Discovery of Novel (Imidazo[1,2-*a*]pyrazin-6-yl)ureas as Antiproliferative Agents Targeting P53 in Non-small Cell Lung Cancer Cell Lines

MARC-ANTOINE BAZIN[1], BÉNÉDICTE ROUSSEAU[2], SOPHIE MARHADOUR[1], CHRISTOPHE TOMASONI[2], PIERRE EVENOU[1], SYLVIE PIESSARD[1], ABRAHAM J. VAISBERG[3], SANDRINE RUCHAUD[4], STÉPHANE BACH[4], CHRISTOS ROUSSAKIS[2] and PASCAL MARCHAND[1]

[1]Medicinal Chemistry Department, [2]Lung Cancer and Molecular Targets Department, Nantes University, Targets and Drugs for Infectious Diseases and Cancer, IICiMed EA 1155, Faculty of Pharmacy, Nantes, France; [3]Department of Pharmaceutical Sciences, Laboratories of Investigation and Development, Faculty of Sciences and Philosophy, Peruvian University Cayetano Heredia, Lima, Peru; [4]Sorbonne Universities, Pierre and Marie Curie University Paris 06, CNRS USR3151, 'Protein Phosphorylation & Human Disease', Roscoff Biology Station, Roscoff, France

Abstract. *A series of (imidazo[1,2-a]pyrazin-6-yl)ureas were synthesized through 6-aminoimidazo[1,2-a]pyrazine as a key intermediate. 1-(Imidazo[1,2-a]pyrazin-6-yl)-3-(4-methoxyphenyl)urea displayed a cytostatic activity against a non-small cell lung cancer cell line and was chosen for further mechanistic studies. Growth kinetics highlighted a selective dose-dependent response of P53-mutant NSCLC-N6-L16 cell line and overexpression of TP53 gene induced by this compound. These pharmacological data suggest a promising reactivation of p53 mutant in NSCLC-N6-L16 cell line.*

Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers and currently remains very resistant to all therapies (1). This underlies the urgent need for the development of new chemotherapeutic agents with more potent antitumor activities and low toxicity. Among the different molecular events involved in carcinogenesis, P53 plays a pivotal role in tumor suppression by inducing cell-cycle arrest, apoptosis or senescence (2). Indeed, the discovery of *TP53* as a tumor-suppressor gene (3, 4) led cancer biologists to study the mechanisms of tumor formation in depth and P53 became a prime target for anticancer drug therapy (5). *TP53* is mutated in 50% of human cancers and in more than 70% of lung cancers, and results in a loss of function of the wild-type (wt) P53 protein (6).

Various strategies have been undertaken for targeting P53 tumor suppressor. Among them, P53 reactivation, that can be achieved by small molecules such as PRIMA-1 (7), has been used to change P53 conformation from the mutant to the wild type (8). This approach has been developed by our laboratory, and we found that triazine A190 was able to restore the transcription factor activity of mutated P53 in an NSCLC cell line, inducing blocking of the cell cycle in the G1 phase and apoptosis (9). Furthermore, more recently, triazine A190 was demonstrated to induce overexpression of neural precursor cell expressed developmentally down-regulated protein 9 (*NEDD9*)/human enhancer of filamentation 1 (*HEF1*)/CRK-associated substrate-related protein (*CASL*) gene, which leads to apoptosis (10).

In the frame of our research line of heterocyclic compounds displaying anti-proliferative activities against NSCLC cell lines (11), we aimed to design original imidazo[1,2-*a*]pyrazines and investigating their antiproliferative activity and selectivity against two NSCLC cell lines. The imidazo[1,2-*a*]pyrazine scaffold is a well-known heterocyclic structure displaying a wide range of pharmacological and biological properties (12), including anticancer activities. Indeed, a literature survey indicated that di-substituted imidazo[1,2-*a*]pyrazines at the C-6 and C-8 positions (13-15) or at the C-3 and C-6 positions (16-18), tri-substituted at the C-3, C-6 and C-8 positions (19), and tetra-substituted at the C-2, C-3, C-6 and C-8 positions (20)

Correspondence to: Professor Pascal Marchand, Laboratoire de Chimie Thérapeutique, IICiMed EA 1155, UFR des Sciences Pharmaceutiques et Biologiques, 1 rue Gaston Veil, 44035 Nantes, France. Tel: +33 240412874, Fax: +33 240412876, e-mail: pascal.marchand@univ-nantes.fr

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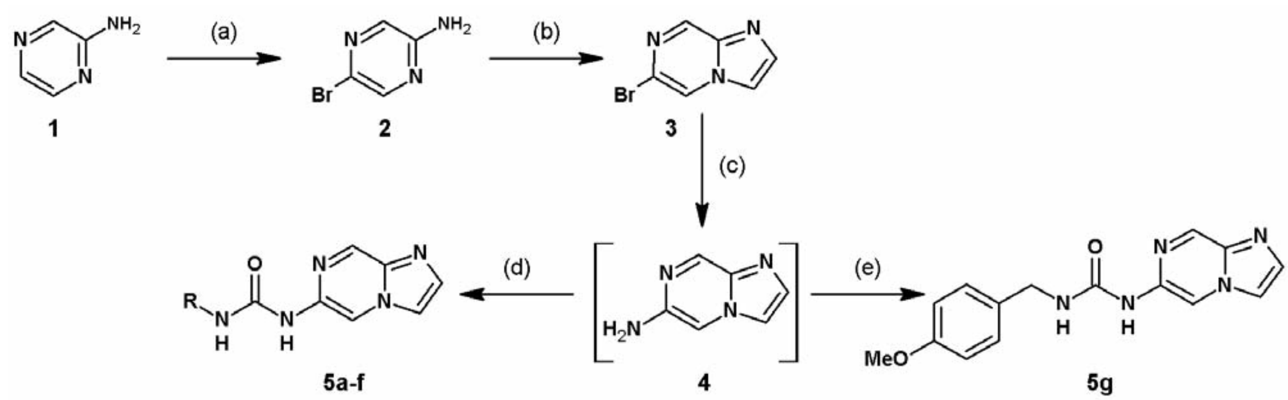


Figure 1. *Schematic chemical synthesis of (imidazo[1,2-a]pyrazin-6-yl)ureas. Reagents and conditions: (a) N-bromosuccinimide, acetonitrile, r.t., 2 h, yield 89%; (b) 2-bromo-1,1-diethoxyethane, HBr 48%, propan-2-ol, 80˚C, 3 h, yield 59%; (c) NH_4OH, $CuSO_4$•$5H_2O$, sealed tube, 90˚C, 2 h; (d) isocyanate, N,N-dimethylformamide, r.t., 0.5-16 h, yield 14-33%; (e) 4-nitrophenyl(4-methoxybenzyl)carbamate, Et_3N, tetrahydrofuran, 60˚C, 16 h, yield 11%.*

possess anticancer activities through kinase inhibition or anti-angiogenic properties. More specifically, imidazo[1,2-*a*]pyrazines bearing urea have been described to display antiproliferative activity (21). Due to the biological significance of imidazo[1,2-*a*]pyrazines and based on our ongoing studies on the synthesis of imidazo[1,2-*a*]azines derivatives (22-25), we report herein the synthesis of novel (imidazo[1,2-*a*]pyrazin-6-yl)ureas **5a-g**, their antiproliferative activities towards cells and their modulation of *TP53* expression (Figure 1).

Materials and Methods

Chemical synthesis of compounds described in this study. Preparation of appropriate (imidazo[1,2-*a*]pyrazin-6-yl)ureas was achieved through obtaining 6-bromoimidazo[1,2-*a*]pyrazine **3**. As a first strategy, Pd-catalyzed cross-couplings between compound **3** and *N*-phenylurea and 1-benzylurea were attempted following the protocol described by Abad *et al*. (26). However, we obtained poor yields (ca <20%) of the desired products. We next decided to synthesize 6-aminoimidazo[1,2-*a*]pyrazine **4** that could react with either an isocyanate or a carbamate to produce the corresponding urea. As depicted in Figure 1, the new (imidazo[1,2-*a*]pyrazin-6-yl)ureas **5a-g** were synthesized in four steps starting from commercially available aminopyrazine **1**. This compound was brominated into 5-bromopyrazin-2-amine **2** using *N-bromosuccinimide*. Subsequent cyclization with 2-bromo-1,1-diethoxyethane and hydrobromic acid afforded imidazo[1,2-*a*]pyrazine scaffold in a satisfactory yield. Ureas **5a-g** were produced through the preparation of the 6-aminoimidazo[1,2-*a*]pyrazine **4** as the key intermediate (27). The first method involved the condensation of isocyanates with heterocyclic amine **4** to provide ureas **5a-f**, unfortunately in low yields (14-33% from **3**). Despite many optimization attempts, the reactions did not work properly due to the weak nucleophilicity of amine **4** and its instability in the medium. Regarding the preparation of urea **5g**, the use of 4-nitrophenyl(4-methoxybenzyl)carbamate was required as described by Manley *et al*. (28).

Solvents and reagents were obtained from commercial suppliers and were used without further purification. Analytical TLC was performed on silica gel 60 F254 plates. Column chromatography was carried out on silicagel Merck 60 (70-230 mesh ASTM. Yields refer to chromatographically and spectroscopically pure compounds. Melting points were determined on an Electrothermal IA 9000 melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a IRAffinity-1 IR-FT spectrophotometer equipped with a MIRacle 10 accessory ATR (Shimadzu, Marne la Vallée, France). NMR experiments were run with a AVANCE 400 spectrometer (Bruker, Wissembourg, France). Spectra were acquired with deuterated dimethylsulfoxide (DMSO-d_6) as a solvent. Chemical shifts are reported as δ values in parts per million (ppm) relative to tetramethylsilane as internal standard and coupling constants (*J*) are given in hertz (Hz). Data are reported as follows: s=singlet, d=doublet, dd=doublet of doublets, ddd=doublet of doublet of doublets, t=triplet, m=multiplet. Mass spectra were recorded using an electrospray ionization method with a ZQ 2000 spectrometer (Water S.A.S, Saint-Quentin en Yvelines, France). Microwave reactions were carried out in a Discover microwave reactor (CEM μ WAVES, Saclay, France) in sealed vessels (monowave, maximum power 300 W, temperature control by IR sensor, fixed temperature). Elemental analyses were performed on a Elemental Analyser Flash EA 1112 (Thermo Scientific, Courtaboeuf, France) and were found to be within 0.4% of the theoretical values.

5-Bromopyrazin-2-amine (2): To a solution of aminopyrazine **1** (20.0 g, 210 mmol) in dry acetonitrile (700 ml) was added *N-bromosuccinimide* (41.2 g, 231 mmol). After stirring for 2 h at room temperature, the resulting mixture was diluted with ethyl acetate. Water was added and the organic layer was extracted with ethyl acetate. The combined organic layers were washed with water, dried over sodium sulfate, filtered and concentrated under vacuum. The crude product was purified by silica-gel chromatography using dichloromethane as eluent to give 5-bromopyrazin-2-amine **2** as a beige powder (32.6 g, 89%). Mp 110-111°C [lit. (29) 112-114°C]; R_f=0.80 (EtOAc); IR ν_{max} (cm^{-1}): 3397 and 3302 (νN-H), 1566 and 1530 (νC=C and νC=N), 646 (νC-Br); ^{1}H NMR (400 MHz) δ 8.06 (s, 1 H, H_6), 7.71 (s, 1 H, H_3), 6.69 (s, 2 H, NH_2); ^{13}C NMR (100 MHz) δ 155.47 (C_2), 143.73 (C_6), 132.27 (C_3), 123.80 (C_5); MS (ESI) m/z (%):174.9 (100) $[M+H]^+$, 176.9 (97) $[M+H+2]^+$; UPLC purity 98%.

6-Bromoimidazo[1,2-a]pyrazine (3): To a solution of 5-bromopyrazin-2-amine **2** (5.0 g, 28.7 mmol) and aqueous HBr 48% (1.25 ml) in propan-2-ol (125 ml) was added 2-bromo-1,1-diethoxyethane (8.9 ml, 57.5 mmol). The reaction mixture was heated at 80°C for 3 h. The reaction mixture was cooled to room temperature and neutralized with an aqueous saturated solution of sodium bicarbonate. The organic layer was extracted twice with dichloromethane. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and concentrated under vacuum. The crude product was purified by silica-gel chromatography using cyclohexane/ethyl acetate (1/1) as eluent to afford 6-bromoimidazo[1,2-*a*]pyrazine **3** as a beige powder (3.3 g, 59%). Mp 159-160°C; R_f=0.50 (EtOAc); IR ν_{max} (cm^{-1}): 3040 (νC-Har), 1601 and 1487 (νC=C and νC=N), 640 (νC-Br); ^{1}H NMR (400 MHz) δ 9.02 (d, 1 H, ^3J=1.0 Hz, H_2), 8.99 (s, 1 H, H8), 8.16 (s, 1 H, H_5), 7.92 (d, 1 H, ^3J=1.0 Hz, H_3); ^{13}C NMR (100 MHz) δ 142.47 (C_8), 139.62 (C_{8a}), 136.84 (C_3), 121.99 (C_6), 120.91 (C_2), 115.44 (C_5); MS (ESI) m/z (%): 199.0 (100) $[M+H]^+$, 201.0 (97) $[M+H+2]^+$; UPLC purity 97%.

Imidazo[1,2-a]pyrazin-6-amine (4): In a sealed tube with a magnetic stir bar was added 6-bromoimidazo[1,2-*a*]pyrazine **3** (500 mg, 2.5 mmol), copper (II) sulfate pentahydrate (946 mg, 3.8 mmol) in a 25% aqueous ammonia solution (25 ml). The suspension was heated at 90°C for 2 h. After cooling, the resulting mixture was diluted with ethyl acetate. Water was added and the organic layer was extracted with ethyl acetate. The combined organic layers were washed with water, dried over sodium sulfate, filtered and concentrated under vacuum. The crude product was used without further purification. Yellow oil; ^{1}H NMR (400 MHz) δ 8.70 (s, 1 H, H_8), 7.93 (s, 1 H, H_5), 7.64 (d, 2 H, ^3J=1.0 Hz, H_2, H_3), 5.41 (s, 2 H, NH_2); MS (ESI) m/z (%): 134.9 (100) $[M+H]^+$.

*General procedure for the synthesis of compounds **5a-f**.* To a solution of crude imidazo[1,2-*a*]pyrazin-6-amine **4** (1 equiv.) in dimethylformamide (3 ml/mmol of **4**) was added isocyanate (1.2 equiv.). The suspension was stirred at room temperature for 30 minutes to 16 h. The solvent was removed under reduced pressure and the crude material was purified by silica gel chromatography.

*1-(Imidazo[1,2-a]pyrazin-6-yl)-3-phenylurea (**5a**):* The reaction proceeded using phenyl isocyanate (330 µl, 3.0 mmol) under stirring for 16 h. The crude product was purified by silica gel chromato-graphy using dichloromethane/ethanol (99/1) as eluent to afford **5a** as a white powder (96 mg, 15% from **3**, two steps). Mp > 350°C; R_f=0.29 (EtOAc); IR ν_{max} (cm^{-1}): 3298 (νN-H), 3042 (νC-Har), 1715 (νC=O), 1549 and 1497 (νC=C and νC=N); ^{1}H NMR (400 MHz) δ 9.10 (s, 1 H, H_2), 9.04-9.03 (m, 2 H, NH), 8.93 (s, 1 H, H_8), 8.26 (s, 1 H, H_5), 7.81 (s, 1 H, H_3), 7.51 (d, 2 H, ^3J=7.2 Hz, H_a), 7.35 (dd, 2 H, ^3J=$^3J'$=7.2 Hz, H_b), 7.04 (t, 1 H, ^3J=7.2 Hz, H_c); ^{13}C NMR (100 MHz) δ 152.01 (C=O), 141.16 (C_8), 139.42 (C), 138.83 (C), 138.02 (C), 135.96 (C_3), 129.10 (2 C_b), 122.38 (C_c), 118.31 (2 C_a), 115.72 (C_5), 106.47 (C_2); MS (ESI) m/z (%): 254.1 (100) $[M+H]^+$; UPLC purity 99%; Anal. Calcd for $C_{13}H_{11}N_5O$: C 61.65; H 4.38; N 27.65. Found: C 61.85; H 4.22; N 27.77%.

*1-(Imidazo[1,2-a]pyrazin-6-yl)-3-(4-methoxyphenyl)urea (**5b**):* The reaction proceeded using 4-methoxyphenyl isocyanate (393 µl, 3.0 mmol) under stirring for 15 h. The crude product was purified by silica-gel chromatography using dichloromethane/ethanol (99/1) as eluent to afford **5b** as a beige powder (165 mg, 23% from **3**, two steps). Mp 230-231°C; R_f=0.28 (EtOAc); IR ν_{max} (cm^{-1}): 3339 (νN-H), 2957 (νC-Hal), 1703 (νC=O), 1555 and 1504 (νC=C and νC=N); ^{1}H NMR (400 MHz) δ 9.08 (d, 1 H, ^3J=1.0 Hz, H_2), 8.94-8.92 (m, 2 H, H_8, NH), 8.86 (s, 1 H, NH), 8.24 (s, 1 H, H_5), 7.80 (d, 1 H, ^3J=1.0 Hz, H_3), 7.42 (d, 2 H, 3J=8.8 Hz, H_a), 6.93 (d, 2 H, ^3J=8.8 Hz, H_b), 3.76 (s, 3 H, OMe); ^{13}C NMR (100 MHz) δ 154.87 (C), 152.17 (C), 141.11 (C_8), 138.81 (C), 138.19 (C), 135.92 (C_3), 132.42 (C), 120.12 (2 C_a), 115.66 (C_5), 114.29 (2 C_b), 106.29 (C_2), 55.37 (OMe); MS (ESI) m/z (%): 284.1 (100) $[M+H]^+$; UPLC purity 96%; Anal. Calcd for $C_{14}H_{13}N_5O_2$: C 59.36; H 4.63; N 24.72. Found: C 59.30; H 4.98; N 25.01%.

*1-(Imidazo[1,2-a]pyrazin-6-yl)-3-(3-methoxyphenyl)urea (**5c**):* The reaction proceeded using 3-methoxyphenyl isocyanate (400 µl, 3.0 mmol) under stirring for 2 h. The crude product was purified by silica-gel chromatography using dichloromethane/ethanol (99/1) as eluent to afford **5c** as a yellow powder (158 mg, 22% from **3**, two steps). Mp 236-237°C; R_f=0.27 (EtOAc); IR ν_{max} (cm^{-1}): 3321 (νN-H), 3099 (νC-Har), 1713 (νC=O), 1545 and 1491 (νC=C and νC=N); ^{1}H NMR (400 MHz) δ 9.11 (d, 1 H, ^3J=1.2 Hz, H2), 9.05 (s, 1 H, NH), 9.01 (s, 1 H, NH), 8.93 (s, 1 H, H_8), 8.26 (s, 1 H, H5), 7.81 (d, 1 H, ^3J=1.2 Hz, H_3), 7.29 (s, 1 H, Ha), 7.24 (dd, 1 H, ^3J=$^3J'$=8.0 Hz, H_c), 6.95 (dd, 1 H, ^3J=8.0 Hz, ^4J=1.6 Hz, H_d), 6.63 (dd, 1 H, ^3J=8.0 Hz, 4J=1.6 Hz, H_b), 3.78 (s, 3 H, OMe); ^{13}C NMR (100 MHz) δ 159.93 (C), 151.94 (C), 141.15 (C8), 140.64 (C), 137.94 (C), 137.86 (C), 135.96 (C_3), 129.86 (C_c), 115.75 (C_5), 110.59 (C_d), 107.92 (C_b), 106.53 (C_2), 103.95 (Ca), 55.12 (OMe); MS (ESI) m/z (%): 284.1 (100) $[M+H]^+$; UPLC purity 95%; Anal. Calcd for $C_{14}H_{13}N_5O_2$: C 59.36; H 4.63; N 24.72. Found: C 59.54; H 4.76; N 24.32%.

*1-(3-Chlorophenyl)-3-(imidazo[1,2-a]pyrazin-6-yl)urea (**5d**):* The reaction proceeded using 3-chlorophenyl isocyanate (367 µl, 3.0 mmol) under stirring for 30 minutes. The crude product was purified by silica-gel chromatography using ethyl acetate/petroleum ether (6/4) as eluent to afford **5d** as a beige powder (157 mg, 22% from 3, two steps). Mp 255-256°C; R_f=0.27 (EtOAc); IR ν_{max} (cm^{-1}): 3292 (νN-H), 3055 (νC-Har), 1722 (νC=O), 1539 and 1481 (νC=C and νC=N), 700 (νC-Cl); ^{1}H NMR (400 MHz) δ 9.23 (s, 1 H, NH), 9.11-9.10 (m, 2 H, H_2, NH), 8.94 (s, 1 H, H_8), 8.26 (s, 1 H, H_5), 7.82-7.80 (m, 2 H, H_3, H_a), 7.37 (dd, 1 H, ^3J=3J'=7.6 Hz, H_c), 7.30 (dd, 1 H, ^3J=7.6 Hz, ^4J=1.4 Hz, H_d), 7.10 (ddd, 1 H, ^3J=7.6 Hz, ^4J=1.4 Hz, ^4J=0.8 Hz, Hb); ^{13}C NMR (100 MHz) δ 151.89 (C=O), 141.18 (C_8), 140.94 (C), 138.85 (C), 137.74 (C), 136.02 (C_3), 133.47 (C), 130.71 (C_c), 122.01 (C_b), 117.72 (C_a), 116.81 (C_d), 115.76 (C_5), 106.80 (C_2); MS (ESI) m/z (%): 288.1 (100) $[M+H]^+$, 290.0 (34) $[M+H+2]^+$; UPLC purity 98%; Anal. Calcd for $C_{13}H_{10}ClN_5O$: C 54.27; H 3.50; N 24.34. Found: C 54.32; H 3.37; N 24.18%.

*1-[4-(Benzyloxy)phenyl]-3-(imidazo[1,2-a]pyrazin-6-yl)urea (**5e**):* The reaction proceeded using 4-benzyloxyphenyl isocyanate (683 mg, 3.0 mmol) under stirring for 16 h. The crude product was purified by silica-gel chromatography using dichloromethane/ethanol (99/1) as eluent to afford **5e** as an orange powder (127 mg, 14% from **3**, two steps). Mp 203-204°C; R_f=0.33 (EtOAc); IR ν_{max} (cm^{-1}): 3219 (νN-H), 3040 (νC-Har), 1680 (νC=O), 1564 and 1495 (νC=C and νC=N); ^{1}H NMR (400 MHz) δ 9.08 (s, 1 H, H_2), 8.95-

8.92 (m, 2 H, H_8, NH), 8.88 (s, 1 H, NH), 8.24 (s, 1 H, H_5), 7.80 (s, 1 H, H_3), 7.49-7.34 (m, 7 H, H_a, $H_{a'}$, $H_{b'}$, $H_{c'}$), 7.01 (d, 2 H, ^3J=8.8 Hz, H_b), 5.11 (s, 2 H, $-CH_2-$); ^{13}C NMR (100 MHz) δ 153.92 (C), 152.16 (C), 141.12 (C_8), 138.81 (C), 138.18 (C), 137.44 (C), 135.93 (C_3), 132.66 (C), 128.59 (2 $C_{b'}$), 127.94 ($C_{c'}$), 127.82 (2 $C_{a'}$), 120.07 (2 C_a), 115.67 (C_5), 115.31 (2 C_b), 106.30 (C_2), 69.59 ($-CH_2-$); MS (ESI) m/z (%): 360.1 (100) $[M+H]^+$; UPLC purity 95%; Anal. Calcd for $C_{20}H_{17}N_5O_2$: C 66.84; H 4.77; N 19.49. Found: C 66.99; H 5.03; N 19.23%.

1-Benzyl-3-(imidazo[1,2-a]pyrazin-6-yl)urea (5f): The reaction proceeded using benzyl isocyanate (377 µl, 3.0 mmol) under stirring for 2 h. The crude product was purified by silica-gel chromatography using dichloromethane/ethanol (99/1) as eluent to afford **5e** as a beige powder (223 mg, 33% from 3, two steps). Mp 190-191˚C; R_f=0.25 (EtOAc); IR ν_{max} (cm^{-1}): 3300 (νN-H), 2968 (νC-Hal), 1661 (νC=O), 1626 (νN-H), 1549 and 1504 (νC=C and νC=N); ^1H NMR (400 MHz) δ 9.02 (d, 1 H, ^3J=1.6 Hz, H_2), 8.93 (s, 1 H, NH), 8.88 (s, 1 H, H8), 8.20 (s, 1 H, H5), 7.78 (d, 1 H, ^3J=1.6 Hz, H_3), 7.40-7.35 (m, 4 H, H_a, H_b), 7.29 (t, 1 H, ^3J=8.8 Hz, Hc), 7.04 (t, 1 H, ^3J=5.8 Hz, NH), 4.38 (d, 2 H, ^3J=5.8 Hz, $-CH_2-$); ^{13}C NMR (100 MHz) δ 154.75 (C=O), 140.92 (C_8), 140.14 (C), 138.73 (C), 138.66 (C), 135.80 (C_3), 128.54 (2 C_b), 127.35 (2 Ca), 127.02 (C_c), 115.48 (C_5), 105.86 (C_2), 42.91 ($-CH_2-$); MS (ESI) m/z (%): 268.1 (100) $[M+H]^+$; UPLC purity 99%; Anal. Calcd for $C_{14}H_{13}N_5O$: C 62.91; H 4.90; N 26.20. Found: C 63.04; H 4.94; N 26.27%.

1-(Imidazo[1,2-a]pyrazin-6-yl)-3-(4-methoxybenzyl)urea (5g): To a solution of 4-nitrophenyl (4-methoxybenzyl)carbamate (1.5 g, 5.1 mmol) in dry tetrahydrofuran (75.0 ml) was added crude imidazo[1,2-*a*]pyrazin-6-amine **4** (339 mg, 2.5 mmol) and triethylamine (70.3 µl, 0.51 mmol). The reaction mixture was purged with argon through the septum inlet for 5 min and the suspension was then heated at 60˚C for 16 h. The solvent was then removed under reduced pressure. The crude product was purified by silica-gel chromatography using ethyl acetate/petroleum ether (7/3) as eluent to give **5g** as a white powder (85 mg, 11%). Mp 193-194˚C; R_f=0.23 (EtOAc); IR ν_{max} (cm^{-1}): 3310 (νN-H), 2957 (νC-Hal), 1697 (νC=O), 1531 and 1512 (νC=C and νC=N); ^1H NMR (400 MHz) δ 9.02 (d, 1 H, ^3J=1.6 Hz, H_2), 8.88-8.87 (m, 2 H, H_8, NH), 8.21 (s, 1 H, H_5), 7.77 (d, 1 H, ^3J=1.6 Hz, H_3), 7.28 (d, 2 H, ^3J=8.4 Hz, H_a), 6.96-6.93 (m, 3 H, H_b, NH), 4.30 (d, 2 H, ^3J=5.6 Hz, $-CH_2-$), 3.77 (s, 3 H, OMe); ^{13}C NMR (100 MHz) δ 158.45 (C), 154.68 (C), 140.92 (C_8), 138.68 (C), 135.80 (C_3), 132.01 (C), 128.75 (2 C_a), 128.29 (C), 115.48 (C5), 113.96 (2 C_b), 105.80 (C_2), 55.25 (OMe), 42.38 ($-CH_2-$); MS (ESI) m/z (%): 298.1 (100) $[M+H]^+$; UPLC purity 97%; Anal. Calcd for $C_{15}H_{15}N_5O_2$: C 60.60; H 5.09; N 23.55. Found: C 60.81; H 4.87; N 23.51%.

Cells for cytotoxicity bioassays. Cytotoxicity of the compounds was evaluated in various tumor cell lines and in a normal cell line using the sulforhodamine B (SRB) assay method (30). The tested cell lines were BALB/3T3 (non-tumorogenic, BALB/c mouse embryo cells), H460 (human large cell lung carcinoma), HuTu 80 (human duodenal carcinoma), DU145 (human prostate carcinoma), MCF-7 (human breast adenocarcinoma), M-14 (human amelanotic melanoma), HT-29 (human colon adenocarcinoma) and K562 (huma chronic myelogenous leukemia cells). All the cell lines were provided from the American Type Culture Collection (Manassas, VA, USA) except M-14 which was from the National Cancer Institute–National Institutes of Health, Bethesda, MA, USA.

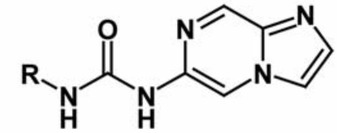
Cytotoxicity assays. To determine the cytotoxicity of the compounds, cells were plated into 96-well tissue culture plates and in their corresponding growth medium at approximately 10% confluency, and incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h to allow cells to attach. A plate containing each of these cells was fixed *in situ* with trichloroacetic acid (TCA) in order to obtain the cell values at cero time before adding the test compounds. The rest of the plates containing the different cell lines received serial 4-fold dilutions of the compound to be tested. The plates were then incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air for 48 h. The assay was terminated by the addition of cold TCA. TCA-treated plates were incubated at 4˚C for 1 h and then washed five times with tap water to remove TCA and then air dried. Background optical densities were measured in wells incubated with growth medium without cells. TCA-fixed cells were stained for 30 min with 0.2% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period unbound dye was removed by washing four times with 1% acetic acid. After air drying the plates, bound dye was solubilized with 10 mM Tris base (pH 10.5) and the absorbance read on an automated plate reader at a wavelength of 510 nm. The 50% growth-inhibitory concentration (GI_{50}) was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and was determined by linear regression analysis. For K562 cells, which grow in suspension, instead of fixing and staining with SRB, cells were counted using a Coulter counter. 5-Fluorouracil (5-FU) was used as a reference compound for the testing.

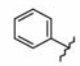
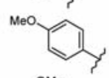
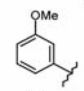
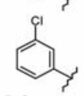
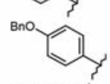
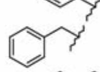
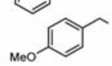
NSCLC cell lines and cultures. Two cell lines, A549 and NSCLC-N6-L16 originating from adenocarcinoma and epidermoid lung cancer, respectively, were used in this study. NSCLC-N6-L16 is a cell line derived from an NSCLC of a previously untreated patient (moderately differentiated classified as T2N0M0) (31). The A549 cell line was obtained from the American Type Culture Collection (reference CCL-185; LGC Standards, Molsheim, France) (32) and is known to have a wild-type TP53 gene, while NSCLC-N6-L16 has a mutant *TP53* gene, similar to tumors *in situ*. The cell lines were cultured in RPMI-1640 enriched with 100 IU penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 5% fetal bovine serum. Cell culture plates were maintained in humidified incubators at 37˚C in a 5% CO_2 atmosphere. NSCLC-N6-L16 has a cell-doubling time of 48 h *in vitro*, and A549 of 24 h.

Cytotoxicity determination under continuous drug exposure. Experiments were performed in 96-well microtiter plates (10^5 cells/ml for NSCLC-N6-L16 and 2×10^4 cells/ml for A549). Cell growth was estimated by a colorimetric assay based on the conservation of tetrazolium dye (MTT) to a blue formazan product by live mitochondria. Eight repeats were performed for each concentration tested. Control growth was estimated from eight determinations. The optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII (LabSystems, Missouri City, TX, USA).

Extraction of total RNA. Total RNA was extracted from NSCLC-N6-L16 and A549 cell lines using the Dynabeads® mRNA Direct™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). The isolation

Table I. *Preliminary antiproliferative activity of ureas* ***5a-g*** *against cancer cell lines.*



Compd	R	Cancer cell line[a] GI50 (μM)							
		3T3	H460	HuTu 80	DU 145	MCF-7	M-14	HT-29	K562
5-FU		1.1	2.3	n.d.	7.7	2.3	5.4	3.1	6.9
5a		>100	>100	>100	>100	>100	>100	>100	>100
5b		>100	>100	>100	>100	>100	>100	>100	>100
5c		>100	>100	>100	>100	>100	95.3	>100	>100
5d		>100	>100	>100	>100	93.8	31.3	41.7	86.9
5e		72.3	36.2	27.8	44.5	19.5	39.0	33.4	27.8
5f		>100	>100	>100	>100	>100	>100	>100	>100
5g		>100	>100	>100	>100	>100	>100	>100	>100

GI_{50}: Concentration required for 50% growth inhibition. [a]Mean from three determinations. 3T3, BALB/c mouse embryo cells; H460, human large cell lung carcinoma; HuTu 80, human duodenal carcinoma; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human amelanotic melanoma; HT-29, human colon adenocarcinoma; K562, human chronic myelogenous leukemia cells; 5-FU: 5-fluorouracil; n.d.: not determined.

protocol relies on base pairing between the polyA residues at the 3' end of most mRNA and the oligo (dT) residues covalently coupled to the surface of the Dynabeads®.

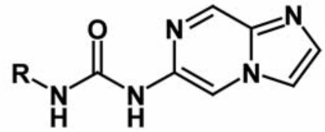
cDNA synthesis. Reverse transcription (RT) was carried out with 1 μg of RNA in a final volume of 25 μl with a mix containing random, dNTP mix, moloney murine leukemia virus (M-MLV) 5X reaction buffer, RNasin ribonuclease inhibitor, M-MLV RT and RNase-free water. In order to check whether the samples were contaminated by genomic DNA, the same mix was made with RNA without the reverse transcriptase.

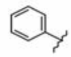
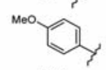
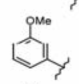
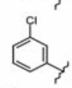
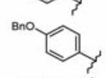
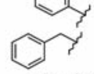
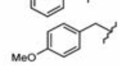
Quantitative real-time RT-polymerase chain reaction (PCR). Real-time RT-PCR was carried out in a final volume of 25 μLl with 1:20 dilution of diluted cDNA mixture, gene-specific forward and reverse primer in 1X SYBR Green PCR master mix (Eurogentec) with the following protocol: 15 s at 95˚C for denaturation, 30 s at 60˚C for annealing and extension on an ABI Prism Sequence Detection System 5700 (Applied Biosystems). The primer sequences were *NEDD9*: forward 5'-CGCTGCCGAAATGAATAT-3', reverse 5'-CCCTGTGTTCTGCTCTATGACG-3'; *TP53*: forward 5'-GTTCC GAGAGCTGAATGAGG-3', reverse 5'-TCTGAGTCAGGCCCT TCTGT-3'. The relative expression of each gene was normalized to that of human β-actin (*ACTB*): forward 5'-ATTCCCTTGCCTTCT TGGAT-3', reverse 5'-CGTGAGGTCTGCCACTACAG-3'. Norma-lization was carried out using the ΔΔ Ct method. The results were analyzed using GenAmp 5700 SDS (Applied Biosystems) software.

Kinase enzymatic assays. Kinase activities were assayed in appropriate kinase buffer, with either protein or peptide as substrate in the presence of 15 μM [γ-^{33}P] ATP (3,000 Ci/mmol; 10 mCi/ml) in a final volume of 30 μl following the assay described before (33). Controls were performed with appropriate dilutions of dimethylsulfoxide. Full-length kinases are used unless specified. Peptide substrates were obtained from Proteogenix (Oberhausbergen, France).

Buffers: A: 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl pH 7.5, 50 μg/ml heparin; B: 60 mM β-glycerophosphate, 30 mM *p*-nitrophenyl-phosphate, 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7), 5 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate; C: 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl-phosphate, 25 mM MOPS (pH 7.2), 5 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT.

Table II. *Antiproliferative activity of ureas* ***5a-g*** *against non-small cell lung cancer (NSCLC) cell lines.*



Compound	R	NSCLC cell line IC_{50} (μM)	
		N6-L16[a]	A549[b]
A190		44.9±2.3	60.0±3.2
5a		65.1±3.6	>100
5b		45.9±2.5	>100
5c		16.2±1.1	>100
5d		34.4±1.7	64.0±3.1
5e		<9.2	<9.2
5f		58.4±3.4	>100
5g		15.5±1.0	53.5±2.4

IC_{50}: Half-maximal inhibitory concentration. [a]Epidermoid lung cancer, P53-mutant. [b]Adenocarcinoma lung cancer, P53 wild-type.

*Mg*CDK1/cyclin B: [extracted from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography] was assayed in buffer C with 1 μg/μl of histone H1 as substrate.

*Hs*CDK2/CyclinA (cyclin-dependent kinase-2, human, kindly provided by Dr. A. Echalier-Glazer, Leicester, UK) was assayed in buffer A (+0.15 mg/ml of bovine serum albumin (BSA) +0.23 mg/ml of DTT) with 0.8 μg/μl of histone H1 as substrate. *Hs*CDK5/p25 (human, recombinant, expressed in bacteria) was assayed in buffer B, with 0.8 μg/μl of histone H1 as substrate. *Ssc*CK1δ/ε (casein kinase 1δ/ε, porcine brain, native, affinity purified) was assayed in buffer B, with 0.022 μg/μl of the following peptide: RRKHAAIGSpAYSITA as CK1-specific substrate. *Rn*DYRK1A-kd (*Rattus norvegicus*, amino acids 1 to 499 including the kinase domain, recombinant, expressed in bacteria, DNA vector kindly provided by Dr. W. Becker, Aachen, Germany) was assayed in buffer A (+ 0.5 mg/ml of BSA + 0.23 mg/ml of DTT) with 0.033 μg/μl of the following peptide: KKISGRLSPIMTEQ as substrate. *Mm*CLK1 (from *Mus musculus*, recombinant, expressed in bacteria) was assayed in buffer A (+ 0.15 mg/ml of BSA + 0.23 mg/ml of DTT) with 0.027 μg/μl of the following peptide: GRSRSRSRSRSR. SscGSK-3α/β (glycogen synthase kinase-3, porcine brain, native, affinity purified) was assayed in buffer A (+ 0.15 mg/ml of BSA + 0.23 mg/ml of DTT), with 0.010 μg/μl of GS-1 peptide, a GSK-3-selective substrate (YRRAAVPPSPSLSRHSSPHQSpEDEEE, where Sp is phosphorylated serine).

Results

Antiproliferative activity against cancer cell lines. Compounds **5a-g** were first subjected to a preliminary screen by determining their antiproliferative activity against a panel of cancer cell lines: H460 (human large cell lung cancer), HuTu80 (human duodenal carcinoma), DU 145 (human prostate carcinoma), MCF-7 (human breast adenocarcinoma), M-14 (human melanoma), HT-29 (human colon adenocarcinoma) and K562 (human chronic myelogenous leukemia cells). The antiproliferative activity was assessed using the GI_{50}, and 5-FU was used as a positive control. Results are shown in Table I. Compounds **5a-d**, **5f** and **5g** exhibited poor activity and compound **5e** displayed slightly better activity. Among the seven synthesized compounds, **5e** displayed the best activity, with GI_{50} values ranging from 19.5 to 72.3 μM. Unfortunately, no selectivity towards the different cell lines emerged for this compound and it should be noted that it had very low activity against 3T3 cell line, which is a standard fibroblast cell line.

Effects of compounds ***5a-g*** *on NSCLC-N6-L16 and A549 cell lines.* We further evaluate the antiproliferative effect of synthesized compounds **5a-g** on two NSCLC cell lines: NSCLC-

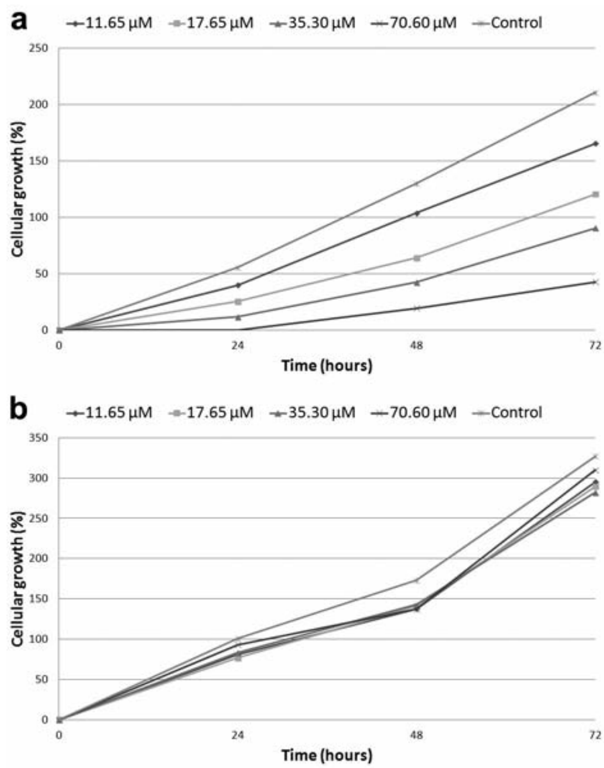


Figure 2. *Effect of **5b** on the growth of NSCLC-N6-L16 (a) and A549 (b) cell lines. The graphs show growth kinetics versus time after continuous exposure to drug at different concentrations.*

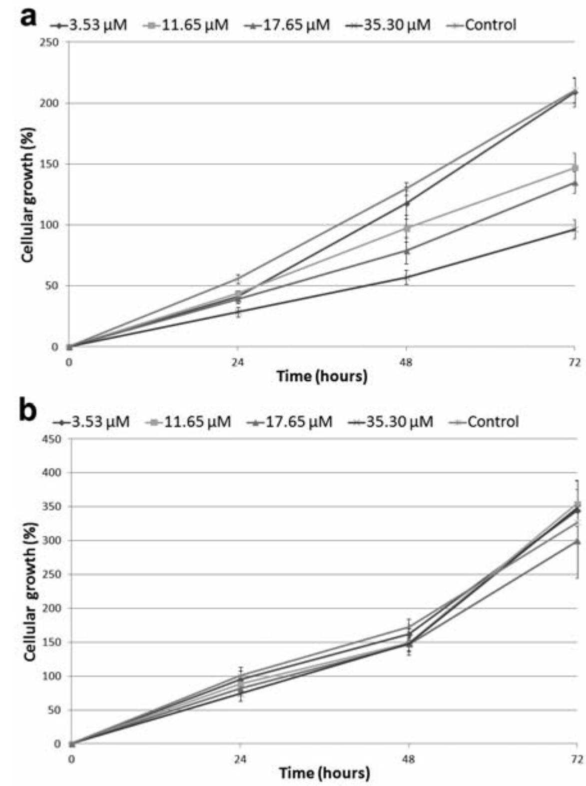


Figure 3. *Effect of **5c** on the growth of NSCLC-N6-L16 (a) and A549 (b) cell lines. The graphs show growth kinetics versus time after continuous exposure to drug at different concentrations.*

N6-L16, an P53-mutant epidermoid lung cancer and A549, a P53 wild-type adenocarcinoma lung cancer. Compound triazine **A190** was used as a reference. Results obtained from ureas **5a-g** are summarized in Table II as IC_{50}. Unlike compound **5e** that exhibited IC_{50} below 9.2 µM, suggesting a cytotoxic effect, the other compounds displayed potent cytostatic effects and/or selectivity against the two NSCLC cell lines.

*Effects of compounds **5b** and **5c** on growth kinetics of NSCLC cell lines*. To determine whether compounds **5b** and **5c** could exert antiproliferative or cytostatic activity, we evaluated the growth kinetics of NSCLC-N6-L16 and A549 cells in the presence and absence (control) of **5b** and **5c** at different concentrations. We highlighted a dose-dependent inhibitory activity of **5b** on NSCLC-N6-L16 but there was no such effect on A549 cell line (Figure 2).

Compound **5b** displayed a kinetic profile which shows a cytostatic effect on the NSCLC-N6- L16 cell line, with a plateau effect at a high dose (70.6 µM) and even at a mean dose (35.3 µM) (Figure 2a). Meanwhile, its counterpart **5c** presented an antiproliferative kinetic profile (Figure 3).

*Effects of compound **5b** on expression of TP53 by qPCR*. The study of the expression of the *TP53* by qPCR gene was carried out on synchronized cells. Indeed, as the expression of genes varies over the cell cycle, it is necessary to study cells that are in the same cell-cycle phase in order to measure accurately the effect of molecules, such as **5b**, on the expression of these genes. Synchronized NSCLC-N6-L16 cells were combined with **5b** from 30 to 42 h (Figure 4). Total RNA was extracted at 30, 32, 40 and 42 h and the expression of *TP53* was quantified by qPCR. For the control, a constant expression at 30, 32 and 40 hours and decline of this expression after 42 h were observed. No significant difference was denoted between control and treated cells at 32, 40 and 42 h, whereas great overexpression was highlighted at 30 h.

Kinase enzymatic assays. In order to obtain more information about the biological profile of compounds **5a-g** and especially to highlight the existence of other target proteins, the compounds were tested for their inhibition of a panel of seven cancer-related protein kinases, which include cyclin-dependent kinases (*Mg*CDK1/cyclin B, *Hs*CDK2/

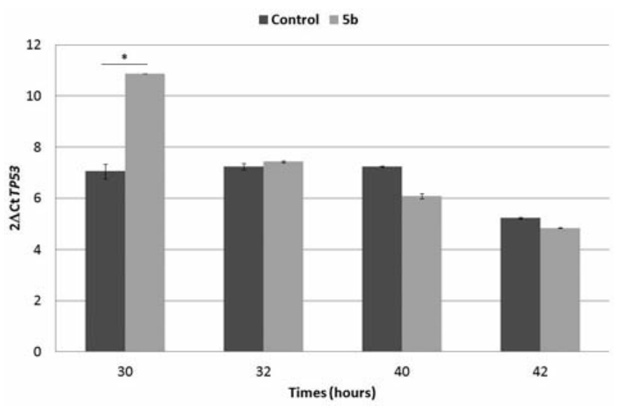


Figure 4. *Expression of TP53 tested by quantitative reverse transcription- polymerase chain reaction at 30, 32, 40 and 42 hours in synchronized NSCLCN6-L16 cells treated or not with* ***5b*** *at the half-maximal inhibitory concentration. The results are expressed as a ratio of mRNA quantity of the genes tested relative to that of β-actin (control gene). The values are mean±S.D. (n=8 for each group). (*p<0.001).*

cyclin A, *Hs*CDK5/p25), casein kinase 1 (*Ssc*CK1δ/ε), cell division control protein 2 homolog-like kinase 1 (*Mm*CLK1), dual-specificity tyrosine phosphorylation-regulated kinase 1A (*Rn*DYRK1A), and glycogen synthase kinase-3 (SscGSK-3α/β). Indeed, the urea appendage at the C-6 position of the imidazo[1,2-*a*]pyrazine ring could mimick the adenine residue of ATP and the compounds were able to act as ATP-competitive kinase inhibitors. Nevertheless, as a general feature, all heterocyclic urea derivatives failed to exhibit significant kinase inhibition (all IC_{50} values >10 μM).

Discussion

We focused our research on cytostatic molecules that stop cell growth in the G_1 phase of the cell cycle, before inducing cells to undergo apoptotic death. In addition, this approach should prevent excessive toxicity *in vivo*. This approach was successfully carried out to discover the anti-NSCLC activity of triazine **A190** (9), by restoring transcription factor activity of mutated P53 protein. In this context, compound **5b**, which displayed selective antiproliferative activity against NSCLC-N6-L16 cell line harboring *TP53* mutant, was chosen as a representative compound for further pharmacological investigations. In addition, its pharmacological profile was similar to the reference compound triazine **A190**.

For compound **5b**, whereas the growth inhibition was dose-dependent in NSCLC-N6-L16 cells, there was no difference between the treated cells and the control A549 cells. The absence of a plateau is typical of antiproliferative activity not cytostatic activity. It is interesting that compound **5b** had an antiproliferative effect on NSCLC-N6-L16 but not on A549 cell line. Both of these cell lines are NSCLC but are different in their expression of *TP53* gene. Molecule **5b** may be effective against *TP53*-mutated cell lines as observed in NSCLC-N6-L16. Thus, our efforts were focused on the capacity of **5b** to influence the expression of *TP53* gene in NSCLC-N6-L16 cells.

The combination of the inhibition of cell proliferation and the overexpression of *TP53*, both induced by **5b**, show the possible capacity of the molecule to reactivate mutant P53 in NSCLC-N6-L16 cell line. Indeed, this cell line has a mutation of *TP53* named His273: a mutation in the DNA-binding domain of P53 protein. It was shown that small molecules may reactivate the transcription factor function of such mutant P53, molecules like PRIMA-1 (7). We believe that, after restoration of the transcription factor function of mutant P53, the overexpression of *TP53* shown at 30 hours, in turn induces an overexpression of the *NEDD9* gene. Indeed, this gene was previously demonstrated by our team to be a new target for transcription factor P53 (10). At 30 hours, all the cells treated with **5b** were blocked in the G_1 phase, therefore the overexpression effect of the gene was more pronounced. Both overexpression of *NEDD9* and *TP53* would then be responsible for apoptosis of the NSCLC-N6-L16 cells (34, 10).

In summary, a series of novel (imidazo[1,2-*a*]pyrazin-6-yl)ureas was synthesized and characterized. Although the target compounds were difficult to obtain, they are the first representatives of imidazo[1,2-*a*]pyrazines functionalized with ureas at position 6. We determined the antiproliferative potencies of these seven different ureas on a panel of cancer cell lines, including NSCLC cell lines. The results of *in vitro* antiproliferative assays indicated that several compounds displayed interesting activities, despite a lack of selectivity. However, compound **5b** was selected since it showed a potent selective antitumor activity against NSCLC-N6-L16 p53 mutant in a dose-dependent response. Furthermore, we proved that the investigated compound was able to promote *TP53* gene overexpression after 30 hours. These findings also suggest that this molecule may be able to reactivate mutant P53 in NSCLC-N6-L16 cell line. Additional experiments are still needed to clarify this hypothesis. The (imidazo[1,2-*a*]pyrazin-6-yl)ureas have also shown promising initial anti-proliferative activities against cancer cell lines, identifying them as a novel scaffold for further pharmacomodulation to design potential antitumour agents, especially in the ongoing research for effective treatments of NSCLC.

Acknowledgements

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