Synthesis and in vitro anticancer activity of certain novel 1-(2-methyl-6-arylpuridin-3-yl)-3-phenylureas as apoptosis-inducing agents

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ABSTRACT

In connection with our research program on the development of novel anticancer candidates, herein we report the design and synthesis of novel series of 1-(2-methyl-6-arylpuridin-3-yl)-3-phenylureas 5a-I. The target pyridines were evaluated for their in vitro anticancer activity against two cancer cell lines: non-small cell lung cancer A549 cell line and colon cancer HCT-116 cell line. Compound 5I emerged as the most active congener towards both A549 and HCT-116 cell lines with IC_{50} values equal to 3.22 ± 0.2 and 2.71 ± 0.16 μM, respectively, which are comparable to those of Doxorubicin: 2.93 ± 0.28 and 3.10 ± 0.22, respectively. Furthermore, compound 5I stood out as the most potent pyridine derivative (mean % GI = 40), at US-NCI Developmental Therapeutic Program anticancer assay, with broad-spectrum antitumor activity against the most tested cancer cell lines from all subpanels. Compound 5I was able to provoke apoptosis in HCT-116 cells as evidenced by the decreased expression of the anti-apoptotic Bcl-2 protein, and the enhanced expression of the pro-apoptotic proteins levels; Bax, cytochrome C, p53, caspase-3 and caspase-9. Moreover, 5I disrupted the HCT-116 cell cycle via alteration of the Sub-G1 phase and arresting the G2-M stage. Also, 5I showed a significant increase in the percent of annexinV-FITC positive apoptotic cells from 1.99 to 15.76%.

Introduction

Apoptosis, a self-automated cell death, represents the principal pathway in tissue homeostasis and in animal development; in addition, it is the main pathway for the clearance of aged or defective cells in the body. Mainly, two major signaling pathways for apoptotic cell death have been signified. The first one is the extrinsic cytoplasmic pathway that is triggered via pro-apoptotic ligands binding to the cell surface death receptor. Whereas, the second is the intrinsic mitochondrial apoptotic pathway that results from an intracellular cascade of events that are mainly produced by cellular stress, in which mitochondrial permeabilization plays a crucial role. Both extrinsic and intrinsic pathways converge onto the activation of effector caspases, resulting in apoptotic cell death program. During cancer pathogenesis, apoptosis deregulation has been widely recognized as a hallmark of cancer. Accordingly, induction of apoptosis in tumor cells has stood out as a successful tactic for combating different human malignancies, in the current medical era.

On the other hand, non-fused pyridines have stood out as a promising class of anticancer agents with efficient pro-apoptotic activity. Regorafenib (Stivarga, Figure 1), a pyridine-based biphenyl urea derivative developed by Bayer, inhibits angiogenickinases VEGFR-1/3, FGFR1, PDGFRb, and Tie-2. Regorafenib was approved by FDA, in September 2012, for the treatment of metastatic colorectal cancer (mCRC). The anticancer effect of Regorafenib is thought to be mediated by apoptosis induction, in addition to its anti-angiogenic and anti-proliferative effects. Crizotinib (Xalkori, Figure 1) is an orally active inhibitor of multiple receptor tyrosine kinases, including anaplastic lymphoma kinase (ALK), Hepatocyte Growth Factor Receptor (HGFR, c-Met), and Recepteur d’Origine Nantais (RON). Crizotinib was approved for the treatment of adults with previously treated, ALK-positive, advanced non-small cell lung cancer (NSCLC). Crizotinib likely exerts its anticancer activity via multiple distinct mechanisms such as apoptosis.

Recently, our research group has explored the anticancer activity for novel series of 1-(2-methyl-6-(4-methoxy/3,4-dimethoxy-phenyl)-puridin-3-yl)-3-phenylureas. All these derivatives were evaluated for their growth inhibitory activity against the proliferation of breast cancer cell line (MCF-7), where they displayed promising anti-proliferative activity. On the other hand, examination of their potential anti-angiogenic activity towards vascular endothelial growth factor receptor 2 (VEGFR-2) tyrosine kinase unveiled their incompetence to inhibit VEGFR-2 significantly.

KEYWORDS

Anticancer agents; apoptosis; cell cycle; pyridine-urea; synthesis

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Based on the aforementioned findings and as a part of our ongoing quest towards developing potent anticancer agents\(^{12-20}\), herein we report the synthesis and biological evaluation of novel series of \(1-(2\text{-methyl-6-arylpurin-3-yl)-3-phenylureas}\) \(5a-i\). Ten selected pyridines \(5a, 5c-j\) and \(5l\) were chosen to be in vitro evaluated for their antitumor activity at one dose (concentration \(10^{-5}\) M) primary anticancer assay towards a panel including 85 cancer lines according to US-NCI protocol. In addition, all pyridines \(5a-i\) were examined for their potential anti-proliferative activity against non-small cell lung cancer A549 cell line and colon cancer HCT-116 cell line. Furthermore, apoptosis induction potential of the target pyridines was examined in HCT-116 cells, in order to acquire more mechanistic insights and to verify and enlighten the antitumor properties of the investigated pyridines.

Materials and methods

Chemistry

Melting points were measured with a Stuart melting point apparatus and were uncorrected. Infrared (IR) Spectra were recorded as KBr disks using Schimadzu FT-IR 8400S spectrophotometer. \(^1\)H NMR and \(^13\)C-NMR experiments were carried out using Bruker NMR spectrometer (400/100 MHz). Chemical shifts (\(\delta\)) were reported relative to TMS as the internal standard. All coupling constant (\(J\)) values are given in hertz. Chemical shifts (\(\delta\)) were reported as follows: s, singlet; d, doublet; m, multiplet. High-reso-lution mass spectra were recorded using a Bruker MicroTOF spectrometer (Bruker Daltonics, Bremen, Germany). All reagents and solvents were dried and purified by the standard techniques.

Compounds 2-methyl-6-arylnicotinohydrazides \(2a-c\)\(^{21-23}\) were previously prepared.

General procedures for preparation of the target pyridines \(5a-l\)

A solution of hydrazides \(2a-c\) (10 mmol) and sodium nitrite (1 g, 14 mmol) in hydrochloric acid was stirred for 1 h in an ice bath, then stirring was continued for an additional 1 h at room temperature. The reaction mixture was poured over crushed ice. The precipitated solid was filtered off and air-dried to yield 2-methyl-6-arylnicotinoyl azides \(3a-c\), which were used in the next step without further purification. Azides \(3a-c\) were heated in refluxing dry xylene for 1 h, then the appropriate aniline derivative was added to this xylene solution. The reaction mixture was heated under reflux temperature for 4 h. After cooling to room temperature, the formed precipitate was filtered, washed with ether and recrystallized from ethanol to afford the target pyridines \(5a-l\).

![Figure 1. Structures of certain pyridine-based approved anticancer drugs, and the target pyridines 5a-l.](image-url)

1-(6-(4-Fluorophenyl)-2-methylpyridin-3-yl)-3-(trifluoromethyl)phenylurea (5a)

White crystals (yield 70%), m.p. 223–225 °C; IR (KBr, \(\nu\) cm\(^{-1}\)) 3393 (NH), 1731 (C=O); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) ppm: 2.64 (s, 3H, CH\(_3\)), 6.50 (s, 1H, NH, D\(_2\)O exchangeable), 6.61 (s, 1H, NH, D\(_2\)O exchangeable), 7.15 (t, 2H, \(J = 8.8\) Hz, Ar-H), 7.38 (d, 1H, \(J = 8.4\) Hz, Ar-H), 7.46 (t, 1H, \(J = 8.0\) Hz, Ar-H), 7.60–7.65 (m, 2H, Ar-H), 7.71 (s, 1H, Ar-H), 7.98 (dd, 2H, \(J = 8.8\) Hz, \(J = 5.6\) Hz, Ar-H), 8.06 (d, 1H, \(J = 8.4\) Hz, Ar-H); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta\) ppm: 21.34 (CH\(_3\)), 115.36, 115.53, 117.69, 121.75, 128.04, 128.67, 130.06, 132.47, 135.00, 135.10, 136.09, 147.93, 148.34, 152.35 (C\(_\alpha\)), 155.75, 173.12, 173.31 (C=O); HRMS (ESI) \(m/z\) calcd for [M + H]\(^+\) (C\(_{20}\)H\(_{16}\)N\(_2\)O\(_4\)F): 390.12240, found: 390.12286.

1-(3,5-Bis(trifluoromethyl)phenyl)-3-(6-(4-fluorophenyl)-2-methylpyridin-3-yl)urea (5b)

White crystals (yield 65%), m.p. 235–237 °C; IR (KBr, \(\nu\) cm\(^{-1}\)) 3390 (NH), 1733 (C=O); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) ppm: 2.58 (s, 3H, CH\(_3\)), 6.31 (s, 1H, NH, D\(_2\)O exchangeable), 6.59 (s, 1H, NH, D\(_2\)O exchangeable), 7.17 (t, 2H, \(J = 8.8\) Hz, Ar-H), 7.59 (s, 1H, Ar-H), 7.63 (d, 1H, \(J = 8.4\) Hz, Ar-H), 7.89 (s, 2H, Ar-H), 8.02–8.10 (m, 3H, Ar-H); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta\) ppm: 21.58 (CH\(_3\)), 115.44, 115.61, 117.75, 128.09, 128.15, 128.66, 132.72, 135.10, 147.79, 148.34, 152.94 (C\(_\alpha\)), 161.49, 163.44 (C=O–C–F).

Ethyl 4-(3-(6-(4-fluorophenyl)-2-methylpyridin-3-yl)ureido)benzoate (5c)

White crystals (yield 73%), m.p. 209–211 °C; IR (KBr, \(\nu\) cm\(^{-1}\)) 3389 (NH), 1733 (C=O); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) ppm: 1.39 (t, 3H, \(J = 7.2\) Hz, –OCH\(_2\)CH\(_3\)), 2.62 (s, 3H, –CH\(_3\)), 4.37 (q, 2H, \(J = 7.2\) Hz, –OCH\(_2\)CH\(_3\)), 6.36 (s, 1H, NH, D\(_2\)O exchangeable), 6.72 (s, 1H, NH, D\(_2\)O exchangeable), 7.14 (t, 2H, \(J = 8.8\) Hz, Ar-H), 7.49 (d, 2H, \(J = 8.4\) Hz, Ar-H), 7.60 (d, 1H, \(J = 8.4\) Hz, Ar-H), 7.98–8.10 (m, 5H, Ar-H); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta\) ppm: 14.30 (CH\(_3\)), 21.37 (CH\(_3\)), 60.39 (CH\(_2\)), 115.41, 115.58, 117.34, 117.75, 122.98, 128.08, 128.50, 130.51, 132.50, 135.04, 144.16, 147.93, 148.47, 152.35 (C\(_\alpha\)), 161.48, 163.43 (C=O–C–F), 165.48 (C=O–CO–), HRMS (ESI) \(m/z\) calcd for [M + H]\(^+\) (C\(_{22}\)H\(_{17}\)N\(_2\)O\(_4\)F\(_3\)): 394.15615, found: 394.15628.

1-(Benzo[d][1, 3]dioxol-5-yl)-3-(6-(4-fluorophenyl)-2-methylpyridin-3-yl)urea (5d)

White crystals (yield 62%), m.p. 254–256 °C; IR (KBr, \(\nu\) cm\(^{-1}\)) 3394 (NH), 1733 (C=O); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) ppm: 2.48 (s, 3H, CH\(_3\)), 6.04 (s, 2H, CH\(_2\)), 6.23 (s, 1H, NH, D\(_2\)O exchangeable), 6.34 (s, 1H, NH, D\(_2\)O exchangeable), 6.84 (d, 1H, \(J = 8.0\) Hz, Ar-H), 6.97–7.02 (m, 2H, Ar-H), 7.12 (t, 2H, \(J = 8.4\) Hz, Ar-H), 7.54–7.57 (m, 1H, Ar-H), 7.94–7.98 (m, 2H, Ar-H), 8.19 (d, 1H, \(J = 8.0\) Hz, Ar-H); \(^13\)C NMR (DMSO-d\(_6\)) \(\delta\) ppm: 21.36 (CH\(_3\)), 100.82 (O–CH\(_2\)–O), 108.20, 110.93,
1-(6-(4-Chlorophenyl)-2-methylpyridin-3-yl)-3-(3-trifluoromethyl)phenyl)urea (5e)

White crystals (yield 68%), m.p. 241-242°C; IR (KBr, ν cm⁻¹) 3378 (NH), 1733 (C=O); ¹H NMR (CDCl₃-d) δ ppm: 2.48 (s, 3H, CH₃), 6.25 (s, 1H, NH, D₂O exchangeable), 6.36 (s, 1H, NH, D₂O exchangeable), 7.38 (d, 1H, J = 8.4 Hz, Ar-H), 7.41 (d, 2H, J = 8.8 Hz, Ar-H), 7.52-7.58 (m, 3H, Ar-H), 7.78 (s, 1H, Ar-H), 7.91 (d, 2H, J = 8.4 Hz, Ar-H), 8.24 (d, 1H, J = 8.4 Hz, Ar-H); ¹³C NMR (DMSO-d₆) δ ppm: 21.57 (CH₃), 111.79, 112.69, 128.41, 128.68, 133.05, 137.32, 147.75, 147.84, 152.82 (C=O); HRMS (ESI) m/z calc'd for [M⁺]+ ([C₂₀H₁₇N₃OClF]⁺): 366.12485, found: 366.12405.

Ethyld 4-(3-(2-methyl-6-(thiophen-2-yl)pyridin-3-yl)ureido)benzoate (5j)

White crystals (yield 69%), m.p. 203–204°C; IR (KBr, ν cm⁻¹) 3393 (NH), 1733 (C=O); ¹H NMR (CDCl₃-d) δ ppm: 1.39 (t, 3H, J = 7.2 Hz, –OCH₃(CH₂)), 2.55 (s, 3H, –CH₃), 4.35 (q, 2H, J = 7.2 Hz, –OCH₂(CH₃)), 6.53 (s, 1H, NH, D₂O exchangeable), 6.98 (s, 1H, NH, D₂O exchangeable), 7.10 (t, 1H, J = 4.4 Hz, Ar-H), 7.38 (d, 1H, J = 5.2 Hz, Ar-H), 7.46 (d, 2H, J = 8.4 Hz, Ar-H), 7.52–7.55 (m, 2H, Ar-H), 7.99–8.02 (m, 3H, Ar-H), 13³C NMR (DMSO-d₆) δ ppm: 14.26 (CH₃), 21.08 (CH₃), 60.32 (O-CH₃), 116.49, 117.28, 122.91, 123.97, 127.21, 128.25, 128.50, 130.45, 132.17, 144.12, 144.57, 145.73, 147.86, 152.26 (C=O), 165.40 (–COO–); HRMS (ESI) m/z calc'd for [M⁺]+ ([C₂₀H₁₇N₃O₂Cl]⁺): 380.10744, found: 380.10764.

1-(Benzodiazolyl-1,3)dioxol-5-yl)-3-(2-methyl-6-(thiophen-2-yl)pyridin-3-yl)urea (5k)

White crystals (yield 58%), m.p. 239–241°C; IR (KBr, ν cm⁻¹) 3388 (NH), 1733 (C=O); ¹H NMR (CDCl₃-d) δ ppm: 2.44 (s, 3H, CH₃), 6.03 (s, 2H, –OCH₂(OH)–), 6.23 (s, 1H, NH, D₂O exchangeable), 6.31 (s, 1H, NH, D₂O exchangeable), 6.77 (dd, 1H, J = 2.0 Hz, Ar-H), 6.83 (d, 1H, J = 8.0 Hz, Ar-H), 6.97 (d, 1H, J = 2.0 Hz, Ar-H), 7.08 (dd, 1H, J = 4.0 Hz, Ar-H), 7.35 (d, 1H, J = 5.0 Hz, Ar-H), 7.53–7.54 (m, 2H, Ar-H), 8.12 (d, 1H, J = 8.4 Hz, Ar-H); ¹³C NMR (DMSO-d₆) δ ppm: 21.08 (CH₃), 100.87 (O-CH₂(OH)), 108.20, 110.96, 116.47, 123.74, 127.01, 127.92, 128.21, 133.26, 135.51, 144.57, 145.20, 147.27, 152.61 (C=O); HRMS (ESI) m/z calc'd for [M⁺]+ ([C₁₈H₁₈N₂O₅S]⁺): 352.07614, found: 352.07642.

2-(3-(2-Methyl-6-(thiophen-2-yl)pyridin-3-yl)ureido)benzenesulfonamide (5m)

White crystals (yield 60%), m.p. 265–266°C; IR (KBr, ν cm⁻¹) 3369, 3207 (NH, NH₂), 1733 (C=O), 1330, 1157 (SO₂); ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 2.50 (s, 3H, CH₃), 7.11 (t, 1H, H-4 of 2-thienyl, J = 4.0 Hz), 7.18 (t, 1H, H-4 of 2-(H₂NO₂S)-C₆H₄, J = 7.6 Hz), 7.52–7.56 (m, 2H, H-5 of 2-(H₂NO₂S)-C₆H₄, and H-5 of 2-thienyl), 7.60 (s, 2H, SO₂NH₂), 7.67 (d, 1H, H-3 of 2-thienyl, J = 4.0 Hz), 7.71 (d, 1H, H-5 pyridine, J = 8.4 Hz), 7.82 (d, 1H, H-6 of 2-(H₂NO₂S)-C₆H₄, J = 7.6 Hz), 7.97 (d, 1H, H-3 of 2-(H₂NO₂S)-C₆H₄, J = 8.0 Hz), 8.04 (d, 1H, H-4 pyridine, J = 8.4 Hz), 8.73 (s, 1H, NH, D₂O exchangeable), 9.15 (s, 1H, NH, D₂O exchangeable).

Biological evaluation

In vitro antitumor activity towards 60 cancer cell lines (NCI, USA)

The antitumor assay was performed according to the protocol of the Drug Evaluation Branch, NCI, Bethesda. A 48 h drug exposure protocol was adopted, and sulforhodamine B (SRB)
In vitro anti-Proliferative activity towards A549 and HCT-116 cell lines

A549 (non-small cell lung cancer cell line) and HCT-116 (human colon cancer cell line), were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St. Louis, MO), and supplemented with 10% heat-inactivated FBS (Hyclone), 10 µg/mL of insulin (Manufacturer, Sigma, St. Louis, MO, USA), and 1% penicillin-streptomycin. MTX assay was adopted to assess the in vitro antitumor activity of the newly synthesized pyridines 5a–l according to the reported procedures, using Doxorubicin as a standard treatment. Experimental conditions were tested using three replicates (three wells of the 96-well plate per experimental condition) and all experiments were carried out in triplicates. IC50 values were calculated by the use of the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models by Graph Pad, Prism version 5 (GraphPad Software Inc., La Jolla, CA).

Chemistry

The method adopted for preparation of the target pyridines 5a–l is depicted in Scheme 1. Firstly, esters 1a–c were hydrazinolized via reaction with hydrazine hydrate in methanol under reflux temperature to furnish 2-methyl-6-arylnicotinohydrazides 2a–c in 75, 71 and 80% yields, respectively. Treatment of hydrazides 2a–c with sodium nitrite in cold hydrochloric acid afforded 2-methyl-6-arylnicotinoyl azides 3a–c, which subsequently subjected to Curtius rearrangement upon heating in xylene to give the corresponding isocyanates derivatives 4a–c. The target hybrids 5a–l was obtained by reaction of isocyanates derivatives 4a–c with the appropriate aniline derivative in xylene with 55–73% yield (Scheme 1).

The structures of the newly prepared pyridines 5a–l were confirmed under the basis of spectral and elemental analyses which
Table 1. Percentage growth inhibition (GI%) of in vitro subpanel tumor cell lines at 10 μM concentration for pyridines 5a and 5c–f.

| Subpanel/Cell Line       | 5a | 5c | 5d | 5e | 5f |
|--------------------------|----|----|----|----|----|
| Leukemia                 | CCRF-CEM | 25 | –  | –  | 22 |
|                          | HL-60(TB) | 11 | 13 | –  | 21 |
|                          | K-562     | 50 | –  | 23 | 20 |
|                          | MOLT-4    | 33 | –  | –  | 12 |
|                          | RPMI-8226 | 52 | 13 | –  | 15 |
|                          | SR        | 44 | 16 | 11 | 41 |
| Non-Small Cell Lung Cancer | AS49/ATCC | 53 | 25 | 13 | 10 |
|                          | EKVX      | 17 | –  | –  | –  |
|                          | HOP-62    | 31 | –  | –  | –  |
|                          | HOP-92    | –  | –  | –  | –  |
|                          | NCI-H226  | 18 | 12 | –  | –  |
|                          | NCI-H223  | 24 | –  | –  | –  |
|                          | NCI-H322M | 15 | –  | –  | –  |
|                          | NCI-H460  | 34 | –  | –  | 34 |
|                          | NCI-H522  | 60 | 24 | 45 | 48 |
| Colon Cancer             | COLO 205  | –  | –  | –  | –  |
|                          | HCC-2998  | –  | –  | –  | –  |
|                          | HCT-116   | 51 | 17 | 26 | 21 |
|                          | HCT-15    | 42 | –  | –  | 18 |
|                          | HT29      | 43 | 18 | 14 | 25 |
|                          | KM12      | 37 | –  | 11 | –  |
|                          | SW-620    | –  | –  | –  | 11 |
| CNS Cancer               | SF-268    | –  | –  | –  | –  |
|                          | SF-295    | 17 | –  | –  | –  |
|                          | SF-539    | –  | –  | –  | 54 |
|                          | SNB-19    | 15 | –  | –  | –  |
|                          | SNB-75    | –  | –  | –  | –  |
| Melanoma                 | LOX IMVI  | 37 | 24 | 34 | 21 |
|                          | MALME-3M  | –  | –  | –  | –  |
|                          | M14       | 45 | 38 | 37 | 38 |
|                          | MDA-MB-435| 19 | –  | –  | –  |
|                          | SK-MEL-2  | –  | –  | –  | –  |
|                          | SK-MEL-28 | 20 | –  | –  | –  |
|                          | SK-MEL-5  | 32 | 38 | 19 | 38 |
|                          | UACC-257  | 38 | 29 | 15 | 26 |
|                          | UACC-62   | 40 | 29 | 20 | 27 |
| Ovarian Cancer           | IGROV1    | –  | –  | –  | –  |
|                          | OVCAR-3   | 41 | –  | –  | –  |
|                          | OVCAR-4   | 30 | –  | –  | –  |
|                          | OVCAR-5   | –  | –  | –  | –  |
|                          | OVCAR-8   | 29 | –  | –  | –  |
|                          | NCI/ADR-RES| 20 | –  | –  | –  |
|                          | SK-OV-3   | 13 | –  | –  | –  |
| Renal Cancer             | 786-0     | –  | –  | –  | –  |
|                          | A498      | –  | –  | –  | –  |
|                          | RXF 393   | –  | –  | –  | –  |
|                          | SN12C     | 26 | –  | –  | –  |
|                          | TR-10     | –  | –  | –  | –  |
|                          | UO-31     | 15 | –  | –  | –  |
| Prostate                 | PC-3      | 55 | –  | 20 | –  |
|                          | DU-145    | 11 | –  | –  | –  |
| Breast Cancer            | MCF-7     | 30 | 20 | –  | 20 |
|                          | MDA-MB-231| 28 | –  | –  | –  |
|                          | HS 578T   | –  | –  | –  | –  |
|                          | BT-549    | 45 | –  | 13 | –  |
|                          | MDA-MB-468| 32 | –  | –  | –  |

aOnly GI% higher than 10% are shown.

were in full agreement with the postulated structures (Supplementary Material).

Biological evaluation

In vitro antitumor activity towards 60 cancer cell lines (NCI, USA)

The structures of all the newly synthesized pyridines 5a–I were submitted to the National Cancer Institute (NCI) Developmental Therapeutic Program (www.dtp.nci.nih.gov). Ten pyridines 5a, 5c–j and 5I were chosen to be in vitro evaluated for their antitumor activity. The selected pyridines 5a, 5c–j and 5I were examined at one dose (concentration 10^{-5} M) primary anticancer assay towards a panel including 85 cancer lines. Nine different types of cancer were tested in this assay: colon, ovarian, prostate, leukemia, melanoma, CNS, renal, breast and lung cancers. A 48 h drug exposure protocol was adopted, and sulforhodamine B (SRB) assay was
cell lines. In particular, cancer (OVCAR-5), renal cancer (A498) and breast cancer (HS 578T) cancer (HOP-92 and NCI-H226), melanoma (MALME-3M), ovarian (OVCA-R-8) and prostate (PC-3) cell lines.

Table 3. In vitro anti-proliferative activity of target pyridines 5a–l against A549 and HCT-116 cell lines.

| Compound | Ar         | R         | A549 (μM) | HCT-116 (μM) |
|----------|------------|-----------|-----------|--------------|
| 5a       | 4-F-C6H4   | 3-CF3     | 6.83 ± 0.42 | 5.49 ± 0.30  |
| 5b       | 4-F-C6H4   | 3,5-(CF3)2 | 24.05 ± 1.78 | 16.03 ± 1.52 |
| 5c       | 4-F-C6H4   | 4-COOEt   | 9.61 ± 1.03 | NTb          |
| 5d       | 4-F-C6H4   | 3,4-Methylenedioxy | 12.48 ± 0.85 | 10.37 ± 0.84 |
| 5e       | 4-Cl-C6H4  | 3-CF3     | 11.87 ± 0.92 | 7.05 ± 0.72  |
| 5f       | 4-Cl-C6H4  | 4-OCH3    | 7.90 ± 0.54 | 12.61 ± 1.08 |
| 5g       | 4-Cl-C6H4  | 3,4-Methylenedioxy | 6.72 ± 0.38 | NTb          |
| 5h       | 2-thienyl  | 4-F       | 10.64 ± 0.86 | 8.25 ± 0.84  |
| 5i       | 2-thienyl  | 4-Cl      | 8.73 ± 0.71 | NTb          |
| 5j       | 2-thienyl  | 4-COOEt   | 8.04 ± 0.59 | 9.38 ± 0.67  |
| 5k       | 2-thienyl  | 3,4-Methylenedioxy | 19.17 ± 2.05 | 16.43 ± 1.30 |
| 5l       | 2-thienyl  | 2-SO2NH2  | 3.22 ± 0.25 | 2.71 ± 0.16  |
| Dox.     | –          | –         | 2.93 ± 0.28 | 3.10 ± 0.22  |

IC50 values are the mean ± SD of three separate experiments.

NA: Not tested.

Utilized to assess the cell growth and viability. The results were reported as mean-graph of the percentage growth of the treated cells, and displayed as percentage growth inhibition (GI%) caused by the test pyridines (Tables 1 and 2). Investigation of data in Tables 1 and 2 revealed that the examined pyridines exhibited distinctive patterns of sensitivity and selectivity against the different NCI cancer cell panels.

Inspecting the GI% values in Tables 1 and 2, highlighted that compound 5l stood out as the most potent pyridine derivative assayed in this study (mean % GI = 40). Pyridine 5l possessed broad spectrum antitumor activity against all tested cancer cell lines from all subpanels with the exception to non-small cell lung cancer (HOP-92 and NCI-H226), melanoma (MALME-3M), ovarian cancer (OVCA-R-5), renal cancer (A498) and breast cancer (HS 578T) cell lines. In particular, 5l showed a potent growth inhibitory activity towards leukemia MOLT-4, non-small cell lung cancer NCI-H460, colon cancer HCT-116 and HCT-15, melanoma LOX IMVI and breast cancer MCF7 cell lines with inhibition % 80, 82, 74, 71, 86 and 75, respectively. In addition, it displayed GI more than 50% over leukemia (CCRF-CEM, K-562, RPMI-8226 and SR), non-small cell lung cancer (A549 and HOP-62), colon cancer (COLO205, HT29, KM12 and SW-620), CNS (SF-295, SF-539 and U251), ovarian (OVCA-R-8 and prostate (PC-3) cell lines, Figure 2.

Furthermore, pyridine 5a was found to be the second most active member (mean % GI = 22) with broad spectrum activity against 42 cell lines represent all subpanels. Compound 5a exerted cytoxic activity with GI more than 40% against leukemia (K-562, RPMI-8226 and SR), non-small cell lung cancer (A549 and NCI-H522), colon cancer (HCT-116, HCT-15 and HT29), melanoma (M14 and UACC-62), ovarian (OVCA-R-3), prostate (PC-3) and breast (T-47D) cell lines (Figure 2).

Further investigation of results in Tables 1 and 2 unveiled that all cell lines of the leukemia subpanel were sensitive to six tested pyridines 5a, 5e, 5f, 5h, 5j and 5l with GI ranging from 10% to 91%. It is noteworthy that only non-small cell lung cancer A549 and NCI-H522 cells were sensitive to all the tested pyridines with GI% range of 10–61% and 24–60%, respectively. Additionally, leukemia SR (except 5l), leukemia HL-60 (except 5d) and colon cancer HT29 (except 5l) cell line were susceptible to nine tested pyridines. The most susceptible cell lines towards the impact of pyridines 5a and 5l are displayed in Figure 2.

In vitro anti-proliferative activity against A549 and HCT-116 cell lines

All newly synthesized pyridines 5a–l were examined for their anti-proliferative activity against two cancer cell lines: non-small cell lung cancer A549 cell line and colon cancer HCT-116 cell line. The MTT colorimetric assay was adopted to assess the anti-proliferative activity as described by Mosmann29. Doxorubicin was used as a control in this assay. The results were expressed as median growth inhibitory concentration (IC50) values that represent the compound concentration required to produce a 50% inhibition of cell growth after 48 h of incubation (Table 3).

The results of the MTT assay listed in Table 3 suggested that the examined pyridines 5a–l exhibited excellent to moderate growth inhibitory activity against the tested A549 and HCT-116 cancer cell lines. Also, HCT-116 cells were found to be more sensitive to the impact of the tested compounds than A549 cells, except compound 5j which is more effective towards A549 cells. Interestingly, compound 5l emerged as the most active one towards both A549 and HCT-116 cell lines with IC50 values equal...
Table 4. Cytotoxicity of pyridines 5a–l towards non-tumorigenic human lung fibroblast WI-38 cell line and their selectivity index (S. I.) towards lung A549 cancer cells.

|                | IC50 (µM) | S. I. |
|----------------|-----------|-------|
|                | WI-38     | WI-38/A549 |
| 5a             | 93.55 ± 5.28 | 13.7 |
| 5b             | 151.37 ± 8.12 | 6.3 |
| 5c             | 122.61 ± 10.17 | 12.8 |
| 5d             | 107.28 ± 7.03 | 8.6 |
| 5e             | 130.44 ± 9.22 | 11.0 |
| 5f             | 115.86 ± 9.61 | 14.7 |
| 5g             | 63.48 ± 5.08 | 9.4 |
| 5h             | 142.60 ± 8.38 | 13.4 |
| 5i             | 129.31 ± 11.95 | 14.8 |
| 5j             | 107.29 ± 7.02 | 13.3 |
| 5k             | 138.74 ± 10.40 | 7.2 |
| 5l             | 67.05 ± 3.82 | 17.6 |

IC50 values are the mean ± SD of three separate experiments.

Table 5. Impact of pyridine 5l on the expression levels of Bax and Bcl-2 in HCT-116 cancer cells treated with the compound at its IC50 concentration.

| Comp. | Bax (ng/mL) | Bcl-2 (pg/mL) | Bax/Bcl-2 ratio |
|-------|-------------|---------------|-----------------|
| Control | 41.9 | 5.11 | 8.2 |
| 5l | 256.7* | 1.24* | 207 |

Data are represented as mean ± SD of three separate experiments.

Table 6. Impact of pyridine 5l on the expression levels of cytochrome C, p53, active caspases-3 and -9, in HCT-116 cancer cells treated with the compound at its IC50 concentration.

| Comp. | Cyt-c (pg/mL) | p53 (ng/mL) | Caspase-9 (ng/mL) | Caspase-3 (pg/mL) |
|-------|--------------|-------------|------------------|------------------|
| Control | 67 | 44.3 | 2.34 | 46.8 |
| 5l | 858* | 961.2* | 21.3* | 458.4* |

Data are mean ± SD of three separate experiments.

Induction of apoptosis in colorectal cancer HCT-116 cells

To investigate the mechanism of antitumor activity of the target pyridines and in continuation of our efforts to develop potent pro-apoptotic anticancer agents, the ability of sulfonamide 5l to provoke apoptosis in HCT-116 cells was evaluated.

Effects on mitochondrial apoptosis pathway proteins Bcl-2 and Bax

Bcl-2 and Bax are two discrete members of a gene family involved in the regulation of cellular apoptosis known as Bcl-2 family, which finely tune the apoptotic switch on/off mechanism and considered as an important gatekeeper to the apoptotic response. While Bcl-2 protein is functionally characterized as an apoptosis-suppressing factor, the Bax protein is more functionally characterized as an apoptosis-promoting factor. So, the intracellular Bax/Bcl-2 ratio can profoundly influence the ability of a cell to respond to an apoptotic signal.

In this study, treatment of HCT-116 cells with the IC50 of pyridine 5l (IC50 = 3.22 ± 0.25 µM) resulted in a significant up-regulation of the expression level of the pro-apoptotic Bax protein by 6-fold compared to untreated control, with a concomitant significant decrease in the expression level of the anti-apoptotic Bcl-2 protein by approximately 75% compared to control (Table 5). These results revealed that pyridine 5l significantly boosted the Bax/Bcl-2 ratio 25-fold in compared to control.

Effect on the level of cytochrome C

The interplay between the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins triggers the activated Bax to bind to the mitochondrial outer membrane which induces the opening of the mitochondrial voltage-dependent anion channel (VDAC), resulting in the release of cytochrome C from mitochondria into cytosol where it activates the caspase-dependent signaling and subsequent apoptosis. Involvement of cytochrome C release from mitochondria is an indicator of activation of the intrinsic apoptotic pathway.

In this study, we assessed the expression level of cytochrome C to assure the adoption of the intrinsic pathway. As shown in Table 6, the level of cytochrome C was induced significantly higher (12-folds) in HCT-116 cells treated with pyridine 5l, compared to untreated control (Table 6).

Effect on the level of p53

One of the major apoptosis signaling pathways involves the p53 tumor suppressor. The ability of p53 to control apoptosis in response to abnormal proliferative signals and stress is crucial for its tumor suppression role. p53 tumor suppressor protein is a nuclear transcription factor that regulates the expression of a wide variety of genes involved in apoptosis. p53 is able to induce Bax oligomerization and cytochrome c release from mitochondria.

The effect of pyridine 5l on p53 expression in HCT-116 cells was evaluated in this study. Results in Table 5 highlighted that treatment of HCT-116 cells with pyridine 5l led to 21-fold enhanced expression levels of p53, compared to control (Table 6).

Effects on the levels of active caspase-3 and caspase-9

Caspases, cysteine-dependent aspartate-directed proteases, are key factors in apoptotic cell death that have been shown to play an important role in cleavage of vital structural and regulatory proteins.

In vitro cytotoxicity towards non-tumorigenic human WI-38 cells

The cytotoxic activity of all synthesized pyridines 5a–l were assessed against non-tumorigenic human lung fibroblast WI-38 cell line to investigate their safety, using the MTT colorimetric assay. The results were expressed as IC50 values and the calculated selectivity index are presented in Table 4.

The examined pyridines 5a–l displayed non-significant cytotoxic impact towards human lung fibroblast WI-38 cell line with IC50 values spanning from 63.48 to 151.08 µM, thereby providing a good safety profile as anticancer agents with selectivity index range (6.3–17.6).
proteins important for cells survival, so activation of caspases is a hallmark for apoptosis induction\(^44\). The leading upstream caspases are caspase-9 in the intrinsic pathway and caspase-8 in the extrinsic pathway, where both converge to caspase-3 which is the key executioner of apoptosis\(^45\).

In comparison with the untreated control, the expression levels of active caspase-3 and caspases-9 in HCT-116 cells were 5.1- and 2.5-fold increased, respectively, in response to pyridine 5l treatment with its IC\(_{50}\) concentration (Table 6).

**Cell cycle analysis**

Targeting the cell cycle of cancer cells has emerged as a promising approach for cancer therapy\(^46\). In the current study, pyridine 5l was examined for its effect on the cell cycle distribution in HCT-116 cells (Figure 3). The results of the DNA flow cytometric assay showed that treatment of HCT-116 cells with pyridine 5l at its IC\(_{50}\) concentration for 24 h resulted in a significant 7.3-fold increased percentage of HCT-116 cells at Sub-G1, with concurrent significant reduction in the G2-M phase by approximately 2.2-fold. Both arrest of G2-M phase and alteration of the Sub-G1 phase are considered significant remarks for pyridine 5l to induce apoptosis in HCT-116 cells.

**AnnexinV-FITC/propidium iodide analysis of apoptosis**

Translocation of phosphatidylserine (PS) from the inner to the outer membrane leaflet of the cell is an early apoptotic event, which could be detected by fluorescein-labeled annexinV (annexinV-FITC), a Ca\(^{2+}\)-dependent phospholipid-binding protein with high affinity for PS. Combined with propidium iodide PI (an indicator of cell integrity), a measure of percentage cell population in early apoptosis can be achieved. Cells displaying increased annexinV-FITC fluorescence without a concurrent increase in PI fluorescence are considered to be in early apoptosis, whereas an increase is seen in both fluorescence channels, signifies a late apoptosis\(^47\).

In this study, AnnexinV-FITC/PI dual staining assay was performed to evaluate the effect of compound 5l on both early and late apoptosis percentages in HCT-116 cells (Figure 4, Table 7). As presented in Figure 4, the assay outcomes clearly indicate that the treatment of HCT-116 cells with 5l resulted in a significant increase in the percentage of annexinV-FITC-positive apoptotic

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**Figure 3.** Effect of compound 5l on the phases of cell cycle of HCT-116 cells. *Significantly different from control at p < 0.05. (Two-way ANOVA test).
Conclusion

In summary, herein we report the synthesis of novel series of 1-(2-methyl-6-arylpyridin-3-yl)-3-phenylureas 5a–l. All the prepared pyridins were evaluated for their in vitro anticancer activity against two cancer cell lines: non-small cell lung cancer A549 cell line and colon cancer HCT-116 cell line. Compound 5l was found to be the most active congener towards both A549 and HCT-116 cell lines with IC50 values equal to 3.22 ± 0.2 and 2.71 ± 0.16 μM, respectively, which are comparable with those of Doxorubicin: 2.93 ± 0.28 and 3.10 ± 0.22, respectively. Furthermore, compound 5l stood out as the most potent pyridine derivative (mean % GI¼ 40), at US-NCI Developmental Therapeutic Program anticancer assay, with broad-spectrum antitumor activity against the most tested cancer cell lines from all subpanels. The ability of sulfonamide 5l to provoke apoptosis in HCT-116 cells was evaluated. Results revealed that pyridine 5l significantly boosted the Bax/Bcl-2 ratio 25-fold compared to control. Also, the expression levels of cytochrome C, p53, active caspase-3 and caspases-9 in HCT-116 cells were 12-, 21-, 5.1- and 2.5-fold increased, respectively, in response to pyridine 5l treatment. Furthermore, treatment of HCT-116 cells with pyridine 5l at its IC50 concentration resulted in a significant 7.3-fold increased percentage of HCT-116 cells at Sub-G1, with concurrent significant reduction in the G2-M phase by approximately 2.2-fold, in addition to a significant increase in the percentage of annexinV-FITC-positive apoptotic cells, including both the early and late apoptotic phases (LR; from 1.18% to 6.79%, and UR; from 0.81% to 8.97%), that represents about eightfold total increase in comparison with control.

Disclosure statement

No potential conflict of interest was reported by the authors.

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