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RESEARCH ARTICLE

# The Development of New Factor Xa Inhibitors Based on Amide Synthesis

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**Abstract:** ***Background:*** Factor Xa (FXa) is known to play a central role in blood coagulation cascade and considered to be one of the most attractive targets for oral anticoagulants of new generation.

***Objective:*** Our approach for the development of directly acting oral anticoagulants (DOAC), FXa inhibitors was demonstrated in this work.

***Method:*** Chemical synthesis is the base of our approach for the development of potential inhibitors. In this work, the substances like $R_1$-(CONH)-$R_2$-(CONH)-$R_3$ are being developed, using previously described docking and screening methods, where $R_1$, $R_2$ and $R_3$ are some chemical groups and (CONH) are amide bonds connecting $R_1$, $R_2$ and $R_3$. The direction of amide bond (CONH) could be arbitrary for $R_1$, $R_2$ and $R_2$, $R_3$.

***Results:*** Chemical modifications were made in the frame of the results, taking into account the structure of FXa, chemical synthesis capabilities, as well as patentability of the target compounds. Subnanomolar potency of several developed compounds was achieved. Several analyzers and various testing-suites have been used to measure the concentration that doubled the prothrombin time (PTx2). Moreover, in human plasma the PTx2 concentration of the compound **217** (DD217) turned out to be 80±20 nM**.** The compound efficacy has proved by *in vivo* assays including oral administrations in rats, rabbits and monkeys.

***Conclusion:*** The pharmacodynamic profile of DD217 for oral administration in cynomolgus monkeys proves the efficacy of the compound, which makes it promising for the future preclinical trials.

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## 1. INTRODUCTION

Thrombus formation in the blood vessels can lead to severe conditions such as acute myocardial infarction, ischemic stroke, unstable angina, deep vein thrombosis and pulmonary embolism. Anticoagulants are among the main medications for the prevention and treatment of these conditions [1]. Vitamin K antagonists were the only class of oral anticoagulants available for decades. Despite proven efficacy, they have plenty of flaws, such as interaction with many other drugs and foods, narrow therapeutic window, unpredictable time course, slow onset and offset effects, and bleeding complications requiring routine monitoring of anticoagulant activity [2-4]. The drawbacks have motivated the development of oral anticoagulants with different mechanisms of action [5]. Numerous studies have shown that FXa is one of the most attractive targets for new antithrombotic agents [6]. FXa is a trypsin-like serine protease, which plays a central role in blood coagulation cascade. Being a part of both the intrinsic and the extrinsic pathways, FXa has the primary function to regulate the conversion of prothrombin to thrombin. The latter is responsible for many coagulation-related reactions, including the transformation of soluble fibrinogen into insoluble strands of fibrin involved in the clotting of blood [7]. Several direct FXa inhibitors have been reported to date [8-10]. In spite of the rapid sales growth of new directly acting oral anticoagulants (DOAC), warfarin is still widely prescribed. In this work, we show the results of development of DOACs, FXa inhibitors, which may become potential anticoagulants. The search for biological active compounds was made on chemical space formed by Lipinsky's rules [11]. The most essential part of the search constriction taken into account immediately in calculations was related with the procedure of chemical synthesis. The choice of the procedure is the key point based on many parameters, including active site peculiarities of the target protein, complexity and cost of chemical synthesis, and patent clearness of the potential chemical substances. The structures of synthesized biologically active compounds, as well as corresponding inhibition constants $K_i$ are represented in the results section. The concentration of 80±20 nM of one of the compounds that doubled the prothrombin time (PTx2) in human plasma is among the best values ever reported.

## 2. MATERIALS AND METHOD

### 2.1. Reagents and Chemicals

4-methylaminobenzoic acid (Aldrich, USA), methyl isocianat (Aldrich, USA) isopropanol (Rushim, Russia), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) (Fluka, USA)), Tetrahydrofuran (THF) (Rushim, Russia), silica gel - normal SILICAGEL KSKG 0.04-0.10 (ChromLab, Russia), Silica gel C8-Reversed phase 35-75 mkm, 150 Å (Anatech, USA), chloroform (Rushim, Russia), ethanol (Ferain, Russia), 5-methoxy-2-nitrobenzoic acid (Aldrich, USA), 2-fluoro-4-(methylamino)benzoic acid (Aldrich, USA), trifluoroacetic acid (Sigma-Aldrich, USA), 5-methyl-2-nitrobenzoic acid (Aldrich, USA), $SOCl_2$ (Chimmed, Russia), 4-chloroaniline (Aldrich, USA), pyridine (Fluka, USA), HCl (Chimmed, Russia), ethyl acetate (Chimmed, Russia), NaOH (Rushim, Russia), $NaHCO_3$ (Bashkir soda company, Russia), 5-fluoro-2-nitrobenzoic acid (Aldrich, USA), trifluoroacetic anhydride (TFAA) (Aldrich, USA), 2-nitrobenzoic acid (Aldrich, USA), dimethyl sulfoxide (DMSO) (Rushim, Russia), $CaCl_2$ (Rushim, Russia), 2-amino-5-chloropyridine (Aldrich, USA), 4-methylaniline (Aldrich, USA), 2-methoxy-4-(methylamino)benzoic acid (Aldrich, USA), 5-chloro-2-nitrobenzoic acid (Aldrich, USA), 2-methoxy-4-aminobenzoic acid (Sigma-Aldrich, USA), methyl iodide (Aldrich, USA), zinc powder (Rushim, Russia), $K_2CO_3$ (Rushim, Russia), Renam's KM-2 control plasma (local manufacturer NPO Renam, Russia), chromogenic substrate and human FXa from the Renaparin-test set (local manufacturer NPO Renam, Russia), thromboplastin Thromborel S (Siemens, Germany), Rivaroxaban, tablet formulation (Xarelto 20 mg tablets of *Bayer* AG, expiry date 02.2013, shipment no. R52004).

### 2.2. Chemistry

**5-chloro-N-(5-chloropyridin-2-yl)-2-[(4-{methyl[(methylamino)carbonyl]amino} benzoyl)**

**amino]benzamide (115).** Reaction 1: 900 mg of 5-chloro-2-nitrobenzoic acid (**1**) was boiled in 20 mL of $SOCl_2$ in a reflux condenser equipped with a calcium chloride tube for 4 h; the obtained solution was cooled, evaporated in a rotary evaporator, twice re-evaporated with anhydrous THF; the residue was dissolved in 10 mL of THF; the obtained solution was added drop wise for 30 min to a stirred solution of 700 mg of 2-amino-5-chloropyridine (**6**) in 20 mL of THF. After 15 h, the reaction mixture was evaporated; the residue was dissolved in 30 mL of chloroform, washed with a saturated aqueous solution of $NaHCO_3$; the chloroform extract was evaporated; the residue was applied on a 20x150 mm column filled with silica gel. The product was eluted with chloroform. Detection was carried out with the aid of an UV-unit at a wavelength of 280 nm. The UV absorbing fractions were collected; the purity of the product was controlled with a TLC technique in chloroform. The Rf of the product was 0.4 and the Rf of the starting 2-amino-5-chloropyridine was 0.7. The yield of 5-chloro-N-(5-chloropyridin-2-yl)-2-nitrobenzamide (**25**) was 750 mg. The mass spectrum (MALDI-VP): M+H312, M+Na334.

Reaction 2: 750 mg of **25** was dissolved in 30 mL of ethyl acetate and mixed with a solution of 4 g of $SnCl_2$ in 20 mL of water acidified with 0.2 mL of concentrated HCl. The reaction mixture was stirred vigorously for 1 h, heated up to boiling, and was boiled for another 3 h. Then the reaction mixture was filtered, the aqueous fraction was extracted with $CHCl_3$, 30 mL of 10% of aqueous ammonia solution was added to it with stirring, and the mixture was allowed to stand for a night to precipitate. The next day, the precipitate was filtered and rinsed with water and chloroform. The aqueous fraction was extracted with chloroform; the extracts were combined and evaporated. The residue was applied on a 20x150 mm column filled with silica gel. The product was eluted with chloroform. Detection was carried out with the aid of an UV-unit at a wavelength of 280 nm; the purity of the product was controlled with a thin-layer chromatography technique in chloroform. The Rf of the product was 0.6; the yield of 2-amino-5-chloro-N-(5-chloropyridin-2-yl)benzamide (**9**) was 300 mg. The mass spectrum (MALDI-VP): M+H 282, M+Na 304.

Reaction 3: A solution of 750 mg of 4-(methylamino)benzoic acid (**16**) in 10 mL of THF was added to 700 mL of methyl isocyanate. The reaction mixture was stirred at room temperature for 72 h, evaporated, and re-evaporated with water. The product was recrystallized from isopropanol. The yield of 4-{methyl[(methylamino) carbonyl]amino}benzoic acid (**19**) was 700 mg.

Reaction 4: 300 mg of **19**, 250 mg of **9**, and 300 mg of EDCI in 2 mL of THF were stirred for 72 h. Then the reaction mixture was evaporated, the residue was separated with a chromatography technique on silica gel. The column was washed with chloroform and then with 9:1 chloroform/ethanol. Rf=0.5 (chloroform). The yield of **115** was 280 mg. Rf=0.3 (8:2 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 472, M+Na 494.

**N-(5-chloropyridin-2-yl)-5-methyl-2-[(4-{methyl[(methylamino)carbonyl]amino} benzoyl) amino]benzamide (117).** By using the procedure described above for reaction 1 of **115**, from 800 mg of 5-methyl-2-nitrobenzoic acid (**2**) were obtained 650 mg of N-(5-chloropyridin-2-yl)-5-methyl-2-nitrobenzamide (**26**). Rf=0.45 (chloroform). The mass spectrum (MALDI-VP): M+H 292, M+Na 314. By using the procedure described above for reaction 2 of **115**, from 600 mg of **26**350 mg of 2-amino-N-(5-chloropyridin-2-yl)-5-methylbenzamide (**10**) was obtained. Rf=0.65 (chloroform). The mass spectrum (MALDI-VP): M+H 262, M+Na 284. By using the procedure described above for reaction 4 of **115**, from 240 mg of **19** and 220 mg of **10**, 250 mg of **117** was obtained. Rf=0.55 (chloroform). The mass spectrum (MALDI-VP): M+H 452, M+Na 474.

**5-chloro-N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl(methyl)amino]benzoyl}amino) benzamide (216).** Reaction 1: 1 g of **16** was mixed with 3 mL of TFAA at 0°C. After 2 hours, the reaction mixture was diluted with 10 ml of chloroform and evaporated at 20°C using rotary evaporator. After that, another 10 ml of chloroform was added to the residue and evaporation was repeated. The residue was dissolved in chloroform and was applied on a 20x150 mm column filled with silica gel. The byproducts were eluted with chloroform and the target product was eluted with a 9:1 chloroform/isopropanol mixture added with 1% acetic acid. The yield of 4-[methyl(trifluoroacetyl)amino]benzoic acid (**22**) was 700 mg. Rf was 0.2-0.4 (9:1 chloro-

form/isopropanol mixture added with a 1% acetic acid). The mass spectrum (MALDI-VP) in negative ions: M-1 246.

By using the procedure described above for reaction 4 of **115**, from 300 mg of **9** and 250 mg of **22** 430 mg of 5-chloro-N-(5-chloropyridin-2-yl)-2-({4-[methyl(trifluoroacetyl)amino]benzoyl} amino)benzamide (**27**) was obtained. Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 511, M+Na 533.

Reaction 2: 400 mg of **27** was dissolved in 5 mL of isopropanol and added to 2 mL of 10% NaOH. The reaction mixture was stirred at room temperature for 3 h; then the excess alkali was neutralized with 5% aqueous solution of HCl; the reaction mixture was evaporated and applied on a 20x150 mm column filled with silica gel. The product was eluted with chloroform; Rf=0.4. The yield of 5-chloro-N-(5-chloropyridin-2-yl)-2-{[4-(methylamino)benzoyl]amino}benzamide (**28**) was 300 mg. The mass spectrum (MALDI-VP): M+H 456, M+Na 478.

Reaction 3: 250 mg of **28** was dissolved with heating in 5 mL of acetonitrile. The obtained solution was cooled on ice and then dry gaseous HCl was passed through it. After 30 minutes, the solution was placed in a refrigerator and kept at 5°C for 48 h. Then, the reaction mixture was neutralized by $NaHCO_3$ (0.5 g). To the resulting solution, 5 mL of water was added and extracted with chloroform (3 × 10 mL). The chloroform extracts were combined and evaporated. The residue was dissolved in water and applied on a 20x250 mm column filled with reversed phase of C2 (RP2). The column was washed with 100 mL of water; then, elution with a gradient of ethanol from 0 to 50% was carried out against a background of 1% acetic acid. The purity control was carried out with a TLC technique in a 9:1 dioxane/aqueous ammonia system; Rf=0.2. The yield of **216** was 130 mg. The mass spectrum (MALDI-VP): M+H 456, M+Na 478.

**N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl(methyl)amino]benzoyl}amino)-5-methylbenzamide (217).** By using the procedure described above for reaction 4 of **115,** from 300 mg of **10** and 250 mg of **22** acid 430 mg of N-(5-chloropyridin-2-yl)-5-methyl-2-({4-[methyl (trifluoroacetyl)amino]benzoyl}amino)benzamide was obtained (**29**). Rf=0.55 (chloroform). The mass spectrum in positively charged ions was M+H 491. By using the procedure described above for reaction 2 of **216**, from 400 mg of **29** 300 mg of N-(5-chloropyridin-2-yl)-5-methyl-2-{[4-(methylamino) benzoyl]amino}benzamide was obtained (**30)**. Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 395, M+Na 417. By using the procedure described above for reaction 3 of **216**, from 250 mg of **30** 130 mg of **217** was obtained. The mass spectrum (MALDI-VP): M+H 436, M+Na 458.

**N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl(methyl)amino]benzoyl}amino)-5-methoxybenzamide (301).** By using the procedure described above for reaction 1 of **115**, from 1.5 g of 5-methoxy-2-nitrobenzoic acid (**3**) 1.1 g of N-(5-chloropyridin-2-yl)-5-methoxy-2-nitrob-enzamide (**31**) was obtained. Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 308, M+Na330. By using the procedure described above for reaction 2 of **115**, from 1 g **31** 300 mg of 2-amino-N-(5-chloropyridin-2-yl)-5-methoxybenzamide was obtained (**11**). Rf=0.7 (chloroform). The mass spectrum (MALDT-VP); M+H 278, M+Na 300. By using the procedure described above for reaction 4 of **115**, from 250 mg **11** and 220 mg **22** 310 mg of N-(5-chloropyridin-2-yl)-5-methoxy-2-({4-[methyl(trifluoroacetyl)amino]benzoyl}amino)benzamid e was obtained (**32**). Rf=0.6 (chloroform). The mass spectrum (MALDI-VP): M+H 507, M+Na 529. By using the procedure described above for reaction 2 of **216**, from 280 mg of **32** 200 mg N-(5-chloropyridin-2-yl)-5-methoxy-2-{[4-(methylamino)benzoyl]amino}benzamide was obtained (**33**). Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 411, M+Na 433. By using the procedure described above for reaction 3 of **216**, from 170 mg of **33** 110 mg of **301** was obtained. Rf=0.2 (9:1 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 452, M+Na 474.

**N-(2-{[(5-chloropyridin-2-yl)amino]carbonyl}-4-methylphenyl)-4-[ethanimidoyl(methyl) amino]-2-fluorobenzamide (302).** Reaction 1: 0.5 mg of 2-fluoro-4-(methylamino)benzoic acid (**17**) was mixed with 1 mL of TFAA. After 1 h, the reaction mixture was evaporated and the residue was applied onto a 20x150 column filled with silica gel. The product was eluted in a 9:1 chloroform/ethanol system. The yield of 22-fluoro-4-[methyl(trifluoroacetyl)amino]benzoic acid (**23**) was 470 mg.

By using the procedure described above for reaction 4 of **115**, from 450 mg of **10** and 480 mg of **23** 470 mg of N-(2-{[(5-chloropyridin-2-yl)amino]carbonyl}-4-methylphenyl)-2-fluoro-4-[methyl(trifluoroacetyl)amino]benzamide was obtained (**34**). Rf=0.5 (methylene chloride). By using the procedure described above for reaction 2 of **216**, from 450 mg of **34** 380 mg of N-(2-{[(5-chloropyridin-2-yl)amino]carbonyl}-4-methylphenyl)-2-fluoro-4-(methylamino)benzamide was obtained (**35**). Rf=0.2 (chloroform). By using the procedure described above for reaction 3 of **216**, from 350 mg of **35** 160 mg of **302** was obtained. Rf=0.2 (9:1 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 454, M+Na 476.

**N-(4-chloro-2-{[(5-chloropyridin-2-yl)amino] carbonyl}phenyl)-4-[ethanimidoyl (methyl) amino]-2-fluorobenzamide (303).** By using the procedure described above for reaction 4 of **115**, from 350 mg **9** and 370 mg **23** 370 mg of N-(4-chloro-2-{[(5-chloropyridin-2-yl)amino] carbonyl} phenyl)-2-fluoro-4-[methyl(trifluoroacetyl) amino]benzamide was obtained (**36**). Rf=0.5 (methylene chloride). By using the procedure described above for reaction 2 of **216**, from 350 mg of **36** 320 mg N-(4-chloro-2-{[(5-chloropyridin-2-yl)amino]carbonyl}phenyl)-2-fluoro-4-(methylamino)benzamide was obtained (**37**). Rf=0.2 (chloroform). By using the procedure described above for reaction 3 of **216**, from 300 mg of **37** 140 mg of **303** was obtained. The mass spectrum (MALDI-VP): M+H 474, M+Na 496.

**N-(4-chlorophenyl)-2-({4-[ethanimidoyl (methyl)amino]benzoyl}amino)-5-methylbenzamide (304).** By using the procedure described above for reaction 1 of **115**, from 1.6 g of **2** and 1.3 g of 4-chloroaniline (**7**) 2.1 g of N-(4-chlorophenyl)-5-methyl-2-nitrobenzamide was obtained (**38**). The mass spectrum (MALDI-VP): M+H 291, M+Na 313. By using the procedure described above for reaction 2 of **115**, from 2.0 g of **38** 1.3 g of 2-amino-N-(4-chlorophenyl)-5-methylbenzamide was obtained (**14**). The Rf of the product was 0.6; The mass spectrum (MALDI-VP): M+H 261, M+Na 283. By using the procedure described above for reaction 4 of **115**, from 500 mg **14** and 400 mg **22** 600 mg of N-(4-chlorophenyl)-5-methyl-2-({4-[methyl(trifluoroacetyl)amino] benzoyl}amino)benzamide was obtained (**39**). Rf=0.5 (methylene chloride). The mass spectrum (MALDI-VP): M+H 490, M+Na 512. By using the procedure described above for reaction 2 of **216**, from 350 mg of **39** 290 mg of N-(4-chlorophenyl)-5-methyl-2-{[4-(methylamino)benzoyl]amino}benzamide (**40**). Rf=0.4 (chloroform). The mass spectrum (MALDI-VP): M+H394,M+Na416. By using the procedure described above for reaction 3 of **216**, from 230 mg of **40** 120 mg of **304** was obtained. The mass spectrum (MALDI-VP): M+H 435, M+Na 457.

**N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl (methyl)amino]benzoyl}amino)-5-fluorobenzamide (306).** By using the procedure described above for reaction 1 of **115**, from 540 mg of 5-fluoro-2-nitrobenzoic acid (**4**) 320 mg of N-(5-chloropyridin-2-yl)-5-fluoro-2-nitrobenzamide was obtained (**41**). Rf=0.4 (chloroform). The mass spectrum (MALDI-VP): M+H 296, M+Na 318. By using the procedure described above for reaction 2 of **115**, from 500 mg of **41** 320 mg of 2-amino-N-(5-chloropyridin-2-yl)-5-fluorobenzamide was obtained (**12**). Rf=0.6 (chloroform). The mass spectrum (MALDI-VP): M+H 266, M+Na 288. By using the procedure described above for reaction 4 of **115**, from 280 mg of **12** and 220 mg of **22** 350 mg of N-(5-chloropyridin-2-yl)-5-fluoro-2-({4-[methyl(trifluoroacetyl)amino]benzoyl}amino)benzamide was obtained (**42**). Rf=0.6 (chloroform). The mass spectrum (MALDI-VP): M+H 495, M+Na 517. By using the procedure described above for reaction 2 of **216**, from 300 mg of **42** were obtained 230 mg of N-(5-chloropyridin-2-yl)-5-fluoro-2-{[4-(methylamino)benzoyl]amino} benzamide (**43**). Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 399, M+Na 421. By using the procedure described above for reaction 3 of **216**, from 200 mg of **43** 110 mg of N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl(methyl) amino]benzoyl}amino)-5-fluorobenzamide was obtained (**306**). Rf=0.25 (9:1 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 440, M+Na 462.

**N-(2-{[(5-chloropyridin-2-yl)amino ]carbonyl}-4-methylphenyl)-4-[ethanimidoyl(methyl) amino]-2-methoxybenzamide (307).** Reaction 1: 400 mg of 2-methoxy-4-aminobenzoic acid (**44**) was mixed with 0.6 mL of 40% formaldehyde, 0.8 g of NaOH in 5 mL of water and 400 mg of zinc powder. The reaction mixture was stirred, heated up to 60°C and allowed to stand for 4 h. Then, the mixture was filtered and the residue was rinsed

with aqueous ethanol. Then the filtrate was acidified with aqueous HCl up to pH 4-5, evaporated, and the residue was applied on a 20x150 column filled with silica gel. The product was eluted with a 9:1 chloroform/ethanol mixture. Rf=0.5 (chloroform/ethanol 9:1). The yield of 2-methoxy-4-(methylamino)benzoic acid (**18**) was 210 mg.

By using the procedure described above for reaction 2 of **216**, from 170 mg of **18** 180 mg of 2-methoxy-4-[methyl(trifluoroacetyl)amino]benzoic acid was obtained (**24**). R=0.2-0.4. The mass spectrum (MALDI-VP) in negative ions: M-1 276. By using the procedure described above for reaction 4 of **115**, from 130 mg of **24** and 150 mg of **10** 150 mg of N-(2-{[(5-chloropyridin-2-yl)amino] carbonyl}-4-methylphenyl)-2-methoxy-4-[methyl (trifluoroacetyl)amino]benzamide were obtained (**45**). Rf=0.6 (chloroform). The mass spectrum (MALDI-VP): M+H 521. By using the procedure described above for reaction 2 of **216**, from 130 mg of **45** 100 mg of N-(2-{[(5-chloropyridin-2-yl)amino]carbonyl}-4-methylphenyl)-2-methoxy-4-(methylamino)benzamide was obtained (**46**). Rf=0.4 (chloroform). The mass spectrum (MALDI-VP): M+H 425, M+Na 447. By using the procedure described above for reaction 3 of **216**, from 90 mg of **46** 70 mg of **307** was obtained. The mass spectrum (MALDI-VP): M+H 466, M+Na 488.

**N-(5-chloropyridin-2-yl)-2-({4-[ethanimidoyl (methyl)amino]benzoyl}amino)benzamide (310).** By using the procedure described above for reaction 1 of **115**, from 1.9 g 2-nitrobenzoic acid (**5**) 2.4 g of N-(5-chloropyridin-2-yl)-2-nitrobenzamide was obtained (**47**). Rf=0.7 (chloroform). The mass spectrum (MALDI-VP): M+H 278, M+Na 300. By using the procedure described above for reaction 2 of **115**, from 1.5 g of **47** 650 mg of 2-amino-N-(5-chloropyridin-2-yl)benzamide was obtained (**13**). The mass spectrum (MALDI-VP): M+H 248, M+Na 270. By using the procedure described above for reaction 4 of **115**, from 300 mg of **13** and 250 mg of **22** 430 mg of N-(5-chloropyridin-2-yl)-2-({4-[methyl (trifluoroacetyl)amino]benzoyl} amino)benzamide was obtained (**48**). Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 477, M+Na 499. By using the procedure described above for reaction 2 of **216**, from 400 mg of **48** 320 mg of N-(5-chloropyridin-2-yl)-2-{[4-(methylamino) benzoyl]amino}benzamide was obtained (**49**). Rf=0.4 (chloroform). The mass spectrum (MALDI-VP): M+H381, M+Na403. By using the procedure described above for reaction 3 of **216**, from 300 mg of **49** 200 mg of **310** was obtained. Rf=0.2 (9:1 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 422, M+Na 444.

**N-(4-chlorophenyl)-5-methyl-2-[(4-{methyl [(methylamino)carbonyl]amino}benzoyl) amino]benzamide (311).** By using the procedure described above for reaction 4 of **115**, from 200 mg of **14** and 180 mg of **19** 200 mg of **311** was obtained. Rf=0.5 (chloroform/ethanol 20:1). The mass spectrum (MALDI-VP): M+H 451, M+Na 473.

**5-methyl-2-[(4-{methyl[(methylamino) carbonyl]amino}benzoyl)amino]-N-(4-methylphenyl)benzamide (312).** By using the procedure described above for reaction 1 of **115**, from 500 mg of **2** and 270 mg of 4-methylaniline (**8**) 310 mg of 5-methyl-N-(4-methylphenyl)-2-nitrobenzamide was obtained (**50**). Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 271, M+Na 293. By using the procedure described above for reaction 2 of **115**, from 250 mg of **50** 190 mg of 2-amino-5-methyl-N-(4-methylphenyl)benzamide was obtained (**15**). Rf=0.7 (chloroform). The mass spectrum (MALDI-VP): M+H 241, M+Na 263. By using the procedure described above for reaction 4 of **115**, from 160 mg of **19** and 140 mg of **15** 160 mg of **312** was obtained. Rf=0.5 (chloroform/ethanol 20:1). The mass spectrum (MALDI-VP): M+H 431, M+Na 453.

**N-(5-chloropyridin-2-yl)-5-methoxy-2-[(4-{methyl[(methylamino)carbonyl]amino} benzoyl)amino]benzamide (313).** By using the procedure described above for reaction 4 of **115**, from 250 mg of **11** and 250 mg of **19** 230 mg of **313** was obtained. Rf=0.5 (chloroform). The mass spectrum (MALDI-VP): M+H 468, M+Na 490.

**N-(5-chloropyridin-2-yl)-5-fluoro-2-[(4-{methyl[(methylamino)carbonyl]amino} benzoyl)amino]benzamide (314).** By using the procedure described above for reaction 4 of **115**, from 280 mg of **12** and 290 mg of **19** 250 mg of **314** was obtained. Rf=0.3 (dioxane/aqueous ammonia 8:2). The mass spectrum (MALDI-VP): M+H 456, M+Na 478.

**N-(2-{[(5-chloropyridin-2-yl)amino] carbonyl}-4-methylphenyl)-2-fluoro-4-{methyl methylamino)carbonyl]amino}benzamide (315).** By

using the procedure described above for reaction 3 of **115**, from 500 mg of 4-amino-2-fluorobenzoic acid (**17**) 320 mg of 2-fluoro-4-{methyl[ (methylamino)carbonyl]amino}benzoic acid was obtained (**20)**. The mass spectrum (MALDI-VP) in negative ions: M-1 168. By using the procedure described above for reaction 4 of **115**, from 300 mg of **20** and 300 of **10** 250 mg of **315** was obtained. Rf=0.3 (8:2 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 470, M+Na 492.

**N-(2-{[(5-chloropyridin-2-yl)amino] carbonyl}-4-methylphenyl)-2-methoxy-4-{methyl[(methylamino)carbonyl] amino}benzamide (316).** By using the procedure described above for reaction 3 of **115**, from 200 mg of **18** 210 mg of 2-methoxy-4-{methyl[(methylamino)carbonyl]amino}benzoic acid was obtained (**21**). The mass spectrum (MALDI-VP) in negative ions: M-1 237. By using the procedure described above for reaction 4 of **115**, from 180 mg of **21** and 190 mg of **10** 250 mg of **316** was obtained. Rf=0.3 (8:2 dioxane/aqueous ammonia). The mass spectrum (MALDI-VP): M+H 482, M+Na 504.

**N-(5-chloropyridin-2-yl)-2-[(4-{methyl[ (methylamino)carbonyl]amino}benzoyl) amino]benzamide (317).** By using the procedure described above for reaction 4 of **115**, from 340 mg of **19** and 350 mg of **13** 350 mg of **317** was obtained. Rf=0.2 (dioxane/aqueous ammonia 8:2). The mass spectrum (MALDI-VP): M+H 438, M+Na 460.

## 2.3. Measurements of Factor Xa (FXa) Inhibition Constants

The $K_i$s were measured using chromogenic substrate and human FXa from the Renaparin-test set. DMSO in aqueous solution (5% v/v; 10 μL) was chosen as the reference standard (RS). The solution of inhibitor in the RS (10 μL) and the solution of human FXa (0.0625 U/mL; 10 μL) were mixed with 40 μL of Tris-BSA-buffer (NaCl 0.2 M; Tris 0.1 M; 0.2% BSA; pH 7.4). The reaction was triggered by introducing the chromogenic substrate (0.75 M) in amount of 40 μL. The mixture was stirred for 10 sec and time evolutions of the optical density of the solution ($OD_{inh}$) and the RS ($OD_{ref}$) were measured at 405 nm. The inhibitory activity was determined by 1- [$OD_{inh}$ /$OD_{ref}$]. Dependence of the activity on inhibitor concentration was allowed to calculate $IC_{50}$ for each inhibitor. Rivaroxaban (as a control) was extracted from tablet formulation by the routine described earlier [12].

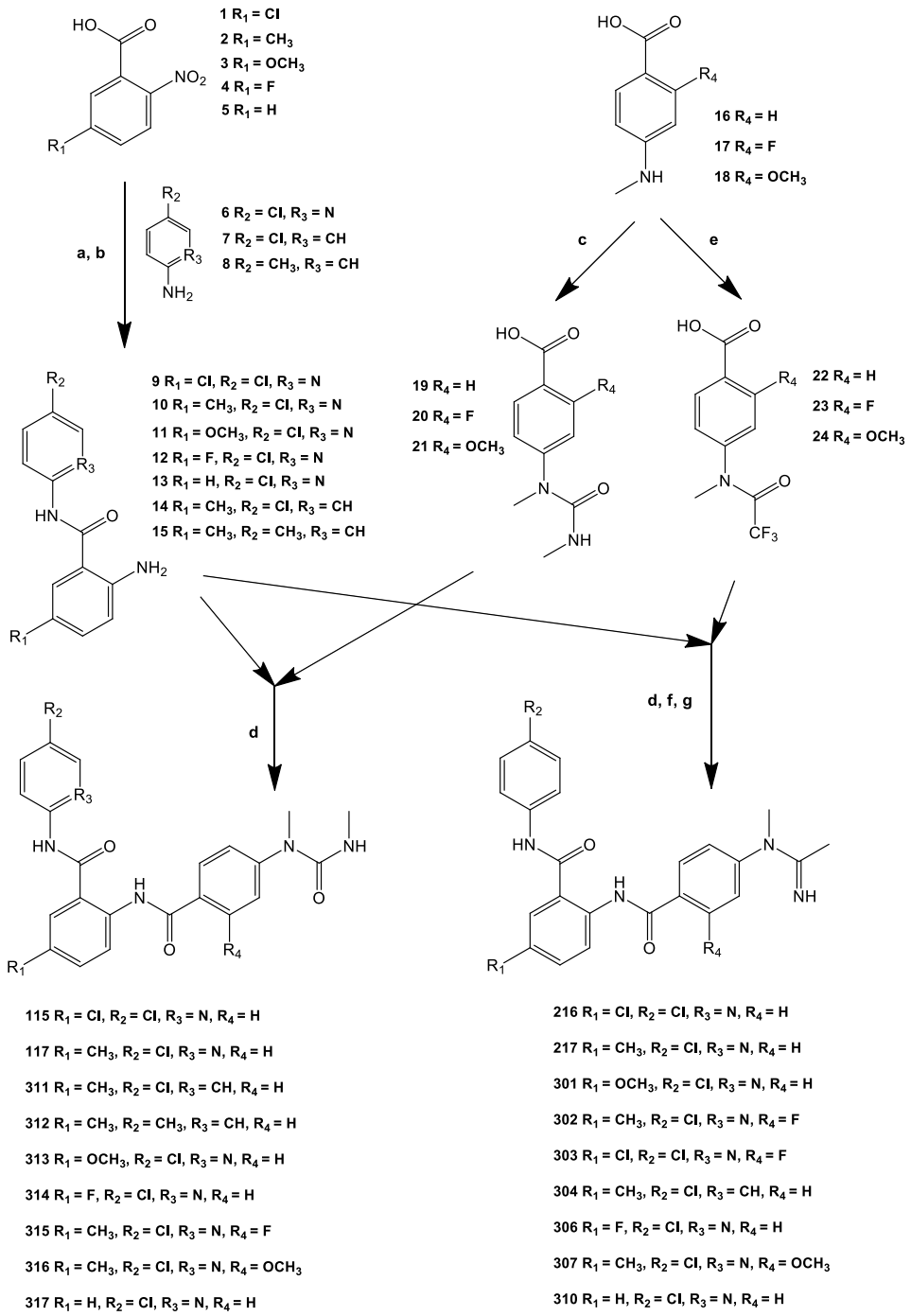
## 2.4. Determination of Human Plasma Prothrombin Time (PT)

PT measurements were made on Sysmex Ca-50 (Sysmex, Japan) coagulometer. Renam's KM-2 control plasma was used for in vitro assays: 50 μL of the plasma was mixed with 1 μL of inhibitor solution and incubated for 1 minute at 37°C. The coagulation process was triggered by thromboplastin (Thromborel S;ISI=1) in amount of 100 μL. PTx2, i.e., the concentration for the PT doubling was estimated by regression analysis from the dose-response curves (available from [13]).

## 2.5. Animal Dosing and Sample Collections

Animal experiments were conducted in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. We chose rats, rabbits and monkeys for the assessment of the pharmacodynamic parameters of the studied compounds according to the recommendation of Scientific and Standardization Committee Communication: On Behalf of the Subcommittee on Animal, Cellular, and Molecular Models of Thrombosis and Haemostasis of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [14]. All animal experiments were conducted at the N.N. Semenov Institute of Chemical Physics Russian Academy of Sciences (Moscow, Russia) following approval by the Biomedicine Ethic Committee of the RF SRC - Institute of Biomedical Problems, Russian Academy of Sciences / Physiology Section of the Russian Bioethics Committee Russian Federation National Commission for UNESCO/ (Approval NO: 396, 397, 398 of June 4, 2015).

3-month-old male "**Soviet Chinchilla**" rabbits (2.40±0.19 kg), 8-week-old outbreeding male rats Wistar (205±16 g) were obtained from the branch of nursery of Scientific center of biomedical technologies of Federal Medical Biological Agency, the Moscow Region, settlement Svetlie Gory, Russian Federation; 2- to 3-year-old male cynomolgus monkeys (*Macaca fascicularis*) (4.0-4.5 kg) were obtained from apery of Federal public budgetary scientific institution" Research institute of medical




**Scheme 1.** General Synthetic Pathways. Reagents and conditions: (**a**) $SOCl_2$, THF, reflux; (**b**) $SnCl_2$, water, boiling; (**c**) methyl isocyanate, THF, room temperature; (**d**) EDCI, THF, room temperature; (**e**) TFAA, 0°C; (f**)** NaOH, isopropanol, room temperature; (**g**) $NaHCO_3$, HCl, acetonitrile, 5°C.

primatology", Adler, Russia. The dispersion on initial weight did not exceed 10%.

Rats were housed in five metabolic polypropylene cages (L×W×H: 460×300×160 mm), rabbits and monkeys were individually maintained in stainless steel cages 645×655×300 mm and 800×700×750 mm, accordingly, under condition of 16-26°C, 40-70% relative humidity, a 12-h light-dark cycle and a room air exchange of 8-10 times per hour. Each rat and rabbit was provided with the granulated all-in-one feed in necessary quantity (on GOST 51849-2001 P.5.) and sterilized water was offered ad libitum. Each monkey

was provided with 200 g of special monkey keeping diet and approximately 150 g of fruit approximately per day, and sterilized water was offered ad libitum. Each monkey was provided with toys (such as mirror) and opportunities for communications with the neighbors, and was monitored daily by animal care staff. Rats, rabbits and monkeys were quarantined for 21 days before the study was conducted. At the end of these experiments, all of the animals were returned in a vivarium.

Solutions of compounds **117**, **216**, **217**, **301**, **302**, **303** and **310** (with 0.9% of NaCl) were injected intravenously (*iv*) without anesthesia to a tail vein of rats (n=6) in doses of 0.5, 1 and 3 mg · $kg^{-1}$. Solution of compound **217** (with 0.9% of NaCl) was injected without anesthesia intravenously to the left regional vein of an ear of rabbits (n=6) in doses of 1 and 1.6 mg · $kg^{-1}$. Before injection of compounds, and after 15, 30, 60, 120, 180, 240 min of injection of compounds, we collected blood (in a plastic test tube with 0.11 M sodium citrate solution in the ratio 9:1) from a tail vein of rats and from the right regional vein of an ear of rabbits.

Solutions of compounds **115**, **216** and **217** once entered into rats without anesthesia (n = 6; in doses 6, 10 mg · $kg^{-1}$), and the tested Amidina Hydrochloride substance **217** (DD217; n=6; in doses of 3.3 and 15 mg · $kg^{-1}$) once intragastric administration to monkeys by means of Gavage Feeding Tube. The individual volume of the entered dose for each animal was counted proceeding from the value of body weight and corrected after weighing. For intragastric administration we prepared for animals a suspension of the studied substance in 1% starched solution just before the beginning of experiment. For rats the volume of the entered solutions made 1-2 ml, for monkeys - 3-5 ml. Before administration of compounds and after 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 25 hours of administration of compounds we collected blood (in a plastic test tube with 0,11 M sodium citrate solution in the ratio 9:1) from a tail vein of rats and from a leg vein (the popliteal vein, the lateral and medial saphenous veins) of monkeys.

Blood from a vein of experimental animals was taken away by means of the device for injection in small veins (a needle butterfly 24G SFM Hospital Products GmbH, Germany). Blood with citrate of sodium were centrifuged by 20 min at 1200 g and 20 °C (used the centrifuge Eppendorf centrifuge 54147R) for receiving plasma of animals. The received plasma was spilled in plastic test tubes and frozen at -45 °C. After defrosting of plasma defined prothrombin time, $T_{max}$ and T ½ were defined with the use the one-channel-ball coagulometer MC 1 [15].

### 2.6. Statistical Analysis

Data are expressed as mean ± SEM. $IC_{50}$ values were calculated using Graph Pad Prism, version 3.02 (Graph Pad Software Inc., San Diego, CA, USA). $ED_{50}$ values were calculated by linear regression analysis using Excel 97 (Microsoft®).

## 3. RESULTS AND DISCUSSION

### 3.1. Chemistry

Chemical synthesis is the base of our approach for the development of potential inhibitors. The reaction of amide bond formation from appropriate acid and amine was chosen to be the main reaction due to its simplicity as well as wide spread of the reactants [16]. A lot of new chemical substances can be obtained in this way especially including the case of multiple amide bonds in one substance. However, the complexity of chemical synthesis grows with the count of amide bonds in a substance. On the other hand, according to Lipinski's rules, the molecular weight of the potential drugs should be less than 500 [17]. In this work, the substances like $R_1$-(CONH)-$R_2$-(CONH)-$R_3$ were developed using previously described docking and screening methods [18], where $R_1$, $R_2$ and $R_3$ are some chemical groups, and CONH is the amide bond connecting $R_1$, $R_2$ and $R_3$. The direction of the amide bond (CONH) could be arbitrary for $R_1$, $R_2$ and $R_2$, $R_3$.

Generally, substances like $R_1$-(CONH)-$R_2$-(CONH)-$R_3$ could not be the best to target FXa, but there are several points why the development of those substances is worthwhile. First reason is, as noted above, abundance of the reactants (acids, amines) and relative simplicity of the chemical synthesis. Secondly, $R_1$-(CONH)-$R_2$-(CONH)-$R_3$ could be a good inhibitor since amide bond is a common participant in protein-protein and ligand-protein interaction. As to active site of FXa, there are some atom groups in it that could form hydrogen bonds with amid groups of the potential inhibitor. Thirdly, there are many existing drugs that have $R_1$-(CONH)-$R_2$-(CONH)-$R_3$ structure.

A minor chemical modification was made to the best substances of the final stage. For these substances additional parameters were measured (on top of the $K_i$s): PTx2, stability, solubility and lipophilicity. Moreover, the patentability was also taken into account during chemical synthesis. General synthetic pathways are shown in Scheme **1**.

### 3.2. Influence on Human Plasma Prothrombin Time (PT) and Factor Xa (FXa) Inhibition Constant of Compounds

Chemical modification was made in the frame of the results of the previous stage, taking into account the structure of FXa, chemical synthesis capabilities, as well as patentability of the target compounds. The $IC_{50}$ and PTx2 of synthesized molecules are represented in Table **1**. The PTx2 of the compound **217** (DD217) is 0.08±0.02 μM, which makes it one of the best FXa inhibitors ever reported.

Several analyzers and various testing-suites have been used to measure the PTx2 concentration. However, one should note that there can be some deviations in PTx2 values of the substance for various testing-suites reaching up one order of magnitude [12, 19, 20]. Particularly, PTx2 =0.40±0.06 μM was obtained for Bayer's rivaroxaban using our routine (see Methods section) as opposite to Bayer's official result of PTx2 =0.3 μM [8].

### 3.3. Anticoagulant Activity of Animal Plasma after Compounds Intake

Compounds **115**, **117**, **216**, **301**, **302**, **303**, **310** were selected for subsequent biological tests with rats and compound **217** - with rabbits and rats. PT pharmacodynamics was studied for various compound doses in *per os* and *iv* (intravenous) assays. Each dose has been tested on at least three different animal models. The results of the assays are summarized in Table **2** and characterized by following quantities.

Table **2** shows the following results: the average time ($T_{max}$) it took to achieve the maximum of therapeutic effect after *per os* administration (which is in phase with dose timing in case of intravenous administration); the average terminal half-life ($T_{1/2}$) according to PT pharmacodynamics; and, finally, the ratio of the PT at $T_{max}$ ($PT_{max}$) to the PT before the administration ($PT_0$). It can be seen from Table **2** that the results shown by compound DD217 are superior to other compounds in terms of half-life and activity ($PT_{max}/PT_0$) under the same doses and routes of administration. Thus, taking into account the activity of DD217 for in vitro assays, others tests were conducted with this compound.

PT pharmacodynamics of DD217 was obtained for *per os* administration in cynomolgus monkeys. Two doses were studied in the assays and 6 monkeys were used for each dose. The PT dependence on time for 3.3 mg/kg live weight of DD217 is shown in Fig. (**1**), where the fitting curve was drawn by the averaged data. $T_{max}$, $T_{1/2}$ and $PT_{max}/PT_0$ values of the assays are summarized in Table **3**.

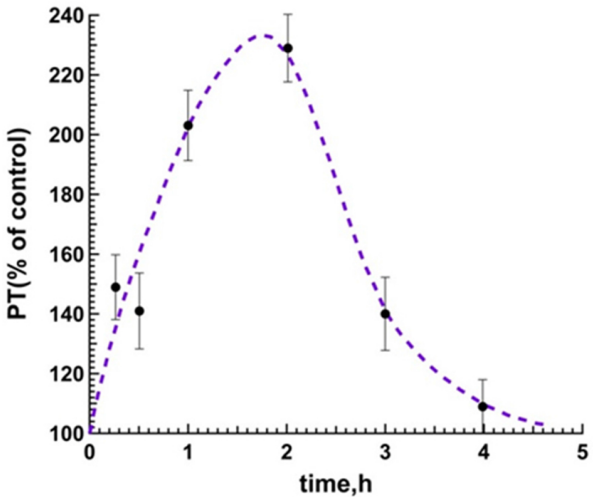



**Fig. (1).** The prothrombin time (PT) after *per os* administration of DD217 (3.3 mg/kg) in monkeys (axis X - the time after *per os* administration, h; axis Y - the ratio of the plasma coagulation time after *per os* administration $PT_{max}$ to the plasma coagulation time before *per os* administration $PT_0$, %).

The obtained class of chemical compounds has been patented as class of "Urethanes, ureas, amidines and related inhibitors of factor Xa" [21]. One important fact is that some groups of the class have certain similarities with those of the known inhibitors toward FXa, although all our described compounds have been developed from scratch. It is not surprising, since there were huge efforts made by many pharmaceutical companies and labs in their attempts to discover new effective inhibitors toward the target. On the contrary, it is rather often the companies which happened to release their inhibitors revealing some structure similarities with those of the competitors.

**Table 1.** ***In vitro*** **potency of experimental compounds.**

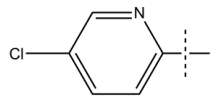
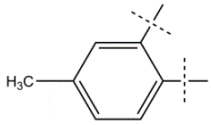
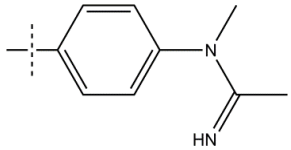
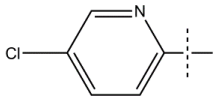
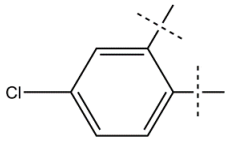
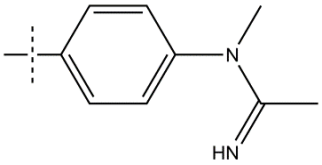
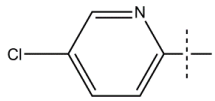
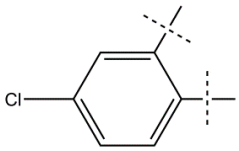
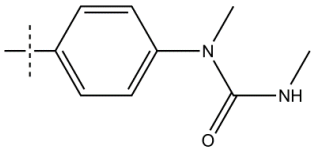
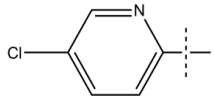
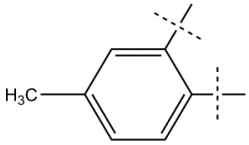
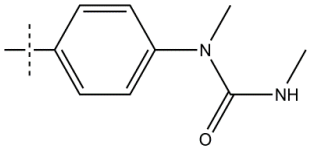
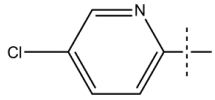
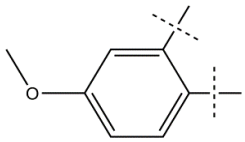
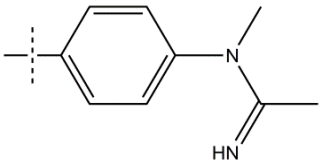
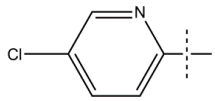
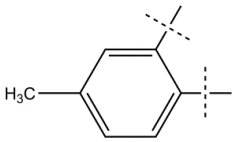
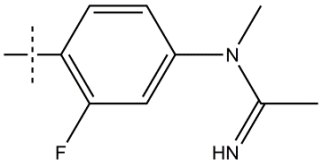
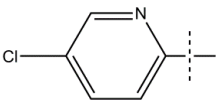
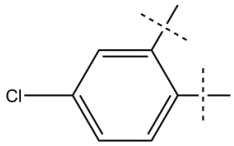
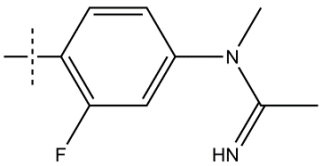
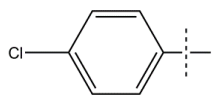
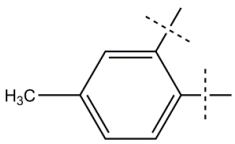
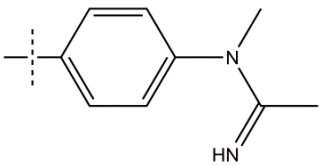
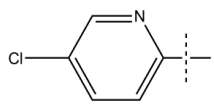
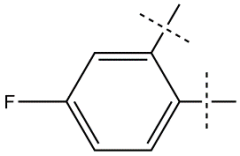
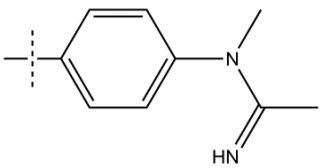
| Com-pound | $R_1$ | $R_2$ | $R_3$ | $IC_{50}$, nM | PTx2, μM |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| 217 |  |  |  | 0.50±0.08 | 0.08±0.02 |
| 216 |  |  |  | 0.30±0.06 | 0.12±0.03 |
| 115 |  |  |  | 0.25±0.05 | 2.5±0.5 |
| 117 |  |  |  | 0.15±0.03 | 2.0±0.4 |
| 301 |  |  |  | 1.0±0.3 | 0.10±0.2 |
| 302 |  |  |  | 0.8±0.2 | 0.09±0.02 |
| 303 |  |  |  | 1.0±0.2 | 0.09±0.02 |
| 304 |  |  |  | 1.0±0.2 | >30 |
| 306 |  |  |  | 0.50±0.12 | 1.5±0.3 |

**Table 1. (Contd...)**

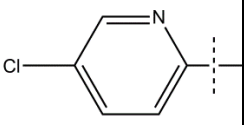
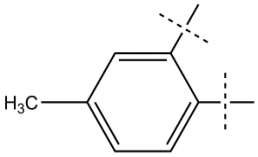
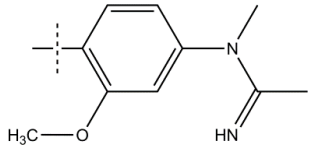
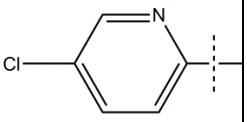
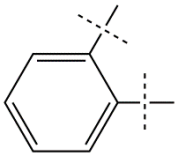
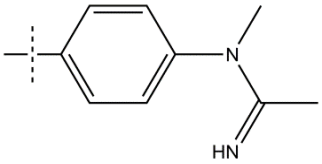
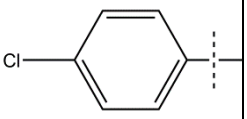
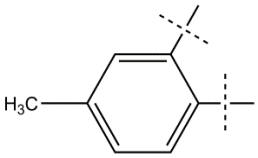
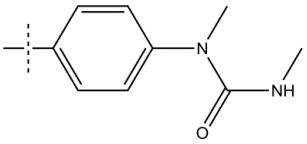
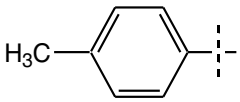
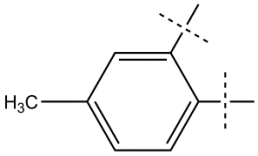
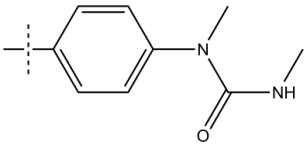
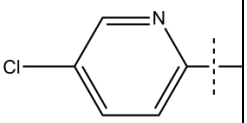
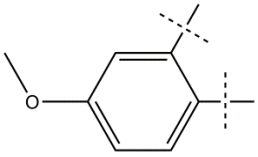
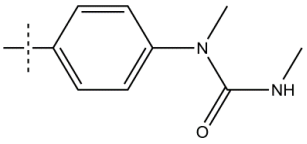
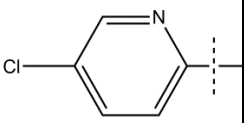
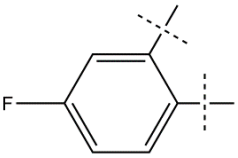
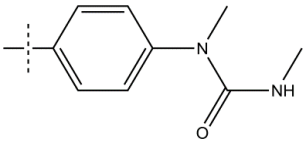
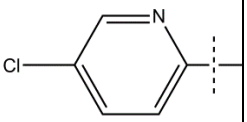
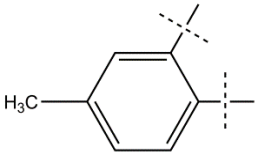
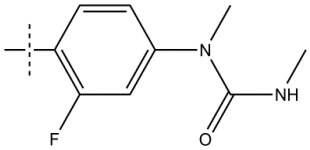
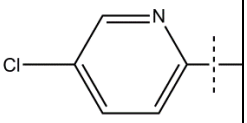
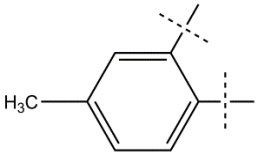
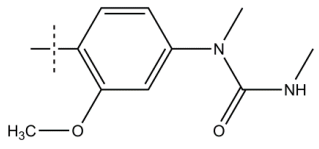
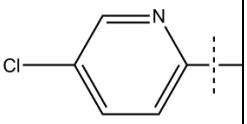
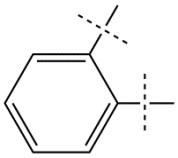
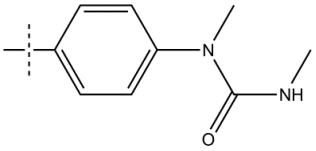
| Com-pound | $R_1$ | $R_2$ | $R_3$ | $IC_{50}$, nM | PTx2, μM |
|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 |
| 307 |  |  |  | 1.0±0.2 | 2.0±0.4 |
| 310 |  |  |  | 2.0±0.2 | 0.50±0.12 |
| 311 |  |  |  | 2.0±0.3 | >30 |
| 312 |  |  |  | 0.15±0.03 | 3.0±0.6 |
| 313 |  |  |  | 0.25±0.05 | 30±6 |
| 314 |  |  |  | 0.50±0.11 | 10±2 |
| 315 |  |  |  | 0.25±0.05 | 10±2 |
| 316 |  |  |  | 10±2 | >30 |
| 317 |  |  |  | 0.25±0.05 | 3.0±0.6 |

**Table 2. Summary of the assays with rabbits and rats.**

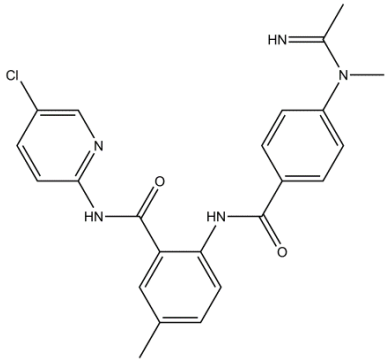
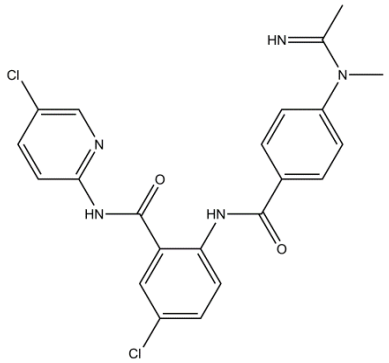
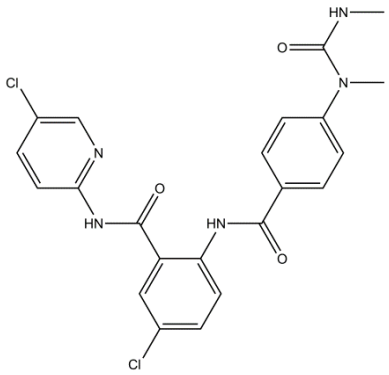
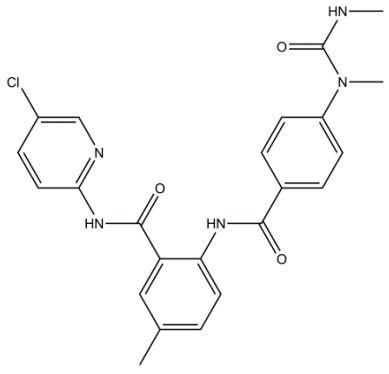
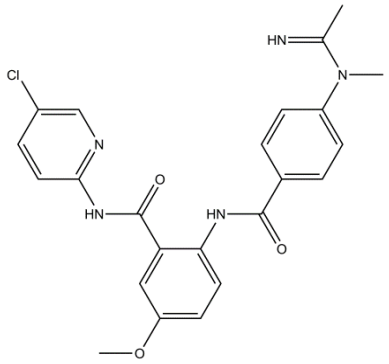
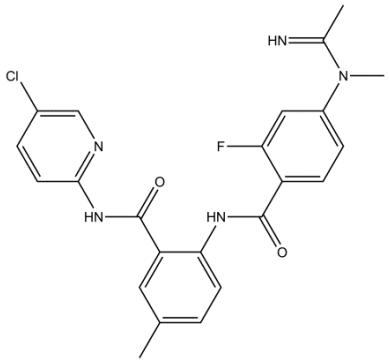
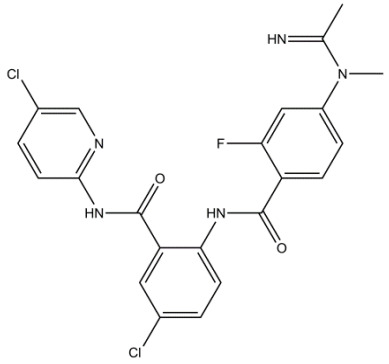
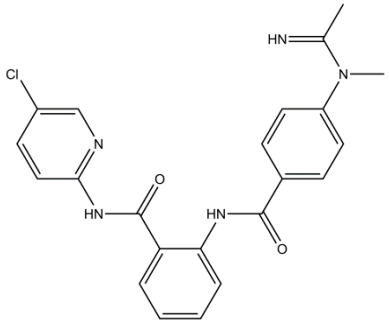
| Com-pound | Structure | Animal | Admin-istration | Dose, mg/kg | $T_{max}$, Min | $T_{1/2}$, Min | $PT_{max}/PT_0$ |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 217 |  | rabbits | *iv* | 1.6 | dd | 25±5 | 2.0±0.3 |
| | | rabbits | *iv* | 1 | dd | 25±5 | 1.8±0.3 |
| | | rats | *per os* | 6 | 10±2 | 30±6 | 1.7±0.2 |
| | | rats | *iv* | 1 | dd | 5.0±1.5 | 3.5±0.6 |
| | | rats | *iv* | 3 | dd | 60±9 | 10±2 |
| 216 |  | rats | *iv* | 0 | dd | | 1.0±0.2 |
| | | rats | *iv* | 3 | dd | 45±9 | 1.7±0.2 |
| | | rats | *per os* | 1 | 20±4 | 50±40 | 1.3±0.2 |
| 115 |  | rats | *per os* | 1 | dd | | 1.0±0.2 |
| 117 |  | rats | *iv* | 1 | dd | 20±5 | 1.3±0.3 |

**Table 2. (Contd...)**

| Com-pound | Structure | Animal | Admin-istration | Dose, mg/kg | $T_{max}$, Min | $T_{1/2}$, Min | $PT_{max}/PT_0$ |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 301 |  | rats | *iv* | 1 | dd | 30±7 | 2.0±0.4 |
| 302 |  | rats | *iv* | 1 | dd | 10±2 | 2.0±0.5 |
| 303 |  | rats | *iv* | 1 | dd | 15±3 | 1.5±0.4 |
| 310 |  | rats | *iv* | 1 | dd | 20±4 | 1.3±0.3 |

Note: dd - didn't define

**Table 3. Summary of the assays with cynomolgus monkeys.**

| Compound | *Per os* dose, mg/kg | $PT_{max}/PT_0$ | $T_{max}$, h | $T_{1/2}$, h |
|---|---|---|---|---|
| DD217 | 3.3 | 2.3±0.4 | 2.0±0.5 | 2.7±0.6 |
| | 15.0 | 6.0±0.9 | 10.0±2.0 | 32.0±6.0 |

The development of oral anticoagulants with new action mechanisms is still a challenging task nowadays, as FXa is considered one of the most effective targets for such agents. We showed that highly potent FXa inhibitors with no patent infringements can be obtained based on the reaction of amide bond formation in compounds like $R_1$-(CONH)-$R_2$-(CONH)-$R_3$, where $R_1$, $R_2$ and $R_3$ are some chemical groups.

## CONCLUSION

Submicromolar concentrations for prothrombin time doubling (PTx2) in human plasma were obtained for 5 compounds like $R_1$-(CONH)-$R_2$-(CONH)-$R_3$, including the compound DD217 having PTx2=80±20 nM, which puts it among the best FXa inhibitors ever reported.

"*In vivo*" experiments conducted for eight compounds showed significant advantages of compound DD217 over others in terms of activity and duration of action. Superiority of DD217 was demonstrated during *per os* and intravenous administration in rabbits and rats. Finally, the pharmacodynamic profile of DD217 for oral administration in cynomolgus monkeys proves the efficacy of the compound, which makes it promising for the future preclinical trials.

## LIST OF ABBREVIATIONS

FXa = Factor Xa

DOAC = Directly Acting Oral Anticoagulants

EDCI = N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride

THF = Tetrahydrofuran

TFAA = Trifluoroacetic anhydride

DMSO = Dimethyl sulfoxide

RS = Reference standard

GOST = Government standard

PT = Prothrombin time

ISI = International Sensitivity Index

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were conducted at the N.N. Semenov Institute of Chemical Physics Russian Academy of Sciences (Moscow, Russia) following approval by the Biomedicine Ethic Committee of the RF SRC - Institute of Biomedical Problems, Russian Academy of Sciences / Physiology Section of the Russian Bioethics Committee Russian Federation National Commission for UNESCO/ (Approval NO: 396, 397, 398 of June 4, 2015).

## HUMAN AND ANIMAL RIGHTS

Animal experiments were conducted in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

## CONSENT FOR PUBLICATION

Not Applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

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