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Discovery of 8-methoxypyrazino[1,2-*a*]indole as a New Potent Antiproliferative Agent Against Human Leukemia K562 Cells. A Structure-Activity Relationship Study

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Abstract

Identification of novel and selective anticancer agents remains an important and challenging goal in pharmacological research. The indole nucleus, frequently encountered as a molecular fragment in natural products and pharmaceutically active compounds, was employed as the initial building block for the synthesis of a series of pyrazino[1,2-*a*]indoles **1a–k**, variably substituted at the 6, 7, 8 and 9-positions. Compound **1e**, bearing the methoxy group at the 8-position of the pyrazino[1,2-*a*]indole nucleus was identified as a novel potent antiproliferative agent against the human chronic myelogenous leukemia K562 cell line, but it was much less active against several other cancer cell lines. Comparison of positional isomers indicated that moving the methoxy group from the 8- to the 7- or 6-position, to furnish compounds **1f** and **1g**, respectively, yielded inactive compounds. The analysis of structure-activity relationships observed in the series of investigated compounds may represent the basis for the design of more active molecules.

Keywords

Pyrazino[1,2-*a*]indole; Human leukemia K562 cell line; Anticancer agents

1. INTRODUCTION

Cancer is a major worldwide problem and is the second leading cause of mortality in developed countries [1]. Since many of the current treatments have problems with toxicity and drug resistance, there is a strong demand for the discovery and development of effective new cancer therapies [2]. Cytotoxic drugs continue to play a major role in cancer therapy. Searching for antineoplastic agents with improved selectivity towards malignant cells remains, therefore, a

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central task for drug development [3]. In particular, great efforts have been made in past years in the search of new compounds for the treatment of leukemias [4]. Leukemia therapy with anticancer compounds is based on cell growth inhibition, induction of cell death through apoptosis or of leukemic blast differentiation. Polycyclic nitrogen heterocycles with a planar structure, such as acridine, phenanthridine and actinomycin derivatives, represent pharmacophores for several classes of antitumor agents [5]. Although the indole ring is found in a wide range of biologically active compounds and is frequently condensed with various heterocycles [6], the synthesis and biological evaluation of the pyrazino[1,2-*a*]indole skeleton has thus far attracted limited attention.

The pyrazino[1,2-*a*]indole nucleus was previously prepared by flash vacuum pyrolysis starting from 2-benzyl- pyrazine-*N*-1-oxide [7]. Although this latter compound was obtained by oxidation of the corresponding pyrazine in satisfactory yield, the reaction gave two isomeric *N*-1 and *N*-4 pyrazine N-oxides, and separation of the desired *N*-1-oxide from the isomeric *N*-4-oxide was necessary.

As a part of our continuing search for nitrogen heterocycles with an indole moiety that have antineoplastic activity, we developed a short and efficient synthetic approach for the preparation of a series of compounds characterized by the presence of the pyrazino[1,2-*a*] indole skeleton. We evaluated these compounds for their antiproliferative activity against the human chronic myelogenous leukemia K562 cell line. We will next undertake studies directed at elucidating the mechanism of action of this new class of compounds.

Structure-activity relationships (SAR) were examined with various substitutions at the 6, 7, 8 and 9-positions of the tricyclic pyrazino[1,2-*a*]indole pharmacophore. Besides hydrogen (compound **1a**), in order to define the structural requirements for antiproliferative activity, we first evaluated the effects of electron-withdrawing (F and Cl) and electron-donating (Me and MeO) substituents at the 8-position of the pyrazino[1,2-*a*]indole nucleus (**1b–e**) and position effects with three methoxyl group isomers (**1e–g**). By the synthesis of compounds **1h–k,** the effects of two (**1h–i**) or three (**1j–k**) methoxy groups at the 6, 7 and 8 positions of the pyrazino [1,2-*a*]indole moiety were also studied.

2. CHEMISTRY

Pyrazino[1,2-*a*]indoles **1a–k** were synthesized following the strategy reported in the Scheme 1.

Condensation of 1*H*-indole-2-carboxylates **2a–k** [8] with chloroacetonitrile in the presence of sodium hydride gave the corresponding 1-cyanomethyl-indole-2-carboxylates **3a–k** in good yields (60–75%) [9,10]. The nitriles **3a–k** were transformed by reductive cyclization with lithium aluminium hydride in dry ethyl ether into the desired 1, 2, 3, 4-tetrahydropyrazino[1,2-*a*]indoles **4a–k** in acceptable yields. The subsequent oxidation of **4a–k** by treatment with manganese dioxide (MnO_2) in toluene under reflux, furnished the pyrazino[1,2-*a*]indole **1a–k** in acceptable yields.

3. RESULTS AND DISCUSSION

Table 1 summarizes the effects of substituted and unsubstituted pyrazino[1,2-*a*]indoles **1a–k** on the growth of human chronic myelogenous K562 leukemia cells. The K562 cell line was used for initial compound screening because of its rapid proliferation, high sensitivity to standard anticancer agents and in order to determine whether these compounds had activity against human transformed cells [11,12]. With the exception of **1e**, all compounds had IC50's over 35 μM. Incubation of K562 cells with 0.0001% (v/v) DMSO showed that the vehicle did not affect cell proliferation at the concentration used in the experiments presented in Table 1.

These results indicated that pronounced inhibition of cell growth of this class of compounds required a methoxy group on the pyrazino[1,2-*a*]indole skeleton at the C-8 position (compound **1e**), while only limited activity was observed with the methoxy at the C-6 or C-7 positions (corresponding to derivatives **1g** and **1f**, respectively). The former compound, however, was almost twice as active as the latter.

SAR studies of **1e** indicated that the C-8 methoxy group was critical for activity. Replacing the methoxy group with a methyl (compound **1d**) or with electron-withdrawing groups (fluoro and chloro, derivatives **1b** and **1c**, respectively) led to a dramatic drop in antiproliferative activity. Moreover, there was major loss of activity when additional methoxy groups were added to compound **1e** (compounds **1h–i**).

Based on the significant antiproliferative activity of compound **1e** against the K562 leukemia cell line, this compound was further tested for growth inhibition against murine leukemia (L1210), murine mammary carcinoma (FM3A), human T-lymphoblastoid (Molt/4 and CEM) and human cervical carcinoma (HeLa) cells. Somewhat surprisingly, **1e** had little antiproliferative activity against these other cell lines, with IC_{50} values of 41, 46, 36, 26 and 20 μM, respectively, against the five tumor cell lines.

To investigate whether the antiproliferative activity of compound **1e** was caused by an interaction with the microtubule system, compound **1e** was evaluated for *in vitro* inhibition of tubulin polymerization [13,14]. Compound **1e** had no effect on the assembly reaction at concentrations as high as 50μM.

Flow cytometry analysis was performed to determine the effect of **1e** on the distribution of K562 cells among the phases of the cell cycle (Table 2). Cells were cultured for 48 h in the presence of compound **1e** at the IC_{50} and examined by flow cytometry [15–17]. The majority of control cells exposed to DMSO of the cell cycle were in the G0–G1 phase (67.8%), with 13.4% of the cells in S phase and 18.8% in G2/M phase. After treatment with **1e** for 24 h, there was a partial block of cells in the G2–M phase: 56.4% of cells were in the G0–G1 phase, 14.6% in the S phase and 29% in the G2–M phase.

4. EXPERIMENTAL SECTION

4.1. Chemistry Materials and Methods

^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents, as indicated. Melting points (mp) were determined on a BuchiTottoli apparatus and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. All reactions were carried out under an inert atmosphere of dry nitrogen, unless otherwise indicated. Standard syringe techniques were applied for transferring dry solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F_{254} Merck plates) and visualized with aqueous $KMnO_4$. Flash chromatography was performed using 230–400 mesh silica gel and the indicated solvent system. Organic solutions were dried over anhydrous Na_2SO_4.

4.1.1. General Procedure (A) for the Synthesis of Compounds 3a–k—Sodium hydride (50% dispersion in mineral oil, 744 mg, 15.52 mmol) was slowly added to a solution of **2a–k** (5.17 mmol) in dry DMF (7 mL) cooled with an ice bath. The mixture was then stirred for 0.5 h at room temperature, and chloroacetonitrile (1.17 mL, 15.52 mmol) dissolved in DMF (2 mL) was added. The reaction mixture was left at room temperature for 18 h, and ice was added to degrade excess of NaH. The reaction mixture was diluted with ethyl acetate (20 mL), and the organic phase was washed with water (3 × 5 mL) and brine (5 mL), dried over

Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (ethyl acetate-petroleum ether as eluent) on silica gel.

4.1.2. General Procedure (B) for the Synthesis of Compounds 4a–k—A suspension of **3a–k** (11 mmol) in dry ethyl ether (70 mL) was slowly added to a well-stirred suspension of $LiAlH_4$ (1.25 g., 33 mmol) in dry ethyl ether (50 mL) cooled in an ice bath. The mixture was then refluxed for 4 h, and water was added to degrade excess of $LiAlH_4$. The precipitate was removed by filtration through a pad of celite, which was washed three times with ethyl acetate (3 × 20 mL). The combined filtrate was washed with water (30 mL), dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography (ethyl acetate-methanol as eluent) on silica gel.

4.1.3. General Procedure (C) for the Synthesis of Compounds 1a–k—A mixture of **4a–k** (0.45 mmol), manganese dioxide (800 mg, 9.1 mmol) and toluene (15 mL) was stirred at reflux for 5 h. The insoluble solid was removed by filtration, and the filter was washed with ethyl acetate (3 × 5 mL). The filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel.

4.1.3.1. Pyrazino[1,2-*a*]indole (1a): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1a** as a yellow solid (54% yield); mp 158–160 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.00 (s, 1H), 7.42 (m 2H), 7.54 (s, 1H), 7.93 (m, 2H), 8.16 (dd, *J*=5.2 and 2.0 Hz, 1H), 9.01 (d, *J*=2.0 Hz, 1H).

4.1.3.2. 8-Fluoro-pyrazino[1,2-a]indole (1b): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate-petroleum ether 8:2 as eluent furnished **1b** as a yellow solid (48% yield); mp 133–135 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.98 (s, 1H), 7.18 (m, 1H), 7.52 (dd, *J*=9.2 and 2.4 Hz, 1H), 7.59 (d, *J*=4.8 Hz, 1H), 7.87 (dd, *J*=9.2 and 4.4 Hz, 1H), 8.14 (m, 1H), 9.01 (d, *J*=1.0 Hz, 1H).

4.1.3.3. 8-Chloro-pyrazino[1,2-a]indole (1c): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate-petroleum ether 9:1 as eluent furnished **1c** as a yellow solid (49% yield); mp 136–138 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.95 (s, 1H), 7.36 (dd, *J*=8.8 and 1.6 Hz, 1H), 7.59 (d, *J*=5.2 Hz, 1H), 7.87 (m, 2H), 8.1 (d, *J*=4.8 Hz, 1H), 9.01 (d, *J*=1.2 Hz, 1H).

4.1.3.4. 8-Methyl-pyrazino[1,2-*a*]indole (1d): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1d** as a brown solid (51% yield); mp 148–150 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.55 (s, 3H), 6.94 (s, 1H), 7.27 (d, *J*=1.5 Hz, 1H), 7.50 (d, *J*=4.8 Hz, 1H), 7.68 (s, 1H), 7.82 (d, *J*=8.8 Hz, 1H), 8.1 (d, *J*=4.8 Hz, 1H), 8.98 (d, *J*=1.6 Hz, 1H).

4.1.3.5. 8-Methoxy-pyrazino[1,2-a]indole (1e): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1e** as a yellow solid (44% yield); mp 162–164 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.92 (s, 3H), 6.95 (s, 1H), 7.09 (dd, *J*=9.2 and 2.8 Hz, 1H), 7.22 (d, *J*=2.4 Hz, 1H), 7.54 (d, *J*=4.8 Hz, 1H), 7.84 (d, *J*=9.2 Hz, 1H), 8.12 (d, *J*=4.8 Hz, 1H), 8.97 (d, *J*=1.2 Hz, 1H).

4.1.3.6. 7-Methoxy-pyrazino[1,2-*a*]indole (1f): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1f** as a yellow solid (45% yield); mp 118–120 °C.^{1}H-NMR ($CDCl_3$) δ: 3.88 (s, 3H), 6.94 (s, 1H), 7.22 (m, 2H), 7.47 (d, *J*=5.0 Hz, 1H), 7.84 (d, *J*=8.8 Hz, 1H), 8.13 (d, *J*=4.6 Hz, 1H), 8.99 (d, *J*=1.2 Hz, 1H).

4.1.3.7. 6-Methoxy-pyrazino[1,2-*a*]indole (1g): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1g** as a brown oil (44% yield). ^{1}H-NMR ($CDCl_3$) δ: 4.09 (s, 3H), 6.76 (d, *J*=7.0 Hz, 1H), 6.96 (s, 1H), 7.22 (m, 2H), 7.49 (m, 2H), 8.83 (d, *J*=4.8 Hz, 1H), 8.96 (d, *J*=1.2 Hz, 1H).

4.1.3.8. 7,8-Dimethoxy-pyrazino[1,2-*a*]indole (1h): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate-methanol 9.5-0.5 as eluent furnished **1h** as a yellow solid (42% yield); mp 156–158 °C. ^{1}H-NMR ($CDCl_3$)δ: 4.00 (s, 3H), 4.03 (s, 3H), 7.22 (m, 3H), 7.52 (d, *J*=5.2 Hz, 1H), 8.01 (d, *J*=5.2 Hz, 1H), 8.91 (d, *J*=1.4 Hz, 1H).

4.1.3.9. 6,8-Dimethoxy-pyrazino[1,2-a]indole (1i): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1i** as a yellow solid (46% yield); mp 133–135 °C. ^{1}H-NMR ($CDCl_3$) δ 3.90 (s, 3H), 4.05 (s, 3H), 6.46 (d, *J*=2.0 Hz, 1H), 6.78 (d, *J*=1.8 Hz, 1H), 6.96 (s, 1H), 7.47 (d, *J*=5.4 Hz, 1H), 8.75 (d, *J*=4.4 Hz, 1H), 8.91 (d, *J*=1.4 Hz, 1H).

4.1.3.10. 6,7,8-Trimethoxy-pyrazino[1,2-*a*]indole (1j): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate as eluent furnished **1j** as a yellow solid (46% yield); mp 92–94 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.96 (s, 3H), 3.97 (s, 3H), 4.19 (s, 3H), 6.92 (s, 1H), 6.97(s, 1H), 7.45 (d, *J*=5.2 Hz, 1H), 8.68 (d, *J*=4.8 Hz, 1H), 8.92 (d, *J*=1.4 Hz, 1H).

4.1.3.11. 7,8,9-Trimethoxy-pyrazino[1,2-*a*]indole (1k): Following general procedure C, the crude residue purified by flash chromatography using ethyl acetate-methanol 9.5-0.5 as eluent furnished **1k** as a brown oil (35% yield). ^{1}H-NMR ($CDCl_3$) δ: 3.94 (s, 3H), 4.00 (s, 3H), 4.17 (s, 3H), 7.00 (s, 1H), 7.08 (s, 1H), 7.46 (d, *J*=5.2 Hz, 1H), 7.97 (d, *J*=5.0 Hz, 1H), 8.92 (d, *J*=1.4 Hz, 1H).

4.2. Biological Assays

4.2.1. Cell Proliferation Analysis—For the evaluation of the effects on cell proliferation, K562 cells were seeded at 20,000 cells/mL in 24-well culture plates. Cell growth was studied by determining the cell number/mL after three days of growth. Cells were counted with a ZF Coulter Counter (Beckman Coulter Electronics, Hialeah, Fla., USA).

Murine leukemia L1210, murine mammary carcinoma FM3A, human T-lymphocyte Molt 4 and CEM and human cervix carcinoma (HeLa) cells were suspended at 300,000–500,000 cells/mL of culture medium, and 100 μL of a cell suspension was added to 100 μL of an appropriate dilution of the test compounds in wells of 96-well microtiter plates. After incubation at 37 °C for two (L1210 and FM3A) or three (Molt 4, CEM and HeLa) days, cell number was determined using a Coulter counter. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

4.2.2. Effects on Tubulin Polymerization—To evaluate the effect of the compounds on tubulin assembly *in vitro* [16], varying concentrations were preincubated with 10 μM tubulin in glutamate buffer at 30 °C and then cooled to 0 °C. After addition of GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30 °C, and the assembly of tubulin was observed turbidimetrically. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after 20 min at 30 °C.

4.2.3. Cell Cycle Analysis—For flow cytometric analysis of DNA content, 5×10^5 K562 cells were centrifuged, fixed in ice-cold ethanol (70%), treated with lysis buffer containing

RNAse A, and stained with propidium iodide. Samples were analyzed on a Becton Coulter Epics XL-MCL flow cytometer. For cell cycle analysis, DNA histograms were analyzed using MultiCycle® for Windows (Phoenix Flow Systems, San Diego, CA).

5. CONCLUSIONS

In conclusion, we have discovered and explored the SAR of a new series of compounds characterized by the presence of a pyrazino[1,2-*a*]indole framework with one, two or three methoxy groups. We found that compound **1e**, bearing a methoxy group at the 8-position of the pyrazino[1,2-*a*]indole system has significant growth inhibitory activity against the K562 cell line. Moreover, it was highly selective for K562 cells in its cytostatic action. From these data, we deduce that methoxy substitution and location plays an important role in affecting antiproliferative activity. Experiments performed to identify a possible interaction of **1e** with microtubules failed to demonstrate an interaction with this cell constituent. Flow cytometric analysis on K562 cells indicated that **1e** can induce cell cycle arrest in the G2-M phase. These results encourage us to explore the activity of **1e** in additional *in vitro* assay systems to gain insights into its mechanisms of action and to prepare additional analogs in hope of further elucidating important SAR features and identifying other active congeners.

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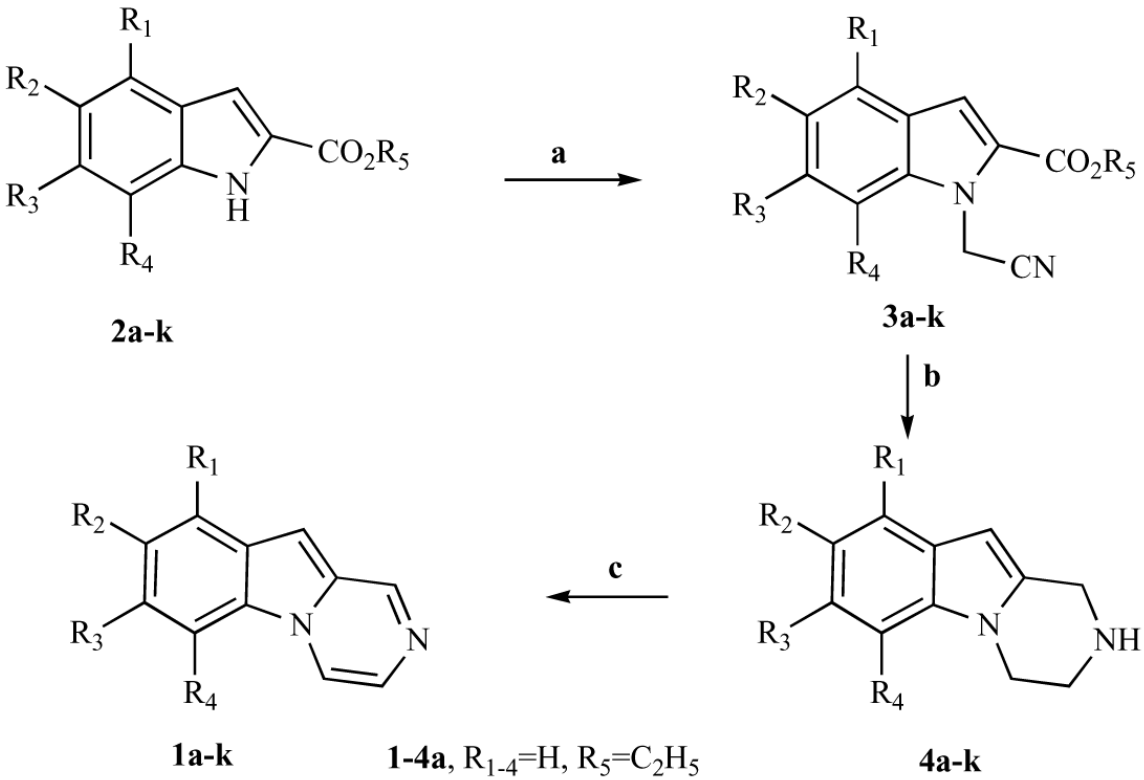
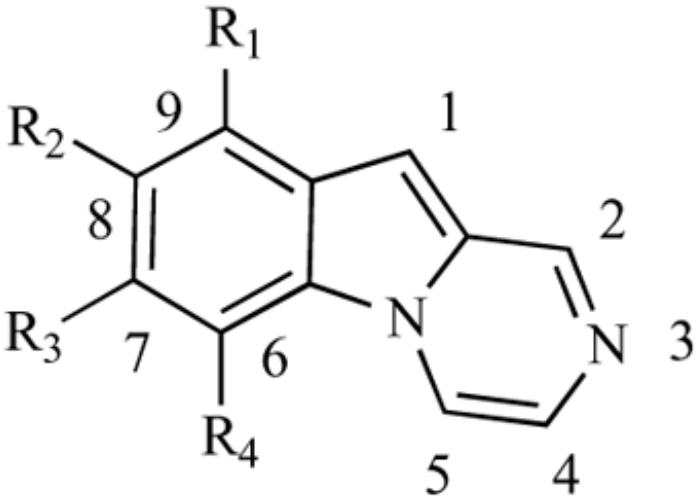


1-4a, $R_{1\text{-}4}$=H, R_5=C_2H_5
1-4b, $R_{1,\,3\text{-}4}$=H, R_2=F, R_5=CH_3
1-4c, $R_{1,\,3\text{-}4}$=H, R_2=Cl, R_5=C_2H_5
1-4d, $R_{1,\,3\text{-}4}$=H, R_2=CH_3, R_5=CH_3
1-4e, $R_{1,\,3\text{-}4}$=H, R_2=OCH_3, R_5=C_2H_5
1-4f, $R_{1\text{-}2,\,4}$=H, R_3=OCH_3, R_5=CH_3
1-4g, $R_{1\text{-}3}$=H, R_4=OCH_3, R_5=C_2H_5
1-4h, $R_{1,\,4}$=H, $R_{2,\,3}$=OCH_3, R_5=CH_3
1-4i, $R_{1,\,3}$=H, $R_{2,\,4}$=OCH_3, R_5=CH_3
1-4j, R_1=H, $R_{2\text{-}4}$=OCH_3, R_5=CH_3
1-4k, R_4=H, $R_{1\text{-}3}$=OCH_3, R_5=C_2H_5

Reagents and conditions. **a**: $ClCH_2CN$, NaH, DMF, rt; **b**: $LiAlH_4$, Et_2O, reflux; **c**: MnO_2, $C_6H_5CH_3$, reflux.

Scheme 1.
Synthesis of compounds **1a–k**.



1a, $R_{1\text{-}4}$=H
1b, $R_{1,\,3\text{-}4}$=H, R_2=F
1c, $R_{1,\,3\text{-}4}$=H, R_2=Cl
1d, $R_{1,\,3\text{-}4}$=H, R_2=CH_3
1e, $R_{1,\,3\text{-}4}$=H, R_2=OCH_3
1f, $R_{1\text{-}2,\,4}$=H, R_3=OCH_3
1g, $R_{1\text{-}3}$=H, R_4=OCH_3
1h, $R_{1,\,4}$=H, $R_{2,\,3}$=OCH_3
1i, $R_{1,\,3}$=H, $R_{2,\,4}$=OCH_3
1j, R_1=H, $R_{2\text{-}4}$=OCH_3
1k, R_4=H, $R_{1\text{-}3}$=OCH_3

Chart 1.
Chemical structures of pyrazino[1,2-*a*]indoles with general structure **1a–k.**

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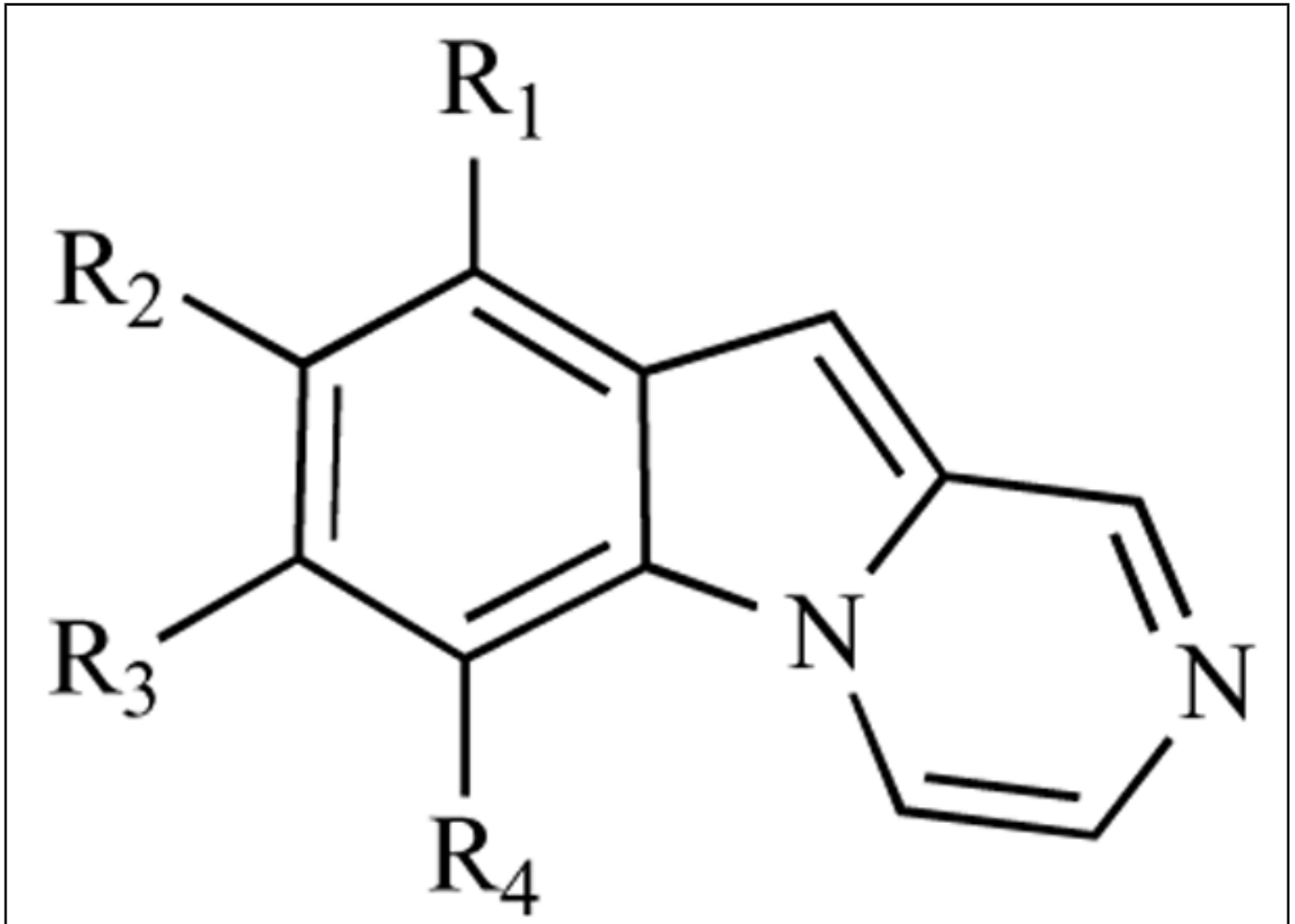
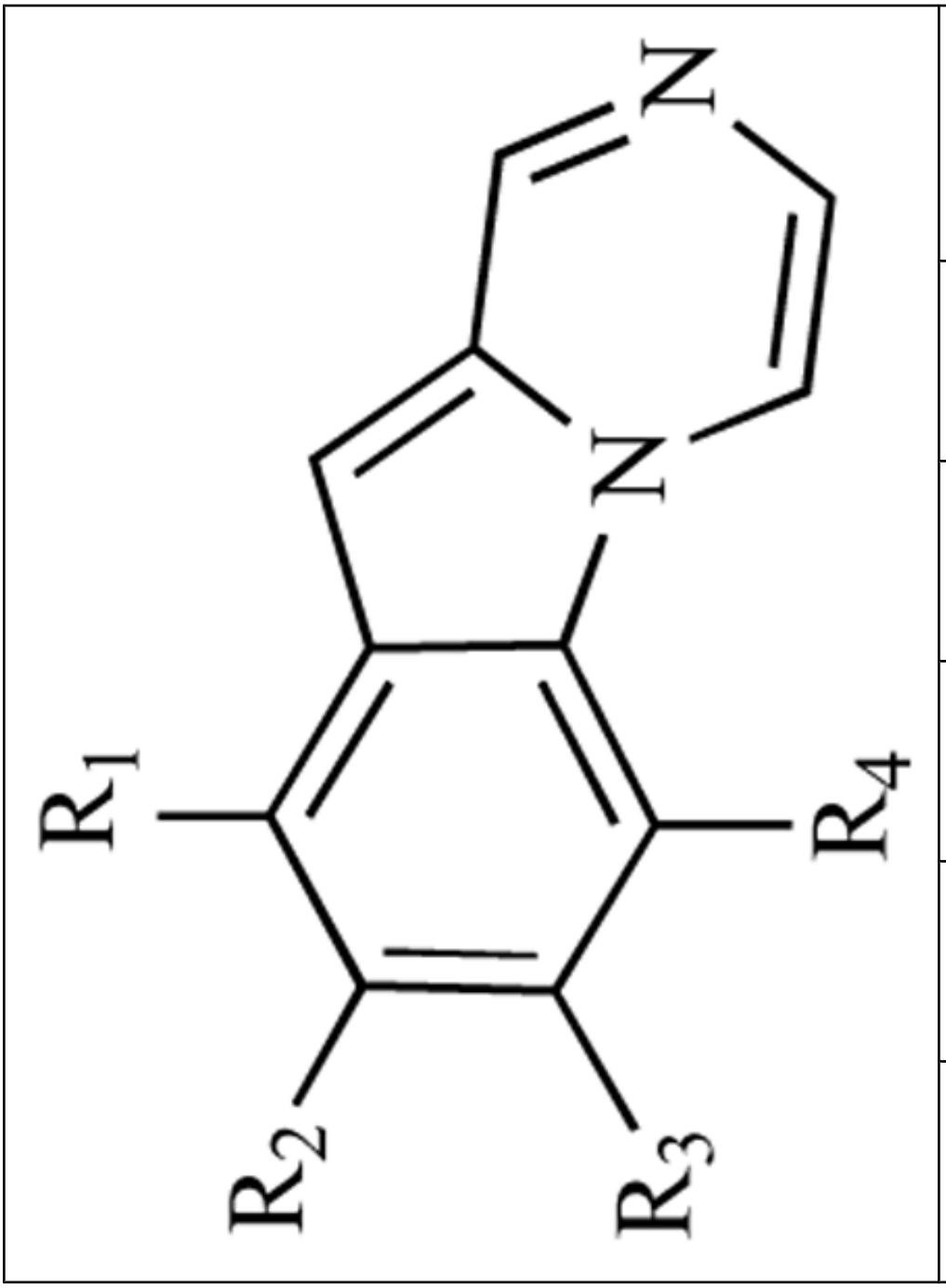
Table 1

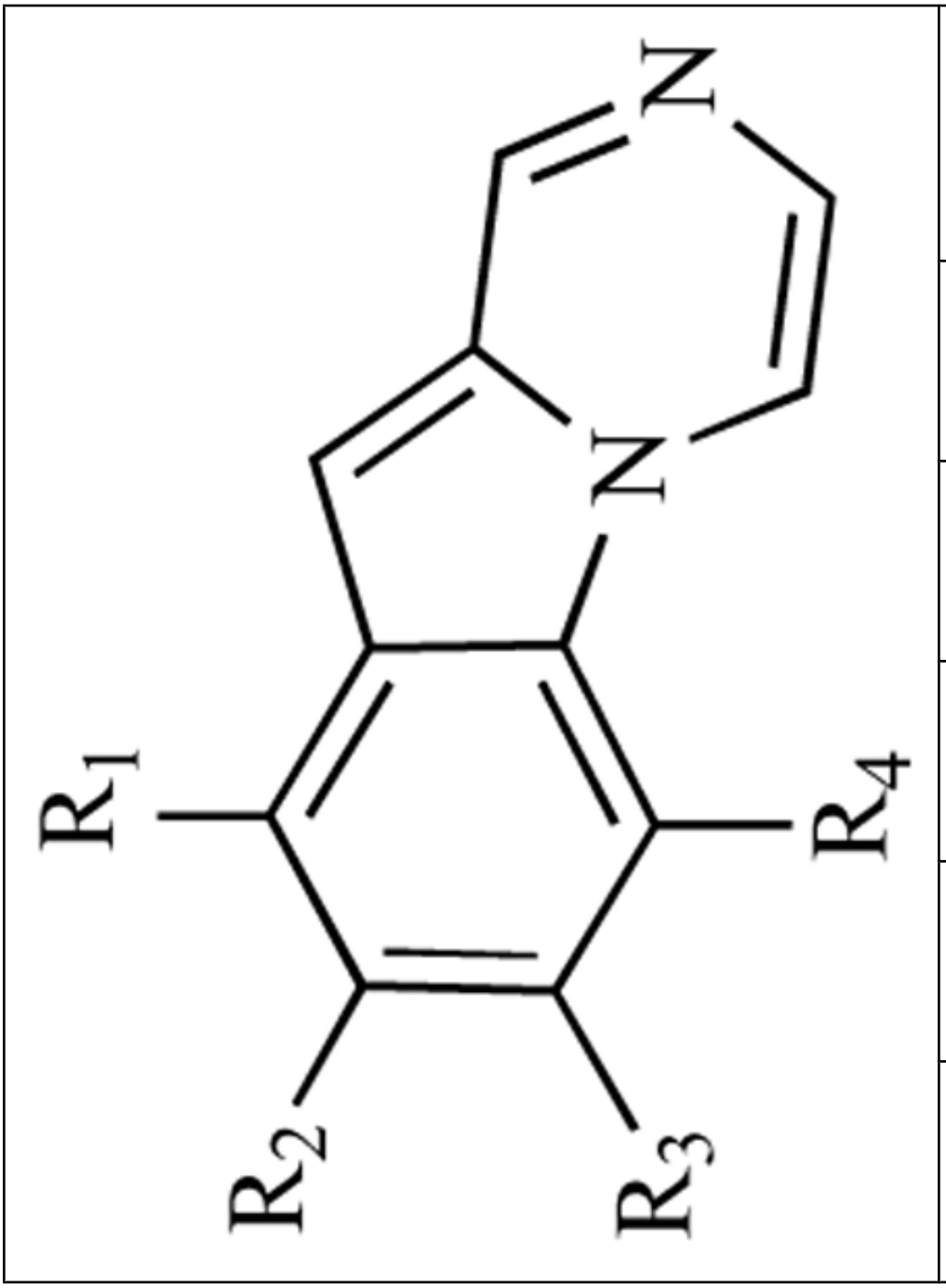
In vitro Inhibitory Effects of Compounds 1a–k Against Proliferation of Human K562 Leukemia Cell Lines



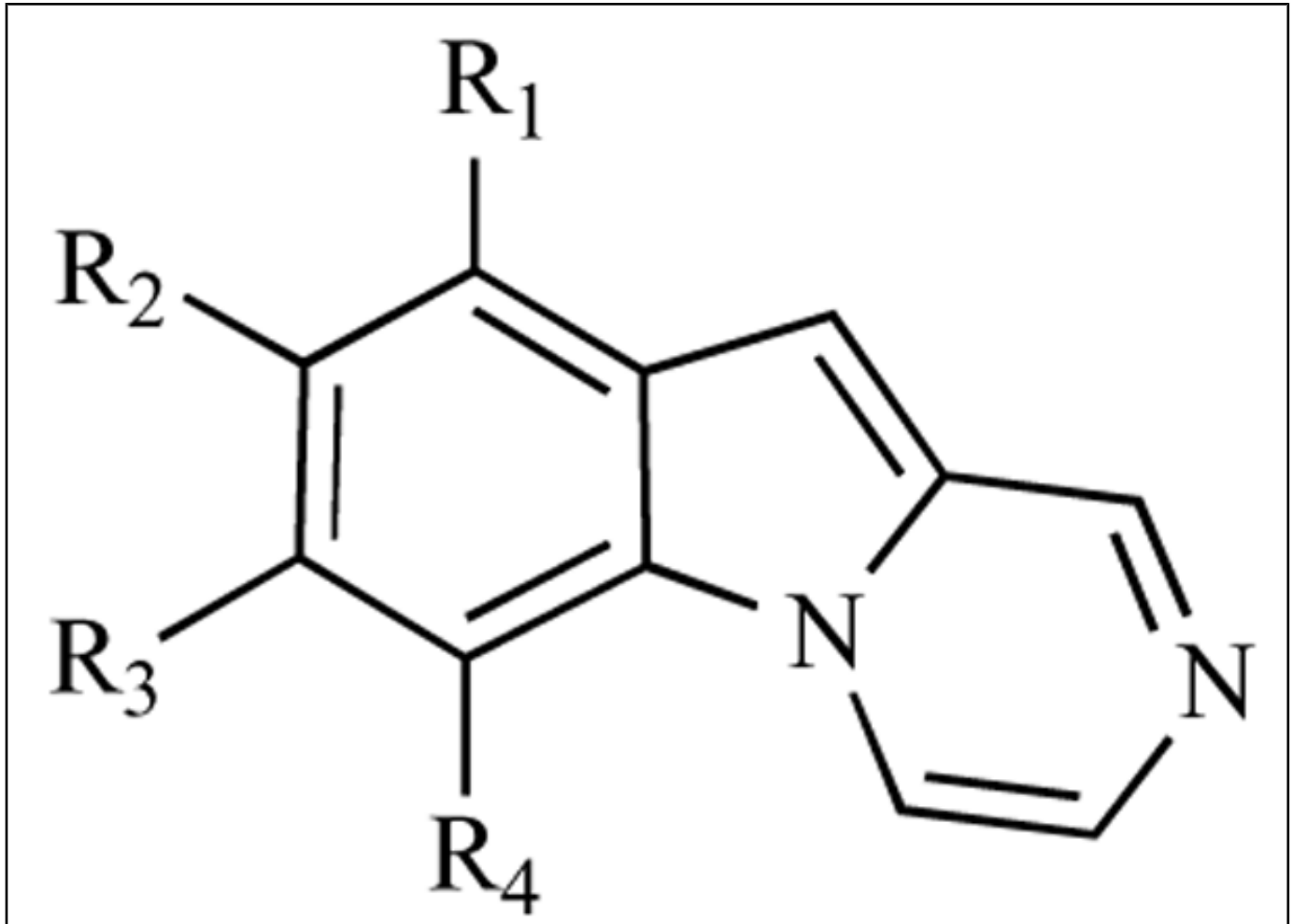
Compd	R_1	R_2	R_3	R_4	[a] IC_{50} (μM)
1a	H	H	H	H	161±12
1b	H	F	H	H	51±3



Compd	R_1	R_2	R_3	R_4	[a] IC_{50} (μM)
1c	H	Cl	H	H	70±3
1d	H	Me	H	H	80±4
1e	H	OMe	H	H	0.07±0.01
1f	H	H	OMe	H	67±3



Compd	R_1	R_2	R_3	R_4	[a] IC_{50} (μM)
1g	H	H	H	OMe	38±3
1h	H	OMe	OMe	H	67±4
1i	H	OMe	H	OMe	54±2
1j	H	OMe	OMe	OMe	83±7



Compd	R_1	R_2	R_3	R_4	[a] IC_{50} (μM)
1k	OMe	OMe	OMe	H	84±5

[a] IC_{50}= compound concentration required to inhibit tumor cell proliferation by 50%. Data are expressed as the mean ± SE from the dose-response curves of at least three independent experiments.

Table 2

Effects of Compounds 1e on Cell Cycle Distribution of Treated K562 Cells

Compd	Cell cycle percentage		
	G0/G1 (%)	S (%)	G2/M (%)
Control	67.8	13.4	18.8
1e	56.4	14.6	29.0