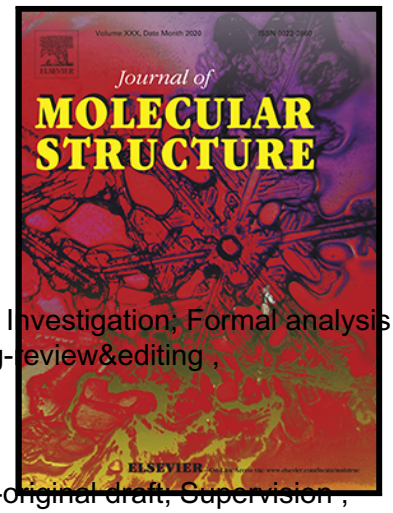

Novel 2,6-disubstituted pyridine hydrazones: Synthesis, anticancer activity, docking studies and effects on caspase-3-mediated apoptosis

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Highlights :

- New 2,6-disubstituted pyridine hydrazone derivatives were synthesized and evaluated for anticancer activity.
- **3f** and **3k** showed remarkable cytotoxic activity against HT-29 with IC_{50} value of 6.78 and 8.88 μM, respectively.
- Also, **3g** showed the best cytotoxic activity against ISH with IC_{50} value of 8.26 μM.
- **3f, 3g** and **3k** caused morphological changes of tumor cells and induced apoptosis
- Molecular docking was performed into BRAF kinase binding site

Novel 2,6-disubstituted pyridine hydrazones: Synthesis, anticancer activity, docking studies and effects on caspase-3-mediated apoptosis

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Abstract

Novel pyridine-based dihydrazones (**3a-l**) were synthesized by the condensation of appropriate aldehydes and pyridine-2,6-dicarbohydrazide (**2**) which was obtained by the treatment of dimethyl pyridine-2,6-dicarboxylate (**1**) with hydrazine hydrate. Structures of all the synthesized compounds were supported by their FTIR, ^{1}H-NMR, ^{13}C-NMR and microanalytical data. The compounds were screened primarily for their antibacterial activities as well as anticancer activities. None of the synthesized compounds had important antibacterial activity. Among the compounds which were tested against human colon cancer cell line (HT-29), compounds **3f** and **3k** showed significant activity (IC_{50}=6.78 µM for compound **3f**, IC_{50}=8.88 µM for compound **3k**). In addition, compound **3g**

exhibited promising activity against Ishikawa human endometrial cancer cell line (ISH) with an IC_{50} value of 8.26 μM. At 10 μM, compounds **3f**, **3k** and **3g** caused morphological changes of HT-29 and ISH cells and caspase-3 activation. In addition, these compounds were evaluated against NIH 3T3 mouse embriyonic fibroblast cell line and all synthesized compounds **(3a-l)** were found to be less toxic than paclitaxel. Moreover, possible inhibition mechanism of compound **3g** was evaluated *in silico* against BRAF kinase enzyme.

Keywords: 2,6-Disubstituted pyridine, hydrazone, BRAF, apoptosis, anticancer, molecular modeling.

1. Introduction

Endometrial cancer (EC) is one of the the most common gynecologic cancers in developed countries, which affects female reproductive organs [1–3]. Endometrial cancer is mostly unexpected clinically or pathologically in women <45 years of age because cases usually happen in patients over 50 [4]. Hormonal factors, obesity, family medical history, diabetes and diet importantly affect the risk of developing endometrial cancer [5]. It has also been reported that women with endometrial cancer have a higher risk of colon cancer [6,7]. Fornasarig *et al.* found that, 13.5% of endometrial cancer cases had colorectal cancers in their family history [8].

Protein kinase family is one of the largest protein families in the human genome. Protein kinases are responsible for maintaining cellular functions and biological balance [9]. There are 538 protein kinase enzymes identified in the body that are responsible for maintaining biological processes [10]. The fact remains that abnormal activity of kinase proteins is linked with several diseases including cancer [11]. After the introduction of imatinib to the clinic in the year 2001, the number of kinase inhibitors as well as efforts to generate novel kinase inhibitors are increasing day by day [12]. Today, there are 52 kinase inhibitor compounds in the clinic approved by the FDA [13].

BRAF is a serine threonine kinase which is considered as a critical therapeutic target in different types of cancer [14]. Especially, V600E mutant form of BRAF is found in many human cancers including lung cancer [15], colorectal cancer [16,17], melanoma [18] and endometrial adenocarcinoma [19].

In medicinal chemistry, compounds bearing hydrazide-hydrazone moiety have received much attention due to their potent biological value in the development of novel antimicrobials, analgesic and antiinflammatory and especially anticancer agents [20]. The compound PAC-1 (Figure 1), bearing hydrazone moiety, was reported to enhance the activity of procaspase-3 *in vitro* and induce apoptosis in tumor cells *in vivo* [21]. Picolinoylhydrazone derivative **(1)** is a potent growth inhibitor of lung, leukemia and ovarian cancer cell lines with with IC_{50} values below 10 μM [22]. A bis-isatin hydrazones of imidazolidine-2,4-dione **(2)** showed good antiproliferative activity against the human colon cancer cell line HCT-116 (Figure 1). Another bis-isatin hydrazone derivative **(3)** displayed a

promising growth inhibitory activity against several distinct cancer cell lines [23]. New 1,4-dihydropyridine-3,5-dicarbohydrazide **(4)** was reported as potential anticancer agents in hepatic HepG2 cancer cell line [24]. Also, our earlier investigations have provided evidence of antitumoral and apoptosis inducing properties of hydrazide-hydrazone derivatives [25–27].

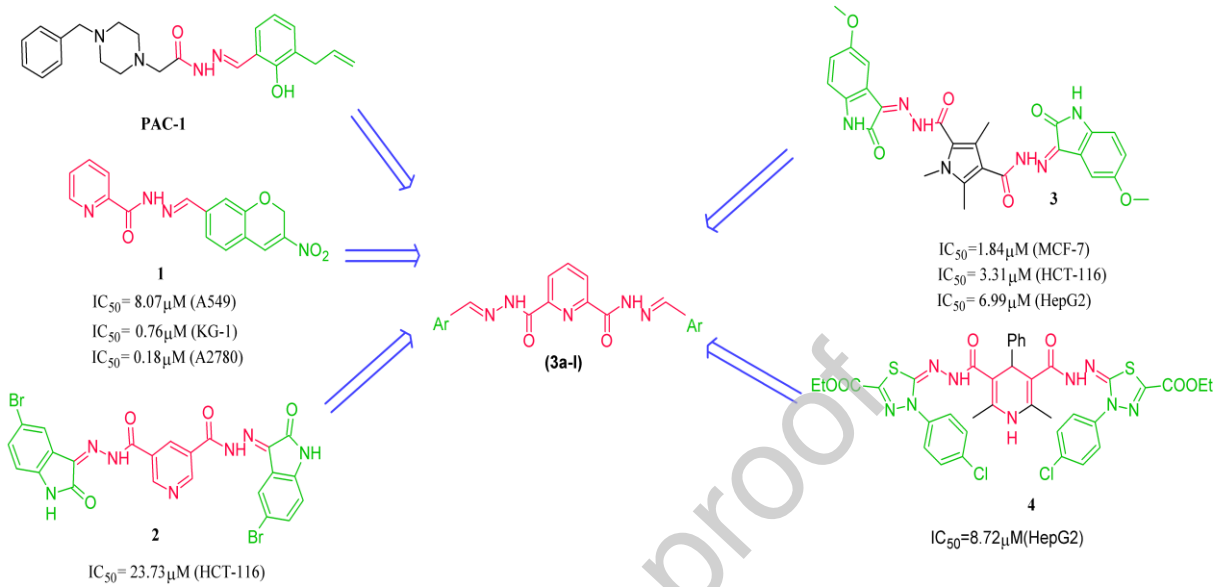


Figure 1. Rational design of novel 2,6-disubstituted pyridines.

Based on their aforementioned biological importance, combining pyridine nucleus with hydrazone pharmacophore has been envisaged to generate new pyridine-2,6-dicarbohydrazide derivatives with anticancer activity. In addition, effects on apoptosis and caspase-3 activation against HT-29 human colon cancer cell line and ISH Ishikawa human endometrial cancer cell line were also investigated for the most active derivatives. Considering the recent reports on BRAF inhibitory activity of hydrazone derivatives [28,29], additional efforts have been made regarding computational studies which include *in silico* docking studies to the target BRAF enzyme.

2. Experimental

2.1. Chemistry

Melting points were determined on an Electrothermal IA9300 melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were obtained on a BRUKER 300 MHz Ultrashield TM spectrometer, DMSO-d_6 was used as a solvent, and TMS was used as an internal standard. Chemical reactions were monitored by thin-layer chromatography under ultraviolet light of 254 nm. The liquid chromatographic system consists of an Agilent Technologies 1100 series instrument equipped with a quaternary solvent delivery system and a model Agilent series G1315 A photodiode array detector. Chromatographic data were collected and processed using Agilent Chemstation Plus software. Chromatographic separation was performed at ambient temperature using a reversed phase Zorbax C8 (4.0×250 mm) column. All

experiments were performed using ACN:H_2O (v/v, 70/30) mobile phase with UV detection at 230 nm. All the reagents and solutions were of analytical reagent grade.

2.1.1. Synthesis of compounds 1 and 2

Dimethyl pyridine-2,6-dicarboxylate **1** was prepared as previously described in the literature [30,31]. Pyridine-2,6-dicarbohydrazide **2** was prepared by heating hydrazine-hydrate (80%, 5 eq.) and **1** in methanol. The reaction mixture was heated at reflux for 4 h and allowed to cool to RT, filtered, dried and recrystallized from methanol to obtain pure product **2** [31].

2.1.2. General procedure for the synthesis of *N*$^{'2}$,*N*$^{'6}$-Bis(substitutedarylidene)pyridine-2,6-dicarbohydrazides (3a-l)

A mixture of compound **2** (2.5 mmol) and substituted aldehydes (5.0 mmol) in 50 mL absolute ethanol was refluxed for 6-12 h. After cooling, the solid precipitate was filtered off, washed with ethanol, dried, and crystallized from ethanol to give compounds **3a-l** [32,33].

***N*$^{'2}$, *N*$^{'6}$-bis {[4-(Trifluoromethyl)phenyl]methylidene}pyridine-2,6-dicarbohydrazide (3a)**

Yield 75%; m.p. 324-326°C; HPLC t_R (min.): 3.74; FT-IR: ν/cm^{-1}: 3190 (NH), 1683, 1668, 1618, (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.88 (d, 4H, *J*= 8.4 Hz, Ar-**H**), 8.05 (d, 4H, *J*= 8.1 Hz, Ar-**H**), 8.29-8.42 (m, 3H, pyridine-**H**), 8.84 (s, 2H, C**H**=N), 12.52 (s, 2H, CON**H**-N=). Anal. calcd for $C_{23}H_{15}F_6N_5O_2$ (507.388): C, 54.44; H, 2.98; N, 13.80; Found: C, 54.40; H, 3.04; N, 13.79.

***N*$^{'2}$,*N*$^{'6}$-bis{[3-(Trifluoromethoxy)phenyl]methylidene}pyridine-2,6-dicarbohydrazide (3b)**

Yield 81%; m.p. 247°C; HPLC t_R (min.): 6.17; FT-IR: ν/cm^{-1}: 3228 (NH), 1668, 1579 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.47-7.86 (m, 8H, Ar-**H**), 8.28-8.40 (m, 3H, pyridine-**H**), 8.81 (s, 2H, C**H**=N), 12.50 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 119.13 (2CH), 120.54 (2OCF_3, d, *J*=255Hz), 123.21 (2CH), 126.17 (2CH), 127.07 (2CH), 131.59 (2CH), 137.06 (2C), 140.56 (CH), 148.54 (2C), 148.70 (2CH=N), 149.30 (2C), 160.11 (2C=O). Anal. calcd for $C_{23}H_{15}F_6N_5O_4.1/2H_2O$ (548.391): C, 50.37; H, 2.94; N, 12.77; Found: C, 50.05; H, 2.78; N, 12.38.

***N*$^{'2}$,*N*$^{'6}$-bis [(4-Fluoro-3-phenoxyphenyl)methylidene]pyridine-2,6-dicarbohydrazide (3c)**

Yield 89%; m.p. 280°C; HPLC t_R (min.): 7.82; FT-IR: ν/cm^{-1}: 3190 (NH), 1680, 1658, 1583 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.07-7.66 (m, 16H, Ar-**H**), 8.24-8.34 (m, 3H, pyridine-**H**), 8.71 (s, 2H, C**H**=N), 12.34 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 118.15 (2CH), 118.43 (2CH), 119.10 (2CH), 124.42 (2CH), 125.20 (2CH), 125.30

(2CH), 126.02 (2CH), 130.73 (2CH), 132.17 (2CH), 132.22 (2C), 140.52 (CH), 144.29 (2C), 144.45 (2C), 148.55 (2CH=N), 148.98 (2C), 155.03 (2CF, d, *J*= 252 Hz), 159.93 (2CO). Anal. calcd for $C_{33}H_{23}F_2N_5O_4.H_2O$ (609.578): C, 65.02; H, 4.13; N, 11.49; Found: C, 65.65; H, 3.78; N, 11.66.

***N*'²,*N*'⁶-bis [(5-Bromo-2-methoxyphenyl)methylidene]pyridine-2,6-dicarbohydrazide (3d)**

Yield 76%; m.p. 269°C; HPLC t_R (min.): 5.96; FT-IR: ν/cm^{-1}: 3159 (NH), 1681, 1668, 1597 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 3.93 (s, 6H, -C**H**$_3$), 7.17 (d, 2H, *J*= 9 Hz, Ar-**H**); 7.63 (dd, 2H, J_1= 9 Hz, J_2= 2.7 Hz, Ar-**H**), 8.02 (d, 2H, *J*= 2.4 Hz, Ar-**H**), 8.26-8.39 (m, 3H, pyridine-**H**), 9.00 (s, 2H, C**H**=N), 12.41 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 56.61 (2OCH_3), 113.04 (2C), 114.99 (2CH), 124.76 (2C), 126.08 (2CH), 128.03 (2CH), 134.45 (2CH), 140.38 (CH), 143.78 (2C), 148.58 (2CH=N), 157.48 (2C), 159.99 (2CO). Anal. calcd for $C_{23}H_{19}Br_2N_5O_4.H_2O$ (607.251): C, 45.49; H, 3.49; N, 11.53; Found: C, 45.77; H, 3.08; N, 11.54.

***N*'²,*N*'⁶-bis{[2-(Trifluoromethoxy)phenyl]methylidene}pyridine-2,6-dicarbohydrazide (3e)**

Yield 81%; m.p. 345°C; HPLC t_R (min.): 4.98; FT-IR: ν/cm^{-1}: 3192 (NH), 1681, 1666, 1602 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.51-8.19 (m, 8H, Ar-**H**), 8.29-8.42 (m, 3H, pyridine-**H**), 8.94 (s, 2H, C**H**=N), 12.52 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 118.53 (2CH), 120.55 (2OCF_3, q, *J*= 273Hz), 126.62 (2CH), 128.67 (2CH), 131.66 (2CH), 131.87 (2C), 134.11 (2CH), 134.57 (CH), 143.82 (2C), 148.13 (2CH=N), 156.25 (2CO), 159.70 (2CO). Anal. calcd for $C_{23}H_{15}F_6N_5O_4.H_2O$ (557.401): C, 49.56; H, 3.07; N, 12.56; Found: C, 49.68; H, 2.78; N, 12.52.

***N*'²,*N*'⁶-bis(Naphthalen-2-ylmethylidene)pyridine-2,6-dicarbohydrazide (3f)**

Yield 76%; m.p. 351°C; HPLC t_R (min.): 5.92; FT-IR: ν/cm^{-1}: 3275 (NH), 1678, 1599 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.61-8.11 (m, 12H, Ar-**H**), 8.33-8.45 (m, 3H, pyridine-**H**), 9.02 (d, 2H, *J*= 8.4 Hz, Ar-**H**), 9.48 (s, 2H, C**H**=N), 12.46 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 120.52 (2CH), 121.20 (2CH), 123.47 (2CH), 124.81 (2CH), 126.17 (2CH), 127.07 (2CH), 129.86 (2CH), 130.54 (2CH), 130.75 (2C), 131.46 (2C), 134.11 (2C), 140.63 (CH), 147.64 (2C), 148.58 (2CH=N), 159.87 (2CO); Anal. calcd for $C_{29}H_{21}N_5O_2.1/2H_2O$ (480.514): C, 72.49; H, 4.61; N, 14.57; Found: C, 72.61; H, 4.47; N, 14.78.

***N*'²,*N*'⁶-bis[(5-Methylthiophene-2-yl)methylidene]pyridine-2,6-dicarbohydrazide (3g)**

Yield 73%; m.p. 260°C; HPLC t_R (min.): 3.96; FT-IR: ν/cm^{-1}: 3524 (OH), 3185 (NH), 1672, 1656, 1622 (C=O and C=N); ^{1}H NMR (600 MHz), (DMSO-d_6/TMS) δ ppm: 2.51 (s, 6H, C**H**$_3$); 6.89 (d, 2H, *J*= 3.6 Hz, thiophene-**H**); 7.38 (d, 2H, *J*= 3.0 Hz, thiophene-**H**); 8.25-8.33 (m, 3H, pyridine-**H**), 8.94 (s, 2H, C**H**=N), 12.21 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 15.90

($2CH_3$), 125.76 (2CH), 127.01 (2CH), 132.52 (2CH), 137.16 (2CH=N), 140.48 (CH), 144.04 (2C), 146.06 (2C), 148.67 (2C), 159.70 (2CO). Anal. calcd for $C_{19}H_{17}N_5O_2S_2.3/2H_2O$ (438.512): C, 52.02; H, 4.59; N, 15.96; S,14.62; Found: C, 51.58; H, 4.57; N, 15.69, S,14.67.

***N*'²,*N*'⁶-bis[(3-Fluorophenyl)methylidene]pyridine-2,6-dicarbohydrazide (3h)**

Yield 79%; m.p. 275°C; HPLC t_R (min.): 3.20; FT-IR: ν/cm^{-1}: 3526 (OH), 3371 (NH), 1668, 1595 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.30-7.69 (m, 8H, Ar-**H**), 8.28-8.40 (m, 3H, pyridine-**H**), 8.78 (s, 2H, C**H**=N), 12.45 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 113.66 (2CH, d, *J*= 22Hz), 117.69 (2CH, d, *J*= 22 Hz), 124.14 (2CH), 126.11 (2CH), 131.54 (2CH), 137.13 (2CH, d, *J*= 7.5 Hz), 140.52 (CH), 148.56 (2C), 149.15 (2CH=N), 160.05 (2CO), 162.91 (2C-F, d, J=243 Hz). Anal. calcd for $C_{21}H_{15}F_2N_5O_2.2H_2O$ (443.403): C, 56.88; H, 4.32; N, 15.79; Found: C, 56.52; H, 4.29; N, 15.60.

***N*'²,*N*'⁶-bis[(4-Fluoro-3-methoxyphenyl)methylidene]pyridine-2,6-dicarbohydrazide (3i)**

Yield 93%; m.p. 304°C; HPLC t_R (min.): 4.72; FT-IR: ν/cm^{-1}: 3159 (NH), 1666, 1649, 1589 (C=O and C=N); ^{1}H NMR (600 MHz), (DMSO-d_6/TMS) δ ppm: 3.95 (s, 6H, C**H$_3$**), 7.34-7.62 (m, 6H, Ar-**H**), 8.29-8.38 (m, 3H, pyridine-**H**), 8.77 (s, 2H, C**H**=N), 12.45 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 56.48 ($2CH_3$), 111.36 (2CH), 116.85 (2CH, d, *J*= 18.75 Hz), 121.70 (2CH, d, *J*=6.75 Hz), 126.03 (2CH), 131.60 (2C, d, *J*= 3.75 Hz), 140.53 (CH), 148.05 (2CH=N), 148.67 (2C), 149.73 (2C),149.96 (2C-F, d, *J*= 251 Hz), 159.94 (2CO). Anal. calcd for $C_{23}H_{19}F_2N_5O_4.1/3H_2O$ (473.429): C, 58.35; H, 4.19; N, 14.79; Found: C, 58.66; H, 4.24; N, 14.70.

***N*'²,*N*'⁶-bis[(4-Bromothiophen-2-yl)methylidene]pyridine-2,6-dicarbohydrazide (3j)**

Yield 78%; m.p. 297°C; HPLC t_R (min.): 4.72; FT-IR: ν/cm^{-1}: 3636 (OH), 3375 (NH), 1668, 1600 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.61 (d, 2H, *J*=1.5 Hz, thiophene-**H**); 7.82 (dd, 2H, J_1= 1.3 Hz, J_2= 0.7 Hz, thiophene-**H**); 8.26-8.36 (m, 3H, pyridine-**H**), 8.99 (s, 2H, C**H**=N), 12.39 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 109.97 (2C), 126.04 (2CH), 127.18 (2CH), 133.37 (2CH=N), 140.60 (2C), 140.92 (2CH), 144.23 (CH), 148.46 (2C), 159.97 (2CO). Anal. calcd for $C_{17}H_{11}Br_2N_5O_2S_2.2H_2O$ (577.270): C, 35.37; H, 2.62; N, 12.13; S,11.11; Found: C, 35.77; H, 2.49; N, 12.07, S,11.06.

***N*'²,*N*'⁶-bis[(4-Phenylthiophen-2-yl)methylidene]pyridine-2,6-dicarbohydrazide (3k)**

Yield 83%; m.p. 360°C; HPLC t_R (min.): 4.89; FT-IR: ν/cm^{-1}: 3257 (NH), 1693, 1658,1599 (C=O and C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.32-8.07 (m, 14H, Ar-**H**); 8.24-8.39 (m, 3H, pyridine-**H**), 9.09 (s, 2H, C**H**=N), 12.39 (s, 2H, CON**H**-N=); ^{13}C NMR (75MHz) (DMSO-d_6/TMS) δ ppm: 124.68 (2CH), 125.95 (4CH), 126.44 (4CH), 128.04 (2CH), 129.46 (2CH), 130.52 (2CH),

134.85 (2CH=N), 136.56 (2C), 140.22 (CH), 142.34 (2C), 145.42 (2C), 148.60 (2C), 159.86 (2CO). Anal. calcd for $C_{29}H_{21}N_5O_2S_2.1/3H_2O$ (541.644): C, 65.03; H, 3.95; N, 13.07; S,11.97; Found: C, 64.66; H, 4.01; N, 13.38, S,11.90.

$N^{'2}$,$N^{'6}$-bis{[4-Fluoro-2-(trifluoromethyl)phenyl]methylidene}pyridine-2,6-dicarbohydrazide (3l)

Yield 79%; m.p. 344-346°C; HPLC t_R (min.): 6.35; FT-IR: ν/cm^{-1}: 3525 (OH), 3188 (NH), 1674, 1656, 1622 (C=O, C=N); ^{1}H NMR (300 MHz), (DMSO-d_6/TMS) δ ppm: 7.69 -8.41 (m, 9H, Ar-**H**), 9.02 (s, 2H, C**H**=N), 12.62 (s, 2H, CON**H**-N=); Anal. calcd for $C_{23}H_{13}F_8N_5O_2.3H_2O$ (597.415): C, 46.24; H, 3.21; N, 11.72; Found: C, 46.36; H, 3.35; N, 11.71.

2.2. Biological Screening

2.2.1. Cell culture and cell viability assay

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human colon cancer cell line (HT-29 cells) and Ishikawa human endometrial cancer cell line (ISH cells) and mouse fibroblast cell line (NIH 3T3 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) or RPMI-1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and kept in a humidified atmosphere at 37°C incubator with 5% CO_2 in air. To determine the cell viability cells were plated onto 96-well plates (1×10^4 cells/well). The cells were treated with different concentrations (0, 0.1, 1, 10, 100, and 1000 µM) of synthesized compounds incubated for 24 h. After the incubation cells were washed with PBS and added to 100 µL DMEM/RPMI-1640. A total of 10 µL of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vybrant, Invitrogen) labeling reagent was added to each well and incubated for 4 h in a humidified atmosphere at 37°C incubator with 5% CO_2 in the air. After the incubation, 100 µL of the SDS buffer was added into each well for the solubilization of formazan precipitate. Then absorbance was measured by a microplate reader at 570 nm and was carried out in triplicate of each assay [34]. Observation of morphological changes of apoptotic cells was performed according to the methods [35,36] with slight modifications.

2.2.2. Caspase 3 activity assay

The caspase activity was measured after cell incubation for 24 h with compounds **3f**, **3g** and **3k** (10 µM), using a Caspase-3 Activity Assay Kit (C10427, Invitrogen), as reported previously [36].

2.2.3. Antibacterial activity test

All synthesized compounds were tested for *in vitro* antibacterial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633 with using micro

broth dilution method according to EUCAST recommendation [37]. The micro-dilution method was performed in cation adjusted Mueller Hinton Broth (CMHB) with serial dilution of compounds in 96 well plates. 100 µL of CMHB with different concentrations of compounds was added into the well. Bacterial suspension was prepared from a fresh culture of bacteria and adjusted using a nephelometer to 0.5 McFarland units and diluted at 1/100 ratio. 100 µL bacterial suspension was added in to the well. Microplates were incubated 35±2°C for 16 to 20 hours. The minimum concentration that inhibits bacterial growth accepted as minimal inhibition concentration (MIC). Moxifloxacin was used as control.

2.2.4. Statistical analysis

All assays were performed in triplicate in three independent assays, and the obtained values were analyzed and expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out by analysis of variance (one-way ANOVA) followed by a Tukey test.

2.3. *In silico* studies

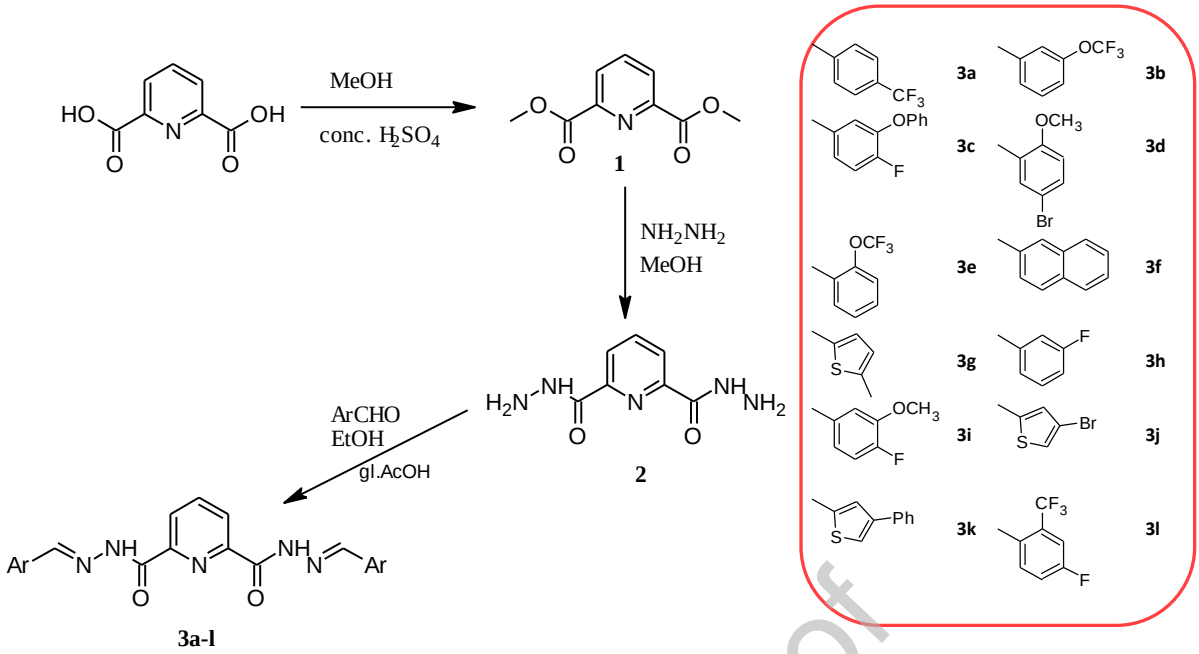
Co-crystal structure of BRAF V600E protein with vemurafenib (PDB ID: 3OG7) was obtained from Protein Data Bank (https://www.rcsb.org/). Vemurafenib and water molecules were removed from the structure first and protein, as well as ligands, were prepared by using Biovia Discovery Studio Visualizer and MGLTools software. AutoDock Vina docking tool was used to estimate possible binding conformations of compounds. Binding modes were evaluated with Biovia Discovery Studio Visualizer.

For validation of the docking process, vemurafenib was re-docked into BRAF. Excellent superimpositioning between co-crystallised structure and re-docked structure was observed. RMSD value, which is expected to be under 3.000 Å after re-docking process, was found as 0.557.

3. Results and Discussion

3.1. Chemistry

The synthesis of the new compounds is illustrated in Scheme 1. The preparation of compound **1** was achieved *via* heating pyridine-2,6-dicarboxylic acid and methanol as previously reported [31]. Compound **2** was obtained by heating compound **1** with hydrazine hydrate [32]. Through the condensation reaction of compound **2** and various aldehydes in the presence of acetic acid, new dihydrazone derivatives **3a-l** were synthesized.



Scheme 1. Synthetic route to compounds **3a-l**.

The IR spectra of compounds **3a-l** showed the disappearance of the absorption band of NH_2 groups and the appearance of absorption bands at 3190–3375cm^{-1} attributed to the NH.. Furthermore, the ^1H NMR spectrum showed a singlet signal at δ 8.71-9.48 ppm corresponding to the azomethine protons, in addition to a singlet signal at δ12.21-12.62 ppm indicating the N-H proton of the hydrazone moiety. As for the ^{13}C NMR spectra, signals for aliphatic and aromatic groups in compounds were observed at expected regions [38,39].

The C=N bond can give rise to the formation of E/Z isomers. Based on literature precedence the isomeric ratio of products was presumed to be mainly E in all cases [40,41]. The presence of a single set of NMR signals in DMSO-d_6 indicates that only one geometric isomer and rotameric form exists in solution. Structural elucidation was supported by a 2D NMR-NOESY spectrum of compound **3f** as shown in Figure 2. The NOESY spectrum, which showed a spatial interaction between CONH and CH=N, supported the formation of the E geometrical isomer. Expectedly, a spatial interaction between the NH and the pyridine hydrogens was not observed which is in good agreement with the crystal structure of $N^{'2}$,$N^{'6}$-bis[(pyridin-2-yl)methylidene]pyridine-2,6-dicarbohydrazide [42]. This is consistent with multiple literature reports which state that the E-isomer is the predominant form in solution due to hindered rotation about the imine bond.

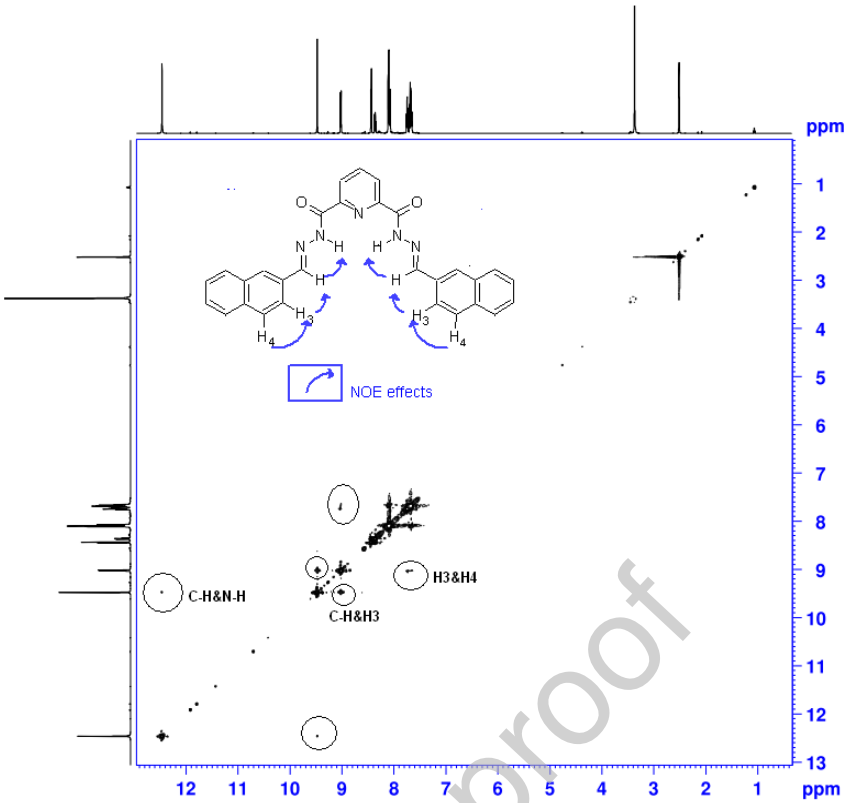


Figure 2. 2D NOESY of **3f** in DMSO-d_6 at 295 K (x- and y-axis, ^{1}H-NMR chemical shift).

3.2. Biological results

Newly synthesized twelve compounds were screened for their *in vitro* growth inhibitory activities against two human cancer cell lines namely HT-29 human colon cancer cell line and ISH Ishikawa human endometrial cancer cell line and mouse embryonic fibroblast cells NIH 3T3 (as healthy cells) by MTT assay method, taking paclitaxel as reference. As shown in Table 1, these derivatives bearing pyridine and hydrazone moiety exhibited remarkable anticancer effects. Among them, compound N'^2,N'^6-bis(naphthalen-2-ylmethylidene)pyridine-2,6-dicarbohydrazide **3f** and N'^2,N'^6-bis[(4-phenylthiophen-2-yl)methylidene]pyridine-2,6-dicarbohydrazide **3k** displayed potent inhibitory activity (IC_{50}=6.78 μM and 8.88 μM, respectively) for HT-29 cell line in comparison to the standard paclitaxel (IC_{50}=0.35 μM). Taken altogether, N'^2,N'^6-bis[(5-methylthiophene-2-yl)methylidene]pyridine-2,6-dicarbohydrazide **3g** was the most potent compound in this series, having an IC_{50} value of 8.26 μM in suppressing ISH cell growth.

The synthesized compounds were evaluated against mouse embryonic fibroblast cells NIH 3T3 for their safety. Their attained IC_{50} values were 80.06-521.78 μM, showing a safe profile and selectivity toward the tested cancer cell lines. All of the compounds were found less toxic in the non-cancerous mouse cell line (NIH 3T3) *in vitro*, as compared to its toxicity over ISH and HT-29, This result is

encouraging, since the well-known drug paclitaxel, which has a much lower IC_{50} value in ISH or HT-29, does not exhibit this property.

Table 1. *In vitro* cytotoxic activities of compounds **3a-l**.

Comp.	IC_{50} (μM)[a]		
	NIH 3T3	**ISH**	**HT-29**
3a	184.67	40.40	32.68
3b	521.78	29.97	132.16
3c	131.56	31.77	32.03
3d	112.38	31.66	31.66
3e	109.71	82.80	31.78
3f	117.78	10.23	6.78
3g	80.13	8.26	12.29
3h	80.06	32.81	31.58
3i	604.17	31.59	118.85
3j	585.43	31.56	31.74
3k	212.74	31.90	8.88
3l	324.91	107.20	383.75
Paclitaxel	74.21	1.025	0.35

[a]IC_{50} compound concentration required to inhibit tumour cell proliferation by 50%.

At 10 μM, compounds **3f**, **3g** and **3k** effectively inhibited the cancer cell viability in a time-dependent manner (Figure 3).

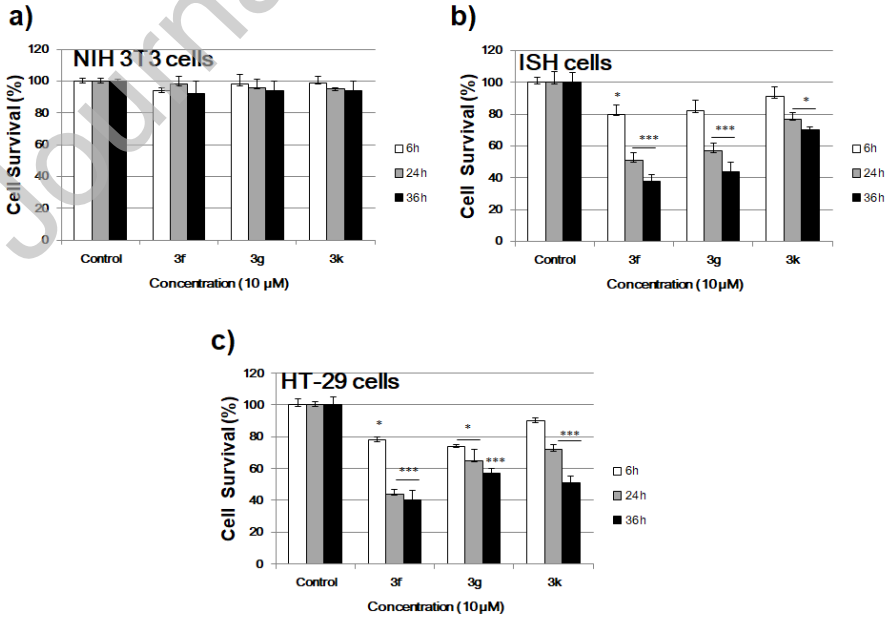


Figure 3. Effects of compounds on cell survival in NIH 3T3, ISH, HT-29 cancer cells. **a)** NIH 3T3 cells **b)** ISH cells **c)** HT-29 cells survival measurement with MTT assay after compounds treatment (10 μM) of cell lines at three different time points. (*p<0.05, **p<0.01, ***p<0.001, compare to control).

Morphological changes in the cancer cells treated with compounds **3f**, **3g** and **3k** using inverted microscopy showed the apoptosis. As shown in Figure 4, 24 h after exposure to 10 μM of the selected compounds, a significant number of HT-29 and ISH cells detached from the plate. When compared to the control, compounds-treated cancer cells began to show cell shrinkage, rounding and fragmentation that were indicative of apoptosis. Apoptosis can be characterized by morphological changes in the nucleus, chromatin condensation and cell shrinkage [43]. The results showed that lead compounds triggered apoptosis as a molecular mechanism. Such morphological changes were not apparent in the control cells.

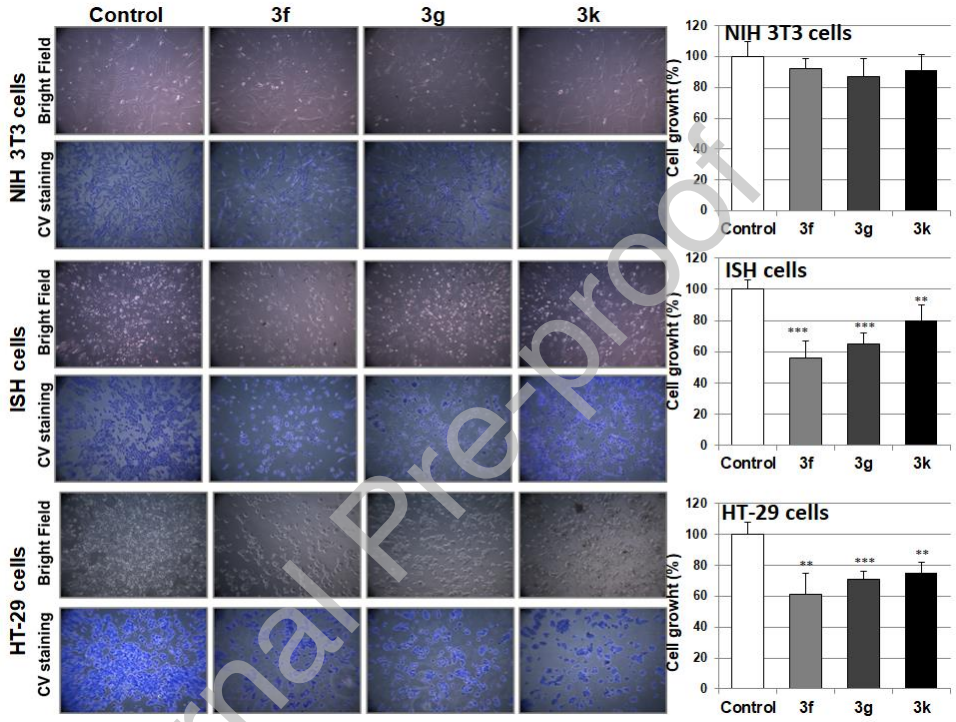


Figure 4. Effects of compounds on cell growing and apoptosis in HT-29, ISH and NIH 3T3 cells. Cell morphological changes with microscobic image (10X). Cells were treated with 10 μM compounds HT-29, ISH and NIH 3T3 cells at 24 well plates and and stained with crystal violet. Cells growing shown as overview images (up), crystal violet staining (down) and percentages of cell growing (right). (**p<0.01, ***p<0.001 compare to control).

Caspase-3 is an effector caspase that has a central role in cell apoptosis. By using DMSO as a negative control, the ability of compounds **3f**, **3g** and **3k** to activate caspase-3 in cancer cells was evaluated (Figure 5). Indeed, these compounds appeared to induce caspase-3 activation as compared to the untreated control cells, as compound-treated cells showed more active caspase-3 expression. This study has shown that compounds might induce apoptotic cell death through a caspase-dependent pathway.

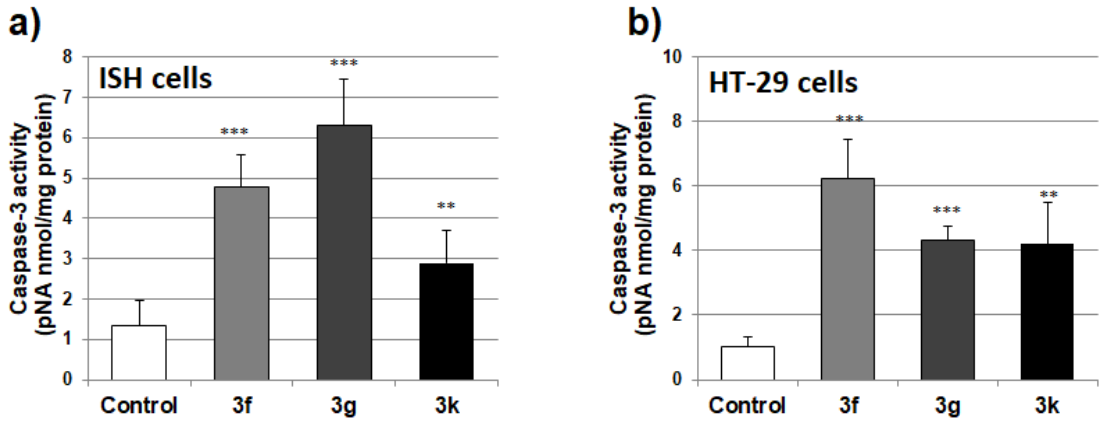


Figure 5. Effects of compounds (10 µM) on the caspase-3 activity in a) HT-29 cells b) ISH cells. (**p<0.01, ***p<0.001 compare to control).

Furthermore, compounds **3a-l** were investigated for their antibacterial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633 with using micro broth dilution method according to EUCAST recommendation. Moxifloxacin was used as the standard reference drug. MIC values of all compounds were found >50 µg/mL. Evaluation of the activity of the synthesized compounds exhibited that, the tested compounds did not exhibit remarkable antibacterial activity.

3.3. Molecular modeling

Regarding that the synthesized compounds were tested against endometrium and colon cancer cell lines, molecular modeling studies were designed with BRAF V600E protein since overactivity of BRAF V600E has been correlated with endometrial and colon cancers [16,17,19]. In this point of view, docking studies were performed to estimate possible inhibition of BRAF with designed compounds. Exemplarily, docking analyses of compound **3g,** which showed superior anticancer activity in endometrium cancer cells is presented here.

The binding energy calculated for the compound **3g** was found as -8.2 kcal/mol while the binding energy of vemurafenib -11.4 was found kcal/mol. The E-isomer of the compound was selected for the docking analysis as E isomers of the compounds were proved with spectral data. According to docking studies, compound **3g** forms two conventional hydrogen bonds in the activation site. One of the H bonds is formed between Cys532 residue, which places in the hinge region of BRAF protein and hydrazone NH atom (Figure 6). This interaction is so fundamental as hydrogen bond interaction with the hinge region is critical for any kinase protein inhibition [44]. The other hydrogen bond is formed between the carbonyl group and Ser536 residue. Besides, compound **3g** makes a carbon-hydrogen bond with Asn580. As seen in Figures 6 and 7, compound **3g** settles properly in the hydrophobic

pocket of BRAF. Both 5-methylthiophene rings of compound **3g** contribute hydrophobic interactions by interacting with Phe468, Val471, Ala481, Lys483 and Leu514 amino acid residues. Sulfur atom of thiophene ring makes pi-sulfur interaction with Phe583. Van der Waals forces are observed between compound **3g** and Thr529, Gly534, His539, Asn581, Asp594 residues.

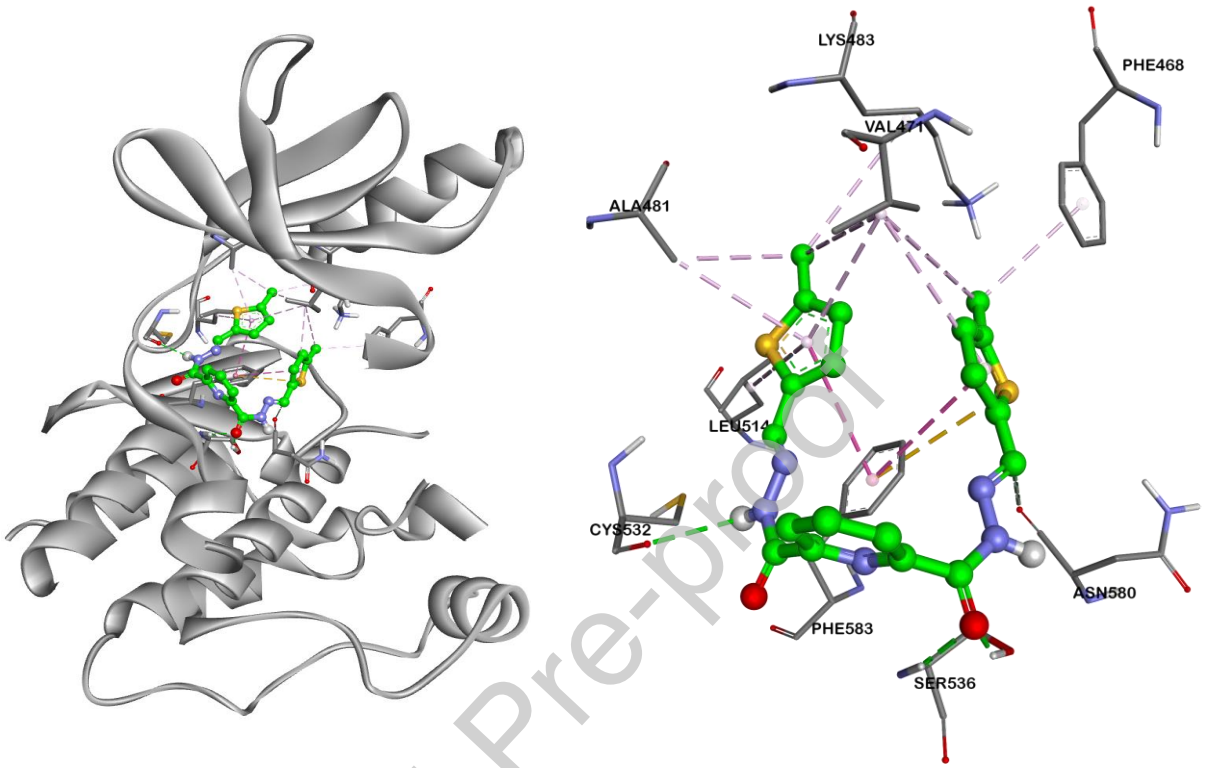


Figure 6. The ligand **3g** (space filling) is embedded into the kinase domain (left panel). Interaction between compound **3g** and V600E- BRAF, hydrogen bonds are shown as green lines (right panel).

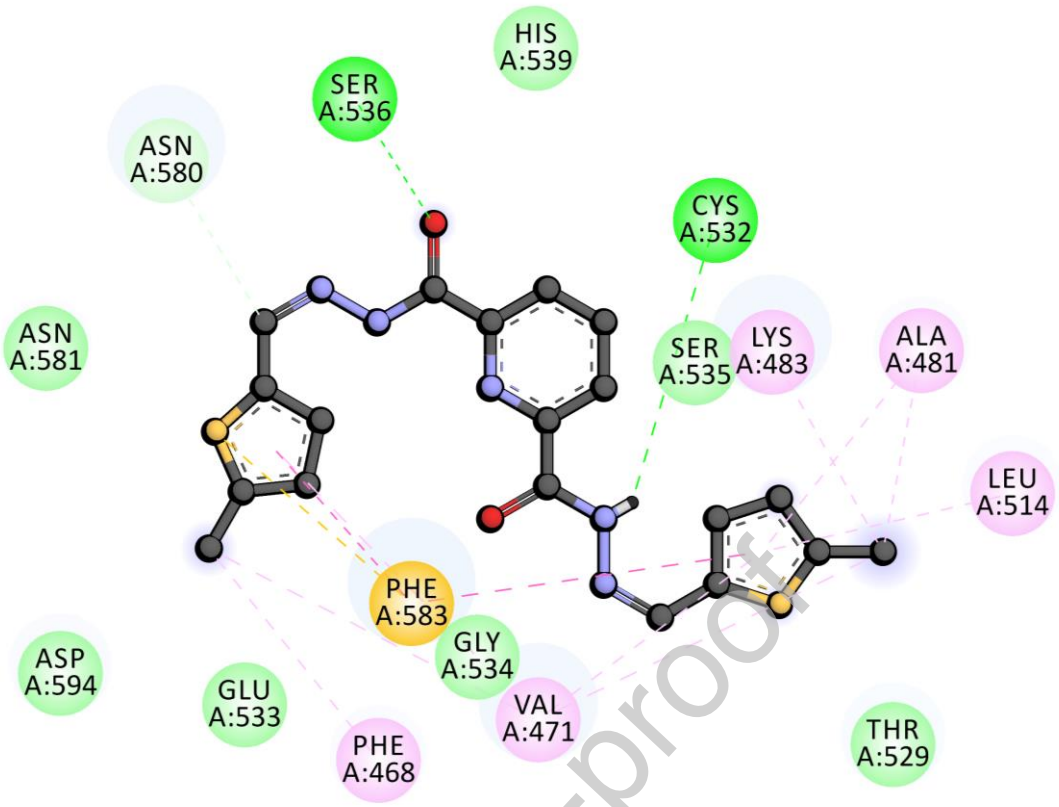


Figure 7. Two-dimentional interaction diagram for compound **3g**. Interaction diagram legends include: 1) pink circles = residues involved in alkyl and pi-alkyl interacitons; 2) light green circles = residues involved in carbon hydrogen bond and van der Waals interactions; 3) green circles = hydrogen bond interactions; 4) yellow circles = pi-sulfur interactions.

4. Conclusion

In the present work, a novel series of hydrazide-hydrazones containing pyridine moiety were synthesized. These compounds were evaluated for their anticancer activity against human colon cancer cell line (HT-29) and Ishikawa human endometrial cancer cell line (ISH). The results showed that the majority of compounds had moderate to high inhibition on cancer cells with low cytotoxicity on normal cells. The hydrazone derivatives **3f**, **3g** and **3k** revealed higher activity than the other substituted hydrazone derivatives. Our results showed that these compounds are capable of inducing apoptosis through caspase-3 activation. The molecular modeling displayed that compound **3g** exhibited similar and additional binding interactions when docked into BRAF kinase, which suggests that **3g** may act as BRAF kinase inhibitors and this may contribute to their anticancer activity.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical Abstract