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Novel urea and *bis*-urea primaquine derivatives with hydroxyphenyl or halogenphenyl substituents: Synthesis and biological evaluation

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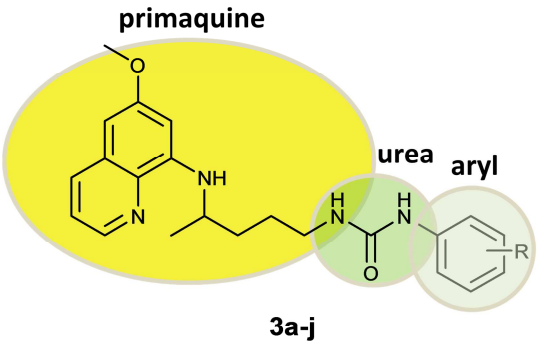
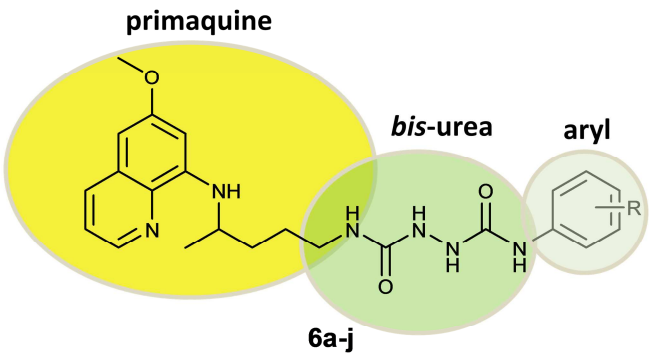
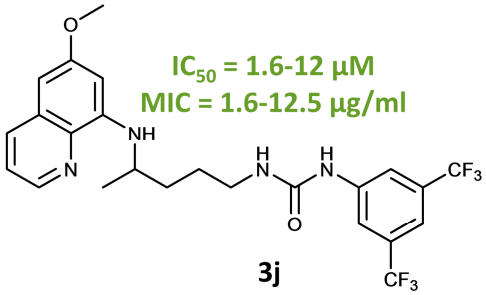


3a-j

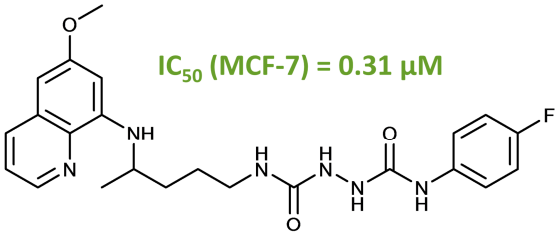


6a-j



IC_{50} = 1.6-12 µM
MIC = 1.6-12.5 µg/ml

3j



IC_{50} (MCF-7) = 0.31 µM

6d

Novel urea and *bis*-urea primaquine derivatives with hydroxyphenyl or halogenphenyl substituents: synthesis and biological evaluation

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Antiproliferative activity

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A B S T R A C T

A series of novel compounds **3a-j** and **6a-j** with primaquine and hydroxyl or halogen substituted benzene moieties bridged by urea or *bis*-urea functionalities were designed, synthesized and evaluated for biological activity. The title compounds were prepared using benzotriazole as the synthon, through several synthetic steps. 3-[3,5-*Bis*(trifluoromethyl)phenyl]-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3j**) was the most active urea and 1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]-3-[3-(trifluoromethyl)phenyl]urea (**6h**) the most active *bis*-urea derivative in antiproliferative screening *in vitro* against eight tested cancer cell lines. Urea derivatives **3a-g** with hydroxy group or one halogen atom showed moderate antiproliferative effects against all the tested cell lines, but stronger activity against breast carcinoma MCF-7 cell line, while trifluoromethyl derivatives **3h-j** showed antiproliferative effects against all the tested cell lines in low micromolar range. Finally, *bis*-ureas with hydroxy and fluoro substituents **6a-d** showed extreme selectivity and chloro or bromo derivatives **6e-g** high selectivity against MCF-7 cells (IC_{50} 0.1-2.6 μM). *p*-Fluoro derivative **6d**, namely 3-(4-fluorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea, is the most promising compound. Further biological experiments showed that **6d** affected cell cycle and induced cell death of MCF-7 cell line. Due to its high activity against MCF-7 cell line (IC_{50} 0.31 μM), extreme selectivity and full agreement with the Lipinski's and Gelovani's rules for prospective small molecular drugs, **6d** may be considered as a lead compound in development of breast carcinoma drugs.

Urea **3b** and almost all *bis*-ureas showed high antioxidant activity in DPPH assay, but urea derivatives were more active in lipid peroxidation test. Only few compounds exhibited weak inhibition of soybean lipoxygenase. Compound **3j** exhibited the strongest antimicrobial activity in susceptibility assay *in vitro* (MIC = 1.6–12.5 μg ml^{-1}).

1. Introduction

Modification of known, clinically approved drugs is one of the possible strategies in drug discovery. In our search for potential anticancer agents we have focused our attention to primaquine (PQ), the antimalarial agent with a pronounced antiproliferative activity [1,2,3]. The potential for discovery of new anticancer agents among antimalarials was first noted in the scientific literature when commonly used antimalarials (artemisinin, artesunate,

tetracyclines, chloroquine and primaquine) were observed to portend significant anticancer activity. Currently, 14 out of 20 agents globally used in the treatment of malaria have reported anticancer effects and 7 have reached a clinical stage of development [4,5]. In several studies coadministration of antimalarial drugs with approved anticancer drugs was studied. It was shown that antimalarial drugs (hydroxychloroquine, chloroquine, primaquine) reduce IC_{50} of anticancer drugs (doxorubicin, cisplatin) and inhibit development of drug resistance of cancer cells [6]. It was also shown that chloroquine is a promising adjuvant together with lidamycin [7], sunitinib [8] or artemisin chemotherapy [9]. Coadministration of primaquine with imatinib showed that primaquine affect imatinib clearance, bioavailability and distribution pattern, which could improve the treatment of renal and brain tumours, but also increase toxicity [10]. Both chloroquine and primaquine, have quinoline moiety in their structure. The widespread use of quinoline heterocycle as an important pharmacophore in medicinal chemistry establishes this moiety as a member of a privileged structures class. Various quinoline derivatives were reported as tubulin polymerization and tyrosine kinase inhibitors [11]. Their potential to develop to anticancer drugs is described recently in review of Afzal *et al*. [12].

In last few years, our research group has focused to primaquine derivatization. We have prepared approximately 90 novel primaquine derivatives and their antiproliferative activity *in vitro* has been well documented. In our previous papers, synthesis and biological evaluation of 1,4-substituted semicarbazides with primaquine moiety bridged by carbonyl group at position 1 and cycloalkyl, aryl, benzyloxy or hydroxy substituent at position 4 [13] or urea and semicarbazide primaquine derivatives with benzhydryl, trityl, phenyl or hydroxyalkyl substituents was reported [14]. Most of the compounds showed significant cytostatic activities in low micromolar concentrations towards all the tested cell lines or high selectivity towards MCF-7 cells (breast carcinoma). The highest selectivity against MCF-7 cells and practically no activity against other 7 tested cancer cell lines showed symmetric derivative with two primaquine residues bind with two urea groups (IC_{50} 0.2 μM). Urea with 5-hydroxypentyl substituent on one nitrogen and primaquine residue on the other one, showed extreme selectivity towards SW 620 colon cancer cells (IC_{50} 0.2 μM) [15]. In order to get better insight to the structural requirements needed for improvement of cytostatic activity of primaquine derivatives, here we report design, synthesis and biological evaluation of a new set of primaquine derivatives bearing urea or *bis*-urea functionalities and hydroxyphenyl or halogenphenyl substituents.

2. Materials and methods

2.1. Chemistry

2.1.1. Materials and general methods

Melting points were measured on a Stuart Melting Point (SMP3) apparatus (Barloworld Scientific, UK) in open capillaries with uncorrected values. IR spectra were recorded on FTIR Perkin Elmer Paragon 500 and UV-Vis spectra on Lambda 20 double beam spectrophotometer (Perkin-Elmer, UK). All NMR (^{1}H and ^{13}C) were recorded at 25 °C on NMR Avance 600 spectrometer (Bruker, Germany) at 300 and 150 MHz for ^{1}H and ^{13}C nuclei, respectively. Chemical shifts (δ) are reported in parts per million (ppm) using tetramethylsilane as reference in the ^{1}H and the dimethyl sulfoxide (DMSO) residual peak as reference in the ^{13}C spectra (39.51 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Mass spectra were recorded on HPLC-MS/MS (HPLC, Agilent Technologies 1200 Series; MS, Agilent Technologies 6410 Triple Quad). Mass determination was realized using electron spray ionization (ESI) in positive mode. Elemental analyses were performed on a CHNS LECO analyzer (LECO Corporation, USA). Analyses indicated by the symbols of the elements were within ± 0.4% of the theoretical values. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates using the following solvent systems: dichloromethane/methanol 97:3, 95:5 and 92:8, petrolether/ethyl acetate/methanol 30:10:5, cyclohexane/ethyl acetate 2:1 and petrolether/ethyl acetate 2:1. Spots were visualized by short-wave UV light and iodine vapour. Column chromatography was performed on silica gel (0.063–0.200 mm), with the same eluents used in TLC. All chemicals and solvents were of analytical grade and purchased from commercial sources. 1*H*-benzo[*d*] [1,2,3]triazole (BtH), triphosgene, primaquine diphosphate, triethylamine (TEA), hydrazine hydrate and hydroxy or halogen substituted aniline derivatives were purchased from Sigma-Aldrich (USA). Primaquine was prepared from primaquine diphosphate prior the use. All reactions with primaquine were run light protected.

*2.1.2. 1-(N-arylcarbamoyl)benzotriazoles (**2a-j**): general procedure*

The title compounds were synthesized from chloride **1** and corresponding hydroxy or halogen substituted aniline derivative, following our previously published procedure [16]. The

solution of 0.908 g (5.0 mmol) chloride **1** in dry dioxane was added dropwise to the solution of 5.0 mmol aniline derivative and 0.507 g (5 mmol) TEA in dry dioxane.

Method A: Reaction mixture was stirred 1 h at room temperature and evaporated under reduced pressure.

Method B: Reaction mixture was stirred 1 h at room temperature, evaporated under reduced pressure, dissolved in ethyl acetate and extracted with 5% NaOH solution (3 × 15 ml) and water (3 × 15 ml). The organic layer was dried over sodium sulfate, filtered and evaporated under reduced pressure.

*2.1.2.1. 1-(N-(3-hydroxyphenyl)carbamoyl) benzotriazole (**2a**).* Method A, from the reaction of 0.546 g 3-aminophenol and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether 0.406 g (32%) of **2a** was obtained; mp 147–149 °C; IR (KBr): ν_{max} 3385, 3164, 1748, 1615, 1604, 1551, 1449, 1251, 1069, 842, 791, 773, 752 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.98 (s, 1H'), 9.55 (s, 1H), 8.28-8.24 (m, 2H), 7.79-7.74 (m, 1H), 7.62-7.57 (m, 1H), 7.38-7.16 (m, 3H), 6.65-6.60 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 158.15, 147.29, 146.03, 138.68, 131.97, 130.54, 129.94, 126.22, 120.36, 114.20, 112.29, 112.28, 108.65; MS/MS *m/z* 120.2 $(BtH+1)^{+}$; Anal. Calcd. for $C_{13}H_{10}N_4O_2$: C, 61.41; H, 3.96; N, 22.04. Found: C, 61.59; H, 4.06, N, 21.77.

*2.1.2.2. 1-(N-(4-hydroxyphenyl)carbamoyl) benzotriazole (**2b**).* Method A, from the reaction of 0.546 g 4-aminophenol and after crystallization from ether 1.004 g (79%) of **2b** was obtained; mp 240 °C (decomp.); Lit. mp 240 °C (decomp.) [17]; IR (KBr): ν_{max} 3456, 3252, 1726, 1544, 1616, 1544, 1618, 1488, 1446, 1370, 1236, 1212, 1060, 926, 825, 146, 583 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.89 (s, 1H), 9.41 (s, 1H), 8.25-8.23 (m, 2H), 7.76-7.73 (m, 1H), 7.60-7.57 (m, 3H), 6.83-6.81 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 154.61, 146.99, 145.50, 131.47, 129.94, 128.40, 125.62, 123.19, 119.81, 115.18, 114.20; MS/MS *m/z* 277.1 $(M+23)^{+}$, 120.1 $(BtH+1)^{+}$; Anal. Calcd. for $C_{13}H_{10}N_4O_2$: C, 61.41; H, 3.96; N, 22.04. Found: C, 61.30; H, 4.04, N, 22.40.

*2.1.2.3. 1-(N-(3-fluorophenyl)carbamoyl) benzotriazole (**2c**).* Method B, from the reaction of 0.556 g 3-fluoroaniline and after crystallization from ether 0.807 g (63%) of **2b** was obtained; mp 162.5–164.5 °C; IR (KBr): ν_{max} 3242, 3073, 1742, 1605, 1543, 1486, 1447, 1362, 1285, 1220, 1131, 1064, 927, 861, 789, 749 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.31 (s, 1H), 8.30-8.25

(m, 2H), 7.79-7.71 (m, 3H), 7.62-7.59 (m, 1H), 7.49-7.43 (m, 1H), 7.06-7.02 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 163.16, 146.95, 145.56, 139.07, 131.45, 130.43, 130.20, 125.84, 119.93, 114.08, 113.71, 108.37, 105.04; MS/MS *m/z* 120.2 $(BtH+1)^+$; Anal. Calcd. for $C_{13}H_9FN_4O$: C, 60.94; H, 3.54; N, 21.87. Found: C, 61.06; H, 3.68, N, 21.69.

*2.1.2.4. 1-(N-(4-fluorophenyl)carbamoyl) benzotriazole (**2d**).* Method B, from the reaction of 0.556 g 4-fluoroaniline and after purification by column chromatography (mobile phase petrolether/ethyl acetate/methanol 3:1:0.5) and crystallization from ether 0.833 g (65%) of **2d** was obtained; mp 167–168 °C; IR (KBr): ν_{max} 3327, 3247, 1742, 1614, 1560, 1552, 1510, 1410, 1365, 1313, 1212, 1129, 1102, 1057, 1000, 929, 835, 770, 747 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.20 (s, 1H'), 8.26-8.24 (m, 2H), 7.78-7.75 (m, 1H), 7.61-7.58 (m, 1H), 7.46-7.44 (m, 2H), 7.29-7.26 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 159.79, 147.11, 145.54, 133.52, 131.47, 130.12, 125.77, 123.23, 119.90, 115.17, 113.70; MS/MS *m/z* 120.2 $(BtH+1)^+$; Anal. Calcd. for $C_{13}H_9FN_4O$: C, 60.94; H, 3.54; N, 21.87. Found: C, 60.66; H, 3.50, N, 21.93.

*2.1.2.5. 1-(N-(3-chlorophenyl)carbamoyl) benzotriazole (**2e**).* Method B, from the reaction of 0.638 g 3-chloroaniline and after crystallization from ether 1.023 g (75%) of **2e** was obtained; mp 179–181.5 °C; IR (KBr): ν_{max} 3287, 3109, 1744, 1594, 1529, 1481, 1427, 1368, 1284, 1239, 1206, 1131, 1092, 1052, 912, 875, 847, 780, 754 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.34 (s, 1H), 8.28-8.24 (m, 2H), 8.00-7.99 (s, 1H), 7.84-7.71 (m, 2H), 7.63-7.58 (m, 1H), 7.46 (t, 1H), 7.29-7.25 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 146.99, 145.52, 138.84, 133.07, 131.45, 130.48, 130.24, 125.88, 124.37, 120.53, 119.96, 119.50, 113.74; MS/MS *m/z* 120.1 $(BtH+1)^+$; Anal. Calcd. for $C_{13}H_9ClN_4O$: C, 57.26; H, 3.33; N, 20.55. Found: C, 57.59; H, 3.66, N, 20.88.

*2.1.2.6. 1-(N-(4-chlorophenyl)carbamoyl) benzotriazole (**2f**).* Method B, from the reaction of 0.638 g 4-chloroaniline and after crystallization from ether 1.104 g (81%) of **2f** was obtained; mp 193–195.5 °C; IR (KBr): ν_{max} 3229, 3105, 3050, 1734, 1599, 1483, 1445, 1360, 1286, 1232, 1046, 920, 820, 718 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.28 (s, 1H), 8.27-8.23 (m, 2H), 7.90-7.87 (d, 2H), 7.80-7.75 (m, 1H), 7.63-7.57 (m, 1H), 7.51-7.48 (d, 2H); ^{13}C NMR (DMSO-d_6) δ 146.96, 145.53, 136.28, 131.43, 130.16, 128.70, 128.46, 125.81, 122.64, 119.91, 113.70; MS/MS *m/z* 120.1 $(BtH+1)^+$; Anal. Calcd. for $C_{13}H_9ClN_4O$: C, 57.26; H, 3.33; N, 20.55. Found: C, 56.93; H, 3.30, N, 20.69.

*2.1.2.7. 1-(N-(4-bromophenyl)carbamoyl) benzotriazole (**2g**).* Method B, from the reaction of 0.860 g 4-bromoaniline and after crystallization from ether 1.300 g (82%) of **2g** was obtained; mp 196–198 °C; IR (KBr): ν_{max} 3248, 3103, 3048, 1741, 1596, 1543, 1534, 1486, 1446, 1400, 1367, 1285, 1238, 1127, 1099, 1053, 928, 827, 808, 750 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.29 (s, 1H), 8.28-8.24 (m, 2H), 7.84-7.75 (m, 3H), 7.65-7.58 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 146.96, 145.55, 136.75, 131.50, 131.29, 130.20, 125.84, 120.20, 119.94, 113.73, 113.36; MS/MS *m/z* 120.1 $(BtH+1)^+$; Anal. Calcd. for $C_{13}H_9BrN_4O$: C, 49.23; H, 2.86; N, 17.67. Found: C, 49.51; H, 3.92, N, 17.55.

*2.1.2.8. 1-(N-(3-(trifluoromethyl)phenyl)carbamoyl) benzotriazole (**2h**).* Method B, from the reaction of 0.806 g 3-(trifluoromethyl)aniline and after crystallization from ether 0.888 g (58%) of **2h** was obtained; mp 176.5–178.5 °C; IR (KBr): ν_{max} 3255, 3075, 1744, 1605, 1548, 1450, 1373, 1333, 1286, 1240, 1164, 1118, 1051, 1000, 921, 897, 850, 793, 750 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.46 (s, 1H), 8.30-8.25 (m, 3H), 8.14 (d, 1H), 7.81-7.75 (m, 1H), 7.70-7.55 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 147.13, 145.55, 138.21, 131.27, 130.22, 130.01, 130.11-128.87, 125.70, 124.52, 121.01-120.06, 119.77, 117.30-117.05, 113.72; MS/MS *m/z* 120.1 $(BtH+1)^+$; Anal. Calcd. for $C_{14}H_9F_3N_4O$: C, 54.91; H, 2.96; N, 18.29. Found: C, 55.09; H, 3.28, N, 18.33.

*2.1.2.9. 1-(N-(4-(trifluoromethyl)phenyl)carbamoyl) benzotriazole (**2i**).* Method B, from the reaction of 0.806 g 4-(trifluoromethyl)aniline and after crystallization from ether 0.918 g (60%) of **2i** was obtained; mp 204.5–207 °C; IR (KBr): ν_{max} 3302, 3121, 3055, 1740, 1606, 1544, 1417, 1330, 1242, 1166, 1103, 1047, 921, 835, 750 cm^{-1}; ^{13}C NMR (DMSO-d_6) δ 152.02, 147.02, 143.02, 131.42, 130.22, 126.00, 125.86, 125.32-123.53, 122.43-121.79, 119.92, 118.08, 113.67; MS/MS *m/z* 120.1 $(BtH+1)^+$; Anal. Calcd. for $C_{14}H_9F_3N_4O$: C, 54.91; H, 2.96; N, 18.29. Found: C, 55.17; H, 3.38, N, 18.00.

*2.1.2.10. 1-(N-(3,5-bis(trifluoromethyl)phenyl)carbamoyl) benzotriazole (**2j**).* Method B, from the reaction of 1.146g 3,5-*bis*(trifluoromethyl)aniline 0.918 g (60%) of crude product **2j** was obtained; it was used in further reaction step without purification.

*2.1.3. Primaquine ureas (**3a-j**): general procedure*

A solution of 0.273 g (0.6 mmol) primaquine bisphosphate and 0.182 g (1.8 mmol) TEA in dichloromethane was stirred 0.25 h at room temperature. Then 6 mmol of corresponding 1-(*N*-arylcarbamoyl)benzotriazole **2** was added to the reaction mixture and stirred 0.5-3.5 h at room temperature. The reaction mixture was evaporated under reduced pressure. The residue was futher purified without extraction (**3a**, **3b**) or was dissolved in ethyl acetate and extracted with 5% NaOH solution (3 × 10 ml) and water (3 × 15 ml), dried over anhydrous sodium sulfate and evaporated (**3c**-**j**). The reactions were run light protected.

*2.1.3.1. 3-(3-Hydroxyphenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3a**).* Reaction time: 3.5 h. From the reaction of 0.153 g **2a** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) 0.156 g (66%) of **3a** was obtained; oil; IR (KBr): ν_{max} 3368, 2936, 2866, 1656, 1610, 1559, 1521, 1452, 1386, 1338, 1225, 1163, 1053, 973, 824, 781 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.17 (s, 1H), 8.54-8.53 (dd, 1H), 8.24 (s, 1H), 8.08-8.06 (dd, 1H), 7.43-7.41 (m, 1H), 6.98 (s, 1H), 6.96 (t, 1H), 6.71 (d, 1H), 6.47 (d, 1H), 6.29-6.28 (m, 2H), 6.14 (d, 1H), 6.08 (t, 1H), 3.82 (s, 3H), 3.66-3.63 (m, 1H), 3.11-3.09 (q, 2H), 1.70-1.49 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.99, 157.61, 155.08, 144.63, 144.23, 143.59, 142.88, 134.77, 134.51, 129.56, 129.16, 122.06, 108.39, 108.08, 104.72, 96.12, 91.64, 54.95, 47.05, 39.02, 33.51, 26.64, 20.22; MS/MS *m/z* 395.3 $(M+1)^+$; Anal. Calcd. for $C_{22}H_{26}N_4O_3$: C, 66.99; H, 6.64; N, 14.20. Found: C, 67.05; H, 6.99, N, 13.98.

*2.1.3.2. 3-(4-Hydroxyphenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3b**).* Reaction time: 3.5 h. From the reaction of 0.153 g **2b** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether 0.173 g (73%) of **3b** was obtained; mp 149–152.5 °C; IR (KBr): ν_{max} 3419, 3326, 2934, 2865, 1683, 1659, 1616, 1576, 1556, 1515, 1455, 1424, 1388, 1223, 1204, 1159, 1101, 1054, 1036, 967, 906, 828, 788 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.91 (s, 1H), 8.55-8.53 (dd, 1H), 8.09-8.06 (dd, 1H), 8.00 (s, 1H), 7.44-7.40 (m, 1H), 7.13 (d, 2H), 6.62 (d, 2H), 6.47 (d, 1H), 6.28 (d, 1H), 6.14 (d, 1H), 5.98 (t, 1H), 3.82 (s, 3H), 3.67-3.63 (m, 1H), 3.11-3.06 (q, 2H), 1.68-1.48 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.98, 155.52, 151.84, 144.62, 144.21, 134.76, 134.49, 132.06, 129.55, 122.05, 119.82, 115.00, 96.11, 91.63, 54.94, 47.05, 39.09, 33.50, 26.72, 20.20; MS/MS *m/z* 395.4 $(M+1)^+$; Anal. Cald. for $C_{22}H_{26}N_4O_3$: C, 66.99; H, 6.64; N, 14.20. Found: C, 66.80; H, 6.90, N, 14.24.

*2.1.3.3. 3-(3-Fluorophenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3c**).* Reaction time: 2 h. From the reaction of 0.154 g **2c** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) 0.152 g (64%) of **3c** was obtained; oil; IR (KBr): ν_{max} 3354, 2961, 2926, 2856, 1651, 1645, 1615, 1557, 1510, 1463, 1424, 1386, 1273, 1221, 1153, 1113, 1052, 967, 940, 863, 822, 791, 773 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.59 (s, 1H), 8.53-8.52 (dd, 1H), 8.08-8.06 (dd, 1H), 7.45-7.41 (m, 2H), 7.23-7.19 (q, 1H), 7.01-7.00 (d, 1H), 6.67 (t, 1H), 6.47 (d, 1H), 6.28 (d, 1H), 6.21 (t, 1H), 6.13 (d, 1H), 3.82 (s, 3H), 3.66-3.63 (m, 1H), 3.13-3.10 (q, 2H), 1.60-1.50 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 163.14, 158.96, 154.89, 144.59, 144.17, 142.39, 134.71, 134.48, 129.92, 129.51, 122.00, 113.20, 107.11, 106.97, 104.26, 104.08, 96.10, 91.64, 54.90, 47.01, 39.02, 33.44, 26.48, 20.17; MS/MS *m/z* 397.2 $(M+1)^+$; Anal. Calcd. for $C_{22}H_{25}FN_4O_2$: C, 66.65; H, 6.36; N, 14.13. Found: C, 66.77; H, 6.50, N, 14.34.

*2.1.3.4. 3-(4-Fluorophenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3d**).* Reaction time: 1 h. From the reaction of 0.154 g **2d** and after purification by column chromatography (mobile phases: dichloromethane/methanol 95:5 and cyclohexane/ethyl acetate 2:1) 0.143 g (60%) of **3d** was obtained; oil; IR (KBr): ν_{max} 3323, 2945, 2926, 2858, 1635, 1568, 1520, 1456, 1424, 1393, 1380, 1292, 1267, 1210, 1160, 1090, 1055, 1033, 829, 819, 788, 768 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.54-8.53 (dd, 1H), 8.36 (s, 1H), 8.08-8.06 (dd, 1H), 7.43-7.41 (m, 1H), 7.39-7.36 (m, 2H), 7.04-7.01 (m, 2H), 6.47 (d, 1H), 6.28 (d, 1H), 6.13 (d, 1H), 6.11 (t, 1H), 3.82 (s, 3H), 3.67-3.63 (m, 1H), 3.12-3.09 (q, 2H), 1.70-1.50 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.95, 155.96, 157.53, 155.18, 144.59, 144.17, 136.86, 134.70, 134.48, 129.51, 121.99, 119.17, 119.13, 114.97, 114.83, 96.08, 91.71, 54.94, 47.02, 39.06, 33.46, 26.56, 20.17; MS/MS *m/z* 397.3 $(M+1)^+$; Anal. Calcd. for $C_{22}H_{25}FN_4O_2$: C, 66.65; H, 6.36; N, 14.13. Found: C, 66.81; H, 6.52, N, 13.95.

*2.1.3.5. 3-(3-Chlorophenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3e**).* Reaction time: 2 h. From the reaction of 0.164 g **2e** and crystallization from ether 0.159 g (64%) of **3e** was obtained; mp 111.5–113.5 °C; IR (KBr): ν_{max} 3338, 2967, 2930, 2871, 1649, 1597, 1574, 1519, 1481, 1455, 1422, 1387, 1311, 1269, 1228, 1205, 1166, 1054, 885, 861, 825, 788 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.58-8.55 (m, 2H), 8.12-8.10 (m, 1H), 7.65 (s, 1H), 7.23-7.17 (m, 2H), 6.91 (d, 1H), 6.49 (m, 1H), 6.37-6.33 (m, 2H), 6.23 (s, 1H), 3.82 (s, 3H), 3.39-3.63 (m, 1H), 3.14-3.09 (m, 2H), 1.71-1.50 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.99, 154.87,

155.18, 144.38, 144.08, 142.08, 134.95, 132.99, 130.05, 129.57, 122.01, 120.39, 116.91, 115.89, 96.39, 91.81, 54.93, 47.11, 39.04, 33.39, 26.49, 20.12; MS/MS *m/z* 413.2 $(M+1)^+$; Anal. Calcd. for $C_{22}H_{25}ClN_4O_2$: C, 63.99; H, 6.10; N, 13.57. Found: C, 64.33; H, 6.40, N, 13.58.

*2.1.3.6. 3-(4-Chlorophenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3f**).* Reaction time: 2 h. From the reaction of 0.164 g **2f** and after purification by column chromatography (mobile phases: dichloromethane/methanol 95:5 and petrolether/ethyl acetate 2:1) and crystallization from ether 0.141 g (57%) of **3f** was obtained; mp 94–96.5 °C; IR (KBr): ν_{max} 3314, 3088, 3047, 2968, 2934, 2862, 1637, 1617, 1591, 1571, 1522, 1492, 1457, 1424, 1388, 1305, 1222, 1205, 1159, 113, 1090, 1051, 1012, 967, 822, 791 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.54-8.53 (dd, 1H), 8.52 (s, 1H), 8.08-8.06 (dd, 1H), 7.43-7.41 (m, 1H), 7.41 (d, 2H), 7.24 (d, 2H), 6.47 (d, 1H), 6.28 (d, 1H), 6.19 (t, 1H, 2), 6.13 (d, 1H), 3.81 (s, 3H), 3.66-3.64 (m, 1H), 3.12-3.09 (q, 2H), 1.69-1.49 (m, 4H), 1.22 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.98, 155.00, 144.62, 144.22, 139.56, 134.76, 134.51, 129.55, 128.36, 124.29, 122.06, 119.04, 96.12, 91.62, 54.94, 47.02, 39.07, 33.46, 26.57, 20.21; MS/MS *m/z* 413.2 $(M+1)^+$; Anal. Calcd. for $C_{22}H_{25}ClN_4O_2$: C, 63.99; H, 6.10; N, 13.57. Found: C, 64.42; H, 6.49, N, 13.71.

*2.1.3.7. 3-(4-Bromophenyl)-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3g**).* Reaction time: 0.5 h. From the reaction of 0.182 g **2g** and crystallization from ether and petrolether 0.181 g (66%) of **3g** was obtained; mp 107–109 °C; IR (KBr): ν_{max} 3324, 2969, 2936, 2861, 1638, 1619, 1595, 1585, 1522, 1490, 1456, 1424, 1388, 1304, 1289, 1238, 1206, 1158, 1112, 1050, 1006, 964, 820, 792 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.54-8.53 (m, 2H), 8.09-8.06 (dd, 1H), 7.44-7.40 (m, 1H), 7.36 (s, 4H), 6.47 (d, 1H), 6.28 (d, 1H), 6.20 (t, 1H), 6.14 (d, 1H), 3.81 (s, 3H), 3.67-3.61 (m, 1H), 3.12-3.07 (q, 2H), 1.69-1.51 (m, 4H), 1.22 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.99, 154.97, 144.62, 144.24, 140.00, 134.80, 134.51, 131.27, 129.57, 122.10, 119.48, 112.11, 96.13, 91.61, 54.96, 47.03, 39.08, 33.46, 26.59, 20.23; MS/MS *m/z* 457.1 $(M+1)^+$, 458.2 $(M+2)^+$; Anal. Calcd. for $C_{22}H_{25}BrN_4O_2$: C, 57.77; H, 5.51; N, 12.25. Found: C, 58.90; H, 5.72, N, 11.91.

*2.1.3.8. 1-{4-[(6-Methoxyquinolin-8-yl)amino]pentyl}-3-[3-(trifluoromethyl)phenyl]urea (**3h**).* Reaction time: 1 h. From the reaction of 0.184 g **2h** and after purification by column chromatography (mobile phase: dichloromethane/methanol 95:5) 0.244 g (91%) of **3h** was

obtained; oil; IR (KBr): ν_{max} 3358, 3082, 2926, 2855, 1654, 1618, 1570, 1522, 1448, 1424, 1388, 1337, 1255, 1221, 1165, 1122, 1068, 1051, 1031, 971, 893, 820, 790 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.79 (s, 1H), 8.54-8.53 (dd, 1H), 8.08-8.07 (dd, 1H), 7.96 (s, 1H), 7.50-7.41 (m, 3H), 7.21 (d, 1H), 6.47 (d, 1H), 6.32 (t, 1H), 6.28 (d, 1H), 6.15 (d, 1H), 3.81 (s, 3H), 3.67-3.62 (m, 1H), 3.14-3.11 (q, 2H), 1.71-1.51 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.99, 155.01, 144.63, 144.23, 141.43, 134.77, 134.52, 129.67, 129.53, 129.53-129.04, 126.96-121.55, 122.07, 121.05, 117.03, 113.46, 96.13, 91.62, 54.93, 47.03, 39.11, 33.45, 26.53, 20.21; MS/MS *m/z* 447.4 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{25}F_3N_4O_2$: C, 61.87; H, 5.64; N, 12.55. Found: C, 61.53; H, 5.68, N, 12.19.

*2.1.3.9. 1-{4-[(6-Methoxyquinolin-8-yl)amino]pentyl}-3-[4-(trifluoromethyl)phenyl]urea (**3i**).* Reaction time: 2 h. From the reaction of 0.184 g **2i** and crystallization from ether and petrolether 0.230 g (86%) of **3i** was obtained; mp 88–91 °C; IR (KBr): ν_{max} 3322, 2968, 2937, 1735, 1699, 1648, 1618, 1597, 1577, 1524, 1459, 1389, 1321, 1243, 1206, 1159, 1113, 1066, 1050, 1014, 965, 823, 792 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.82 (s, 1H), 8.54-8.52 (dd, 1H), 8.09-8.05 (dd, 1H), 7.59-7.52 (m, 4H), 7.44-7.40 (m, 1H), 6.47 (d, 1H), 6.32 (t, 1H), 6.28 (d, 1H), 6.13 (d, 1H), 3.81 (s, 3H), 3.69-3.61 (m, 1H), 3.15-3.10 (q, 2H), 1.70-1.50 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.96, 154.76, 144.60, 144.26, 144.20, 134.74, 134.49, 129.53, 125.89-125.75, 122.04, 117.12, 96.11, 91.62, 54.92, 47.01, 39.06, 33.43, 26.46, 20.19; MS/MS *m/z* 447.5 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{25}F_3N_4O_2$: C, 61.87; H, 5.64; N, 12.55. Found: C, 61.56; H, 5.63, N, 12.26.

*2.1.3.10. 3-[3,5-Bis(trifluoromethyl)phenyl]-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3j**).* Reaction time: 0.5 h. From the reaction of 0.225 g **2j** and after purification by column chromatography (mobile phase: dichloromethane/methanol 95:5) 0.200 g (65%) of **3j** was obtained; mp 171–173.5 °C; IR (KBr): ν_{max} 3348, 3230, 3118, 2936, 1661, 1618, 1577, 1521, 1476, 1456, 1425, 1390, 1275, 1222, 1180, 1132, 1051, 1032, 1002, 946, 888, 874, 824, 792 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.17 (s, 1H), 8.54-8.52 (dd, 1H), 8.07-8.05 (m, 3H), 7.52 (s, 1H), 7.44-7.40 (m, 1H), 6.51 (t, 1H), 6.47 (d, 1H), 6.28 (d, 1H), 6.14 (d, 1H), 3.81 (s, 3H), 3.69-3.62 (m, 1H), 3.17-3.11 (q, 2H), 1.71-1.52 (m, 4H), 1.23 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.96, 154.72, 144.60, 144.18, 142.63, 134.81, 134.50, 131.16-129.86, 129.53, 128.75-117.91, 122.03, 117.15, 113.25, 96.13, 91.60, 54.89, 47.00, 39.22, 33.38,

26.38, 20.18; MS/MS *m/z* 515.3 $(M+1)^+$; Anal. Calcd. for $C_{24}H_{24}F_6N_4O_2$: C, 56.03; H, 4.70; N, 10.89. Found: C, 55.88; H, 4.63, N, 10.70.

*2.1.4. 1-Primaquinecarbonyl-4-substituted bis-ureas (**6a-j**): general procedure*

A solution of 0.190 g (0.6 mmol) primaquine semicarbazide **5**, 0.061 g (0.6 mmol) TEA and 0.6 mmol corresponding 1-(*N*-arylcarbamoyl)benzotriazole (**2**) in 10 ml dichloromethane was stirred at room temperature for 1.5–24 h. The reaction mixture was evaporated under reduced pressure. The residue was further purified without extraction (**6a**, **6b**) or was dissolved in ethyl acetate and extracted with 5% NaOH solution (3 × 10 ml) and water (3 × 15 ml), dried over anhydrous sodium sulfate and evaporated (**6c**-**j**).

*2.1.4.1. 3-(3-Hydroxyphenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6a**).* Reaction time: 4 h. From the reaction of 0.153 g **2a** and after purification by column chromatography (mobile phase: dichloromethane/methanol 9:1) and crystallization from ether and petrolether 0.166 g (70%) of **6a** was obtained; mp 196.5–198.5 °C; IR (KBr): ν_{max} 3323, 3097, 2934, 1669, 1611, 1523, 1455, 1386, 1311, 1206, 1160, 1053, 1032, 973, 823, 776 cm^{-1}; ^1H NMR (DMSO-d_6) δ 9.24 (s, 1H), 8.54-8.53 (dd, 1H), 8.47 (s, 1H), 8.08-8.07 (dd, 1H), 7.73, 7.60 (2s, 2H), 7.43-7.41 (m, 1H), 7.06 (s, 1H), 6.99 (t, 1H), 6.80 (d, 1H), 6.47 (m, 2H), 6.35-6.34 (d, 1H), 6.27-6.26 (d, 1H), 6.11 (d, 1H), 3.83 (s, 3H), 3.65-3.61 (m, 1H), 3.08-3.02 (m, 2H), 1.64-1.48 (m, 4H), 1.21 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.99, 158.64, 157.57, 155.82, 144.63, 144.23, 140.71, 134.77, 134.51, 129.56, 129.16, 122.07, 109.16, 108.89, 105.47, 96.08, 91.61, 54.97, 47.04, 39.23, 33.41, 26.66, 20.20; MS/MS *m/z* 453.3 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{28}N_6O_4$: C, 61.05; H, 6.24; N, 18.57. Found: C, 60.70; H, 6.43, N, 18.41.

*2.1.4.2. 3-(4-Hydroxyphenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6b**).* Reaction time: 4 h. From the reaction of 0.153 g **2b** and after purification by column chromatography (mobile phase dichloromethane/methanol 9:1) and crystallization from ether 0.166 g (70%) of **6b** was obtained; mp 119–121.5 °C; IR (KBr): ν_{max} 3289, 3098, 2936, 1666, 1614, 1553, 1518, 1454, 1386, 1330, 1223, 1166, 1051, 827, 790 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.91 (bs, 1H), 8.56-8.54 (dd, 1H), 8.34 (s, 1H), 8.12-8.09 (dd, 1H), 7.69, 7.59 (2s, 2H), 7.46-7.42 (m, 1H), 7.23 (d, 2H), 6.64 (d, 2H), 6.49-6.48 (d+t, 2H), 6.28 (d, 1H), 6.18 (bs, 1H), 3.82 (s, 3H), 3.67-3.60 (m, 1H), 3.08-3.02 (q, 2H), 1.65-1.46 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 159.05,

158.72, 156.28, 152.40, 144.43, 144.10, 135.15, 134.14, 131.15, 129.66, 122.11, 120.55, 114.96, 96.39, 91.75, 55.02, 47.13, 39.23, 33.36, 26.67, 20.17; MS/MS *m/z* 453.3 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{28}N_6O_4$: C, 61.05; H, 6.24; N, 18.57. Found: C, 61.42; H, 6.21, N, 18.40.

*2.1.4.3. 3-(3-Fluorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6c**).* Reaction time: 4 h. From the reaction of 0.154 g **2c** and crystallization from ether and ethanol 0.243 g (89%) of **6c** was obtained; mp 207–208.5 °C; IR (KBr): ν_{max} 3307, 3220, 3106, 2930, 1668, 1615, 1568, 1551, 1521, 1495, 1454, 1388, 1325, 1325, 1280, 1201, 1157, 1052, 966, 865, 816, 785 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.86 (s, 1H), 8.53-8.52 (dd, 1H), 8.07-8.06 (dd, 1H), 7.93, 7.65 (2s, 2H), 7.50 (d, 1H), 7.42-7.40 (m, 1H), 7.26-7.23 (m, 2H), 6.73 (t, 1H), 6.50 (t, 1H), 6.46 (d, 1H), 6.26 (d, 1H), 6.10 (d, 1H), 3.82 (s, 3H), 3.64-3.61 (m, 1H), 3.07-3.03 (m, 2H), 1.65-1.46 (m, 4H), 1.19 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 163.02, 158.98, 158.52, 155.87, 144.62, 144.22, 144.19, 141.71, 141.64, 134.76, 134.50, 130.01, 129.94, 129.55, 122.06, 114.19, 114.16, 108.02, 107.88, 104.99, 96.07, 91.59, 54.96, 47.03, 39.23, 33.39, 26.63, 20.19; MS/MS *m/z* 455.3 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{27}FN_6O_3$: C, 60.78; H, 5.99; N, 18.49. Found: C, 60.51; H, 5.70, N, 18.41.

*2.1.4.4. 3-(4-Fluorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6d**).* Reaction time: 4 h. From the reaction of 0.154 g **2d** and after purification by column chromatography (mobile phase dichloromethane/methanol 92:8) and crystallization from ether and ethanol 0.145 g (53%) of **6d** was obtained; mp 168–170 °C; IR (KBr): ν_{max} 3296, 3228, 3095, 2934, 1686, 1652, 1616, 1575, 1501, 1456, 1415, 1387, 1330, 1212, 1159, 1051, 1031, 831, 791 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.67 (s, 1H), 8.54-8.53 (dd, 1H), 8.08-8.06 (dd, 1H, 13), 7.84, 7.62 (2s, 2H), 7.51-7.48 (m, 2H), 7.43-7.41 (m, 1H), 7.08-7.04 (m, 2H), 6.49-6.47 (d+t, 2H), 6.26 (d, 1H), 6.11 (d, 1H), 3.82 (s, 3H), 3.64-3.60 (m, 1H), 3.08-3.03 (m, 2H), 1.66-1.47 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.98, 155.58, 158.03, 156.46, 156.12, 144.62, 144.21, 136.05, 134.77, 134.50, 129.55, 122.07, 120.26, 120.21, 114.99, 114.85, 96.07, 91.59, 54.96, 47.04, 39.23, 33.29, 26.64, 20.18; MS/MS *m/z* 455.3 $(M+1)^+$; Anal. Calcd. $C_{23}H_{27}FN_6O_3$: C, 60.78; H, 5.99; N, 18.49. Found: C, 60.30; H, 6.45, N, 18.58.

*2.1.4.5. 3-(3-Chlorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6e**).* Reaction time: 2 h. From the reaction of 0.164 g **2e** and after purification by column chromatography (mobile phases

dichloromethane/methanol 97:3, 95:5, 9:1) and crystallization from ether 0.173 g (61%) of **6e** was obtained; mp 152–154.5 °C; IR (KBr): ν_{max} 3289, 3233, 3102, 2933, 1669, 1616, 1595, 1541, 1519, 1483, 1455, 1423, 1387, 1319, 1220, 1201, 1158, 1051, 882, 821, 790, 778 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.87 (s, 1H), 8.54-8.52 (dd, 1H), 8.09-8.06 (dd, 1H), 7.98, 7.74 (2s, 2H), 7.67 (s, 1H), 7.44-7.37 (m, 2H), 7.24 (t, 1H), 6.97 (d, 1H), 6.52 (t, 1H), 6.47 (d, 1H), 6.26 (d, 1H), 6.11 (d, 1H), 3.82 (s, 3H), 3.66-3.58 (m, 1H), 3.09-3.03 (m, 2H), 1.68-1.48 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 159.00, 158.54, 155.92, 144.64, 144.23, 141.38, 134.79, 134.52, 132.91, 130.11, 129.57, 122.09, 121.30, 117.90, 116.82, 96.09, 91.59, 54.98, 47.04, 39.23, 33.41, 26.67, 20.21; MS/MS *m/z* 471.3 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{27}ClN_6O_3$: C, 58.66; H, 5.78; N, 17.84. Found: C, 58.40; H, 5.61, N, 17.52.

*2.1.4.6. 3-(4-Chlorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6f**).* Reaction time: 4 h. From the reaction of 0.164 g **2f** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether 0.241 g (85%) of **6f** was obtained; mp 171–173 °C; IR (KBr): ν_{max} 3292, 3228, 3098, 2934, 1668, 1616, 1597, 1541, 1520, 1495, 1456, 1388, 1329 1288, 1237, 1220, 1202, 1158, 1094, 1051, 1031, 1014, 824, 790 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.78 (s, 1H), 8.53-8.52 (dd, 1H), 8.08-8.06 (dd, 1H), 7.89, 7.63 (s, 1H), 7.52 (d, 2H), 7.43-7.41 (m, 1H), 7.26 (d, 2H), 6.49 (t, 1H), 6.47 (d, 1H), 6.26 (d, 1H), 6.10 (d, 1H), 3.82 (s, 3H), 3.63-3.61 (m, 1H), 3.07-3.03 (m, 2H), 1.64-1.48 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.98, 158.54, 155.93, 144.62, 144.21, 138.76, 134.76, 134.50, 129.55, 128.30, 125.21, 122.06, 120.02, 96.07, 91.60, 54.96, 47.03, 39.21, 33.39, 26.61, 20.19; MS/MS *m/z* 471.2 $(M+1)^+$; Anal. Calcd. for $C_{23}H_{27}ClN_6O_3$: C, 58.66; H, 5.78; N, 17.84. Found: C, 58.73; H, 5.73, N, 17.66.

*2.1.4.7. 3-(4-Bromophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6g**).* Reaction time: 24 h. From the reaction of 0.190 g **2g** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether 0.211 g (77%) of **6g** was obtained; mp 172.5–174 °C; IR (KBr): ν_{max} 3289, 3236, 3101, 2932, 1672, 1595, 1526, 1457, 1389, 1325, 1211, 1161, 1053, 822, 789 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.78 (s, 1H), 8.53-8.52 (dd, 1H), 8.08-8.06 (dd, 1H), 7.89, 7.63 (s, 1H), 7.47 (d, 2H), 7.43-7.41 (m, 1H), 7.38 (d, 2H), 6.78 (t, 1H), 6.47 (d, 1H), 6.26 (d, 1H), 6.11 (d, 1H), 3.82 (s, 3H), 3.64-3.60 (m, 1H), 3.07-

3.03 (m, 2H), 1.66-1.47 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.97, 158.52, 155.88, 144.61, 144.20, 139.18, 134.75, 134.49, 131.19, 129.54, 122.05, 120.43, 113.11, 96.06, 91.59, 54.95, 47.03, 39.20, 33.39, 26.63, 20.18; MS/MS *m/z* 516.2 $(M+1)^+$, 517.2 $(M+2)^+$; Anal. Calcd. for $C_{23}H_{27}BrN_6O_3$: C, 53.60; H, 5.28; N, 16.31. Found: C, 53.41; H, 5.11, N, 15.99.

*2.1.4.8. 1-[({4-[(6-Methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]-3-[3-(trifluoromethyl)phenyl]urea (**6h**).* Reaction time: 1.5 h. From the reaction of 0.184 g **2h** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether and petrolether 0.197 g (65%) of **6h** was obtained; mp 120–122 °C; IR (KBr): ν_{max} 3286, 3102, 2936, 1672, 1618, 1574, 1548, 1521, 1451, 1424, 1388, 1335, 1262, 1221, 1203, 1167, 1125, 1072, 1052, 1031, 973, 930, 890, 821, 791 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 9.05 (s, 1H), 8.54-8.52 (dd, 1H), 8.09-8.02 (m, 3H), 7.74 (d, 1H), 7.67 (s, 1H), 7.48-7.40 (m, 2H), 7.26 (d, 1H), 6.52 (t, 1H), 6.47 (d, 1H), 6.26 (d, 1H), 6.11 (d, 1H), 3.82 (s, 3H), 3.67-3.58 (m, 1H), 3.10-3.03 (q, 2H), 1.66-1.48 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.98, 158.49, 156.03, 144.63, 144.19, 140.68, 134.75, 134.50, 129.67-128.61, 129.53, 129.53, 129.48-118.57, 122.05, 117.89, 117.84, 114.60, 96.13, 91.59, 54.94, 47.04, 39.23, 33.40, 26.62, 20.17; MS/MS *m/z* 505.3 $(M+1)^+$; Anal. Calcd. for $C_{24}H_{27}F_3N_6O_3$: C, 57.14; H, 5.39; N, 16.66. Found: C, 57.00; H, 5.49, N, 16.90.

*2.1.4.9. 1-[({4-[(6-Methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]-3-[4-(trifluoromethyl)phenyl]urea (**6i**).* Reaction time: 3.5 h. From the reaction of 0.184 g **2i** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether 0.215 g (71%) of **6i** was obtained; mp 158–160.5 °C; IR (KBr): ν_{max} 3284, 3102, 2936, 1672, 1618, 1604, 1542, 1521, 1458, 1418, 1388, 1326, 1245, 1221, 1203, 1164, 1116, 1070, 1018, 971, 840, 822, 791 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 9.08 (s, 1H), 8.54-8.52 (dd, 1H), 8.09-8.03 (m, 2H), 7.73-7.70 (m, 3H), 7.58 (d, 2H), 7.44-7.40 (m, 1H), 6.54 (t, 1H), 6.47 (d, 1H), 6.27 (d, 1H), 6.12 (d, 1H), 3.82 (s, 3H), 3.65-3.61 (m, 1H), 3.09-3.04 (q, 2H), 1.67-1.48 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.97, 158.53, 155.83, 144.64, 144.23, 143.57, 134.79, 134.52, 129.96-119.19, 129.58, 125.82, 125.77, 122.28-121.02, 122.09, 118.16, 96.09, 91.58, 54.97, 47.05, 39.23, 33.41, 26.67, 20.20; MS/MS *m/z* 505.3 $(M+1)^+$; Anal. Calcd. for $C_{24}H_{27}F_3N_6O_3$: C, 57.14; H, 5.39; N, 16.66. Found: C, 56.93; H, 5.01, N, 16.80.

*2.1.4.10. 3-[3,5-Bis(trifluoromethyl)phenyl]-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6j**).* Reaction time: 2 h. From the reaction of 0.225 g **2j** and after purification by column chromatography (mobile phase dichloromethane/methanol 95:5) and crystallization from ether and petrolether 0.165 g (48%) of **6j** was obtained; IR (KBr): ν_{max} 3316, 3108, 2962, 2935, 2870, 1684, 1652, 1616, 1576, 1520, 1475, 1456, 1424, 1388, 1337, 1279, 1222, 1171, 1128, 1051, 1030, 947, 884, 821, 791 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 9.53 (s, 1H), 8.53-8.52 (dd, 1H), 8.41, 7.74 (2s, 2H), 8.32 (s, 2H), 8.09-8.05 (dd, 1H), 7.55 (s, 1H), 7.44-7.40 (m, 1H), 6.58 (t, 1H), 6.47 (d, 1H), 6.26 (d, 1H), 6.11 (d, 1H), 3.82 (s, 3H), 3.66-3.58 (m, 1H), 3.10-3.03 (m, 2H), 1.66-1.48 (m, 4H), 1.20 (d, 3H); ^{13}C NMR (DMSO-d_6) δ 158.97, 158.38, 156.05, 144.62, 144.23, 142.09, 134.74, 134.49, 131.03-129.74, 129.54, 128.77-117.93, 122.03, 118.31, 114.08, 96.04, 91.58, 54.92, 47.05, 39.23, 33.40, 26.59, 20.16; MS/MS *m/z* 573.3 $(M+1)^+$; Anal. Calcd. for $C_{25}H_{26}F_6N_6O_3$: C, 52.45; H, 4.58; N, 14.68. Found: C, 52.68; H, 4.69, N, 14.81.

2.2. Biological evaluation

2.2.1. Materials and general methods

The cancer cells were seeded in 6-well or 96-well microtiter plates (Starlab GmbH, Germany). The cell cycle distribution was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA) in the total population (20 000 cells). FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, USA) was used for the assessment of cell death mode. Protein concentrations were measured using BCA Protein Assay kit (Santa Cruz Biotechnology, USA). Absorbance was measured using microplate reader GloMax-Multi (Promega, USA) at 560 nm. SDS-PAGE electrophoresis was performed on horizontal Amersham ECL Gel Box System (GE Healthcare Life Sciences, UK) and protein transfer on XCell Blot module (Invitrogen, USA). Hyperfilm ECL film (GE Healthcare Life Sciences, United Kingdom) and Kodak processing chemicals (Sigma-Aldrich, Germany) were used for film development. All chemical and biochemical reagents were of analytical grade and purchased from commercial sources. Eagle's Minimum Essential Medium (EMEM), penicillin, streptomycin, DMSO, cisplatin, propidium iodide (PI), CelLytic M extraction reagent, protease inhibitor cocktail, anti-GAPDH antibody, soybean lipoxygenase (LOX), linoleic acid sodium salt, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nordihydroguaiaretic acid (NDGA), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-

carboxylic acid (Trolox), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tetracycline hydrochloride, 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC) and 2*H*-tetrazolium, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-hydroxide (XTT) were purchased from Sigma-Aldrich (Germany), fetal bovine serum (FBS), L-glutamine and phosphate-buffered saline (PBS) from Gibco Laboratories (USA), sodium pyruvate from Biological Industries (Israel), human insulin, BCA Protein Assay kit and anti-PARP-1 antibody from Santa Cruz Biotechnology (USA), doxorubicin (DOX) from Farmitalia Research Laboratories (Italy), RNase A from Invitrogen (USA), FITC Annexin V Apoptosis Detection Kit I from Becton Dickinson (USA) and TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 from GE Healthcare Life Sciences (UK), anti-rabbit HRP-linked secondary antibody from Cell Signaling Technology (USA), Clarity Western ECL Substrate from Bio-Rad (USA) and voriconazole from Pfizer (USA). All microbiological media were purchased from Merck (Germany). Bacteria and one fungus from stock-cultures of Collection of microorganism of Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb, Croatia (MFBF), ATCC cell culture collection (LGC Standards Ltd, Germany) and National Collection of Type cultures (Public Health, England, UK) were used in antimicrobial susceptibility assay.

2.2.2. Antiproliferative activity assay

The antiproliferative activity experiments were carried out on murine leukemia (L1210) and 7 human cell lines derived from cancer types: acute lymphoblastic leukemia (Molt4/C8, CEM), cervical carcinoma (HeLa), pancreatic carcinoma (Mia PaCa-2), lung carcinoma (NCI-H460), colon carcinoma (SW 620) and breast carcinoma (MCF-7). Cytostatic activity against L1210, Molt4/C8 and CEM cell lines was measured essentially as originally described for the mouse leukemia cell lines [18]. The HeLa, Mia PaCa-2, NCI-H460, SW 620 and MCF-7 cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. L1210, Molt4/C8 and CEM were cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

The growth inhibition activity of NCI-H460, SW 620 and MCF-7 cell lines was assessed according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program [19]. The cell lines were seeded into a series of standard 96-well

microtiter plates on day 0, at 1×10^4 to 3×10^4 cells/ml, depending on the doubling times of the specific cell line. Test compounds were then added in five, 10-fold dilutions (10^{-8} to 10^{-4} M) and incubated for 72 hours. Working dilutions were freshly prepared on the day of testing. The solvent was also tested for eventual inhibitory activity by adjusting its concentration as in the working concentrations. After 72 hours of incubation the cell growth rate was evaluated by performing the MTT assay, as described previously. Each test point was performed in quadruplicate in three individual experiments. The results were expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound were calculated from dose-response curves using linear regression analysis by fitting the test concentrations that gave percentage of growth (PG values) above and below the reference value (i.e. 50%).

2.2.3. Cell culture conditions for MCF-7 and drug treatments

MCF-7 cells were grown in EMEM, containing 1% penicillin/streptomycin, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.01 mg/ml human insulin and incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were treated with 0.31 µM solution of compound **6d** (IC_{50}). DMSO (0.022%) was used for additional negative control. For positive controls cells were treated with 13 µM cisplatin (IC_{50}) and 1 µM doxorubicin (IC_{95}) for 24, 48 and 72 h, respectively.

2.2.4. Analysis of cell cycle distribution by propidium iodide (PI) staining

MCF-7 were seeded in 6-well plates and treated as indicated. Following the incubation period of 24, 48 or 72 h, respectively, cell medium was collected; cells were harvested, washed twice with ice-cold PBS and fixed with ice-cold 70% ethanol in PBS for 24 h at –20 °C. After centrifugation at 400 × *g* for 10 min, cells were washed with 500 µl PBS. RNA was digested with RNase A (20 µg/ml) for 30 min at 37 °C while DNA was stained with PI (5.5 µg/ml) for 10 min in dark at room temperature. Stained cells were analyzed by flow cytometry in the total population (20 000 cells). Data analyses were performed using FCS 3 Express (De Novo Software). Results are represented in a form of histogram as an average of three independent experiments.

2.2.5. Analysis of Annexin-V binding by flow cytometry

FITC Annexin V Apoptosis Detection Kit I was used for assessment percentage of viable, early apoptotic, late apoptotic/necrotic and necrotic cell population. Following drug treatment

cells were washed twice in ice-cold PBS and centrifuged for 5 minutes at 350 × *g*, 4 °C. Cells were resuspended in 1 x Annexin V Binding Buffer to a final concentration of 1×10^6 cells per 100 µl and then stained with 5 µl of FITC Annexin V and 5 µl of PI. Samples were gently vortexed and incubated for 15 min at room temperature in the dark. After incubation, 400 µl of Annexin V Binding Buffer was added to each tube and samples were analyzed by flow cytometry in the total population (10 000 cells). Data analyses were performed using FCS 3 Express (De Novo Software). Excitation was at 488 nm with emission wavelength of 530 nm for green (fluorescein) fluorescence and 580 nm for red (PI) fluorescence. Results are represented in a form of histogram as an average of three independent experiments.

2.2.6. Total protein extraction and quantification

Following drug treatments at indicated time points, cells were collected, washed twice in ice-cold PBS and centrifuged for 5 min at 400 × *g*, 4 °C. The pellet was resuspended in 50 µl of chilled CelLytic M extraction reagent with protease inhibitor cocktail in ratio 1:100. Samples were incubated on ice for 10 min and then centrifuged for 15 min at 15 000 × *g*, 4 °C. Supernatant was collected and stored at –80 °C for further analysis. Protein concentrations were measured using BCA Protein Assay kit following manufacturer's manual. Absorbance was measured using microplate reader at 560 nm.

2.2.7. Analysis of protein expression by western blotting

Following protein isolation and determination of protein concentration, 5 µg of proteins were separated by gel electrophoresis on 4-20% gels using horizontal Amersham ECL Gel Box System. Proteins were electrotransferred to a nitrocellulose membrane, pore size 0.45 µm (GE Healthcare Life Sciences) using XCell Blot module. Transfer was run overnight at constant voltage of 10 V on ice. Membrane was blocked in 5% low-fat milk diluted in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 1 h on shaker at room temperature. Membrane was washed in TBST buffer and incubated overnight at 4 °C with anti-PARP-1 or anti-GAPDH antibodies, diluted in 0.5% low-fat milk in TBST buffer in ratio 1:1000 and 1:5000, respectively. Membrane was washed again in TBST buffer and incubated for 1 h at room temperature with anti-rabbit HRP-linked secondary antibody diluted to 1:2000 in 0.5% low-fat milk in TBST buffer. After incubation, membrane was washed, over-layered with Clarity Western ECL Substrate (Bio-Rad, USA) and left in dark for 3 min. Membrane was covered with Hyperfilm ECL film and film was developed using Kodak processing chemicals for autoradiography films.

2.2.8. Statistical analysis

A statistical analysis of the data was performed using a one-way ANOVA analysis of variance followed by Newman-Keuls test and Duncan test using the STATISTICA 12 program (StatSoft, Inc.). Statistical significance was set at $p < 0.05$.

2.2.9. Interaction of the new derivatives with the stable radical DPPH

To a solution of DPPH in absolute ethanol the appropriate volume of the compound solution (0.1 mM final concentration) dissolved in DMSO was added. The absorbance was recorded at 517 nm after 20 and 60 min at room temperature. The experiments were repeated at least in triplicate and the standard deviation of absorbance was less than 10% of the mean [14].

2.2.10. LOX inhibition study in vitro

LOX inhibitory assay *in vitro* was accomplished as described previously [14]. The test compounds (stock solutions 10 mM in DMSO) were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of LOX enzyme solution ($1/9 \times 10^{-4}$ *m/V* in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was measured at 234 nm and compared with the reference inhibitor. Several concentrations were used for the IC_{50} determination. The assays were repeated at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

2.2.11. Inhibition of linoleic acid peroxidation

For initiating the free radical AAPH was used [14]. The final solution in the UV cuvette consisted of ten microliters of the 16 mM linoleate sodium dispersion 0.93 ml of 0.05 M phosphate buffer, pH 7.4, thermostated at 37 °C. AAPH solution (50 µl of 40 mM) was added as a free radical initiator at 37 °C under air and 10 µl of the tested compounds. The oxidation of linoleic acid sodium salt was monitored at 234 nm. The assays were repeated at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

2.2.12. The in vitro antimicrobial susceptibility assay

The *in vitro* antibacterial activity of compounds **3a-j** and **6a-j** were carried out against a panel of five Gram-positive (*Staphylococcus aureus, Enterococcus faecalis, Bacillus pumilus, Bacillus cereus, Bacillus subtilis*) and two Gram-negative bacteria (*Pseudomonas aeruginosa, Echerichia coli*). Three methicillin-susceptible *S. aureus* (MSSA) and two methicillin-

resistant *S. aureus* (MRSA) and two strains of *E. faecalis* were used: vancomycin-susceptible (VSE) and vancomycin-resistant (VRE). Antifungal susceptibility assay for *Candida albicans* was performed as well. Serial microdilution broth assay based on the EUCAST reference documents for aerobic bacteria was used to determine minimal inhibitory concentrations (MIC) [20]. Briefly, cell suspension of bacteria was prepared from stock cultures kept at –30 °C in 25% (*V/V*) glycerol-nutrient broth and maintained in trypticase soy broth for 18 h at 37 °C. Inoculum was prepared using PBS pH 7.4 and adjusted using nephelometer (ATB 1550, *BioMérieux*, France) to 0.5 MacFarland units and diluted 1:10 (*V/V*) with Mueller-Hinton broth. Stock solutions of tested compounds were prepared in DMSO at concentration 10 mg/ml before testing. According to EUCAST recommendations, microdilution was performed in Mueller-Hinton broth with serial dilution in microtiter flat-bottom 96-well plates from 1000 µg/ml to 0.78125 µg/ml. After inoculation and incubation during 18 h aerobically at 35 °C in dark, MICs were determined by addition of 0.5 mg/ml TTC (redox-indicator). After 3 h of incubation at 35 °C in dark, 0.04 M HCl in isopropanol was added to all wells and absorbance was recorded at 490 nm. For determination of MIC for fungal species tested microdilution was performed in RPMI 1640 broth with glutamine supplemented with 2% glucose and 10 mg ml^{-1} XTT was added in combination with menadione (1 mg ml^{-1} in acetone) (7:1, *V/V*) [21]. MIC values for yeast species were determined at 540 nm. Tetracycline hydrochloride and voriconazole dissolved in PBS pH 7.4 were used as quality control of susceptibility of strains and MIC determination. MICs were recorded as the lowest concentrations resulting in 50% growth inhibition (IC_{50}) in comparison to control (untreated cells). All tests were performed in duplicate and expressed as $MIC_{averrage}(EC_{50})$.

2.2.13. Hemolytic activity on human red blood cells (hRBCs) ex situ

Fresh venous human blood (AB+ type) donor (45 years old healthy male) was centrifuged at 2500 rpm for 5 min. The supernatant and buffy coats were removed, and cells were washed 3 times in PBS pH 7.4. After washing, hRBCs were resuspended in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS pH 7.4) at concentration 4% (*v/v*). Hemolytic activity of compounds **3a**, **3g**, **3i** and **3j** on hRBCs ex situ was determined in the concentration ranged from 100 to 0.156 µg/ml [22]. Intact 4% hRBCs were used as a negative control and 4% hRBCs plus 0.1% (*v/v*) Triton X-100 was used as a positive control. After incubation (1 h at 37 °C in dark) the solutions were centrifugated at 2500 rpm for 5 minutes. Absorbance of the supernatant was determined at 540 nm. Hemolysis was calculated according to the equation:

Hemolysis (%) = 100 [($A_p - A_s$)/($A_p - A_n$) × 100)], where A_p, A_s and A_n were the absorbances of the positive control, test sample and negative control, respectively. The results are given as the ratio between the hemolysis percentage of the test condition and control. The experiments were performed three independed times and presented as mean ± SD of a change in absorbance vs. concentration ($x–y_1$) and hemolysis percentage according the equation ($x–y_2$).

2.2.14. Cytotoxic effect of selected compounds on human embryonic kidney (HEK) cells

The cytotoxic effect of compounds **3a**, **3g**, **3i** and **3j** on HEK cells was determined using MTT assay. Briefly, 5×10^3 cells per well were cultured in 96-well plates and the following day incubated with specific compound for 24 h. Used doses ranged from 200 to 1.5625 μg/ml, depending of antimicrobial activity of each compound. The following day, 40 μl of 0.5 mg/ml MTT solution in DMEM containing 10% FBS was added to each well and incubated at 37 °C under 5% CO_2. After 4 h, formazan was dissolved by adding 170 μl of DMSO. The absorbance was measured at 570 nm using microplate reader. All analyzes were performed in triplicates and results are presented as the percentage of untreated control.

3. Results and discussion

3.1. Chemistry

Novel compounds **3a-j** and **6a-j** described in this paper are derived from primaquine. They differ in central functional group and/or in substituents in aromatic region. Compounds **3** are ureas and compounds of general formula **6** are *bis*-ureas. Products **3a,b** and **6a,b** possess hydroxy substituent (electron donating group), while compounds **3c-g** and **6c-g** bear halogen atoms (electron withdrawing group) either in *meta* or *para* position. Finally, compounds **3h-j** and **6h-j** have one or two trifluoromethyl groups. The strategy for the preparation of the title compounds is analogue to the previously published one [14] and is outlined in Scheme 1.

Scheme 1.

1-Benzotriazole carboxylic acid chloride BtcCl (**1**) [23,24] was the common starting material for synthesis of *N*-1-benzotriazole carboxylic acid amides **2a-j** and (*N*-(4-((6-methoxyquinolin-8-yl)amino)pentyl)-1*H*-benzo[*d*][1,2,3]triazole-1-carboxamide) (**4**) [25]

Compounds **2a-j** were prepared from **1** and corresponding hydroxy- or halogenaniline and product **4** from **1** and primaquine base. The following aromatic amines were used: 3-aminophenol, 4-aminophenol, 3-fluoroaniline, 4-fluoroaniline, 3-chloroaniline, 4-chloroaniline, 4-bromoaniline, 3-trifluoromethylaniline, 4-trifluoromethylaniline and 3,5-*bis*(trifluoromethyl)aniline. Compounds **2a-j** (active ureas) readily reacted with primaquine (nucleophile) affording urea derivatives **3a-j**. On the other hand, compound **4** and hydrazine hydrate gave semicarbazide **5**, which in reaction with **2a-j** gave the target *bis*-ureas **6a-j**.
All new compounds are fully characterized by the usual spectroscopic methods (IR, ^{1}H-, ^{13}C-NMR and MS) and elemental analyses. Spectral data are consistent with the proposed structures and are given in short in the Experimental section and in detail in Suporting information.
Majority of the urea and *bis*-urea primaquine derivatives are fully in agreement with the Lipinski's and Gelovani's rules for prospective small molecular drugs (MW ≤ 500, log *P* ≤ 5, number of H-bond donors ≤ 5, number of H-bond acceptors ≤ 10, polar surface area (PSA) < 140 Å^2, molar refractivity (MR) within the range of 40 and 130 cm^3/mol, the number of atoms 20-70) [26]. Few analogues showed minimal aberrations of the rules. The parameters are calculated with Chemicalize.org program and presented in Table 1.

Table 1 ref. [27]

3.2. Biological evaluation

3.2.1. Cytostatic activity

The results of antiproliferative screening *in vitro* of urea **3a-j** and *bis*-urea derivatives **6a-j** on murine lymphocytic leukemia (L1210) and seven human tumour cell lines (Molt4/C8, CEM, HeLa, Mia Paca-2, NCI-H460, SW620, MCF-7) are given in Table 2. Urea derivatives **3a-g** bearing hydroxy, fluoro, chloro or bromo substituent either in *meta* or *para* position showed moderate antiproliferative effects against all the tested cell lines, but stronger activity against MCF-7. Regioisomers with the same substituent either in *meta* or *para* position **3a/3b**, **3c/3d** and **3e/3f** showed comparable results. Trifluoromethyl derivatives **3h** and **3i** were more active than **3a-g** and showed antiproliferative effects against all the tested cell lines in low micromolar range. Additional trifluoromethyl group enhanced antiproliferative activity: 3-[3,5-*bis*(trifluoromethyl)phenyl]-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3j**) was the most active urea.

Table 2

In *bis*-urea series hydroxy **6a,b** and fluoro derivatives **6c,d**, especially *p*-fluoro derivative **6d**, showed extreme selectivity and chloro or bromo derivatives **6e-g** high selectivity against MCF-7 cells (IC_{50} for MCF-7 ranged from 0.1 to 2.6 μM; practically no activity or very weak activity towards the other cancer cell lines was observed). Comparison of regioisomer pairs in *bis*-urea series showed that *meta* isomers showed slightly higher activity than *para*. Trifluoromethyl derivatives **6h-j** exerted high activity towards all tested cell lines and again the highest activity towards MCF-7. Derivative **6h** with *meta* substituent was the most active compound.

*3.2.2. Induction of cell cycle arrest by compound **6d***

Since compound **6d** showed extreme selectivity against MCF-7 cells and practically no activity or very weak activity towards the other cancer cell lines we have investigated the mechanisms through which **6d** mediates pharmacological actions on MCF-7 human breast cancer cells. To examine whether **6d**-induced growth inhibition was associated with cell cycle regulation, the cell cycle distribution was analyzed by flow cytometry. Analyzed phases were: Gap1 (G_1), DNA syntheses (S) and Gap2/Mitosis (G_2/M). MCF-7 cells were incubated with **6d** (IC_{50} 0.31 μM) and DMSO for 24, 48 and 72 h, respectively. Cisplatin, one of the most commonly used anticancer drug in oncology, was used as a positive control (IC_{50} 13 μM) [28].

Figure 1

The effect of **6d** on G_1, S and G_2/M phase was shown in Fig. 1. The G_2/M population of cells treated with **6d** for 24 h increased in comparison to control, with a corresponding reduction of G_1 phase population (Fig. 2). It is important to stress that IC_{50} value of cisplatin [29] was 42-fold higher than IC_{50} value of **6d**, indicating the high potency of **6d**.

Figure 2

*3.2.3. Apoptosis-inducing effect of compound **6d***

To investigate the ability of compound **6d** to promote apoptosis and/or necrosis in MCF-7 cells, we preformed FITC Anexin V/PI staining after 24, 48 and 72 hours of treatment, respectively (Fig. 3).

Figure 3

As positive control we used doxorubicin (IC_{95} 1 μM) [30], currently considered to be one of the most effective agent in the treatment of breast cancer. Compound **6d** promotes the induction of cell death, as it was observed by increase in number of late apoptotic/necrotic cells as well as only necrotic cells when applied for 72 h (Fig. 4). Therefore, we wanted to further elucidate the specific mode of cell death.

Figure 4

Full-length poly (ADP-ribose) polymerase 1 (PARP-1) is an 116 kDa protein involved in the repair of DNA, differentiation and chromatin structure formation. During apoptosis this protein is cleaved by caspase-3 and caspase-7 into a stable 89 kDa fragment containing the active site and a 25 kDa fragment [31]. Therefore, we investigated possible **6d**-induced inhibition of PARP-1 by immunoblotting of PARP-1 (116 kDa) and its 89 kDa fragment. The results showed that **6d** did not trigger apoptosis in MCF-7 cells 24 and 48 h after treatment because expression levels of cleaved PARP-1 (C-PARP-1) were not detected (Fig. 5). These results were in line with results obtained by FITC Anexin V/PI staining at same time points (Fig. 3A and 3B). The expression levels of 89 kDa fragment increased at incubation period of 72 h, when compared to negative controls (Fig. 5). The results indicate that **6d** promotes apoptotic death of MCF-7 cells after incubation period of 72 h, in addition to cell cycle arrest.

Figure 5

Induction of apoptosis is precisely mediated by caspases cascade via both intrinsic and extrinsic pathway. Caspases play essential role in apoptosis which mainly included two main groups of initiators and executioners. Caspases-8 and -9 are known as initiator caspases through extrinsic and intrinsic pathway, respectively, and facilitate the activation of executioners, such as caspases-3/7 [32]. MCF-7 cells have functional deletion of *caspase-3* gene [33] providing a slightly higher threshold for the induction of apoptosis and probably shifting a part of apoptotic death mode into necrotic. Potential solution would be treatment with IC_{95} concentration as in case of doxorubicin. Since cancer cells, such as MCF-7, have a defect in apoptotic pathway, cell death needs to be triggered by alternative mechanisms [34].

3.2.4. Antioxidative activity

Carcinogenesis is a multistage process consisting of at least three separate but closely linked processes: initiation, promotion and progression. Promotion is closely linked to oxidative and inflammatory tissue damage. Conversely, substances with potent antiinflammatory and/or antioxidative activities are anticipated to exert chemopreventive effects on carcinogenesis, particularly in the stage of promotion and act as antitumor promoters. Taking under

consideration the above mentioned facts, antioxidative potential of new urea and *bis*-urea primaquine derivatives **3a-3j** and **6a-6j** was studied and compared to the well-known antioxidant agents such as NDGA and Trolox. Two different antioxidant assays were used: (*i*) interaction with the stable free DPPH radical and (*ii*) interaction with the water-soluble azo compound AAPH, used as a source of peroxyl radicals [14]. DPPH interaction of the tested compounds was examined at 100 μM concentration after 20 and 60 min. Among the urea derivatives compound **3b** with *p*-hydroxy substituent showed the best DPPH interaction value (95.5%), close to that of the reference compound NDGA (83%) at the same concentration, followed by **3j** (with two trifluoromethyl substituents), **3a** (*m*-hydroxy) and **3g** (*p*-bromo) (Table 3). The nature of the halogen atom in *para* position affected the antioxidant result. Thus, derivative **3g** (*p*-bromo) was found more potent than **3d** (*p*-fluoro) and **3f** (*p*-chloro). Derivatives **3c** and **3d** with fluoro atoms either in *meta* or *para* position and *m*-chloro derivative **3e** exerted activities only after 60 min. Compound **3f** with *p*-chloro substituent gave a slightly better result. The attachment of CF_3 group (**3h** and **3i**) gave the products with low activity, but introduction of the second trifluoromethyl group enhanced the antioxidant ability. In *bis*-urea series only compound **6d** with *p*-fluoro substituent showed a very weak interaction with DPPH, whereas all others presented high antioxidant activity (75.7-99.3%). For both groups of compounds no correlation between lipophilicity and activity was found.

Table 3

In our studies, AAPH was used as a free radical initiator to follow oxidative changes of linoleic acid to conjugated diene hydroperoxide. The results showed that all the analogues within the group **3** are significant anti-lipid peroxidation agents (54-90.2%). Phenolic derivative **3b** with the lowest log *P* (3.07) presented the lowest activity (54%), whereas the most lipophilic compound **3j** (log $P = 5.13$) with two CF_3 groups exhibited the highest activity (90.2%). Chloro and bromo derivatives **3e**, **3f**, **3g** with similar lipophilicity were almost equivalent in activity, as well as **3c** and **3h**. *Bis*-ureas generally presented lower inhibition of lipid peroxidation (LP) (52.2-75.3%) and two compounds (**6d** and **6g**) showed no effect under the reported conditions. A correlation between lipophilicity and LP inhibition was found only in urea series. In *bis*-urea series, the most active derivatives (**6i**, **6e** and **6b**) were not the most lipophilic ones. Our results also indicated that LP inhibition was not always accompanied by DPPH radical scavenging activity. It is worth to note, that the most active LP

inhibitor **3j** (derivative with two CF_3 groups) was the strongest antiproliferative agent as well, but such a strong correlation for other compounds was not found.

LOX inhibitors have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, certain types of cancer, and cardiovascular diseases [35]. In order to diminish the pro-cancerous mechanisms a key treatment strategy is to reduce the free radical load. Antioxidant activity by scavenging of reactive oxygen species is important in preventing potential damage of cellular compounds such as DNA, proteins and lipids. Moreover, it has been found that LOX inhibitors may have chemopreventive activity in lung carcinogenesis [36]. Reports published over the past three decades support a growth-regulatory role of arachidonic acid metabolites in the aetiology of mammary carcinogenesis [37]. Studies have demonstrated that levels of several eicosanoids are increased in breast cancer in comparison to benign breast tumours [38]. In this context, we evaluated the newly prepared primaquine derivatives for their ability to inhibit soybean LOX. From the tested compounds, ureas **3a**, **3b** and **3h** and *bis*-ureas **6c** and **6i** exhibited limited activity in LOX inhibition assay (48.3-67.4%), significantly lower than the reference NDGA (83%). The compounds with high log *P* values were not the most active, so the idea that lipophilicity was an important physicochemical property for LOX inhibition was not supported.

3.2.4. Antimicrobial activity

The *in vitro* antimicrobial susceptibility assay was carried out against a panel of 15 bacterial strains and the most common cause of fungal infections *C. albicans* and compared to parent compound primaquine and tetracycline hydrochloride (TC) or voriconazole (VOR) as standard antimicrobial drugs. Out of twenty newly synthesized compounds, three compounds from series **3**, e.g. **3a**, **3g** and **3i**, showed weak antibacterial activity against selected bacterial strains, with MIC values lower than primaquine (12.5-50 μg ml^{-1} vs. 70 μg ml^{-1}). However, one compound, *bis*(trifluoromethyl)phenyl]-1-{4-[(6-methoxyquinolin-8-yl)amino]pentyl}urea (**3j**), exhibited very strong antibacterial activity towards five bacterial strains, with MIC values ranged from 1.6 to 12.5 μg ml^{-1}. It was noticed that *S. aureus* ATCC 6538 was the most sensitive Gram-positive and *P. aeruginosa* ATCC 27853 the most sensitive Gram-negative bacteria. The results of antimicrobial screening are presented in Table 4 (only data for compounds that showed antibacterial/antifungal activity are given).

Table 4

In order to better estimate potential of compounds **3a**, **3g**, **3i** and **3j** as antimicrobial agents two tests were performed: haemolytic activity test on fresh hRBc *ex situ* and cytotoxicity test on human embryonic kidney (HEK) 293 cell line measured by MTT assay. All tested compounds exhibited low haemolytic activity after 1 h of aerobic incubation (Fig. 6). Haemolysis was a dose-depended process. The highest haemolysis was observed for compound **3i** (7.85% at 100 μg/ml concentration). Other compounds showed very low haemolysis potential, less than 1% at the highest concentration.

Figure 6

The cytotoxic effect of selected compounds from series **3** on HEK cells showed low toxicity (**3a** and **3i**) or no toxicity (**3g**) in the range of MIC values, except compound **3j**. Compound **3j** showed strong cytotoxicity in the range of MIC values, starting from concentration 3.125 μg ml^{-1}. Our data support the potential of primaqine-urea scaffold for development of lead compounds with antimicrobial activities.

Figure 7

4. Conclusions

Twenty novel compounds with the primaquine scaffold/urea or *bis*-urea motif/hydroxyl or halogen substituted phenyl ring have been prepared and tested for biological activity. Most of the compounds showed significant cytostatic activities in low micromolar concentrations towards all the tested cancer cell lines or high selectivity towards the human breast carcinoma MCF-7 cell line. Our results strongly support previous findings of MCF-7 susceptibility towards primaquine derivatives. *Bis*(trifluoromethyl)phenyl urea (**3j**) and (trifluoromethyl)phenyl *bis*-urea (**6h**) were the most active derivatives in antiproliferative screening *in vitro*. 3-(4-Fluorophenyl)-1-[({4-[(6-methoxyquinolin-8-yl)amino]pentyl}carbamoyl)amino]urea (**6d**) showed extreme selectivity: high activity against MCF-7 cell lines (IC_{50} 0.31 μM) and practically no activity against other tested cancer cell lines. Our studies demonstrated that **6d** induced growth inhibition in MCF-7 cell line through at least two different mechanisms: by induction of cell cycle arrest in G1 phase and inhibition of cell cycle progression through transient M phase accumulation and subsequent G2 arrest, and by inducing apoptosis. In conclusion, due to its high activity and selectivity compound **6d** may be considered as a potential scaffold for development of more effective and safer drugs for breast carcinoma treatment.

Almost all *bis*-ureas and only one urea (**3b**) showed high antioxidant activity in DPPH assay. On the other hand, ureas were superior in LP inhibition test. Only few compounds exhibited limited LOX inhibition activity. Information obtained from the antioxidative tests could help partly to understand anticancer properties of the tested primaquine derivatives. Antimicrobial activity in susceptibility assay *in vitro* showed that only four compounds from the series **3** have antibacterial/antifungal effect. Again, the strongest activity exhibited compound **3j**, with a MIC value ranged from 1.6 to 12.5 μg ml^{-1} against the selected bacterial strains. This compound showed low haemolytic activity on fresh hRBc ex situ but significant cytotoxicity on HEK cell line. However, compounds **3a** and **3g** showed selective antimicrobial activity without haemolytic and cytotoxic effects and have potential for development of antimicrobial agents. The results obtained from biological evaluation of here described novel compounds will contribute to QSAR study of a set of primaquine derivatives which is currently in progress.

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List of abbreviations

AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; BtcCl, 1-benzotriazole carboxylic acid chloride; BtH, 1*H*-benzo[*d*][1,2,3]triazole; CEM, acute lymphoblastic leukemia cells; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DOX, doxorubicin; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; EMEM, Eagle's Minimum Essential Medium; FBS, fetal bovine serum; HEK, human embryonic kidney 293 cells; HeLa, cervical carcinoma cells; hRBCs, human red blood cells; IC_{50}, the concentration that causes 50% growth inhibition; L1210, murine lymphocytic leukemia cells; LOX, lipoxygenase; LP, lipid peroxidation; MCF-7, breast carcinoma cells; Mia PaCa-2, pancreatic carcinoma cells; MDR, multi-drug resistant strain; MIC, minimal inhibitory concentration; Molt4/C8, acute

lymphoblastic leukemia cells; MR, molecular refractivity; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCI-H460, non small cell lung carcinoma cells; NDGA, nordihydroguaiaretic acid; PBS, phosphate buffered saline; PG, percentage of growth, PI, propidium iodide; PSA, polar surface area; SW620, metastatic colorectal adenocarcinoma cells; TBST buffer, 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20; TC, tetracycline hydrochloride TEA, triethylamine, TTC, 2,3,5-triphenyl-2*H*-tetrazolium chloride; XTT, 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-hydroxide; VOR, voriconazole; VRE, vancomycin-resistant *E. faecalis*; VSE, vancomycin-susceptible *E. faecalis*.

Appendix. Supporting information

Supplementary data related to this article can be found online at http://.....

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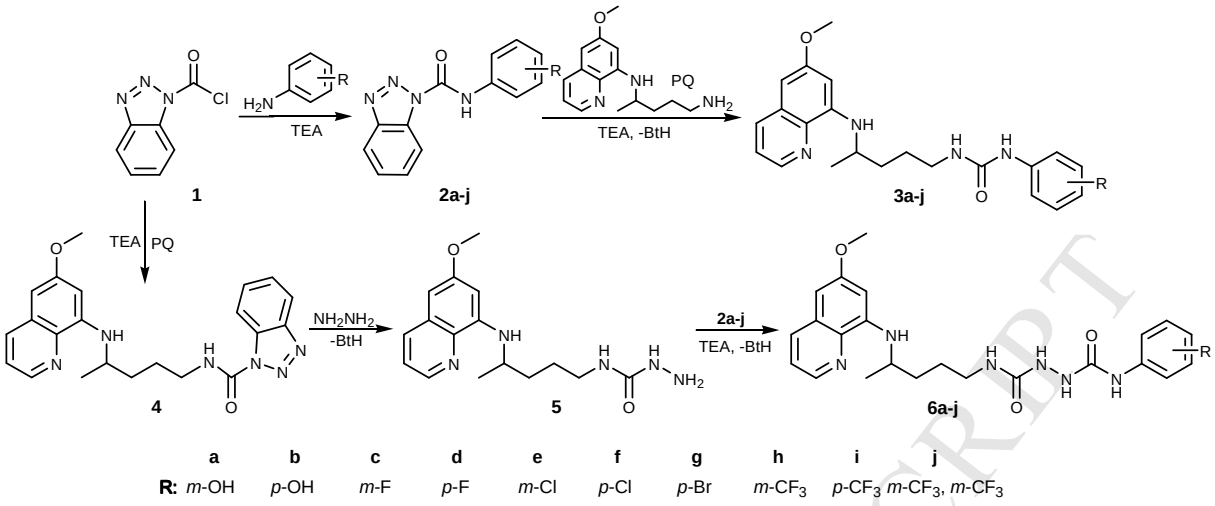


Scheme 1. Synthetic pathways of intermediates **2a-j**, **4**, **5** and target compounds **3a-j** and **6a-j**.

Figure legends

Fig. 1. Effect of **6d** on cell cycle distribution of MCF-7 cells. MCF-7 cells were treated with compound **6d** (IC_{50}) for 24 (A), 48 (B), and 72 (C) hours, respectively. DNA content and cell cycle were analyzed using PI staining and detected by flow cytometry. The percentage of cells in each phase (G_1, S and G_2/M) is presented in the histogram. Data are represented as an average of three independent experiments.

Fig. 2. Compound **6d** induces G_1 to G_2/M phase arrest 24 h after treatment of MCF-7. The percentage of cells in G_1 (A), S (B) and G_2/M phase (C) is presented in the histogram**.** Data are represented as an average of three independent experiments and error bars represent mean value ± SD. In the columns marked with asterisk * the mean values are significantly different compared to control, according to Newman-Keuls test ($p < 0.05$).

Fig. 3. Effect of compound **6d** on induction of cell death of MCF-7 cells. MCF-7 cells were treated with **6d** (IC_{50}) for 24 (A), 48 (B), and 72 (C) h, respectively. Percentages of viable, apoptotic, apoptotic /necrotic and necrotic cells were illustrated as histogram. Data are represented as an average of three independent experiments.

Fig. 4. **6d** promotes apoptosis and necrosis of MCF-7 cells. MCF-7 cells were treated with compound **6d** (IC_{50}) and DMSO for 72 h. Cell death type was analyzed using FITC Annexin V Apoptosis Detection Kit I and detected by flow cytometry. Data are represented as an average of three independent experiments and error bars represent mean value ± SD. In the columns marked with asterisk * the mean values are significantly different compared to control, according to Duncan test ($p < 0.05$).

Fig. 5. Western blot analysis of PARP-1 cleavage after **6d** treatment. The bands corresponding to PARP-1 and cleaved PARP-1(C-PARP-1) are indicated. MCF-7 cells were treated with **6d** (IC_{50}), DMSO and DOX for 24, 48 and 72 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Results are representative of three independent experiments.

Fig. 6. Hemolysis potential of compounds **3a**, **3g**, **3i** and **3j** evaluated on hRBSc ex situ.

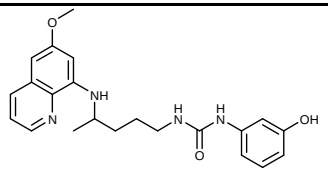
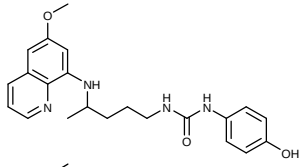
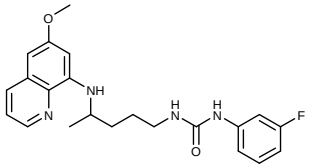
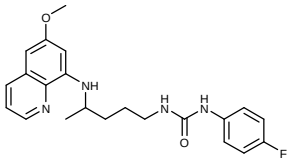
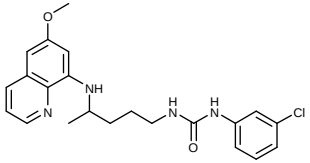
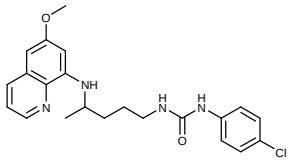
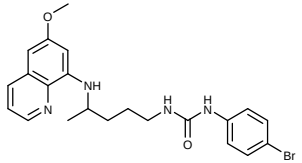
Fig. 7. The cytotoxic effect of compound **3a**, **3g**, **3i** and **3j** on human embryonic kidney (HEK) 293 cells, measured by MTT assay. The cells (5×10^3 cells/well) were cultured in 96-well plates and incubated for 24 h with specific compound (concentration range from 200 to 1.5625 µg/ml). The results are presented as percent of untreated control (100 % viability). Data are represented as an average of three independent experiments and error bars represent mean value + SD. In the columns marked with asterisk * the mean values are significantly different compared to DMSO control, according to Duncan test ($p < 0.05$).

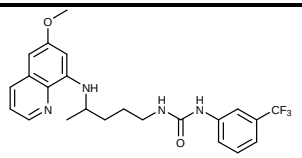
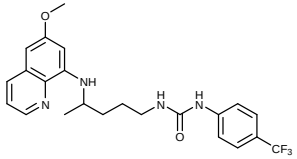
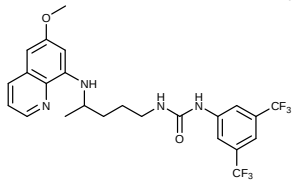
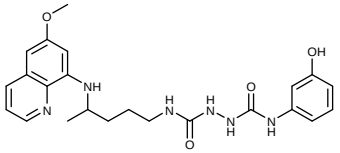
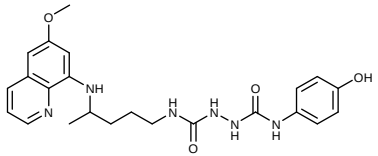
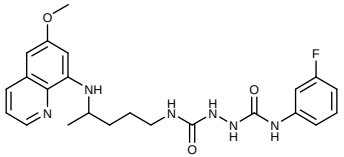
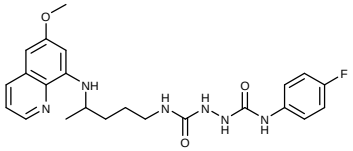
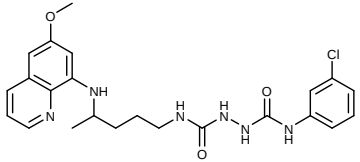
Table 1
Properties of ure and *bis*-urea derivatives **3a-j** and **6a-j**: the Lipinski's and Gelovani's parameters[a].

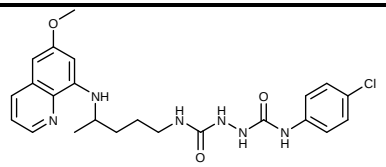
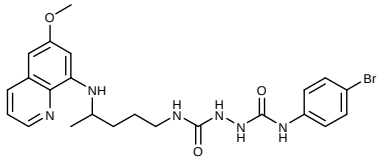
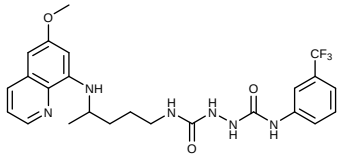
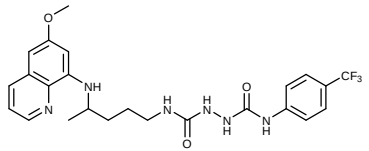
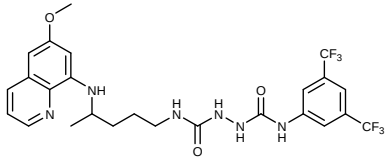
Compd.	Molecular formula	Number of atoms	MW	log *P*	H-bond donor	H-bond acceptor	Lipinski score[b]	MR cm^3/mol	PSA ($Å^2$)
3a	$C_{22}H_{26}N_4O_3$	55	394.47	3.07	4	5	4	115.071	95.51
3b	$C_{22}H_{26}N_4O_3$	55	394.47	3.07	3	4	4	115.071	95.51
3c	$C_{22}H_{25}ClN_4O_2$	54	396.46	3.51	3	4	4	113.307	75.28
3d	$C_{20}H_{25}FN_4O_2$	54	396.46	3.51	3	4	4	113.307	75.28
3e	$C_{22}H_{25}ClN_4O_2$	54	412.91	3.97	3	4	4	117.895	75.28
3f	$C_{22}H_{25}ClN_4O_2$	54	412.91	3.97	3	4	4	117.895	75.28
3g	$C_{22}H_{25}BrN_4O_2$	54	457.36	4.14	3	4	4	120.713	75.28
3h	$C_{23}H_{25}F_3N_4O_2$	57	446.47	4.25	3	4	4	119.064	75.28
3i	$C_{23}H_{25}F_3N_4O_2$	57	446.47	4.25	3	4	4	119.064	75.28
3j	$C_{24}H_{24}F_6N_4O_2$	60	514.46	5.13	3	4	2[c]	125.038	75.28
6a	$C_{23}H_{28}N_6O_4$	61	452.51	2.14	6	6	4	126.614	136.64
6b	$C_{23}H_{28}N_6O_4$	61	452.51	2.14	6	6	4	126.614	136.64
6c	$C_{23}H_{27}FN_6O_3$	60	454.50	2.59	5	5	4	124.849	116.41
6d	$C_{23}H_{27}FN_6O_3$	60	454.50	2.59	5	5	4	124.849	116.41
6e	$C_{23}H_{27}ClN_6O_3$	60	470.95	3.05	5	5	4	129.438	116.41
6f	$C_{23}H_{27}ClN_6O_3$	60	470.95	3.05	5	5	4	129.438	116.41
6g	$C_{23}H_{27}BrN_6O_3$	60	515.40	3.22	5	5	3[c]	132.256	116.41
6h	$C_{24}H_{27}F_3N_6O_3$	63	504.50	3.32	5	5	4	130.607	116.61
6i	$C_{24}H_{27}F_3N_6O_3$	63	504.50	3.32	5	5	4	130.607	116.61
6j	$C_{25}H_{26}F_6N_6O_3$	66	572.5	4.20	5	5	3	136.580	116.41

[a] calculated with Chemicalize.org program [27]; [b] out of four; [c] minimal aberrations of the rules; MR – molecular refractivity; PSA – polar surface area

Table 2
Growth inhibition of tumor cell lines *in vitro*: IC_{50} (μM)[a].

Compd.	Structural formula	Cell line							
		L1210	Molt4/C8	CEM	HeLa	Mia PaCa-2	NCI-H460	SW 620	MCF-7
3a		25 ± 2	24 ± 0	32 ± 10	48 ± 12	46 ± 16	25 ± 11	27 ± 10	11 ± 4
3b		21 ± 2	21 ± 3	23 ± 1	70 ± 4	57 ± 16	34 ± 2	30 ± 4	5.2 ± 0.6
3c		19 ± 2	23 ± 2	21 ± 2	18 ± 1	22 ± 3	24 ± 1	27 ± 10	18 ± 5
3d		18 ± 4	24 ± 0	26 ± 4	20 ± 2	21 ± 2	26 ± 2	25 ± 10	26 ± 10
3e		17 ± 3	21 ± 1	22 ± 0	17 ± 1	21 ± 2	37 ± 1	26 ± 8	11 ± 4
3f		19 ± 2	24 ± 1	24 ± 3	18 ± 7	22 ± 0	34 ± 2	28 ± 8	12 ± 2
3g		18 ± 2	25 ± 0	23 ± 5	20 ± 5	23 ± 2	30 ± 9	23 ± 7	2.6 ± 2.5

3h		9.2 ± 2.9	20 ± 3	19 ± 1	14 ± 1	17 ± 1	21 ± 2	22 ± 1 0	3.3 ± 0.6
3i		7.4 ± 3.3	20 ± 2	14 ± 7	8.3 ± 1.1	8.4 ± 3.3	24 ± 4	22 ± 4	1.9 ± 1.1
3j		3.5 ± 0.3	12 ± 7	4.1 ± 0.4	3.1 ± 0.0	4.7 ± 0.6	8.3 ± 2.5	3.9 ± 0.1	1.6 ± 0.6
6a		160 ± 71	85 ± 6	143 ± 8	180 ± 100	129 ± 4	> 100	17 ± 0.0	1.1 ± 0.2
6b		95 ± 2	104 ± 2	111 ± 7	85 ± 18	142 ± 1	> 100	> 100	2.6 ± 0.7
6c		108 ± 35	≥ 250	98 ± 18	> 250	69 ± 3	50 ± 4	9.5 ± 0.9	0.15 ± 0.02
6d		104 ± 27	≥ 250	118 ± 1	≥ 250	≥ 250	> 100	> 100	0.31 ± 0.07
6e		27 ± 1	93 ± 13	81 ± 14	44 ± 14	74 ± 7	32 ± 21	25 ± 9	0.22 ± 0.11

6f		58 ± 7	186 ± 13	108 ± 13	120 ± 19	≥ 250	63 ± 2	48 ± 17	0.18 ± 0.11
6g		51 ± 16	209 ± 10	110 ± 8	101 ± 15	231 ± 27	27 ± 3	17 ± 5	0.10 ± 0.01
6h		4.0 ± 0.5	36 ± 15	6.9 ± 1.0	4.9 ± 0.2	5.6 ± 0.0	20 ± 0.7	21 ± 4	0.19 ±0.07
6i		18 ± 3	24 ± 1	18 ± 1	15 ± 4	19 ± 0	12 ± 0.2	12 ± 4	0.10 ± 0.01
6j		18 ± 1	22 ± 3	13 ± 2	17 ± 2	20 ± 1	18 ± 3	16 ± 5	2.4 ± 0.6

[a] IC_{50} – the concentration that causes 50% growth inhibition

Table 3
DPPH-reducing ability (RA), *in vitro* inhibition of soybean lipoxygenase (LOX) and lipid peroxidation (LP) of compounds **3a-j** and **6a-j**.

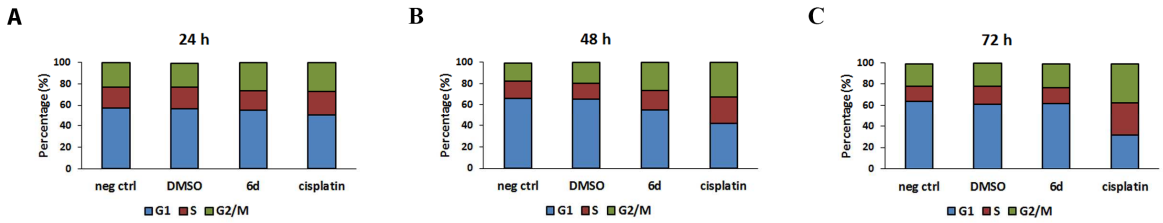
Compd.	RA (%)		LOX inhibition[a] (%)(IC_{50} μM)	LP inhibition[a] (%)
	20 min	60 min		
3a	48.6	52.3	50.8	66.9
3b	95.5	94.5	49.9	54.0
3c	na	26.0	44.6	74.3
3d	na	24.7	39.8	64.4
3e	na	35.7	11.8	84.1
3f	24.0	37.0	21.6	81.9
3g	46.2	46.4	37.1	80.4
3h	31.3	47.7	48.3	71.8
3i	38.9	36.4	22.4	67.0
3j	51.7	55.2	42.3	90.2
6a	96.5	100	41.6	52.2
6b	99.3	100	33.4	61.9
6c	75.7	100	67.4 (40 μM)	58.2
6d	7.6	na	31.9	na
6e	97.9	95.1	48.4	70.8
6f	97.6	100	39.0	42.1
6g	96.9	99.0	28.0	na
6h	98.3	91.2	51.4	52.6
6i	99.0	96.1	55.8 (85 μM)	75.3
6j	86.8	88.0	33.9	39.9
NDGA	83.0	97.0	83.0 (4.5 μM)	
Trolox				76.0

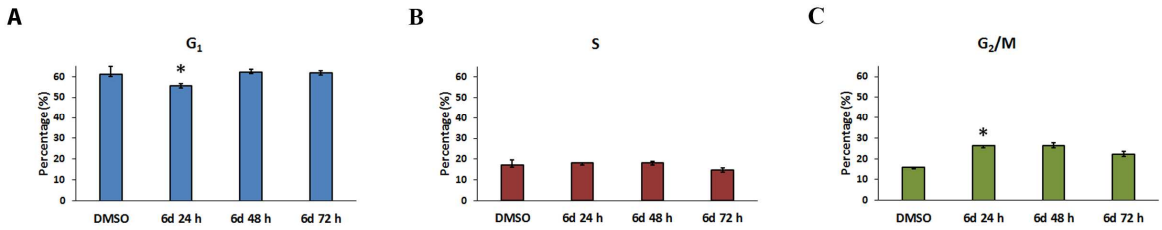
[a] Concentration of the tested compound: 1×10^{-4} M
na – no activity

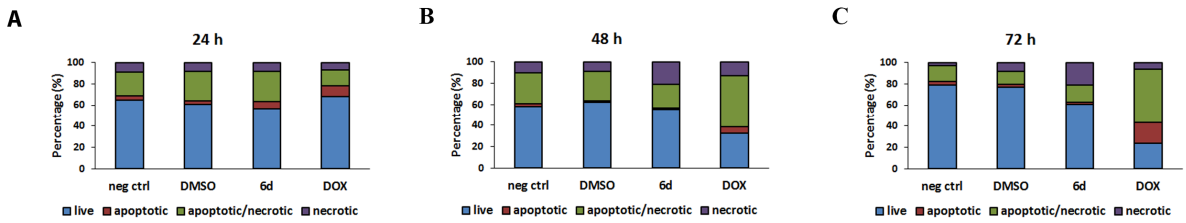
Table 4
Antimicrobial activity of selected compounds from series **3** by microdilution assay.

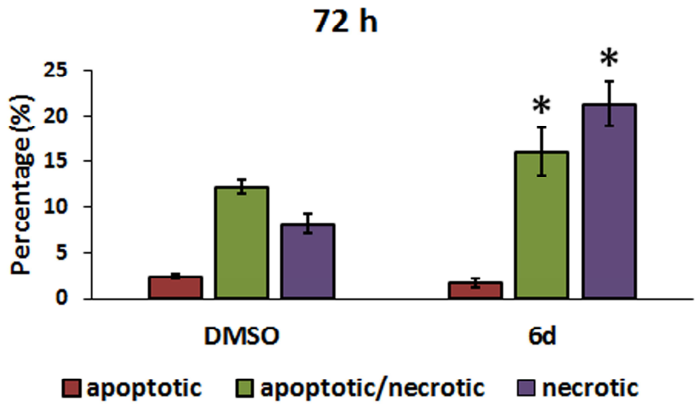
Compd.	MIC (μg ml^{-1})														
	S. aureus ATCC 6538	*S. aureus* MSSA ATCC 25923	*S. aureus* MSSA ATCC 25213	*S. aureus* MRSA MFBF 10682	*S. aureus* MRSA MFBF 10679	*E. faecalis* VSE ATCC 29212	*E. faecalis* VRE MFBF 11419	*B. pumil.* NCTC 8241	*B. cereus* ATCC 11778	*B. subtilis* ATCC 6633	*P. aerug.* ATCC 9027	*P. aerug.* ATCC 27853	*P. aerug.* MFBF (MDR)	*E. coli* ATCC 10535	*C. albicans* ATCC 90028
3a	20	50	50	50	100	>100	>100	12.5	50	50	100	50	100	100	>100
3g	50	>100	>100	>100	>100	>100	>100	100	50	25	100	25	100	>100	>100
3i	25	35	50	>100	>100	>100	>100	>100	>100	>100	100	25	150	>100	>100
3j	1.6	6.3	12.5	>100	>100	>100	>100	12.5	100	>100	100	12.5	>100	>100	>100
PQ	70	70	70	60	70	>100	>100	70	70	80	70	65	70	65	350
TC	0.156	0.312	0.156	0.312	0.312	0.312	>128	0.156	0.312	0.312	3	3	>128	2.5	nd
VOR	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.5

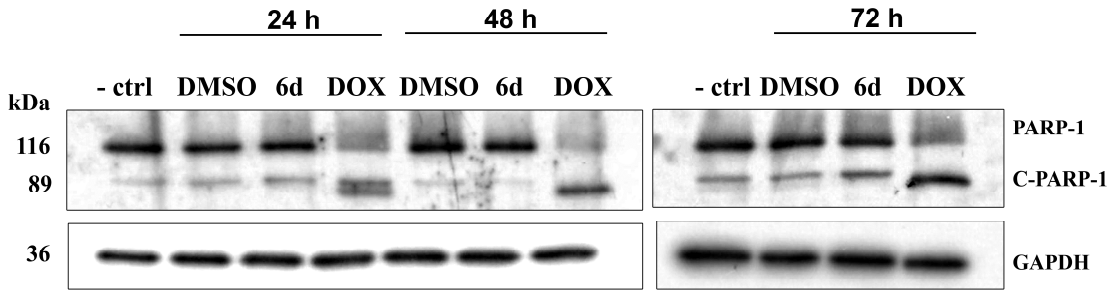
MIC – minimum inhibitory concentration (EC_{50}), average value (n = 2); MSSA – methicillin-susceptible *S. aureus*, MRSA – methicillin-resistant *S. aureus*; VSE – vancomycin-susceptible *E. faecalis*; VRE – vancomycin-resistant *E. faecalis*; ATCC – code of the strain from ATCC cell culture collection (LGC Standards Ltd, Germany); MFBF – code of the strain from Collection of microorganisms (Faculty of Pharmacy and Biochemistry, Croatia); NCTC – code of the strain from National Collection of Type Cultures (Public Health, England, UK); MDR – multi-drug resistant strain; nd – not determined; PQ – primaquine; TC – tetracycline×HCl; VOR – voriconazole.

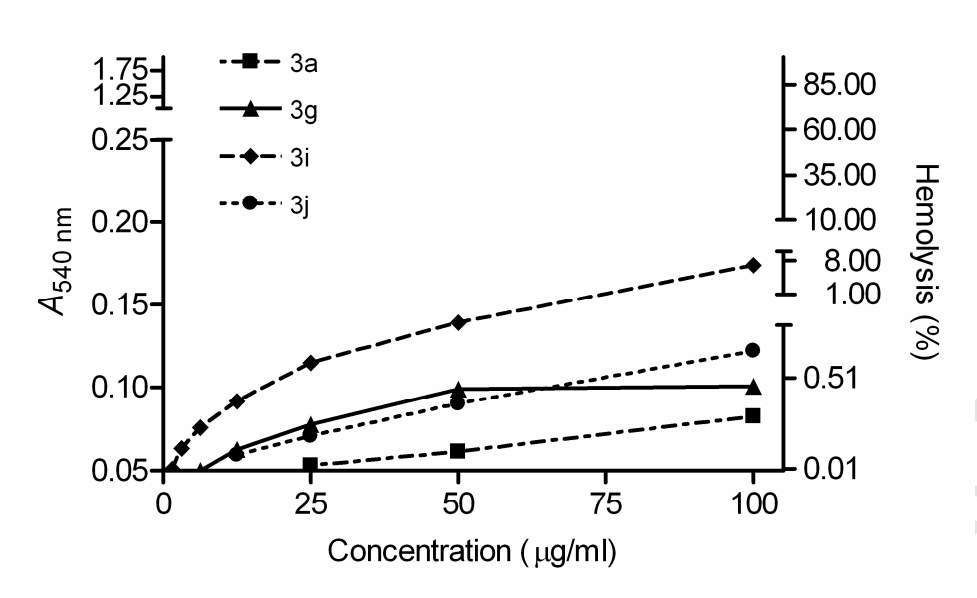


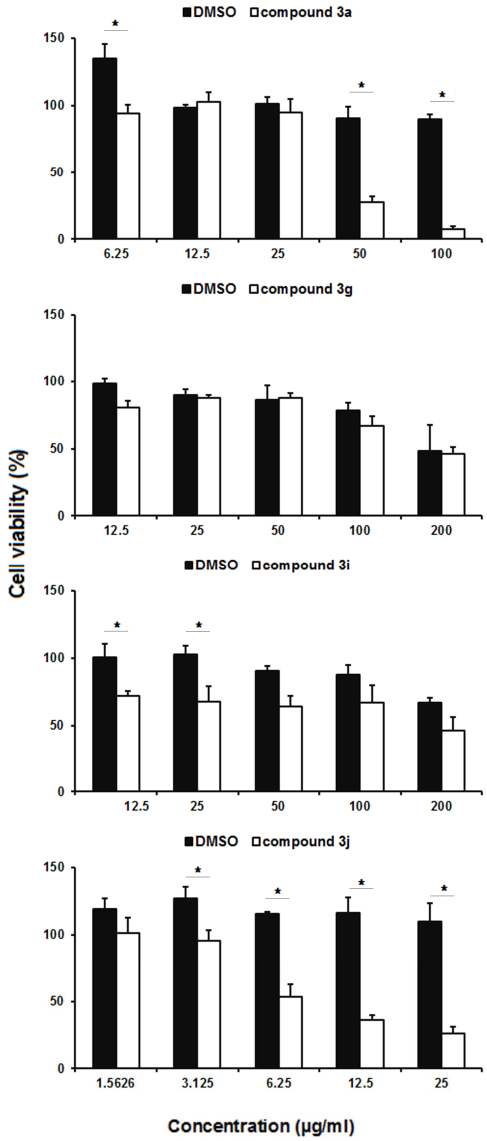












Research Highlights

- Novel hydroxyl- and halogenphenyl primaquine ureas and *bis*-ureas were synthesized.
- Most of the compounds exerted potent cytostatic activity.
- Several hits displayed extremely high selectivity against MCF-7.
- Most derivatives showed high antioxidant activity in DPPH and LP assays.
- Three compounds with PQ-urea scaffold exhibited weak antimicrobial activity.