Cytotoxic Effects of Newly Synthesized Heterocyclic Candidates Containing Nicotinonitrile and Pyrazole Moieties on Hepatocellular and Cervical Carcinomas

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Academic Editor: Qiao-Hong Chen
Received: 28 April 2019; Accepted: 16 May 2019; Published: 22 May 2019

Abstract: In this study, a series of newly synthesized substituted pyridine 9, 11–18, naphthopyridine derivative 10 and substituted pyrazolopyridines 19–23 by using cycnopyridone 8 as a starting material. Some of the synthesized candidates are evaluated as anticancer agents against different cancer cell lines. In vitro cytotoxic activities against hepatocellular and cervical carcinoma cell lines were evaluated using standard MTT assay. Different synthesized compounds exhibited potential in vitro cytotoxic activities against both HepG2 and HeLa cell lines. Furthermore, compared to standard positive control drugs, compounds 13 and 19 showed the most potent cytotoxic effect with IC50 values of 8.78 ± 0.7, 5.16 ± 0.4 µg/mL, and 15.32 ± 1.2 and 4.26 ± 0.3 µg/mL for HepG2 and HeLa cells, respectively.

Keywords: cyanopyridone; substituted pyridine; pyridotriazine; pyrazolopyridine; thioxotriazopyridine; anticancer activity; HepG2; HeLa

1. Introduction

Multicomponent reactions (MCR) “in which three or more starting materials react to form a product” play a significant role in the synthesis of heterocyclic compounds with pharmaceutical and chemical importance [1]. Several nicotinonitriles have been constructed via (MCR) and showed antitumor [2], antimicrobial [3], and antioxidant [4] activities. Also nicotinonitriles have been utilized as a scaffold for the synthesis of heterocyclic compounds containing a pyridine moiety with antimicrobial and antiviral activities [5]. A series of nicotinonitriles 1–3 (Figure 1) and have been synthesized and anti-proliferative [6], anti-Alzheimer’s [7], and anti-inflammatory [8] activities.
The pyrazole moiety is both pharmacologically and medicinally significant [9]. A series of pyrazoles 4–7 (Figure 2) has been reported as anti-inflammatory activity by Bekhit et al. [10], they observed that the synthesized pyrazoles showed more anti-inflammatory activity than the standard indomethacin [11]. Trisubstituted pyrazoles have been constructed by Christodoulou et al. (2010) [11] and evaluated as anti-angiogenic agents; these derivatives showed a potent anti-angiogenic efficacy and moreover inhibited the growth of Mammary gland breast cancer (MCF-7) and cervical carcinoma (Hela) [12]. Recently novel derivatives of pyrazoles 5,6 have been prepared as antimicrobial [13] and anticonvulsant [14] agents. The pyrazole 7 has been prepared by Bonesi et al. (2010) [15] and showed effective Angiotensin -1-Converting Enzyme (ACE) inhibitor activity [15].

Based on the previous facts about the importance of pyrazoles and nicotinonitriles in medicinal chemistry, we have herein synthesized of some novel heterocyclic candidates containing nicotinonitrile and pyrazole moieties and tested their anticancer activity.

2. Results

2.1. Chemistry

The nicotinonitriles were obtained by two different ways, from the reaction of chalcone with ethylcyanoacetate, ammonium acetate and drops of piperidine as a base and from one pot four components reaction of methylketone, aldehyde, ethylcyanoacetate, ammonium acetate and drops of piperidine as a base [15]. In prolongation of our work in the synthesis of heterocyclic compounds and evaluation of their medicinal importance [16–27] and based on the literature survey about the

Figure 1. Nicotinonitriles with anti-proliferative, anti-Alzheimer’s anti-inflammatory activities.

Figure 2. Pyrazoles as anti-inflammatory antimicrobial and anticonvulsant activities.
pharmacological and medicinal importance of pyrazoles and nicotinonitriles, we have devoted our efforts to design and synthesize novel heterocyclic compounds containing pyrazol and nicotine-nitrile moieties, 4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-hydroxy-6-(naphthalen-1-yl)-nicotinonitrile 8 has been obtained by reacting of 1-acetylnapthalene (A), 3-(4-fluorophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (B), ethyl 2-cyanoacetate, ammonium acetate and piperidine (Scheme 1).

![Scheme 1. Synthesis of compound 8 as starting material.](image)

The structure of the nicotinonitrile 8 has been confirmed from its spectral data. IR spectrum showing absorption frequencies at \(\nu = 3159 \text{ cm}^{-1}, 2220 \text{ cm}^{-1}\) and \(\nu = 1647 \text{ cm}^{-1}\) for OH, C≡N and C=N groups, respectively. Also, \(^1\)H-NMR spectrum of the assigned compound displayed signals at \(\delta = 12.89 \text{ ppm}\) (disappeared with D\(_2\)O) corresponding to acidic OH. A compelling evidence for the structure of 8 was provided by \(^13\)C-NMR spectrum that showed a singlet signal at \(\delta = 149.8, 139.3\) and \(139.3 \text{ ppm}\) for C-OH, C=N and C≡N groups respectively. Mass spectra of 8 showed [M\(^+\)] at \(m/z\) (%) 482 (22). Treatment of 8 with ethylchlorooacetate afforded compound 9, which was hydrazinolysis with NH\(_2\)NH\(_2\) to give the corresponding cyclized product 10.

Remediation of the nicotinonitrile derivative 8 with malonitrile in the presence of few drops of piperidine afforded 1,8-naphthyridine-3-carbonitrile derivative 11. Chlorination of 8 by a mixture of (POCl\(_3/\)PCl\(_5\)) afforded 2-chloronicotinonitrile derivative 12, which was reacted with malonitile as a carbon nucleophile gave the nicotinonitrile derivative 13. Reaction of 12 with primary and secondary amines, namely, o-aminothiophenol, morpholine, 1-methylpiperazine and hydrazine hydrate gave novel nicotinonitriles 14, 15a, b and 16 (Scheme 2). The mechanism formation route of compound 11 has been shown in Figure 3.

![Figure 3. The mechanism formation route of compound 11.](image)
Scheme 2. Synthetic route for compounds 9–16.

Compound 16 was utilized as a building block for novel nicotinonitriles containing two pyrazole moieties. 2-Pyrazolyl nicotinonitrile derivatives 17 and 18 were prepared by treatment of 16 with acetyl acetone and 4,4,4,-trifluoro-1-(thiophen-2-yl)butane-1,3-dione, respectively. Treatment of 16 with acetic anhydride and acetic acid afforded pyrazolopyridine derivative 19. The derivative 16 was treated with acetic anhydride to afford the N-acetyl pyrazolopyridine as a sole product 20. The structure of compound 20 was confirmed chemically by acetylation of the amino pyrazopyridine 19 (Scheme 3).

Treatment of 16 with 4-chlorobenzaldehyde and/or tetrachlorophthalic anhydride in the presence of acetic acid afforded the cyclized 19 followed by condensation to give the Schiff’s base 21 and tetra chloroisodindoline 22, respectively. The structures of 21 and 22 were confirmed chemically by condensation of compound 19 with 4-chlorobenzaldehyde and/or tetrachlorophthalic anhydride to provide compounds 21 and 22, respectively. Treatment of hydrazinyl derivative 16 with CS₂ in the presence of alcoholic KOH provided thioxotriazolo pyridine derivative 23 (Scheme 3).
Compound 16 was utilized as a building block for novel nicotinonitriles containing two pyrazole moieties. 2-Pyrazolyl nicotinonitrile derivatives 17 and 18 were prepared by treatment of 16 with acetyl acetone and 4,4,4,-trifluoro-1-(thiophen-2-yl)butane-1,3-dione, respectively. Treatment of 16 with acetic anhydride and acetic acid afforded pyrazolopyridine derivative 19. The derivative 16 was treated with acetic anhydride to afford the N-acetyl pyrazolopyridine as a sole product 20. The structure of compound 20 was confirmed chemically by acetylation of the amino pyrazopyridine 19 (Scheme 3).

Treatment of 16 with 4-chlorobenzaldehyde and/or tetrachlorophthalic anhydride in the presence of acetic acid afforded the cyclized 19 followed by condensation to give the Schiff`s base 21 and tetra chloroisoindoline 22, respectively. The structures of 21 and 22 were confirmed chemically by condensation of compound 19 with 4-chlorobenzaldehyde and/or tetrachlorophthalic anhydride to provide compounds 21 and 22, respectively. Treatment of hydrazinyl derivative 16 with CS2 in the presence of alcoholic KOH provided thioxotriazolo pyridine derivative 23 (Scheme 3).

2.2. Cytotoxic Activity

The newly synthesized compounds were screened for their anticancer potentials against hepatocellular carcinoma HepG2 and cervical carcinoma HeLa. The cytotoxicity of the compounds was determined using MTT assay and DOX as a positive control [28–31]. The cytotoxic activities of the novel synthesized compounds 8–23 were estimated and the obtained results are presented in Figure 4. In general, it can be seen that all synthesized compounds exhibited cytotoxic activities against both tested cancer cell lines. Moreover, it can be seen that both cells reacted in a dose-dependent manner toward the applied concentrations. Additionally, both tested cell lines varied in their response toward different synthesized compounds. Furthermore, based on the IC50 values (Table 1) obtained for the tested compounds, it can be seen that cytotoxic activities ranged from very strong to non-cytotoxic. Compounds 13 and 19 exhibited the most potent cytotoxic effect (very strong activity) with IC50 8.78 ± 0.7, 5.16 ± 0.4 µg/mL, and 15.32 ± 1.2 and 4.26 ± 0.3 µg/mL for HepG2 and HeLa cells, respectively. Furthermore, it can be noticed that Cpd. 19 exhibited more or less stronger activity similar to DOX towards HepG2 cells, (IC50 5.16 ± 0.4 and 4.50 ± 0.2 µg/mL, respectively). On the other hand, it was stronger by about 23.5% than DOX against HeLa cells (4.50 ±
0.2 and 5.57 ± 0.4 μg/mL, respectively). Additionally, Cpd. 18 showed very strong activity towards HeLa cells with IC₅₀ value of 7.67 ± 0.6 μg/mL, while it exhibited strong activity towards HepG2 cells (IC₅₀ 16.70 ± 1.3 μg/mL). Moreover, Cpd. 14 showed strong cytotoxic activities towards both tested cell lines (IC₅₀ values 12.20 ± 1.0 and 19.44 ± 1.4 μg/mL for HepG2 and HeLa cells, respectively). Meanwhile, Cpd. 16 and 22 showed moderate and strong activities towards both cell lines. Cpd. 16 showed IC₅₀ value of 33.45 ± 2.3 and 10.37 ± 0.9 μg/mL against HepG2 and HeLa cells, respectively. Also, Cpd. 22 showed IC₅₀ of 26.64 ± 1.9 and 9.33 ± 0.8 μg/mL for HepG2 and HeLa cells, respectively. On the other hand, Cpd. 17 showed strong activity towards HepG2 cells (IC₅₀ 20.00 ± 1.7 μg/mL) and moderate activity towards HeLa cells (IC₅₀ 35.58 ± 2.6 μg/mL). Finally, Cpd. 9, 10, 11, 12, 15a, b, 17, 20, 21 and 23 showed activities ranging from moderate to non-cytotoxic, with IC₅₀ values ranging from 24.83 ± 1.8 to >100 μg/mL.

![Figure 4](image-url)
Table 1. IC₅₀ values obtained for the tested compounds against both HepG2 and HeLa cell lines.

| Compound | IC₅₀ (µM) * |
|----------|------------|
|          | HepG2      | HeLa       |
| 8        | 20.00 ± 1.7 | 35.58 ± 2.6 |
| 9        | 42.95 ± 3.2 | 55.00 ± 3.7 |
| 10       | 56.57 ± 3.4 | 47.02 ± 3.4 |
| 11       | 30.22 ± 2.1 | 43.64 ± 3.3 |
| 12       | 83.82 ± 4.5 | 89.72 ± 4.7 |
| 13       | 8.87 ± 0.70 | 15.32 ± 1.2 |
| 14       | 12.20 ± 1.0 | 19.44 ± 1.4 |
| 15a      | 90.05 ± 5.1 | >100       |
| 15b      | 68.19 ± 3.7 | 75.05 ± 4.5 |
| 16       | 33.45 ± 2.3 | 10.37 ± 0.9 |
| 17       | 49.66 ± 3.2 | 65.91 ± 4.1 |
| 18       | 16.70 ± 1.3 | 7.67 ± 0.60 |
| 19       | 5.16 ± 0.40 | 4.26 ± 0.30 |
| 20       | 64.39 ± 3.6 | 28.15 ± 2.2 |
| 21       | 37.42 ± 2.5 | 24.83 ± 1.8 |
| 22       | 26.64 ± 1.9 | 9.33 ± 0.80 |
| 23       | 73.48 ± 4.0 | 62.07 ± 3.9 |
| Doxorubicin| 4.50 ± 0.20 | 5.57 ± 0.40 |

* IC₅₀: 1–10 is (very strong), 11–20 is (strong), 21–50 is (moderate), 51–100 is (weak) and above is 100 (non-cytotoxic).

3. Discussion

During current work, multi-component reaction strategy was used to synthesize of compound 8, which was used as a building block for preparing 16 new derivatives. The cytotoxic potential of the new prepared compounds has been evaluated against HepG2 and HeLa cells. Results obtained showed potential cytotoxic activities against both cell lines. Compounds 13 and 19 showed the most cytotoxic effects (IC₅₀ 8.78 ± 0.7 and 5.16 ± 0.4 µg/mL, for HepG2 cells, and 15.32 ± 1.2 and 4.26 ± 0.3 µg/mL for HeLa cells, respectively). Also, results showed that both tested cell lines varied in their response toward different synthesized compounds. This can be attributed to the inherent differences in both cell lines in terms of membrane structure and organization, hence different cell lines react differently towards different compounds [32–35].

Different activities of the prepared compounds may be attributed to the structure–activity relationship of these compounds. It can be seen that conversion of Cpd. 12 to 13, 14 and 16, 18, 19 and 22 altered the cytotoxicity from weak to moderate and strong activity towards two cell lines. This explained due to the introduction of two more nitrile groups, which significantly increased the activity. Compound 14 exhibited very strong activity due to the entity of the SH and NH groups, which may be added to any unsaturated group in DNA (thia or aza Michael addition) or the formation of hydrogen bonds with either one of the nucleo-bases of the DNA, thus causing DNA damage. Furthermore, the cytotoxicity of Cpd. 16 may be due to the intermolecular hydrogen bonding of NH and NH₂ groups with DNA moieties. Additionally, conversion of Cpd. 16 to 18, 19 and 22 increased their cytotoxic activities against both cell lines. Introducing thiophene ring increases the cytotoxic effect of Cpd. 18 beside the effect of the pyrazole ring and the trifluoromethyl group. Additionally, introducing pyrazole ring bearing NH₂ group to Cpd. 16 increases the cytotoxic effect of Cpd. 19 to very strong effect against both cell lines. The introduction of chloroisoi- indoline-1,3-dione increases the cytotoxic effects of Cpd. 22. The chloro- group, with more electron withdrawing properties, may be the crucial for tumor cell inhibition beside the effect of the isoindoline-1,3-dioneas moderate cytokine inhibitor in cancer cells.
4. Materials and Methods

4.1. Chemistry

"Melting points reported are inaccurate. IR spectra were registered on Shimadzu FT-IR 8300 E (Shimadzu Corporation, Kyoto, Japan) spectrophotometer using the (KBr) disk technique. 1H-NMR spectra were determined on a Varian Spectrophotometer at 400 MHz using (TMS) as an internal reference and DMSO-d6 as solvent using (TMS) as internal standard. All chemical shifts (δ) are uttered in ppm. The mass spectra were determined using (MP) model MS-5988 and Shimadzu single focusing mass spectrophotometer (70 eV). Elemental analysis was investigated by Elemental analyzer Vario EL III.”

4.1.1. Synthesis of 4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-hydroxy-6-(naphthalen-1-yl)-nicotinonitrile (8)

A mixture of 1-acetyl naphthalene (A) (1.7 g, 0.01 mol), ethyl cyanoacetate (1.3 g, 0.01 mol), aldehyde (B) (3.6 g, 0.01 mol), ammonium acetate (5.40 g, 0.07 mol) and three drops of piperidine in ethanol (20 mL) was heated under reflux for 3 h. The obtained precipitate was filtered off, washed with cold water, dried and crystallized from ethanol/dioxane to give compound 8. Yield 75%, yellow powder, m.p. > 300 °C; IR (KBr): ν (cm−1) 3159 (OH), 2220 (C≡N), 1647 (C=O); 1H-NMR (DMSO-d6): δ (ppm) 12.89 (s, 1H, OH, disappeared by D2O), 9.80 (s, 1H, pyrazole-H), 8.39–7.78 (m, 7H, Ar-H for naphthalene), 7.75–7.37 (m, 10H, Ar-H). 13C NMR (DMSO-d6): δ (ppm) 149.8 (C=O-H), 139.3 (C=N), 119.3 (C≡N), 139.4, 134.3, 133.8, 133.5, 131.6, 131.2, 131.0, 130.9, 130.4, 130.3, 130.2, 129.9, 129.4, 129.3, 129.2, 129.8, 128.2, 127.8, 127.6, 127.0, 125.6, 125.1, 117.4, 114.8 (Ar-CH), 40.6, 39.9 (aliph-C); MS m/z (ESI): 482 [M+H]+ (22), 465 (21), 440 (12), 237 (100), 204; Anal. Calcd. for C31H19FN4O (482.50): C, 77.17; H, 3.97; N, 11.61. Found C, 76.98; H, 3.78; N, 11.52%.

4.1.2. Synthesis of ethyl 2-(3-cyano-4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-2-oxopyridin-1(2H)-yl)acetate (9)

A mixture of 8 (4.84 g, 0.01 mol), ethyl chloroacetate (1.22 g, 0.01 mol) and K2CO3 (2.2 g, 0.015 mol) in (CH3)2O (40 mL) was heated under reflux for 24 h, concentrated and poured on water; the obtained precipitate was collected by filtration off, dried and crystallized from EtOH/dioxane to give 9. Yield 74%, m.p. 158–160 °C; IR (KBr): ν (cm−1) 2204 (C≡N), 1751 (C=O ester), 1651 (C=O pyridine); 1H-NMR (DMSO-d6): δ (ppm) 9.15 (s, 1H, pyrazole-5H), 8.10–7.49 (m, 7H, Ar-H for naphthalene), 7.48–7.33 (m, 10H, Ar-H). 13C NMR (DMSO-d6): δ (ppm) 3159 (OH), 2204 (C≡N), 1751 (C=O ester), 1651 (C=O pyridine); MS m/z (ESI): 568 [M+H]+ (2.5), 495 (65), 237 (80), 127 (100); Anal. Calcd. for C33H25FN4O (568.60): C, 73.93; H, 4.43, N, 9.85. Found C, 73.80; H, 4.21; N, 9.64%.

4.1.3. Synthesis of 8-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-3-oxo-3,4-dihydro-2H-pyrido[2,1-c][1,2,4]triazine-9-carbonitrile (10)

A mixture of 9 (5.7 g, 0.01 mol), NH2NH2·H2O (2 mL, 0.04 mol) and EtOH (20 mL) was heated under reflux for 3 h. The outward appearance solid was filtered off, dried and crystallized from EtOH/dioxane to give 10. Yield 71%, yellow powder, m.p. > 300 °C; IR (KBr): ν (cm−1) 3209 (NH), 2218 (C≡N), 1647 (C=O); 1H-NMR (DMSO-d6): δ (ppm) 12.89 (s, 1H, NH, disappeared in D2O), 9.13 (s, 1H, pyrazole-5H), 8.87–7.65 (m, 7H, Ar-H for naphthalene), 7.63–6.85 (m, 10H, Ar-H), 6.10 (s, 2H, CH2). 13C-NMR (DMSO-d6): δ (ppm) 165.8 (C=O), 139.7 (C≡N), 136.1 (C≡N), 133.8, 133.4, 131.7, 130.9, 130.8, 130.7, 130.6, 130.3, 130.2, 129.9, 129.7, 129.2, 129.1, 128.8, 128.5, 128.1, 127.3, 126.8, 125.8, 125.6, 119.2, 119.1, 118.9, 118.5, 117.6 (Ar-CH), 119.3 (C≡N), 40.5, 39.9 (2CH), 17.6 (CH2); MS m/z (ESI): 519 [M+H+−OH] (82), 393 (64), 284 (100), 237 (68), 127 (56); Anal. Calcd. for C33H21FN6O (536.50): C, 73.87; H, 3.94; N, 15.66. Found C, 73.68; H, 3.24; N, 15.06%.
4.1.4. Synthesis of 5-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-7-(naphthalen-1-yl)-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carbonitrile (11)

Refluxing of compound 8 (4.84 g, 0.01 mol) with malononitrile (0.015 mol) in ethanol (20 mL) in the presence of solid of TEA for 5 h, then cooled, poured on ice/water, neutralized with drops of conc. HCl. The obtained solid was collected by filtration, crystallized from EtOH/dioxane to afford 11. Yield 71%, pale brown powder, m.p. > 300 °C; IR (KBr): ν (cm⁻¹) 3386, 3273 (NH₂), 3158 (NH), 2218 (C≡N), 1646 (C=O), 1H-NMR (DMSO-d₆): δ (ppm) 12.89 (s, 1H, NH, disappeared by D₂O), 9.08 (s, 1H, pyrazole-5H), 8.07–7.61 (m, 7H, Ar-H for naphthalene), 7.60–7.37 (m, 10H, Ar-H), 6.22 (s, 2H, NH₂, disappeared in D₂O). 13C-NMR (DMSO-d₆): δ (ppm) 149.9 (C=O), 139.3 (C=N), 133.8, 133.5, 131.0, 131.1, 131.00 (2), 130.9, 130.4, 130.3 (2), 130.2, 129.9 (2), 129.4, 129.1, 128.9 (2), 128.2, 127.8(2), 127.6, 127.1 (2), 125.6, 125.2 (2), 117.4, 116.8, 110.0 (Ar-CH), 119.3 (C≡N), 40.6, 39.9 (2CH); MS m/z (ESI): 532 [M⁺ – NH₃] (82), 516 (76), 440 (28), 310 (20), 237 (100); Anal. Calcd. for C₃₄H₂₁FN₄O (548.50): C, 74.44; H, 3.89; N, 15.32. Found C, 74.24; H, 3.25; N, 14.98%.

4.1.5. Synthesis of 2-chloro-4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-nicotinonitrile (12)

A mixture of 8 (4.82 g, 0.01 mol), PCl₅ (3 g, 0.03 mol) and POCl₃ (5 mL, 0.03 mol) was heated under reflux for 8 h, then it was poured on crushed ice. The formed solid was filtered off, dried and crystallized from EtOH/dioxane to give 12. Yield 61%, yellow powder, m.p. 164–166 °C; IR (KBr): ν (cm⁻¹) 2227 (C≡N), 1628 (C=O), 1484, 1392 (C=N), 133.8, 131.5, 131.0, 130.4, 130.3, 130.2, 129.8, 129.5, 129.2 (2), 129.1, 127.9, 127.6, 126.9, 125.8 (2), 125.4, 125.1, 119.3 (C≡N), 116.6, 115.5, 107.8 (Ar-CH), 40.6, 39.9 (2CH); MS m/z (ESI): 503 [M⁺ – NH₃] (6), 501 [M⁺] (50), 465 (100), 237 (82); Anal. Calcd. for C₃₁H₁₈FN₄O (500.90): C, 74.32; H, 3.62; N, 11.84. Found C, 74.12; H, 3.26; N, 11.42%.

4.1.6. Synthesis of 2-[4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-3-cyano-pyridinyl]malononitrile (13)

To a solution of 12 (5.0 g, 0.01 mol) in EtOH (20 mL), malononitrile (0.01 mol) and TEA (1 mL) were added. The reaction mixture was heated under for 3 h. After cooling, it was poured on water and neutralized with diluted HCl. The obtained solid was separated by filtration, washed with water, dried and crystallized from EtOH/dioxane to yield 13. Yield 76%, pale brown powder, m.p. 194–196 °C; IR (KBr): ν (cm⁻¹) 2203 (C≡N), 1527, 150.0, 1484, 1392 (C=N), 133.8, 131.5, 131.0, 130.4, 130.3, 130.2, 129.8, 129.5, 129.2 (2), 129.1, 127.9, 127.6, 126.9, 125.8 (2), 125.4, 125.1, 119.3 (C≡N), 116.6, 115.5, 107.8 (Ar-CH), 40.6, 39.9 (2CH); MS m/z (ESI): 530 [M⁺] (12), 440 (100), 237 (76), 204 (31); Anal. Calcd. for C₃₄H₁₉FN₆ (530.50): C, 76.97; H, 3.61; N, 15.84. Found C, 76.78; H, 3.42; N, 15.24%.

4.1.7. Synthesis of 14 and 15a,b

A mixture of 2-chloronicotinonitrile 12 (5.0 g, 0.01 mol) and the appropriate amine, namely, o-aminothiophenol, morpholine or 2-methylpiperidine (0.01 mol) in EtOH (20 mL) was heated under reflux for 3 h, then it was poured on cold water, filtered off and crystallized from EtOH/dioxane to afford 14 and 15a,b, respectively.

4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-[2-mercapto-phenylamino]-6-(naphthalen-1-yl) nicotinonitrile (14). Yield 74%, brown powder, m.p. 108–110 °C; IR (KBr): ν (cm⁻¹) 3330 (NH), 2208 (C≡N), 1H-NMR (DMSO-d₆): δ (ppm) 9.29 (s, 1H, pyrazole-5H), 9.06–8.54 (m, 4H, Ar-H, thionyl-H), 8.26–7.66 (m, 7H, Ar-H for naphthalene), 7.60–6.66 (m, 10H, Ar-H), 3.34 (s, 1H, NH, disappeared in D₂O), 1.20 (s, 1H, SH, disappeared in D₂O). MS m/z (ESI): 589 [M⁺] (32), 465 (82), 441 (62), 237 (100), 127(12), 124 (20); Anal. Calcd. for C₃₅H₂₄FN₈O (589.60): C, 75.36; H, 4.10; N, 11.88. Found C, 75.18; H, 4.05; N, 11.73%.
4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-morpholino-6-(naphthalen-1-yl)nicotinonitrile (15a). Yield 65%, pale brown powder, m.p. 130–133 °C; IR (KBr): ν (cm⁻¹) 2226 (C≡N), 1H-NMR (DMSO-d₆): δ (ppm) 9.16 (s, 1H, pyrazole-5H), 8.71–7.56 (m, 7H, Ar-H for naphthalene), 7.55–7.15 (m, 10H, Ar-H), 4.82 (s, 1H, H-N). MS m/z (ESI): 564 [M⁺] (52), 465 (28), 237 (100), 230 (7), 127 (12), 87 (22); Anal. Calcd. for C₃₅H₂₅FN₅O (551.60): C, 76.58, H, 5.18; N, 14.88. Found C, 75.99; H, 4.75; N, 14.70.

4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-(4-methylpiperazin-1-yl)-6-(naphthalen-1-yl)nicotinonitrile (15b). Yield 61%, brown powder, m.p. 156–158 °C; IR (KBr): ν (cm⁻¹) 2918 (aliph-H), 2227 (C≡N), 1H-NMR (DMSO-d₆): δ (ppm) 9.18 (s, 1H, pyrazole-5H), 8.71–7.65 (m, 7H, Ar-H for naphthalene), 7.64–7.12 (m, 10H, Ar-H), 3.30–3.25 (m, 4H, CH₂), 2.43–2.23 (m, 4H, 2CH₂), 2.24 (s, 3H, CH₃), MS m/z (ESI): 564 [M⁺] (27), 538 (25), 439 (12), 237 (100), 100 (23); Anal. Calcd. for C₃₅H₂₅FN₅O (564.60): C, 76.58, H, 5.18; N, 14.88. Found C, 75.98; H, 4.26; N, 14.72%.

4.1.8. Synthesis of 4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-hydrazinyl-6-(naphthalen-1-yl)nicotinonitrile (16)

A mixture of the 2-chloronicotinonitrile 12 (5.0 g, 0.01 mol) and NH₂NH₂·H₂O (0.04 mol) in EtOH (20 mL) was heated under reflux for 4 h. The obtained solid was collected by filtration, dried and crystallized from EtOH/dioxane to yield 16. Yield 86%, yellow powder, m.p. 164–168 °C; IR (KBr): ν (cm⁻¹) 3145 (NH₂), 2936, 2880, 2240 (C≡N), 1617 (C=N), 1517, 1492 (CH₂=CH₂), 1472 (CH₂), 1260, 1167, 1123 (CH₃), 794, 745 (C-H). MS m/z (ESI): 552 [M⁺] (13), 465 (28), 237 (100), 100 (23); Anal. Calcd. for C₃₃H₂₃FN₅O (540.65): C, 74.85; H, 4.84; N, 16.89. Found C, 74.68; H, 4.12; N, 14.78%.

4.1.9. Synthesis of 17 and 18

A mixture of 16 (4.9 g, 0.01 mol), acetylarone or 4,4,4-trifluoro-1-(thiophen-2-yl)butane-1,3-dione (0.01 mol) in EtOH (20 mL) and AcOH (4 mL) was heated for reflux for 3 h. After cooling, the solid obtained was filtered off, dried and crystallized from EtOH/dioxane to afford 17 and 18, respectively.

2-(5,5-Dimethyl-1H-pyrazol-1-yl)-4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)nicotinonitrile (17). Yield 85%, pale orange powder, m.p. 300–302 °C; IR (KBr): ν (cm⁻¹) 2226 (CH=N), 1600 (C=N), 1H-NMR (DMSO-d₆): δ (ppm) 9.24 (s, 1H, pyrazole-5H), 8.71–7.96 (m, 7H, Ar-H for naphthalene), 7.66–7.35 (m, 10H, Ar-H), 7.25 (s, 1H, pyrazole-4H), MS m/z (ESI): 560 [M⁺] (13), 465 (26), 438 (62), 237 (15), 95 (100); Anal. Calcd. for C₃₅H₂₅FN₅O (560.60): C, 77.13; H, 4.49; N, 14.99. Found C, 76.92; H, 4.32; N, 14.81%.

4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)nicotinonitrile (18). Yield 82%, dark yellow powder, m.p. 117–119 °C; IR (KBr): ν (cm⁻¹) 2209 (C≡N), 1H-NMR (DMSO-d₆): δ (ppm) 8.92 (s, 1H, pyrazole-5H), 8.03–7.89 (m, 7H, Ar-H for naphthalene), 7.59–7.54 (m, 3H, thionyl-H), 7.53–7.33 (m, 10H, Ar-H), 6.88 (s, 1H, pyrazole-4H), MS m/z (ESI): 583 [M⁺] (10), 465 (72), 237 (100), 299 (8), 217 (5); Anal. Calcd. for C₃₉H₂₅FN₅O (682.60): C, 68.61; H, 3.25; N, 12.31. Found C, 68.02; H, 3.12; N, 12.03%.

4.1.10. Synthesis of 19 and 20

A solution of 16 (4.9 g, 0.01 mol) in a mixture of Ac₂O/AcOH (10 mL) or in glacial AcOH (10 mL) was refluxed for 2 h, poured on ice/water, filtered off and crystallized from EtOH/dioxane to give 19 and 20, respectively. Also, refluxing of 19 (0.5 g, 0.01 mol) in acetic anhydride (7 mL) afforded compound 20.

4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)1H-pyrazolo[3,4-b]pyridin-3-amine (19). Yield 84%, pale yellow powder, m.p. 140–143 °C; IR (KBr): ν (cm⁻¹) 3425–3354 (NH₂),
3198 (NH), $^1$H-NMR (DMSO-d$_6$): $\delta$ (ppm) 8.92 (s, 1H, pyrazole-5H), 8.22–7.90 (m, 7H, Ar-H for naphthalene), 7.66–7.34 (m, 10H, Ar-H), 5.02 (s, 2H, NH$_2$, disappeared in D$_2$O), 4.63 (s, 1H, NH, disappeared in D$_2$O); MS $m/z$ (ESI): 496 [M$^+$] (28), 479 (76), 244 (50), 237 (100); Anal. Calcd. for C$_{31}$H$_{32}$F$_6$N$_6$ (496.52): C, 74.99; H, 4.26; N, 16.93. Found C, 74.70; H, 4.15; N, 16.82%.

$\text{N}-(4-(3-(4-Fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)acetamide (20).$ Yield 78%, yellow powder, m.p. 138–140 °C; IR (KBr): $\nu$ (cm$^{-1}$) 3196 (NH), 1690 (C=O), $^1$H-NMR (DMSO-d$_6$): $\delta$ (ppm) 12.37 & 10.31 (s, NH, OH), 8.88 (s, 1H, pyrazole-5H), 7.98–7.59 (m, 7H, Ar-H for naphthalene), 7.57–6.88 (m, 10H, Ar-H), 4.82 (s, 1H, NH, disappeared in D$_2$O), 2.73 (s, 3H, acetyl); MS $m/z$ (ESI): 538 [M$^+$] (20), 479 (36), 244 (20), 237 (100); Anal. Calcd. for C$_{33}$H$_{33}$F$_6$N$_6$O (538.59): C, 73.59; H, 3.82; N, 13.27%. Found C, 73.28; H, 3.49; N, 13.52%.

4.1.11. Synthesis of $\text{N}-(4-Chlorobenzylidene)-4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-1H-pyrazolo[3,4-b]pyridine-3-amine (21)$

A solution of 16 or 19 (0.01 mol) in AcOH (10 mL) in the presence of 4-chlorobenzaldehyde (0.01 mol) was heated under reflux for 2 h, left to precipitate, filtered off and crystallized from EtOH/dioxane to afford 21. Yield 58%, yellow powder, m.p. 158–160 °C; IR (KBr): $\nu$ (cm$^{-1}$) 3192 (NH), $^1$H-NMR (DMSO-d$_6$): $\delta$ (ppm) 9.89 (s, 1H, pyrazole-5H), 9.06 (s, 1H, N=C=O), 8.87–7.56 (m, 7H, Ar-H for naphthalene), 7.52–6.88 (m, 14H, Ar-H), 4.82 (s, 1H, NH, disappeared in D$_2$O); MS $m/z$ (ESI): 621 [M$^+$] (15), 619 (48), 479 (20), 237 (80), 139 (35), 137 (100); Anal. Calcd. for C$_{38}$H$_{24}$ClF$_6$N$_6$ (619.10): C, 73.72; H, 3.91; N, 13.57. Found C, 73.25; H, 3.82; N, 13.27%.

4.1.12. Synthesis of 2-(4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-6-(naphthalen-1-yl)-1H-pyrazolo[3,4-b]pyridin-3-yl)isoindoline-1,3-dione (22)$

A mixture of 16 or 19 (0.01 mol) and tetrachlorophthalic anhydride (0.01 mol) in glacial acetic acid (10 mL) was refluxed for 1 h, poured on ice water, filtered off and crystallized from EtOH/dioxane to yield 22. Yield 94%, yellow powder, m.p. 115–117 °C; IR (KBr): $\nu$ (cm$^{-1}$) 3196 (NH), 1785, 1731 (C=O); $^1$H-NMR (DMSO-d$_6$): $\delta$ (ppm) 8.87 (s, 1H, pyrazole-5H), 8.04–7.56 (m, 7H, Ar-H for naphthalene), 7.55–7.33 (m, 10H, Ar-H), 4.28 (s, 1H, NH, disappeared in D$_2$O); Anal. Calcd. for C$_{39}$H$_{19}$Cl$_4$F$_6$N$_6$O$_2$ (764.42): C, 61.28; H, 2.51; N, 10.99. Found C, 61.00; H, 2.42; N, 10.89%.

4.1.13. Synthesis of 7-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-5-(naphthalen-1-yl)-3-thioxo-2,3-di hydro[1,2,4]triazolo[4,3-a]pyridine-8-carbonitrile (23)$

Solution of hydrazinyl derivative 16 (4.9 g, 0.01 mol) in alcoholic KOH (10%, 20 mL) and CS$_2$ (0.01 mol) was refluxed for 2 h, lift overnight, then poured on ice water, filtered off the solid obtained and crystallized from EtOH/dioxane to afford 23. Yield 47% yellow powder, m.p. 288–290 °C; IR (KBr): $\nu$ (cm$^{-1}$) 3192 (NH), 2218 (C=N), 1240 (C=S); $^1$H-NMR (DMSO-d$_6$): $\delta$ (ppm) 8.73 (s, 1H, pyrazole-5H), 7.97–7.63 (m, 7H, Ar-H for naphthalene), 7.53–6.77 (m, 10H, Ar-H), 3.76 (s, 1H, NH, disappeared in D$_2$O); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ (ppm) 148.1 (C=S), 142.3, 138.7 (C=N), 133.8 (2), 133.4 (C=N), 131.7 (2), 131.2, 130.6 (2), 130.1, 129.9 (2), 129.4, 129.2 (2), 128.9, 128.4 (2), 126.9, 126.4 (2), 126.3 (2), 125.9 (2), 119.1 (C=S), 110.0 (Ar-CH), 40.5, 39.9 (2CH); MS $m/z$ (ESI): 538 [M$^+$] (45), 494 (18), 479 (10), 453 (50), 237 (100); Anal. Calcd. for C$_{32}$H$_{19}$F$_6$N$_6$S (538.60): C, 71.36; H, 3.56; N, 15.60. Found C, 71.31; H, 3.52; N, 15.58%.

4.2. Cytotoxicity Assay

4.2.1. Materials and Cell Lines

Hepatocellular carcinoma (HepG2) and cervical Carcinoma (HeLa) cell lines, ATCC, VA, USA, were used throughout the work. All used chemicals and reagents were of high purity-cell culture grade.
4.2.2. MTT Assay

Cytotoxic assay depends on the formation of purple formazan crystals by the action of dehydrogenase in living cells. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, antibiotic solution (100 units/mL penicillin, 100 µg/mL streptomycin) at 37 °C in a 5% CO2 incubator. Cells were seeded in a 96-well plate (10^4 cells/well), and the plates were incubated for 48 h. Afterwards, cells were exposed to variable concentrations of prepared derivatives and incubation proceeded for further 24 h. After treatment, 20 µL of MTT solution (5 mg/mL) was added and incubated for 4 h. DMSO (100 µL/well) is added and the developed color density was measured at 570 nm using a plate reader (ELx 800, BioTek, Winuski, VT, USA). Relative cell viability was calculated as (Atreated/Auntreated) ×100 [36,37]. Results were compared with doxorubicin as a positive control.

5. Conclusions

During the current investigation, we synthesized a new building block; namely 4-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-2-hydroxy-6-(naphthalen-1-yl)nicotinonitril, with the help of multicomponent reaction systems. From that compound, a series of 16 different nicotinonitril derivatives were synthesized, and their structural and spectral data were elucidated. Furthermore, in vitro cytotoxic activities against hepatocellular and cervical carcinoma cell lines were investigated. Obtained results revealed that different synthesized compounds showed promising in vitro cytotoxic activities against both HepG2 and HeLa cell lines. Compounds 13 and 19 showed the most potent cytotoxic effect (IC_{50}: 8.78 ± 0.7, 5.16 ± 0.4 µg/mL, and 15.32 ± 1.2 and 4.26 ± 0.3 µg/mL for HepG2 and HeLa cells, respectively.

Author Contributions: The listed authors contributed to this work as described in the following: A.A.E.-S. and A.K.E.-Z. synthesis, and interpreted the spectroscopic identification of the synthesized compounds, A.E.-G.E.A. and E.A.E. are interpreted the results, the experimental part and E.A.E. performed the revision before submission. All authors read and approved the final manuscript.

Funding: The authors are grateful to the Deanship of Scientific Research, king Saud University for funding through Vice Deanship of Scientific Research Chairs.

Acknowledgments: The authors are appreciative to Faculty of Science, Ain Shams University where the experimental part carried out in its laboratories and Faculty of Pharmaceutical, El-Masoura University to carry the anticancer activity in it.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.