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2,2′-Pyridoin derivatives protect HL-60 cells against oxidative stress

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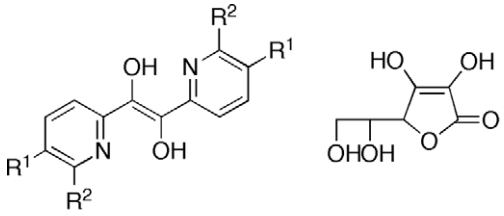
ABSTRACT

Focusing on 2,2′-pyridoin (**1**, 1,2-di(2-pyridyl)-1,2-ethenediol) and its synthetic derivatives as the lead compound of the potent antioxidative enediol, their protective effect against oxidative stress was evaluated on the HL-60 cell system. 2,2′-Pyridoins showed no remarkable cytotoxic effect on HL-60 cells. The derivatives **1**, **2**, **3**, **5**, and **6** inhibited H_2O_2-induced cell death and intracellular oxidative stress more significantly than ascorbic acid. Since 2,2′-pyridoins are oxidized to the diketones, 2,2′-pyridils, in a protic solvent, the antioxidant activity of 2,2′-pyridils was also investigated. 2,2′-Pyridils showed antioxidant activity in the cell; however, the activity was lower than that of 2,2′-pyridoins. These results suggested that 2,2′-pyrdoin derivatives can be good cytoprotective agents against oxidative stress.

Reactive oxygen species (ROS) and free radicals are considered to be implicated in a variety of pathological events, such as cancer and aging.[1–3] ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical, are thought to be generated by subsequent reduction of molecular oxygen in aerobic organisms.[4,5] Under normal conditions, cells and tissues are protected against ROS by an array of defense systems of enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase or free radical scavengers.[6] Among these radical scavengers, ascorbic acid (AsA, Fig. 1) shows very effective activity[7] and has often served as a lead compound for the design and synthesis of pharmacologically effective antioxidants.[8]

In our previous study, we elucidated that 2,2′-pyridoin (**1**, Fig. 1), the enediol compound possessing two pyridine rings, and



AsA

1: $R^1 = H$, $R^2 = H$
2: $R^1 = H$, $R^2 = CH_3$
3: $R^1 = H$, $R^2 = OCH_3$
4: $R^1 = H$, $R^2 = COCH_3$
5: $R^1 = CH_3$, $R^2 = H$
6: $R^1 = OCH_3$, $R^2 = H$
7: $R^1 = COCH_3$, $R^2 = H$

Figure 1. Structures of 2,2′-pyridoin derivatives and AsA.

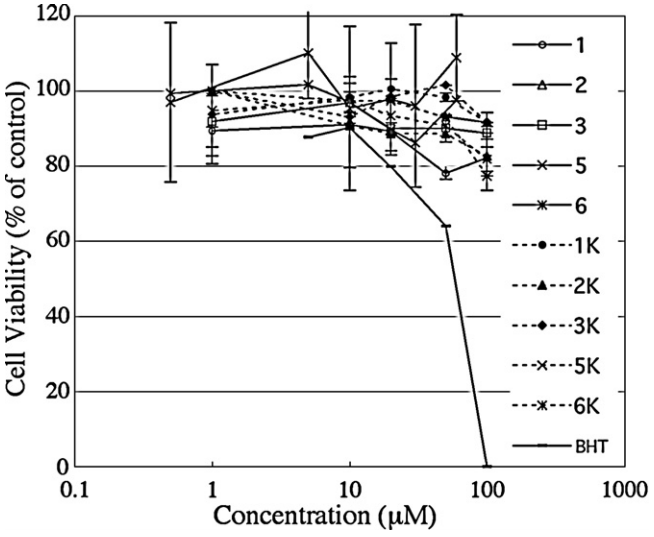


Figure 2. Cytotoxicity of 2,2′-pyridoins and of 2,2′-pyridils. Data are means ± SD of three independent experiments.

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its synthetic derivatives **2**–**7** exerted DPPH (1,1′-diphenyl-2-picrylhydrazil) radical scavenging activity. We also elucidated that **3**, **5**, and **6** inhibited lipid peroxidation more strongly than AsA and that the inhibitory activity of **1** and **2** was comparable to that of AsA but that of **4** and **7** was lower than that of AsA.[9] Therefore, it was assumed that 2,2′-pyridoin derivatives were pharmacologically useful lead compound of antioxidants.

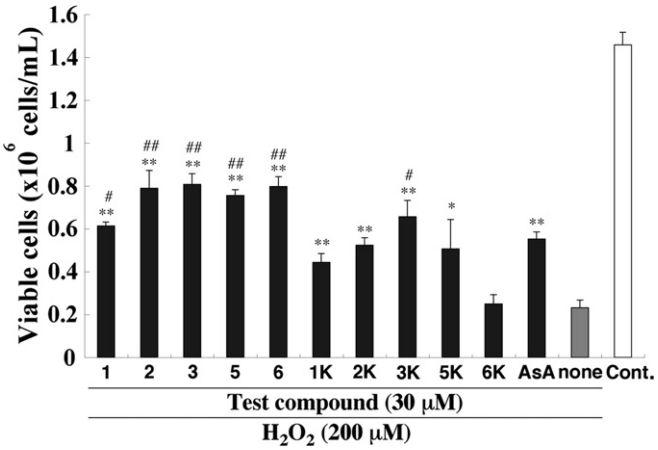


Figure 3. Inhibitory effect of 2,2′-pyridoin derivatives on H_2O_2-induced cell death. Data are means ± SD of 3–6 independent experiments ($^{**}p < 0.01$ vs none, $^{\#}p < 0.05$ vs AsA, $^{\#\#}p < 0.01$ vs AsA, Student's *t*-test).

In the present study, we selected **1**, **2**, **3**, **5**, and **6** as candidates of excellent antioxidants and investigated the protective effect of the 2,2′-pyridoin derivatives against oxidative stress under physiological condition using human promyeloid leukemia cell lines (HL-60). HL-60 cells are commonly used in ROS-mediated studies because they can produce large amounts of ROS following stimulation.[10] Hence, we selected the HL-60 cell line for the antioxidant assay in the present study.

Prior to the antioxidant assay, HL-60 cells were exposed to 2,2'-pyridoins for 24 h, and the cytotoxicity of 2,2′-pyridoins was checked.[11] Since the solubility of **5** and **6** in the incubation medium was not particularly good, the highest concentration of **5** and **6** was set to 60 μM, and that of the other 2,2′-pyridoins was set to 100 μM. As shown in Figure 2, 60–100 μM of 2,2′-pyridoins did not cause a remarkable decrease in the cell viability. In contrast, butylated hydroxytoluene (BHT), a synthetic phenolic antioxidant, exerted potent cytotoxicity at 100 μM. Therefore, it was confirmed that up to 60 μM of 2,2′-pyridoin and its derivatives has no cytotoxicity for HL-60 cells.

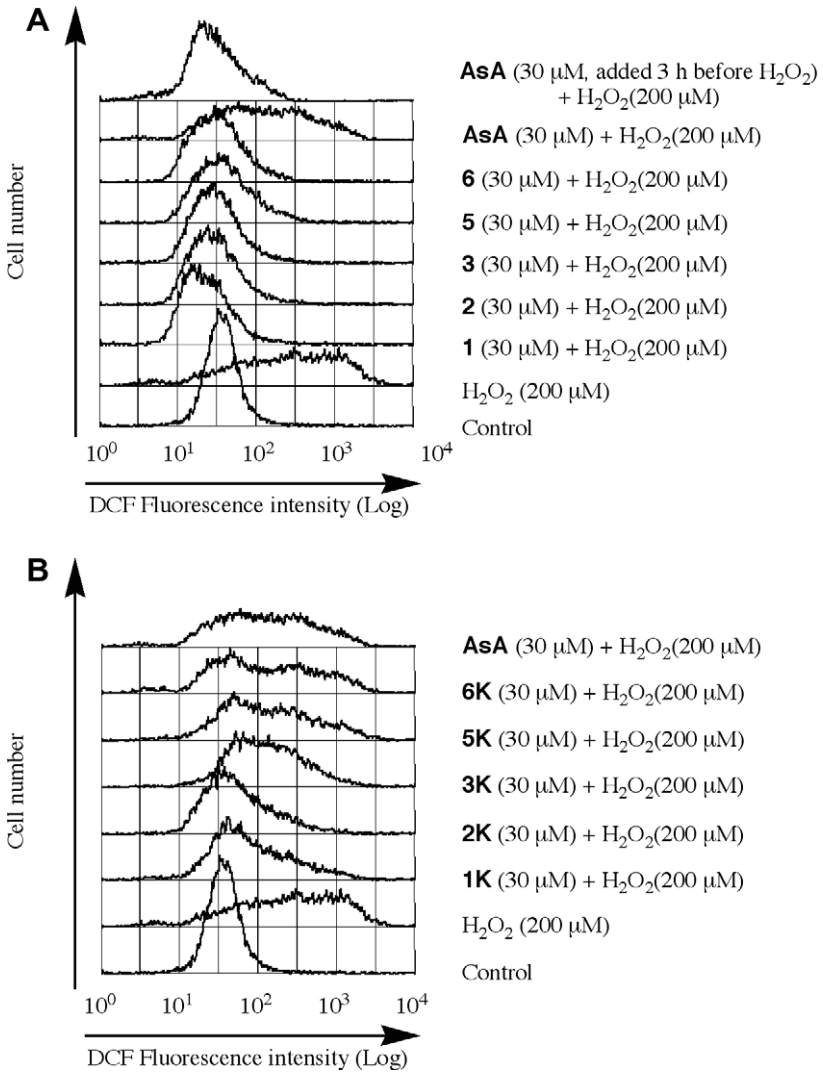


Figure 4. Scavenging effect on intracellular ROS. ((A) 2,2′-pyridoins, (B) 2,2′-pyridils.)

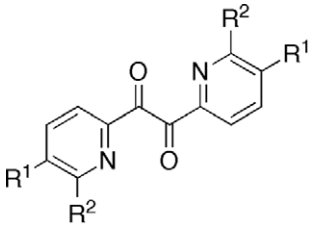
To investigate the antioxidant activity in the cell lines, 2,2′-pyridoins and AsA were added to H_2O_2-treated HL-60 cells, and the viable cells after 24 h incubation were determined (Fig. 3).[11,12] AsA, a conventional enediol antioxidant, was used as a positive control. The number of viable cells was decreased to 15.8% of the non-treated control cells by treatment of the cells with H_2O_2 (200 μM). The cell viability was recovered to 37.9% with the addition of 30 μM of AsA. 2,2′-Pyridoin (**1**) showed a more significant increase in cell viability than AsA (42.1%, $p < 0.05$ vs AsA, Student's *t*-test). Moreover, pyridoin derivatives **2**, **3**, **5**, and **6** showed a higher protective effect than **1** (51.9–55.4%, $p < 0.01$ vs AsA, Student's *t*-test). These results suggested that 2,2′-pyridoin derivatives have a more potent cytoprotective effect against oxidative stress than AsA. As showed in our previous results,[9] the most potent DPPH radical scavengers, **5** and **6**, inhibited lipid peroxidation effectively in a dose-dependent manner. Similarly, **3** inhibited lipid peroxidation effectively; nevertheless, **3** showed lower DPPH radical scavenging activity than AsA. The high activity of **3** may have been due to its high lipophilicity. The cytoprotective effects were nearly correlated with the inhibitory activities of lipid peroxidation and the log k_w, lipophilicity parameter,[13] together with the DPPH radical scavenging activity.[9] Therefore, we speculated that both the radical scavenging activity and the lipophilicity are important in cytoprotection.

The scavenging effect of 2,2′-pyridoins on intracellular oxidative stress was measured by using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), an intracellular ROS-sensitive fluorescence probe. DCFH-DA penetrates into cells and is hydrolyzed by intracellular esterase to non-fluorescent 2′,7′-dichrolofluorescin (DCFH). DCFH is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular ROS.[14] In this study, the total amount of the fluorescence of DCF, the relative intracellular ROS level, after 1 h of incubation was determined by flow cytometry.[15] The DCF fluorescence on HL-60 cells was markedly increased by the treatment of the cells with H_2O_2 (200 μM). The DCF production caused by H_2O_2 was slightly suppressed by AsA (30 μM). The pyridoin derivatives decreased the DCF fluorescence to the level of the non-treated control cells (Fig. 4A). These results suggested that the cytoprotection of 2,2′-pyridoin derivatives was accompanied by suppression of the intracellular oxidative stress. However, in the AsA case, the DCF production did not correlate with the cytoprotective effect. When the cells were treated with AsA (30 μM) before the addition of H_2O_2, the DCF production caused by H_2O_2 was markedly decreased (Fig. 4A), and the cytoprotective effect was increased (data not shown). These observations indicated that 2,2'-pyridoin derivatives were more incorporated into the cell than AsA.

It has been reported that 2,2′-pyridoin (**1**) is quickly oxidized to 1,2-diketone, 2,2′-pyridil (**1K**, Fig. 5), in protic solvent.[16] 2,2′-Pyridoin is supposed to be oxidized in the incubation medium, and its antioxidant activity is assumed to be attenuated. Therefore, to elucidate the antioxidant activity of 2,2′-pyridoin in the cell system, the cytoprotective effect and the suppressive effect on intracellular ROS of **1**–**3K** and **5**–**6K** (Fig. 5) were investigated. Compound **1K** was a commercially available material. **2**–**3K**, **5K**, and **6K** were prepared by the oxidation of **2**–**3**, **5**, and **6** using iodine according to a previous report (Scheme 1). [17,18] As shown in Figure 3, **1K**, **2K**, and **5K** showed a slight cytoprotective effect, and the effect of **3K** was significantly higher than that of AsA, while **6K** did not show a remarkable effect. In the DCF fluorescent assay, the H_2O_2-induced DCF production was slightly decreased by 2,2′-pyridil (Fig. 4B). These results suggested that 2,2′-pyridil derivatives, except **6K**, suppressed intracellular oxidative stress to some extent. However, the DPPH radical scavenging activities of 2,2′-pyridils were about 1000 times lower than that of 2,2′-pyridoins.

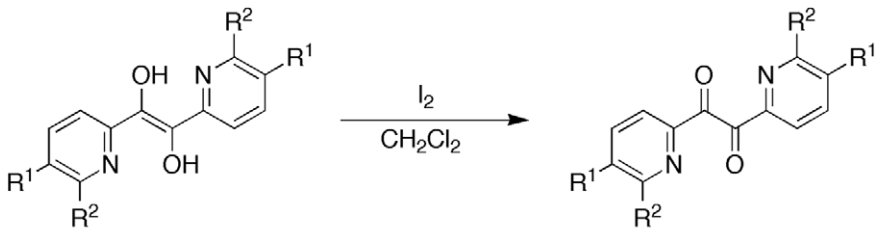
Therefore, it is speculated that 2,2′-pyridoins themselves, not 2,2′-pyridils, contributes to the antioxidant activity of 2,2′-pyridoins in the cell. And it is also speculated that 2,2′-pyridils were reduced to 2,2′-pyridoins in the cell and exerted antioxidant activity. These observations suggested that a large proportion of 2,2′-pyridon derivatives **1**–**3** and **5**–**6** was incorporated into the cell more quickly than they were oxidized and exerted antioxidant activity. We are now investigating the antioxidant mechanism of 2,2′-pyridils themselves.

In conclusion, we investigated the antioxidant effect of 2,2′-pyridoin derivatives in the cell system in the present study. These results demonstrated that 2,2′-pyridoins have no cytotoxicity and



1K: $R^1 = H$, $R^2 = H$
2K: $R^1 = H$, $R^2 = CH_3$
3K: $R^1 = H$, $R^2 = OCH_3$
5K: $R^1 = CH_3$, $R^2 = H$
6K: $R^1 = OCH_3$, $R^2 = H$

Figure 5. Structures of 2,2′-pyridils.



2: $R^1 = H$, $R^2 = CH_3$
3: $R^1 = H$, $R^2 = OCH_3$
5: $R^1 = CH_3$, $R^2 = H$
6: $R^1 = OCH_3$, $R^2 = H$

2K: $R^1 = H$, $R^2 = CH_3$
3K: $R^1 = H$, $R^2 = OCH_3$
5K: $R^1 = CH_3$, $R^2 = H$
6K: $R^1 = OCH_3$, $R^2 = H$

Scheme 1. Synthesis of 2,2′-pyridil derivatives.

exert better activity than AsA. Furthermore, it was suggested that 2,2′-pyridoins are incorporated more rapidly than AsA into the cell and react with intracellular ROS. Among 2,2′-pyridoin derivatives, **2**, **3**, **5**, and **6** are expected to be good lead compounds of cytoprotective agents against oxidative stress.

Acknowledgments

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11. Determination of cytotoxicity and cytoprotective effect: HL-60 cells (5×10^5 cells/mL) were plated onto a six-well multi-plate and incubated with the test compound in DMSO (0.5–100 μM) and/or aqueous H_2O_2 (200 μM) at 37 °C for 24 h under a 5% CO_2 atmosphere. Only the cells treated with DMSO were used as a non-treated control. The concentration of DMSO was set at 1 v/v%. The incubation mixture was centrifuged at 1000 rpm for 5 min, and the pellet was suspended in 2 mL of PBS (−). The cells were stained with trypan blue, and the viable cells were counted by a Vi-CELL™ cell viability analyzer (Beckman Coulter Inc.). Cell viability was calculated by the following equation. Cell Viability (%) = (treated viable cells)/(non-treated control viable cells) × 100.
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15. DCF fluorescence assay: HL-60 cells (5×10^5 cells/mL) were plated onto a six-well multi-plate and pretreated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 μM) for 15 min, and the medium was exchanged. These cells were incubated with the test compound in DMSO (0 or 30 μM) and aqueous H_2O_2 (200 μM) at 37 °C for 1 h under a 5% CO_2 atmosphere. The cells treated with DCFH-DA and DMSO were used as a control. The incubation mixture was centrifuged at 1000 rpm for 5 min, the pellet was suspended in 1 mL of BD FACS flow™, and the cells were quantified using a BD FACSCalibur™ flow cytometer (BD, Japan) with excitation and emission settings of 488 and 530 nm, respectively. The obtained data were analyzed by BD CellQuest™ Pro.
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18. Synthesis of **2K**: I_2 (147 mg, 578 μmol in 8 mL CH_2Cl_2) was added to a solution of 6.6′-dimethyl-2,2′-pyridoin (**2**, 200 mg, 826 μmol) in CH_2Cl_2 (8 mL). The reaction mixture was stirred for 1 h at ambient temperature. Then, saturated aqueous $NaHCO_3$ was added to the reaction mixture, and the whole mixture was extracted with CH_2Cl_2 (3× 20 mL). The combined organic layer was washed with brine, dried with $MgSO_4$, and concentrated. The obtained crude product was chromatographed on a silica-gel column using *n*-hexane/AcOEt (5:1) to give 117 mg of **2K** as a pale-yellow solid (59%). Other substituted 2,2′-pyridils (**3K**, **5K**, **6K**) were also synthesized with slightly modified procedure in 67–79% yield. **2K**; ^{1}H NMR (CD_3OD, 500 MHz): δ 2.39 (s, 6H, CH_3), 7.45 (d, J = 7.6 Hz, 2H, H-5 and 5′), 7.89 (t, J = 7.6 Hz, 2H, H-4 and 4′), 7.94 (d, J = 7.6 Hz, 2H, H-3 and 3′); ^{13}C NMR (CD_3OD, 125 MHz): δ 23.93 (CH_3), 120.32, 128.99, 138.74, 152.59, 160.13, 198.72 (C=O); MS (EI): m/z 240 (M^+), 183, 92, 65. **3K**; ^{1}H NMR (CD_3OD, 500 MHz): δ 3.52 (s, 6H, OCH_3), 7.00 (d, 2H, J = 8.2 Hz, H-5 and 5′), 7.74 (d, J = 7.0 Hz, 2H, H-3 and 3′), 7.89 (t, J = 8.2 Hz, 2H, H-4 and 4′); ^{13}C NMR (CD_3OD, 125 MHz): δ 53.72 (OCH_3), 115.97, 117.46, 140.99, 150.41, 164.92, 198.29 (C=O); MS (EI): m/z 272 (M^+), 229, 215, 201, 136, 108, 93. **5K**; ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 2.37 (s, 6H, CH_3), 7.92 (d, 2H, J = 8.2 Hz, H-4 and 4′), 8.06 (d, 2H, J = 7.6 Hz, H-3 and 3′), 8.43 (s, 2 H, J = 7.9 Hz, H-6 and 6′); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 18.31 (CH_3), 121.53, 138.09, 139.51, 148.66, 150.05, 197.03 (C=O). **6K**; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.92 (s, 6H, –OCH_3), 7.63 (dd, J = 8.5 Hz, 2.7 Hz, 2H, 4 and 4′-H), 8.14 (d, J = 8.5 Hz, 2H, H-3 and 3′), 8.30 (d, J = 2.7 Hz, 2H, H-6 and 6′); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 56.20 (–OCH_3), 120.99, 123.86, 138.02, 143.94, 158.99, 195.84 (C=O); MS(EI): m/z 272 (M^+), 244, 215, 201, 186, 173, 146, 136, 108, 93.