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CWJ-081, a novel 3-arylisoquinoline derivative, induces apoptosis in human leukemia HL-60 cells partially involves reactive oxygen species through c-Jun NH_2-terminal kinase pathway

So-Jung Won [a,b], Kyung-Sook Chung [a,c], Yo Sook Ki [a,c], Jung-Hye Choi [d], Won-Jea Cho [e], Kyung-Tae Lee [a,b,c,*]

[a] Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea
[b] Department of Life and Nanopharmaceutical Science, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea
[c] Department of Biommedical Science, College of Medical Science, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea
[d] Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea
[e] College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Yongbong-dong, Gwangju 500-757, Republic of Korea

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ABSTRACT

In the present study, we investigated the effect of a novel 3-arylisoquinoline derivative 3-(6-ethyl-benzo[1,3]dioxol-5-yl)-7,8-dimethoxy-2-methyl-2*H*-isoquinolin-1-one (CWJ-081) on the induction of apoptosis and the putative molecular mechanism of its action in human leukemia cells. Treatment with CWJ-081 exhibited a characteristic feature of apoptosis including externalization of phosphatidylserine and formation of DNA fragmentation in human leukemia cell lines (HL-60, U-937, K-562). In addition, stimulation of HL-60 cells with CWJ-081 induced a series of intracellular events: (1) the activations of caspase-8, -9, and -3; (2) the cleavage of poly (ADP-ribose) polymerase-1 (PARP-1); (3) the loss of mitochondrial membrane potential ($\Delta\Psi_m$); (4) the release of cytochrome *c*; and (5) the modulation of Bcl-2 family proteins. We further demonstrated that CWJ-081 induces reactive oxygen species (ROS) production and c-Jun NH_2-terminal kinase (JNK) activation. Pretreatment with the antioxidant *N*-acetyl-L-cysteine (NAC) markedly inhibited the CWJ-081-induced JNK activation and apoptosis. Moreover, CWJ-081-induced apoptosis was suppressed in the presence of SP600125, a specific JNK inhibitor. Taken together, these data suggest that CWJ-081 induces apoptosis via the mitochondrial apoptotic pathway in HL-60 cells, and ROS-mediated JNK activation plays a key role in the CWJ-081-induced apoptosis.

Cancer is a disease state characterized by a loss of apoptosis and accelerated proliferation. Therefore, compounds that block or suppress the proliferation of cancer cells by inducing cell cycle arrest and apoptosis are considered to have potential as anti-cancer agents.[1] Apoptosis is a highly regulated death process, by which cells undergo inducible non-necrotic cell death. In general, the activations of a family of cysteine proteases, called caspases, is an important mechanism in triggering apoptosis.[2] In addition, the inductions of pro-apoptotic Bcl-2 family proteins and the suppressions of anti-apoptotic family proteins have been associated with chemotherapeutic agent-induced apoptosis.[3] Leukemia is an aggressive disease that responds poorly to conventional therapy. The primary cause of treatment failure is the emergence of multi-drug resistance, which modifies drug targets and reduces drug-induced apoptosis.[4] Accordingly, chemotherapeutic agents to induce apoptosis of the cancer cells could be useful for improving the prognosis of this cancer.

Isoquinolines and structurally related compounds, represent an important class of biologically and medicinally useful agents. Many isoquinolines have been reported to possess diverse biological effects such as anti-inflammatory, anti-tumor, and anti-malarial effects.[5–8] In particular, isoquinolines have attracted research interest as a potential anti-cancer agent. For example, benzo[*c*]-phenanthridine derivatives have been found to possess great anti-proliferative activity in various cancer cells.[9–11] NK314, a novel benzo[*c*]phenanthridine was found to inhibit topoisomerase IIα and G_2 cell cycle arrest.[10] In a previous study, we found that a 3-arylisoquinoline derivative exhibited very strong anti-tumor activity against human cancer cell lines.[12] In the present study, we evaluated the anti-cancer effect of 3-(6-ethyl-benzo[1,3]dioxol-5-yl)-7,8-dimethoxy-2-methyl-2*H*-isoquinolin-1-one (CWJ-081, structure shown in Fig. 1), and its molecular mechanism of apoptosis induction in human promyelocytic leukemia HL-60 cells.

We examined the cytotoxicity of CWJ-081 in various cancer cells (HL-60, U-937, K-562, HeLa, and Caski) and in normal cells (Chang, L-132, and IOSE-80PC) using MTT assays. Among these cancer cell lines, leukemia cells were significantly sensitive to CWJ-081 with an IC_{50} value of 5.62–38.14 μM (Table 1). Interest-

* Corresponding author. Tel.: +82 2 9610860; fax: +82 2 9620860.
E-mail address: ktlee@khu.ac.kr (K.-T. Lee).

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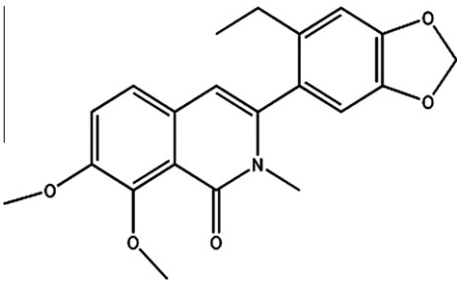

Figure 1. The chemical structure of CWJ-081, 3-(6-ethyl-benzo[1,3]dioxol-5-yl)-7,8-dimethoxy-2-methyl-2*H*-isoquinolin-1-one.

Table 1
Cytotoxic activity of CWJ-081 on various cell lines in vitro

Cell lines	Origin	IC_{50}[a] (μM)	
		CWJ-081	Cisplatin
HL-60	Human leukemia cells	5.62	6.72
U-937	Human leukemia cells	33.18	50.56
K-562	Human leukemia cells	38.14	41.22
HeLa	Human cervical cancer cells	42.22	33.63
Caski	Human cervical cancer cells	47.33	14.46
Chang	Human liver epithelial cells	39.86	28.74
L-132	Human lung epithelial cells	59.42	24.78
IOSE-80PC	Human ovarian epithelial cells	68.00	38.42

[a] IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared to that of the control cultures in the absence of CWJ-081. The values represent the means of three independent experiments with similar patterns. Cisplatin is treated as a positive control.

ingly, HL-60 cells were found to be most vulnerable to CWJ-081 with an IC_{50} value of 5.62 μM. In addition, the cytotoxicity of CWJ-081 was greater than that of cisplatin in HL-60 cells.

In order to determine whether CWJ-081-induced cytotoxic effect is associated with apoptosis in leukemia cells, we investigated the translocation of phosphatidylserine and DNA fragmentation using Annexin V and PI double staining and agarose gel electrophoresis, respectively, in HL-60, U-937, and K-562 cells. As shown in Figure 2A, proportions of Annexin V-positive cells (early apoptotic cells) in these leukemia cells were found to increase in a time-dependent manner after treatment with CWJ-081. Typical ladder patterns of internucleosomal DNA fragmentation were time-dependently observed after treating the leukemia cells with CWJ-081 (Fig. 2B). Since HL-60 was the most vulnerable apoptosis-inducing leukemia cells with CWJ-081 among the tested leukemia cells, further experiments were performed using HL-60 cells to identify molecular mechanism of apoptotic activity of CWJ-081.

The activation of the caspase cascade is a central mechanism of apoptosis in response to death-inducing signals from cell surface receptors or mitochondria.[13,14] To determine whether CWJ-081-induced apoptosis involves caspase activation, we investigated caspases activity using Western blot analysis. As a result, proteolytic cleavages of procaspase-8, -9, -3 and PARP-1 (a substrate of caspase-3) was observed at 36 h after CWJ-081 (10 μM) treatment (Fig. 3A).

The mitochondria act as a point of convergence for numerous apoptotic signaling pathways.[15] While the precise mechanisms of mitochondrial involvement in apoptosis are still the subject of ongoing research, the loss of the $\Delta\Psi_m$ is associated with the release of pro-apoptotic factors, such as cytochrome *c*, into the cytosol and the balance of Bcl-2 family proteins on the mitochondrial membrane.[16,17] In this study, we examined $\Delta\Psi_m$ perturbation

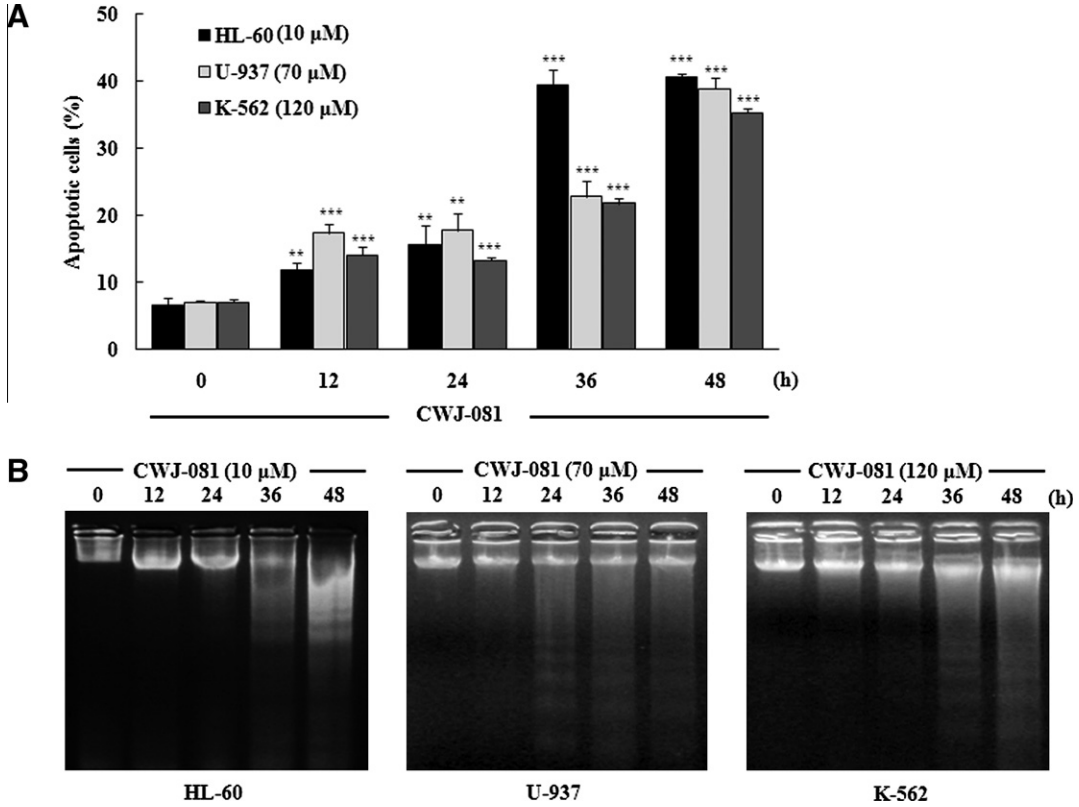

Figure 2. Induction of apoptosis by CWJ-081 in human leukemia cells (HL-60, U-937, and K-562). (A) Cells were treated with CWJ-081 for the indicated concentrations and times. The cell were co-stained with PI and FITC-conjugated Annexin V, and analyzed by flow cytometry. Data are presented as means ± SD of the results of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated control group. (B) DNA fragmentation was analyzed by agarose gel electrophoresis.

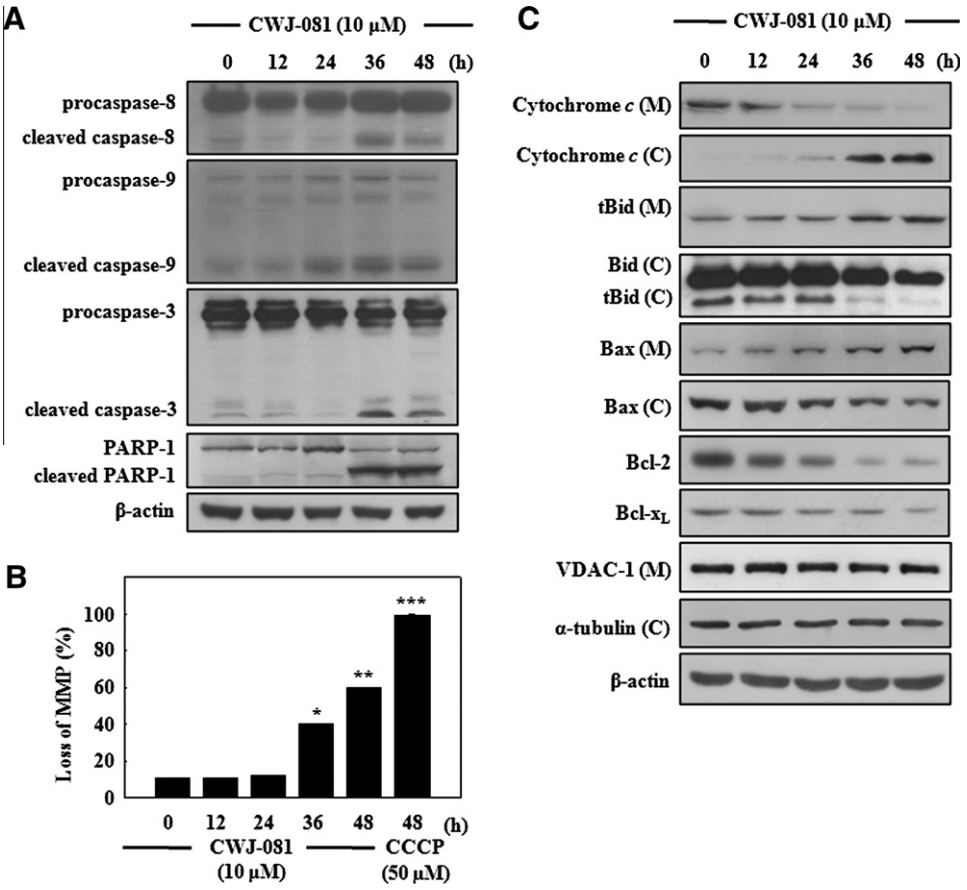

Figure 3. Changes in the levels of apoptosis-related proteins and the disruption of $\Delta\Psi_m$ during CWJ-081-induced apoptosis in HL-60 cells. (A) HL-60 cells were treated with or without CWJ-081 (10 μM) for the indicated times, and the caspase-8, -9, -3, and PARP-1 expressions were examined using Western blotting. β-Actin was used as an internal control. (B) Cells were treated with CWJ-081 for the indicated times, stained with $DiOC_6$, and analyzed by flow cytometry. CCCP (50 μM) was used as a positive control. Data are presented as means ± SD of results of the three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus non-treated control group; significances were determined by the Student's *t*-test. (C) Cells were treated with CWJ-081 (10 μM) for the indicated times, and expressions of cytochrome *c* and Bcl-2 family proteins were examined by Western blotting. VDAC-1, α-tubulin, and β-actin were used as internal control. (M) Mitochondrial fraction, (C) cytosolic fraction.

using the mitochondrial membrane specific dye, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$). As shown in Figure 3B, a loss of $\Delta\Psi_m$ was slightly observed at 24 h and a significant loss of $\Delta\Psi_m$ was detected in CWJ-081-treated cells. The positive control carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) also induced mitochondrial membrane depolarization. In addition, obvious changes of Bax and Bcl-2 expressions were observed at 12 h after CWJ-081 treatment, and release of cytochrome *c* from mitochondria were slightly observed at 24 h. These time-dependent changes on the levels of Bcl-2 family members during CWJ-081-induced apoptosis suggested the apoptosis of mitochondrial pathway via changes of Bcl-2 family members/reduction of mitochondrial membrane potential/cytochrome *c* release from mitochondria/activation of caspase-9 pathway.

Recent reports have documented that many forms of apoptosis are associated with ROS production.[18,19] Many bioactive agents have been shown to eliminate cancer cells by ROS production.[20,21] We first examined the effect of CWJ-081 on ROS production in HL-60 cells using H_2DCF-DA staining. H_2O_2 (200 μM, 2 h) was used as a positive control for ROS production. When cells were treated with CWJ-081 for the indicated time (0.5, 1, 3, 6, or 48 h), ROS production was found to increase in a time-dependent manner and to peak at 48 h (data not shown). It is of note that pretreatment with NAC (10 mM) completely blocked CWJ-081-induced ROS production in HL-60 cells (Fig. 4A). To determine whether the production of ROS is required for the induction of cell death by CWJ-081, we pretreated HL-60 cells with NAC and then treated them with CWJ-081 for 48 h. As shown in Figure 4B, CWJ-081-induced apoptosis (50.5%) was inhibited by NAC pretreatment (33.8%), indicating that CWJ-081-induced apoptotic cell death is, at least in part, due to ROS generation. ROS can cause apoptotic cell death via a variety of mechanisms, which include the activation of the JNK. In fact, a number of anti-cancer drugs have been reported to induce apoptosis in cancer cells via the JNK activation.[22] We found that CWJ-081 markedly increased the level of phosphorylation of JNK at 6 h, and then decreased toward base line at 12 h in HL-60 cells (Fig. 4C). Since the phosphorylation of JNK was also increased during 24–48 h (data not shown), it is likely that CWJ-081 induces the activation of JNK in a biphasic manner. Notably, cells were found to be significantly rescued from CWJ-081-induced apoptosis when they were pretreated with the JNK inhibitor SP600125 (10 μM) (Fig. 4D). Moreover, our data showed CWJ-081-induced phosphorylation of JNK was completely blocked by NAC pretreatment (Fig. 4E). These results suggested that CWJ-081 induces ROS production, which then regulates the activation of JNK in HL-60 cells. These results indicated that CWJ-081-induced apoptosis might be involved another additional mechanism. Endoplasmic reticulum (ER)-specific apoptotic pathway, it is induced by accumulation of unfolded/misfolded protein aggregating in ER or by excessive protein traffic. Under these conditions, the modulation of unfolded protein response (UPR) signaling leads to the activation of the pro-apoptotic kinase JNK.[23] Furthermore, in the mitochondria

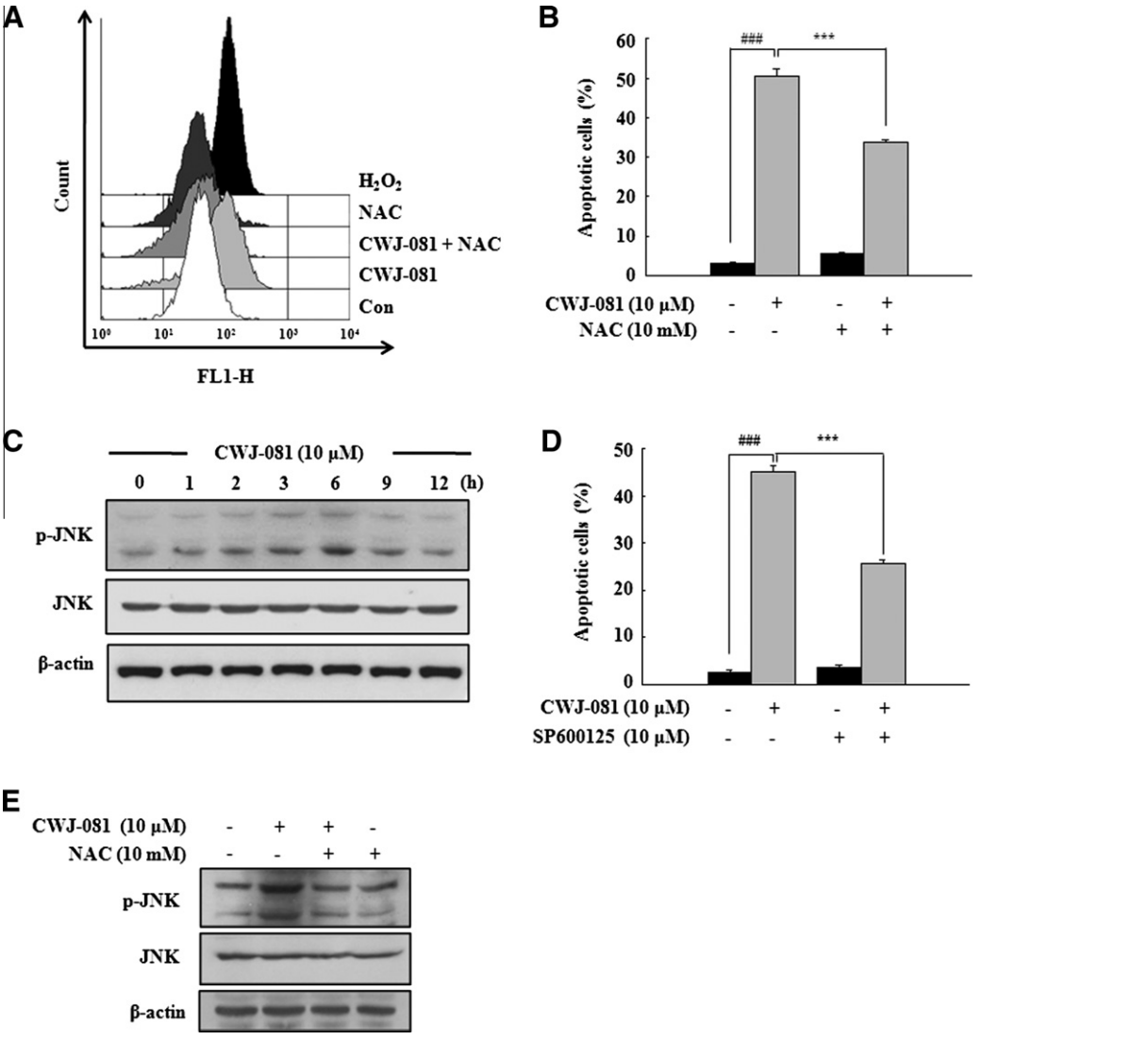

Figure 4. Involvement of ROS production and JNK activation in CWJ-081-induced apoptosis in HL-60 cell. Cells were pretreated with NAC (10 mM) and then 1 h later treated with CWJ-081 (10 μM) for 48 h. They were then stained with (A) H_2DCF-DA or (B) Annexin V and PI, and then analyzed by flow cytometry. H_2O_2 (200 μM, for 2 h) was used as a positive control. $^{###}p$ <0.001 versus the control group, $^{***}p$ <0.001 versus CWJ-081-treated group. (C) Cells exposed to CWJ-081 for the indicated times and then subjected to Western blot analysis using specific p-JNK and JNK antibodies. β-Actin was used as an internal control. (D) Cells were pretreated with SP600125 (10 μM) for 1 h, and then treated with CWJ-081 for 48 h. Cells were then harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. (E) Cells were pretreated with NAC for 1 h and then with CWJ-081 for 48 h. Protein extracts were then harvested and subjected to Western blot analysis using specific p-JNK and JNK antibodies.

apoptotic pathway, NF-κB pathway is one of the critical transcription factors that regulate the transcription of many genes associated with tumorigenesis.[24] Further ER stress and NF-κB examination of CWJ-081 will provide more clarified information on the revealing the underlying molecular mechanism.

Taken together, a novel 3-arylisoquinolin derivative CWJ-081 exhibits an anti-proliferative effect in HL-60 cells by induction of apoptosis that is associated with activation of caspases and mitochondrial dysfunction. Furthermore, CWJ-081-induced apoptosis is significantly associated with ROS production and JNK activation in HL-60 cells. Our data suggest that CWJ-081 could be a potent chemotherapeutic agent that is worthy of further development to improve leukemia.

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