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Relationship Between Structure and Antiproliferative Activity of Novel 5-amino-4-cyanopyrazole-1-formaldehydehydrazono Derivatives on HL-60RG Human Leukemia Cells

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Abstract. *Background: Pyrazole derivatives have been reported to have potent antimicrobial and anticancer activity. We recently synthesized and determined the effects of analogs, benzamidoxime derivatives, on mammalian cells and discovered that benzamidoximes had an antiproliferative effect. Here we synthesized and determined the anticancer effects of hydrazonopyrazole derivatives on a mammalian cancer cell line. Materials and Methods: We synthesized 12 hydrazonopyrazole derivatives with several constant alkyl chain length or branched chains at the side chain to investigate their anticancer cell activity, using the human myelogenous leukemia cell line HL-60RG. Results: Among all hydrazonopyrazole derivatives we synthesized, the hydrazonopyrazole derivative with a branched chain at the side chain rather than a constant alkyl chain significantly inhibited cell viability. The strongest hydrazonopyrazole derivative, 5-amino-4-cyanopyrazole-1-formaldehydehydrazono-3'-pentanal, tended to damage cells dose-dependently. This cell growth attenuation was a result of apoptosis, activating caspase-3 and fragmented DNA. Conclusion: Hydrazonopyrazole derivatives induced apoptosis of HL-60RG leukemia cells.*

Pyrazole derivatives have been reported to have potent antimicrobial activity (1-3). Pyrazoles also effect mammalian cells and have anti-inflammatory and anticancer activity (4-6). These pyrazole derivatives antibiotics have also been reported to possess a broad spectrum of biological activities, not only in microorganisms, but also mammalian cells. Moreover, it was revealed that pyrazole derivatives induce the apoptosis of mammalian cancer cells (4, 7, 8).

Many chemicals have been developed to attack cancer cells. For example, the anticancer effect of some chemicals is to attack DNA, inhibit microtubule function, or block metabolism. However, most drugs induce multidrug resistance in cancer cells (9-12), and the development of new classes of anticancer drugs is, thus, necessary. The modes of action of such novel synthesized drugs should also be determined for a better understanding of the component chemicals and for more effective uses in cancer treatments (13, 14).

Many chemical anticancer drugs induce apoptosis (15). During apoptosis, mainly caspases, which specifically activate proteases, are involved in processing apoptosis. Caspases are classified as "initiator" caspases and "effector" caspases. The activity of initiator caspases (caspase-8 and caspase-9) is provoked by various apoptotic stimuli, *e.g.*, mitochondria damage, UV, and environmental stress (16). Once initiator caspases are activated, they in turn activate the effector caspases (caspase-3, -6, and -7) to execute apoptosis by means of chromatin DNA fragmentation, morphological changes, and cell volume loss.

A caspase inhibitor blocks the above phenomena. However, recent studies revealed that apoptotic cell death is not only caspase-dependent; there is also a caspase-independent pathway. It was reported that pyrazoles also perturb mitochondria directly or indirectly during apoptosis (17, 18).

We recently synthesized and determined the effect of analogs, *i.e.*, benzamidoxime derivatives, on mammalian cells, and discovered that the benzamidoximes had an antiproliferative effect (6). In the present study, we determined the effects of hydrazonopyrazole derivatives on a mammalian leukemia cell line, HL-60RG. We synthesized 12 1-formaldehydehydrazonopyrazole derivatives (19) with a several-constant-alkyl-chain length or branched chains at the side chain, and we studied their anticancer cell effects.

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Key Words: Hydrazonopyrazole, cancer, apoptosis, HL-60RG.

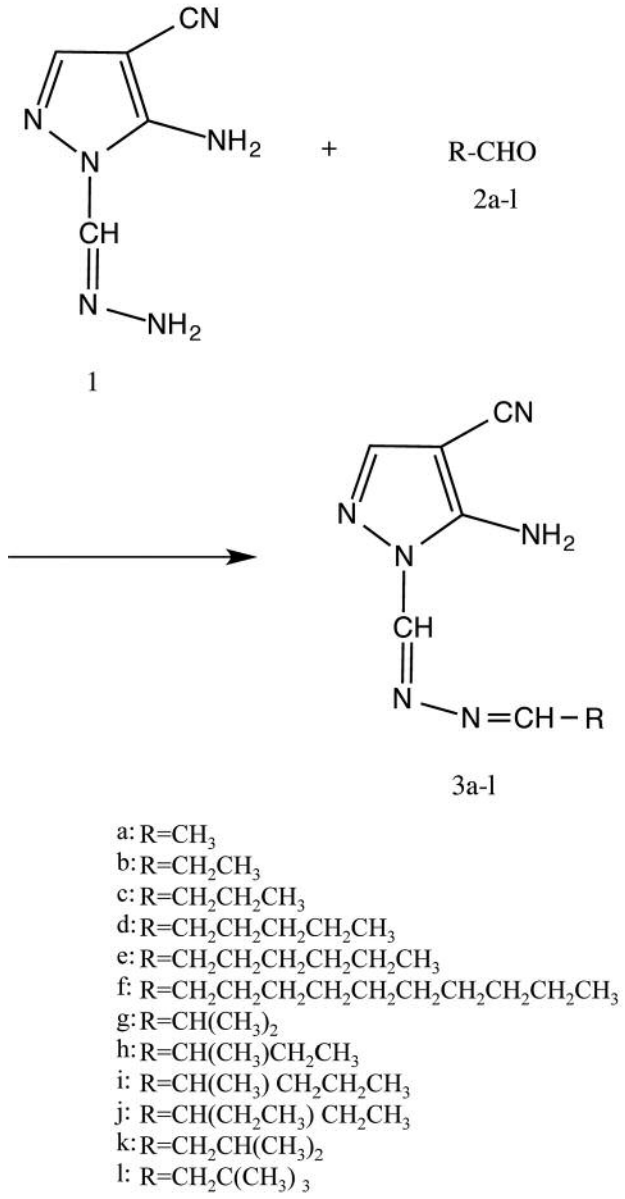

Figure 1. *Chemical structures of the hydrazonopyrazole derivatives.*

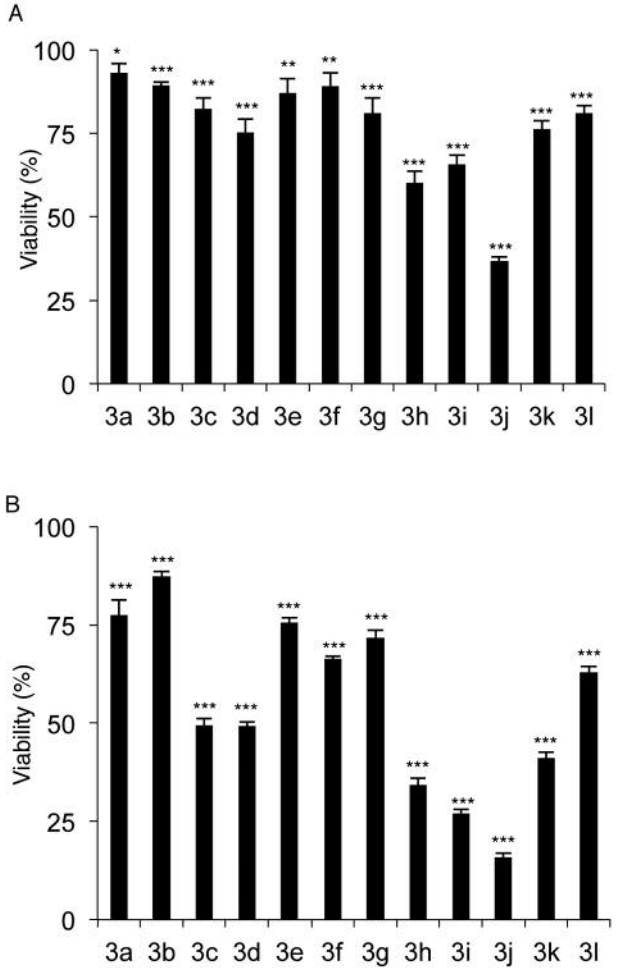

Figure 2. *Hydrazonopyrazole derivatives induced damage in HL-60RG cells. HL-60RG cells were incubated with 100 μM of hydrazonopyrazole derivatives for 24 h (A) or 48 h (B). Cell viability was estimated by MTT assay. The data are presented as a comparison to the untreated control. Each bar denotes the SD (n=4). *p<0.05, **p<0.01, and ***p<0.001 compared to the untreated control groups.*

Materials and Methods

Cells. The human myelogenous leukemia cell line HL-60RG was provided by Human Science Resources Bank (Osaka, Japan).

Reagents. The reaction of 5-amino-4-cyano-1-hydrazinomethyl-pyrazole (**1**) with an equivalent amount of alkylaldehyde (**2a–l**) afforded 5-amino-4-cyanopyrazole-1-formaldehydehydrazono derivatives (**3a–l**), as shown in Figure 1.

Cell culturing. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 75 mg/l kanamycin sulfate, and maintained at 37˚C in a humidified chamber under an atmosphere of 95% air and 5% CO_2.

Cell viability assay. The cells were incubated in 96-well plates at 37˚C with test agents for 23 h or 47 h. Then, 10 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Wako, Osaka, Japan) was added to each well, and the plates were incubated at 37˚C for 1 h. The media were discarded, and 100 μl of dimethyl sulfoxide was added to dissolve MTT formazan. The absorbance of each well was measured using a microplate reader (Awareness Technology, Palm City, FL, USA) at 570 nm. The absorbance of the culture wells without test agents was set as 100%.

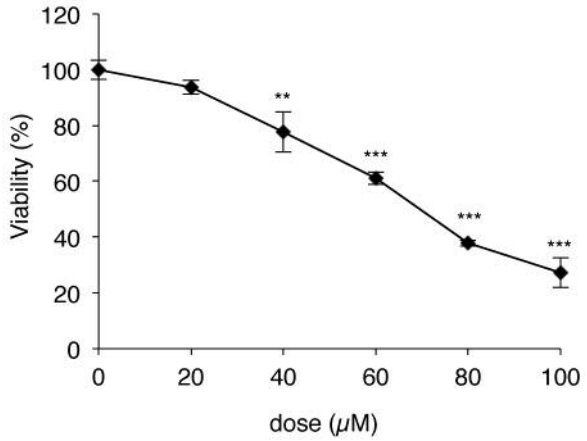

Figure 3. ***3j*** *induced dose-dependent cell damage in HL-60RG cells. HL-60RG cells were incubated with the indicated doses of hydrazonopyrazole derivatives for 24 h. Cell viability was estimated by MTT assay. The data are presented as a comparison to the untreated control. Each bar denotes the SD (n=4). **p<0.01 and ***p<0.001 compared to the untreated control groups.*

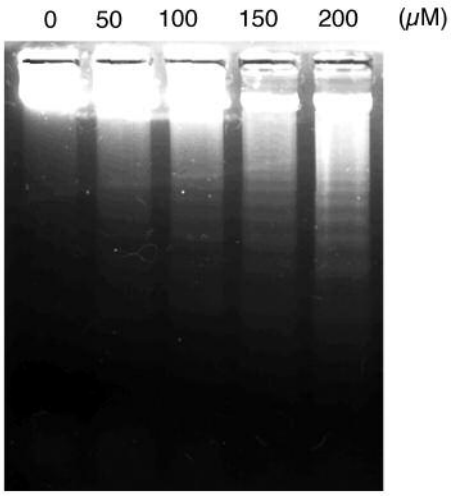

Figure 4. ***3j*** *fragmented genomic DNA. HL-60RG cells were incubated with the indicated doses of* ***3j*** *for 24 h. The cells were then lysed and DNA was prepared. DNA fragmentation was analyzed by agarose gel electrophoresis. Data are representative of three independent experiments.*

Agarose gel electrophoresis. Apoptosis was determined by DNA fragmentation, which was assessed by agarose gel electrophoresis. Cells (1×10^6) were rinsed once with 10 mM Tris-HCl buffer, pH 8.7, containing 3 mM $MgCl_2$ and 2 mM 2-mercaptoethanol and were dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 10 mM EDTA, 0.5% sodium lauryl sarcocinate, and 1 mg/ml proteinase K. After incubation at 50˚C for 30 min, RNase A was added at a concentration of 0.5 mg/ml and further incubated at 50˚C for 15 min. Lysates were mixed with an equal volume of loading buffer containing TBE buffer (89 mM Tris, pH 8.4, 2.5 mM EDTA, 89 mM boric acid), 20% glycerol, and 0.01% bromophenol blue. Samples were electrophoresed on 1.8% agarose gels in TBE containing 0.5 mg/ml ethidium bromide.

Western blotting. Cells were washed with phosphate-buffered saline (PBS) and placed on ice for 20 min in lysis buffer: 50 mM HEPES-NaOH, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% proteinase inhibitor cocktail (Sigma, St. Louis, MO), pH 7.5, 0.1 mM sodium orthovanadate. Cell lysates were centrifuged at 4˚C for 15 min at 13,000 × *g*. The protein concentrations of the supernatant were determined using a Bicinchoninic Acid protein assay (Thermo Scientific, Waltham, MA, USA). Cell lysates (30 μg) were mixed in the same volume of sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 125 mM Tris, pH 6.8, 10% glycerol, 0.02 mg/ml bromophenol blue, 10% 2-mercaptoethanol) and heated at 100˚C for 3 min. Proteins were separated by 15% polyacrylamide gel SDS-electrophoresis and electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After the membrane was blocked with use of 3% skimmed milk, caspase-3 and β-actin were immunodetected using specific antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). Thereafter, horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) was applied as the second antibody, and positive bands were detected by enhanced chemiluminescence (Thermo Scientific). Visualization was done with an Image Quant LAS-4000 (GE Healthcare, Buckinghamshire, UK).

Statistical analysis. All statistical analyses were performed using Student's *t*-test. Significance was established at the $p<0.05$ level.

Results and Discussion

Hydrazonopyrazole derivatives induced the cell damage of leukemia cells. We synthesized 12 hydrazonopyrazole derivatives which were then used throughout this study (Figure 1). Compounds **3a–f** were hydrazonopyrazoles with a straight chain at the side chain, and compounds **3g–l** were hydrazonopyrazoles with a branched chain at the side chain. To estimate the potency of hydrazonopyrazole derivatives in mammalian cells, we performed an MTT assay to determine the extent of cell injury in HL-60RG leukemia cells. Synthesized hydrazonopyrazole derivatives were incubated with 100 μM for 24 h on HL-60RG cells. Among our newly-synthesized compounds, the hydrazonopyrazole with a branched chain at the side chain, that is, 5-amino-4-cyanopyrazole-1-formaldehydehydrazono-3'-pentanal, **3j**, was the most effective compound (Figure 2A).

Moreover, the longer treatment time (48 h) of the compounds resulted in a more damaging effect on HL-60RG cells, which suggests that hydrazonopyrazoles with a branched chain at the side chain compounds tended to induce cell damage compared to the compounds with a straight chain

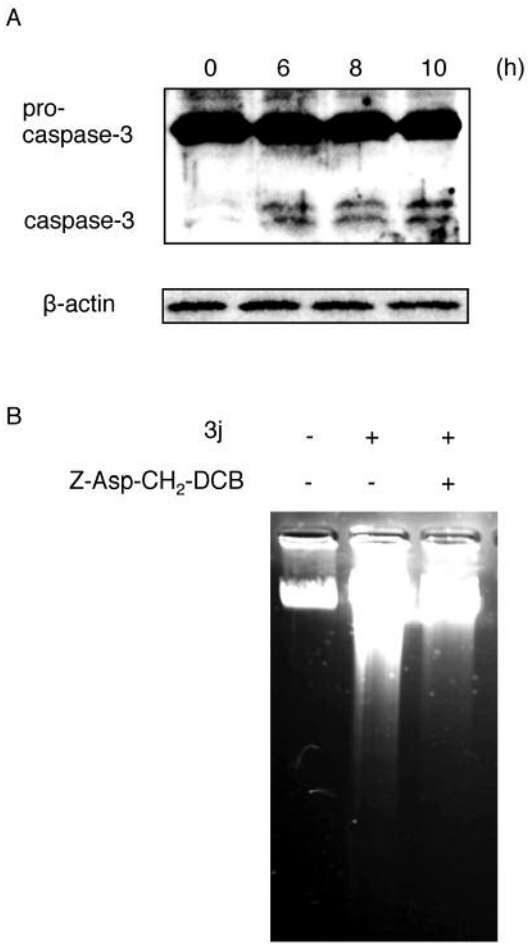

Figure 5. *Caspase-dependent apoptosis induced by **3j** in HL-60RG cells. A: HL-60RG cells were incubated with 100 μM **3j** for the indicated times. The cells were then lysed, and caspase-3 was detected by western blotting. Data are representative of three independent experiments. B: HL-60RG cells were incubated with or without 40 μM Z-Asp-CH_2-DCB, then 100 μM **3j** for 24 h. The cells were then lysed and DNA was prepared. DNA fragmentation was analyzed by agarose gel electrophoresis. Data are representative of three independent experiments.*

at the side chain, in a time-dependent manner (Figure 2B). Moreover, our newly-synthesized compounds with an alkyl chain with 4-6 carbon atoms had a strong cell-damaging effect, and the compounds with shorter or longer than 4-6 carbon atoms had less toxicity. These results suggest that a bulky substituent, not the electrical charge, is important for cell damage and that cell membrane permeability would affect the compounds' toxicity because too long a straight chain may decrease the membrane permeability.

In order to determine the half-maximal inhibitory concentration (IC_{50}) of the most effective compound, **3j**, we treated HL-60RG cells with 0-100 μM **3j** for 24 h. The estimated IC_{50} dose of 3j was approximately 73 μM (Figure 3). Taken together, these results indicate that hydrazonopyrazole derivatives induce cell damage in HL-60RG cells and that the branched chain at the side chain increases the derivatives' potency.

Hydrazonopyrazole derivatives induced apoptosis of HL-60RG cells with caspase activation. With the above findings we further examined how this cell damage effect occurred in response to hydrazonopyrazole treatment, using the drug-susceptible cell line HL-60RG. First, we assayed DNA fragmentation, which is a hallmark of apoptosis. As shown in Figure 4, doses over 100 μM of **3j** fragmented DNA in a 24-h treatment. Moreover, one of the effector caspases, *i.e.*, caspase-3 was detected by western blotting. As shown is Figure 5A, pro-caspase-3 was decreased and cleaved to an active form by 100 μM **3j** in a <6-h treatment, suggesting that **3j** induced apoptosis with the activation of caspase-3. Moreover, the pan-caspase inhibitor Z-Asp-CH_2-DCB was administered prior to the incubation with 100 μM **3j**. The Z-Asp-CH_2-DCB treatment blocked DNA fragmentation, suggesting that the **3j**-induced apoptosis was caspase-dependent. Many chemical anticancer drugs induce apoptosis by provoking mitochondria (15). Damaged mitochondria release cytochrome *c*. The released cytochrome *c* then binds to Apaf-1 and activates one of the initiator caspase, caspase-9, with formation of apoptosome. Activated caspase-9 further activates effector caspase, caspase-3. Caspase-3 then execute apoptotic cell death. Compound **3j** might induce apoptosis by above-mentioned pathway. Further studies are warranted to investigate the precise mechanism of apoptosis induction by **3j**.

Overall, our findings indicate that the novel synthesized compounds are promising drugs that may be used to damage cancer cells at very low treatment doses.

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