Curcumin Blocks Kv11.1 (*erg*) Potassium Current and Slows Proliferation in the Infant Acute Monocytic Leukemia Cell line THP-1

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Key Words

Curcumin • *erg* • Resting potential • AML • Proliferation

Abstract

Acute Myeloid Leukemia (AML) accounts for approximately one fifth of all childhood leukemia yet is responsible for a significant proportion of morbidity and mortality in this population. For this reason, research to identify novel targets for the development of effective AML therapeutics has intensified in the recent past. The THP-1 cell line, which was originally established from an infant diagnosed with AML, provides an experimental model for functional, pre-clinical therapeutics and target identification studies of AML. Here we show the expression of the voltage gated potassium channel Kv11.1 in THP-1 cells as opposed to normal hematopoietic stem cells. In addition, curcumin, a natural polyphenol derived from the plant *Curcuma longa*, effectively blocked Kv11.1 activity and also inhibited the proliferation of these cells. Curcumin was rapidly internalized by THP-1 cells and possibly exerts potential growth inhibitory activity by interacting with intracellular epitopes of the ion channel. Inhibition of ionic currents carried by Kv11.1

resulted in depolarization of cell membrane potential. We propose that the inhibition of Kv11.1 activity by curcumin may lead to interference with leukemic cell physiology and consequently the suppression of survival and proliferation of AML cells.

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Introduction

Acute myeloid leukemia (AML), that accounts for approximately 20% of all leukemia diagnosed in children, develops de novo or as a consequence of myelodysplasia or prior chemotherapy. With current treatment regimens, most of the children with AML achieve a first complete remission (CR1), but the relapse rate remains unacceptably high with a 5-year event-free survival (EFS) of about 50% [1]. Among AML patients, certain subgroups, such as infants of less than one year of age, have even more unfavorable outcome. As such, there is an urgent need to identify novel therapeutic agents to treat refractory and high risk AML patients. The identification of critical cell growth and survival pathways

in AML blasts has led to the development of newer targeted therapeutic agents including monoclonal antibodies and receptor tyrosine kinase inhibitors and has led to the evaluation of a number of small-molecular-weight targeted inhibitors for AML [2].

There is growing evidence to indicate that membrane ion channels are critical for cell survival and play a key role in the regulation of cellular proliferation, differentiation and apoptosis [3]. It has been hypothesized that under pathological conditions such as malignancy, the expression and activity of ion channels are changed and such changes may promote the growth and proliferation of tumor cells [4]. Thus, these molecules present an opportunity to identify effective targets and pathways for the development of novel therapeutics against certain malignancies [4, 5]. In particular, functional roles of ion channels in leukemia malignancy have been reported [6-10].

Consequently, evaluation of the expression and inhibition of ion channels in relevant *in vitro* models is a practical approach in the initial drug screening and proof-of-concept studies for AML.

The THP-1 cell line was established from AML blasts from a one year old child and carries the *MLL-AF9* oncogene as a consequence of t(9;11)(p22;q23) translocation [11, 12]. The THP-1 cell line has been used in *in vitro* studies to evaluate the activity of targeted and conventional antileukemic agents [13, 14]. THP-1 cells have also been used by a number of investigators to evaluate the structure and function of ion channels under a wide range of normal and abnormal conditions [15-17].

Extensive research over the past several years has indicated that curcumin (a natural polyphenol derived from the plant *Curcuma longa*) is capable of preventing and treating various cancers [18, 19]. The cytotoxic effect of curcumin specific to AML cells has also been described [20-22]. A wide range of mechanisms have been proposed in these studies to explain the observed activity of curcumin against AML cells. The present study describes a potential role for curcumin in the ion channel mediated growth and survival of the THP-1 experimental model of AML.

Our data show that the addition of curcumin to the extracellular medium strongly depolarized these cells. This finding points to a likely involvement of membrane transport proteins (ion channels) in THP-1 proliferation. We have carried out parallel electrophysiology and target identification experiments to further characterize the ion channels controlling membrane potential in these cells. Our results show that at the transcriptional level, the

voltage gated potassium channel Kv11.1 (also referred to as *ether a go-go related channel*, or *erg*) is highly expressed. Curcumin potently blocks currents carried by Kv11.1.

The analysis of confocal microscopy images of curcumin intrinsic fluorescence, showed that curcumin, once added to the extracellular medium is quickly internalized by the cells, suggesting that its inhibitory effect could result from an intracellular interaction with the target channel. Comparison with normal hematopoietic stem cells CD34+ showed that expression of Kv11.1 and membrane current inhibition by curcumin is more pronounced in the leukemic cell population.

Materials and Methods

Cell culture

THP-1 cells (TIB-202, ATCC) were grown in OPTI-MEM (Gibco) medium containing 10% heat inactivated fetal calf serum (FCS) and 100 units/ml each of penicillin and streptomycin (Gibco) and plated onto glass cover slips in six wells culture plates (Gibco, Carlsbad, CA). Peripheral CD34+ cells were obtained from residual material from mobilized affinity purified cells prepared for hematopoietic stem cell transplant. This material was used after local Institutional Review Board approval and informed consent.

Cell proliferation assay

Cells were cultured in 96 well plates at a density of 10⁴ cells/well. After three days in culture, cell viability was quantified by Alamar Blue Assay (Medicorp, Montreal, QC, Canada) according to the protocol suggested by the manufacturer. Briefly, Alamar Blue is a molecule which changes color according to its oxidation state. When added to the culture in its oxidized form, a fraction of it is reduced by living cells metabolism and consequently changes its color. The ratio between light absorbance at wavelengths corresponding to the colors of the oxidized and reduced form respectively, gives a value proportional to the percentage of Alamar blue reduction and therefore to the proportion of living cells in the culture. Inhibitors were initially dissolved in DMSO. All data was normalized to corresponding DMSO controls.

Quantitative RT-PCR for KCNH isoforms expression and Western Blot

mRNA expression levels of KCNH1, KCNH2, KCNH4 and KCNH5 in THP-1 and CD34+ stem cells were determined using a real-time PCR automated detection system (Biorad, Mississauga Ontario). Quantitect human primer pairs and the Quantitect SYBR Green PCR kit for the Biorad icycler were obtained from Qiagen (Mississauga, Ontario). Harvesting of mRNA from THP-1 cells was accomplished using the Qiagen RNeasy extraction kit. RNA isolated from THP-1 cells was converted to cDNA using the Quantitect Reverse Transcription

kit from Qiagen. Quantitative real time PCR was performed using the SYBR Green PCR kit with GAPDH serving as the reference gene. Three samples were measured for mRNA expression levels. All procedures were made following the manufacturer's protocols. Antibody targeted against KCNH2 were purchased from Abcam (Cambridge, MA, USA, catalog number ab85398) and used in western blot analysis of channel expression in THP-1 and CD34+ cells. Western blots were performed in triplicate.

Solutions and chemicals

The control extracellular solution contained (in mM): NaCl 150, KCl 5.4, CaCl₂ 2, MgCl₂ 1, HEPES 5, Glucose 10 (pH was titrated to 7.4 with NaOH). The K⁺-rich solution was obtained replacing 30 mM NaCl with KCl in the control. Curcumin was purchased from Sigma (Oakville, Ont. Canada). Stock solutions were prepared dissolving curcumin powder in DMSO (50 mM) or ethanol (25 mM, for confocal imaging experiments) and kept at -30°C protected from light. The pipette solution contained: K-aspartate 126, KCl 10, CaCl₂ 0.1, MgCl₂ 1, HEPES 10, EGTA 10, Na-GTP 0.3, Na-ATP 4 (pH was titrated to 7.2 with KOH).

Electrophysiology

Single cell electrophysiological measurements were made with the patch-clamp technique in the whole cell configuration. Patch clamp recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes (resistance 2-5 MΩ) were pulled from borosilicate capillaries using a Narishige pipette puller (Model PP-10). Data acquisition was performed using a Digidata 1322A (Molecular Devices) at a sampling rate of 2.0 KHz with filtering at 1 KHz. Data analysis was carried using OriginPro software (OriginLab, Northampton, MA). Monitoring of membrane cellular potential changes was achieved in continuous recordings of intracellular voltage in zero-current clamp protocols with cells bathed in the control solution, in the K⁺-rich solution and after addition of curcumin respectively.

Confocal imaging

Confocal images were taken with a Zeiss LSM-DUO microscope (Carl Zeiss, Jena, Germany). Curcumin fluorescence was excited at 458 nm using an argon laser at 0.2% maximal power (25 mW). The time course of cellular curcumin uptake was monitored acquiring images at 5 sec intervals after application of curcumin to the extracellular solution. In order to rule out an eventual contribution of DMSO to the fluorescence signal, two series of control experiments were carried out. (1) No fluorescence was observed when cells were exposed to corresponding concentrations of DMSO without curcumin, (2) whereas identical results were obtained when ethanol was used as polar solvent for curcumin instead of DMSO. Due to the weak adhesion of leukemia cells to the culture support, solutions flow during curcumin application often resulted in displacements of the cells out of the visual field, thus preventing the monitoring of fluorescence changes as a function of time.

For this reason, Fluorescence Recovery after photobleaching (FRAP) experiments were also performed as a complementary approach to assess curcumin uptake. Cells

incubated in the presence of curcumin were imaged prior to photobleaching. One cell in the visual field was optically isolated and its fluorescence was photobleached by exposure to 60% intensity laser light for 45 sec. Image sampling of the entire visual field with 0.2% laser power at 5 to 10 sec intervals was then started in order to monitor fluorescence recovery in the photobleached cell. The unbleached cells in the visual field were used as reference to compensate for observed time dependent fluorescence decay.

Statistics

The results are expressed as mean ± standard error. Comparisons of means were made using the two tailed Student's T-test. When applicable, paired T-tests were performed and use of paired on unpaired T-test is indicated in the text. Comparison of current inhibition and curcumin uptake time courses was performed applying the Fisher-Snedecor test [23] to the best fitting exponential functions. The accepted statistical significance level was p<0.05.

The equation used to fit the curcumin dose response relationships was:

$$y = \frac{A_2 + (A_1 - A_2)}{1 + \left(\frac{[c]}{IC_{50}}\right)^p}$$

where A₁ and A₂ are the upper and lower asymptotes respectively, [c] is curcumin concentration, IC₅₀ is the half maximal effect concentration and p represents the slope of the curve at IC₅₀.

Time courses of curcumin uptake and current inhibition were fitted to exponential functions (the sum of two exponential in the case of CD34+ curcumin uptake) of the form:

$$y = 1 - A e^{-\left(\frac{t}{\tau}\right)}$$

in which A is the exponential amplitude and τ is the time constant.

Results

Curcumin and THP-1 cell proliferation

The first aim of this study was to assess the growth inhibitory effects of curcumin on THP-1 cells. Curcumin at increasing concentrations, ranging between 10 pM and 10 μM, was added to the cells and growth inhibition was measured as indicated in materials and methods. Fig. 1A shows the dose response curve obtained. Curcumin elicited cell death and inhibited cell proliferation with an IC₅₀ value of 0.34 ± 0.03 μM.

Curcumin and cell membrane potential

It has been shown previously that cellular membrane potential is a key factor in modulating cell proliferation. Membrane potential in mitotically active cells is generally more depolarized (less negative) than in quiescent cells

[24, 25]. In THP-1 cells the resting membrane potential was -38 ± 3 mV ($n=9$, Fig. 1B). When the K^+ -rich medium was perfused (external solution containing 30 mM K^+), the resting potential depolarized to -29 ± 4 mV ($n=5$, unpaired T-test $p=0.15$). Subsequent addition of 10 μ M curcumin elicited a further depolarization to an average value of -10 ± 2 mV ($n=3$, unpaired T-test $p=0.01$, Fig. 1B,C). As shown in Fig. 1C, at the end of the experiment control of the quality of the recording was made by superfusing the cells with an external solution in which all NaCl was replaced by KCl (KCl 155), which was expected to bring membrane potential values close to 0 mV. As explained in the next section, the K^+ -rich solution was used to enhance the size of K^+ currents and better evidence the effects of curcumin. However, when curcumin was added directly to the control NaCl solution, it elicited an equivalent cell depolarization (data not shown). In non-tumor CD34+ (used as control), the resting potential was -46 ± 3 mV ($n=10$) in control conditions (Fig. 1B). Replacement of the external medium with the K^+ -rich solutions depolarized membrane potential to -29 ± 6 mV ($n=7$, unpaired T-test $p=0.02$) and after addition of curcumin (10 μ M) CD34+ cells membrane potential was -31 ± 2 mV ($n=7$, paired T-test $p=0.70$, Fig. 1B). Thus curcumin had no significant effect on these cells, even in the K^+ -rich condition.

Electrophysiology of the ion channels controlling membrane potential in THP-1

The identity of the ion channel(s) responsible for the control of membrane potential in these cells was then investigated. In a series of whole cell voltage clamp experiments, all THP-1 cells tested showed activity of a voltage gated potassium selective channel as the major membrane current. Fig. 2A shows a typical pattern of currents recorded in cells superfused with the K^+ -rich (30 mM) external solution. This experimental condition was chosen to enhance currents size. The voltage clamp protocol consisted of a 100 ms conditioning pre-pulse to +40 mV from the holding potential of -40 mV, followed by series of 600 ms rectangular voltage steps ranging between -120 and +20 mV with 10 mV increments (Fig. 2A, inset). This protocol activated a voltage and time dependent membrane current (Fig. 2A). These currents activated rapidly, reached a peak and inactivated to reach a steady-state late current level at the end of the 600 ms pulse. Kinetics of activation and inactivation were faster at hyperpolarizing voltage pulses: the time between the stimulus onset and the peak was 6 ms at -120 mV and gradually increased to 20 ms at -50 mV. This behavior is

Fig. 1. Effect of curcumin on cell proliferation and membrane potential. (A) Dose response relationship of curcumin effects on proliferation of THP-1 cells in culture. The dashed line is the best fit curve to a dose response equation (see Methods). Data was normalized to corresponding DMSO controls. (B) Resting membrane potential in THP-1 and CD34+ cells in control (physiological extracellular NaCl solution, CTL), in K^+ -rich (30 mM KCl containing external solution) conditions and after addition of 10 μ M curcumin to the K^+ -rich solution (* = $p < 0.05$). (C) Whole cell current clamp recording of a single THP-1 cell membrane potential in K^+ -rich conditions, after addition of 10 μ M curcumin to the K^+ -rich solution and after complete replacement of NaCl with KCl in the external medium (KCl 155). Note that NaCl for KCl replacement, brings membrane potential close to 0 mV. This was used as a control maneuver for the quality of the recording.

Fig. 2. Characterization of ion channels in THP-1 and CD34+ cells. (A) Voltage clamp recording of typical THP-1 whole cell currents after these cells were exposed to the K⁺-rich (30 mM) extracellular solution. Under these conditions, inwardly directed currents, reflecting net influx of K⁺ ions into the cytoplasm, were enhanced with respect to the control NaCl condition. Time and voltage dependent currents could then be observed in 100% recordings from THP-1 under these conditions. The inset describes the voltage clamp protocol. (B) Whole cell currents recorded from CD34+ cells under the same conditions. (C) Quantitative RT-PCR results illustrating transcript expression levels of Kv10.1 (KCNH1), Kv11.1 (KCNH2), Kv12.3 (KCNH4) and Kv10.2 (KCNH5) in THP-1 and CD34+ cells. (D) Western blot analysis performed on THP-1 and CD34+ cells using a monoclonal antibody directed against the KCNH2 gene product Kv11.1. The arrow indicates the Kv11.1 signal at 127 kDa.

characteristic of the voltage gated potassium channel Kv11.1, also referred to as *ether a go-go related* channel or *erg*. In similar electrophysiological voltage clamp experiments performed on the non-tumor bone marrow stem cells CD34+ (Fig. 2B) no such time and voltage dependent K⁺ current was observed.

Molecular identity of the THP-1 K⁺ channel

Quantitative RT-PCR was used to assess the mRNA expression levels of channels of the *ether a go-go* family in the THP-1 and the non-tumor bone marrow cells (Fig. 2C). Primer pairs targeted against KCNH1 (Kv10.1),

KCNH2 (Kv11.1), KCNH4 (Kv12.1) and KCNH5 (Kv10.2) potassium channels, belonging to the K⁺ channel families Kv10, Kv11 and Kv12 were used. These results revealed that Kv11.1 (gene KCNH2) was the most highly expressed transcript in THP-1. Note that Kv10.2 (KCNH5) is also expressed but at a level 40-fold lower than Kv11.1. In the bone marrow non-tumor cells, mRNA expression levels of all of these channels were undetectable (Fig. 2C). These results therefore point to Kv11.1 as the major contributor to ionic permeability of THP-1 cell membranes. As such, it may be the major player in the control of the membrane potential in these cells.

Fig. 3. Effect of curcumin on whole cell membrane currents in THP-1 and CD34+ cells. (A) Voltage clamp recording showing block of Kv11.1 currents in THP-1 cells by 10 μ M curcumin as recorded in the K⁺-rich (30 mM) external solution. The voltage clamp protocol consisted of a conditioning prepulse to +40 mV from the initial holding potential of -40 mV, followed by a hyperpolarizing pulse to -80 mV. The current recorded in physiological control solution (K⁺ concentration 5.4 mM) is used as control level. (B) Dose response relationships of the differential effect of curcumin on peak and steady state late Kv11.1 currents in THP-1 cells. Dashed lines are best fits to dose response curves. (C) Voltage clamp recordings of currents from CD34+ cells in K⁺-rich conditions and after application of 10 μ M curcumin. (D) Comparison of current densities recorded on THP-1 and CD34+ cells at -80 mV (steady state late currents are reported for THP-1) in control, K⁺-rich (KCl 30) conditions and after addition of 10 μ M curcumin to the K⁺-rich external medium (* = $p < 0.05$).

Western blots were also run on THP-1 and CD34+ cells to test the expression of Kv11.1 protein. As shown in Fig. 2D, a band at a molecular weight close to 130 kDa was identified in THP-1. This was not present in CD34+. This band is consistent with the predicted molecular weight for Kv11.1 which is 127 kDa.

Effect of Curcumin on Kv11.1 activity

Addition of curcumin to the external K⁺-rich solution blocked Kv11.1 currents in a dose dependent manner. Fig. 3A shows that 10 μ M curcumin significantly reduced the overall Kv11.1 current. Peak current was reduced

by approx 70% and the steady state or late current was reduced to a level close to the control NaCl solution. Fig. 3B shows the outcome of dose response experiments. Curcumin inhibited peak currents with an IC_{50} of 2.0 ± 0.5 μ M, and late currents with an IC_{50} of 1.3 ± 0.1 μ M ($n=5$). Similar electrophysiology experiments showed that curcumin did not significantly affect membrane currents in CD34+ cells (Fig. 3C). In Fig. 3D, the current density at -80 mV in THP-1 (steady state currents) and CD34+ cells are compared under three different experimental conditions. In control, current densities were of 0.8 ± 0.2 pA/pF in CD34+ and of 0.5 ± 0.2 pA/pF in THP-1. A

change to the K⁺-rich external medium increased the current densities to approx. 2 pA/pF in both cells lines (2.2±0.5 in CD34+ and 1.9±0.2 in THP-1, paired T-test p=0.03 and 0.01 respectively). This observation and the fact that the K⁺-rich solution depolarized CD34+ membrane potential, indicates the activity of some background time and voltage independent und K⁺ channels in these cells, whose identity is yet to be assessed. Addition of 10 μM curcumin had no effect on CD34+ (2.5±0.7 pA/pF, paired T-test p=0.48) but significantly reduced the current density in THP-1 cells (1.1±0.2 pA/pF, paired T-test p=0.01).

Curcumin uptake by cells

To begin to understand the mechanism of this ion channel block, the site of action of block needs to be identified. This site can be either intracellular or extracellular. To explore the localization of Kv11.1 interaction with curcumin, we took advantage of the intrinsic fluorescence of curcumin [26]. Confocal microscopy imaging (Fig. 4) showed that when applied to THP-1 (14 cells were observed) or CD34+ (16 cells observed) cells, curcumin is internalized by the cells. This is expected based on its high hydrophobicity. Soon after application by superfusion, its distribution appears essentially intracellular. It is therefore likely that the inhibitory effect of curcumin on Kv11.1 takes place via an interaction with an intracellular epitope of these K⁺ channels. The time course of curcumin internalization was also studied by confocal microscopy. Fig. 5A shows images from one representative experiment in which the dynamics of curcumin uptake by THP-1 cells was monitored at five seconds intervals (the * symbol indicates the moment of curcumin application). In Fig. 5C, the average time course of fluorescence intensity increases within the cytoplasm was plotted for THP-1 (n=4) and CD34+ (n=5) cells and these data are compared with the time course of K⁺ current inhibition by curcumin at equal concentratin (50 μM) in THP-1 cells. These experimental results were fitted to exponential functions. In THP-1 cells, a single exponential function with a time constant of 26±1 s (n=4) provided a good fit of the experimental data. This value was not significantly different from the time constant of current inhibition which was 34±3 s (n=5, Fisher-Snedecor test p=0.12). For the CD34+ cells, a double exponential function was required, with time constants of 16±1 and 200±81 s respectively (n=5). From the analysis of the data presented in Fig. 5C, it can be seen that in both cases 80% of the maximal curcumin uptake occurred in approx 45 seconds.

Fig. 4. Internalization of curcumin. The intrinsic fluorescence of curcumin allowed the cellular distribution of this compound to be monitored using confocal microscopy imaging. After exposure of THP-1 (image A) and CD34+ (image D) cells to solutions containing 50 μM curcumin, intracellular fluorescence could be detected and increased rapidly with time of exposure. Panels B and E show fluorescence signals in THP-1 (n=14) and CD34+ (n=16) cells respectively approx. 5 min after curcumin application. Note that the intracellular distribution of curcumin appears to be perinuclear, as shown by the merged fluorescence and transmitted light images in panels C (THP-1) and F (CD34+).

As mentioned in the Methods Section, in order to avoid cell movements during curcumin perfusion, the FRAP (Fluorescence Recovery After Photobleaching) approach was also used to monitor curcumin internalization. This approach entails monitoring of uptake of fluorescent curcumin after the fluorescence of this compound internalized in a selected cell is bleached by short high intensity illumination. Typically, fluorescence

Fig. 5. Time course of curcumin uptake. (A) Sequence of confocal images of fluorescence signal from two THP-1 cells (shown in panel B under transmitted light) illustrating progressive curcumin uptake. Images were acquired at 5 s interval. The * symbol indicates the time point when curcumin (50 μ M) was added to the external medium. (C) Time course of curcumin uptake in THP-1 (black, n=4) and CD34+ (red, n=5) cells as calculated by the increase in intracellular fluorescence intensity. Continuous lines are best fits to exponential functions. The blue open circles represent the average time course of K⁺ current inhibition by 50 μ M curcumin in THP-1 cells (n=5). The arrow indicates curcumin application.

recovery was monitored with images taken at five second intervals. Selected representative images are shown in Fig. 6A. The graph in Fig. 6C shows averaged results from three independent experiments on THP-1 cells. This experimental data was best fit to a single exponential function characterized by a time constant of 87 ± 3 s.

In CD34+ cells, fluorescence recovery signals did not reach a steady state value and increased to levels even higher than the control before photobleaching. This could probably be ascribed to damages to cell membranes resulting from the intense laser light exposure. These results therefore were not taken into account.

Fig. 6. FRAP imaging of curcumin uptake. (A) Sequence of fluorescence recovery after photobleaching (FRAP) images of a THP-1 cell (panel B). Curcumin fluorescence in the selected cell was photobleached by exposure to 60% power laser light for 45 s. Fluorescence recovery therefore represents the subsequent progressive increase in new fluorescent intracellular curcumin molecules. (C) Time course of curcumin (50 μ M) uptake after photobleaching in THP-1 cells (n=3). Continuous line is the best fit to a single exponential function. Signals were normalized to the intensity of fluorescence in the selected cells before photobleaching.

Discussion

The role of ion channels in the development of tumors is receiving increasing emphasis in the field of oncology [5, 27, 28]. Ion channels play distinct and critical functions in cellular proliferation [29], migration [30] and in the crosstalk between cells and the tumor microenvironment [31]. Recent studies have shown that the ion channel Kv11.1 is important in the pathogenesis of various malignancies. For example, a close correlation between

density of expression of Kv11.x channel and cell cycle progression has been reported in various tumor cells [32–34]. Inhibition of Kv11.1 by specific blockers such as imipramine and astemizole, has been shown to affect cancer cell proliferation [35, 36].

In this study we used the pediatric AML cell line THP-1 cells, derived originally from an infant diagnosed with AML, as an experimental model for this disease. These cells have been found to express Kv11.1 as the main potassium selective channel. As such, it is a principal

contributor to the control of membrane potential. This channel was not found in the non-tumor hematopoietic stem cells used as control. These findings are consistent with a previous report in which Kv11.1 expression was identified in primary human acute myeloid leukemia cells and not in circulating progenitor CD34+ cells [37].

Data from our studies clearly show that curcumin inhibits the proliferation of the AML cell line. Curcumin is known to have many therapeutic properties. These include its activity as an antioxidative, anti-inflammatory and antiviral agent (reviewed in [38]) as well as its chemoprotective and anticancer characteristics [18, 19, 39]. Recent studies have shown the effect of curcumin on various ion channels [40-42]. We also showed that curcumin has important effects on the electrophysiological activity of THP-1 cells but not on non-tumor CD34+ cells. Curcumin potently blocked Kv11.1 and consequently strongly depolarized THP-1 cell membrane potential. Important changes in membrane potential controlled by the activation of specific ion channels occur during the different phases of cell cycle and thus participate in the signaling mechanisms underlying cell proliferation. Block of specific ion channels may then affect cell cycle in different ways. The physiological membrane potential oscillations that take place during the cell cycle are interrupted and the intracellular signaling pathways, such as intracellular calcium modulation and signaling, can be altered. Alternatively, direct interactions between the channel and cell cycle proteins can be compromised [24, 25]. This possibility is supported by the finding that the expression of Kv11.1 appears to correlate with stages of cell cycle progression [32].

In general, cells which are dividing quickly can be characterized by more depolarized resting potentials than non-dividing cells [24, 25]. Our results are consistent with this finding. Curcumin further depolarized THP-1 cells but failed to show this effect on control cells, indicating a selective interaction of curcumin with Kv11.1. Our confocal microscopic imaging results clearly demonstrate that curcumin can be very quickly internalized by the cells, probably due to its high hydrophobicity. The time course of cytoplasmic fluorescence intensity increase indicates that uptake occurred in less than a minute and it correlated with the time course of current inhibition. In the FRAP experiments however, the time course of fluorescence recovery was approximately 4-fold slower. This is due to the fact that cells were already loaded with non-fluorescent curcumin and the uptake of new fluorescent molecules took place against a strongly unfavorable concentration gradient. These results do not allow the

assessment of the position of channel-curcumin interaction site but, although an extracellular target cannot be ruled out, they might suggest that blocking of Kv11.1 could rely on the interaction between curcumin and an intracellular epitope of this channel. This phenomenon becomes an important issue when safety and potential side effects are considered [38, 43, 44]. In fact, Kv11.1 channels are widely expressed in excitable cells. They contribute to the repolarization phase of the action potential in the heart, smooth muscles and neurons. In the heart, curcumin was reported to have antiarrhythmic properties and cardioprotective effects in cases of heart failure, diabetic cardiovascular complications and hypertrophy [44-48]. A drawback was also reported though, in which curcumin was shown to increase doxorubicin-induced apoptosis in embryonic rat heart cells [49].

A plausible explanation for the differential effect of curcumin in cells expressing Kv11.1 could be related to the fact that two splice variants of this protein exist. These isoforms are commonly termed *erg1a* (the full length protein of 127 kDa molecular weight) and *erg1b* (an n-terminus truncated isoform of 90 kDa), and often Kv11.1 channels are *erg1a/erg1b* heteromers [50-52]. Our results show that only *erg1a* was expressed in THP-1 cells and Kv11.1 in these cells is therefore likely to be a homotetramer of *erg1a* subunits. Since in all voltage dependent potassium channels both the n- and c-terminus regions of the protein are intracellular, it is possible that small structural differences in the cytoplasmic region arising from varied stoichiometric combinations of *erg1a* and *erg1b* subunits give rise to different affinities for curcumin. Alternatively, Kv11.1 channels could be part of tissue specific multiprotein complexes (e.g. with integrins and chemokine receptors [6]) leading to differential sensitivities to curcumin. It is also possible however that curcumin could simultaneously affect other tissue specific ion channels, thus producing compensatory effects.

We show experimental evidence that the resting membrane potential of THP-1 cells is controlled mainly by the activity of Kv11.1. Curcumin, a highly hydrophobic compound, is quickly internalized by the cells, and it appears to interact with an intracellular epitope of Kv11.1 channels. This interaction results in blocking of the channel activity which, in turn depolarizes the resting potential. In summary, we provide, as a proof-of-concept, a potential mechanistic explanation for the activity of curcumin in AML cells. Further studies are needed to validate this finding in conditions of the tumor microenvironment, such as higher serum concentrations and pH values of the

tumor milieu. Finally, experiments using relevant primary leukemia specimens and xenograft studies are needed to provide additional information on the stability, bioavailability and non-specific toxicity of curcumin in the pediatric clinical setting. Nevertheless, the data presented herein provides essential primary information to further explore the utility of targeting anomalous ion channels in novel therapeutics development for AML.

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