N-(2-Amino-5-chlorobenzoyl)benzamidoxime Derivatives Inhibit Human Leukemia Cell Growth

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Abstract. *Background/Aim: Amidoxime derivatives have been previously reported to have potent anti-microbial and anti-tumor activity. Little is known about the tumor cell growth-inhibition mechanism of amidoximes, especially benzamidoxime derivatives. Herein we determined the effects of N-(2-amino-5-chlorobenzoyl)benzamidoxime analogs on mammalian cancer cells. Materials and Methods: We synthesized four chloride-substituted benzamidoxime analogs from the original benzamidoxime to investigate their anticancer cell activity using the Jurkat T-cell lymphoma cell line and the human leukemia cell line HL-60RG. Results: All amidoxime derivatives inhibited Jurkat and HL-60RG cell viability dose-dependently. Benzamidoximes tended to damage HL-60RG cells to a greater extent compared to Jurkat cells. Benzamidoximes with chloride substitutes caused a strong decrease in cell growth, and this cell growth attenuation was transient at 5 μM (below the half-maximal inhibitory concentration, IC_{50}) but long-lasting at 10 μM (greater than the IC_{50}). Conclusion: Benzamidoxime derivatives caused a transient cell-cycle delay at a low dose and cell death at a high dose.*

Various chemicals with distinct cellular targets have been developed to attack cancer cells. For example, 5-deoxyuridine inhibits RNA synthesis and function, blocks thymidylate synthase activity, and is incorporated into DNA, leading to DNA strand breaks (1). The taxanes paclitaxel and docetaxel interfere with microtubule function, leading to altered mitosis and cellular death (2). However, most drugs induce multi-drug resistance in cancer cells (3-5), and the development of new classes of anticancer drugs is necessary. The modes of action of such drugs should also be determined for a better understanding of the component chemicals and for more effective and safe use of these drugs to treat cancer (6). Indeed, combination cancer chemotherapy can work when tumor-prevention mechanisms that are different from those of pre-existing drugs are developed (7, 8).

It has been reported that amidoxime derivatives have potent antimicrobial activity, including activity against African trypanosomes, pneumocystis, and *Aspergillus* (9, 10). Amidoximes also have an antitumor activity (11, 12). Benzene-substituted amidoximes, *i.e.*, benzamidoxime derivatives, have been reported to have a genotoxic effect on mammalian cells (13), suggesting that these analogs could also have a potent effect on mammalian tumor cells. However, little is known about the mechanism of mammalian cell growth inhibition by benzamidoxime derivatives.

In the present study, we determined the effects of *N*-(2-aminobenzoyl)benzamidoxime analogs on mammalian cells. The reaction of 5-chloroisatoic anhydride (Ib) with benzamidoxime analogs (IIa-d) afforded *N*-(2-amino-5-chlorobenzoyl)benzamidoxime derivatives (IIIb-e). We synthesized four chloride-substituted benzamidoximes analogs from the original benzamidoxime (IIIa), because chlorinated benzenes cause oxidative stress and apoptosis in mammalian cells (14, 15), and studied their anticancer cell effects on Jurkat and HL-60RG cells.

Materials and Methods

Cells. Jurkat cells, a human T-cell lymphoma cell line, were provided by Dr. T. Miyashita of Kitasato University (Kanagawa, Japan). The human myelogenous leukemia cell line HL-60RG was provided by the Human Science Research Resources Bank (Osaka, Japan).

Reagents. The synthesis of these compounds (IIIa-e) was considered in reference to the amidoxime derivatives method of Nagahara *et al.* (16, 17), shown in Figure 1.

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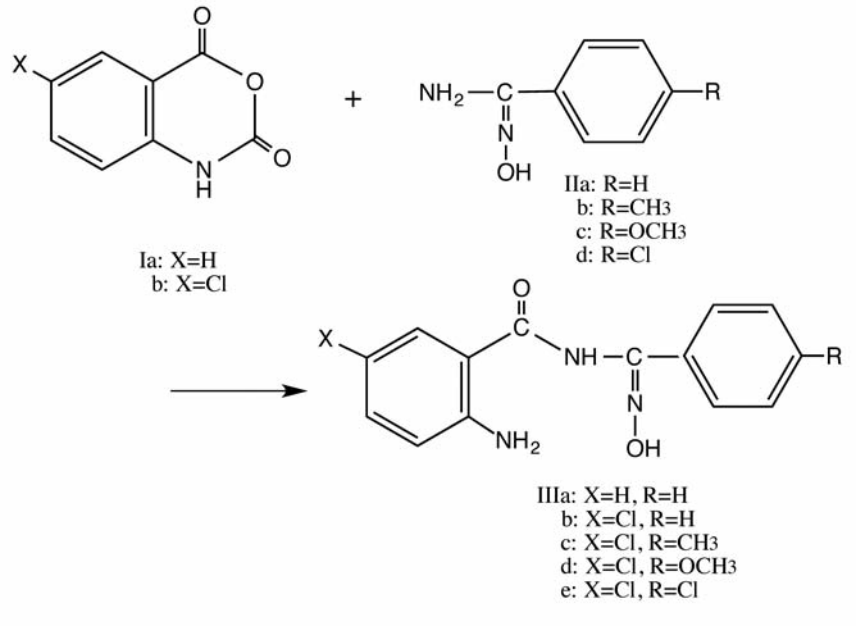

Figure 1. *Chemical structures of the benzamidoxime derivatives.*

Medium and cell cultures. 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. 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 culture wells without test agents was set as 100%.

Cell growth count. Cells were incubated with or without (control) compound IIIc at 37˚C for 24 h or 48 h. At each time point, the number of cells was counted under phase-contrast microscopy.

Assessment of DNA content. Cells (1×10^6) were washed with phosphate-buffered saline (PBS) and suspended in permeabilizing buffer (0.1% Triton-X 100 in PBS). Next, 0.5 mg/ml RNase A and 2 μg/ml propidium iodide were added and flow cytometric analysis was conducted (FACS Calibur; Becton Dickinson, Mountain View, CA, USA). Data were analyzed using Cell Quest (Becton Dickinson).

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

Results

N-(2-aminobenzoyl)benzamidoxime derivatives induced cell damage in leukemia cells. We synthesized five benzamidoxime derivatives which were used throughout this study. Compound IIIa, which was the original compound, was substituted with a chloride atom (compounds IIIb, IIIc, IIId, IIIe). To estimate the potency of benzamidoxime derivatives in mammalian cells, we performed an MTT assay to determine the extent of cell injury in the Jurkat and HL-60RG cells. Synthesized benzamidoxime

Table I. *Viability of Jurkat cells at 10 μM and half maximal inhibitory concentration (IC_{50}) of benzamidoximes.*

Compound	Viability at 10 μM (%)*	IC_{50} (μM)
IIIa	106	65
IIIb	68	37
IIIc	55	19
IIId	66	34
IIIe	43	9.1

*Relative to the untreated control.

Table II. *Viability of HL-60RG cells at 10 μM and half maximal inhibitory concentration (IC_{50}) of benzamidoximes.*

Compound	Viability at 10 μM (%)*	IC_{50} (μM)
IIIa	108	59
IIIb	36	8.4
IIIc	19	6.9
IIId	27	7.2
IIIe	23	7.5

*Relative to the untreated control.

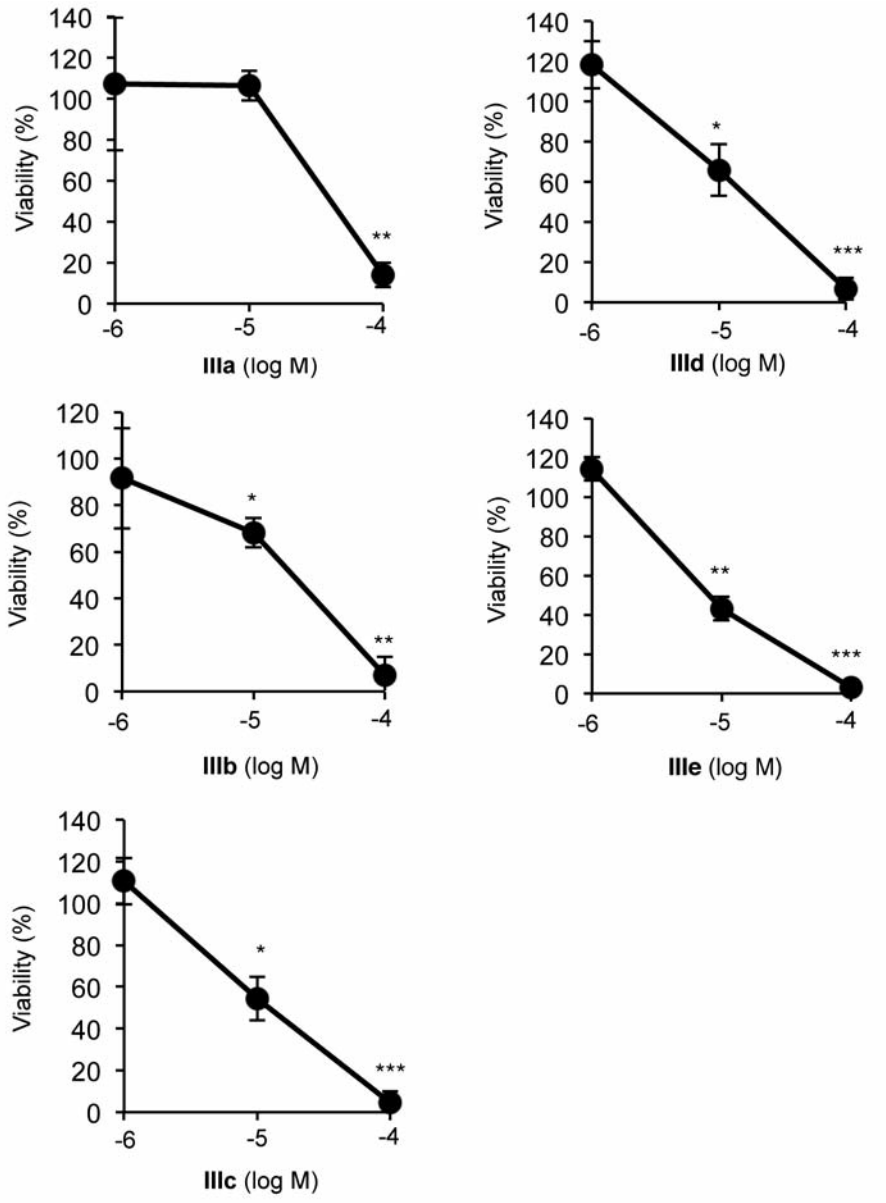

Figure 2. *Benzamidoximes induced damage in Jurkat cells. Jurkat cells were incubated with the indicated doses of benzamidoximes for 24 h. Cell viability was estimated by an MTT assay. Data are presented as a comparison to the untreated control. Each bar denotes the standard deviation (SD; n=4). *p<0.05, **p<0.01, ***p<0.001 compared to the untreated control group.*

derivatives were incubated with a dose of 10 μM for 24 h on Jurkat and HL-60RG cells. At 10 μM, compound IIIa treatment did not damage Jurkat cells or HL-60RG cells (Tables I and II). Surprisingly, treatment with benzamidoxime with chloride substitutes resulted in significant damage to both cell lines.

In order to determine the half-maximal inhibitory concentration (IC_{50}), we treated both cell lines with 1-100 μM benzamidoxime derivatives (Figures 2 and 3). All of benzamidoxime derivatives inhibited Jurkat and HL-60RG cell viability dose-dependently. In addition, the benzamidoximes with chloride substitutes caused a strong decrease in the growth of Jurkat and HL-60RG cells. The estimated IC_{50} doses of compound IIIa (no chloride) and compound IIIc (chloride-substituted) are 65 μM and 19 μM, respectively, for Jurkat cells (Table I) and 59 μM and 6.9 μM, respectively, for HL-60RG cells (Table II). Taken together, these results indicate that benzamidoxime derivatives induce cell damage in leukemia cells and that chloride substitution increases their potency.

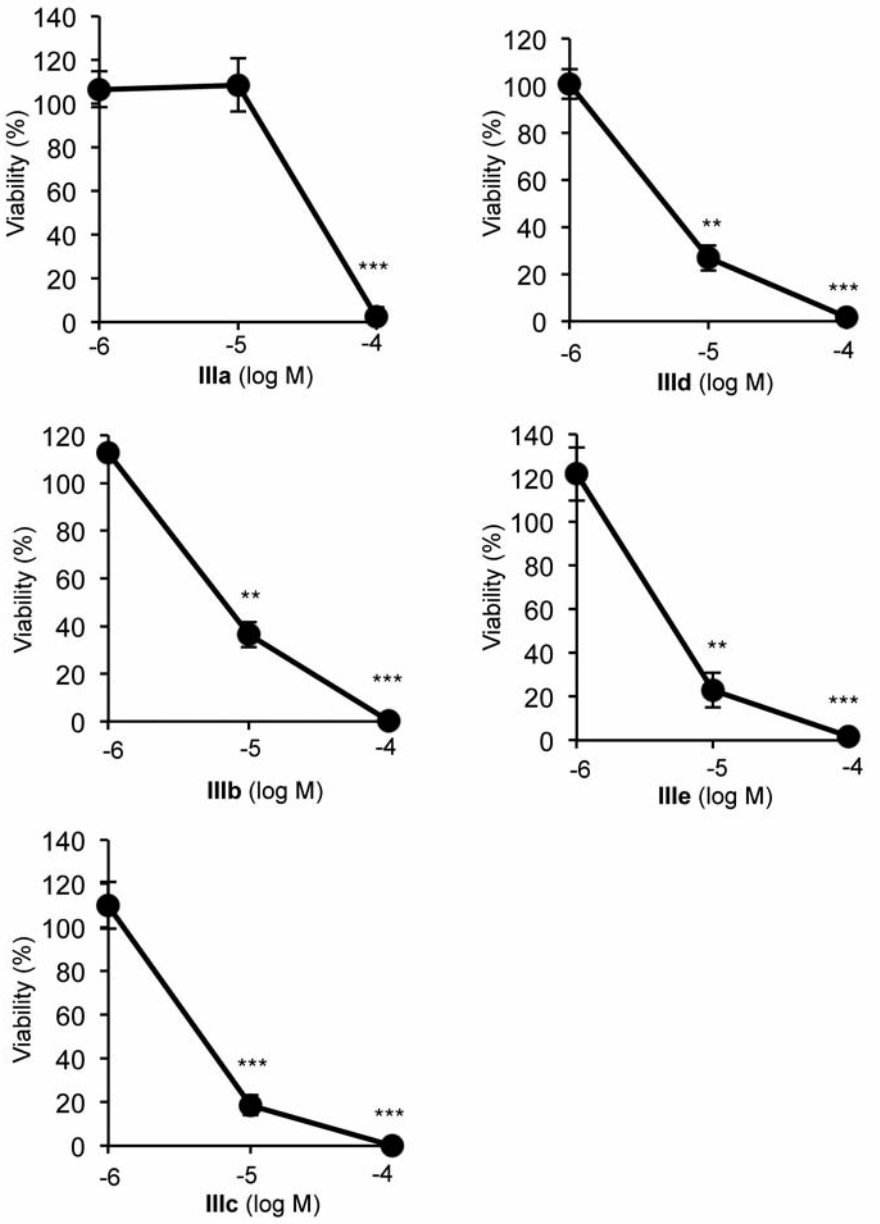

Figure 3. *Benzamidoximes induced damage in HL-60RG cells. HL-60RG cells were incubated with the indicated doses of benzamidoximes for 24 h. Cell viability was assessed by the MTT assay. The data are presented as a comparison to the untreated control. Each bar denotes the SD (n=4). **p<0.01 and ***p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

N-(2-Aminobenzoyl)benzamidoxime derivatives induced cell growth delay and death of HL-60RG cells. With the above findings we further examined how this cell damage effect occurred in response to benzamidoxime treatment, using the drug-susceptible HL-60RG cell line. Firstly, we counted the number of HL-60RG cells during their treatment with the most effective synthesized compound, IIIc at around the IC_{50} doses (approximately 7 μM), 5 and 10 μM. Compound IIIc treatment inhibited HL-60RG cell growth compared to control cells (Figure 4). The 24-h treatment with IIIc at 5 μM inhibited cell growth, as the cell number was slightly increased compared to the start of the incubation (0-h treatment); however, inhibition of cell growth was transient and cell growth began to increase upon 48-h treatment. In contrast, 10 μM of IIIc clearly reduced the cell number in 24-h treatment, and the cell number dropped further upon 48-h treatment.

We also explored the type of cell injury of HL-60RG cells responding to benzamidoximes, by measuring the DNA

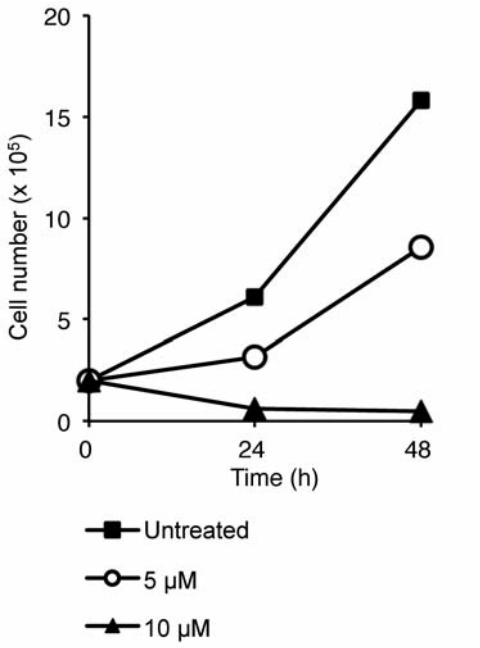

Figure 4. *Compound IIIc attenuated cell growth. HL-60RG cells were incubated with 5 μM or 10 μM compound IIIc for the indicated times. The cell number was counted by phase-contrast microscopy.*

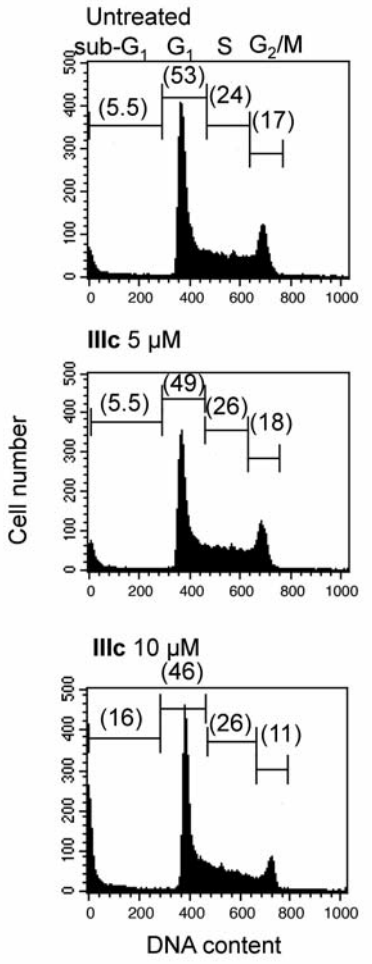

Figure 5. *Compound IIIc inhibited HL-60RG cell proliferation by increasing the proportion of sub-G_1 cells. HL-60RG cells were incubated with 5 μM or 10 μM of compound IIIc for 24 h. The cells were assayed flow cytometrically as described in the Materials and Methods. Data are representative of three independent experiments. The numbers in parentheses indicate the percentages of sub-G_1, G_1, S, and G_2/M phase cells as those of the total.*

content by flow cytometry. As shown in the histogram of DNA content in Figure 5, treatment with compound IIIc induced little change of the cell-cycle phase rate, and the subset of sub-G_1 cells also seemed to be unchanged at the 5 μM dose, but the 10-μM dose of IIIc induced a significant increase of sub-G_1 cells, that is, fragmented DNA (Figure 5).

Overall, these results indicate that compound IIIc caused inhibition of the growth of HL-60RG cells as a result of a transient cell-cycle delay at the low dose (below the IC_{50} dose) and as a result of cell death induction at the higher dose (greater than the IC_{50} dose).

Discussion

In the present study, we synthesized four chloride-substituted benzamidoxime analogs from the original benzamidoxime, compound IIIa, to determine their anticancer effects on human leukemia Jurkat and HL-60RG cells. All the benzamidoxime derivatives inhibited Jurkat and HL-60RG cell viability dose-dependently. We also observed that treatment with the benzamidoximes with chloride substitutes markedly inhibited Jurkat and HL-60RG cell growth. We further examined the mechanism underlying the damage induced by compound IIIc of drug-susceptible HL-60RG cells, and our findings suggest that the inhibition of the growth of HL-60RG cells by compound IIIc was the result of a transient cell-cycle delay at the low dose (below the IC_{50} dose) and the result of cell death induction at the high dose (above the IC_{50} dose).

Our newly-synthesized compounds have two rings, and chloride was substituted in both rings. When chloride was substituted to benzamide at the C-5 position, the cell damage effect was generally activated. We also compared the cell inhibition induced by substituting the functional group at the C-4 position. Methyl-, methoxyl-, and chloride substitutions reduced the cell growth of Jurkat and HL-60RG cells compared to the unsubstituted compound. These results suggest that a bulky substituent, not the electrical charge, is important for cell damage.

Other synthesized chloride benzenes induce apoptosis of tumor cells (15). Our newly-synthesized compounds induced a strong cell growth-inhibition effect. This effect was a result of transient cell-cycle delay at the under-IC_{50} dose and cell death induction at the over-IC_{50} dose. We performed a time-course MTT assay with 5 μM compound IIIc and found that the 48-h treatment with compound IIIc did not significantly inhibit cell viability compared to 24-h treatment (the time point of Figure 3 data) (data not shown), similar to the cell growth data shown in Figure 4, suggesting that compound IIIc could have been degraded or been bound by a component in the medium.

Consistently, when 5 μM of compound IIIc were added to the medium for 24-h and this medium was used to incubate HL-60RG cells for 24-h, cell viability was only weakly inhibited (data not shown). We also observed that higher doses of compound IIIc caused sub-G_1 DNA fragmentation, which seemed to induce apoptosis at treatment over 10 μM.

Further studies are warranted to investigate the precise mechanism of apoptosis induction by compound IIIc. Overall, novel synthesized compounds are promising drugs that may be used to damage cancer cells at very low treatment doses.

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