New 1,4-Dihydropyrazolo[4,3-b]indoles Induce Antiproliferation of Acute Myeloid Leukemia Cells and Inhibition of Selective Inflammatory Cytokines

Vo Ngoc Binh\textsuperscript{1,2}, Sabrina Adorisio\textsuperscript{3}, Domenico V. Delfino\textsuperscript{3} and Quoc Anh Ngo\textsuperscript{1,2,}\textsuperscript{E}

Abstract

Research on multitargeting drugs is emerging, focusing on the discovery of agents that simultaneously act on more than one biological target. Here, a novel synthetic route to access the fused-heterocycles 1,4-dihydropyrazolo[4,3-b]indoles (4) from pyrazolo[4,3-c][2,1]benzothiazine 4,4-dioxide (3) via \(\text{H}_2\text{O}–\text{SO}_2\) elimination and an intramolecular ring-closing reaction is reported. Two lead compounds 3b and 4b were found to show significant inhibition of cell growth by suppressing cell cycle progression at the G\(_0\)/G\(_1\) phases and inducing apoptosis of the acute myeloid leukemia OCI-AML3 cell line. Both compounds also significantly decreased tumor necrosis factor-\(\alpha\) and transforming growth factor-\(\beta\) (at all tested concentrations), whereas no effect was seen on other cytokines (interleukin-4, interferon-\(\gamma\), interleukin-9, interleukin-12). Thus, these compounds are promising leads in the discovery of novel anticancer agents.

Keywords
antiinflammation, anticancer, 1, 4-dihydropyrazolo[4, 3-b]indoles, cytokine, antiproliferation

Introduction

Indoles are widely distributed in nature and are attractive biological molecules.\textsuperscript{1} Hybridization of indoles with other active moieties would be an important approach to generating new therapeutic agents with higher potency. Sunitinib, indomethacin, pindolol, physostigmine, delavirdine, metralindole, vincristine, and sumatriptan are indole-containing drugs approved by the Food and Drug Administration for the treatment of cancer, cardiovascular and neurologic disorders.\textsuperscript{2} Among these drugs, heteroaryl-substituted or fused-indoles frequently form privileged architectural units of the molecules.\textsuperscript{3} Recently, 1,4-dihydropyrazolo[4,3-b]indoles have been designed as structural scaffolds of new Topo I/II inhibitors based on the merging of potent anticancer pharmacophores carbazoles, pyrazolocarbazoles, and N-acetyl pyrazolines.\textsuperscript{4-6} This structural architecture was also confirmed by their preliminary docking into the proteins, which account for their proposed inhibitory activity.\textsuperscript{4}

Inflammation is a complex pathophysiological process in which a variety of signaling molecules in excess levels are produced by macrophages, mononuclear phagocytes, eosinophils, and neutrophils.\textsuperscript{5} These are inflammatory mediators such as nitric oxide (NO), prostaglandin E\(_2\), and cytokines, such as interleukin-1\(\beta\) (IL-1\(\beta\)), interleukin-6, and tumor necrosis factor (TNF-\(\alpha\)).\textsuperscript{9} An overproduction of proinflammatory cytokines and NO characterizes the pathogenesis of many inflammation-derived diseases such as rheumatoid arthritis, inflammatory bowel diseases, atherosclerosis, diabetes, and cancer.\textsuperscript{10}

Effective cancer management often recommends a combination of multiple drugs with different mechanisms. Thus, research on multitargeting drugs is emerging, focused on the discovery of agents that simultaneously act on more than one biological target. Here, we report the synthesis of a new 1,4-dihydropyrazolo[4,3-b]indole and its antiproliferation effect on acute myeloid leukemia cells, and selective inflammatory cytokines inhibition in comparison with pyrazolo[4,3-c][2,1]benzothiazine 4,4-dioxide.

1Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam
2Institute of Chemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam
3Department of Medicine and Surgery, Foligno Nursing School and Section of Pharmacology, University of Perugia, Perugia, Italy

Corresponding Author:
Quoc Anh Ngo, Institute of Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.
Email: ngoanhdichv@ich.vast.vn

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**Scheme 1.** Synthesis of compounds 4a and 4b. **Reagents and conditions:** (a) H₂O₂, H₂O/MeOH (1:4 v/v), 2 M NaOH, room temperature, 6 h; (b) phenylhydrazine or 4-(trifluoromethyl)phenylhydrazine, 3 drops CH₃COOH, CH₂Cl₂, room temperature, 72 h; (c) I₂, CH₃COOH, reflux, 24 h.

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**Results and Discussion**

Only a few synthetic methods to access either pyrazolo[4,3-\(\beta\)] or [3,4-\(\beta\)]indole derivatives have been reported.\(^{11-13}\) Here, a new synthetic route for the novel 1,4-dihydropyrazolo[4,3-\(\beta\)]indole is shown in Scheme 1. 2,1-Benzothiazine 2,2-dioxide (1) is conveniently synthesized via the unique neutral redox reaction of 2-nitrochalcone with elemental sulfur in the presence of either 3-picoline or N-methyl morphine under catalyst-free conditions.\(^{14}\) Epoxidation of the \(\alpha\beta\)-unsaturated ketone scaffold in 1 was carried out in the water at 0 °C–2 °C using H₂O₂ and a catalytic amount of NaOH to yield epoxide 2, with a conversion of 95%. Crude epoxide 2 was used in the next step without further purification. The pyrazolo[4,3-\(\alpha\)][2,1]benzothiazine 4,4-dioxides (3a, 3b) were obtained from the condensation reaction of epoxide 2 with either phenylhydrazine or 4-(trifluoromethyl)phenylhydrazine, catalyzed by acetic acid. Finally, 1,4-dihydropyrazol[4,3-\(\beta\)]indoles (4a, 4b) were obtained by a dehydration reaction of 3 using I₂, and refluxing in CH₃COOH. The proton nuclear magnetic resonance (\(^1\)H NMR), carbon-13 nuclear magnetic resonance (\(^{13}\)C NMR), and mass spectra of compound 4a coincide with the published spectral data.\(^{7}\) In the \(^1\)H NMR spectrum of 4b, the singlet at 9.63 ppm of a hydroxyl proton (–OH) and the singlet at 6.19 ppm of a methine proton (–CH) of 3b also disappeared. In the infrared (IR) spectrum, stretching bands assigned to the sulfonamide group (belonging to pyrazolo[4,3-\(\beta\)][2,1]benzothiazine 4,4-dioxide) at 1356 and 1173 cm\(^{-1}\) (3a) and 1322 and 1180 cm\(^{-1}\) (3b) are absent in compounds 4a and 4b. The molecular weights obtained by high-resolution mass spectrometry correspond with the assigned molecular formulas of 4a and 4b (Figures S9 and S11).

A large number of biological studies have been undertaken on pyrazolobenzothiazines, such as on their antiinflammatory and anticancer activities, but nothing has been reported so far about the cytotoxic/antiproliferative/antiinflammatory activity of these compounds, which prompted us to investigate the novel pyrazolobenzothiazine (3b), together with 1,4-dihydropyrazolo[4,3-\(\beta\)]indole (4b) against the acute myeloid leukemia OCI-AML3 cell line.\(^{18}\) The choice of the cell line was due to the need to find the effective concentrations in dose-response experiments and also because this cell line is representative of not only myeloid leukemic cells, but possibly also of myeloid cells (granulocytes, macrophage/monocytes) that are in part responsible for inflammatory response.

In Figure 1, it is shown that 24 h of treatment with 10 and 20 µg/mL of compound 3b and with 5 and 10 µg/mL of 4b induced a significant decrease in OCI-AML3 cell numbers, as counted by hemocytometer (left). Analysis of apoptotic cell death showed a significant increase in apoptosis with 10 and 20 µg/mL of compound 3b and with 5 and 10 µg/mL of compound 4b (right). The decrease in cell number was due, at least in part, to a significant increase in apoptosis.

Analysis of the cell cycle revealed a significant increase in cells in the G0/G1 phase with 5, 10, and 20 µg/mL of compound 3b and with 10 µg/mL of compound 4b (Figure 2), and fewer cells in S (with 10 and 20 µg/mL of compound 3b and with 10 µg/mL of compound 4b) and G2/M (with 10 and 20 µg/mL of compound 3b and with 5 and 10 µg/mL of compound 4b) phases of the cell cycle. In the case of referential chemotherapies, such as with azacitidine (AZA) and decitabine (DAC), EC\(50\) values obtained in the similar cell line were 0.8 ± 0.1 and 0.7 ± 0.4 µM, respectively. Both drugs increased the sub-G1 fraction and apoptosis markers, with AZA decreasing all cell cycle phases and DAC causing an increase in G2/M.\(^{19}\)

The antiinflammatory activity of pyrazolobenzothiazines prompted us to test, by real-time polymerase chain reaction (PCR), the ability of compounds 3b and 4b to affect the transcription of inflammatory cytokines. As shown in Figure 3, the expression of TNF-\(\alpha\), transforming growth factor-\(\beta\) (TGF-\(\beta\)), and IL-9 was tested by real-time PCR, and results indicate that both compounds were able to decrease significantly the expression of both TNF-\(\alpha\) and TGF-\(\beta\) (with 10 and 20 µg/mL for compound 3b and 2.5, 5, and 10 µg/mL for compound 4b), whereas no significant decrease was detected for the expression of IL-9. TNF-\(\alpha\) is not only a well-known antiinflammatory cytokine but also a major effector and regulatory cytokine in the pathogenesis of several immune-regulated diseases and hematologic malignancies, including AML. The presence of TNF-\(\alpha\) in leukemic cells has been related to the percentage of cells in the S phase and associated with resistance to induction therapy. Therefore, either inactivating or blocking TNF-\(\alpha\) are emerging therapeutic approaches for hematological malignancies.\(^{20}\) TGF-\(\beta\) induces...
the production of T-helper 17 (Th17) and regulatory T-cells (Treg) cells. Th17 cells are pathogenic in rheumatoid arthritis, multiple sclerosis, and a glucocorticoid-resistant form of asthma. Treg cells can be either detrimental (they can protect cancer cells) or beneficial (they can inhibit autoimmune diseases). TGF-β is a potent inhibitor of human myeloid leukemia cells.7 Paradoxically, elevated levels of TGF-β can be an essential mediator of myelofibrosis in myeloproliferative diseases and hairy-cell leukemia.8 Thus, understanding the role of the TGF-β signaling pathway in hematologic malignancies should be established for effective strategies of the treatment.

Conclusion

We report here a novel synthetic approach to access 1,4-dihydropyrazolo[4,3-β]indols from pyrazolo[4,3-c][2,1]benzothiazine 4,4-dioxide via [H2O–SO2] elimination and intramolecular ring-closing reaction. The significance of compounds 3b and 4b resides in their capacity to affect different processes involved in cancer such as cell proliferation and the immune response/inflammation. They were able to decrease the number of acute myeloid leukemia cells by acting on both apoptosis and cell cycle since they increased apoptosis and blocked cells in the G0/G1 phase of the cell cycle. Moreover, both compounds also simultaneously decreased the level of TNF-α and TGF-β (at all tested concentrations), whereas no effect was seen on other cytokines (IL-4, interferon-γ [IFN-γ], IL-9, and IL-12). Thus, the role of these novel compounds in cancer treatment is promising and will be the focus of future studies.

Materials and Methods

General

All the reagents were purchased from commercial suppliers and used without further purification. Melting points were measured with a Krüss M5000 melting-point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer
Spectrum Two Fourier transform-infrared spectrometer by dispersion of the sample in KBr. The 1H NMR (500/600 MHz) and 13C NMR (125/150 MHz) spectra were recorded in CDCl₃ or dimethylsulfoxide (DMSO)-d₆ using Bruker AV-500 and AV-600 spectrometers with trimethylsilane as an internal reference. The J values are given in Hertz. High-resolution electrospray ionization (ESI) mass spectra were obtained on a SCIEX-X500R QTOF LC/MS system. Column chromatography was performed using silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck), and thin-layer chromatography was performed using precoated silica gel 60 F254 (0.25 mm, Merck).

**Synthesis**

(2,2-dioxido-1H-benzo[e][1,2]thiazin-3-yl)(p-tolyl)methanone (I) was prepared using the literature procedure. 14

(2,2-Dioxido-3,7b-Dihydro-1aH-Benzocyclob避ene[2,3-ε][1,2]thiazin-1a-yl)(p-Tolyl)methanone (2). Aroyl sulfox 1 (409.71 mg, 1.37 mmol) and 2 M aqueous solution of NaOH (1.16 ml, 1.7 equiv.) were mixed with 4 ml of H₂O/MeOH solution (1/4, v/v) and stirred for 10 min at 0 °C to 2 °C. Then 0.28 ml (2 equiv.) of H₂O₂ (35 wt%) was added to the mixture and stirred at room temperature (RT) for 6 h. At the end of the reaction, the mixture was extracted with CH₂Cl₂ (3 × 30 ml). The organic phase was washed with saturated NaCl (20 ml), then dried over sodium sulfate and evaporated under reduced pressure to give 390 mg of epoxide 2 as an orange-red solid with 95% purity. The crude product was used in the next step without further purification. 1H NMR (CDCl₃, 500 MHz): δ (ppm) 7.70 (d, J = 8.2 Hz, 2H), 7.31–7.25 (m, 1H), 7.22–7.17 (m, 3H), 7.13 (t, J = 7.6 Hz, 1H), 7.01 (d, J = 7.9 Hz, 1H), 6.96 (s, 1H), 2.39 (s, 3H). 13C NMR (CDCl₃, 125 MHz): δ (ppm) 188.97, 143.74, 142.85, 133.98, 133.45, 131.80, 129.83, 129.08, 126.41, 125.29, 124.54, 124.19, 21.65. High-resolution mass spectrometry (HR-MS; ESI) calcd. For C₁₆H₁₃NNaO₄S [(M + Na)⁺]: 338.0463; found: 338.0444.

**General Procedure for the Synthesis of 3a-hydroxy-1,3a,5,9b-tetrahydroprazolo[4,3-ε][2,1]benzothiazine 4,4-dioxides (3)**

A mixture of epoxide 2 (1.0 mmol), phenylhydrazine derivatives (1.2 mmol), and 3 drops of acetic acid in 12 ml of
dichloromethane was stirred for 72 h at RT. At the end of the reaction, the mixture was added to NH₄OH solution (10 ml) and extracted with CH₂Cl₂ (3 x 30 ml). The organic phase was dried over sodium sulfate and concentrated under reduced pressure to obtain the crude product, which were purified by column chromatography on silica gel (n-hexane eluate with 3%-12% EtOAc) to obtain the product (3).

3a-Hydroxy-1-Phenyl-3-(p-Tolyl)-1,3a,5,9b-Tetrahydropyrazolo[4,3-c][2,1]Benzothiazine-4,4-Dioxide (3a). Applying the above experimental procedure using phenylhydrazine, compound 3a was obtained as a light yellow solid, yield: 75%; m.p. 206.4-207.6; IR (KBr, cm⁻¹): ν 3318, 1599, 1422, 1356, 1173; ¹H NMR (DMSO, 600 MHz): δ (ppm) 10.96 (s, 1H), 9.16 (s, 1H), 7.43 (d, J = 7.7 Hz, 2H), 7.35 (dd, J = 7.9, 6.3 Hz, 1H), 7.28 (d, J = 8.1 Hz, 2H), 7.26–7.23 (m, 2H), 7.23–7.19 (m, 4H), 7.16 (d, J = 7.4 Hz, 1H), 6.81 (tt, J = 7.1, 1.4 Hz, 1H), 6.12 (s, 1H), 2.43 (s, 3H); ¹³C NMR (125 MHz, DMSO) δ 147.88, 145.47, 139.49, 139.12, 133.91, 131.56, 130.13, 129.62, 125.09, 115.96, 113.28, 21.06. HR-MS (ESI) calcd. For C₂₂H₂₀N₃O₃S [(M + H)⁺]: 406.1225; found: 406.1201.

3a-Hydroxy-3-(p-Tolyl)-1-(4-(Trifluoromethyl)Phenyl)-1,3a,5,9b-Tetrahydropyrazolo[4,3-c][2,1]Benzothiazine-4,4-Dioxide (3b). Applying the above experimental procedure using 4-(trifluoromethyl)phenylhydrazine, compound 3b was obtained as a light yellow solid, yield: 43%; m.p. 222.3-223.1; IR (KBr, cm⁻¹): ν 3328, 3220, 1599, 1422, 1356, 1173; ¹H NMR (DMSO, 600 MHz): δ (ppm) 10.96 (s, 1H), 9.16 (s, 1H), 7.43 (d, J = 7.7 Hz, 2H), 7.35 (dd, J = 7.9, 6.3 Hz, 1H), 7.28 (d, J = 8.1 Hz, 2H), 7.26–7.23 (m, 2H), 7.23–7.19 (m, 4H), 7.16 (d, J = 7.4 Hz, 1H), 6.81 (tt, J = 7.1, 1.4 Hz, 1H), 6.12 (s, 1H), 2.43 (s, 3H); ¹³C NMR (125 MHz, DMSO) δ 145.88, 144.65, 138.94, 137.02, 133.82, 131.41, 130.21, 129.38, 129.17, 129.05, 128.11, 127.75, 126.40, 124.73, 120.13, 114.63, 113.53, 21.10. HR-MS (ESI) calcd. For C₂₃H₁₇F₃N₃O₃S [(M + H)⁺]: 472.0943; found: 472.0932.

General Procedure for the Synthesis of 1,4-dihydropyrazolo[4,3-b]indoles (4)

A solution of 3 (0.1 mmol) and I₂ (0.1 mmol) in CH₃COOH (4 ml) was refluxed for 24 h. The mixture was then neutralized with 2 M NaOH solution, and extracted with EtOAc (3 x 20 ml). The combined organic phases were washed with 10% Na₂S₂O₃ solution and dried over sodium sulfate. The solvent was removed and the residue was purified by column chromatography on silica gel using n-hexane/ethyl acetate (98:2) as eluent to yield 1,4-dihydropyrazolo[4,3-b]indoles (4).

1-Phenyl-3-(p-Tolyl)-1,4-Dihydropyrazolo[4,3-b]Indole (4a). Light yellow solid, yield: 43%; m.p. 222.3-223.1; IR (KBr, cm⁻¹): ν
3144, 1596, 1503, 1249; $^1$H NMR (DMSO, 600 MHz): $\delta$ (ppm) 11.51 (s, 1H), 8.02 (d, $J = 8.0$ Hz, 2H), 7.95 (d, $J = 7.4$ Hz, 2H), 7.90 (d, $J = 8.0$ Hz, 1H), 7.66 (t, $J = 8.0$ Hz, 1H), 7.59 (d, $J = 8.2$ Hz, 1H), 7.43–7.35 (m, 4H), 7.17 (t, $J = 7.5$ Hz, 1H), 2.40 (s, 3H); $^{13}$C NMR (150 MHz, DMSO) $\delta$ (ppm) 144.82, 140.92, 137.59, 134.24, 131.85, 130.31, 129.95, 126.47, 126.02, 125.05, 120.56, 119.39, 119.31, 113.63, 112.83, 21.39; HR-MS (ESI) calcd. For C$_{23}$H$_{17}$F$_{3}$N$_{3}$ [(M+H)$^+$]: 324.1501; found: 324.1485.

### OCI-AML Culture Conditions

The OCI-AML3 cell line was from the German Collection of Microorganisms and Cell Cultures (Braunschweig), cultured in 24-well plates at logarithm growth. RPMI 1640 medium was used with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO$_2$. Cultures were grown at a concentration of 2 × 10$^5$ cells/ml, and treated with various doses of DMSO (the higher concentration 0.6 µl/ml). The compounds were added at concentrations of 2.5, 5, 10, and 20 µg/ml, and cells were analyzed after 24, 48, and 72 h.

### Analysis of Cell Number, Apoptotic Cell Death, and Cell Cycle Progression

Cells were counted using a hemocytometer. Cell viability and cell cycle were assessed by flow cytometry to determine the propidium iodide (PI)-stained DNA content of cell nuclei after gating out of necrotic cells by size (forward scatter [FSC]). Brieﬂy, cells were centrifuged and washed in phosphate-buffered saline (PBS). Cells were incubated in PBS containing 50 µg/ml PI for 30 min at 4 °C. Cells were then analyzed by flow cytometry using Coulter Epics XL-MCL equipment (Beckman Coulter Inc.). Doublet discrimination was made with the Coulter Epics XL-MCL™ Flow Cytometer SYSTEM IITM Software, which can detect >90% cellular doublets in cells ≥7 µm, using peak versus integral discrimination. A peak fluorescence signal was assigned to AUX to measure peak versus integral fluorescence.

### Quantitation of Cytokine Levels by Enzyme-Linked Immunosorbent Assay

Cytokine secretion was evaluated by enzyme-linked immunosorbent assay in a medium in which OCI-AML cells, untreated or treated with experimental compounds, were cultured. High-binding 96-well microtiter plates (Nunc Maxisorb) were adhered with 100 µl anticytokine capture antibody (diluted in 0.1 M NaHCO$_3$, pH 8.3) and incubated overnight at 4 °C. Plates were ﬂushed three times with PBS–0.05% Tween and treated with 200 µl of PBS–10% fetal calf serum (FCS) for 1 h at RT. After having washed the plates, standard samples were added at 100 µl/well, and incubated for 2 h at RT. After washing with PBS–0.05% Tween plates were incubated with a biotinylated anti-cytokine antibody for 60 min at RT. After additional washes with PBS–0.05% Tween, horseradish peroxidase avidin D (Vector Laboratories) (1:2000 dilution in PBS–10% FCS) was added (100 µl/well), and plates were incubated for 30 min at RT, followed by additional washes in PBS–0.05% Tween. The antibodies utilized for the IL-4, IFN-γ, IL-9, IL-12, TNF-α, and TGF-β assays were rat anti-mouse cytokine mAbs (capture antibody) and biotinylated rat antimouse cytokine mAb (detection antibody) (Pharmingen).

### Statistical Analysis

Statistical analysis was performed by the Mann–Whitney U-test. Statistically significant differences were in agreement with the criteria: *p < 0.05; **p < 0.01; ***p < 0.001.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Supplemental Material

Supplemental material for this article is available online.

### ORCID iD

Quioc Anh Ngo https://orcid.org/0000-0002-2319-2880

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