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Design, synthesis and in vitro apoptotic mechanism of novel pyrrolopyrimidine derivatives

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ABSTRACT

In this work we described the synthesis and evaluation of cytotoxic and apoptotic activity of novel pyrrolopyrimidine derivatives against A549, PC3 and MCF-7 cells. Among the synthesized compounds, **6b**, **8a**, **9a** and **7a**, **8b** displayed the significant cytotoxic activities against A549 and PC3 cells with IC_{50} value of 0.35, 1.48, 1.56 and 1.04, 1.89 μM, respectively. It was found that A549 cells were more sensitive to synthesized compounds than PC3 and MCF-7 cells. In order to evaluate the mechanism of cytotoxic activity in A549, compounds **6b**, **8a** and **9a** were selected for further studies. Annexin V binding assay and western blot analysis results revealed that **6b**, **8a** and **9a** induced apoptosis in A549 cells by intrinsic apoptotic pathway through the activation pro-apoptotic proteins such as Bim, Bax, Bak, Puma and deactivation of anti-apoptotic proteins including Bcl-2, Mcl-1 and Bcl-XL accompanied by the activation of caspase-3, caspase-9 and cleavage of PARP. Also, compounds **6b**, **8a** and **9a** triggered apoptosis in HCT116 wt cells via activation of caspase-3 and caspase-9, but not in HCT116 Bax/Bak KO cells, indicating resistance to **6b**, **8a** and **9a** treatment.

1. Introduction

Apoptosis, programmed cell death, plays essential role in the cellular development and differentiation (homeostasis) [1]. Dysregulation of apoptosis lead to a variety of human diseases, such as cancer, autoimmune disease and neurodegenerative disorders [2]. Two types apoptotic pathways have been extensively described, including the extrinsic and intrinsic pathway. These two process are highly regulated by Bcl-2 family of proteins which are structurally and functionally classified as either anti-apoptotic (e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1) and pro-apoptotic (e.g. Bak, Bax, Bok) or BH-3 only pro-apoptotic Bcl-2 proteins (Bad, Bim, Bmf, Bik, Hrk, Bid, Puma, Noxa) [3–6]. Overexpression of anti-apoptotic proteins or the downregulation of pro-apoptotic proteins is associated with resistance to cell death in cancer cells. Developing small molecules that activate and induce apoptosis is promising strategy for the treatment of cancer [7].

A number of promising new compounds with pyrrolo[2,3-*d*]pyrimidine scaffold have been reported to possess anti-inflammatory [8], antimicrobials [9,10], and antiviral [11–13] and anticancer [14–24] activities. Over the past years pyrrolo[2,3-*d*]pyrimidine-based compounds have become one of the most extensively studied classes of heterocycles in cancer drug discovery and development compounds. Well-known anticancer agent pyrrolopyrimidine derivative **Pemetrexed** (Fig. 1) is clinically used as thymidylate synthase (TS) inhibitors in malignant pleural mesothelioma and first-line treatment in combination with cisplatin for the treatment of non-small cell lung cancer (NSCLC) [14]. **Toyocamycin** [15] and **Sangivamycin** [16] (Fig. 1), naturally occurring nucleoside antibiotics, have been reported as inhibitors of protein kinase C (PKC) and/or protein kinase A (PKA). **Tofacitinib** [17] and **Ruxolitinib** [18] (Fig. 1) bearing pyrrolopyrimidine scaffold are approved as small-molecule Janus-associated kinase (JAK) inhibitors by FDA for the treatment of rheumatoid arthritis (RA) and myelofibrosis, respectively. **EC144** was found as a second generation inhibitor of heatshock protein 90 (Hsp90) by Bimonte group with improved in vitro and in vivo potency (IC_{50} = 1.1 nM) [19]. **MLN4924** is a small-molecule inhibitor of NEDD8-activating enzyme (NAE) with IC_{50} value of 4 nM and suppressed the growth of human tumor xenografts in mice at well-tolerated concentrations [20–23]. Furthermore, it was shown to inhibit tumor angiogenesis in *in vivo* models and to overcome resistance of cancer cells to cell death. Compound **I**, 6-substituted pyrrolo[2,3-*d*]pyrimidine with a thienoyl side chain, were reported as an inhibitor of KB and IGROV1 cell proliferation and of catalytic activity of the target enzyme GARFTase both in vitro and *in situ* [24]. Compound **II**, 4,6-bis-anilino-1*H*-pyrrolo[2,3-*d*]pyrimidine derivative was discovered as a nonselective inhibitor of the insulin-like growth factor 1 receptor (IGF-1R) [25]. Moreover, there are many pyrrolopyrimidine-based

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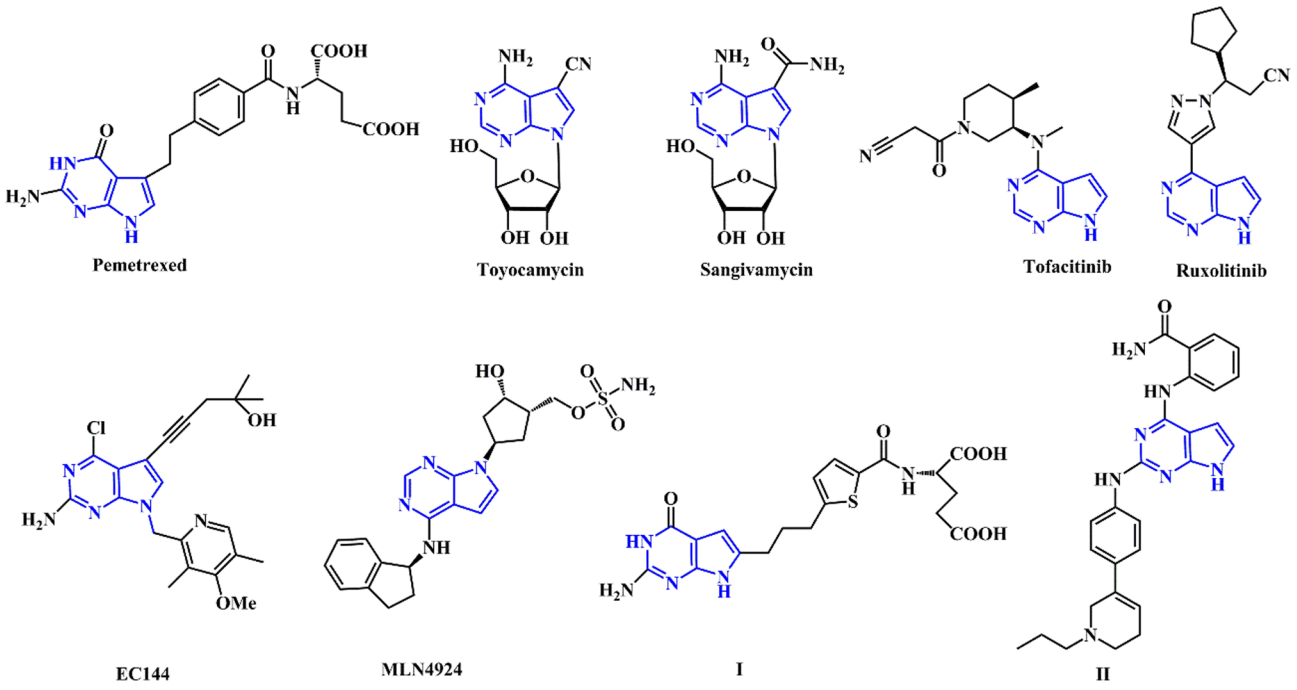


Fig. 1. Pyrrolopyrimidine derivatives having anticancer activities.

compounds showing anticancer activity through different mechanism have been reported in the literatures [26–29].

Urea derivatives have received great attention as potent anticancer agents. It has been known that some urea-based compounds are used in treatment of various cancers through a variety of mechanisms. Nitrosoureas, DNA alkylating agents, and **Nilutamide**, androgen receptor agonist, are well-known compounds among the anticancer agents [30]. Multikinase inhibitors bearing urea moiety such as **Sorafenib** (Fig. 2, VEGFR, PDGFR and Raf inhibitor) [31] and **Regorafenib** (Fig. 2, VEGFR1-3, Tie-2, PDGFR-β, FGFR, Kit, Ret, and Raf inhibitor) [32] have been clinically approved for renal cell and metastatic colorectal cancer treatment, respectively. **Linifanib (**Fig. 2, VEGFR and PDGFR inhibitor) [33] is currently in active clinical trials for non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer and acute myeloid leukemia treatment. In addition, several urea based multikinase ihibitors (Fig. 2), including **Quizartinib** [34], **Tandutinib** [35], **BIRB-796** [36], **CP-547632** [37], compounds **III-V** [38–40] were reported in the literature.

Recently, several pyrrolopyrimidine derivatives which were substituted with urea motif at 4-position of pyrrolopyrimidine ring were reported as anticancer agents [29,41]. Understanding that urea moiety and pyrrolopyrimidine scaffold are found in compounds which are approved and in clinical trials for cancer treatment, and pyrrolopyrimidine derivatives containing urea motif at 2-position have not been explored as anticancer compounds in published reports encourage us to design novel pyrrolo[2,3-*d*]pyrimidine derivatives with urea moiety at 2-position and to evaluate their anticancer activity against breast (MCF-7), lung (A549), prostate (PC3) and colon (HCT116) cancer cell lines.

2. Materials and methods

2.1. Chemistry

2.1.1. Synthesis of 2-amino-3,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one (3)

The mixture of 2,4-diamino-6-hydroxypyrimidine (**1**, 5.14 g;

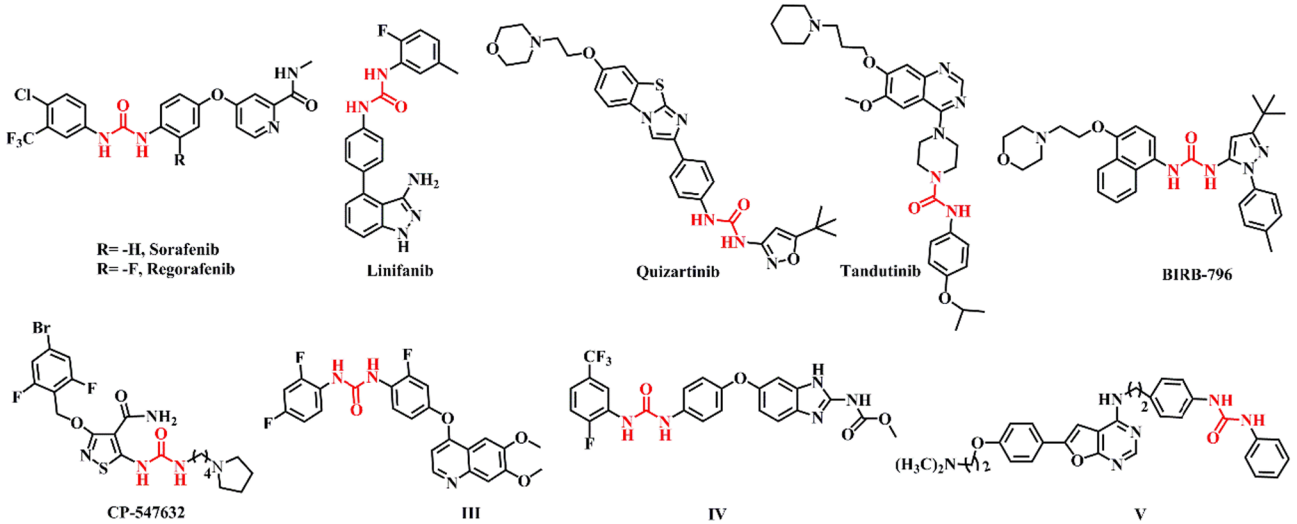


Fig. 2. Urea derivatives with anticancer activity.

0.04 mol) and sodium acetate (3.34 g; 0.04 mol) was dissolved in the mixture of DMF:H_2O (5:1) at room temperature. Then, chloroacetaldehyde (5.22 mL) was added and the mixture was stirred for 48 h at room temperature. After completion of the reaction, solvent was evaporated under vacuum and the brown solid was dissolved in water (25 mL). The resulting precipitate was filtered. The filtrate was evaporated until one third of the solvent volume was remained and the ethanol was added. The resulting precipitate was collected and washed with little cold water and acetone to afford the compound **3** (5.28 g, 74%) as beige solid [42].

*2.1.2. Synthesis of 4-chloro-7H-pyrrolo[2,3-d]pyrimidin-2-amine (**4**)*

Compound **3** (2.95 g; 0.02 mol) was suspended in $POCl_3$ (25 mL) and the mixture was refluxed for 2 h. After cooling to room temperature, excess of $POCl_3$ was evaporated under vacuum and the residue was treated with ice water. The resulting precipitate was filtered off and the pH of the filtrate was adjusted to 7 with aqueous ammonia. Then, the filtrate was extracted with EtOAc (3 × 50 mL). The organic phase was dried over sodium sulfate and evaporated to afford compound **4** (0.33 g, 10%), which was used for the next reaction without further purification [42].

*2.1.3. Synthesis of 4-chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-amine (**5**)*

4-Chloro-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine (**4**, 0.248 g; 1.47 mmol) was dissolved in anhydrous DMF (3 mL) and cooled to 0 °C, NaH (35 mg; 1.47 mmol) was added into the mixture and stirred for 1 h. Then, MeI (0.1 mL; 1.62 mmol) was added dropwise to the reaction mixture and stirring was continued overnight at room temperature. The reaction was quenched with water and the solution was extracted with EtOAc (3 × 20 mL). The organic phase was dried with anhydrous Na_2SO_4 and evaporated under vacuum. Crude product was purified by column chromatography with 9:1 hexane/ethylacetate to afford pure compound **5** (0.159 g; 38%), [43].

*2.1.4. General procedure for the synthesis of compounds **6-10***

4-Chloro-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine (**5**, 1 eq) was dissolved in anhydrous dioxane under nitrogen atmosphere. Et_3N (2.5 eq) and triphosgene (1 eq) in anhydrous dioxane were added to the mixture dropwise at 0 °C and stirred for 5 h at the room temperature. Then, corresponding anilines were added and the stirring was continued overnight. At the end of the time, the solvent was evaporated and acetone was added. Resulting precipitate was filtered and washed with cold water to afford desired compounds **6-10** in moderate yield.

*2.1.4.1. 1-(4-Chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(3-(trifluoromethyl)phenyl) urea (**6**).* Yield 47%; mp: 216 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.84 (s, 3H, CH_3), 6.54 (d, 1H, J = 3.2 Hz, H-5), 7.39 (d, 1H, J = 8 Hz), 7.51 (d, 1H, J = 4 Hz, H-6), 7.57 (t, 1H), 7.70 (d, 1H, J = 8.8 Hz), 8.15 (s, 1H), 10.28 (s, 1H, NH), 11.38 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.37, 98.66, 112.07, 114.99, 119.15, 122.68, 125.39, 129.61, 130.01, 130.75, 139.40, 150.62, 150.93, 151.18, 151.81. MS (ESI, 70 eV) *m/z*: 370.30 (M + H), 372.28 (M + H + 2).

*2.1.4.2. 1-(4-Chloro-3-(trifluoromethyl)phenyl)-3-(4-chloro-7-methyl-7H-pyrrolo[2,3-d] pyrimidin-2-yl)urea (**7**).* Yield 41%; mp: 244 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.84 (s, 3H, CH_3), 6.52 (d, 1H, J = 3.2 Hz, H-5), 7.52 (d, 1H, J = 3.6 Hz, H-6), 7.65 (d, 1H, J = 8.4 Hz), 7.72 (dd, 1H, J_o = 8.4 Hz, J_m = 2.4 Hz), 8.22 (d, 1H, J_m = 2.4 Hz), 10.38 (s, 1H, NH), 11.43 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.38, 98.64, 112.09, 117.53, 121.28, 123.40, 123.80, 126.74, 130.80, 132.04, 138.08, 150.46, 150.92, 151.12, 151.63. MS (ESI, 70 eV) *m/z*: 404.12 (M^+), 406.62 (M + 2).

*2.1.4.3. 1-(4-Chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(4-fluoro-3-(trifluoromethyl) phenyl)urea (**8**).* Yield 75%; mp: 248–250 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.81 (s, 3H, CH_3), 6.51 (d, 1H, J = 3.6 Hz, H-5), 7.43–7.49 (m, 2H), 7.72–7.74 (m, 1H), 8.12 (d, 1H, J = 4 Hz), 10.29 (s, 1H, NH), 11.31 (s, 1H, NH). ^{13}C NMR spectra could not be measured since compound **8** is not enough soluble in DMSO-d_6. MS (ESI, 70 eV) *m/z*: 388.20 (M + H), 389.77 (M + 2).

*2.1.4.4. 1-(4-Chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(4-fluorophenyl)urea (**9**).* Yield 51%; mp: 260 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.75 (s, 3H, CH_3), 6.75 (d, 1H, J = 3.6 Hz, H-5), 7.13 (t, 2H), 7.65 (d, 1H, J = 4.0 Hz, H-6), 7.80–7.96 (m, 2H), 10.27 (s, 1H, NH), 11.99 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.40, 99.35, 110.75, 114.27, 115.49, 120.94, 121.6, 126.41, 130.05, 150.95, 151.60, 152.86. MS (ESI, 70 eV) *m/z*: 320.15 (M + H), 322.26 (M + H + 2).

*2.1.4.5. 1-(4-Chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(2,4-difluorophenyl)urea (**10**).* Yield 68%; mp: 268 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.77 (s, 3H, CH_3), 6.51 (d, 1H, J = 3.6 Hz, H-5), 7.03–7.08 (m, 1H), 7.32–7.38 (m, 1H), 7.48 (d, 1H, J = 4.0 Hz, H-6), 8.25–8.32 (m, 1H), 10.39 (s, 1H, NH), 11.46 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.12, 98.77, 103.57, 103.80, 111.13, 112.08, 121.37, 123.55, 130.71, 150.44, 150.56, 150.96, 151.05, 151.89. MS (ESI, 70 eV) *m/z*: 338.40 (M + H).

*2.2. General procedure for the synthesis of compounds **6-8a, 9a, 10a***

Compounds **6-10** (1 eq) were dissolved in isopropanol and corresponding anilines (2 eq) and 2–3 drops of conc. HCl was added and refluxed for 10–24 h. The reaction mixture was cooled to room temperature and resulting precipitate was filtered and washed with isopropanol. The crude product was purified by recrystallization from ethanol to give pure compounds **6-8a**, **9a**, **10a**.

*2.2.1. 1-(4-((3-Bromophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d] pyrimidin-2-yl)-3-(3-(trifluoromethyl)phenyl)urea (**6a**)*

Yield 36%; mp: 262–264 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.75 (s, 3H, CH_3), 6.77 (d, 1H, J = 2.8 Hz, H-5), 7.16 (d, 1H, J = 3.6 Hz, H-6), 7.23–7.30 (m, 2H), 7.35 (d, 1H, J = 7.2 Hz), 7.44–7.52 (m, 2H), 7.99–8.0 (m, 3H), 10.08 (s, 2H, NH), 11.77 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.05, 99.37, 99.53, 114.86, 119.0, 120.33, 121.67, 122.57, 123.39, 125.80, 126.05, 129.39, 129.71, 129.89, 130.75, 139.44, 140.57, 149.17, 152.32. MS (ESI, 70 eV) *m/z*: 507.07 (M + 2). Anal. calcd for $C_{21}H_{16}BrF_3N_6O{\cdot}1.2$ HCl: C 46.05, H 3.16, N 15.35, found: C 45.99, H 3.08, N 15.53.

*2.2.2. 1-(4-((3-Chloro-4-fluorophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(3-(trifluoromethyl)phenyl)urea (**6b**)*

Yield 63%; mp: 258 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.78 (s, 3H, CH_3), 6.77 (d, 1H, J = 3.2 Hz, H-5), 7.18 (d, 1H, J = 3.2 Hz, H-6), 7.34 (t, 1H), 7.38 (d, 1H, J = 7.2 Hz), 7.49–7.55 (m, 2H), 7.91–7.95 (m, 1H), 8.01 (s, 1H), 8.18 (dd, 1H, J_o = 6.4 Hz, J_m = 2.8 Hz), 9.92 (s, 1H, NH), 10.10 (s, 1H, NH), 11.90 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.08, 99.54, 99.95, 115.33, 117.07, 117.29, 119.49, 119.76, 119.95, 121.87, 122.80, 123.05, 126.04, 129.90, 130.21, 130.40, 137.10, 140.04, 149.67, 152.75, 153.35. MS (ESI, 70 eV) *m/z*: 479.30 (M + H), 481.27 (M + H + 2). Anal. calcd for $C_{21}H_{16}ClF_4N_6O{\cdot}1.5$ H_2O: C 49.86, H 3.58, N 16.61, found: C 49.94, H 3.23, N 16.76.

*2.2.3. 1-(4-((3-Bromophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d] pyrimidin-2-yl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (**7a**)*

Yield 86%; mp: 265 °C.; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.75 (s,

3H, CH_3), 6.71 (d, 1H, J = 3.6 Hz, H-5), 7.14 (d, 1H, J = 3.6 Hz, H-6), 7.18 (d, 1H, J = 8.0 Hz), 7.24 (t, 1H), 7.54 (d, 1H, J_o = 8.8 Hz), 7.59 (d, 1H, J = 8.8 Hz), 7.99 (s, 1H), 8.02 (d, 1H, J = 8.4 Hz), 9.63 (s, 1H, NH), 9.97 (s, 1H, NH), 12.02 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.91, 99.15, 99.86, 117.50, 119.64, 121.51, 122.67, 123.03, 123.77, 125.12, 125.29, 126.53, 130.50, 131.86, 138.40, 141.41, 149.57, 152.01, 152.13, 153.26. MS (ESI, 70 eV) m/z: 540.73 (M + H), 542.15 (M + H + 2). Anal. calcd for $C_{21}H_{15}BrClF_3N_6O{\cdot}0.1H_2O$: C 46.57, H 2.82, N 15.51, found: C 46.22, H 2.94, N 15.51.

2.2.4. 1-(4-Chloro-3-(trifluoromethyl)phenyl)-3-(4-((3-chloro-4-fluorophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)urea (**7b**)

Yield 79%; mp: 276–278 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.74 (s, 3H, CH_3), 6.68 (d, 1H, J = 3.6 Hz, H-5), 7.13 (d, 1H, J = 3.6 Hz, H-6), 7.29 (t, 1H), 7.57–7.63 (m, 2H), 7.86–7.90 (m, 1H), 7.96 (d, 1H, J = 2 Hz), 8.13 (d, 1H, J_o = 6.8 Hz, J_m = 2.8 Hz), 9.62 (s, 1H, NH), 10.02 (s, 1H, NH), 12.0 (s, 1H, NH). MS (ESI, 70 eV) m/z: 513.16 (M^+), 515.69 (M + 2). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.93, 98.60, 99.56, 116.55, 117.49, 119.24, 120.87, 121.80, 123.08, 123.75, 125.38, 126.71, 131.88, 136.88, 138.32, 149.33, 151.64, 152.04, 152.17, 153.20, 154.04. Anal. calcd for $C_{21}H_{14}Cl_2F_4N_6O{\cdot}2H_2O2HCl$: C 40.53, H 3.23, N 17.50, found: C 40.85, H 3.32, N 17.78.

2.2.5. 1-(4-((3-Bromophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(4-fluoro-3-(trifluoromethyl)phenyl)urea (**8a**)

Yield 58%; mp: 262–264 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.73 (s, 3H, CH_3), 6.77 (d, 1H, J = 2.8 Hz, H-5), 7.16 (d, 1H, J = 3.6 Hz, H-6), 7.22–7.29 (m, 2H), 7.41 (t, 1H), 7.52–7.56 (m, 1H), 7.90 (dd, 1H, J_o = 6.4 Hz, J_m = 2.8 Hz), 7.95–7.99 (m, 2H), 10.11 (s, 2H, NH), 11.76 (s, 1H, NH). MS (ESI, 70 eV) m/z: 523.21(M^+), 525.05 (M + 2). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.11, 99.41, 99.55, 116.77, 117.53, 117.74, 120.37, 121.76, 123.43, 125.03, 125.11, 125.86, 126.10, 130.82, 135.46, 140.61, 149.24, 151.15, 152.43, 152.90, 155.38. Anal. calcd for $C_{21}H_{15}BrF_4N_6O{\cdot}1.2HCl$: C 44.58, H 2.88, N 14.86, found: C 44.48, H 2.97, N 14.97.

2.2.6. 1-(4-((3-Chloro-4-fluorophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(4-fluoro-3-(trifluoromethyl)phenyl)urea (**8b**)

Yield 53%; mp: 279 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.74 (s, 3H, CH_3), 6.75 (d, 1H, J = 3.2 Hz, H-5), 7.15 (d, 1H, J = 3.6 Hz, H-6), 7.31 (t, 1H), 7.42 (t, 1H), 7.59–7.63 (m, 1H), 7.86–7.89 (m, 2H), 8.14 (dd, 1H, J_o = 6.4 Hz, J_m = 2.8 Hz), 9.98 (s, 1H, NH), 10.10 (s, 1H, NH), 11.81 (s, 1H, NH). MS (ESI, 70 eV) m/z: 497.18 (M + H), 499.27 (M + H + 2). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.08, 99.19, 99.44, 116.63, 116.85, 117.51, 117.72, 119.32, 119.49, 121.49, 122.46, 125.03, 125.11, 125.65, 135.52, 136.48, 149.19, 152.37, 152.67, 152.88. Anal. calcd for $C_{21}H_{14}ClF_5N_6O{\cdot}1.2HCl$: C 46.73, H 2.84, N 15.58, found: C 46.71, H 2.95, N 15.73.

2.2.7. 1-(4-((4-Chlorophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(4-fluorophenyl)urea (**9a**)

Yield 85%; mp: 307 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.75 (s, 3H, CH_3), 6.72 (d, 1H, J = 3.6 Hz, H-5), 7.09–7.13 (m, 3H), 7.31 (d, 2H, J = 8.8 Hz), 7.39–7.43 (m, 2H), 7.97 (d, 2H, J = 8.8 Hz), 9.62 (s, 1H, NH), 9.69 (s, 1H, NH), 11.75 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 31.10, 99.07, 99.71, 115.22, 115.44, 120.63, 120.71, 123.58, 125.73, 128.76, 134.73, 137.42, 148.69, 152.39, 156.60, 158.97. MS (ESI, 70 eV) m/z: 411.39 (M + H), 413.24 (M + H + 2). Anal. calcd for $C_{20}H_{16}ClFN_6O{\cdot}0.3H_2O$: C 57.71, H 4.01, N 20.19, found: C 57.87, H 4.12, N 19.95.

2.2.8. 1-(4-((3-Bromophenyl)amino)-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-2-yl)-3-(2,4-difluorophenyl) urea (**10a**)

Yield 40%; mp: 308–309 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.76 (s, 3H, CH_3), 6.78 (d, 1H, J = 3.6 Hz, H-5), 7.09 (t, 1H), 7.15 (d, 1H, J = 3.2 Hz, H-6), 7.18 (d, 1H, J = 8.0 Hz), 7.25 (t, 1H), 7.34–7.40 (m, 1H), 8.07 (t, 1H), 8.27 (d, 1H, J = 8.4 Hz), 8.33–8.38 (m, 1H), 9.54 (s, 1H, NH), 10.11 (s, 1H, NH), 11.96 (s, 1H, NH). MS (ESI, 70 eV) m/z:473.24 (M^+), 474.79 (M + H), 475.18 (M + 2). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 30.05, 99.24, 100.35, 111.50, 119.67, 121.86, 122.39, 125.29, 125.76, 131, 139.11, 142.08, 149.34, 152.47, 153.03, 154.09, 154.60, 155.88, 156.19, 159.28. Anal. calcd for $C_{20}H_{15}BrF_2N_6O{\cdot}0.03H_2O$: C 50.69, H 3.20, N 17.73, found: C 50.59, H 3.24, N 17.68.

2.3. Biological activity

2.3.1. Cytotoxic activity determined by MTT assay

In vitro cytotoxic effects of the synthesized compounds were evaluated using MTT assay. Briefly, a 180 µl of cultured cell suspension (5×10^4 cell/ml) was seeded in 96 well plates and incubated for 24 h. Following incubation, various amounts of the synthesized compounds (0.1, 0.5, 2.5, 5, 10, 25, 50 and 100 µM) were added to the cells and incubated for 24 h in a CO_2 incubator. The cells were than treated with 5 mg/ml MTT reagent for 2 h and the viable cell amount was determined by metabolic conversion of the soluble MTT dye. The reduced crystals were dissolved in 180 µl of DMSO and the absorbance at 540 nm was measured in a spectrophotometer. The effect of compounds on cell viability was calculated as a percentage of control cell growth obtained from untreated cells. Imatinib was used as positive control. Each experiment wasperformed in quadruplicate to assess the consistency of results. IC_{50} values were calculated by using linear regression equations obtained from the concentration vs viable cell amount % graphs.

2.3.2. Annexin V binding assay

The effects of synthesized compounds on apoptosis were analyzed using the Muse Annexin V/Dead Cell (Merck Millipore) assay, according to the manufacturer's instructions. Briefly, the cells were seeded and incubated for 24 h for attachment. The synthesized compounds were then applied with a final concentration of IC_{50} values of each compound, which were determined from the MTT measurements for 24 h. The cells were then harvested and incubated with Annexin V and 7-aminoactinomycin (7-AAD) dye, for 20 min, at room temperature, in the dark. Four populations were differentiated by using Annexin V and/or 7-AAD positivity on Muse Cell Analyzer (Merck, Millipore): non-apoptotic live (7-AAD negative, apoptosis negative), non-apoptotic dead (7-AAD positive, apoptosis negative), apoptotic live (7-AAD negative, apoptosis positive), and apoptotic dead (7-AAD positive, apoptosis positive) cells.

2.3.3. Western blot analysis

Total cell lysates were separated on 10% SDS-PAGE gels. Following SDS-PAGE, proteins were transferred onto PVDF membranes, blocked with 5% dried milk in PBS-Tween20 and incubated with corresponding primary and secondary antibodies. Immunoblots were developed by using Luminata Crescendo Western HRP substrate (Millipore, MA, USA) and imaged with C-DiGit Blot Scanner (LI-COR Biosciences, Bad Homburg, Germany). The following antibodies were used for immunoblotting: BCL-2 (#2872, Cell Signaling), BCL-XL (#2762, Cell Signaling), MCL-1 (#5453, Cell Signaling), BIM (#2819, Cell Signaling), NOXA (FL-54, Santa Cruz Biotechnology), PUMA (#4967, Cell Signaling), BAX (#2774, Cell Signaling), BAK (#3814, Cell Signaling), Cleaved Caspase-9 (Asp315) (#9505, Cell Signaling), Cleaved Caspase-3 (Asp175) (#9661, Cell Signaling), Cleaved PARP (Asp214) (#9541, Cell Signaling) and Actin (#8457, Cell Signaling).

2.3.4. Cell culture

HCT-116 wild-type and HCT-116 BAX/BAK knockout cells grown in McCoy's 5A medium (ThermoFisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Thermo Scientific) in a humidified incubator at 37 °C and 5% CO_2.

2.3.5. Caspase activation assays

The activity of caspase-3 and caspase-9 was determined by Apo Alert Caspase Assay Plates (Clontech, Takara) as described by the manusfacturer. The release of fluorochrome AMC was analyzed at 380 nm excitation and 460 nm emission using Spectramax Gemini microplate fluorometer. Data shown are mean ± SEM of three independent experiments and expressed in arbitrary fluorescence units.

2.3.6. Statistical analysis

The statistical significance for the assays were determined using GraphPad Prism Software 6.0e version (Software MacKiev, Inc.). Data obtained from the cell culture experiments were expressed as mean ± SD and one-way ANOVA test was applied for multiple comparisons. A p-value of less than 0.05 was considered as statistically significant.

3. Result and discussion

3.1. Chemistry

The synthesis of the target pyrrolo[2,3-*d*]pyrimidine derivatives (see Scheme 1) begins with reaction of commercially available 2,4-diamino-6-hydroxypyrimidine with chloroacetaldehyde in the mixture of DMF: H_2O to obtain compound **3** [42]. Then, carbonyl group of compound **3** was chlorinated by $POCl_3$ in poor yield (10%) [42]. Alkylation of pyrrole-nitrogen of compound **4** was afforded with MeI in the presence of NaH [43]. Converting the exocyclic amino at 2-position of pyrrolopyrimidine ring to the corresponding urea (**6-10**) using triphosgene and appropriate anilines was firstly performed by our group. Finally, target compounds (**6-8a**, **9a**, **10a**) were obtained via nucleophilic displacement of 4-chloro moiety of urea intermediates (**6-10**) with corresponding anilines in the presence of 2–3 drops of conc. HCl at reflux [44]. The structures of synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, Mass-ESI and elemental analysis data.

3.2. Evaluation of biological activity

3.2.1. In vitro cytotoxic activity

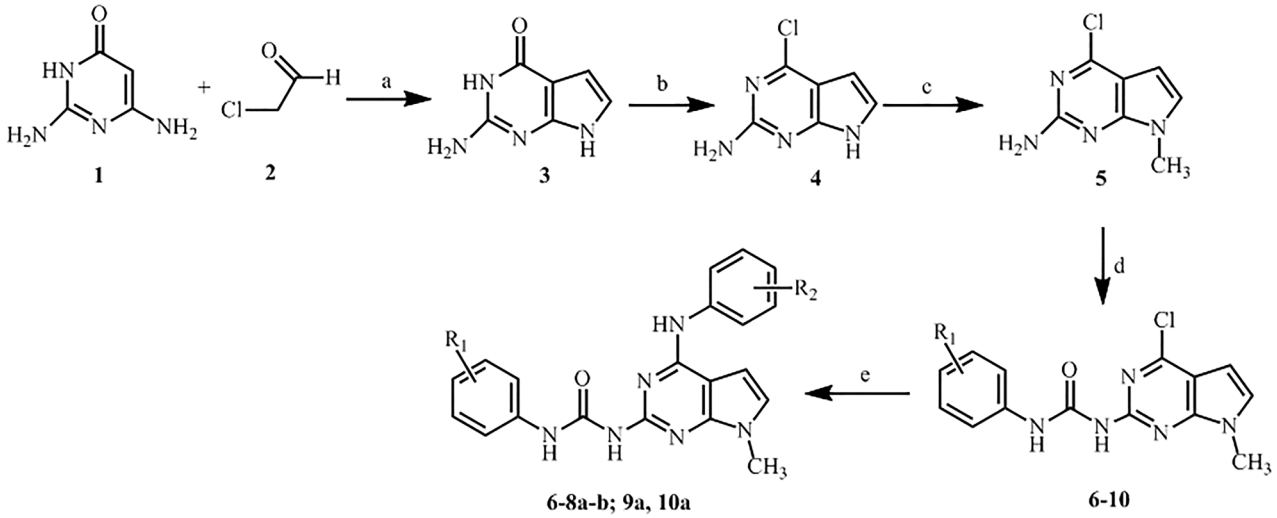
Target compounds (**6-8a-b**; **9a**, **10a**) have been evaluated for in vitro cytotoxic activity in various human cancer cell lines, including PC3 (prostat), A549 (lung) and MCF-7 (breast), using MTT assay. The results are summarized in Table 1. Imatinib was used as positive control. Majority of the synthesized compounds showed more cytotoxic activity than imatinib. Among the tested carcinoma cell lines, A549 cells seemed to be more sensitive in response to the growth inhibition with IC_{50} values between 0.35 and 5.68 μM. Synthesized compounds were found to be moderately active against MCF-7 cell line (23.29–71.90 μM). Compound **6b**, **8a** and **9a** exhibited the remarkably cytotoxic activity against A549 with IC_{50} value of 0.35, 1.48 and 1.56 μM, respectively. Compound **7a**, bearing 4-chloro-3-(trifluoromethyl)phenyl)urea moiety was more active than corresponding **6a**, **8a** and **10a** on PC3 cells (IC_{50} = 1.04 μM) suggesting that bulky urea moiety enhance the activity. In addition, **7a** was less cytotoxic against A549 (IC_{50} = 5.68 μM) and MCF-7 (IC_{50} = 24.85 μM) cells. Among the compounds substituted with 3-chloro-4-fluorophenylamino, **8b** bearing 3-(trifluoromethyl)phenylurea moiety, substituted at 4-position with an electron withdrawing fluoro atom showed better cytotoxic activity against PC3 (IC_{50} = 1.89 μM) and A549 (IC_{50} = 5.17 μM) cell lines than MCF-7 (IC_{50} = 26.19 μM).

3.2.2. Determination of apoptosis

The apoptotic efficiency of the most active compounds including **6b**, **8a** and **9a** were determined by Annexin V binding assay in A549 cells. The obtained results are shown in Fig. 3. Flow cytometry analysis revealed that A549 cells treated with compounds **6b**, **8a** and **9a** showed significant increase in the percentage of late apoptotic cells by 2.8-, 2.7- and 2.5-folds compared to control, respectively. These results clearly demonstrated that the compounds **6b**, **8a** and **9a** had strong cytotoxic activity against A549 cell line through induction of apoptosis.

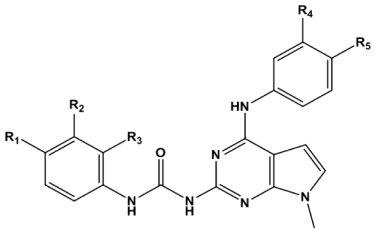
3.2.3. Effects on the level of Bcl-2 family members

We tested whether treatment with **6b**, **8a** and **9a** altered BCL-2 protein family members in A549 cells. As shown in Fig. 4, treatment with **6b**, **8a** and **9a** led to decreased levels of BCL-XL and MCL1, although only **9a** treatment reduced BCL-2 levels. We detected increased levels of BIM with **8a** and **9a** treatment as well as increased BAX



Scheme 1. Synthesis of target pyrrolo[2,3-*d*]pyrimidine derivatives. Reagent and conditions: (a) NaOAc, DMF:H_2O (5:1), 48 h, rt. (b) $POCl_3$, 110 °C, 2 h (c) CH_3-I, NaH, 0 °C → rt. (d) triphosgene, Et_3N, anhydrous dioxane, 0 °C → rt, 5 h, then appropriate aniline, rt. (e) isopropanol:DMF (1:1), three drops conc. HCl, appropriate aniline, 10–24 h, reflux.

Table 1
In vitro cytotoxicity of synthesized compounds **6-8a-b**; **9a**, **10a** against A549, PC3 and MCF-7 cells (IC_{50} in μM).



Compd	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)		
						A549	PC3	MCF-7
6a	−H	$-CF_3$	−H	−Br	−H	5.26	32.30	23.29
6b	−H	$-CF_3$	−H	−Cl	−F	0.35	35.69	23.84
7a	−Cl	$-CF_3$	−H	−Br	−H	5.68	1.04	24.85
7b	−Cl	$-CF_3$	−H	−Cl	−F	5.36	27.56	39.01
8a	−F	$-CF_3$	−H	−Br	−H	1.48	44.63	43.24
8b	−F	$-CF_3$	−H	−Cl	−F	5.17	1.89	26.19
9a	−F	−H	−H	−H	−Cl	1.56	31.01	24.85
10a	−F	−H	−F	−Br	−H	4.09	12.46	71.90
Imatinib						30.65	39.71	3.01

The cells were treated with different concentrations of compounds ranged between 0.1 and 100 μM and the cytotoxicity was determined by MTT assay. IC_{50} values of experiments conducted in triplicates.

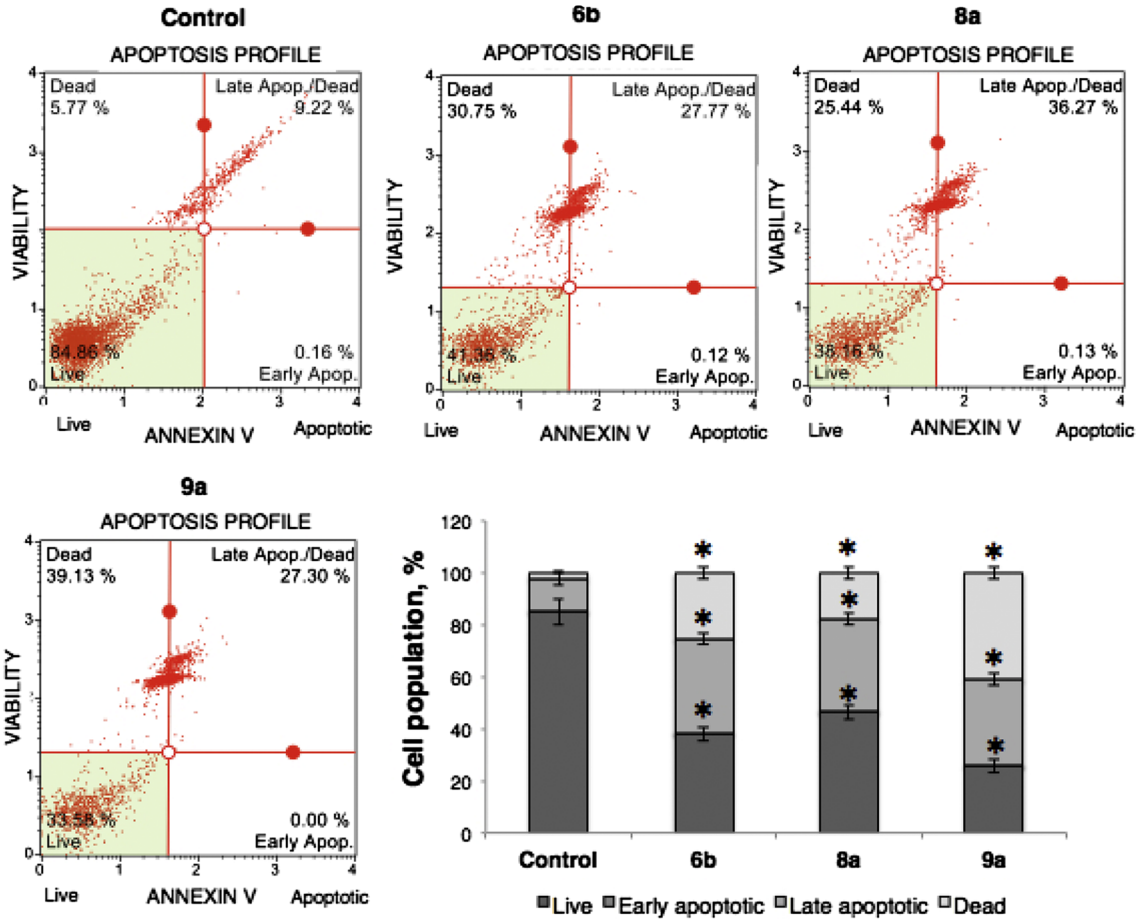


Fig. 3. The apoptotic effects of compound **6b**, **8a** and **9a** on A549 cells. The cells were treated with IC_{50} concentrations of compounds for 24 h and the cell population % were determined by Muse cell analyzer (Merck Millipore). The apoptotic cell population were determined by the Annexin V positivity and dead cells were determined by the nuclear dye 7-aminoactinomycin D (7-AAD) positivity. The cell analyzer determined four different group of population via cytofluorometric separation as follows: non-apoptotic live (7-AAD negative, apoptosis negative), non-apoptotic dead (7-AAD positive, apoptosis negative), apoptotic live (7-AAD negative, apoptosis positive), and apoptotic dead (7-AAD positive, apoptosis positive) cells. The figure represents cytofluorimetric dot plots of three independent experiments performed for annexin V detection and mean ± SD of late apoptotic population %. Statistical analysis was performed with one-way ANOVA for three independent data and the differences are identified as * from control, ($p < 0.01$).

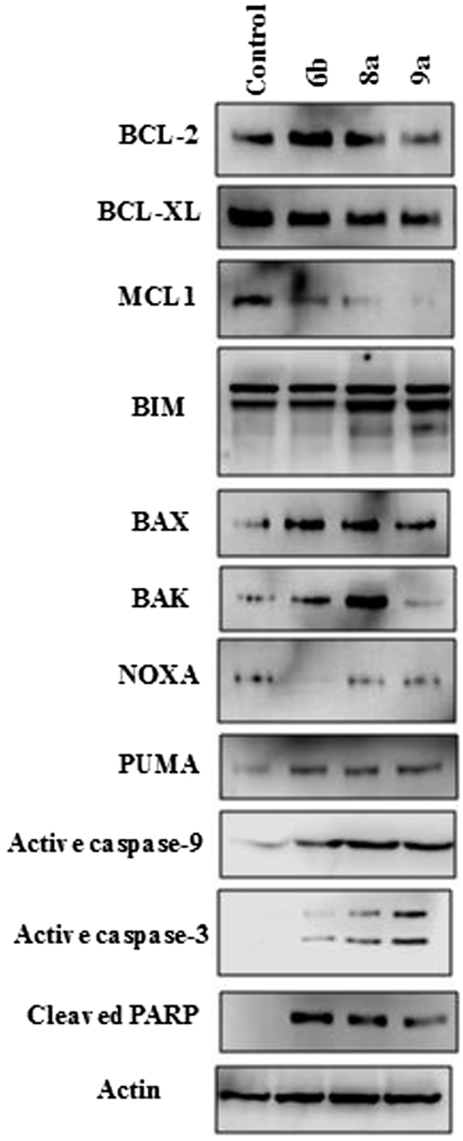


Fig. 4. A549 cells were treated with **6b**, **8a** and **9a** for 24 h. BCL-2, BCL-XL, MCL1, BIM, NOXA, PUMA, BAX, BAK, active caspase-9, active caspase-3, cleaved PARP levels were detected by using immunoblot analysis. Actin was probed as loading control.

expression upon treatment with all three molecules. **6b** and **8a** treatment resulted in increased BAK levels, which was more prominent following **8a** exposure. NOXA was not altered in response to **8a** and **9a** treatment, **6b** treatment led to decreased NOXA levels. In addition, we observed increased PUMA expression following treatment with all three molecules. Likewise, **6b**, **8a** and **9a** induced activation of caspase-3, caspase-9 and PARP cleavage, indicating the activation of apoptosis in A549 cells.

3.2.4. Apoptosis effect on HCT116 wt and HCT116 BAX/BAK KO cells

To further confirm the involvement of intrinsic apoptotic pathway in **6b**, **8a** and **9a**-induced cell death, we took advantage of HCT116 wt and HCT116 BAX/BAK KO cells. We initially verified the absence of BAX and BAK in HCT116 BAX/BAK KO by immunoblot analysis (Fig. 5A). As demonstrated in Fig. 5B, **6b**, **8a** and **9a** induced apoptosis in HCT116 wt cells although HCT116 BAX/BAK KO cells were significantly resistant to **6b**, **8a** and **9a** treatment. Correspondingly, **6b**, **8a** and **9a** treatment triggered activation of caspase-3 and caspase-9 in HCT116 wt cells but not in HCT116 BAX/BAK KO cells (Fig. 5C).

4. Conclusions

In this work, we have designed and synthesized novel pyrrolo[2,3-*d*]pyrimidine derivatives and analyzed their cytotoxic activities against three cancer cell lines (MCF-7, A549 and PC3). Most of the synthesized compounds showed more cytotoxicity against A549 cell than other tested cell lines. Compounds **6b**, **8a** and **9a** displayed the highest activity against A549 with IC_{50} value of 0.35, 1.48 and 1.56 μM, respectively and they induced intrinsic apoptosis in A549 cells confirmed by western blot analysis of pro-apoptotic and anti-apoptotic protein levels. In order to verify the intrinsic apoptotic induction of compounds **6b**, **8a** and **9a**, apoptotic effects of them on HCT116 wt and HCT116 BAX/BAK KO cells were evaluated. Although HCT116 wt cells treated with all of three compounds showed significant increase in the percent of annexin V-FTIC-positive apoptotic cells, HCT116 BAX/BAK KO cells were found significantly resistant to compounds **6b**, **8a** and **9a**. These results were consistent with the levels of caspase-3 and caspase-9 activation in HCT116 wt and HCT116 BAX/BAK KO cells.

The obtained potent cytotoxic effects through activation of the mitochondrial apoptotic pathway encourage further modifications of the compounds **6b**, **8a** and **9a** and to develop more potent anticancer agents. Also, further investigation to reveal mechanism of action of these compounds will be conducted.

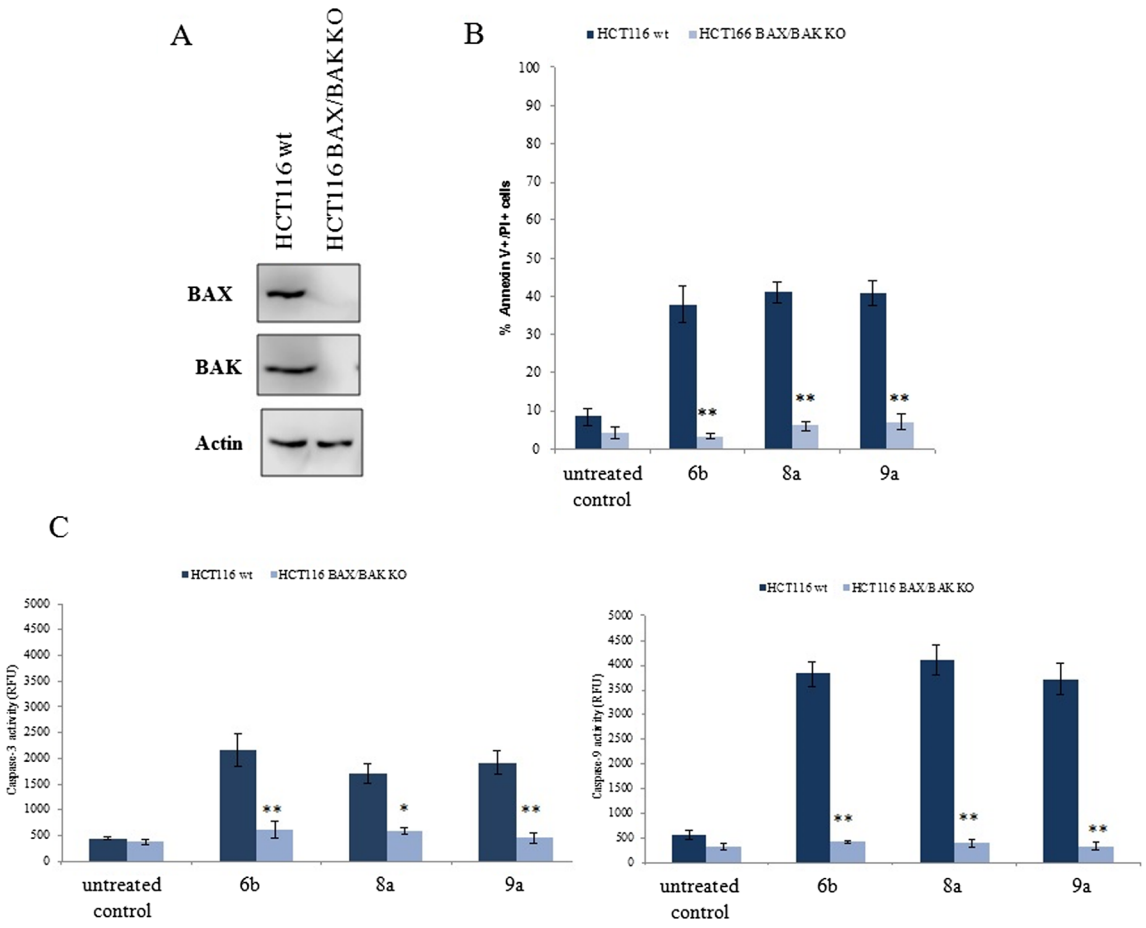


Fig. 5. (A) Expression of BAX and BAK in HCT116 wt and HCT116 BAX/BAK KO cells were analyzed by using immunoblot analysis. Actin was probed as loading control. (B) HCT116 wt and HCT116 BAX/BAK KO cells were treated with **6b**, **8a** and **9a** for 48 h. Cell death was evaluated by Annexin V/PI staining and flow cytometry. Columns, mean % Annexin V/PI positive cells from three independent experiments; bars, SE;**P < 0.01. (C) HCT116 wt and HCT116 BAX/BAK KO cells were treated with **6b**, **8a** and **9a** for 48 h. Activation of caspase-3 and caspase-9 was evaluated by fluorometric caspase activation assays. Columns, mean relative fluorescence units from three independent experiments; bars, SE; *P < 0.05, **P < 0.01.

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Disclosure statement

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

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