Received Date : 23-Apr-2016

Revised Date : 08-Oct-2016

Accepted Date : 31-Oct-2016

Article type : Research Article

Synthesis and biological evaluation of novel imidazopyrimidin-3-amines as anticancer agents

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Keywords: imidazo[1,2-*a*]pyrimidine, anticancer agents, apoptosis, Groebke-Blackburn-Bienayme reaction, isocyanide

Running title: Synthesis and anticancer activity of imidazopyrimidines

Abstract: Groebke-Blackburn-Bienayme reaction (GBB) has been utilized for the synthesis of new imidazo[1,2-*a*]pyrimidine derivatives as novel anticancer agents. The cytotoxic activities of compounds were evaluated against human cancer cell lines including MCF-7, T-47D and MDA-MB-231, compared with etoposide as the standard drug. Among the tested compounds, hydroxy- and/or methoxy-phenyl derivatives (**6a-c** and **6k**) with IC_{50} values of 6.72-14.36 µM were more potent than etoposide against all cell lines. The acridine-orange/ethidium bromide double staining and DNA-fragmentation studies demonstrated that the cytotoxic effect of 3-hydroxy-4-methoxyphenyl derivative **6c** is associated with apoptosis in cancer cells.

The cells constitute the building block of the body, growing, dividing and dying through a regular mechanism which are necessary for normal human's life. The uncontrolled cell proliferation led to severe health consequences, called in the worst case, cancer (1). The life style

choices (2), environmental factors (3) and family history are responsible risk factors for mutations (4), resulted in the increased risk of cell malfunction and the subsequent new cancer cases. Being raised as a worldwide killer (5), many therapeutic strategies have been devised to date like surgery, chemotherapy, radiotherapy and many others. None of these treatment types exhibited 100% efficacy.

Programmed cell death or apoptosis normally occurs to maintain cell populations in tissues. Any abnormalities in this case causes different kinds of diseases. Producing excessive amounts of anti-apoptotic proteins in cancer cells, regarded as a sign of cancer, interfering in normal cell cycle and led to tumor progression (6). Caspase-induced activation of apoptosis is one of the most important treatment methods confronting the vicious cell cycle (7, 8). In this context, exploring for new chemotherapeutic strategies with the goal of tumor regression by inducing apoptosis has gained considerable attention (9).

Pyrimidine-fused heterocycles constitute a pivotal framework in the field of anticancer drug development and discovery (10). In particular, imidazopyrimidines are well-known structures for their cytotoxic activities. For example, Fig. 1 depicts some 2,3-disubstituted-imidazopyrimidines (structures **A**-**C**) which have been previously reported as cytotoxic agents (11). Pal-Bhadra group have synthesized imidazopyrimidine-chalcones (structure **A**) with promising activity (GI_{50} values = 0.28 to 30.0 μM). A number of imidazo[1,2-*a*]pyrimidine Mannich bases (structure **B**) were synthesized and tested against three different human cancer cell lines. Most of the Mannich bases displayed GI_{50} values ranging from 0.01 to 79.4 μM. Furthermore, some imidazopyrimidine-benzimidazole conjugates were designed by Kamal et al. and evaluated against the human cervical (Hela), lung (A549), prostate (DU-145) and melanoma (B-16) cancer cell lines. Among them, the compound with structure **C** exhibited significant anti-proliferative

activity against lung cancer cell line A549 (IC_{50} = 1.48 μM) (11). On the other hand, the 5,7-diphenyl-triazolo[1,5-*a*]pyrimidine scaffold (structure **D**) has been introduced as a potent lead compound for designing novel anti-cancer agents (12).

In continuation of our ongoing program to design new anticancer agents (13), the prominent activity of pyrimidine-fused heterocycles (structures **A-D**) paid our attention on 5,7-diphenylimidazo[1,2-*a*]pyrimidine scaffold as a hybrid of structures **A-C** and **D**. Thus, we report here, the synthesis of 2-aryl-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amines **6a-o** (Fig. 1) as new cytotoxic compounds and evaluation of their biological activity as apoptosis-inducing agents.

Methods and Materials

General chemistry

Melting points are uncorrected and were determined with a Kofler hot-stage apparatus (Reichert, Vienna, Austria). Bruker 500 spectrometers and Nicolet Magna FT-IR 550 spectrophotometer (potassium bromide disks) were used to record ^{1}H, ^{13}C NMR spectra and IR spectra, respectively. Agilent Technology (HP) mass spectrometer operating at an ionization potential of 70 eV was utilized to determine mass spectra. The elemental analysis for C, H, N was carried out with an Elementar Analysen system GmbH VarioEL. All needed chemicals and solvents were commercially bought and used without further purification.

Synthesis of 4,6-diphenylpyrimidin-2-amine 5

A solution of chalcone (**3**, 10 mmol), and guanidine hydrochloride (**4**, 10 mmol) in 15 mL ethanol was refluxed for 15 min. To this mixture, NaOH solution (4 mL, 50%) was added dropwise and the reaction was refluxed for another 6 h. Then, the reaction mixture was concentrated under the reduced pressure and kept at 0 ºC. The resulting crystals were filtered and recrystallized from ethanol to give compound **5** as a yellow solid.

General procedure for the synthesis of *N*-substituted-5,7-diphenyl-imidazo[1,2-*a*]pyrimidin-3-amines (6a-o)

To the stirred solution of compound **5** (1 mmol), aldehyde derivatives (1 mmol) and NH_4Cl (1 mmol) in toluene (5 mL), isocyanide (1 mmol) was added. The mixture was refluxed for 18 h. Then, the reaction was cooled and the obtained solid was filtered, washed with water and recrystallized from petroleum ether/ethyl acetate to give corresponding compounds **6a-o**.

4-(3-(Cyclohexylamino)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-2-yl) phenol (6a)

Yellow solid; yield: 0.33 g (71 %); m.p. 233-236 ˚C. IR (KBr) (ν_{max}, cm^{-1}): 3358, 3059, 2926, 1610. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.51 (s, OH), 8.28 (d, J = 6.5 Hz, 2H, H-Ph), 8.12 (d, J = 8.5 Hz, 2H, $H_{2,6}$-OHC_6H_4), 7.78-7.80 (m, 2H, H-Ph), 7.50-7.55 (m, 6H, H-Ph), 7.43 (s, 1H, H_6-pyrimidine), 6.81 (d, J = 8.5 Hz, 2H, $H_{3,5}$-OHC_6H_4), 3.88 (s, 1H, NH), 2.03 (s, 1H, NCH), 0.59-1.32 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 156.9, 153.4, 146.0, 145.2, 140.1, 136.9, 132.8, 130.0, 129.7, 129.2, 128.8, 127.8,126.9, 125.1, 123.0, 115.8, 114.8,

107.5, 56.5, 31.9, 25.3, 24.0. Anal. Calcd. for $C_{30}H_{28}N_4O$: C, 78.23; H, 6.13; N, 12.16. Found: C, 78.15; H, 6.25; N, 12.31.

_N_-Cyclohexyl-2-(2-methoxyphenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6b)

Yellow solid; yield: 0.40 g (87 %); m.p. 148–149 °C. IR (KBr) (ν_{max}, cm^{-1}): 3303, 2920, 1604, 1483, 1242. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.29 (d, J = 7.9 Hz, 2H, H-Ph), 7.70-7.77 (m, 3H, H_6-$MeOC_6H_4$, H-Ph), 7.50-7.58 (m, 6H, H-Ph), 7.49 (s, 1H, H_6-pyrimidine), 7.40 (t, J = 7.4 Hz, 1H, H_4-$MeOC_6H_4$), 7.17 (td, J = 8.4, 1.5 Hz, 1H, H_3-$MeOC_6H_4$), 7.10 (t, J = 7.5 Hz, 1H, H_5-$MeOC_6H_4$), 3.89 (s, 3H, OMe), 3.71 (s, 1H, NH), 1.80 (s, 1H, NCH), 0.28–1.17 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 155.5, 152.4, 146.1, 144.8, 136.9, 135.6, 135.5, 132.0, 131.6, 130.2, 130.0, 129.4, 129.3, 128.9, 127.1, 126.8, 123.5, 121.0, 112.0, 107.8, 55.9, 55.8, 32.0, 25.1, 23.9. MS: *m/z* (%) = 474 (28) [M^+], 443 (100), 345 (23), 268 (57), 192 (17), 77 (65).

5-(3-(Cyclohexylamino)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-2-yl)-2-methoxyphenol (6c)

Yellow solid; yield: 0.37 g (76 %); m.p. 285-287 °C. IR (KBr) (ν_{max}, cm^{-1}): 3369, 1602, 1243. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.99 (s, OH), 8.29 (d, J = 6.9 Hz, 2H, H-Ph), 7.79-7.81 (m, 3H, H_2-3-OH-4-$MeOC_6H_3$, H-Ph), 7.76 (d, J = 8.4 Hz, 1H, H_6-3-OH-4-$MeOC_6H_3$), 7.50-7.60 (m, 6H, H-Ph), 7.46 (s, 1H, H_6-pyrimidine), 6.96 (d, J = 8.4 Hz, 1H, H_5-3-OH-4-$MeOC_6H_3$), 3.80 (s, 3H, OMe), 3.12 (s, 1H, NH), 2.03 (s, 1H, NCH), 0.59-1.30 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.1, 146.4, 145.4, 137.1, 136.7, 136.2 (2C), 132.4, 130.3 (2C),

129.8, 129.5, 128.9, 127.7, 127.6, 127.0, 126.2, 125.3, 122.7, 107.8, 56.9, 56.8, 32.1, 25.3, 24.3. Anal. Calcd. for $C_{31}H_{30}N_4O_2$: C, 75.89; H, 6.16; N, 11.42. Found: C, 75.71; H, 6.21; N, 11.40.

***N*-Cyclohexyl-5,7-diphenyl-2-(*m*-tolyl)imidazo[1,2-*a*]pyrimidin-3-amine (6d)**

Yellow solid; yield: 0.40 g (89 %); m.p. 200-201 °C. IR (KBr) (ν_{max}, cm^{-1}): 3312, 2923, 1600, 1483. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.29 (d, *J* = 7.8 Hz, 2H, H-Ph), 8.13 (s, 1H, H_2-MeC_6H_4), 8.10 (d, *J* = 7.9 Hz, 1H, H_6-MeC_6H_4), 7.80-7.81 (m, 2H, H-Ph), 7.58-7.63 (m, 3H, H-Ph), 7.51-7.56 (m, 3H, H-Ph), 7.48 (s, 1H, H_6-pyrimidine), 7.31 (t, *J* = 7.6 Hz, 1H, H_5-MeC_6H_4), 7.11 (d, *J* = 7.4 Hz, 1H, H_4-MeC_6H_4), 3.38 (s, 1H, NH), 2.36 (s, 3H, Me), 2.03 (s, 1H, NCH), 0.55-1.28 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.0, 146.4, 145.2, 139.2, 137.0, 136.8, 134.1, 132.7, 130.2, 129.8, 129.3, 128.9, 128.2, 128.1, 128.0, 127.8, 126.9, 124.7, 124.4, 107.8, 56.5, 31.9, 25.2, 24.0, 21.2. MS: *m/z* (%) = 458 (41) [M^+], 443 (100), 345 (35), 269 (29), 194 (28), 98 (44), 77 (66). Anal. Calcd. for $C_{31}H_{30}N_4$: C, 81.19; H, 6.59; N, 12.22. Found: C, 81.15; H, 6.71; N, 12.28.

***N*-Cyclohexyl-2-(2-fluorophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6e)**

Light Yellow solid; yield: 0.31 g (68 %); m.p. 134-135 °C. IR (KBr) (ν_{max}, cm^{-1}): 3408, 2924, 1606, 1483, 1096.^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.30 (d, *J* = 7.5 Hz, 2H, H-Ph), 7.81 (m, 1H, H_6-FC_6H_4), 7.77 (d, *J* = 6.9 Hz, 2H, H-Ph), 7.52-7.59 (m, 6H, H-Ph), 7.51 (s, 1H, H_6-pyrimidine), 7.42-7.45 (m, 1H, H_4-FC_6H_4), 7.33 (m, 1H, H_3-FC_6H_4), 7.30 (t, *J* = 7.7 Hz, 1H, H_5-FC_6H_4), 3.22 (s, 1H, NH), 1.92 (s, 1H, NCH), 0.35-1.20 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 158.2 (d, $J_{C\text{-}F}$ = 224 Hz), 153.7, 146.5, 145.2, 136.7, 134.4, 132.2,

131.9, 130.2, 129.9, 129.7, 128.9, 127.3, 127.0, 126.3, 124.4, 122.2, 122.1, 115.7, 108.0, 56.0, 31.8, 25.1, 23.8. Anal. Calcd. for $C_{30}H_{27}FN_4$: C, 77.90; H, 5.88; N, 12.11. Found: C, 77.76; H, 5.79; N, 12.18.

_N_-Cyclohexyl-2-(4-fluorophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6f)

Yellow solid; yield: 0.32 g (69 %); m.p. 220-222 °C. IR (KBr) (ν_{max}, cm^{-1}): 3349, 2928, 1740, 1489, 1211.^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.29 (d, J = 7.1 Hz, 2H, H–Ph), 8.26 (d, J = 8.0 Hz, 2H, $H_{2,6}$-FC_6H_4), 7.82 (d, J = 6.5 Hz, 2H, H-Ph), 7.53-7.62 (m, 6H, H-Ph), 7.49 (s, 1H, H_6-pyrimidine), 7.26 (dd, J = 8.0, 1.5 Hz, 2H, $H_{3,5}$-FC_6H_4), 4.02 (s, 1H, NH), 1.99 (s, 1H, NCH), 0.60-1.31 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 159.3 (d, $J_{C\text{-}F}$ = 221 Hz), 154.3, 146.5, 145.4, 138.6, 136.7, 132.6, 130.7, 130.2, 129.7, 129.6, 129.2, 128.8, 127.8, 126.9, 124.0, 114.8 (d, $J_{C\text{-}F}$ = 20 Hz), 107.8, 56.6, 31.9, 25.2, 24.0. Anal. Calcd. for $C_{30}H_{27}FN_4$: C, 77.90; H, 5.88; N, 12.11. Found: C, 77.83; H, 5.96; N, 12.08.

2-(3-Chlorophenyl)-_N_-cyclohexyl-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6g)

Deep Yellow solid; yield: 0.41 g (88 %); m.p. 186-188 °C. IR (KBr) (ν_{max}, cm^{-1}): 3364, 2922, 1604, 1481, 687.^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.30 (d, J = 7.3 Hz, 2H, H-Ph), 8.13 (s, 1H, H_2-ClC_6H_4), 8.11 (d, J = 7.9 Hz, 1H, H_6-ClC_6H_4), 7.80-7.82 (m, 2H, H-Ph), 7.50-7.62 (m, 6H, H-Ph), 7.48 (s, 1H, H_6-pyrimidine), 7.30 (t, J = 7.8 Hz, 1H, H_5-ClC_6H_4), 7.11 (d, J = 7.3 Hz, 1H, H_4-ClC_6H_4), 4.03 (s, 1H, NH), 2.49 (s, 1H, NCH), 0.55-1.28 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.5, 146.6, 145.4, 138.2, 136.7, 133.1, 132.5, 131.9 (2C), 130.3, 129.9 (2C), 129.3, 129.2, 128.9, 128.1, 127.9, 127.0, 124.5, 108.0, 56.7, 31.9, 25.2,

24.1. Anal. Calcd. for $C_{30}H_{27}ClN_4$: C, 75.22; H, 5.68; N, 11.70. Found: C, 75.30; H, 5.56; N, 11.79.

***N*-Cyclohexyl-2-(2,4-dichlorophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6h)**

Yellow solid; yield: 0.30 g (60 %); m.p. 97–98 °C. IR (KBr) (ν_{max}, cm^{-1}): 3393, 2926, 1735, 1481, 695. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.28 (d, *J* = 7.1 Hz, 2H, H-Ph), 7.75 (d, *J* = 8.0 Hz, 2H, H-Ph), 7.72 (d, *J* = 1.8 Hz, 1H, H_3-2,4-$Cl_2C_6H_3$), 7.67 (d, *J* = 8.0 Hz, 1H, H_6-2,4-$Cl_2C_6H_3$), 7.51-7.60 (m, 7H, H–Ph, H_5-2,4-$Cl_2C_6H_3$), 7.50 (s, 1H, H_6-pyrimidine), 4.02 (s, 1H, NH), 1.91 (s, 1H, NCH), 0.34-1.17 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 153.9, 146.5, 144.8, 136.7, 134.0, 133.8, 133.3, 132.4, 132.2, 130.3, 129.7, 129.6, 129.0, 128.9, 127.5, 127.0, 126.1, 108.0, 55.8, 31.6, 25.1, 23.7. Anal. Calcd. for $C_{30}H_{26}Cl_2N_4$: C, 70.18; H, 5.10; N, 10.91. Found: C, 70.24; H, 5.16; N, 10.85.

***N*-Cyclohexyl-2-(3-nitrophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6i)**

Deep Yellow solid; yield: 0.31 g (65 %); m.p. 250-251 °C. IR (KBr) (ν_{max}, cm^{-1}): 3377, 2924, 1522, 1345. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.42 (d, *J* = 1.9 Hz, 1H, H_2-$NO_2C_6H_4$), 8.80 (dd, *J* = 7.9 Hz, 1H, H_4-$NO_2C_6H_4$), 8.26 (d, *J* = 7.7 Hz, 2H, H-Ph), 8.13 (d, *J* = 8.2 Hz, 1H, H_6-$NO_2C_6H_4$), 7.65-7.66 (m, 5H, H-Ph), 7.59 (t, *J* = 7.9 Hz, 1H, H_5-$NO_2C_6H_4$), 7.50-7.52 (m, 3H, H-Ph), 7.22 (s, 1H, H_6-pyrimidine), 2.34 (s, 1H, NH), 2.19 (s, 1H, NCH), 0.67-1.41 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 156.2, 148.2, 146.4, 145.8, 136.6, 133.6, 135.8, 133.6, 132.9, 130.58, 130.53, 129.0 (2C), 128.87, 128.84, 128.3, 127.2, 125.2, 122.6,

122.0, 108.3, 58.2, 32.6, 25.4, 24.6. Anal. Calcd. for $C_{30}H_{27}N_5O_2$: C, 73.60; H, 5.56; N, 14.31. Found: C, 73.51; H, 5.46; N, 14.40.

***N*-Cyclohexyl-5,7-diphenyl-2-(thiophen-2-yl)imidazo[1,2-*a*]pyrimidin-3-amine (6j)**

Yellow solid; yield: 0.34 g (76 %); m.p.: 232-234 °C. IR (KBr) (ν_{max}, cm^{-1}): 3323, 2922, 1604. ^1H NMR (500 MHz, DMSO-d_6): δ = 8.29 (d, *J* = 7.6 Hz, 2H, H-Ph), 7.84 (d, *J* = 3.5 Hz, 1H, H_5-thiophene), 7.80 (d, *J* = 6.7 Hz, 2H, H-Ph), 7.57-7.61 (m, 3H, H-Ph), 7.52-7.54 (m, 4H, H-Ph, H_3-thiophene), 7.51 (s, 1H, H_6-pyrimidine), 7.13 (t, *J* = 4.0 Hz, 1H, H_4-thiophene), 3.50 (s, 1H, NH), 1.92 (s, 1H, NCH), 0.72-1.34 (m, 10H, $5CH_2$, cyclohexyl). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.1, 146.4, 145.4, 137.1, 136.7, 136.2, 132.3, 130.2, 129.8, 129.4, 128.9, 127.7, 127.5, 126.9, 126.2, 125.2, 122.7, 107.8, 56.9, 32.1, 25.3, 24.3. MS: *m/z* (%) = 450 (29) [M^+], 352 (100), 269 (47), 192 (25), 82 (39), 77 (71). Anal. Calcd. for $C_{28}H_{26}N_4S$: C, 74.63; H, 5.82; N, 12.43. Found: C, 74.51; H, 5.96; N, 12.40.

***N*-(*tert*-Butyl)-2-(2-methoxyphenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6k)**

Light Yellow solid; yield: 0.37 g (85 %); m.p. 194-196 °C. IR (KBr) (ν_{max}, cm^{-1}): 3336, 2956, 1482. ^1H NMR (500 MHz, DMSO-d_6): δ = 8.31 (d, *J* = 7.0 Hz, 2H, H-Ph), 7.78 (dd, *J* = 7.5, 1.5 Hz, 1H, H_6-$MeOC_6H_4$), 7.72-7.74 (m, 2H, H-Ph), 7.53-7.56 (m, 6H, H-Ph), 7.52 (s, 1H, H_6-pyrimidine), 7.40 (dt, *J* = 7.2, 1.5 Hz, 1H, H_4-$MeOC_6H_4$), 7.16 (d, *J* = 8.5 Hz, 1H, H_3-$MeOC_6H_4$), 7.11 (t, *J* = 7.2 Hz, 1H, H_5-$MeOC_6H_4$), 3.92 (s, 3H, OMe), 3.65 (s, 1H, NH), 0.30 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 155.8, 153.4, 146.9, 145.5, 139.4, 136.8, 132.5, 131.5, 130.5, 130.1, 129.5, 129.4, 128.9, 126.9, 126.8, 126.0, 124.1, 120.9, 111.8, 108.4,

55.9, 54.9, 28.7. Anal. Calcd. for $C_{29}H_{28}N_4O$: C, 77.65; H, 6.29; N, 12.49. Found: C, 77.58; H, 6.36; N, 12.40.

***N*-(*tert*-Butyl)-5,7-diphenyl-2-(*m*-tolyl)imidazo[1,2-*a*]pyrimidin-3-amine (6l)**

Yellow solid; yield: 0.39 g (90 %); m.p. 273-275 °C. IR (KBr) (ν_{max}, cm^{-1}): 3326, 2963, 1596, 1484. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.32 (d, *J* = 6.8 Hz, 2H, H-Ph), 8.02 (s, 1H, H_2-MeC_6H_4), 7.96 (d, *J* = 7.4 Hz, 1H, H_6-MeC_6H_4), 7.76-7.77 (m, 2H, H-Ph), 7.54-7.59 (m, 6H, H-Ph), 7.52 (s, 1H, H_6-pyrimidine), 7.31 (t, *J* = 7.6 Hz, 1H, H_5-MeC_6H_4), 7.13 (d, *J* = 7.0 Hz, 1H, H_4-MeC_6H_4), 3.54 (s, 1H, NH), 2.38 (s, 3H, CH_3), 0.38 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.1, 147.0, 145.4, 142.3, 137.3, 134.6, 133.1, 130.4, 130.2, 129.6, 129.0, 128.9, 128.6, 128.2, 127.7, 127.6, 126.9, 125.6, 123.9, 108.3, 55.4, 29.0, 21.0. Anal. Calcd. for $C_{29}H_{28}N_4$: C, 80.52; H, 6.52; N, 12.95. Found: C, 80.51; H, 6.59; N, 12.84.

***N*-(*tert*-Butyl)-2-(4-fluorophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6m)**

Light Yellow solid; yield: 0.30 g (69 %); m.p. 291-293 °C. IR (KBr) (ν_{max}, cm^{-1}): 3392, 1608, 1482, 1204.^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.32 (d, *J* = 7.8, 2H, H-Ph), 8.21 (m, 2H, $H_{2,6}$-FC_6H_4), 7.79 (m, 2H, H-Ph), 7.56-7.60 (m, 6H, H-Ph), 7.55 (s, 1H, H_6-pyrimidine), 7.26-7.28 (m, 2H, $H_{3,5}$-FC_6H_4), 3.53 (s, 1H, NH), 0.39 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 162.3 (d, $J_{C\text{-}F}$ = 218 Hz), 147.1, 136.5, 132.8, 130.4, 129.6, 128.8, 127.6, 126.9, 114.8, 114.6, 108.4, 55.2, 28.8. Anal. Calcd. for $C_{28}H_{25}FN_4$: C, 77.04; H, 5.77; N, 12.83. Found: C, 77.10; H, 5.81; N, 12.91.

***N*-(*tert*-Butyl)-2-(4-chlorophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6n)**

Light Yellow solid; yield: 0.38 g (84 %); m.p. 290-292 °C. IR (KBr) (ν_{max}, cm^{-1}): 3390, 1610, 1480, 755. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.32 (d, *J* = 7.0 Hz, 2H, H-Ph), 8.21 (d, *J* = 8.2 Hz, 2H, $H_{2,6}$-ClC_6H_4), 7.76-7.78 (m, 2H, H-Ph), 7.57-7.59 (m, 3H, H-Ph), 7.55 (s, 1H, H_6-pyrimidine), 7.52-7.54 (m, 3H, H-Ph), 7.48 (d, *J* = 8.3 Hz, 2H, $H_{3,5}$-ClC_6H_4), 3.55 (s, 1H, NH), 0.38 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 154.6, 147.1, 145.6, 140.9, 136.6, 133.7, 132.9, 132.1, 131.2, 130.1, 129.7, 129.3, 128.9, 128.0, 127.7, 127.0, 124.1, 108.5, 55.5, 28.9. Anal. Calcd. for $C_{28}H_{25}ClN_4$: C, 74.24; H, 5.56; N, 12.37; Found: C, 74.30; H, 5.61; N, 12.40.

***N*-(*tert*-Butyl)-2-(3-nitrophenyl)-5,7-diphenylimidazo[1,2-*a*]pyrimidin-3-amine (6o)**

Yellow solid; yield: 0.32 g (70 %); m.p. 248-249 °C. IR (KBr) (ν_{max}, cm^{-1}): 3392, 1605, 1520, 1344. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.08 (d, *J* = 1.7 Hz, 1H, H_2-$NO_2C_6H_4$), 8.66 (d, *J* = 7.7 Hz, 1H, H_4-$NO_2C_6H_4$), 8.33 (d, *J* = 7.8 Hz, 2H, H-Ph), 8.17 (d, *J* = 8.0 Hz, 1H, H_6-$NO_2C_6H_4$), 7.80-7.82 (m, 2H, H-Ph), 7.74 (t, *J* = 8.0 Hz, 1H, H_5-$NO_2C_6H_4$), 7.62-7.63 (m, 3H, H-Ph), 7.58 (s, 1H, H_6-pyrimidine), 7.54-7.57 (m, 3H, H-Ph), 3.56 (s, 1H, NH), 0.42 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ = 155.2, 147.8, 147.4, 145.8, 139.5, 136.5, 136.4, 134.4, 132.8, 130.5, 129.9, 129.6, 128.9, 127.9, 127.1, 124.7, 122.6, 122.5, 122.1, 108.8, 55.6, 29.0. MS: *m/z* (%) = 463 (27) [M^+], 406 (100), 284 (51), 122 (39), 207 (28), 77 (35), 57 (64). Anal. Calcd. for $C_{28}H_{25}N_5O_2$: C, 72.55; H, 5.44; N, 15.11. Found: C, 72.51; H, 5.46; N, 15.20.

Cell lines and cell culture

The human breast cancer cell lines including MCF-7, MDA-MB-231 and T-47D were received from Pasture Institute, Tehran (Iran). The cultured cells in RPMI 1640 medium (containing 10% FBS, 1% L-Glutamine, and Penicillin-Streptomycin) were incubated under humidified atmosphere with 5% CO_2 at 37 ºC.

MTT assay

The stock solutions of compounds were prepared in DMSO. The cells were treated with different concentrations of the compounds and incubated for 48 h. Etoposide and DMSO were used as reference drug and control, respectively. The effect of compounds **6a-o** on cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the reported method (14). The optical density was read at 492 nm with an ELISA plate reader (Exert 96, Asys Hitch, Ec Austria).

Acridine orange/ethidium bromide staining method

The cancer cells (MCF-7 and T47D) grown in 12-well plates (50,000 cells/well) were treated with and without IC_{50} concentration of compounds **6c** and **6k** for 24 h. After treatment, cells were harvested and washed three times with phosphate buffer saline (PBS). Then, the cells were stained with 100 mL of a mixture of acridine orange and ethidium bromide (1:1, 100 mg/mL) solutions. The stained cell suspension (10 mL) was placed on a clean microscope slide and

covered with a coverslip. The cells were immediately analyzed by Axoscope 2 plus fluorescence microscope (Zeiss, Germany).

DNA-fragmentation assay

DNA fragmentation was measured by a slight modification of the diphenylamine assay described by Gercel-Taylor (15). The diphenylamine assay is a very useful method for measuring apoptosis and is based on the notion that extensively fragmented double-stranded DNA can be separated from apoptotic nuclei into the cytoplasm and constitutes a quantitative measure of soluble DNA (16).

Briefly, MCF-7 and T-47D cells treated with test compounds for 24 h were harvested and centrifuged at 2000 RPM, for 10 min (4°C) to obtain a cell pellet. The pellets were resuspended in ice-cold hypotonic lysis TTE buffer (10 mM tris-base, 1 mM EDTA, 0.2% Triton X-100, pH=8.0) and samples were allowed to lyse for 30 min at 4°C. Then, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation at 13000 g for 15 min (4°C). The pellets were then resuspended in TTE buffer. Both the pellets and the supernatant samples were precipitated with trichloroacetic acid (TCA; 25%). Samples were then centrifuged at 13000 g for 15 min (4°C) and the supernatant was removed. The precipitates were resuspended in 5% TCA, boiled at 90 ºC for 20 min and centrifuged at 4 ºC to remove proteins. The resulting supernatants, whether containing whole or fragmented DNA, were left to react with freshly prepared diphenylamine for 16–20 h at room temperature, and absorbance was measured at 620 nm. DNA fragmentation was expressed as a percentage of fragmented to total DNA by the formula:

% DNA fragmentation= [OD of supernatant/(OD of supernatant + OD of pellet)] × 100

Results and Discussion

Chemistry

The reaction pathway toward target compounds **6a-o** namely *N*-substituted-5,7-diphenyl-imidazo[1,2-*a*]pyrimidin-3-amines is depicted in Scheme 1. Initially, we prepared chalcone **3** according to the reported method (17), *via* the reaction of acetophenone **1** and benzaldehyde **2** in the presence of NaOH in ethanol. In the next step, the cyclization reaction of guanidine hydrochloride **4** with chalcone **3** provided 4,6-diphenylpyrimidin-2-amine **5** as a yellow powder in 75% yield identified according to the literature (m.p. 139 ^{o}C; m.p. lit. 138-139 ^{o}C) (18). The resulting solid was used in the subsequent step after a simple recrystallization from ethanol. Groebke-Blackburn-Bienayme reaction (GBB) of compound **5** with aromatic aldehydes and isocyanides afforded *N*-substituted-5,7-diphenyl-imidazo[1,2-*a*]pyrimidin-3-amines **6a-o** in 60-90% yields. The reaction was performed in refluxing toluene (19). Accordingly, this condition was applied for the synthesis of 15 different imidazopyrimidines which were fully characterized by ^{1}H, ^{13}C-NMR, and IR spectroscopy. The NMR spectra are provided as Appendix 1 file.

Pharmacology

In vitro anticancer screening (MTT assay)

The cytotoxic activities of compounds **6a-o** were tested against three different cell lines MCF-7, T-47D and MDA-MB-231 by using MTT assay (14). The IC_{50} values were given in Table 1.

Most of the compounds showed remarkable activity against tested cell lines. Particularly, compound **6c** bearing a methoxyphenolic group showed the most potent activity against MCF-7 and T-47D cells. Its IC_{50} value against T-47D cell line was 6.72 µM, being 5-fold lower than that of etoposide as reference drug. The most potent compound against MDA-MB-231 cell line was compound **6e** with IC_{50} value of 9.90 µM. Besides **6e**, compound **6c** also showed good activity against the latter cell line, being more potent than etoposide. The obtained IC_{50} values revealed that better results were obtained with hydroxy- and methoxy-phenyl derivatives (compounds **6a-c** and **6k**). The IC_{50}s of these compounds were in the range of 6.72-14.36 µM. Their activity was higher than that of etoposide against all cell lines. These results demonstrated that the presence of electron-donating groups such as OH and OMe could be favorable for cytotoxic activity. In contrast, since the compounds such as **6g**, **6i**, **6n** and **6o** were less active or inactive derivatives, it could conclude that the electron-withdrawing groups such as chlorine or nitro diminish the anti-proliferative activity. Exceptionally, the 2-fluorophenyl derivative **6e** exhibited significant activity against all cell line (IC_{50}s = 8.88-16.97 µM). However, the displacement of fluoro group to *para* position resulted in compound **6f** with diminished activity. On the other hand, the activity of 2,4-dichloro derivative **6h** was better than that of 3-chloro analog **6g**. The comparison of cyclohexyl and *tert*-butyl series revealed that the pendent resides (R= cyclohexyl or *tert*-butyl) didn't have same effect on different cell lines. For example, while the cyclohexyl derivative **6d** was inactive against MCF-7, its *tert*-butyl analog **6l** showed moderate activity against this cell line. In contrast, compound **6d** was more active than *tert*-butyl derivative **6l** against MDA-MB-231 and T-47D cells.

Acridine orange/ethidium bromide double staining test

The morphological assessment of MCF-7 and T-47D cells by acridine orange/ethidium bromide double staining method was used to indicate the potential of compounds **6c** and **6k** as apoptotic inducers. The test cells were treated with the IC_{50} concentration of compounds **6c** and **6k** for 24 h and then stained with acridine orange/ethidium bromide. Using fluorescence microscopy, uniformly stained green cells can be distinguished as live cells and orange cells are characterized as apoptotic cells. The observed results demonstrated that the selected compounds can induce apoptosis in MCF-7 and T-47D cells (Fig. 2 and 3). Also, chromatin condensation and nuclear fragmentation were occurred following the treatment of cancer cells with compounds **6c** and **6k**.

DNA-fragmentation assay

DNA fragmentation using diphenylamine assay is a useful tool in measuring apoptosis. The cells exhibiting the morphological characteristics associated with DNA fragmentation are also referred to as apoptotic cells (15, 16). The MCF-7 and T-47D cells were treated with selected compounds **6c** and **6k** for 24 h and subjected to the assay. The obtained results were summarized in Fig. 4 as mean percentages of DNA fragmentation. Based on the results, the compounds **6c** and **6k** induced DNA fragmentation more significantly in the MCF-7 cells compared to the T-47D cells.

Conclusion

In conclusion, we synthesized a series of functionalized imidazo[1,2-*a*]pyrimidin-3-amines bearing poly-aromatics and a bulky residue (cyclohexyl or *tert*-butyl) on the amine, as new cytotoxic agents. The results of cytotoxicity assay against different tumor cell lines revealed that

most of the compounds have significant activity against MCF-7, T-47D and MDA-MB-231 cells. Especially, compounds **6a-c** and **6k** bearing hydroxy- and/or methoxy group on the 2-phenyl ring showed more promising results against all cell lines, with IC_{50} values in the range of 6.72-14.36 μM. Their activities were superior to that of etoposide as reference anticancer agent. In particular, the 3-hydroxy-4-methoxyphenyl derivative **6c** with IC_{50} value of 6.72 μM against T-47D cells was 5 times more potent than etoposide. Further biological assays by using acridine orange/ethidium bromide double staining test and DNA-fragmentation study demonstrated that the promising compound **6c** exhibits its cytotoxic effect partly by the induction of apoptosis in cancer cells.

Acknowledgment

† In the memory of Professor Abbas Shafiee, a leading Iranian medicinal chemist and the first corresponding author of this article, who was passed away during the reviewing process. This study was supported and funded by grants from the Tehran University of Medical Sciences (TUMS) and Iran National Science Foundation (INSF).

Conflict of interest

The authors declare no conflict of interest

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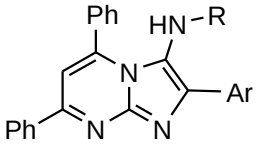
Table 1: *In vitro* cytotoxic activities of compounds **6a-o** against cancer cell lines.



Compound	Ar	R	(IC_{50}, μM)		
			MCF-7	MDA-MB-231	T-47D
6a	4-HOC_6H_4	Cyclohexyl	11.56±0.03	14.08±0.26	14.36±0.43
6b	2-$MeOC_6H_4$	Cyclohexyl	13.65±0.007	10.47±0.005	8.42±0.016
6c	3-OH-4-$MeOC_6H_3$	Cyclohexyl	10.92±0.04	10.43±0.18	6.72±0.81
6d	3-MeC_6H_4	Cyclohexyl	>100	33.54±0.01	69.73±1.02
6e	2-FC_6H_4	Cyclohexyl	16.97±0.001	9.90±0.01	8.88±0.06
6f	4-FC_6H_4	Cyclohexyl	81.43±0.006	>100	21.11±0.02
6g	3-ClC_6H_4	Cyclohexyl	>100	>100	>100
6h	2,4-*di*ClC_6H_3	Cyclohexyl	73.59±0.05	34.00±0.004	15.44±0.68
6i	3- $O_2NC_6H_4$	Cyclohexyl	>100	>100	61.23±0.29
6j	2-Thienyl	Cyclohexyl	77.33±0.02	>100	27.59±1.48
6k	2-$MeOC_6H_4$	*tert*-Butyl	11.41±0.54	12.64±0.09	7.93±0.49
6l	3-MeC_6H_4	*tert*-Butyl	27.95±0.27	42.53±0.40	72.53±0.78
6m	4-FC_6H_4	*tert*-Butyl	35.21±0.49	69.21±1.02	9.87±0.35
6n	4-ClC_6H_4	*tert*-Butyl	>100	84.11±0.25	46.98±0.39
6o	3- $O_2NC_6H_4$	*tert*-Butyl	93.00±0.08	>100	93.98±0.39
Etoposide	-	-	39.41±0.63	26.01±0.09	36.60±0.71