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Synthesis of Novel Substituted Pyridines from 1-(3-Aminophenyl)-3-(1*H*-indol-3-yl)prop-2-en-1-one and Their Anticancer Activity[1]

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Abstract—A series of novel substituted pyridine derivatives have been synthesized via the reaction of 3-indole carboxaldehyde with 3-aminoacetophenone. The products structures have been elucidated from elemental analysis as well as IR, ^{1}H NMR, ^{13}C NMR, and MS spectroscopy data. All the synthesized compounds have shown anticancer activity against HEPG2 and MCF-7 in vitro; some of them have exhibited the in vivo activity.

Keywords: pyridine derivative, triazolo[4,3-*a*]pyridine**,** anticancer activity

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A series of publications have discussed synthesis and biological activity (such as antifertility [1], antimicrobial [2, 3], anti-inflammatory [4], and anti-convulsant [5] effects) of heterocyclic nitrogen-containing compounds. Indole, a constituent of proteins in the form of tryptophan, is an important heterocyclic system; on top of that, certain drugs (i.e., indomethacin) and alkaloids (i.e., strychnine and LSD) contain indole as a constituent part. Indole derivatives have been synthesized and tested for anti-inflammatory [6, 7], 5a-reductase inhibition [8], antidiabetic [9] and antihistaminic [10], and anticancer [11–13] activity.

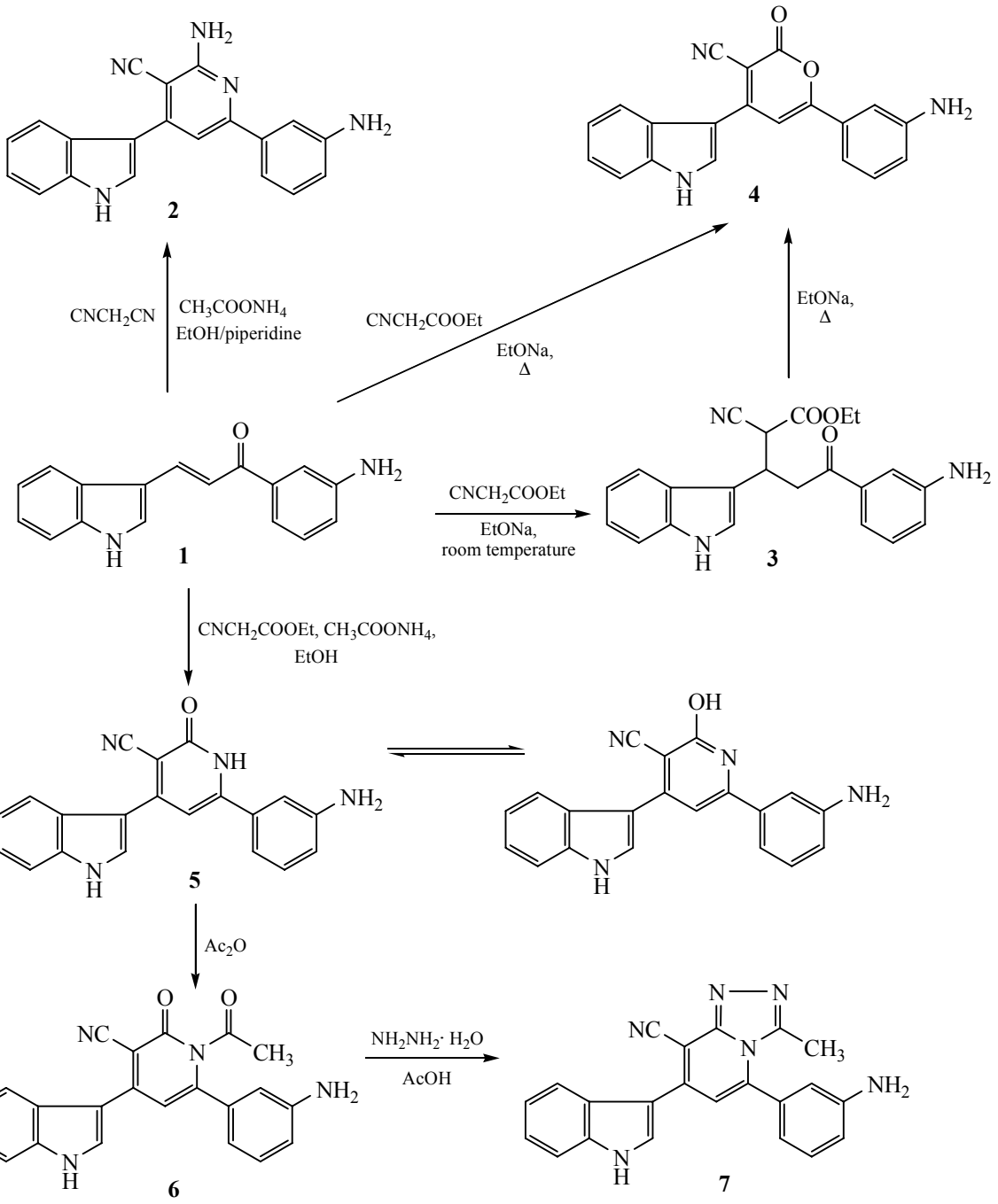
In the present study we prepared several novel substituted pyridine derivatives from 1-(3-aminophenyl)-3-(1*H*-indol-3-yl)prop-2-en-1-one (**1**) [14]. In detail, the reaction with malononitrile in the presence of ammonium acetate gave the corresponding cyano-amino pyridine derivative **2**. Compound **1** was also introduced in the reaction with ethyl cyanoacetate affording the corresponding compound **3** that was further cyclized into the corresponding pyran-3-carbonitrile derivative **4** with sodium ethoxide. Compound **4** was also prepared directly from compound **1** by the reaction with ethyl cyanoacetate in the presence of sodium ethoxide. Furthermore, chalcone **1** was treated with ethyl cyanoacetate in the presence of ammonium acetate to give the cyano pyridine derivative **5** that was acylated with anhydrous acetic acid anhydride to afford the *N*-acetyl derivative **6**. Triazolo[4,3-*a*]pyridine **7** was synthesized via refluxing of compound **6** with hydrazine hydrate in glacial acetic acid (Scheme 1).

The reaction of pyran-3-carbonitrile derivative **4** with hydrazine hydrate or 2,4-dinitrophenyl hydrazine afforded the corresponding *N*-aminocyanopyridone **8** and *N*-dinitrophenylamino pyridone **9**, respectively. Additionally, treatment of compound **4** with 2-amino-benzimidazole or 4-amino-benzene sulphonamide afforded the corresponding *N*-benzo[d]imidazole pyridone **10** and *N*-phenyl-4-sulfonalide pyridine derivatives **11**, respectively. Finally, compound **4** was reacted with 6-(4-aminophenyl)-1,6-dihydro-4-(1*H*-indol-3-yl)pyrimidin-2-amine or 2-aminobenzoic acid to give compounds **12** and **13**, respectively (Scheme 2).

Many indole derivatives linked to pyridines and others bioisosteric heterocyclic systems show anti-cancer activity, especially against MCF7 and HEPG2.

[1] The text was submitted by the authors in English.

Scheme 1. Synthetic routes for compounds **2–7**.



In view of the structural similarity between the structures of these compounds and the compounds prepared in this work, we screened the new derivatives for the anticancer activity against MCF7 and HEPG2 in vitro and in vivo.

Compounds **2** and **4**–**13** exhibited potential in vitro activity against MCF-7 and HEPG4 (cf. the IC_{50} values listed in Tables 1 and 2, respectively). Screening of the most active compounds in vivo revealed their potential activity against HEPG4 and MCF-7 (cf. Tables 3 and 4, respectively).

EXPERIMENTAL

Melting points were determined in open glass capillary tubes with an Electro Thermal Digital

Scheme 2. Synthetic routes for compounds **8–13**.

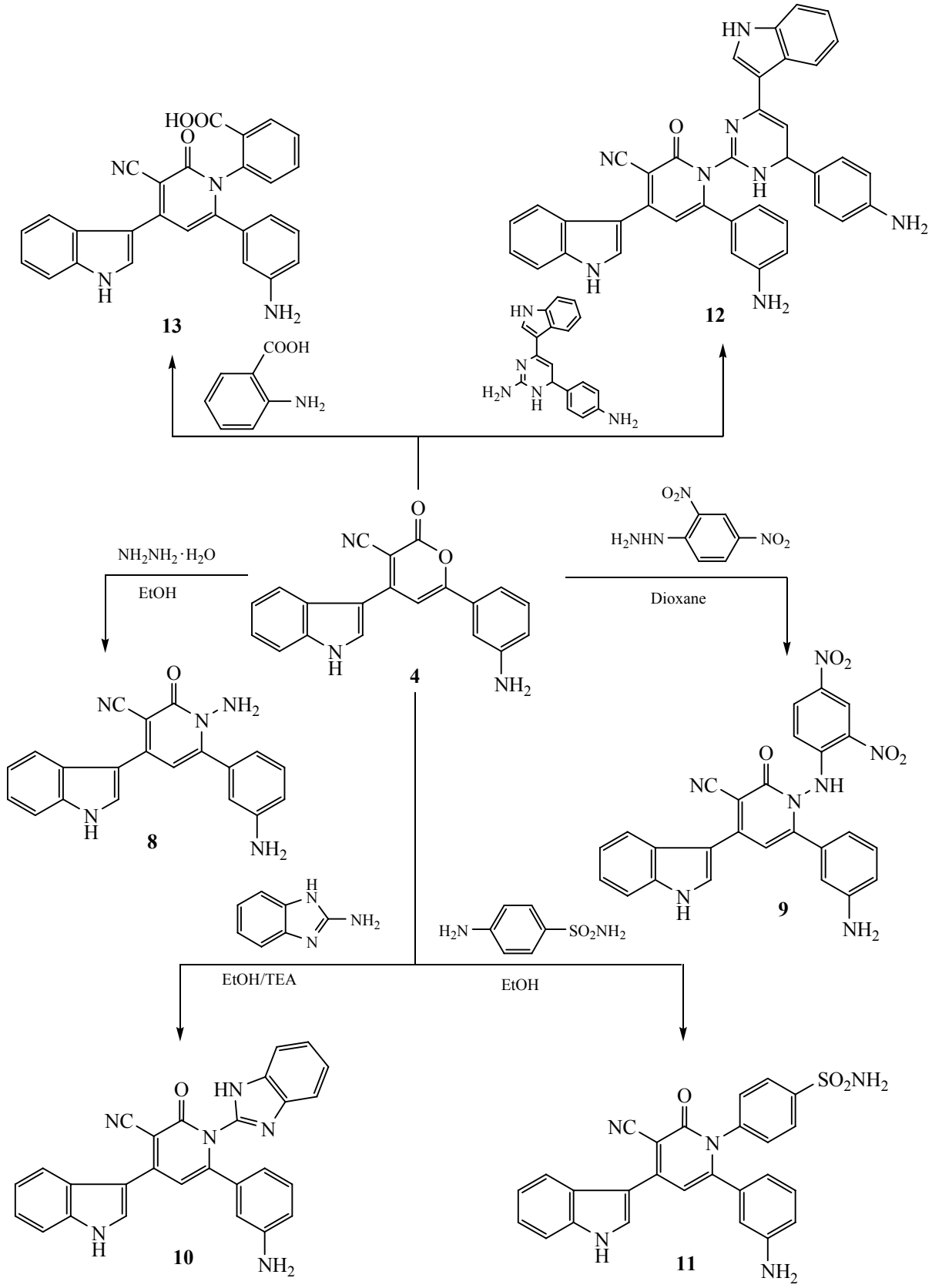


Table 1. In vitro anti-MCF-7 activity of the selected tested compounds[a]

Comp. no	IC_{50}, nM
2	0.056±0.001
4	0.078±0.001
5	0.054±0.001
6	0.043±0.001
7	0.035±0.001
8	0.024±0.001
9	0.013±0.001
10	0.0056±0.0001
11	0.0089±0.0001
12	0.0034±0.0001
13	0.0012±0.0001

[a] Mean values of the data from three separate experiments are given. All results are significant different from control values at $p \leq 0.005$. All results are significant different from reference standard values at $p \leq 0.005$.

Table 2. In vitro anti-HEPG2 activity of the selected tested compounds[a]

Comp. no	IC_{50}, nM
2	0.91±0.01
4	1.7±0.1
5	0.89±0.01
6	0.76±0.01
7	0.68±0.01
8	0.56±0.01
9	0.44±0.01
10	0.29±0.01
11	0.31±0.01
12	0.23±0.01
13	0.17±0.01

[a] Mean values of the data from three separate experiments are given. All results are significant different from control values at $p \leq 0.005$. All results are significant different from reference standard values at $p \leq 0.005$.

melting point apparatus (model IA9100) and are given uncorrected. Elemental microanalysis for carbon, hydrogen, and nitrogen (Microanalytical Unit, NRC) was found within the acceptable limits of the calculated values. Infrared spectra (KBr) were recorded using a Nexus 670 FTIR Nicolet infrared spectrometer. ^{1}H NMR spectra of the solutions in DMSO-d_6 were recorded using Jeol 500 MHz instrument. EI mass spectra were registered using a MAT Finnigan SSQ 7000 spectrometer. Analytical thin layer chromatography (TLC) was performed on silica gel aluminum sheets, 60 F_{254} (E. Merck). Starting material **1** was prepared as described in [14].

2-Amino-6-(3-aminophenyl)-4-(1*H*-indol-3-yl)-pyridine-3-carbonitrile (2). 0.01 mol of malononitrile, 0.01 mol of ammonium acetate and a few drops of piperidine were added to a solution of 0.01 mol of

Table 3. In vivo anti-HEPG2 activity of the selected tested compounds[a]

Comp. no.	Tumor growth V_t/V_0			
	16 days	20 days	24 days	28 days
Control	3.66±0.01	4.38±0.01	5.07±0.01	7.12±0.01
8	1.19±0.01	1.61±0.01	2.01±0.01	2.79±0.01
9	1.17±0.01	1.55±0.01	1.92±0.01	2.28±0.01
10	1.13±0.01	1.30±0.01	1.77±0.01	1.98±0.01
11	1.15±0.01	1.45±0.01	1.88±0.01	2.23±0.01
12	1.11±0.01	1.27±0.01	1.61±0.01	1.90±0.01
13	1.10±0.01	1.22±0.01	1.45±0.01	1.77±0.01

[a] Mean values of the data from three separate experiments are given. All results are significant different from control values at $p \leq 0.005$. All results are significant different from reference standard values at $p \leq 0.005$.

Table 4. In vivo anti-MCF-7 activity of the selected tested compounds[a]

Comp. no.	Tumor growth V_t/V_0										
	0 day	2 days	4 days	6 days	8 days	10 days	12 days	14 days	16 days	18 days	20 days
Control	1.00	1.38	1.81	4.75	9.88	12.64	24.75	27.66	29.00	38.90	40.21
8	1.00	1.28	1.41	3.11	3.89	4.77	5.98	6.89	7.90	8.90	11.99
9	1.00	1.27	1.35	2.98	3.25	4.56	5.89	6.78	7.77	8.89	11.12
10	1.00	1.22	1.27	2.11	2.56	3.56	4.88	5.67	6.57	7.56	8.23
11	1.00	1.23	1.32	2.56	2.78	3.90	5.11	6.12	7.21	8.19	10.20
12	1.00	1.18	1.22	1.90	2.45	3.12	3.56	5.11	6.12	7.18	8.16
13	1.00	1.12	1.19	1.89	2.12	2.89	3.22	4.34	5.12	6.55	7.13

[a] Mean values of the data from three separate experiments are given. All results are significant different from control values at $p \le 0.005$. All results are significant different from reference standard values at $p \le 0.005$.

chalcone **1** in 30 mL of anhydrous ethanol. The reaction mixture was refluxed during 3 h, cooled, and dropped into acidified icy water. The formed precipitate was filtered off, washed with water, dried, and recrystallized from methanol. Yield 78%, mp 187–189°C. IR spectrum, ν, cm^{-1}: 3285 (NH), 3240 (NH_2), 2152 (NH_2), 2223 (C≡N). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 7.28 s (2H, NH_2, D_2O exchangeable), 7.32 s (2H, NH_2, D_2O exchangeable), 7.58 s (1H, CH pyridine), 7.70–8.72 m (9H, Ar), 12.74 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 111.2, 112.6, 113.4, 113.7, 114.5, 114.7, 115.6, 117.2, 118.3, 119.4, 119.7, 121.2, 123.5, 123.8, 124.2, 124.3, 126.8, 128.4, 134.3, 137.6 (20C). Mass spectrum: *m/z* 325 [M^+]. Found, %: C 73.72; H 4.60; N 21.44. $C_{20}H_{15}N_5$. Calculated, %: C 73.83; H 4.65; N 21.52.

Ethyl 5-(3-aminophenyl)-2-cyano-3-(1*H*-indol-3-yl)-5-oxopentanoate (3). 0.01 mol of ethyl cyanoacetate in sodium ethoxide solution prepared from 0.01 mol of metal sodium and 50 mL of anhydrous ethanol was added to a stirred solution of 0.01 mol of chalcone **1** in 30 mL of anhydrous ethanol. The mixture was stirred during 8 h; the formed solid was filtered off, washed with diluted hydrochloric acid, dried, and recrystallized from dioxane. Yield 76%, mp 165–167°C. IR spectrum, ν, cm^{-1}: 3316 (NH), 3262 (NH_2), 2213 (CN), 1736 (C=O), 1698 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 1.29–1.45 t (3H, CH_3 of ethyl), 3.35 d (2H, CH_2), 4.28 q (2H, CH_2 of ethyl), 4.31 s (2H, NH_2, D_2O exchangeable), 7.26 d (1H, CH), 7.29–7.31 m (1H, CH), 7.57–8.58 m (9H, Ar), 12.59 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 17.60, 62.20, 62.30, 92.40, 92.50, 112.10, 112.30, 114.80, 114.90, 119.20, 119.60, 120.20, 120.80, 121.30, 126.30, 126.50, 128.30, 128.40, 143.30, 143.50, 164.40, 164.50 (22C). Mass spectrum: *m/z* 375 [M^+]. Found, %: C 70.30; H 5.56; N 11.10. $C_{22}H_{21}N_3O_3$. Calculated, %: C 70.38; H 5.64; N 11.19.

6-(3-Aminophenyl)-4-(1*H*-indol-3-yl)-2-oxo-2*H*-pyran-3-carbonitrile (4). *a*. 0.01 mol of chalcone **1** and 0.01 mol of ethyl cyanoacetate were added to a solution of sodium ethoxide prepared from 0.01 mol of metal sodium in 30 mL of anhydrous ethanol. The mixture was refluxed during 5 h; the formed solid was filtered off, dried, and recrystallized from ethanol. Yield 77%.

b. 0.01 mol of compound **3** in sodium ethoxide solution prepared from 0.01 mol of metal sodium in 30 mL of anhydrous ethanol was refluxed during 3 h; the formed solid was filtered off, dried, and recrystallized from ethanol. Yield 84%, mp 205–207°C. IR spectrum, ν, cm^{-1}: 3321 (NH), 3225 (NH_2), 2212 (C≡N), 1698 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 4.32 s (2H, NH_2, D_2O exchangeable), 7.15 s (1H, CH), 7.18–8.59 m (9H, Ar), 12.91 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 10.40, 113.50, 114.20, 118.20, 119.80, 121.10, 122.40, 123.30, 124.70, 125.40, 128.20, 129.10, 130.60, 134.30, 134.90, 136.20, 136.60, 138.10, 148.70, 165.60 (20C). Mass spectrum: *m/z* 328 [M^+]. Found, %: C 73.30; H 3.85; N 12.76. $C_{20}H_{13}N_3O_2$. Calculated, %: C 73.38; H 4.00; N 12.84.

6-(3-Aminophenyl)-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxopyridine-3-carbonitrile (5). 0.01 mol of

ammonium acetate was added to a solution of 0.01 mol of chalcone **1** in 50 mL of anhydrous ethanol and 0.01 mol of ethyl cyanoacetate. The mixture was refluxed during 6 h. The formed precipitate was filtered off, washed with ethanol, dried, and recrystallized from dioxane/methanol mixture. Yield 88%, mp 172–174°C. IR spectrum, ν, cm^{-1}: 3318 (NH), 3258 (NH), 3240 (NH_2), 2212 (C≡N), 1697 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 4.29 s (2H, NH_2, D_2O exchangeable), 7.27 s (1H, CH pyridine), 7.30–7.97 m (9H, Ar), 8.57 s (1H, NH, D_2O exchangeable), 12.52 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 110.10, 110.30, 113.30, 113.50, 113.90, 118.20, 118.40, 119.10, 119.20, 122.40, 122.60, 124.20, 124.50, 127.30, 133.20, 137.10, 137.20, 147.30, 147.50, 163.90 (20C). Mass spectrum: *m/z* 326 [M^+]. Found, %: C 73.53; H 4.25; N 17.10. $C_{20}H_{14}N_4O$. Calculated, %: C 73.61; H 4.32; N 17.17.

1-Acetyl-6-(3-aminophenyl)-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxopyridine-3-carbonitrile (6). 0.01 mol of compound **6** was refluxed in 30 mL of acetic anhydride during 3 h, cooled, and dropped into icy water. The formed precipitate was filtered off, dried, and crystallized from methanol. Yield 67%, mp 112–114°C. IR spectrum, ν, cm^{-1}: 3333 (NH), 3116 (NH_2), 2215 (CN), 1716 (C=O), 1694 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 2.80 s (3H, CH_3), 4.35 s (2H, NH_2, D_2O exchangeable), 7.37 s (1H, CH pyridine), 7.46–8.83 m (9H, Ar), 12.58 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 25.30, 111.50, 112.80, 113.40, 115.20, 123.50, 126.60, 129.20, 130.80, 131.60, 135.60, 137.80, 138.40, 141.50, 144.20, 145.60, 147.70, 148.20, 149.30, 150.60, 194.60, 208.60 (22C). Mass spectrum: *m/z* 368 [M^+]. Found, %: C 71.65; H 4.30; N 15.15. $C_{22}H_{16}N_4O_2$. Calculated, %: C 71.73; H 4.38; N 15.21.

5-(3-Aminophenyl)-7-(1*H*-indol-3-yl)-3-methyl-[1,2,4]triazolo[4,3-*a*]pyridine-8-carbonitrile (7). A solution of 0.01 mol of compound **6** and 0.01 mol of hydrazine hydrate in 30 mL of glacial acetic acid was refluxed during 6 h, cooled, and dropped into icy water. The formed precipitate was filtered off, dried, and recrystallized from ethanol. Yield 75%, mp 147–149°C. IR spectrum, ν, cm^{-1}: 3350 (NH), 3274 (NH_2), 2217 (C≡N). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 1.94 s (3H, CH_3), 4.48 s (2H, NH_2, D_2O exchangeable), 6.85 s (1H, CH pyridine), 7.13–8.69 m (9H, Ar), 10.18 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 21.90, 102.30, 108.50, 111.20, 114.30, 115.60, 117.20, 118.40, 121.60, 123.40, 125.60, 126.10, 128.40, 129.70, 130.20, 130.90, 132.40, 132.80, 136.50, 137.80, 142.40, 157.50 (22C). Mass spectrum: *m/z* 364 [M^+]. Found, %: C 72.44; H 4.35; N 23.00. $C_{22}H_{16}N_6$. Calculated, %: C 72.51; H 4.43; N 23.06.

***N*-Aminocyanopyridone derivative (8).** 0.01 mol of hydrazine hydrate was added to a solution of 0.01 mol of the cyanopyranone **4** in 30 mL of anhydrous ethanol. The mixture was refluxed during 3 h. The formed precipitate was filtered off, dried, and recrystallized from ethanol. Yield 86%, mp >300°C. IR spectrum, ν, cm^{-1}: 3215 (NH), 3190 (NH_2), 3110 (NH_2), 2218 (C≡N), 1670 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 7.17 s (2H, NH_2, D_2O exchangeable), 7.23 s (2H, NH_2, D_2O exchangeable), 7.48 s (1H, CH pyridine), 7.92–8.92 m (9H, Ar-H), 11.68 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 111.90, 112.60, 120.50, 121.30, 122.50, 122.90, 123.70, 124.80, 125.60, 125.90, 126.10, 127.50, 130.10, 132.40, 133.80, 135.30, 135.80, 136.80, 137.30, 156.30 (20C). Mass spectrum: *m/z* 341 [M^+]. Found, %: C 70.28; H 4.32; N 20.43. $C_{20}H_{15}N_5O$ (341.37). Calculated, %: C 70.37; H 4.43; N 20.52.

1-(2,4-Dinitrophenylamino)-6-(3-aminophenyl)-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxopyridine-3-carbonitrile (9). 0.01 mol of 2,4-dinitrophenylhydrazine was added to a solution of 0.01 mol of the cyanopyranone **4** in 30 mL of anhydrous ethanol and 10 mL of dioxane. The mixture was refluxed during 5 h. The formed precipitate was filtered off, dried, and recrystallized from dioxane. Yield 78%, mp 212–214°C. IR spectrum, ν, cm^{-1}: 3363 (NH), 3319 (NH), 3100 (NH_2), 2223 (C≡N), 1646 (C=O). ^1H NMR spectrum (DMSO-d_6), δ_H, ppm: 5.06 s (2H, NH_2, D_2O exchangeable), 7.23 s (1H, CH pyridine), 7.48–8.92 m (12H, Ar), 10.01 s (1H, NH, D_2O exchangeable), 11.13 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 113.10, 113.60, 114.30, 115.30, 116.50, 118.40, 118.90, 119.80, 120.70, 121.60, 123.10, 123.90, 124.80, 125.60, 126.50, 128.50, 129.70, 130.20, 131.10, 133.60, 135.30, 135.80, 136.80, 137.30, 138.70, 157.20 (26C). Mass spec-trum: *m/z* 507 [M^+]. Found, %: C 61.47; H 3.30; N 19.26. $C_{26}H_{17}N_7O_5$. Calculated, %: C 61.54; H,3.38; N 19.32.

6-(3-Aminophenyl)-1-(1*H*-benzo[*d*]imidazol-2-yl)-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxopyridine-3-carbonitrile (10). 0.01 mol of 2-aminobenzimidazole was added to a solution of 0.01 mol of the cyanopyranone **4** in 50 mL of anhydrous ethanol and few drops of

triethylamine. The mixture was refluxed during 8 h and cooled. The formed solid was filtered off, dried, and crystallized from ethanol. Yield 77%, mp 223–225°C. IR spectrum, ν, cm^{-1}: 3383 (NH), 3225 (NH), 3167 (NH_2), 2208 (C≡N), 1692 (C=O) .^{1}H NMR spectrum (DMSO-d_6), δ_H, ppm: 6.11 s (2H, NH_2, D_2O exchangeable), 6.83 s (1H, CH pyridine), 7.09–8.29 m (13H, Ar), 9.94 s (1H, NH, D_2O exchangeable), 12.22 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_H, ppm: 112.20, 112.80, 113.20, 113.80, 117.60, 118.50, 118.70, 119.20, 119.40, 120.10, 120.40, 121.50, 121.80, 122.50, 123.70, 124.50, 124.90, 131.50, 132.10, 136.20, 136.70, 138.20, 140.10, 144.60, 153.20, 155.30, 185.50 (27C). Mass spectrum: *m/z* 442 [M^+]. Found, %: C 73.20; H 4.02; N 18.90. $C_{27}H_{18}N_6O$. Calculated, %: C 73.29; H 4.10; N 18.99.

6-(3-Aminophenyl)-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxo-1-(phenyl-4-sulfonamide)pyridine-3-carbonitrile (11). 0.01 mol of sulfonamide was added to a solution of 0.01 mol of the cyanopyridone **4** in 40 mL of anhydrous ethanol and 5 mL of DMF. The mixture was refluxed during 6 h and cooled. The formed precipitate was filtered off, dried, and recrystallized from ethanol. Yield 82%, mp >300°C. IR spectrum, ν, cm^{-1}: 3461 (NH), 3373 (NH_2), 3264 (NH_2), 2212 (C≡N), 1698 (C=O). ^{1}H NMR spectrum (DMSO-d_6), δ_H, ppm :4.29 s (2H, NH_2, D_2O exchangeable), 5.82 s (2H, NH_2, D_2O exchangeable), 6.58 s (1H, CH pyridine), 6.89–8.58 m (13H, Ar), 12.76 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 110.80, 112.40, 112.80, 113.50, 113.70, 118.20, 118.60, 119.10, 119.40, 122.20, 122.70, 123.80, 127.30, 127.7, 127.90, 130.50, 130.8, 133.70, 136.50, 136.90, 147.20, 147.60, 152.30, 157.80, 158.20, 164.00 (26C). Mass spectrum: *m/z* 481 [M^+]. Found, %: C 64.75; H 3.90; N 14.48; S, 6.60. $C_{26}H_{19}N_5O_3S$. Calculated, %: C 64.85; H 3.98; N 14.54; S, 6.66.

6-(3-Aminophenyl)-1-[6-(4-aminophenyl)-1,6-dihydro-4-(1*H*-indol-3-yl)-pyrimidin-2-yl]-1,2-dihydro-4-(1*H*-indol-3-yl)-2-oxopyridine-3-carbonitrile (12). 0.01 mol of 6-(3-aminophenyl)-1,6-dihydro-4-(1*H*-indol-3-yl)pyrimidin-2-amine was added to a solution of 0.01 mol of the cyanopyranone **4** in a mixture of 50 mL of anhydrous ethanol and 5 mL of glacial acetic acid. The mixture was refluxed during 4 h and cooled. The formed precipitate was filtered off, dried, and recrystallized from ethanol. Yield 82%, mp 165–167°C. IR spectrum, ν, cm^{-1}: 3404 (NH), 3380 (NH), 3327 (NH), 3290 (NH_2), 3213 (NH_2), 2213 (C=N), 1699 (C=O). ^{1}H NMR spectrum (DMSO-d_6), δ_H, ppm: 4.29 s (2H, NH_2, D_2O exchangeable), 4.31 s (2H, NH_2, D_2O exchangeable), 6.85 d (1H, CH)), 6.93 d (1H, CH pyridine), 7.04 s (1H, CH), 7.15–8.81 m (18H, Ar), 10.33 s (1H, NH, D_2O exchangeable), 11.59 s (1H, NH, D_2O exchangeable), 12.59 s (1H, NH, D_2O exchangeable). Found, %: C 74.40; H 4.54; N 18.20. $C_{38}H_{28}N_8O$. Calculated, %: C 74.49; H 4.61; N 18.29.

2-[6-(3-Aminophenyl)-3-cyano-4-(1*H*-indol-3-yl)-2-oxopyridin-1(2*H*)-yl]benzoic acid (13). 0.01 mol of anthranilic acid was added to a solution of 0.01 mol of cyanopyranone **4** in 30 mL of anhydrous ethanol and 10 mL of glacial acetic acid. The mixture was refluxed during 10 h, cooled, and dropped into icy water. The formed precipitate was filtered off, dried, and recrystallized from methanol. Yield 84%, mp 231–233°C. IR spectrum, ν, cm^{-1}: 3490–3100 (OH), 3429 (NH), 3325 (NH_2), 2211 (CN), 1720 (C=O), 1698 (C=O). ^{1}H NMR spectrum (DMSO-d_6), δ_H, ppm: 3.54 br.s (1H, OH D_2O exchangeable), 4.28 s (2H, NH_2 D_2O exchangeable), 6.28 s (1H, CH pyridine), 6.37–8.63 m (13H, Ar), 9.95 s (1H, NH, D_2O exchangeable). ^{13}C NMR spectrum (DMSO-d_6), δ_C, ppm: 111.60, 114.10, 114.20, 115.50, 115.70, 118.20, 118.60, 118.90, 121.20, 121.50, 122.60, 122.70, 123.10, 125.20, 126.30, 126.90, 129.20, 129.40, 132.10, 138.60, 140.30, 146.20, 151.70, 151.90, 152.50, 164.40, 172.20 (27C). Mass spectrum: *m/z* 446 [M^+]. Found, %: C 72.55; H 4.00; N 12.50. $C_{27}H_{18}N_4O_3$. Calculated, %: C 72.64; H 4.06; N 12.55.

In vitro anti-MCF-7 activity [15–17]. The cytotoxicity of the synthesized compounds against cancer cell lines in vitro was performed using the MTT assay according to the Mosmann's method. The MTT assay is based on the reduction of the soluble 3-(4,5-methyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) into a blue-purple formazan product, mainly due to the mitochondrial reductase activity inside living cells. The cells used in cytotoxicity assay were cultured in the RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2×10^4 mL) were seeded in a 96-well culture plate and incubated at 37°C in a 5% CO_2 incubator. After 12 h, the test sample (2 μL) was added to the cells (2×10^4) in 96-well plates and cultured at 37°C for 3 days.

The cultured cells were mixed with 20 μL of the MTT solution and incubated during 4 h at 37°C. The supernatant was carefully removed from each well, and 100 μL of DMSO was added to each well to dissolve the formazan crystals which were formed via the

cellular reduction of MTT. After mixing using a mechanical plate mixer, the absorbance of each well was measured with a microplate reader using a test wavelength of 570 nm. The results were expressed as the IC_{50}, concentration inducing a 50% inhibition of growth of the treated cells as compared to the growth of the control cells. Each experiment was performed at least 3 times. There was a good reproducibility between replicate wells with standard errors below 10%.

Inhibition of HepG2 cell growth and proliferation by tested compounds. Cytotoxicity assay with HepG2 cells was performed to determine the IC_{50} of the tested compounds. HepG2 cells were trypsinized and washed with Ca^{2+}/Mg^{2+}-free PBS (pH = 7.2). The cells were adjusted to 4×10^4 cells/mL with DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) and plated (50 µl/well) in a 96-well cell culture plate (Corning, Corning, NY, USA) and incubated overnight at 37°C with 5% CO_2 and 95% humidity. 50 µL of serial 2-fold diluted sterile tested compounds were added to the final concentrations of 0–0.50 µg/mL. The culture medium was used as negative control. The cultures were incubated during further 2 d. The supernatants were discarded, added to 20 µl/well of methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Promega, Madison, WI, USA), and incubated during 4 h at 37°C with 5% CO_2. Sterile SDS (10% v/v in PBS) was added at 25 µl/well, and the plate was incubated at room temperature during 18 h before measuring the absorbance at 490 nm. Each assay was performed in triplicate, and DMEM was used as a blank control. The percentage of viable cells was calculated as [(O.D. of cell control–O.D. of treated cells)/(O.D. of cell control–O.D. of initiated cells)]×100, O.D. being the corresponding absorbance value.

The IC_{50} values were defined as the concentration resulting in 50% cell survival. The dose–response curve was plotted between viable cells (%) (*Y*-axis) and the final concentration of tested compounds (*X*-axis) to determine the IC_{50} value.

Animal experiments for studying the in vivo anti HEPG2 activities [18]. The animal study was approved by the Experimental Animal Committee of Faculty of Medicine, Chiang Mai University. All the animal experiments met the Animal Welfare guidelines. Male BALB/c nude mice (6 weeks old) were purchased from National research Center (Giza, Egypt). Mice were housed in laminar-flow cabinets under specific pathogen-free conditions at room temperature with a 24-h night-day cycle and fed with pellets and water ad libitum. Log growth-phase of HepG2 cells (1×10^5 cells in 0.1 mL of PBS) were injected subcutaneously into the right flank of athymic nude mice ($n = 4$) to establish a model of tumor-bearing mice. On day 4 after the implanting, the tested compounds was injected subcutaneously (10 µg/50 µL) daily in 4 doses. Monoclonal anti-platelet antibody (PY-13) and sterile PBS were used as isotype and negative controls, respectively.

Tumor growth was observed every 3 days by measuring its diameter with Vernier calipers. Tumor weight (TW) was calculated as TW (g) = tumor volume (cm^3) = $d^2 \times D/2$, where d is the shortest and D is the longest diameter, respectively. Mice were sacrificed when the tumor size reached 2.0 cm in diameter, and samples were collected.

Human breast cancer xenograft models and animal treatment [19]. The animal protocol was approved by the Institutional Animal Use and Care Committee of the University of Alabama at Birmingham. Female athymic pathogen-free nude mice (nu/nu, 4–6 weeks) were purchased from National research Center (Giza, Egypt). To establish MCF-7 human breast cancer xenografts, each of the female nude mice was first implanted with a 60-day sc slow release estrogen pellet (SE-121, 1.7 mg 17β-estradiol/pellet; Innovative Research of America, Sarasota, FL). The next day, cultured MCF-7 cells were harvested from confluent monolayer cultures, washed twice with serum-free medium, resuspended, and injected subcutaneously (s.c.) (5×10^6 cells, total volume 0.2 mL) into the left inguinal area of the mice. All animals were monitored for activity, physical condition, body weight, and tumor growth. Tumor size was determined by caliper measurement in two perpendicular diameters of the implant every other day. Tumor weight (TW) was calculated as TW (g) = tumor volume (cm^3) = $d^2 \times D/2$, where d is the shortest and D is the longest diameter, respectively.

The animals bearing human cancer xenografts were randomly divided into various treatment groups and a control group (7–10 mice/group). The untreated control group received the vehicle only. For the MCF-7 xenograft model, the tested compounds was dissolved in PEG400 : ethanol : saline (57.1 : 14.3 : 28.6, v/v/v), and administered by intraperitoneal (i.p.)

injection at doses of 5 and 10 mg/kg/day, 3 day/week during 3 weeks.

Statistical analysis. Statistical comparison of the difference between the control and the treated groups was performed via one-way ANOVA and Duncan's multiple comparison test $p < 0.05$.

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