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INFLUENCE OF *N*,*N*′-SUBSTITUTED PIPERAZINES ON CYTOLYSIS

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The hemolytic activity of *N*,*N*′-substituted piperazines and their influence on the hemolytic process initiated by radiation (red LED, 653 nm) in the presence of radachlorin (RADA-FARMA, Russia) photosensitizer and on complement-dependent hemolysis were studied. All compounds did not cause human RBC lysis in the studied concentration range (0.15 – 3.0 mM) although dose-dependent inhibition of photo-induced hemolysis that manifested as an increase of the 50% lysis time was observed. The piperazine derivatives did not substantially influence RBC lysis by the activated complement. Therefore, the antihemolytic effect of the *N*,*N*′-substituted piperazines was due to their antioxidant activity.

Keywords: *N*,*N*′-substituted piperazines, photo-induced hemolysis, photosensitizer, antioxidants, complement system.

Piperazine derivatives have broad spectra of biological activity and are used successfully in various medical applications [1, 2]. The design of novel drugs based on substituted piperazines and the comprehensive study of their properties are of definite interest. Thus, we showed earlier [3] that *N*,*N*′-substituted piperazines exhibited antiaggregant, anticoagulant, and vasodilating activity and could be used to design drugs. Thus, the cytolytic activity of drugs intended for therapy of hemostatic diseases must be assessed because cytolysis, in particular hemolysis, caused by administering the drugs is a serious therapeutic problem [4, 5].

Slight structural changes of physiologically active compounds are known to alter considerably their biological activity [6].

The goal of the present work was to study the cytolytic activity of novel *N*,*N*′-substituted piperazines (**I**, **II**, **III**; see Table 1) with different structures and also to assess their influence on various types of hemolysis, i.e., photo-induced hemolysis due to oxidative stress and complement-dependent hemolysis that does not involve reactive oxygen species.

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The *N*,*N*′-substituted piperazines were synthesized at Vertex Co.

EXPERIMENTAL CHEMICAL PART

The course of reactions and purity of intermediates in each synthetic step were monitored by TLC on TLC Silicagel 60 F_{254} (Merck) or Alugram Sil G/UV_{254} (Macherey-Nagel) plates with detection by UV light. PMR and ^{13}C NMR spectra were recorded on a DRX-500 spectrometer (Bruker, Germany) at operating frequency 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C. Residual solvent resonances ($CDCl_3$, 7.28 ppm; D_2O, 4.80; DMSO-d_6, 2.50) were used as standards. Mass spectra were taken on an AmaZon mass spectrometer (Bruker) using electrospray ionization. The purity of the compounds was evaluated by HPLC on an Alliance chromatograph (Waters, USA) using a Zorbax Eclipse XDB-C18 column (3.5 μm, 3 × 100 mm) (Agilent Technologies, USA). The mobile phase was a mixture (75:25) of buffer (pH 3.0) containing sodium octanesulfonate (0.0125 M) (Merck, #118307) and sodium dihydrogen phosphate (0.03 M) (Merck, #106342) and MeCN (J. Baker, #9012) at flow rate 0.5 mL/min. The elution was isocratic with detection at 210 nm.

1-Carbimidamido-4-(2,3,4,5-tetramethoxybenzoyl)piperazine hemifumarate (I). 1-Carbimidamido-4-(2,3,4,5-tetramethoxybenzoyl)piperazine acetate (**IV**) was prepared as described earlier by us [3]. Compound **IV** (39.0 g, 95 mmol) was dissolved in hot H_2O (125 mL, ~60°C) and treated with a hot (~60°C) solution of fumaric acid (5.2 g, 45 mmol) in H_2O (75 mL). The resulting precipitate was filtered off, rinsed on the filter with H_2O (3 × 75 mL), and recrystallized from hot H_2O to afford **I** as colorless crystals (30.3 g, 78%). PMR spectrum (DMSO-d_6), δ, ppm (J, Hz): 3.25 (m, 2H, C$\underline{H}_2$-piperazine C$\underline{H}_2$), 3.39 (m, 4H, C$\underline{H}_2$-piperazine C$\underline{H}_2$ and $\underline{H}_2O$), 3.51 (m, 2H, C$\underline{H}_2$-piperazine C$\underline{H}_2$), 3.65 (m, 5H, C$\underline{H}_2$-piperazine C$\underline{H}_2$ and C$\underline{H}_3$O-), 3.76 (m, 3H, C$\underline{H}_3$O-), 3.78 (m, 3H, C$\underline{H}_3$O-), 3.83 (m, 3H, C$\underline{H}_3$O-), 6.21 (s, 1H, $HO_2CC\underline{H}$=C$\underline{H}CO_2H$), 6.67 (s, 1H, H-Ar), 9.43 (br.s, 4H, C(N$\underline{H}_2$)($N^+\underline{H}_2$)). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 40.64 (CH_2-piperazine C$\underline{H}_2$), 44.29 (CH_2-piperazine C$\underline{H}_2$), 44.82 (CH_2-piperazine C$\underline{H}_2$), 45.63 (CH_2-piperazine C$\underline{H}_2$), 56.17 (CH_3O-), 60.68 (CH_3O-), 61.06 (CH_3O-), 61.52 (CH_3O-), 105.43 (C6, Ar), 124.54 (C1, Ar), 136.56 ($HO_2C\underline{C}H$=$\underline{C}HCO_2H$), 143.04 (C2, Ar), 143.20 (C5, Ar), 146.48 (C4, Ar), 149.51 (C3, Ar), 157.43 ($\underline{C}(NH_2)(N^+H_2)$), 166.16 ($\underline{C}ON$), 172.12 ($HO_2\underline{C}CH$= $CH\underline{C}O_2H$). Mass spectrum: MH^+ found, 353.20; MH^+ calc., 353.18. The product was 100% pure according to HPLC.

1-Carbimidamido-4-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine hemifumarate (II)

2-Bromo-3,4,5-tributoxybenzoic acid (V). A solution of 3,4,5-trimethoxybenzoic acid (106.0 g, 0.50 mol) in $CHCl_3$ (500 mL) was treated with H_2O (10 mL), refluxed, stirred, treated dropwise with a solution of Br_2 (32.2 mL, 99.8 g, 0.626 mol) in $CHCl_3$ (100 mL) over 30 min, refluxed for 10 h, cooled, washed with H_2O (3 × 200 mL), and dried over anhydrous $MgSO_4$. The desiccant was filtered off. The filtrate was evaporated in a rotary evaporator at reduced pressure to afford the intermediate as pale-cream-colored crystals (120.8 g, 83%) that were used in the next step without further purification. PMR spectrum ($CDCl_3$), δ, ppm (J, Hz): 3.92 (3H, CH_3O-), 3.93 (3H, CH_3O-), 3.98 (3H, CH_3O-), 7.42 (s, 1H, H-Ar), 11.00 (br.s, 1H, CO_2H). The intermediate was 95% pure according to PMR spectroscopy and TLC.

2-Hydroxy-3,4,5-trimethoxybenzoic acid (VI). A hot solution of **V** (60.0 g, 0.206 mol) and NaOH (50.0 g, 1.25 mol) in H_2O (400 mL) was stirred, treated with $CuSO_4 \cdot 5H_2O$ (51.4 g, 0.206 mol, 100 mol%), refluxed for 3.5 h [1-BuOH (10 mL) was added to the reaction mixture 2 h after the start of refluxing in order to suppress foaming], and cooled. The resulting precipitate was filtered off, suspended in NaOH solution (5%) in H_2O (200 mL), and stirred for 1 h. The solid was separated by filtration (3×), suspended in H_2O (200 mL), and acidified with conc. HCl (50 mL, 60.0 g, 0.6 mol). The resulting precipitate was separated by filtration, rinsed on the filter with H_2O (3 × 100 mL), and dried in vacuo to afford pale-cream-colored crystals (8.46 g, 18%).

PMR spectrum ($CDCl_3$), δ, ppm (J, Hz): 3.87 (3H, CH_3O-), 3.95 (3H, CH_3O-), 4.05 (3H, CH_3O-), 5.5 (br.s, 1H, HO-Ar), 7.17 (s, 1H, H-Ar), 10.31 (s, 1H, $CO_2\underline{H}$).

Acid **VI** was 99.5% pure according to PMR spectroscopy and TLC.

Methyl 2-hydroxy-3,4,5-trimethoxybenzoate (VII). A solution of **VI** (18.2 g, 0.080 mol) in refluxing Me_2CO (150 mL) was stirred, treated in portions with anhydrous Na_2CO_3 (20.4 g, 0.192 mol) and dimethylsulfate (9.1 mL, 12.1 g, 0.096 mol), refluxed with stirring for 3 h, and cooled. The precipitate of inorganic salts was filtered off. The filtrate was evaporated in a rotary evaporator at reduced pressure. The obtained solid was dissolved in EtOAc (200 mL); washed successively with saturated $NaHCO_3$ solution in H_2O (2 × 50 mL), H_2O (2 × 50 mL), and saturated NaCl solution (50 mL); and dried over anhydrous $MgSO_4$. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator at reduced pressure. The solid was recrystallized from heptane to afford **VII** as sandy-colored large crystals (11.6 g, 60%). PMR spectrum ($CDCl_3$), δ, ppm (J, Hz): 3.84 (s, 3H, CH_3O_2C-), 3.93 (3H, CH_3O-), 3.95 (3H, CH_3O-), 4.01 (3H, CH_3O-), 7.08 (s, 1H, H-Ar), 10.68 (s, 1H, HO-Ar).

Methyl 2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoate (VIII). Ester **VII** (4.95 g, 0.20 mol), 2-bromobutane (2.74 mL, 3.43 g, 0.025 mol), anhydrous K_2CO_3 (4.15 g, 0.030 mol), and KI (0.33 g, 0.002 mol) were dissolved in DMF (15 mL), stirred, heated to 65°C for 5 h, cooled, treated with H_2O (50 mL), acidified with conc. HCl (20 mL, 24 g, ~0.240 mol), and extracted with methyl-*tert*-butylether (MTBE, 3 × 25 mL). The combined extracts were washed successively with H_2O (2 × 30 mL), saturated $NaHCO_3$ solution in H_2O (2 × 30 mL), $Na_2S_2O_3$ solution (10%) in H_2O (2 × 20 mL), and saturated NaCl solution (30 mL) and dried over anhydrous $CaCl_2$. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator at reduced pressure. The intermediate was obtained as a yellow oil (5.90 g, 97%) and used in the next step without further purification.

2-(1-Methylpropyl)oxy-3,4,5-trimethoxybenzoic acid (IX). A solution of **VIII** (5.90 g, 0.020 mol) in aqueous EtOH (95%, 20 mL) was treated with KOH (1.40 g, 0.025 mol) and refluxed with stirring for 1 h. The mixture was evaporated in a rotary evaporator at reduced pressure. The resulting solid was refluxed in Et_2O (50 mL) and cooled. The solid was isolated by filtration, rinsed on the filter with Et_2O (40 mL), and dried in air. The resulting cream-colored solid was dissolved in H_2O (50 mL), acidified with conc. HCl (3 mL, 3.6 g, 0.036 mol), and extracted with Et_2O (2 × 25 mL). The combined extracts were washed with H_2O (2 × 20 mL) and saturated NaCl solution (20 mL) and dried over anhydrous $MgSO_4$. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator at reduced pressure to afford a yellow liquid (5.17 g, 89%) that was used in the next step without further purification. PMR

spectrum ($CDCl_3$), δ, ppm (J, Hz): 1.06 (t, 3H, 6 Hz, $C\underline{H}_3CH_2C \cdot HCH_3$), 1.29 (d, 3H, 6 Hz, $CH_3CH_2C \cdot HC\underline{H}_3$), 1.8 (m, 2H, $CH_3C\underline{H}_2C \cdot HC\underline{H}_3$), 3.90 (s, 6H, 2x CH_3O-), 4.00 (s, 3H, CH_3O-), 4.73 (m, 1H, $CH_3CH_2C \cdot \underline{H}CH_3$), 7.45 (s, 1H, H-Ar), 11.56 (br.s 1H, $CO_2\underline{H}$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 9.92 ($\underline{C}H_3CH_2C \cdot HCH_3$), 19.01 ($CH_3CH_2C \cdot H\underline{C}H_3$), 29.54 ($CH_3\underline{C}H_2C \cdot HCH_3$), 56.19 ($CH_3O$-), 61.22 ($CH_3O$-), 61.33 ($CH_3O$-), 83.51 ($CH_3CH_2\underline{C} \cdot HCH_3$), 108.00 (C1, Ar), 117.20 (C6, Ar), 144.49 (C2, Ar), 145.96 (C5, Ar), 147.92 (C3, Ar), 149.70 (C4, Ar), 165.36 ($\underline{C}O_2H$). Acid **IX** was 99.8% pure according to PMR spectroscopy and TLC.

2-(1-Methylpropyl)oxy-3,4,5-trimethoxybenzoylchloride (X). A solution of acid **IX** (5.0 g, 0.018 mol) in CH_2Cl_2 (25 mL) was treated with thionylchloride (2.50 mL, 4.18 g, 0.035 mol) and DMF (0.01 mL), stirred at room temperature for 1 h, refluxed for 2 h, and evaporated in a rotary evaporator at reduced pressure. The intermediate was obtained in quantitative yield (5.32 g, 100%) as a yellow oil and used in the next step without further purification.

1-(*tert*-Butoxycarbonyl)-4-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine (XI). A solution of 1-(*tert*-butoxycarbonyl)piperazine (3.76 g, 0.020 mol) and Et_3N (4.20 mL, 3.02 g, 0.030 mol) in CH_2Cl_2 (25 mL) was stirred, treated dropwise with a solution of **X** (5.32 g, 0.018 mol) in CH_2Cl_2 (25 mL), and stirred for 3 h. The separated precipitate of $Et_3N \cdot HCl$ was filtered off. The filtrate was washed successively with H_2O (2 × 20 mL), saturated NH_4Cl solution (2 × 20 mL), H_2O (2 × 20 mL), saturated $NaHCO_3$ solution (2 × 20 mL), and saturated NaCl solution (20 mL) and dried over anhydrous $MgSO_4$. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator at reduced pressure. The intermediate was obtained in quantitative yield (7.95 g, 100%) as a yellowish-brown oil that crystallized slowly upon standing into cream-colored crystals and used in the next step without further purification. PMR spectrum ($CDCl_3$, δ, ppm, J, Hz) (two isomers appeared in the PMR spectrum because of hindered rotation around the amide bond): 0.87 and 1.01 (two t, 7.4 and 7.6 Hz respectively, 3H total, $C\underline{H}_3CH_2C \cdot HCH_3$), 1.09 and 1.27 (two d, 5.9 and 5.9 Hz respectively, 3H, $CH_3CH_2C \cdot HC\underline{H}_3$), 1.49 (s, 9H, $(C\underline{H}_3)_3C$-), 1.66 (m, 2H, $CH_3C\underline{H}_2C \cdot HCH_3$), 3.0 – 4.0 (m, 17H, 4×$C\underline{H}_2$-piperazine $C\underline{H}_2$ and 3×$C\underline{H}_3O$-), 4.19 (m, 1H, $CH_3CH_2C \cdot \underline{H}CH_3$), 6.60 and 6.62 (two s, 1H total, H-Ar)

1-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine hydrochloride (XII). Compound **XI** (7.95 g, 0.018 mol) was stirred with conc. HCl (50 mL, 60.0 g, ~0.6 mol) at room temperature until totally dissolved (3 h). The mixture was evaporated in a rotary evaporator at reduced pressure. The solid was heated with *i*-PrOH (100 mL). The insoluble impurities were removed by filtration through Celite. The filtrate was evaporated in a rotary evaporator at reduced pressure. Intermediate **XII** (6.63 g, 97%) was used in the next step without further purification.

1-Carbimidamido-4-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine acetate (XIII). A solution of **XII** hydrochloride (6.48 g, 17 mmol) and 1*H*-pyrazole-1-carboximidamide hydrochloride (3.66 g, 25 mmol) in DMF (20 mL) was heated to 50°C, treated with di-isopropylethylamine (8.00 mL, 5.92 g, 46 mmol), stirred at 50°C for 10 h, and evaporated in a rotary evaporator at reduced pressure. The solid was dissolved in H_2O (120 mL) and extracted with MTBE (3 × 30 mL). The organic extracts were discarded. The transparent aqueous layer was made basic with NaOH solution (50%) in H_2O (35 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with H_2O (2 × 20 mL) and dried over anhydrous KCO_3. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator at reduced pressure. The solid 1-carbimidamido-4-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine (3.90 g, 10 mmol) was dissolved in CH_2Cl_2 (20 mL) and treated with glacial HOAc (0.56 mL, 0.59 g, 10 mmol). The resulting precipitate was filtered off, rinsed on the filter successively with CH_2Cl_2 (2 × 5 mL) and MTBE (3 × 10 mL), suspended in a mixture of Me_2CO (20 mL) and H_2O (2 mL), stirred, refluxed for 1 h, and cooled. The precipitate was separated by filtration, rinsed on the filter with Me_2CO (2 × 5 mL), and dried in air to afford **XIII** as fine colorless crystals (4.00 g, 53%). PMR spectrum (DMSO-d_6), δ, ppm (J, Hz): 0.90 (t, 3H, 6 Hz, $C\underline{H}_3CH_2C \cdot HCH_3$), 1.09 (d, 3H, 6 Hz, $CH_3CH_2C \cdot HC\underline{H}_3$), 1.64 (m, 5H, $CH_3C\underline{H}_2C \cdot HCH_3$ and $C\underline{H}_3CO_2$-), 3.0 – 4.0 (m, 17H, 4×$C\underline{H}_2$-piperazine $C\underline{H}_2$ and 3×$C\underline{H}_3O$-), 4.12 (m, 1H, $CH_3CH_2C \cdot \underline{H}CH_3$), 6.62 (s, 1H, H-Ar), 8.20 (br.s, 4H, $C(N\underline{H}_2)(N^+\underline{H}_2)$). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 10.31 ($\underline{C}H_3CH_2C \cdot HCH_3$), 20.02 ($CH_3CH_2C \cdot H\underline{C}H_3$), 25.83 ($\underline{C}H_3CO_2$-), 30.16 ($CH_3\underline{C}H_2C \cdot HCH_3$), 41.50 ($CH_2$-piperazine $C\underline{H}_2$), 45.05 (CH_2-piperazine $C\underline{H}_2$), 45.63 (CH_2-piperazine $C\underline{H}_2$), 46.27 (CH_2-piperazine $C\underline{H}_2$), 56.99 (CH_3O-), 61.49 (CH_3O-), 61.72 (CH_3O-), 81.38 ($CH_3CH_2\underline{C} \cdot HCH_3$), 106.84 (C6, Ar), 126.32 (C1, Ar), 141.43 (C2, Ar), 144.31 (C5, Ar), 147.59 (C4, Ar), 150.18 (C3, Ar), 158.42 ($\underline{C}(NH_2)(N^+H_2)$), 167.44 ($\underline{C}ON$), 176.66 ($CH_3\underline{C}O_2H$). The product was 99.6% pure according to HPLC with a maximum of 0.1% of an unidentified impurity and 0.3% of total unidentified impurities.

1-Carbimidamido-4-[2-(1-methylpropyl)oxy-3,4,5-trimethoxybenzoyl]piperazine hemifumarate (II). Acetate **XIII** (3.5 g, 7.7 mmol) was dissolved in hot H_2O (10 mL, ~60°C) and treated with a hot solution (~60°C) of fumaric acid (0.45 g, 3.85 mmol) in H_2O (5 mL). The resulting precipitate was separated by filtration, rinsed on the filter with H_2O (3 (5 mL), and recrystallized from hot H_2O to afford **II** hemifumarate as colorless crystals (3.21 g, 92%).

PMR spectrum (DMSO-d_6), δ, ppm (J, Hz): 0.92 (t, 3H, 6 Hz, $C\underline{H}_3CH_2C \cdot HCH_3$), 1.11 (d, 3H, 6 Hz,

$CH_3CH_2C \cdot HC\underline{H}_3$), 1.66 (m, 2H, $CH_3C\underline{H}_2C \cdot HCH_3$), 3.0 – 4.0 (m, 17H, 4×$C\underline{H}_2$-piperazine $C\underline{H}_2$ and 3×$C\underline{H}_3O$-), 4.15 (m, 1H, $CH_3CH_2C \cdot \underline{H}CH_3$), 6.20 (s, 1H, $HO_2CC\underline{H}$=$C\underline{H}CO_2H$), 6.61 (s, 1H, H-Ar), 8.30 (br.s, 4H, $C(N\underline{H}_2)(N^+\underline{H}_2)$). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 10.41 ($\underline{C}H_3CH_2C \cdot HCH_3$), 20.12 ($CH_3CH_2C \cdot H\underline{C}H_3$), 30.22 ($CH_3\underline{C}H_2C \cdot HCH_3$), 41.51 ($CH_2$-piperazine $C\underline{H}_2$), 45.15 (CH_2-piperazine $C\underline{H}_2$), 45.66 (CH_2-piperazine $C\underline{H}_2$), 46.37 (CH_2-piperazine $C\underline{H}_2$), 57.00 (CH_3O-), 61.59 (CH_3O-), 61.75 (CH_3O-), 81.40 ($CH_3CH_2\underline{C} \cdot HCH_3$), 106.88 (C6, Ar), 126.33 (C1, Ar), 136.55 ($HO_2C\underline{C}H$=$\underline{C}HCO_2H$), 141.45 (C2, Ar), 144.32 (C5, Ar), 147.60 (C4, Ar), 150.21 (C3, Ar), 158.44 ($\underline{C}(NH_2)(N^+H_2)$), 167.41 ($\underline{C}ON$), 172.13 ($HO_2\underline{C}CH$=$CH\underline{C}O_2H$). Mass spectrum: MH^+ found, 395.22; MH^+ calc., 395.23. The product was 99.7% pure according to HPLC with a maximum of 0.1% of an unidentified impurity and 0.2% of total unidentified impurities.

1-Carbimidamido-4-(3,4,5-trimethoxyphenylsulfonyl)-piperazine fumarate (III). 1-Carbimidamido-4-(3,4,5-trimethoxyphenylsulfonyl)piperazine hydrochloride (5 g, 12.7 mmol) that was prepared as described by us earlier [3] was treated with NaOH solution (50%) in H_2O (35 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with H_2O (20 mL) and dried over anhydrous K_2CO_3. The desiccant was separated by filtration. The filtrate was evaporated in a rotary evaporator. The solid was dissolved in H_2O and treated with fumaric acid (1.47 g, 12.7 mmol). The H_2O was evaporated in a rotary evaporator at reduced pressure. The resulting solid was suspended in a mixture of Me_2CO (40 mL) and H_2O (2 mL), stirred, refluxed for 1 h, and cooled. The solid was separated by filtration, rinsed on the filter with Me_2CO (2 × 10 mL), and dried in vacuo to afford the product as fine colorless crystals (3.3 g, 55%).

PMR spectrum (DMSO-d_6), δ, ppm (J, Hz): 3.03 (4H, m, 2×$C\underline{H}_2$-piperazine $C\underline{H}_2$), 3.50 (4H, m, 2×$C\underline{H}_2$-piperazine $C\underline{H}_2$), 3.77 (s, 3H, $C\underline{H}_3O$-), 3.88 (s, 6H, 2×$C\underline{H}_3O$-), 6.41 (s, 2H, $HO_2CC\underline{H}$=$C\underline{H}CO_2H$), 6.89 (s, 2H, H-Ar), 8.50 (br.s, 5H, $C(N\underline{H}_2)(N^+\underline{H}_2)$). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 44.18 (CH_2-piperazine $C\underline{H}_2$), 45.22 (CH_2-piperazine $C\underline{H}_2$), 56.36 (2×CH_3O-), 104.97 (C2, Ar), 129.56 (C1, Ar), 135.34 ($HO_2C\underline{C}H$=$\underline{C}HCO_2H$), 141.36 (C4, Ar), 153.12 (C3, Ar), 156.92 ($\underline{C}(NH_2)(N^+H_2)$), 168.63 ($HO_2\underline{C}CH$=$CH\underline{C}O_2H$). Mass spectrum: MH^+ found, 358.15; MH^+ calc., 358.13. The product was 99.7% pure according to HPLC with a maximum of 0.1% of an unidentified impurity and 0.2% of total unidentified impurities.

The log P value (logarithm of the octanol—water concentration ratio of un-ionized compound) was used as an indicator of the hydrophobicity of the synthesized compounds; the pKa value, as an indicator of basicity (Table 1). The pKa values were calculated using the program at the website www.chemicalize.org (chemAxon); log P, the program at the website http://www.organic-chemistry.org/prog/peo/ (OSIRIS Property Explorer).

EXPERIMENTAL BIOLOGICAL PART

Blood from practically healthy people (20 – 30 years old) and rabbits (2.5 – 3.0 kg, Rappolovo nursery) was used.

Erythrocytes were obtained from citrated blood by centrifugation at 1,500 rpm for 10 min followed by rinsing (3×) with normal saline. Then, the cells were stabilized for at least 1 d at 4°C in Alsever's solution. Erythrocytes were rinsed three times with normal saline before use. A standard cell suspension in Veronal—Medinal buffer (pH 7.4) was prepared. The optical density of the standard suspension was (0.560 ± 0.020) at 800 nm after dilution in eight times the amount of buffer. Measurements were made on an SF 2000 spectrophotometer (LOMO) in a 5-mm cuvette. The resulting standard cell suspension was used in the studies.

Cytolytic activity of the compounds was recorded at 37°C in a thermostatted spectrophotometer cuvette (5-mm optical path length) into which a solution of the studied compound (from 0.01 to 0.2 mL) that was diluted beforehand in normal saline was placed. The volume of the mixture was adjusted to 0.7 mL using Veronal—Medinal buffer. Standard erythrocyte suspension (0.1 mL) was added to the mixture after heating for 3 min. The decrease of optical density of the suspension was recorded at 800 nm at 5-second intervals until the hemolysis was complete.

Antioxidant properties of the compounds were assessed using an apparatus for studying photo-induced cytolysis by the previously published method [7]. According to this method, an incubation mixture containing standard erythrocyte suspension (0.1 mL); Veronal—Medinal buffer (pH 7.2 – 7.4); various amounts of studied compounds; and radachlorin photosensitizer (0.35% solution for i.v. injection, RADA-FARMA, Russia), the principal drug substance of which was (7*S*,8*S*)-13-vinyl-5-(carboxymethyl)-7-(2-carboxyethyl)-2,8,12,17-tetramethyl-18-ethyl-7*H*,8*H*-porphyrin-3-carboxylic acid, was prepared in a shielded 5-mm cuvette. The control contained normal saline instead of the studied drug. The final radachlorin concentration in the sample was 6.25 μg/mL. The incubation mixture (total volume 0.8 mL) was thermostatted in the spectrophotometer cuvette holder for 3 min at 37°C with constant stirring and then irradiated with monochromatic light (red LED, 653 nm, output power 12 mW, irradiation dose 1.4 J/cm^2). After the irradiation was finished, the decrease of the solution optical density at 750 nm was recorded.

The T_{50} value, i.e., the time for 50% erythrocyte lysis in the incubation mixture, was determined using computer software from the recorded smooth S-shaped hemolytic curve [7]. The rate of hemolysis was judged from the change of T_{50}.

Complement-dependent hemolysis was recorded in a 5-mm spectrophotometer cuvette thermostatted at 37°C into

which human blood serum (0.05 mL) was added. The volume was adjusted to 0.7 mL using Veronal—Medinal buffer (5 mM, pH 7.4). The mixture was thermostatted for 3 min and treated with standard rabbit erythrocyte suspension (0.1 mL). The decrease of suspension optical density at 800 nm was recorded at 5-second intervals. Complement-dependent hemolysis parameters, namely the induction period duration (T-lag) in seconds and the hemolysis rate (V) expressed in millions of erythrocytes lysed per minute [8], were determined from the hemolysis curves.

Various amounts of compound in normal saline were added to the incubation mixture during the study of the influence of the compound on complement-dependent hemolysis. The same amount of normal saline was added as a control to the incubation mixture.

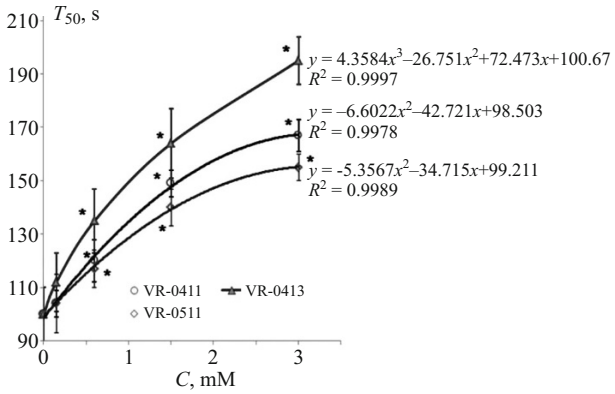


Fig. 1. Time for 50% erythrocyte lysis (T_{50}) as a function of compound concentration, % vs. control (normal saline). Control T_{50} value, 87 ± 15 sec. * Differences statistically significant vs. control, $p < 0.05$ ($n = 5$).

RESULTS AND DISCUSSION

The *N*,*N*′-substituted piperazines (Table 1) were basic compounds, the hydrophobicity (log P) of which increased in the order **III**, **I**, **II**. The compounds had practically the same basicity (pKa).

All compounds in the studied concentration range (0.15 – 3.0 mM) did not cause lysis of human erythrocytes.

It is well known [9] that erythrocyte lysis is initiated by irradiation with UV or visible light in the presence of photosensitizers, the most efficient of which are porphyrins and their derivatives [10], in particular radachlorin. It was found that generation of primarily singlet oxygen and then other reactive oxygen species was responsible for the photodynamic effect [11]. Binding of porphyrins to cell membranes reduced the photolytic stability of their membranes [10, 12].

N,*N*′-Substituted piperazines **I**, **II**, and **III** inhibited radachlorin-induced hemolysis statistically significantly compared with the control. This was evident in the increased 50% erythrocyte hemolysis time (T_{50}) (Fig. 1).

The results showed that the inhibitory activity of all three compounds was polynomial and dose-dependent in nature. The EC_{50} values for **II**, **I**, and **III** were 1.0, 1.6, and 2.3 mM, respectively. A comparison of the physicochemical properties and activities (Table 1, Fig. 1) suggested that the inhibitory activity of the compounds for photo-induced hemolysis increased as their hydrophobicity and the number of alkoxy substituents on the aromatic ring increased.

The influence of the *N*,*N*′-substituted piperazines on complement-dependent hemolysis was studied in order to determine if their antioxidant activities or membrane-protective properties were responsible for the reduced hemolysis (Table 2).

Complement-dependent hemolysis does not involve reactive oxygen species. The complement system is activated

TABLE 1. Structures and Properties of Studied *N*,*N*′-Substituted Piperazines

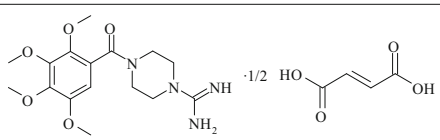
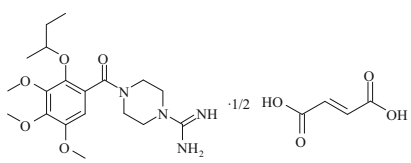
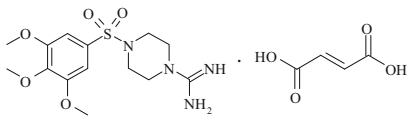
Compound	Structural formula	Mol. wt.	log P	pKa
I		410.15	0.2	11.55
II		452.50	1.42	11.53
III		474.49	– 0.36	11.51

TABLE 2. Human Complement Activation Parameters in the Presence of *N*,*N*′-Substituted Piperazines, % vs. Control (Normal Saline)

Compound	Concentration, mM					
	0.15 (n = 5)		0.6 (n = 5)		1.5 (n = 5)	
	T-lag	V	T-lag	V	T-lag	V
I	101 ± 10	102 ± 6	99 ± 9	107 ± 7	110 ± 10	99 ± 5
II	97 ± 11	101 ± 4	104 ± 16	101 ± 3	110 ± 14	98 ± 12
III	99 ± 13	106 ± 9	107 ± 12	100 ± 7	105 ± 10	103 ± 7

by foreign agents (rabbit erythrocytes) and attacks the membrane to form complexes that are incorporated into foreign cell membranes and cause lysis [13].

Studies of the influence of the compounds on complement-dependent hemolysis did not change T-lag or the hemolysis rate (V).

The results indicated that all studied *N*,*N*′-substituted piperazines possessed primary antioxidant activity that manifested in the inhibition of oxidative processes at the membrane level during photodynamic treatment of the erythrocytes.

The antioxidant activities of the *N*,*N*′-substituted piperazines that were studied in the present work could be due to the three (**III**) or four (**I** and **II**) alkoxy groups on the aromatic ring (Table 1). The higher antioxidant activity of **II** as compared with **I** and **III** could be related to the fact that adding a *sec*-butoxy substituent to the aromatic ring enhances the hydrophobic and membranotropic properties of the compound and, as a result, increases the antioxidant activity.

Compounds containing aromatic rings with alkoxy substituents, e.g., trimethoxybenzoic acid, are known to be radical traps [14].

It was shown experimentally that the cytoprotective activity of trimetazidine [1-(2,3,4-trimethoxybenzyl)piperazine-hydrochloride] [15] was due to its ability to trap free radicals.

Antioxidant properties were also confirmed for substituted *N*-(aryloxyethyl)-*N*′-(2-methoxyphenyl)piperazines [16].

The method used by us made it possible to determine the mechanism of the antioxidant activity of the compounds based on suppression of erythrocyte lysis as a result of oxidative photo-induced degradation of membrane components in the presence of radachlorin [17].

The experimental results led to the conclusion that all tested *N*,*N*′-substituted piperazines possessed antioxidant activity that was apparently due to protection of erythrocyte membranes from damage by reactive oxygen species.

All studied *N*,*N*′-substituted piperazines in the concentration range 0.15 – 3.00 mM did not exhibit hemolytic activity for donor blood. Induced hemolysis models established that all compounds slowed photo-induced hemolysis and did not influence hemolysis caused by complement system activation. The activities of the compounds were not related to a direct membrane-protective effect. It could be assumed based on the photo-induced hemolysis mechanism that the studied *N*,*N*′-substituted piperazines protected cells from lysis as a result of their antioxidant activities that were manifested upon interaction with cell membranes. Compound **II** had the greatest protective effect.

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