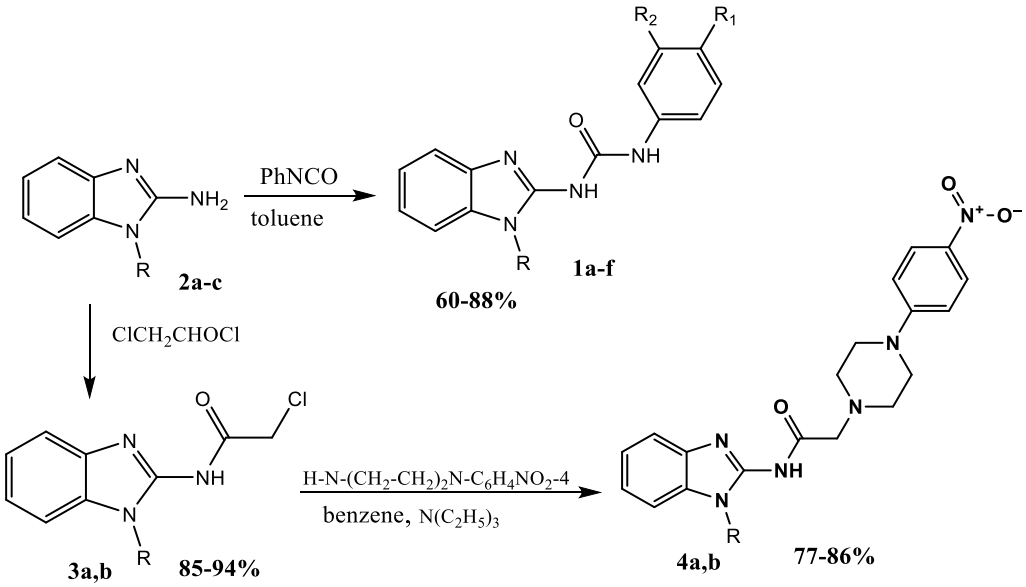
Chemical synthesis

This work included three stages. The first stage included the synthesis of 18 new benzimidazole derivatives, confirmation of the structure of all obtained compounds using IR, 1H, 13C spectroscopy, elemental analysis (C, H, Hal, N) and determination of the melting point. The second stage: studying the ophthalmic hypotensive activity of the obtained substances and

analyzing the dependence of the activity on the chemical structure of the substances. The third stage: studying the inhibition of Rho-kinase-2 of the most active compounds.

The compounds under study

The synthesis of 1H-benzo[d]imidazol-2-yl)urea salts **(1a-f)** and 1H-benzo[d]imidazol-2-yl)-2-(4-nitrophenyl)piperazin-1-yl)acetamides (4a,b) is shown in **Scheme 1**. Compounds **1a-f** were obtained by prolonged refluxing of a mixture of starting amines 2a-c and isocyanates in toluene. Urea derivatives **1a-f** are colorless substances with high melting points. The ^{1}H NMR spectra of these compounds contain proton signals from two NH groups of the urea moiety. Intermediates **3a,b** were synthesized by acylation of amines **2d,e** with chloroacetyl chloride in absolute benzene in the presence of pyridine. Compounds **3a** and **3b**, 1-(4-nitrophenyl)piperazine, and triethylamine were then refluxed for 4 hours in dimethylformamide. The resulting compounds **4a** and **4b** are colorless substances whose ^{1}H NMR spectra contain characteristic signals from the NH protons of the carbamide moiety.

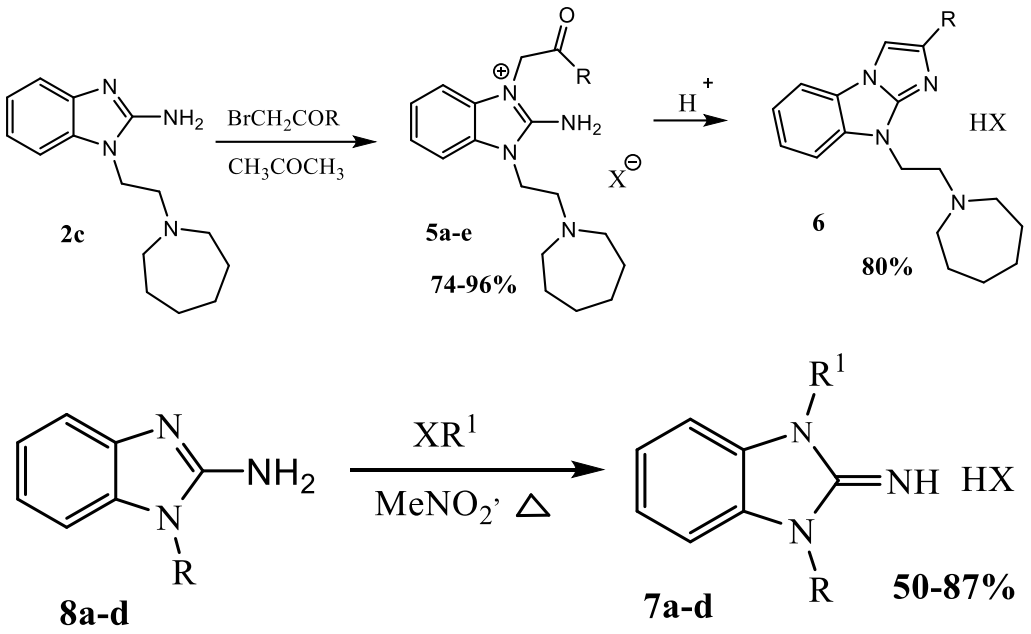


Scheme 1. Synthesis of 1H-benzo[d]imidazol-2-yl)urea and 1H-benzo[d]imidazol-2-yl)-2-(4-nitrophenyl)piperazin-1-yl)acetamide derivatives. ***Note:*** **1: R= $(CH_2)_2N(CH_2)_4$,** R_1=Cl, R_2=H (**a**); R_1=H, R_2=CF3 (**b**); R_1=H, R_2=Cl (**c**). **R= $(CH_2)_2N(CH_2)_6$,** R_1=H, R_2=CF_3 **(d); R= 4-(2'-MeOCO)$C_6H_4C_6H_4CH_2$,** R_1=H, R_2=Cl **(e); CF_3 (f). 2: R**=$(CH_2)_2N(CH_2)_4$ **(a),** 4-(2'-MeOCO)$C_6H_4C_6H_4CH_2$ **(b),** $(CH_2)_2N(CH_2)_6$ **(c). 3,4: R=**All **(a);** CH_2-*(4-tert*-butyl)C_6H_4 **(b).**

Synthesis of 2-aminobenzimidazolium bromides 5 (**a-f**), 9-(2-azepan-1-yl)ethyl)-2-(3-methoxyphenyl)-9H-benzo[d]imidazo[1,2-a]-imidazole (**6**) and 2-iminobenzimidazole derivatives **7** (**a-d**) is shown in Scheme 2.

2-Phenoxyethyl bromide, 4-fluorobenzyl chloride, 2-bromo-4-(trifluoromethyl)acetophenone, 1-(4-nitrophenyl)piperazine, isocyanates, and biphenyl-containing reagents used in this work were purchased from Alfa Aesar (Great Britain).

2-Aminobenzimidazole was obtained by alkaline hydrolysis of methyl N-benzimidazole-2-carbamate (Pozharsky et al. 1988); its N-substituted derivatives 2a (Di Braccio et al. 2013), 2b (Spasov et al. 2020), 2c (Zhao et al. 2021), 2d (Anisimova and Tolpygin 2011), 2e (Divaeva et al. 2015) were obtained according to the indicated methods, and phenacyl bromides were obtained by bromination of the corresponding acetophenones in alcohol. The reaction progress and the individuality of the compounds were monitored by TLC on Al_2O_3 plates. IR spectra (ν/cm^{-1}) of the obtained compounds were recorded on a Varian Excalibur 3100 FTIR IR spectrophotometer (Varian, USA) using the attenuated total reflectance method in powder; NMR spectra were recorded on a Bruker Avance 600 spectrometer (USA) (600 MHz for ^{1}H and 150 MHz for ^{13}C). Chemical shifts are given relative to residual proton signals deuterated solvent (2.49 for ^{1}H, 39.7 for ^{13}C in DMSO-d_6). Melting points were measured on a Fisher-Johns melting point apparatus (Fisher Scientific, USA). Elemental analysis was performed using the classical method (Gelman et al. 1987).



Scheme 2. Scheme of the synthesis of 2-aminobenzimidazolium bromides **5** (a-e), 9-(2-azepan-1-yl)ethyl)-2-(3-methoxyphenyl)-9H-benzo[d]imidazo[1,2-a]imidazole (**6**) and 2-iminobenzimidazole derivatives **7** (a-d). ***Note*: 5: R=** C_6H_5 **(a),** 3-$MeOC_6H_4$**(b),** 4-$CF_3C_6H_4$**(c),** 4- $C_6H_5C_6H_4$ **(d),** X=Br**; (4-MeOC6H4)NH (e),** X=Cl**; 6: R= 3-MeOC6H4, X=** Cl; **7: R=** $CH_2C_6H_4F$**-4,** X=Cl **(a),** $C_6H_5OCH_2CH_2$**(b),** R_1= $CH_2CH_2N(CH_2)_4$; **R= 4-(2´-MeOOCC$_6$H$_4$)C$_6$H$_4$,** R_1= $C_6H_5OCH_2CH_2$ **(c); R=** $C_6H_5CH_2$**,** R_1= $CH_2C_6H_4C_6H_5$(d), X=Br. **8: R=** $CH_2C_6H_4F$-4 **(a),** $C_6H_5OCH_2CH_2$**(b, c),** $CH_2C_6H_5$ **(d).**

General procedure for synthesizing (scheme 1)

5 mmol of the appropriate isocyanate was added to a solution of 5 mmol of the appropriate 2-aminobenzimidazole 2a-c in 5-10 mL of toluene, and the mixture was refluxed for 8-10 hours. After refluxing, the reaction mixture was cooled in an ice bath, the precipitate was filtered, and washed with petroleum ether. The resulting compounds 1a-f were air-dried and crystallized from the appropriate solvent. Compounds 1a-f were then dissolved in a minimal amount of 2-PrOH, concentrated hydrochloric acid was added to pH 1, and the reaction mixture was maintained at room temperature for 1 hour.

The precipitated salt product was filtered, washed with acetone and ether, and air-dried.

1-(4-chlorophenyl)-3-(1-(2-pyrrolidin-1-yl)ethyl)-1H-benzo[d]imidazole-2-yl)urea (1a HCl)

Yield 81 %, m.p.167-169º C. IR spectrum, ν/cm^{-1}: 3225, 3160 (2NH), 1702 (C=O). Found (%): C 57.04, H 5.63; Cl 16.74; N 16.54. $C_{20}H_{22}ClN_5O\cdot$ HCl. Calculated (%): C 57.15, H 5.52; Cl 16.87; N 16.66. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.94 (c, 4H, 2CH_2^{endo}), 3.52-3.64 (m, 6H, CH_2NCH_2 + $CH_2^{exo}N$), 4.81 (c, 2H, CH_2), 7.36-7.95 (m, 8H, H_{Ar}), 10.54 (broad c, 1H, NH), 11.48 (c, 1H, NH), 13.25 (broad c, 1H, N^+H). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 22.82, 38.92, 50.21, 53.12, 110.73, 113.06, 119.93, 123.79, 124.17, 126.68, 128.88, 128.95, 129.12, 137.47. M.p. of base 120-121 ºC (benzene/hexane, 1:1).

1-(1-(2-pyrrolidin-1-yl)ethyl)-1H-benzo[d]imidazole-2-yl)-3-(3-(triflоromethyl) phenyl)urea (1b HCl)

Yield 66 %, m.p. 125-126º C. IR spectrum, ν/ cm^{-1}: 3225, 3160 (2NH), 1702 (C=O). Found (%): N 15.32. $C_{21}H_{22}F_3N_5O\cdot$HCl. Calculated (%): N 15.43. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.86 (t. 4H, 2CH_2^{endo}, J=5.97), 3.18 (t, 2H, $CH_2^{exo}N$, J= 5.43), 3.51-3.63 (m, 4H, CH_2NCH_2), 4.78 (c, 2H, N-CH_2^{exo}), 7.26-7.28 (m, 1H, H_{Ar}), 7.34-7.41 (m, 3H, H_{Ar}), 7.57-7.66 (m, 2H, H_{Ar}), 7.80-7.94 (m, 1H, H_{Ar}), 8.2 (c, 1H, H_{Ar}), 10.65 (b.c, 1H, NH), 11.69 (c, 1H, NH), 12.96 (c, 1H, N^+H). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 22.82, 22.85, 38.29, 39.02, 49.76, 50.31, 52.85, 53.13, 110.37, 112.87, 119.14, 121.90, 123.66, 123.75, 124.01, 124.99, 128.97, 129.74, 130.21, 139.65, 150.1.

1-(3-chlorophenyl)-3-(1-(2-(pyrrolidin-1-yl)ethyl)-1H-benzo[d]imidazole-2-yl)urea (1c HCl)

Yield 65 %, m.p.166-168 º C. IR spectrum, ν/ cm^{-1}: 3225, 3160 (2NH), 1702 (C=O). Found (%): C 57.04; H 5.63; Cl 16.74; N 16.54. $C_{20}H_{22}ClN_5O\cdot$ HCl. Calculated (%): C 57.15; H 5.52; Cl 16.87; N 16.66. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.95 (t, 4H, 2CH_2^{endo},

J= 1.2), 3.24-3.27 (m, 2H, CH_2 ^{exo}N), 3.64-3.61 (k, 4H, $(CH_2^{endo})_2N$, J=6.4), 4.78 (c, 2H, NCH_2^{exo}), 7.12 (d, 1H, H^4_{Ph}), 7.35-7.40 (m, 4H, H_{Bzm}), 7.61 (d, 1H, H^5_{Ph}, J=7.3), 7.81 (c, 1H, H^6_{Ph}), 7.93 (b.c, 1H, H^2_{Ph}), 10.54-10.53 (m, 1H, NHPh), 11.35 (t, 1H, $C^2_{Bzm}NH$, J=3.2), 13,3 (d.c, 1H, N^+H).^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 22.80, 38.85, 50.38, 110.61, 112.91, 116.77, 117.76, 122.52, 123.68, 124.05, 128.89, 129.16, 130.68, 133.33, 140.29. M.p. of base 119-120 ºC (benzene/hexane, 1:1).

1-(1-(2-(azepan-1-yl)ethyl)-1H-benzo[d]imidazole-2-yl)-3-(3-trifloromethyl) phenyl)urea (1d HCl)

Yield 86%, m.p.218-219 ºC. IR spectrum, ν/ cm^{-1}: 3225, 3160 (2NH), 1734 (C=O). Found (%): 14.41 $C_{23}H_{26}F_3N_5O$· HCl. Calculated (%): N 14.53. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.54 (c, 4H, CH_2), 1.88 (c, 4H, CH_2), 3.44 (c, 4H, CH_2), 3.56 (t, 2H, CH_2, J=1.6), 4.87 (t, 2H, CH_2, J=7.23), 7.36-7.61 (m, 6H, H_{Ar}), 8.04-8.16 (m, 2H, H_{Ar}), 10.59 (c, 1H, NH), 11.34 (c, 1H, NH), 13.26 (br.c, 1G, N^+H). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J.

Methyl-4'-((2-(3-(3-chlorophenyl)ureido)-1H-benzo[d]imidazole-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylate (1e HCl)

Yield 60 %, m.p. 199-200º C. IR spectrum, ν/ cm^{-1}: 3225, 3160 (2NH), 1736 (COOMe), 1710 (C=O). Found (%): C 63.52, H 4.53; Cl 12.82; N 10.11. $C_{29}H_{23}ClN_4O_3$· HCl. Calculated (%): C 63.63, H 4.42; Cl 12.95; N 10.23. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 3.52 (c, 3H, OMe), 5.7 (c, 1H, N-CH_2), 7.11, 7.12 (c, c, 1H, H_{Ar}), 7.25 (c, 1H, H_{Ar}), 7.26 (c, 1H, H_{Ar}), 7.31-7.41 (m, 6H, H_{Ar}), 7.42-7.46 (m, 4H, H_{Ar}), 7.47-7.51 (m, 1H, H_{Ar}), 7.52-7.57 (m, 1H, H_{Ar}), 7.59-7.72 (m, 1H, H_{Ar}), 7.80 (c, 1H, NH), 10.63 (b.c, 1H, NH), 13.36 (b.c, 1H, N^+H). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 45.83, 51.79, 110.79, 112.93, 113.11, 116.81, 117.81, 122.60, 123.84, 127.35, 127.39, 127.50, 128.49, 129.00, 129.12, 129.29, 130.43, 130.56, 130.65, 131.46, 133.27, 133.31, 133.78, 140.14, 140.20, 140.72, 168.22. M.p. of base m.p. 152-153ºC.

Methyl 4'-((2-(3-(3-(trifluoromethyl)phenyl)ureido)-1H-benzo[d]imidazole-1-yl)methyl)-[1,1'-bithenyl]-2-carboxylate (1f)

Yield 88 %, m.p. 128-129º C. IR spectrum, ν/ cm^{-1}: 3225, 3160 (2NH), 1736 (COOMe), 1710 (C=O). Found (%): 9.55. $C_{30}H_{23}F_3N_4O_3$ HCl. Calculated (%): N 9.67. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 3.52 (c, 3H, MeO), 5.72 (c, 2H, N-CH_2), 7.25 (d, 2H, H_{Ar}, J=6.0), 7.28-7.35 (m, 2H, H_{Ar}), 7.37-7.38 (m, 2H, H_{Ar}), 7.44-7.49 (m, 4H, H_{Ar}), 7.56-7.59 (m, 2H, H_{Ar}), 7.63 (d, 1H, H_{Ar}, J=7.26), 7.67 (d, 1H, HAr, J= 8.34, 7.70-7.72 (m, 1H, H_{Ar}), 8.2 (c, 1H, H_{Ar}), 9.00 (c, 1H, NH), 10.52 (b.c, 1H, NH). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 45.89, 51.76, 110.74, 112.90, 114,27, 118.95, 121.93, 123.58, 125.01, 126.96, 127.36, 127.48, 128.46, 128.99, 129.14, 129.27, 129.50, 129.70, 130.08, 130.40, 130.63, 131.44, 133.95, 139.80, 140.13, 140.71, 168.20, 124.19, 124.94, 126.74, 128.82, 129.03, 129.59, 129.81, 130.28, 139.37, 146.22, 153.07. M.p. of base 168-169ºC.

General procedure for *synthesizing* of compounds 3a,b and 4a,b

To a boiling solution of 0.015 mol of 2-aminobenzimidazole 2 d,e in 80 mL of absolute benzene, add 1-2 mL of pyridine. A solution of 0.018 mol of chloroacetyl chloride in 5 mL of absolute benzene is added dropwise over 1 hour with stirring. The mixture is refluxed for 8-10 hours. By the end of this time, a white fibrous precipitate begins to separate from the solution. The reaction mixture is cooled, the precipitate is filtered, and washed repeatedly with water until the washings test negative for ionic halide, crystallized from 2-PrOH, DMF, $MeNO_2$.~~a suitable solvent.~~ Next, a solution of 0.002 mol of the above-described compounds 3a or 3b, 0.3 mL of triethylamine, and 0.002 mol of 1-(4-nitrophenyl)piperazine in 5-10 mL of dimethylformamide was refluxed for 4 hours. The reaction mixture was poured onto ice and left overnight at room temperature. The following day, the resulting precipitate was filtered, washed with water, and air-dried. The mixture was crystallized from nitromethane.

N-(1-allyl-1H-benzo[d]imidazole-2-yl)-2-chloroacetamide (3a)

Yield 85 %, m.p. 161-161.5º C (2-PrOH). IR spectrum, ν/ cm^{-1}: 3172 (NH), 1735 (C=O), 1625, 1585 (C=N). Found (%): C 57.65; H 4.95; Cl 14.07; N 16.71. $C_{12}H_{12}ClN_3O$. Calculated (%): C 57.77; H 4.84; Cl 14.20; N 16.83. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 4.25 (c, 2H, CH_2Cl), 4.76 (d, 2H, N-CH_2, J= 5.1), 5.04-5.18 (m, 2H, =CH_2), 5.87-5.96 (m, 1H, CH), 7.19-7.22 (m, 2H, H_{Ar}), 7.38-7.52 (m, 2H, H_{Ar}), 12.62 (c, 1H, NH). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 42.7, 46.9, 110.0, 118.0, 117.2, 123.0, 123.0, 132.9, 134.2, 141.3, 152.8, 166.5.

N-(4-(tert-butyl)benzyl)-1H-benzo[d]imidazole-2-chloroacetamide (3b)

Yield 94 %, m.p. 208-210ºC (DMF). IR spectrum, ν/ cm^{-1}: 3210 (NH), 1732 (C=O), 1623, 1583 (C=N). Found (%): C 67.39; H 6.32; Cl 9.83; N 11.69. $C_{20}H_{22}ClN_3O$. Calculated (%): C 67.50; H 6.23; Cl 9.96; N 11.81. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.20 (c, 9H, $C(Me)_3$, 4.36 (c, 2H, CH_2Cl), 5.39 (c, 2H, N-CH_2), 7.21-7.24 (m, 2H, H_{Ar}), 7.31-7.39 (m, 4H, H_{Ar}), 4.44-7.47 (m, 1H, H_{Ar}), 7.52-7.55 (m, 1H, H_{Ar}). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 31.3, 31.3, 31.3, 34.2, 42.7, 49.7, 110.0, 118.5, 123.0, 123.0, 124.9, 124.9, 126.9, 126.9, 134.2, 134.2, 141.3, 148.3, 152.0, 166.5.

N-(1-allyl-1H-benzo[d]imidazole-2-yl)-2-(4-(4-nitrophenyl)piperazin-1-yl)acetamide (4a)

Yield 77%, m.p. 201-202ºC ($MeNO_2$). IR spectrum, ν/ cm^{-1}: 3423 (NH), 1725 (C=O), 1633, 1591 (C=N). Found (%): C 62.73; H 5.86; N 19.87. $C_{22}H_{24}N_6O_3$. Calculated (%): C 62.84; H 5.75; N 19.99. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 2.79 (t, 4H, CH_2NCH_2, J=4.95), 3.39-3.52 (m, 6H, CH_2NCH_2+ CH_2N), 4.75 (c, 2H, N-CH_2), 5.14-5.26 (m, 2H, CH_2^{All}), 5.85-5.95 (m, 1H, CH), 6.82 (d, 2H, H_{Ph}, J=9.0), 7.22 (t, 3H, H_{Bzm}, J=4.05), 7.31 (c,1H, H_{Bzm}), 8.11 (d, 2H, H_{Ph}, J=9.3), 12.0 (b.c, 1H, NH). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 43.32, 44.14, 50.85, 58.57, 110.55, 112.73, 113.35, 113.42, 117,51, 123.41, 123.45, 125.60, 128.80, 131.94, 137.99, 149.50, 153,74, 170.89.

***N*-(1-(4-(*tert*-butyl)benzyl)-1*H*-benzo[d]imidazole-2-yl)-2-(4-(4-nitrophenyl) piperazin-1-yl)acetamide (4b)**

Yield 86 %, m.p. 199-200ºC ($MeNO_2$). IR spectrum, ν/ cm^{-1}: 3423 (NH), 1725 (C=O), 1633, 1591 (C=N). Found (%): C 68.31; H 6.62; N 15.84. $C_{30}H_{34}N_6O_3$. Calculated (%): C 68.42; H 6.51; N 15.96. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.21 (c, 9H, $(Me)_3$), 2.78 (c, 4H, CH_2NCH_2), 3.46 (m, 6H, CH_2NCH_2+ CH_2N), 5.28 (c, 2H, N-CH_2), 6.81 (d, 2H, H_{Ph}, J=9.3), 7.17-7.32 (m, 8H, $4H_{Bzm}$+$4H_{Ph}$), 8.11 (d, 2H, H_{Ph}, J= 9.3), 12.1 (b.c, 1H, NH).), NH). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 31.0, 34.17, 43.32, 44.57, 50.79, 58.79. 110.53, 112.65, 113.41, 123.35, 123.39, 125.39, 125.58, 127.05, 128.80, 128.85, 133.09, 138.02, 150.09, 150.43, 153.72, 171.51.

General procedure for the synthesis of 2-aminobenzimidazolium bromides 5 (a-f) and tricyclic compound 6 (Scheme 2)

5 mmol of the corresponding α-bromoketone was added to a hot solution of 5 mmol of amine (2c) in acetone. The mixture was stirred until the reagent dissolved, heated until the quaternary salt began to precipitate, and then kept at room temperature for 6-8 hours. The precipitate was filtered, washed thoroughly with acetone, and dried at 40-45°C. Chromatographically pure salts were obtained, which were used without further purification. ~~General procedure for synthesizing~~

2-Amino-1-(2-(azepan-1-yl)ethyl)-3-(2-oxo-2-phenylethyl)-1H-benzo[d] imidazole-3-ium bromide (5a)

Yield 86 %, m.p. 177-178ºC. IR spectrum, ν/ cm^{-1}: 3202, 3154 (NH_2),1710 (C=O), 1633, 1591 (C=N). Found (%): C 60.28; H 6.50; Br 17.34; N 12.13. $C_{23}H_{29}BrN_4O$. Calculated (%): C 60.39; H 6.39; Br 17.47; N 12.25. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.46, 1.52 (d, c, 8H, $(CH_2)_4^{endo}$, J=2.34), 2.68 (c, 4H, $(CH_2)_2^{endo}$, 2.86 (c, 2H, CH_2^{exo}), 4.32 (c, 2H, $N_{Bzm}CH_2$), 5.99 (c, 2H, CH_2CO), 7.26-7.35 (m, 2H, H_{Ar}), 7.60, (d, 1H, H_{Ar}, J= 7.98), 7.63-7.66 (k, 3H, H_{Ar}, J= 5.78), 7.75-7.78 (k, 1H, H_{Ar}, J= 5.36), 8.09-8.11 (m, 2H, H_{Ar}), 9.3 (b.c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 26.22, 27.65, 41.96, 49.85, 55.15, 55.27, 110.39, 110.62, 123.39, 123.49, 128.42, 128.81, 129.80, 130.21, 134.02, 134.22, 150.91, 191.07.

2-Amino-1-(2-(azepan-1-yl)ethyl)-3-(2-(3-methoxyphenyl)-2-oxoethyl)-1H-benzo[d]imidazole-3-ium bromide (5b)

Yield 80 %, m.p. 182-183ºC. IR spectrum, ν/ cm^{-1}: 3205, 3154 (NH_2), 1710 (C=O), 1633, 1591 (C=N). Found (%): C 59.03; H 6.52; Br 16.26; N 11.37. $C_{24}H_{31}BrN_4O_2$. Calculated (%): C 59.14; H 6.41; Br 16.39; N 11.49. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.45-1.5 (k, 8H, $(CH_2)_4^{endo}$, J=12.2), 2.67 (c, 4H, $(CH_2)_2^{endo}$, 2.87 (c, 2H, CH_2^{exo}), 3.86 (c, 3H, OMe), 4.31 (t, 2H, $N_{Bzm}CH_2$, J=5.58), 5.98 (c, 2H, CH_2CO), 7.25-7.35 (m, 3H, H_{Ar}), 7.55-7.63 (m, 4H, H_{Ar}), 7.70 (d, 1H, H_{Ar}, J=74), 9.3 (b.c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 26.24, 27.70, 42.02, 49.95, 55.18, 55.29, 55.56, 110.37, 110.64, 113.14, 120.14, 120.89, 123.39, 123.51, 129.84, 130.08, 134.18, 135.33, 150.92, 159.44, 190.98.

2-amino-3-(2-(azepan-1-yl)ethyl)-1-(2-oxo-2-(4-(trifluoromethyl)phenyl)ethyl)-1H-benzo[d]imidazole-3-ium bromide (5c)

Yield 96 %, m.p. 205-207ºC. IR spectrum, ν/ cm^{-1}: 3207, 3154 (NH_2),1703 (C=O), 1660, 1618, 1584 (C=N). Found (%): N 10.54. $C_{24}H_{28}BrF_3N_4O$. Calculated (%): 10.66. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.47 (c, 4H, $(CH_2)_2^{endo}$), 1.52 (c, 4H, $(CH_2)_2^{endo}$), 2.68 (c, 4H, $(CH_2)_2^{endo}$, 2.88 (c, 2H, CH_2^{exo}), 4.33 (c, 2H, $N_{Bzm}CH_2$), 6.05 (c, 2H, CH_2CO), 7.28 (t, 1H, H_{Ar}, J=7.68), 7.34 (t, 1H, H_{Ar}, J=7.74), 7.65 (t, 2H, H_{Ar}, J=4.32), 8.04 (d, 2H, H_{Ar}, J=8.28), 8.30 (t, 2H, H_{Ar}, J=8.16), 9.27 (b.c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 26.23, 27.66, 42.02, 50.26, 55.27, 110.48, 110.68, 120.97, 122.77, 123.43, 123.56, 124.57, 125.75, 126.38, 129.32, 129.81, 130.16, 132.99, 133.20, 133.41, 133.62, 137.28, 150.84, 190.82.

3-(2-([1,1'-biphenyl]-4-yl)-2-oxoethyl)-2-amino-1-(2-(azepan-1-yl)ethyl)-1H-benzo[d]imidazole-3-ium bromide (5d)

Yield 75 %, m.p. 185-187ºC. IR spectrum, ν/ cm^{-1}: 3207, 3240 (NH_2), 1687 (C=O) 1663 (C=N). Found (%): C 65.18; H 6.34; Br 14.77; N 10.38. $C_{29}H_{33}BrN_4O$. Calculated (%): C 65.29; H 6.23; Br 14.98; N 10.50. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.48, 1.54 (c, c, 8H, $(CH_2)_2^{endo}$), 2.69 (c, 4H, $(CH_2)_2^{endo}$, 2.90 (c, 2H, CH_2^{exo}), 4.34 (c, 2H, $N_{Bzm}CH_2$), 6.02 (c, 2H, CH_2CO), 7.29 (t, 1H, H_{Ar}, J=7.7), 7.35 (t, 1H, H_{Ar}, J=7.71), 7.45-7.47 (m. 1H, H_{Ar}), 7.62 (d, 1H, H_{Ar}, J=7.9), 7.65 (b.d, 1H, J=6.9), 7.81 (c, 2H, H_{Ar}), 7.97 (d, 2H, H_{Ar}, J=8.2), 8.18 (d, 2H, H_{Ar}, J=8.22), 8.30 (t, 2H, H_{Ar}, J=8.16), 9.25 (b.c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 25.23, 27.63, 41.93, 49.87, 55.24, 110.43, 110.55, 123.46, 123.54, 126.91, 127.04, 128.64, 129.14, 129.18, 129.78, 130.25, 132.86, 138.63, 145.53, 150.90, 190.65.

Azepan-1-yl)ethyl)-1-(2-((4-methoxyphenyl)amino)-2-oxoethyl)-1H-benzo[d]imidazole-3-ium chloride (5e)

Yield 74 %, m.p. 242-243ºC. IR spectrum, ν/ cm^{-1}: 3332, 3240, 3207(NH+NH_2), 1657 (C=O) 1663 (C=N). Found (%): C 62.83; H 7.15; Cl 7.61; N 15.17. $C_{24}H_{32}ClN_5O_2$. Calculated (%): C 62.94; H 7.04; Cl 7.74; N 15.29. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.45, 1.54 (c, c, 8H, $(CH_2)_2^{endo}$), 2.68 (t, 4H, $(CH_2)_2^{endo}$, J=5.31), 2.85 (t, 2H, CH_2^{exo}, J=6.0), 3.70 (c, 3H, MeO), 4.30 (t, 2H, $N_{Bzm}CH_2$, J=5.73), 5.20 (c, 2H, CH_2CO), 6.88 (d, 2H, H_{Ar}, J=9.0), 7.26-7.33 (m, 2H, H_{Ar}), 7.52-7.57 (m, 4H, H_{Ar}), 9.4 (b.c, 2H, N^+H_2), 10.84 (c, 1H, NH). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 26.22, 27.64, 41.83, 45.61, 55.07, 55.11, 55.22, 110.19, 110.45, 113.86, 120.53, 123.3, 123.36, 129.82, 130.14, 131.7, 151.1, 155.41, 163.04.

9-(2-(azepan-1-yl)ethyl)-2-(3-methoxyphenyl)-9H-benzo[d]imidazo[1,2-a]imidazole (6 HCl)

0.002 mol of bromide 5b is thoroughly stirred in 5 mL of 20% NaOH solution for 45 min. After cooling, the resulting imine precipitate is filtered off, washed with ice water until the washings are neutral, and then used in the cyclization reaction without additional purification and drying. Imine 5b is dissolved in 7.5 mL of 7.5% concentrated hydrochloric acid solution and refluxed for 3.5-4 hours, monitoring the reaction progress by TLC (Al_2O_3), eluent chloroform, visualization with iodine vapor in a humid chamber. Rf of the starting imine is 0.16, Rf of the reaction product is 0.27. After cooling to 10-15ºC, the reaction mixture is maintained at this temperature for 3-4 hours. The resulting precipitate of compound 6 is filtered off, washed with ethanol, then with acetone. The compound is dried in a desiccator over P_2O_5 for 10 hours.
Yield 80%, m.p. 238-240ºC. Found (%): C 67.72; H 6.99; Cl 8.21; N 12.98. C24H28N4O.HCl. Calculated (%): C 67.83; H 6.88; Cl 8.34; N 13.18. 1H NMR spectrum (600 MHz, DMSO-d6), δ, ppm, J (Hz): 1.65 (s, 4H, (CH2)2endo), 1.88 (s, 4H, (CH2)2endo), 3.38 (s, 2H, CH2exo), 3.63 (s, 2H, CH2endo), 3.67 (s, 2H, CH2endo, J=15), 3.85 (s, 3H, MeO), 5.12 (s, 2H, NBzmCH2), 6.95 (k, 1H, HAr, J=3.5), 7.42 (t, 1H, HAr, J=7.95), 7.54-7.56 (m, 2H, HAr), 7.66 (s, 1H, HAr), 7.98 (d, 1H, HAr, J=7.98), 8.1 (d, 1H, HAr, J=8.1), 8.69 (s, 1H, HAr), 11.2 (s, 1H, N+ H). 13C NMR spectrum (150 MHz, DMSO-d6), δ, ppm, J (Hz): 23.07, 25.94, 37.98, 52.85, 53.25, 53.82, 55.09, 55.47, 105.22, 110.72, 111.92, 112.19, 112.9, 117.17, 122.83, 124.15, 125.21, 130.21, 133.08, 134.22, 134.79, 159.77.

General Method for solts 2-imino-2,3-dihydro-1H-benzimidazole (7a-d)

A solution of 3 mmol of the appropriate 2-aminobenzimidazole and 3 mmol of an alkyl halide in 5–12 mL of dioxane is refluxed for 5.5 hours. During the reaction, the solution turns yellow-red, and a precipitate forms. The precipitate is filtered off, washed with dioxane and acetone until the filtrate becomes colorless. The mixture is dried and crystallized from ethanol or 2-propanol.

1-(4-flurobenzyl)-3-(2-(pyrrolidin-1-yl)ethyl)-1,3-dihydro-2H-benzo[d]imidazole-2-imine hydrogen chloride (7a)

Yield 87%, m.p. 239-240ºC. IR spectrum, ν/ cm-1: 3332 (NH). Found (%): N 14.83. C20H23FN4.HCl. Calculated (%): N 14.95. 1H NMR spectrum (600 MHz, DMSO-d6), δ, ppm, J (Hz): 1.66 (s, 4H, (CH2)2endo), 2.63 (s, 4H, (CH2)2endo), 3.80 (s, 2H, CH2 exo), 5.02 (s, 2H, NBzmCH2), 5.56 (s, 2H, NBzmCH2), 7.18-7.38 (m, 7H, HAr), 7.39 (d, 1H, J=6.0), 7.8 (s, 1H, NH). 13C NMR spectrum (150 MHz, DMSO-d6), δ, ppm, J (Hz): 21.38, 23.12, 44.73. 44.83, 53.50, 56.46, 56.46, 110.5, 110.6, 115.9, 115.95, 118.0, 118.0, 128.50, ~~129.50,~~ 129.50, 130.9, 131.0, 150.27, 160.8.

1-(2-phenoxyethyl)-3-(2-pyrrolidin-1-yl)ethyl)-1,3-dihedro-2H-benzo[d]imidazole-2-imine hydrogen bromide (7b)

Yield 50 %, m.p. 201-202º C (2-PrOH). IR spectrum, ν/ cm^{-1}: 3495, 3442 (NH_2). Found (%): C 58.36; H 6.41; Br 18.37; N 12.85 $C_{21}H_{26}N_4O$ HBr. Calculated (%): C 58.47, H 6.31; Br 18.52; N 12.99. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 1.62 (c, 4H, $2CH_2^{endo}$), 2.52 (c, 4H, CH_2NCH_2), 2.79 (c, 2H, $CH_2^{exo}N$), 4.29 (c, 4H, N^1-CH_2, N^3-CH_2), 4.62 (c, 2H, CH_2-O), 6.8 (~~д~~,d, 2H, H_{Ar}, J=7.86), 6.91 (t,1H, H_{Ar}, J=6.87), 7.23 (t, 2H, H_{Ar}, J=7.17), 7.32 (c, 2H, $H^{5,6}$), 7,64-7.54 (m, 2H, H_{Ar},). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 23.12, 42.26, 42.40, 52.74, 53.54, 64.92, 110.32, 110.82, 114,21, 120.97, 123.23, 123.36, 129.41, 129.70, 129.67, 150.90, 157.70.

Methyl 4'-((2-imino-3-(phenoxyethyl)-2,3-dihydro-1H-benzo[d]imidazol-1-yl)methyl)-[1,1'-biphenyl]-2-carboxylate hydrobromide (7c)

Yield 72 %, m.p. 230-231º C (EtOH). IR spectrum, ν/ cm^{-1}: 3344 (NH), 1726 ($COOCH_3$), 1655 (C=N). Found (%): C 64.41; H 5.17; Br 14.18, N 7.39. $C_{30}H_{27}N_3O_3$ HBr**.** Calculated (%): C 64.52; H 5.05; Br 14.31, N 7.52. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 3.52 (c, 3H, OCH_3), 4.35 (t, 2H, N^3-CH_2, J=5.13), 4.69 (t, 2H, O-CH_2, J=5.13), 5.54 (c, 2H, N^1-CH_2), 6.81, 6.81 (c, c, 2H, H_{Ar}), 6.89-6.92 (m, 1H, H_{Ar}),7.22-7.31 (m ,7H, $H^{5,6}$, $2H_{Ar}$), 7.34-7.37 (m, 2H, $H^{4,7}$), 7.46-7.49 (m, 2H, H_{Ar}), 7.58-7.61 (m, 1H, H_{Ar}), 7.71-7.73 (m, 2H, H_{Ar}), 9.12 (c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 42.62, 45.32, 51.73, 64.83, 110.56, 111.09, 114,25, 121.02, 123.64, 123.66, 126.72, 127.52, 128.50, 129.28, 129.33, 128.44, 129.90, 130.39, 130.56, 131.45, 133.42, 140.15, 140.56, 150.14, 157.72, 168.08.

1-([1,1'-biphenyl]-4-yl-methyl)-3-benzyl-1,3-dihydro-2H-benzo[d]imidazole-2-imine hydrobromide (7d)

Yield 84 %, m.p. 286-287ºC (EtOH/H_2O). Found (%) C 68.83; H 5.25; N 8.81. $C_{27}H_{23}N_3$·HBr. Calculated (%): C 68.94; H 5.14; N 8.93. ^{1}H NMR spectrum (600 MHz, DMSO-d_6), δ, ppm, J (Hz): 5.55 (c, 2H, CH_2), 5.59 (c, 2H, CH_2), 7.27-7.29 (m, 2H, $H^{5,6}{}_{Bzm}$), 7.32, 7,34 (c c, 3H, H_{ph}+$H^{4,7}{}_{Bzm}$), 7.35-7.55 (m, 9H, $4H_{ph}$ + $5H_{Bif}$), 7.62-7.64 (m, 2H, H_{Bif}), 7.67 (c, 1H, H_{Bif}), 7.69 (c, 1H, H_{Bif}), 9.26 (c, 2H, N^+H_2). ^{13}C NMR spectrum (150 MHz, DMSO-d_6), δ, ppm, J (Hz): 45.51, 45.80, 110.89, 123.87, 126.62, 127.01, 127.10, 127.61, 127.66, 128.03, 128.86, 128.92, 129.57, 133.67, 134.50, 139.40, 139.91, 149.99.

Effect of compounds on intraocular pressure

The ophthalmic hypotensive effect of the compounds was studied on adult outbred rats using a tonometer mounted on a TonoVet veterinary tonometer (Finland) (Du Sert et al. 2020). All animals were divided into 20 groups, with each group containing 6 rats: 2 groups of reference drugs (timolol and melatonin), and 18 experimental groups instilled with solutions of the test compounds. The compounds were tested using the method of Marcus et al. (Pease et al. 2006), according to which one drop of the test compound solution was instilled into the right (test) eye of the laboratory animal, and the solvent (deionized water) was instilled into the left eye. IOP was measured in both eyes. The left eye served to assess the possible resorptive effect of the test compounds.

The studied melatonin isosteres – benzimidazole derivatives – were instilled into the test eye at a screening concentration of 0.4% once in a volume of 30 μL. IOP measurements were performed at four time points (0, 1, 2, and 3 hours), with 0 hours being the baseline value. Ophthalmic hypotensive activity was assessed by the maximum reduction in IOP relative to baseline. A 0.5% timolol solution (a drug used in clinical practice) and a 0.4% melatonin solution (SigmaAldrich, USA) were used as comparators.

Effect of compounds on Rho-kinase-2 activity in vitro

For the study, a 15 ng/mL Rho-kinase-2 standard solution was prepared.
The test compounds were diluted to the desired concentration ($1*10^{-4}$ mol/L).
The test compound solutions were mixed with the standard solution at a ratio of 1:9 and incubated for 30 minutes at 37°C to initiate the enzyme inhibition reaction. The effect of the test compounds on Rho-kinase-2 activity was then assessed using the Rho Associated Coiled Coil Containing Protein Kinase 2 ELISA Kit (Cloud-Clone Corp., China). The enzymatic-substance reaction is assessed by the color change spectrophotometrically at a wavelength of 450 nm ± 10 nm with Infinite 200 PRO multiplate reader (Tecan, Austria).

Statistical analysis

Statistical analysis was performed in Prism 8.0 (GraphPad Inc.), with calculation of the mean and standard deviation. Student's t-test was used to assess changes in IOP relative to baseline values. The nonparametric Mann-Whitney test was used to compare groups.

Results and Discussion

The study revealed that after instillation of the comparator drugs timolol and melatonin, IOP decreased in the test eye by 26.84% and 30.95%, respectively, relative to baseline values. Similar changes were observed in the contralateral (control) eye, which may indicate an undesirable resorptive effect, likely related to the absorption of the test substance solutions into the bloodstream (Table 1).

Melatonin, known Rho-kinase-2 inhibitors, and the new compounds synthesized for the study are heterocyclic nitrogen-containing structures. When synthesizing the new benzimidazole-based compounds, the structure of Rho-kinase-2 inhibitors was taken into account. As a result, the structures of the studied compounds included certain fragments (radicals) that could theoretically be responsible for the corresponding mechanism of action and the ophthalmic hypotensive effect.

All synthesized compounds were divided into five groups.

The first group included six compounds **1a-f**, which are urea derivatives where one nitrogen atom contains benzimidazole and the other a substituted phenyl. In compounds **1a-d**, the N^1 nitrogen atom in the benzimidazole contains a dialkylaminoethyl substituent, and the phenyl moiety contains a p-Cl, m-Cl, or m-CF_3 group. Instillation of 1-(4-chlorophenyl)-3-(1-(2-pyrrolidin-1-yl)ethyl)-1*H*-benzo[*d*]imidazole-2-yl)urea hydrogen chloride (**1a**) resulted in a 16.26±0.43% decrease in IOP in the study animals. In the structure of compound **1c**, the Cl atom is transferred to the meta position, resulting in a significant increase in ophthalmic hypotensive activity and a 22.98±7.53% decrease in IOP relative to baseline values. At the same time, replacing m-Cl with the m-CF_3 group (compound **1b**) led to a decrease in ophthalmic hypotensive activity to 12.50±12.50%. However, replacement of the pyrrolidine ethyl substituent with 1-ethylazepane in compound **1d** (1-(1-(2-((azepan-1-yl)ethyl)-1*H*-benzo[*d*]imidazole-2-yl)-3-(3-trifloromethyl) phenyl)urea hydrogen chloride) significantly increased the IOP-lowering activity, and instillation of a solution of this compound decreased the ophthalmotonus by 23.96±6.78% ($p \leq 0.05$, t-test). In compounds **1e** and **1f**, only replacement of the diethylaminoethyl radical at the N^1 atom of benzimidazole with methyl biphenylcarboxylate negatively affected the ophthalmohypotensive effect. Thus, compound **1e**, with one electronegative m-Cl atom, reduced IOP by 11.36±2.66% when instilled into normotensive animals, while compound **1f**, with three more electronegative F atoms, reduced IOP by 17.17±4.84%.

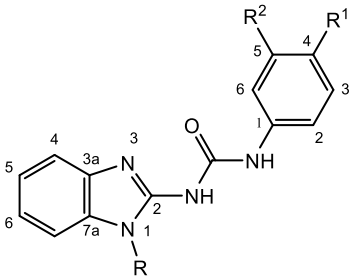
Among the studied subgroup, only compound **1c** demonstrated a decrease in IOP in the contralateral eye, which may indicate a systemic effect, which is an undesirable effect. The remaining compounds – **1a**, **1b**, **1d**, **1e**, and **1f** – had no effect on the ophthalmotonus of the control eye.

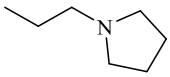
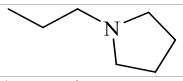
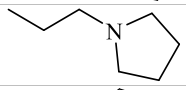
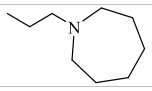
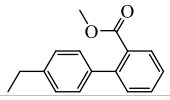
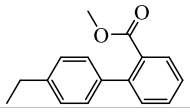
The second group is represented by benzimidazole acetamides **4a** and **4b**, containing 4-nitrophenylpiperazine. Compound N-(1-allyl-1H-benzo[*d*]imidazole-2-yl)-2-(4-(4-nitrophenyl)piperazin-1-yl)acetamide (**4a**) did not affect IOP when instilled into normotensive animals. This may be due to the negative effect of the allyl radical at the N^1 nitrogen of benzimidazole on the biological activity. Instillation of compound **4b** resulted in a decrease in ophthalmotonus by 12.93±2.46% relative to the initial values; similar dynamics were not observed in the contralateral eye, which may indicate the absence of the resorptive effect of the test substance.

Table 1. Effect of 0.5% solution of timolol, melatonin and new benzimidazole derivatives on IOP of normotensive rats after a single instillation at a concentration of 0.4%

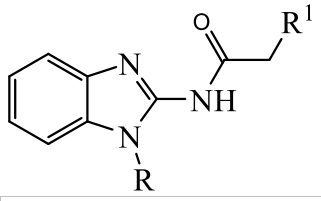
Compound	Max Δ IOP, %	Effect on the contralateral eye
Timolol	-26.84±0.94*	+
Melatonin	-30.33±2.91*	+

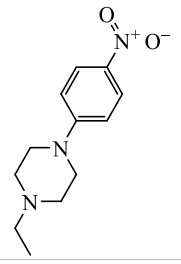
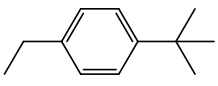
Base structure



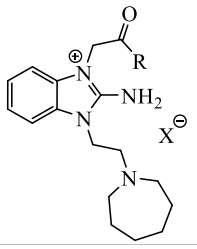
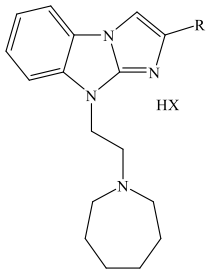
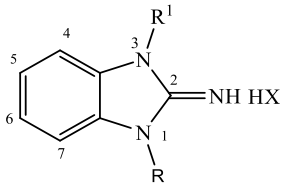
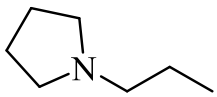
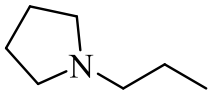
	R	R^1	R^2	Max Δ IOP, %	Effect on the contralateral eye
1a		Cl	H	16.26±0.43*	-
1b		H	CF_3	12.50±12.50	-
1c		H	Cl	-22.98±7.53	+
1d		H	CF_3	**-23.96±6.78***	-
1e		H	Cl	-11.36±2.66	-
1f		H	CF_3	-17.17±4.84	-

Base structure



	R	R^1	Max Δ IOP, %	Effect on the contralateral eye
4a	$-CH_2-CH=CH_2$		no effect	-
4b			-12.93±2.46	-

End of table 1

	Base structure 			
	R	$X^{\ominus}$	Max Δ IOP, %	Effect on the contralateral eye
5a	Ph	**Br** $^{\ominus}$	-14.43±8.93	+
5b	3-$MeOC_6H_4$	**Br** $^{\ominus}$	no effect	-
5c	4-$CF_3C_6H_4$	**Br** $^{\ominus}$	-10.74±6.43	+
5d	4-PhC_6H_4	**Br** $^{\ominus}$	-22.71±10.84	-
5e	NH(4-$MeOC_6H_4$)	Cl $^{\ominus}$	-16.48±1.19*	+
	Base structure 			
	R	**HX**	Max Δ IOP, %	Effect on the contralateral eye
6	3-$MeOC_6H_4$	2HCl	no effect	-
	Base structure 			
	R	**R^1**	Max Δ IOP, %	Effect on the contralateral eye
7a	$CH_2C_6H_4F$-4		**-28.21±2.56***	-
7b	$CH_2CH_2OC_6H_5$		-14.47±10.85	-
7c	4-(2′-$MeOOCC_6H_4$)C_6H_4	$CH_2CH_2OC_6H_5$	-19.10±8.56	-
7d	Ph	$CH_2C_6H_4C_6H_5$	-17.81±9.47	-

Note: "-" – absence of effect; "+" – presence of effect; * – differences are statistically significant relative to baseline values (Student's t-test, $p<0.05$).

The third group of the studied compounds are benzimidazolium halides **5-a-e**, obtained by quaternization of 1-(2-(azepan-1-yl)ethylbenzimidazoleamine with haloketones. After instillation of bromide solution **5a** (R=Ph), there was a decrease in IOP in the test eye by 14.43±8.93%. The inclusion of a methoxy group in the meta-position of phenyl R (compound **5b**) resulted in the absence of an ophthalmohypotensive effect, thus, instillation of the test compound had no effect on IOP. At the same time, the replacement of phenyl in the R radical with biphenyl – compound **5d** – on the contrary, significantly increased the IOP-lowering activity. Thus, in animals that were instilled with a solution of compound **5d** in the test eye, ophthalmotonus decreased by 22.71±10.84%. Instillation of solutions of compounds **5c** (R=CF_3) and **5e** (R= 4-MeOC6H4NH-) resulted in a decrease in IOP by 10.74±6.43% and 16.48±1.19%, respectively. Among the compounds in this group, only substance **5d** showed no effect on the contralateral eye, which may indicate the absence of a systemic effect.

Compound **6** was isolated into a separate group, since the basic structure is imidazobenzimidazole. Cyclization of the inactive benzimidazolium bromide **5b** to the tricyclic structure of imidazo[1,2-*a*]benzimidazole 6, unfortunately, did not contribute to the appearance of ophthalmic hypotensive activity.

The fifth group is represented by derivatives of 1,3-dihydro-2*H*-benzo[*d*]imidazole-2-imine, in which the radicals R = 4-fluorobenzyl (**7a**), phenoxyethyl (**7b**), methyl biphenylcarboxylate (**7c**), benzyl (**7d**) are located at the nitrogen atom N^1, and the radical R^1 = ethylpyrrolidine (**7a,b**), ethylphenoxy (**7c**) and 1.1'-biphenylmethyl (**7d**) are in position N^3.

After instillation of 1-(4-flurobenzyl)-3-(2-(pyrrolidin-1-yl)ethyl)-1,3-dihydro-2H-benzo[d]imidazole-2-imine hydrogen chloride (**7a**) solution, IOP in the test eye was significantly reduced by 28.21±2.56% ($p \leq 0.05$, t-test) relative to baseline, which is the highest effect among all the studied substances. Replacing the 4-fluorobenzyl substituent with ethylphenoxy decreases the IOP-lowering activity by 2-fold to 14.47±10.85% in the case of compound 7**b**. Replacing the pyrrolidinoethyl fragment with methyl biphenylcarboxylate, as in the structure of compound **7c**, slightly increases the ophthalmic hypotensive effect to 19.10±8.56%. In compound **7d**, the R and R^1 radicals are represented by benzyl and 1.1'-biphenylmethyl, respectively. However, this structure has virtually no effect on the strength of the ophthalmic hypotensive effect, and instillation of a solution of this compound reduces IOP by 17.81±9.47%. No effect on the contralateral eye was observed for the studied compounds of this subgroup, which may indicate the absence of a systemic effect.

Thus, following the screening, compounds with laboratory codes **7a** and **1d** emerged as the most promising, as they reliably reduced IOP, slightly inferior to the reference drugs timolol and melatonin, and did not exhibit a systemic effect. For these compounds, the probable mechanism of action – impact on Rho kinase activity – was assessed (Table 2).

Table 2. Effect of benzimidazole derivatives at a concentration of 1*10-4 mol/L on the activity of Rho-kinase-2 in the ELISA test in vitro.

Test compound	% ROCK-2 inhibition
1d	18.11
7a	26.72

The study revealed that both compounds exhibit inhibitory activity against Rho kinase. Compound **1d** inhibited this enzyme by 18.11%, while compound **7a** inhibited it by 26.72%.

Conclusion

Thus, based on the results of the studies, newly synthesized benzimidazole derivatives containing fragments of known Rho-kinase-2 inhibitors have proven to be a promising class for the search for new IOP-lowering compounds. According to the results of a study on normotensive outbred rats, with a single instillation at a concentration of 0.4%, the most active compounds are compound **7a**, which has higher ophthalmic hypotensive activity than the reference drug timolol, and compound **1d**, which is slightly inferior to the reference drug timolol. However, unlike timolol, these compounds do not affect IOP dynamics in the contralateral eye, indicating the absence of an undesirable resorptive effect.

Based on the structural analysis, it can be suggested that the most promising compounds for the development of ophthalmic hypotensive agents are those containing dimethylbenzimidazolimine (compounds **7a-d**) and benzimidazole urea (compounds **1a-f**) in their structure, as all compounds exhibited IOP-lowering activity. The presence of strong electronegative atoms and heterocyclic structures – hexamethyleneimine and pyrrolidine – in the radicals (compounds **7a** and **1d**) also leads to increased activity.

Based on the ophthalmic hypotensive activity results and the assessment of the effects of new benzimidazole derivatives, it can be assumed that there is a relationship between these activities: the stronger the inhibitory activity against ROCK, the greater the ophthalmic hypotensive effect.

Additional Information

Conflict of interest

The authors declare no conflict of interest.

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Ethics statement

The experiments were approved by the Biomedical Ethics Committee of Volgograd State Medical University (extract from minutes № 2022/043 dated December 2, 2022).

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Data Availability

All data supporting the results of this study are available in the main text.

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