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Original article

Synthesis and bioactivity of novel amino-pyrazolopyridines

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ABSTRACT

Here we describe the synthesis and biological activity of novel amino-pyrazolopyridines with anti-NF-κB and pro-apoptotic potential. α-Methylene ketones were used as a starting point for synthesis of amino-pyrazolopyridine **3**. The alkylidene malononitriles **1** were obtained by the Knoevenagel reaction of ketones with malononitriles. Vilsmeier–Haack reaction allowed direct access to 2-chloro-3-cyanopyridines **2**. Those products, by refluxing with hydrazine hydrate, allowed cyclization to amino-pyrazolopyridines **3a–g**, which were not previously described in the literature.

Bioactivity results indicated that amino-pyrazolopyridines **3a**, **3b** and **3g** induced apoptotic cell death in K562 cancer cells with an IC_{50} of 36.5 ± 3.9 μM, 27.6 ± 4.5 μM and 35.0 ± 2.3 μM, respectively, after 72 h. In addition, compounds **3a**, **3b** and **3g** exerted NF-κB inhibition activity with an IC_{50} of 4.7 ± 1.6 μM, 6.9 ± 1.9 μM and 39.8 ± 3.9 μM, respectively, after 8 h in K562 cells activated with TNFα. Compounds **3b** and **3g** showed interesting differential toxicity as viability of peripheral blood mononuclear cells (PBMCs) from healthy donors remained largely unaffected by this treatment.

1. Introduction

Cancer is one of the most deadly diseases worldwide and therefore synthesis of novel and potent small molecule anti-cancer agents is intensifying [1–3]. Chemotherapeutic drugs including cisplatin or etoposide induce selective cell death in cancer cells [4–6]. In this research field, transcription factor NF-κB is an important target for the development of anti-cancer therapies as the pathological overexpression of the NF-κB pathway is a leading cause of resistance towards chemo- and radiotherapy [7,8]. More specifically, pyrazolopyridine derivatives have been reported in the literature to exert anti-inflammatory [9] and anti-cancer [10,11] properties in addition to their anti-viral [12] and anti-leishmanial [13] bioactivity.

Considering these potent reported bioactivities, we describe here a synthesis of amino-pyrazolopyridines based on our previous synthetic studies with 2-chloro-3-cyanopyridine. Two major key reactions were used in the synthetic route: first, the Knoevenagel reaction, applied in our synthetic route, has been well described [14–17] and the synthetic parameters were optimized to give a good yield in our laboratory [18]. Second, cyclization reaction was carried out by a Vilsmeier–Haack reaction, as previously described in our laboratory [18,19]. Treatment of 2-chloro-3-cyanopyridine with hydrazine monohydrate provided amino-pyrazolopyridine [20].

Based on the reported bioactivity data, we evaluated the cytotoxic and NF-κB inhibition potential of the novel synthesized products in human leukemia cells and demonstrated for the first time that amino-pyrazolopyridines repress TNFα-induced NF-κB signaling and induce apoptotic cell death in leukemia cells whereas healthy blood cells remain largely unaffected by this treatment.

2. Material and methods

2.1. Chemistry

The used chemical reagents were analytically pure. All solvents and liquids were dried and distilled prior to use. Yields were

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optimized. All melting points were measured in open capillary tubes. NMR spectra were recoded on a Bruker AC 250 (250 MHZ) spectrometer in deuteron-chloroform $CDCl_3$ or hexadeuterodimethylsulfoxide DMSO. Chemical shifts (δ) are reported in ppm and coupling constant (J) in Hz. Mass spectrometry has been realized *via* an Agilent GC–MS system. After chromatographic separation by a gas phase on a capillary column, the mass analysis was performed with a Bruker MICROTOF-Q ESI/QqTOF or a Varion-Ion spectrometer ESI-FTICR/MS QFT-9 4T. Microanalysis was performed on a Thermo Finnigan EA 1112.

2.1.1. Synthesis of 2-chloro-3-cyanopyridine substituted in position 4 and 5 ***2a–g***

These compounds were synthesized according to a known procedure described by Aadil and et al. [18].

2.1.2. General procedure of synthesis of amino-pyrazolopyridine ***3a–g***

Compound **3** (1.5 mmol) was dissolved in hydrazine hydrate (3.1 g), the reaction mixture was heated for 2 h at 150 °C. After cooling, the mixture is poured into water and ice. The obtained solid was filtered, washed with water twice and crystallized from cyclohexane.

2.1.2.1. Synthesis7-methyl- 6-hydro-3H-benzo[f]pyrazolo[3,4-C]isoquinolin-1-amine ***3a***. Aspect Colorless solid, yield 92%, Mp = 209 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ = 1.69–1.19 (m, 3H, CH_3), 2.70–2.78 (m, 1H, CH), 3.02–3.18 (m, 2H, CH_2), 4.24 (s, 2H, NH_2), 7.33–7.47 (m, 3H, $3H_{ar}$), 8.12–8.15 (m, 1H, H_{ar}), 8.43 (s, 1H, H_{ar}), 10.66 (s, 1H, NH). ^{13}C NMR ($CDCl_3$, 250 Hz) δ = 19.08, 30.39, 36.89, 127.00, 128.86, 129.13, 129.54, 129.81, 131.01, 137.77, 138.26, 147.02, 148.39, 153.83, 174.32. Anal. Calcl: C, 71.97; H, 5.63; N, 23.38. Found: C, 71.63; H, 5.02; N, 23.12. m/z [$C_{15}H_{14}N_4$ + H^+] calcd: 251.12; Found: 251.11.

2.1.2.2. Synthesis 6,7-dihydro-3H-benzo[f]pyrazolo[3,4-C]isoquinolin-1-amine ***3b***. Aspect: colorless solid; yield: 85%; Mp = 204 °C; ^{1}H NMR: (DMSO-d_6, 250 MHz) δ = 2.80 (m, 4H, CH_2CH_2), 4.24 (s, 2H, NH_2), 7.37–7.48 (m, 3H, H_{ar}), 8.12–8.15 (d, J = 7.5 Hz, 1H, H_{ar}), 8.40 (s, 1H, H_{ar}), 10.30 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 250 MHz) δ = 25.02, 28.75, 100.90, 124.21, 126.75, 127.86, 129.00, 129.32, 131.29, 137.07, 139.30, 146.66, 148.33, 153.51. Anal. Calcl: C, 71.169; H, 5.11; N, 23.712. Found: C, 71.15; H, 5.42; N, 23.22. HRMS (APCI): m/z [$C_{14}H_{12}N_4$ + H^+] calcd: 273.11; Found: 273.11.

2.1.2.3. Synthesis of 3,6,7,8-tetrahydrocyclopenta[d]pyrazolo[3,4-b]pyridin-1-amine ***3c***. Aspect Colorless solid; yield 99%, Mp = 240 °C; ^{1}H NMR: (DMSO-d_6, 250 MHz) δ = 2.02–2.11 (m, 2H, CH_2), 2.84–2.90 (t, J = 7.5 Hz, 2H, t, CH_2), 3.15–3.21 (t, J = 7.5 Hz, 2H, t, CH_2), 5.15 (s, 2H, NH_2), 8.18 (s, 1H, H_{ar}), 11.79 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 250 MHz) δ = 24.77, 29.07, 30.75, 103.86, 129.50, 144.66, 146.83, 147.39, 152.31. Anal. Calcl: C, 62.053; H, 5.786; N, 32.161. Found: C, 62.01; H, 5.26; N, 32.13. HRMS (APCI): m/z [$C_9H_{10}N_4 + H^+$] calcd: 175.09; Found: 175.09.

2.1.2.4. Synthesis of 4-ethyl-5-methyl-1H-pyrazolo[3,4-b]pyridin-3-amine ***3d***. Aspect Colorless solid, yield 62%, Mp = 206 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ = 1.12–1.18 (m, 3H, CH_3), 2.26 (s, 3H, CH_3), 2.87–2.96 (m, 2H, CH_2), 5.02 (s, 2H, NH_2), 8.08 (s, 1H, H_{ar}). ^{13}C NMR (DMSO-d_6, 250 Hz) δ = 14.38, 14.51, 105.04, 120.48, 145.67, 147.29, 150.44, 152.41. Anal. Calcl: C, 61.343; H, 6.86; N, 31.794. Found: C, 61.15; H, 6.81; N, 31.86. HRMS (APCI): m/z [$C_9H_{12}N_4 + H^+$] calcd: 177.11; Found: 177.11.

2.1.2.5. Synthesis 7-methyl-6,7,8,9-tetrahydro-3H-pyarazolo[3,4-C]isoquinolin-1-amine ***3e***. (Colonn. Chromat.: AcOEt 30%/C_6H_{12}), Aspect Colorless solid, yield 75%, Mp = 243 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ = 1.02–1.04 (d, J = 2.5 Hz, 3H, CH_3), 1.33–1.35 (m, 1H, CH), 1.84 (m, 2H, CH_2), 2.24–2.34 (m, 1H, CH), 2.76–2.2.83 (m, 1H, CH), 3.00 (m, 1H, CH), 3.22 (s, 1H, CH), 5.00 (s, 2H, NH_2), 7.99 (s, 1H, H_{ar}), 11.77 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 250 MHz) δ = 21.55, 25.05, 28.25, 29.76, 34.02, 104.84, 121.95, 140.00, 147.97, 150.02, 151.65. Anal. Calcl: C, 65.32; H, 6.97; N, 27.70. Found: C, 65.46; H, 6.69; N, 27.07. HRMS (APCI): m/z [$C_{11}H_{14}N_4$ + H^+] calcd: 203.12; Found: 203.13.

2.1.2.6. Synthesis of 6,7,8,9-tetrahydro-3H-pyrazolo[3,4-C]isoquinolin-1-amine ***3f***. Aspect Colorless solid, yield 88%, Mp = 230 °C; ^{1}H NMR (DMSO-d_6, 250 MHz) δ = 1.69–1.75 (m, 4H, CH_2CH_2), 3.06–3.09 (t, J = 7.5 Hz, 2H, CH_2), 3.30–3.33 (t, 2H, J = 7.5 Hz, CH_2), 5.00 (s, 2H, NH_2), 7.99 (s, 1H, H_{ar}), 11.77 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 250 MHz) δ = 21.67, 22.29, 25.10, 25.57, 104.93, 122.16, 140.39, 147.95, 150.09, 151.59. Anal. Calcl: C, 63.81; H, 6.425; N, 29.765. Found: C, 62.61; H, 6.26; N, 30.27. HRMS (APCI): m/z [$C_{10}H_{12}N_4$ + Na^+] calcd: 211.11; Found: 211.09.

2.1.2.7. Synthesis of 5-methyl-4-phenyl-1H-pyrazolo[3,4-b]pyridin-3amine ***3g***. Aspect Colorless solid, yield 76%, Mp = 217 °C; ^{1}H NMR ($CDCl_3$, 250 MHz) δ = 2.208 (s, 3H, CH_3), 3.66 (s, 2H, NH_2), 7.35–7.39 (m, 2H, H_{ar}), 7.48–7.57 (m, 3H, H_{ar}), 8.41 (s, 1H, H_{ar}). ^{13}C NMR ($CDCl_3$, 250 Hz) δ = 15.97, 30.92, 104.93, 122.11, 128.50, 128.54, 128.61, 128.73, 135.93, 143.54, 147.31, 151.39, 152.11. Anal. Calcl: C, 69.624; H, 5.393; N, 24.982. Found: C, 69.23; H, 5.81; N, 24.46. HRMS (APCI): m/z [$C_{13}H_{12}N_4$ + H^+] calcd: 225.11; Found: 225.11.

2.2. Biological assays

2.2.1. Cell culture

TNFα was purchased from Sigma (Bornem, Belgium) and dissolved to a concentration of 10 mg/mL in phosphate buffered saline (PBS) supplemented with 0.5% (w/v) bovine serum albumin (BSA; Sigma) according to the manufacturer's instructions. K562 (human chronic myelogenous leukemia) cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany) were cultured in RPMI medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (Lonza) and 1% (v/v) antibiotic-antimycotic (Bio-Whittaker, Verviers, Belgium) at 37 °C and 5% of CO_2. The cells were harvested every 3 days. After stock defrosting, cells were kept in normal culture conditions for 10 days before experimental application.

Cells were pre-treated with synthesized amino-pyrazolopyridines at indicated concentrations and time periods. Amino-pyrazolopyridines were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Bornem, Belgium) and the amount of DMSO in cell culture did not exceed 0.4%.

Healthy blood samples from donors were kindly provided as buffy coats by the Red Cross (Luxembourg). A 1-1 dilution of blood and RPMI Medium was added to a Ficoll (Paque PRENIUM, GE Healthcare, Diegem, Belgium) layer and then subjected to a centrifugation (400 g for 20 min). Then, after 2–3 washes of the pellet with RPMI medium, the isolated peripheral blood mononuclear cells (PBMCs) were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 24 h prior to treatment with the synthesized compounds.

2.2.2. Cell viability assessment

Percentages of cell survival were evaluated using Promega's CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Leiden, Netherlands) kit, according to the manufacturer's instructions.

Alternatively, Trypan Blue staining was used to determine cell integrity (Supplementary data). Data were normalized to the control and reported as percentage of viable cells. Apoptosis estimation was performed as previously described by analyzing nuclear morphology upon staining with Hoechst 33342 (1 μg/mL) [21]. Briefly, cells were incubated at 37 °C for 15 min with the DNA-specific dye and analyzed by fluorescence microscope.

2.2.3. Transient transfection and luciferase reporter gene assay

K562 cells were transiently transfected as described previously [22]. For each electroporation, we used 5 μg of a luciferase reporter gene construct containing five repeats of a consensus NF-κB site (pGL4.32[luc2P/NF-κB-RE/Hygro], Promega, Leiden, Netherlands) and 5 μg of a *Renilla* luciferase plasmid (phRG-TK, Promega, Leiden, Netherlands). After electroporation cells were re-suspended in normal culture medium (RPMI, 10% FCS) and cultured at 37 °C and 5% CO_2 for 24 h. Then, cells were harvested and re-suspended in fresh growth medium (RPMI, 0.1% FCS) to a final concentration of 1×10^6 cells/mL and pre-treated for 2 h with the indicated concentrations of amino-pyrazolopyridines. After 2 h of pre-treatment, cell were activated by 20 ng/mL of TNFα, followed by an additional incubation period of 6 h. After 8 h of total treatment time, 75 μL of Dual-Glo™ luciferase reagent (Promega, Leiden, Netherlands) were added to 75 μL of the cellular suspension for 10 min incubation at 22 °C before luciferase activity measurement. Afterwards, 75 μL of Dual-Glo™ Stop&Glo Reagent (Promega, Leiden, Netherlands) were added for 10 min at 22 °C to the cell suspension to assay Renilla activity. An Orion microplate luminometer (Berthold, Pforzheim, Germany) was used to measure luciferase and Renilla activity. The results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to Renilla luciferase activity.

2.2.4. Human CXCL8/IL-8 immunoassay

IL-8 concentrations in culture supernatants of activated K562 cells were measured by sandwich ELISA (R&D Systems, Abingdon, United Kingdom). According to the manufacturer's guide, 50 μL of cell supernatants were added with 100 μL of assay diluent to anti-IL-8 pre-coated wells followed by 2 h incubation at RT. After washing, a polyclonal peroxidase-conjugated anti-IL-8 antibody was added for another 60 min at RT. Colorimetric visualization and protein dosage were developed by addition of the H_2O_2 + TMB (tetramethylbenzidine) containing substrate. After a 30 min reaction at room temperature (RT) in the dark, the enzymatic reaction was stopped by addition of H_2SO_4 and optical densities were measured at a wavelength of 450 nm.

2.2.5. Caspase-glo® 3/7 assay

Measurement of caspase 3/7 activities was assessed by homogeneous Caspase-Glo®3/7 luminescent assay (Promega) following manufacturer's guide. Briefly, 3×10^5 cells/mL were pre-treated or not for 1 h with 50 μM of Caspase Inhibitor I (Z-VAD (OMe)-FMK, Calbiochem), following treatments with amino-pyrazolopyridines **3a**, **3b** and **3g** at 40 μM for 8 h, 24 h and 48 h. After treatment period, 75 μL of cell culture were mixed with 75 μL of Caspase-Glo®3/7 reagent and incubated at room temperature for 1 h. Addition of Caspase-Glo®3/7 reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present. The results are expressed as fold change compared to the control.

2.2.6. Statistical analysis

Data are expressed as mean ± S.D. and their signification degrees were analyzed by Student's *T*-tests. *P*-values below 0.01 were considered as statistically significant.

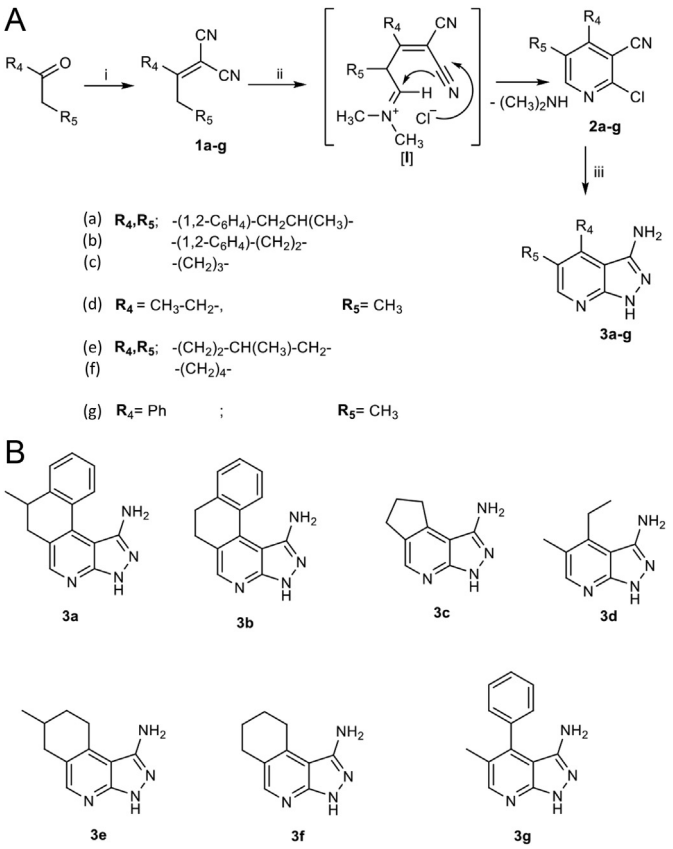

Fig. 1. Synthesis and chemical structures of amino-pyrazolopyridines. A) Synthesis of 2-chloro-3-cyanopyridines substituted in position 4 and 5 **2a**–**g** through Knoevenagel reaction followed by a Vilsmeier–Haack reaction and Condensation of 2-chloro-3-cyanopyridines with hydrazine hydrate leading to the formation of amino-pyrazolopyridines **3a**–**g**. Reagent and conditions: (i) malononitrile, ammonium acetate, acetic acid, toluene, reflux, 24 h. (ii) phosphorus oxychloride, dimethylformamide, NP/KF, 70–80 °C, 3 h. (iii) hydrazine hydrate, reflux, 2–3 h. B) Chemical structures of synthesized amino-pyrazolopyridines **3a**–**g**.

3. Results

The goal of this article was the synthesis of previously unknown amino-pyrazolopyridine **3** *via* cyclization of intermediary 2-chloro-3-cyanopyridines **2**, prepared from α-methylene ketones through a Knoevenagel reaction and Vilsmeier–Haack reaction [14–19]. A cyclization of the 2-chloro-3-cyanopyridines **2** with hydrazine hydrate led to the formation of amino-pyrazolopyridines **3**. In a second part, a bioactivity study of the synthesized products was carried out. In detail, this study evaluated differential cytotoxic activity of novel amino-pyrazolopyridines and determined inhibitory potential of novel amino-pyrazolopyridines on TNFα-induced NF-κB pathway in human leukemia cells.

3.1. Chemistry

The alkylidene-malonodinitrile **1** prepared by Knoevenagel reaction from α-methylene ketones, which has a methylene active in α position, can serve as intermediates for the synthesis of desired heterocycles **3a**–**g** (Fig. 1) [14–18].

The treatment of alkylidene malononitriles **1a**–**g** by Vilsmeier–Haack reagent at 70–80 °C leads to the formation of 2-chloro-3-cyanopyridines **2a**–**g** substituted in position 4 and 5 (Fig. 1) [18,19]. Reaction of the reactive methylene group of the alkylidene malononitrile **1** with the Vilsmeier–Haack reagent gave

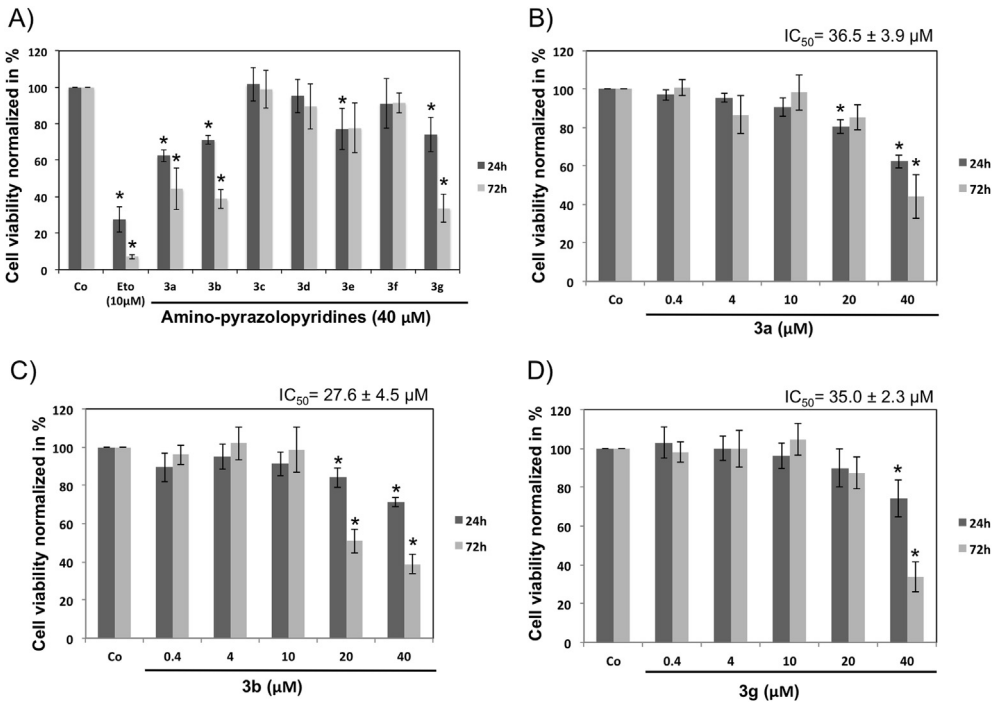

Fig. 2. Effects of amino-pyrazolopyridines on K562 cell viability. A) Effect of amino-pyrazolopyridine analogues on metabolic activity of K562 cells after 24 and 72 h at 40 μM. Control (Co) corresponds to untreated cells. Etoposide (Eto) served as a positive control. Each value is a mean ± SD of three determinations. The asterisk indicates a significant difference compared to control analyzed by *t*-test (*$p < 0.01$). B) Dose-dependent inhibition of K562 cell viability by compound **3a**, C) by compound **3b** and D) by compound **3g** is shown.

an intermediate iminium salt. The latter cyclizes spontaneously with loss of dimethylamine to the α-chloro β-cyano pyridines **2a**–**g**. However, this reaction has been accomplished, in general with low yields from 10 to 15%. The use of natural phosphate (NP) alone or natural phosphate modified by potassium fluoride (KF/NP) as new efficient solid catalysts in the Vilsmeier–Haack reaction can increase the yield to 60%.

Aminopyrazolopyridine **3a**–**g** substituted in position 4 and 5 with either an aliphatic or cyclic system, however, were not previously known. We describe here their preparation from 2-chlorocyanopyridines and hydrazine hydrate as shown in Fig. 1A. The aromatic nucleophilic substitution of the chlorine by hydrazine followed by the attack of the cyano group led to the amino pyrazoles [20].

Hence, the described synthetic route led to the synthesis of novel amino-pyrazolopyridines substituted in position 4 and 5 previously not described in literature (Fig. 1B).

3.2. Biological activity

3.2.1. Differential toxicity of amino-pyrazolopyridines

We first evaluated the effect of amino-pyrazolopyridines on viability of chronic myeloid leukemia K562 cells. Cells were treated with amino-pyrazolopyridines at 40 μM for 24 and 72 h. Compounds **3a**, **3b** and **3g** decreased more than 50% of K562 cell viability after 72 h and were selected for further studies (Fig. 2A). Compound **3a**, **3b** and **3g** dose-dependently down-regulated K562 metabolic activity after 24 and 72 h. (Fig. 2B–D, IC_{50}s are indicated). Results were confirmed by trypan blue assays (Fig. S1). To assess for differential toxicity, PBMCs from healthy donors were treated under the same conditions with compounds **3a**, **3b** and **3g**. Results show no strong effect on PBMC viability for **3b** and **3g** whereas compound **3a** induced levels of toxicity comparable to K562 cells (Fig. 3B–D, IC_{50}s are indicated). Altogether, these results indicate a selective cytotoxic effect of compounds **3b** and **3g**.

3.2.2. Amino-pyrazolopyridines induce apoptotic cell death

To further assess cell death mechanisms, morphology of nuclei of K562 cells treated with 40 μM of amino-pyrazolopyridines **3a**, **3b** and **3g** for 72 h was assessed. Results showed condensed nuclei typical of apoptosis (Fig. 4). In order to strengthen our results, we evaluated activation of executioner caspases 3/7. Amino-pyrazolopyridines increased caspase 3/7 activity in K562 cells reaching a maximum after 24 h (Fig. 5A). In order to generalize our results, we tested the effects of amino-pyrazolopyridines in two additional leukemia cell lines (Jurkat and U937). Our results showed that all three amino-pyrazolopyridines significantly induced caspase 3/7 activity in Jurkat and U937 cells at 40 μM (Fig. 5B and C). Compounds **3a** and **3b** reached the activation peak after 8 h in both cell lines, while compound **3g** induced caspase 3/7 activity after 48 h and 24 h in Jurkat and U937 cells, respectively. In order to ascertain involvement of caspase-dependent apoptotic mechanisms, we pre-treated cells with 50 μM of Z-VAD caspase inhibitor for 1 h. Under these conditions, induction of caspase 3/7 activity was completely abrogated (Fig. 5A–C). Caspase 3/7 activities after amino-pyrazolopyridines treatments are summarized in Table 1.

3.2.3. TNFα-induced NF-κB pathway is repressed by amino-pyrazolopyridines

Our results showed that amino-pyrazolopyridines **3a**, **3b** and **3g** inhibited TNFα-induced NF-κB transactivation at 40 μM (Fig. 6A). These three compounds dose-dependently inhibited NF-κB activation in K562 cells (Fig. 6B–D, IC_{50}s are indicated). Importantly, compounds **3a**, **3b** and **3g** inhibit NF-κB at sub-cytotoxic concentrations. This includes compound **3g** as the IC_{50} value for NF-κB inhibition (39.8 ± 3.9 μM) was evaluated after 8 h of treatment

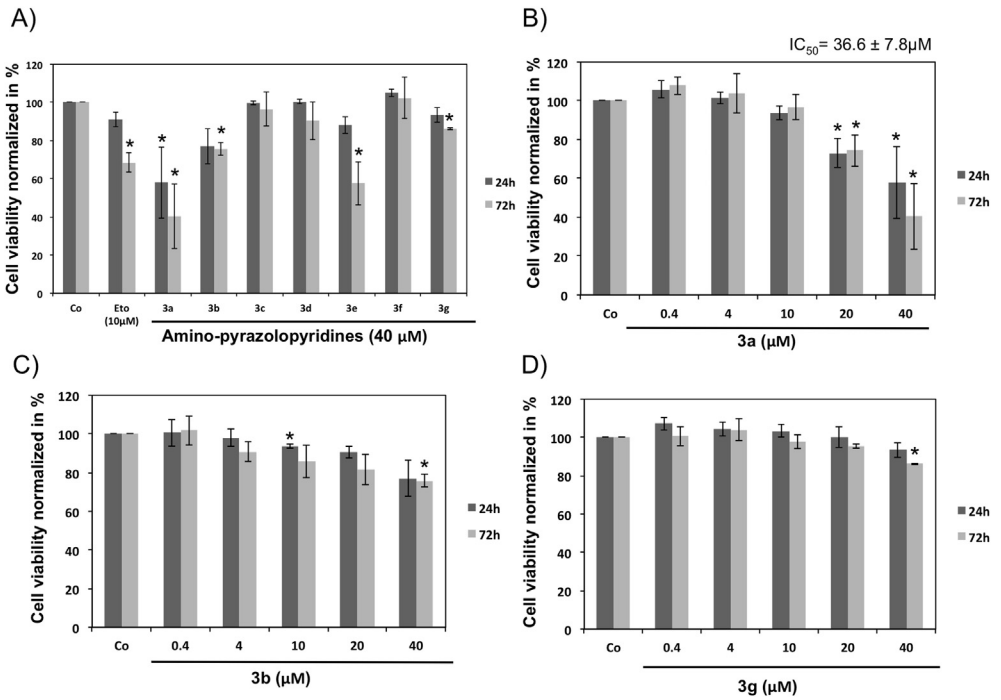

Fig. 3. Effects of amino-pyrazolopyridines on PMBC viability. A) Effects of amino-pyrazolopyridine analogues on metabolic activity of healthy PBMCs after 24 and 72 h at 40 μM. Control (Co) corresponds to untreated cells. Etoposide (Eto) served as a positive control. Each value is a mean ± SD of three determinations. The asterisk indicates a significant difference compared to control analyzed by *t*-test (*$p < 0.01$). B) Dose-dependent inhibition of PBMCs viability by compound **3a**, C) by compound **3b** and D) by compound **3g** is displayed.

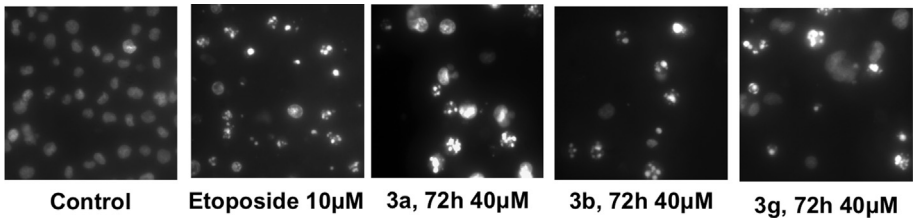

Fig. 4. Induction of apoptotic morphology by amino-pyrazolopyridines. K562 cells were stained with Hoechst in order to detect nuclear morphology, non-treated (control) vs. cells treated with 40 μM of amino-pyrazolopyridines **3a**, **3b** or **3g** for 72 h. 10 μM of etoposide served as a positive control.

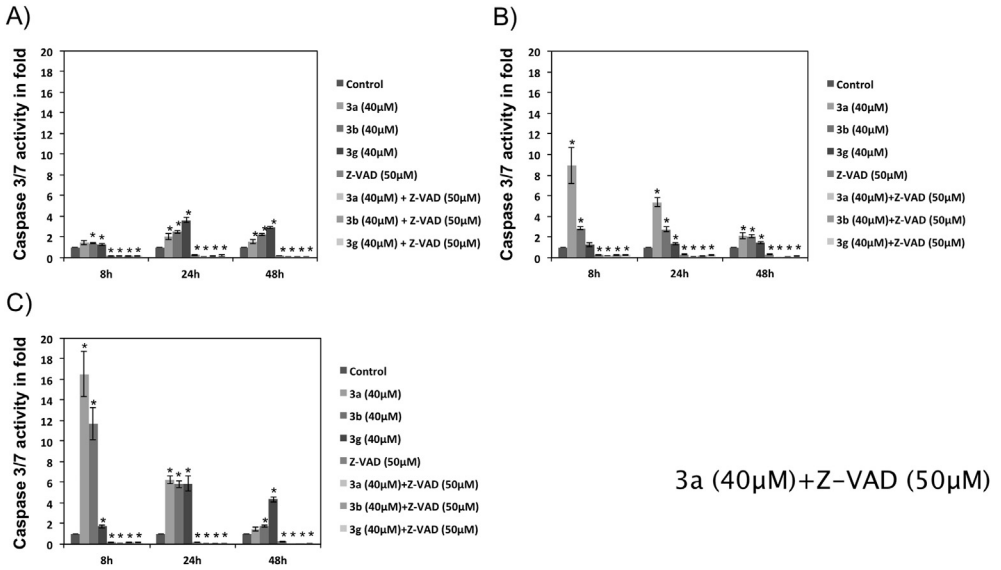

Fig. 5. Induction of caspase 3/7 activity in leukemia cell lines. K562 (A), Jurkat (B) and U937 cells (C) were pre-treated or not with Z-VAD at 50 μM for 1 h, followed by amino-pyrazolopyridines treatment at 40 μM for 8, 24 and 48 h. Control corresponds to untreated cells. Asterisk indicates a significant difference compared to control analyzed by *t*-test (*$p < 0.05$).

Table 1
Activation of caspase 3/7 activity by amino-pyrazolopyridines in leukemia cancer cells. Results are expressed as fold-change increase compared to untreated cells.

	3a (40 μM)			**3b** (40 μM)			**3g** (40 μM)		
	8 h	24 h	48 h	8 h	24 h	48 h	8 h	24 h	48 h
K562	1.44	2.04	1.55	1.37	2.50	2.23	1.26	3.62	2.90
Jurkat	8.89	5.38	2.11	2.86	2.73	2.05	1.27	1.31	1.44
U937	16.51	6.27	1.48	11.68	5.84	1.74	1.73	5.87	4.35

whereas IC_{50} value for cell viability (35.0 ± 2.3 μM) was determined after 72 h. Moreover, after 24 h of treatment with compound **3g** at 40 μM, we did not observe 50% inhibition of cell viability, indicating that this concentration is sub-toxic up to 24 h.

To further confirm the inhibition potential of amino-pyrazolopyridines on TNFα-induced NF-κB activation, we investigated the effect of amino-pyrazolopyridines on interleukin 8 (IL-8), a gene under the control of NF-κB. Results show that compound **3a** dose-dependently inhibited IL-8 release in K562 cells (Fig. 7). Importantly, K562 cell viability was not affected after 24 h at concentrations corresponding to the IC_{50} of the inhibition of IL-8 release (8.2 ± 0.9 μM) (Figs. 3B and S1).

4. Discussion

The goal of this bioactivity study was the determination of cytotoxicity and NF-κB inhibition potential of newly synthesized amino-pyrazolopyridines.

Over the last years, pyrazolopyridines were published to impact a large variety of cancer signaling pathways: pyrazolopyridines showed AT_1 receptor affinity [23], anti-inflammatory [9] and cytotoxic effects [11,24], inhibition of Aurora A kinase [24,25], PI3-kinase [26], protein kinase B/Akt [27], VEGFR kinase [28] and Polo-like kinase [29]. Considering these results, it was not surprising that viability assays confirmed the cell-death inducing properties of amino-pyrazolopyridines derivatives **3a**, **3b** and **3g** in the leukemia cell line K562. Hoechst staining showed condensed nuclei witnessing apoptosis induction in K562 cells further validated by caspase assays. Viability assays on PBMCs from healthy donors showed that compounds **3b** and **3g** present interesting differential toxicity in line with previous reports [10,24].

Interestingly, to our best knowledge, no NF-κB inhibition property of pyrazolopyridines has been reported in literature so far. Our results, shown by the NF-κB assays, indicate that amino-pyrazolopyridine derivatives **3a**, **3b** and **3g** inhibited TNFα-induced NF-κB activity in K562 cells with an IC_{50} of 4.7 ± 1.6 μM, 6.9 ± 1.9 μM and 39.8 ± 3.9 μM, respectively. This new reported activity of amino-pyrazolopyridine derivatives is not surprising as previously synthesized pyrazolopyridines were reported to affect signaling pathways tightly associated to the NF-κB transcription factor. The later is linked to anti-inflammatory activity [9], Aurora A kinase [25,30], AT_1 receptor [23,31], protein kinase B/Akt [27], VEGFR kinase [28,32] Polo-like kinase [29]. These cell signaling pathways were all reported to be targets of various pyrazolopyridine derivatives.

We hypothesize that inhibition of NF-κB pathway by amino-pyrazolopyridines may be responsible for the induction of apoptotic cell death in K562 cells, as concentrations necessary for NF-κB inhibition were lower and/or appeared at the shorter time points compared to cytotoxic concentrations. These observations indicate that inhibition of NF-κB activity occurs prior to induction

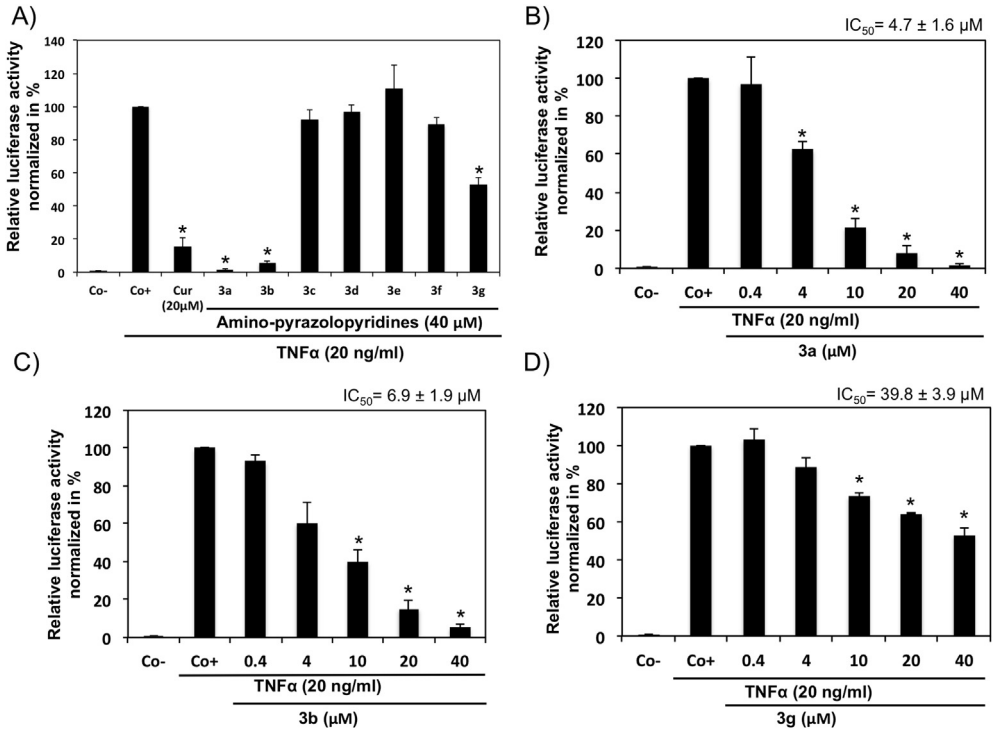

Fig. 6. Inhibitory effects of amino-pyrazolopyridines on TNFα-induced NF-κB activity. A) K562 cells were transiently transfected with firefly luciferase vector (NF-κB pGL4) and ph-RG-tk Renilla plasmid for 24 h. After transfection, K562 cells were treated with amino-pyrazolopyridines at 40 μM for 2 h followed by TNFα stimulation (20 ng/ml) during 6 h. Results are expressed as a ratio of the measured luminescence of the firefly luciferase vector and the luminescence of *Renilla* plasmid. Negative control corresponds to untreated cells, positive control refers to TNFα-treated cells. Curcumin (Cur) has been used as positive inhibitory control. Results are presented as a mean ± S.D. of three independent experiments. The asterisk indicates a significant difference compared to the positive control analyzed by *t*-test (*$p < 0.01$). B) Dose-dependent inhibition of TNFα-induced NF-κB activity in K562 cells by compound **3a**, C) by compound **3b** and D) by compound **3g** is shown.

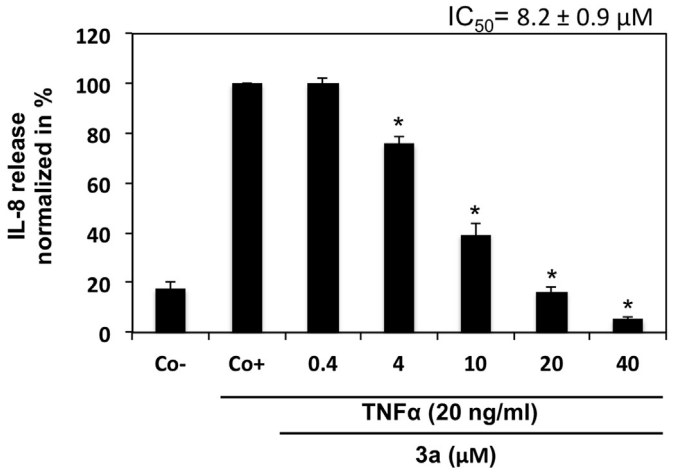

Fig. 7. Down-regulation of NF-κB target gene interleukin-8. K562 cells were treated or not at indicated concentrations with compound **3a** for 2 h, before being activated with TNFα (20 ng/ml) during 22 h. After 24 h of incubation, IL-8 concentration was measured in supernatants. Negative control corresponds to untreated cells, positive control refers to TNFα treated cells. Each value is a mean ± SD of three determinations. Asterisks indicate a significant difference compared to control positive as analyzed by *t*-test ($^*p < 0.01$).

of cell death. NF-κB activation leads to transcription of large battery of genes including those involved in apoptosis resistance. Thus, blocking of NF-κB activity potentiates apoptosis induction. The latter has been reported for variety of natural products and anticancer drugs in leukemia cells including K562 cells [21,22,33–37].

5. Conclusion

In summary, our results show that amino-pyrazolopyridine derivatives **3a**, **3b** and **3g** exert anti-NF-κB properties first reported for this chemical class. Inhibition of NF-κB activity may potentiate the cytotoxic character of studied compounds by inhibition of target genes including IL-8. Furthermore, our study shows that amino-pyrazolopyridine analogues **3b** and **3g** express selective cytotoxic activity towards K562 cancer cells compared to cells from healthy donors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.008.

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