4-aminopyridine Induces Apoptosis of Human Acute Myeloid Leukemia Cells via Increasing [Ca\^{2+}]_{i} Through P\textsubscript{2}X\textsubscript{7} Receptor Pathway

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Key Words
4-aminopyridine • Acute myeloid leukemia • Apoptosis • Mitochondria • Purinergic receptors

Abstract
4-AP, a voltage-gated potassium channel blocker, was identified to exert critical pro-apoptotic properties in various types of cancer cells. The present study aims to explore the effect of 4-AP on the apoptosis of human AML cells and the underlying mechanism. We found 4-AP inhibited the proliferation and induces apoptosis in both AML cell lines and primary cultured human AML cells. The apoptosis of AML cells after 4-AP treatment was further confirmed by the disruption of mitochondrial membrane potential (MMP) and activation of caspase 3 and 9. 4-AP inhibited Kv currents in NB\textsubscript{4}, HL-60 and THP-1 cells. Furthermore, 4-AP induced significant increment in \([Ca^{2+}]_{i}\), which were inhibited by KN-62, a specific blocker of \(P_{2}X_{7}\) receptors. KN-62 also abrogated 4-AP induced apoptosis. Knockdown of \(P_{2}X_{7}\) receptor by small interfering RNA blocked the effect of 4-AP. Conclusively, this study indicated that 4-AP promotes apoptosis in human AML cells via increasing \([Ca^{2+}]_{i}\) through \(P_{2}X_{7}\) receptor.

Introduction
Acute myeloid leukemia (AML) is a malignancy of the myeloid lineage of white blood cells, characterized by clonal expansion of abnormal myeloid progenitor cells that accumulate in the bone marrow and interfere with normal hematopoiesis. A recent study reported that the 5-year survival rate of those diagnosed with AML age =55 years was 23%, whereas the corresponding rate for those age >55 years was 11% [1, 2]. Even when the most aggressive therapeutic approaches are applied early, the cure rate remains at 35% to 40% [3-6] and <10% of all AML patients survive past 3 years [7]. Thus, there is an urgent need for the discovery of novel therapeutic agents for AML.

The aim of anti-cancer therapy is to induce apoptosis of tumor cells. Studies demonstrated that 4-aminopyridine (4-AP), one of the most commonly used K\textsuperscript{-} channel inhibitors, suppresses proliferation and induces apoptosis in various types of cancer cells [8-12], such as neuroblastoma cells [8], NF1 Schwann cells [9], human melanoma cells [10], LNCaP human prostate cancer cells [11], and malignant astrocytoma cell lines [12]. These anti-cancer activities were mostly attributed to its inhibition on voltage-gated potassium channels.
Recently, 4-AP was linked to the activation of calcium influx and apoptosis [13-15]. Guse et al. showed that 4-AP could cause a transient increase in $[\text{Ca}^{2+}]$ [14], which act as a common mediator of cell death [16] and a signal transducer of apoptosis [17]. Kim et al. showed that 4-AP induced apoptosis in HepG2 human hepatoblastoma cells by activating Ca$^{2+}$ influx pathway through nonselective cation channel NSCC induced by membrane potential change [15]. 4-AP was further confirmed to induce increases in intracellular calcium influx through modulation of the activity of purinergic cationic channels in human mononuclear cells [18].

Currently, the effect of 4-AP in AML is largely unknown, except that Xu B et al demonstrated that 4-AP induces G$_1$ arrest in the human myeloblastic ML-1 cell line [19]. As ML-1 cell line is derived from a patient diagnosed as T-cell malignant lymphoma (Stage IV) terminating in AML [19], it cannot represent the general features of AML. Therefore, in this study we investigated the effect of 4-AP on AML cell lines and primary AML cells, and the involvement of P$_2$X$_7$ receptor mediated calcium influx in this process. We found 4-AP induces apoptosis of the AML cell lines NB$_4$, HL-60 and THP-1 as well as primary AML cells, which were prevented by inhibition of P$_2$X$_7$ pore mediated $[\text{Ca}^{2+}]$ increment.

**Materials and Methods**

**Cell culture**

THP-1 (Permanent human cell lines representing acute monocytic leukemia), NB$_4$ (acute promyelocytic leukemia) and HL-60 (acute promyelocytic leukemia) were obtained from the Peking Union Medical College cell bank (Beijing, China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), and maintained at 37°C in a humid atmosphere of 5% CO$_2$ in air. The cells were counted each day with a hemacytometer and then passaged every 2 days to ensure that no significant cell death occurred.

**Isolation of leukemic blasts**

Normal peripheral lymphocytes and leukemic bone marrow cells were obtained from 3 volunteer donors and 21 patients treated at the Hematology Unit of First Clinical Medical College of Harbin Medical University for newly diagnosed AML before chemotherapy. According to the French–American–British committee classification, there are 3 cases M1, 5 cases M2, 4 cases M3, 5 cases M4 and 4 cases M5. Enriched populations of blast cells (more than 95%) were obtained by Ficoll–Hypaque (Pharmacia) density gradient centrifugation [20]. Peripheral lymphocytes were cultured with IL-2 (200 U/ml, Yope Biotech Limited Company, Shanghai, China).

**Annexin-V Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Double Staining Assay**

Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) was used to distinguish and quantitatively determine the percentage of dead, viable, apoptotic and necrotic cells after treatment with drugs according to the manufacturer's instructions. The cells were seeded at 4x10$^5$ cells/ml in 6-well plates. After treatment with drugs, the cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 100 μl of binding buffer. A total of 5 μl of Annexin V-FITC and 10 μl of PI were added and the mixture was incubated in the dark for 30 min. Then, 400 μl of binding buffer was added to the cells. The labeled cells (10,000/sample) were analyzed by measuring fluorescence intensity using FACS can flow cytometry (BD Bioscience) in conjunction with CellQuest software (BD Biosciences, San Jose, CA).

**Measurement of nuclear fragmentation**

Apoptotic death was evaluated by nuclear fragmentation in 4′,6-diamidino-2-phenylindole-2 HCl (DAPI)-staining cells. After fixation in 70% ethanol for 10 min, cells were stained with DAPI (0.5 μg/ml) for 10 min, and observed under UV-light using a confocal microscope (R2100AG2, Bio-Rad, Melville, NY). Ten different fields were randomly selected for counting 300 cells. The percentage of cells with fragmented nuclei was then calculated.

**Mitochondrial membrane potentials assay**

JC-1 probe (Sigma, St Louis, MO, USA) was employed to measure mitochondrial depolarization. Briefly, cells were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min and rinsed twice with PBS. Mitochondrial membrane potential ($\Delta$Ψm) was determined using FACSscan flow cytometer (BD Bioscience), and the mean fluorescence intensities were calculated and employed to express $\Delta$Ψm alteration.

**Patch-clamp recording of Kv current**

Cells were transferred to the recording chamber, which was attached on the stage of an inverted phase-contrast microscope (IX71, Olympus, Tokyo, Japan). To immobilize the cells, they were adhered to the chamber with a bottom glass coated with poly-L-lysine (Sigma, St Louis, MO, USA). Cells were bathed at room temperature (20-25°C) and then superfused by gravity at a rate of about 2-4ml/min (bath volume 2ml) with an extracellular solution containing (in mM) (Sigma, St Louis, MO, USA) 136.5 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.53 MgCl$_2$, 5.5 glucose and 5 HEPES-NaCl buffer, pH 7.4. The patch pipettes were made from Kimax capillary tubes (Kimble Glass, Vineland, NJ) using a vertical two-step electrode puller (PB-7, Narishige, Tokyo, Japan) and the tips were fire-polished with a microforge (MF-83, Narishige, Tokyo, Japan). The tip solution contained (in mM) (Sigma, St Louis, MO, USA) 135 K-aspartic acid, 2 MgCl$_2$, 1.1 EGTA, 0.1 CaCl$_2$ and 10 HEPES-KOH buffer, pH 7.2. The resistance of the patch tip was 3-5 MΩ when it was
immerged in extracellualr solution. When the seal resistance exceeded 4GΩ, the whole cell membrane currents were recorded. Whole-cell voltage-clamp recordings were performed to record the voltage-gated potassium currents in NB4, HL-60 and THP-1 cells using Axopatch 700B patch-clamp magnifying instrument (Axon Instruments, Foster City, CA, USA). For the voltage-gated potassium currents recording, the membrane voltage was stepped to −90mV for 1s followed by a ramp to + 60 mV from a holding potential of -80 mV. All the records were stored on hard disk for the post-experiment analysis.

**Transmission electron microscopy**

NB4, HL-60, and THP-1 cells were cultured in Petri dishes with 4mM 4-AP for 72h. 5 × 10^6 cells were collected by centrifugation at 12 000 g for 5 min and washed twice with PBS. The pelleted cells were fixed in 2.5% cold glutaraldehyde supplemented with 0.1 M of sodium cacodylate/ 1% sucrose buffer for 24 h. The cells were washed three times with PBS, then postfixed in 1% osmium tetroxide (60 min), encapsulated in 1% agar, stained with uranyl acetate and phosphotungstic acid, and dehydrated in a series of graded ethanol solutions. Propylene oxide was added before the cells were finally embedded in Epon 812-Araldite mixture. Ultrathin sections (50 nm) were cut using LKL-208 ultramicrotome, placed under 200 mesh standard copper grids and examined under JEM-1200 transmission electron microscope.

**Measurement of Intracellular Ca^{2+} Concentration [Ca^{2+}]**

Cells were loaded with Fluo-3/AM (5μmol/L). Fluorescent intensity of Fluo-3/AM-loaded cells were detected using laser scanning confocal microscope (Olympus FV-300) with 488 nm intensity of Fluo-3/AM-loaded cells were detected using laser scanning confocal microscope (Olympus FV-300) with 488 nm for excitation from an argon ion laser and 530 nm for emission. Acquisition rate was 1 frame per 20 s and [Ca^{2+}]i was monitored for at least 600 s. Drugs were added between the 1st and 2nd scan. The fluorescent intensities before (FI_0) and after (FI) drug administration were recorded. Quantitative changes in [Ca^{2+}]i were inferred from the ratio of FI/ FI_0.

**Reverse transcriptase-PCR (RT-PCR)**

Total RNA was extracted with TRlzol (BRL Life and Technologies,MD). The cDNAs were amplified from 2 μg of total RNA using ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). The following primer pairs were used for RT-PCR: P2X7, 5'-TCT GCA AGA TGT CAA GGG C-3', 5'-TCA CTC TTC GGA AAC TCT TTC C-3'; ApoAlert® caspase fluorescent and colorimetric assays (BD Biosciences Clontech,CA, U.S.A.) were used to determine the enzyme activity of caspase-3, -8 and -9. After incubation with 4mM 4-AP for 3.5, 7 and 14h, cells were collected and lysed in ice-cold buffer. The homogenate was centrifuged at 12000g for 10 min at 4 °C. Caspase activities in the supernatant were determined by cleavage of the specific chromophore-conjugated substrates. The substrate peptides of caspase-3, -8 and -9 were conjugated to 7-amino-4-trifluoromethyl coumarin (AMC), respectively. Caspase-3, -8 and -9 were measured quantitatively by comparing fluorescent intensity on a fluorescence spectrophotometer (Hitachi F-2500, Japan). Caspase-3, -8 and -9 activity was determined by absorbance of p-NA at 405 nm in a microplate reader(BIO-TEK ELX800,USA).

**Small RNA interference of P2X, receptor**

P2X, siRNA was designed using online program from Invitrogen, siDirector, and Wistar. These quences were as the followings: P2X, siRNA, 5'-CGA UGG ACU UCA CAG AUU U-3' and 5'-ACA UCC UGC CAG GUU UAA ATT-3'; control siRNA, 5'-CGA CAU ACU GUU CAG GCC UTT-3'. The duplex of each siRNA were synthesized by Sigma-Prologic. The siRNA(50 μM) was mixed with 100μl HiPerFectin and added into 8ml NB4 cells (5×10^6/ml) for 48 h in OPTI-MEM medium with 5% FCS. Cells were kept in culture for another 48h before collection for Western blotting, laser scanning confocal microscope and FITC/PI double staining assay.

**Measurement of ATP concentration**

NB4 cells were treated with 4-AP (4mM) for 6h. After centrifugation, the supernatant was collected to detect extracellular ATP concentration by luciferin-based ENLITEN ATP Assay (Promega, Madison, USA) following the manufacturer’s instructions.

**Western blots**

Cells were lyzed by RIPA buffer (100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% NP-40 and 50 mM Tris- HCl [pH 7.2]). Western blots were performed by a standard enhanced chemiluminescence (ECL) method (Amersham) using the following antibodies: GAPDH (Santa Cruz Biotechnology; Santa Cruz, CA); anti-P2X, antibodies (BD Biosciences Pharmingen).

**Statistical analysis**

Data are presented as mean ± SEM. Comparisons between controls and treatments were analyzed by two-tailed analyses of variance (ANOVA) followed by Dunnett’s test. Difference was considered significant when p<0.05.

**Results**

**Effects of 4-AP on the Growth of Human AML Cell Lines**

We first measured the effects of 4-AP on continuously growing AML cell lines. The AML cell lines, NB4, HL-60 and THP-1, were treated with incremental

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4-AP Induced Apoptosis of AML Cells

Cell Physiol Biochem 2011;28:199-208

201
doses up to 8 mM of 4-AP for 72 h. 4-AP suppressed cell viability in a dose-dependent manner in all cell lines (Fig 1A). The IC50 of 4-AP was 3.74 mM (NB4), 3.61 mM (HL-60) and 3.68 mM (THP-1), respectively.

As continuously growing cell lines exert different proliferative activity and survival requirements than do primary leukemic cells, their susceptibility to antileukemic compounds may be different as well [21]. We thus tested the effects of 4-AP on cell viability of primary cultured AML cells of 21 cases of M1-M5 patients. At the concentration of 8 mM, 4-AP markedly reduced cell viability in all 21 cases of AML patients, and the median cell viability is 7% (ranging from 1% to 18%) of that measured in parallel untreated cultures (Fig 1B). The effects of 4-AP remained evident at 4 mM (median cell viability is 48%) and 2 mM (median cell viability is 82%) (Fig 1B).

4-AP induces apoptosis in AML cells

Previous studies indicated that 4-AP inhibits cell proliferation, but does not cause cytotoxicity in ML-1 cells [19]. In this study, we found that 4-AP induced significant apoptosis in AML cells. NB4, HL-60, and THP-1 cells treated with 4 mM 4-AP for 72 h revealed typical nuclear features of apoptosis including the pycnosis of cell nucleus, and the appearance of apoptotic body with nuclear membrane observed using the electron microscopy (Fig 2A). We also employed Annexin V-FITC/PI and DAPI staining to explore the induction of apoptosis by 4-AP. Treatment with 4 mM 4-AP significantly increased apoptotic cells (NB4: p<0.001 vs control; HL-60: p<0.001 vs control; THP-1: p<0.001 vs control) (Fig 2B, C, D). We also observed the apoptosis of 21 primary cells samples of AML patients treated with 4 mM 4-AP for 72 h by Annexin-V/PI staining, and 4-AP induced apoptosis in all the 21 cases (Fig 2E).

Effects of 4-AP on mitochondrial membrane potential (ΔΨm) and Caspase activity

The ΔΨm disruption is critical for apoptosis. When NB4, HL-60, and THP-1 cells were treated with 4-AP (4 mM), the ΔΨm disruption began to increase at 24 h and further increased in a time-dependent manner (Fig 3A), further indicating the induction of apoptosis.

Caspases play key roles for both commitment and execution of apoptosis [22]. Caspase-3 is one of the downstream effectors of the caspase family and is involved in both the mitochondrial apoptotic pathway and the death receptor pathway. Caspase-3 is synthesized through cleavage in response to apoptotic stimuli by initiator caspases (including caspase-2, -8, -9, -10, -11 and -12) and then becomes activated [23]. As shown in Fig 3B, 4-AP enhanced the activation of caspase-3 in a time-dependent manner. Treatment with 4-AP (4 mM) for 14 h caused a 2.5 fold increase in caspase-3 activity (P<0.05 vs control group). Moreover, 4-AP (4 mM) treatment dramatically increased caspase-9 activity by 2.9 fold, while caspase-8 activity was not changed, illustrating the activation of caspase-3 and -9 but not caspase-8 in the apoptotic process of NB4, HL-60 and THP-1 cells induced by 4-AP.

Effects of 4-AP on voltage-gated potassium currents in AML cells

A typical outward rectifier current was recorded by using patch-clamp technique in NB4 cells (Fig 4A). The current recorded was activated rapidly and did not inactivate during 1000 ms test pulses, and was blocked by 4 mM 4-AP (Fig 4B). The current was not blocked by the specific inhibitor of Ca2+-activated K+ channels (KCa), 1 μM charybdotoxin (Fig 4C). The electrophysiological features of outward rectifier currents appeared clearly similar to those reported in other tumor cell lines [12, 24]. The effect of 4-AP on the current-voltage relations in
the range of −90mV and +60mV was demonstrated in the 3 different cell lines NB4, HL-60, and THP-1 (Fig 4D, E, F). At +60 mV, 4-AP(4mM) reduced the peak amplitudes of Kv current by 47.5% (NB4), 48.2% (HL-60) and 42.2% (THP-1) of their respective controls (Fig 4D, E, F).

Effects of 4-AP on intracellular Ca2+ concentration
Mobilization and concentration of intracellular calcium are an essential and well-established step in cell proliferation, activation and apoptosis [22]. To determine...
whether 4-AP affected intracellular Ca\textsuperscript{2+} signaling in the leukemic cell lines, we measured the changes in cytosolic Ca\textsuperscript{2+} concentration. Exposure to 4 mM 4-AP caused a rapid and sustained rise in intracellular Ca\textsuperscript{2+} concentration of NB\textsubscript{4} cells (Fig 5A). In nominal Ca\textsuperscript{2+}-free medium containing 1 mM EGTA (Fig 5B,G), a chelator of extracellular calcium, 4-AP induced [Ca\textsuperscript{2+}]\textsubscript{i} increment was inhibited. While verapamil (100μmol/L) in normal Ca\textsuperscript{2+}-containing medium (Fig 5C,G), a voltage-operated Ca\textsuperscript{2+} channel blocker produced no effect on 4-AP induced [Ca\textsuperscript{2+}]\textsubscript{i} increment. Flufenamic acid (200μmol/L), a nonselective cation channel blocker, completely blocked 4-AP induced Ca\textsuperscript{2+} influx (Fig 5D,G). These results illustrate that 4-AP induced Ca\textsuperscript{2+} influx through the activation of NSCCs, a finding that is consistent with previous reports [21]. Furthermore, the presence of ionotropic P\textsubscript{2}X\textsubscript{7} receptors, belonging to nonselective cation channel, has been found in AML cells [25]. We found that KN-62 (1 μmol/L), a specific blocker of P\textsubscript{2}X\textsubscript{7} receptors, completely blocked 4-AP induced Ca\textsuperscript{2+} influx (Fig 6A). The increment of [Ca\textsuperscript{2+}] evoked by 4-AP (4mM) was inhibited by si-P\textsubscript{2}X\textsubscript{7} and si-random produced no effect (Fig 6B). Furthermore, suppressing P\textsubscript{2}X\textsubscript{7} expression also abrogated 4-AP induced apoptosis of NB\textsubscript{4} cells (Fig 6C). These results indicated that P\textsubscript{2}X\textsubscript{7} receptor plays a crucial role in the effect of 4-AP on AML cells. Moreover, 4-AP (4mM) treatment increased ATP release from NB\textsubscript{4} cells (Fig 6D).

**Effect of P\textsubscript{2}X\textsubscript{7} knockdown on the response of NB\textsubscript{4} to 4-AP and Release of ATP induced by 4-AP**

To further confirm the involvement of P\textsubscript{2}X\textsubscript{7} receptor in the pro-apoptotic effect of 4-AP in AML cells, we employed siRNA technique to knockdown P\textsubscript{2}X\textsubscript{7} receptor. The siRNA used in this study suppressed the expression of P\textsubscript{2}X\textsubscript{7} receptor, with the protein expression decreased by about 78% after adding siRNA-P\textsubscript{2}X\textsubscript{7} (Fig 6A). The increment of [Ca\textsuperscript{2+}] evoked by 4-AP (4mM) was inhibited by si-P\textsubscript{2}X\textsubscript{7} and si-random produced no effect (Fig 6B). Furthermore, suppressing P\textsubscript{2}X\textsubscript{7} expression also abrogated 4-AP induced apoptosis of NB\textsubscript{4} cells (Fig 6C). These results indicated that P\textsubscript{2}X\textsubscript{7} receptor plays a crucial role in the effect of 4-AP on AML cells. Moreover, 4-AP (4mM) treatment increased ATP release from NB\textsubscript{4} cells (Fig 6D).
**Discussion**

4-AP, a specific inhibitor of Kv channels, was shown to induce apoptosis in HepG2 hepatoma cells [15]. In this study, we found 4-AP induced apoptosis of AML cells (NB4, HL-60, and THP-1). Depolarization of the mitochondrial membrane potential and release of apoptotic proteins are the characteristic changes during cell apoptosis. We observed that 4-AP treatment resulted in disruption of ΔΨm. Disruption of ΔΨm undergoes a series of changes during apoptotic death of cells, and loss of MMP together with the open of permeability transition pore induces the cytosolic release of cytochrome c and activation of initiator caspases-9, which activates the...
apoptosis executioner protease, caspase-3 through proteolytic cleavage at specific internal aspartate residues [26]. In this study caspase-9 and 3 activity increased dramatically after treatment with 4-AP while the activity of caspase-8 did not change. In this study the concentrations required for proapoptotic effect of 4-AP are less than those that induce toxic effects in normal lymphocytes. 4-AP also caused significant apoptosis of primary cultured AML cells of clinical patients, which indicates the potential of 4-AP in treating acute myelocytic leukemia. These results are consistent with Xu B et al’s study, just that we found 4-AP caused apoptosis, while they showed that 4-AP inhibited proliferation of human myeloblastic ML-1 cell line [19].

In cancer cells, K⁺ channels play crucial roles in cancer cell development, proliferation and migration [27] and are an excellent target for diagnosis and therapy [27]. Many agents inhibiting K⁺ channels have been shown to suppress cell proliferation, including leukemic cells [28-30]. In this study we found NB₄, HL-60 and THP-1 cells all express functional Kv currents, which can be inhibited by 4-AP, indicating that 4-AP might exert its proapoptotic action via inhibition of potassium channels. However, as the resting potential of the cell lines is around -40 mV, and 4-AP inhibited Kv current at +40mV and +50mV, implying that at the resting state inhibition of potassium channel may not contribute to its pro-apoptotic action on tumor cells at least at the resting state. As the resting potential of tumor cells are not constant in the phases of proliferation, and a transient hyperpolarization is required for the progression of the early G1 phase of the cell cycle [31]. Under this situation, it would be reasonable for 4-AP to take action by inhibiting potassium channel.

4-AP was shown to affect calcium mobilization [13-15]. Guse et al. demonstrated that 4-AP caused a transient increase of intracellular calcium [14]. 4-AP has been shown to block K⁺ channels at the concentration used in this study, and thus depolarize membrane potential in many cell types [32-34], which caused calcium influx. As calcium overload has been shown to act as a common mediator of cell death [16] and a signal transducer of apoptosis [17], the proapoptotic action of 4-AP on AML cells may be due to its effect on [Ca²⁺]. In this study, we found 4-AP induced a rapid and sustained increase of intracellular Ca²⁺ in AML cells, which is consistent with those previously described in other cell types [15]. We further explored the mechanism by which 4-AP increased intracellular Ca²⁺, and found that deprivation of extracellular calcium using EGTA diminished [Ca²⁺], increase, suggesting that extracellular influx plays a major
role in this process. As in AML cells extracellular calcium mainly influx via voltage-dependent calcium channel and nonselective cation channel, we explored which of them accounts for 4-AP induced calcium influx. The fact that FA (a nonselective cation channel blocker) but not verapamil blocked 4-AP induced \([\text{Ca}^{2+}]_{i}\) increase means that nonselective cation channel is responsible for the influx of extracellular calcium. Furthermore, we identified mRNA expression of \(P_{X7}\) receptor in NB4, HL-60 and THP-1 cells, which is an ATP-gated cation channel expressed in hemopoietic cells and participates in both cell proliferation and apoptosis [35]. Interestingly, inhibition of \(P_{X7}\) activity and knock down of \(P_{X7}\) receptor expression abrogated 4-AP induced apoptosis, which strongly support the involvement of \(P_{X7}\) receptor in this process. Consistently, Kim et al. showed that 4-AP induced apoptosis of HepG2 human hepatoblastoma cells by activating \(\text{Ca}^{2+}\) influx through NSCCs by changing membrane potential. However, Dong et al. demonstrated that blocking of Kv currents by 4-AP induced apoptosis in HepG2 cells, but not GH3 cells [36]. Wu et al. found that 4-AP directly stimulates high voltage-activated calcium channels in acutely dissociated neurons [37]. These studies indicated that blocking of Kv current may not always be the cause of cell apoptosis, and 4-AP can target on calcium channels directly. Therefore, when interpreting our data, we can not rule out the possibility that 4-AP induced apoptosis may be caused by the direct activation of mediated calcium influx, which requires further study to clarify it.

Taken together, we found 4-AP induced apoptosis in both AML cell lines and primary cultured AML cells of patients. Inhibiting of Kv channel and the subsequent calcium influx mediated \(P_{X7}\) receptors account for 4-AP’s anticancer activity against AML cells. This study indicated that 4-AP represents a promising candidate for the development of novel agents for treating AML.

**Abbreviations**

4-AP (4-aminopyridine); AML (Acute myeloid leukemia); EGTA (Ethylene glycol bis(2-aminoethyl ether)tetraacetic acid); FA (Flufenamic acid); Ver (Verapamil); \(P_{X7}\) (Purinergic receptor \(P_{X7}\), ligand-gated ion channel, 7); MMP (Mitochondrial membrane potentials).

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