Magnesium lithospermate B extracted from *Salvia miltiorrhiza* elevates intracellular Ca\(^{2+}\) level in SH-SY5Y cells

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**Aim:** To examine if magnesium lithospermate B (MLB), a potent inhibitor of Na\(^+\)/K\(^+\)-ATPase, leads to the elevation of intracellular Ca\(^{2+}\) level as observed in cells treated with cardiac glycosides.

**Methods:** Viability of SH-SY5Y neuroblastoma cells treated with various concentrations of ouabain or MLB was measured. Intracellular Ca\(^{2+}\) levels were visualized using Fluo4-AM (fluorescent dye) when cells were treated with ouabain or MLB in the presence or absence of KB-R7943 (Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor) and 2-APB (IP\(_3\) receptor antagonist). Molecular modeling was conducted for the docking of ouabain or MLB to Na\(^+\)/K\(^+\)-ATPase. Changes of cell body and dendrite morphology were monitored under a microscope.

**Results:** Severe toxicity was observed in cells treated with ouabain of concentration higher than 1 µmol/L for 24 h while no apparent toxicity was observed in those treated with MLB. Intracellular Ca\(^{2+}\) levels were substantially elevated by MLB (1 µmol/L) and ouabain (1 µmol/L) in similar patterns, and significantly reduced in the presence of KB-R7943 (10 µmol/L) or 2-APB (100 µmol/L). Equivalent interaction with the binding cavity of Na\(^+\)/K\(^+\)-ATPase was simulated for ouabain and MLB by forming five hydrogen bonds, respectively. Treatment of ouabain (1 µmol/L), but not MLB (1 µmol/L), induced dendritic shrink of SH-SY5Y cells.

**Conclusion:** Comparable to ouabain, MLB leads to the elevation of intracellular Ca\(^{2+}\) level presumably via the same mechanism by inhibiting Na\(^+\)/K\(^+\)-ATPase. The elevated Ca\(^{2+}\) levels seem to be supplied by Ca\(^{2+}\) influx through the reversed mode of the Na\(^+\)/Ca\(^{2+}\) exchanger and intracellular release from endoplasmic reticulum.

**Keywords:** intracellular calcium; cardiac glycoside; magnesium lithospermate B; Na\(^+\)/K\(^+\)-ATPase; ouabain; SH-SY5Y neuroblastoma cells

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**Introduction**

Gradients of Na\(^+\) and K\(^+\) across the plasma membrane of animal cells are important for maintaining membrane potentials, cell volume, and active transport of other solutes. Na\(^+\)/K\(^+\)-ATPase, an intrinsic ion transporter on the plasma membrane of animal cells, belongs to the family of P-type cation transporters, and generally consists of a heterodimer of α- and β-subunits. It pumps 3 Na\(^+\) ions out of and 2 K\(^+\) ions into the cells at the expense of hydrolyzing one ATP, and thus maintains the gradients of Na\(^+\) and K\(^+\) ions across the cell membrane. For the continuous exchange of Na\(^+\) and K\(^+\) across the membrane, Na\(^+\)/K\(^+\)-ATPase actively consumes 20%–30% of ATP energy generated in animal cells at rest.

Cardiac glycosides, such as ouabain and digoxin, are steroid-like compounds and have been used in the treatment of congestive heart failure. The therapeutic effect of cardiac glycosides lies in their reversible inhibition on the Na\(^+\)/K\(^+\)-ATPase of myocardium. An inhibition on Na\(^+\)/K\(^+\)-ATPase leads to the elevation of intracellular Na\(^+\) concentration, which in turn activates a Na\(^+\)/Ca\(^{2+}\) exchanger resulting in an increase of intracellular Ca\(^{2+}\) level. The elevated intracellular Ca\(^{2+}\) level induces positive inotropy that eventually accentuates the force of myocardial contraction.

Danshen, the dried roots of medicinal plant *Salvia miltiorrhiza*, is one of the most popular Chinese herbal products. Traditionally regarded as an effective medicine for the promotion of blood circulation, danshen has been extensively used in the treatment of cardiac and cerebrovascular diseases. Magnesium lithospermate B (MLB), a derivative of caffeic...
acids and the major soluble ingredient in danshen, has been demonstrated to possess several medicinal effects, such as vasodilating, antihypertensive, antioxidantive, and free radical scavenging activities\(^7,8\). Recently, MLB was suggested to be responsible for the cardiac therapeutic effect of danshen by its effective inhibition on Na\(^+\)/K\(^-\)-ATPase via the same molecular mechanism triggered by cardiac glycosides\(^9\). Whether MLB leads to the elevation of intracellular Ca\(^{2+}\) level in cells as observed when they are treated with cardiac glycosides has not been verified.

In this study, SH-SY5Y neuroblastoma cells were employed to examine if MLB treatment may lead to an elevation of intracellular Ca\(^{2+}\) level. Moreover, a Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor, KB-R7943, and an IP\(_3\) receptor antagonist, 2-APB, were utilized to assess possible intracellular and extracellular sources for the fluctuating cytosolic Ca\(^{2+}\) level in SH-SY5Y cells treated with MLB and ouabain. Molecular modeling and docking of ouabain and MLB to Na\(^+\)/K\(^-\)-ATPase were exhibited to compare their inhibitory potency at molecular level. Cell viability (toxicity) as well as changes of cell body and dendrite morphology was observed in SH-SY5Y cells.

**Materials and methods**

**Chemicals and reagents**

Penicillin, streptomycin, RPMI (Roswell Park Memorial Institute) medium 1640, and calcium free HBSS (Hanks Balanced Salt Solution) buffer were purchased from Gibco (Grand Island, NY, USA). Ouabain, dimethyl sulfoxide (DMSO) and 2-aminomethyl diphenylborinate (2-APB) were supplied from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), fluo-4 acetoxyethyl ester (Fluo4-AM), and 2-[2-(4-(nitrobenzyloxy)phenyl)ethyl]isothiourea methanesulfonate (KB-R7943) were purchased from USB corporation (Cleveland, Ohio, USA), Biological Industries (Israel), Molecular Probe (Eugene, Oregon, USA), and Calbiochem (Darmstadt, Germany), respectively. Magnesium l-thiospermine B (MBL) was extracted from roots of danshen (Salvia miltiorrhiza) and purified as described previously\(^9\). Glass bottom culture dishes (35 mm) were obtained from MatTek (Ashland, MA, USA).

**Cell cultures**

The human adrenergic neuroblastoma cell line, SH-SY5Y\(^10\) was kindly provided by Dr Tin-ynu HO of the Graduate Institute of Chinese Medical Science, China Medical University, Taiwan, China. SH-SY5Y cells grown in RPMI-1640 culture medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin were maintained at 37°C in a humidified atmosphere of 95% air/5% CO\(_2\) and passages were performed every other day by trypsinization. For MTT staining, cells were plated into 24-well culture plates at a density of 1×10\(^5\) cells. For fluorescence imaging of Ca\(^{2+}\) level and calculation of cell volume, cells were plated in 35 mm glass bottom culture dishes and grown to 80% confluency (approximately 48 h).

**Cell viability assay**

SH-SY5Y cells treated with ouabain or MLB of concentrations ranging from 10 nmol/L to 100 µmol/L for 5, 10, 30, 60 min, and 24 h were subjected to cell viability assay by MTT staining\(^11\). Cells were added with MTT to a final concentration of 500 µg/mL, and incubated at 37°C for 2 h. After MTT removal, cells were lysed with DMSO. Absorbance was measured using SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. Control cells were treated in the same way without adding ouabain or MLB. Viability was expressed in percentage as the absorbance value of cells treated with ouabain or MLB over that of control cells.

**Intracellular Ca\(^{2+}\) imaging**

Fluctuation of the intracellular Ca\(^{2+}\) level of SH-SY5Y cells was tracked and visualized by a preloaded fluorescent Ca\(^{2+}\)-sensitive dye, Fluo4-AM\(^12\). Cell-permeable Fluo4-AM was dissolved in DMSO to a concentration of 3 mmol/L, and then further diluted to 3 µmol/L in cell media. The cells were washed once with the culture medium (145 mmol/L NaCl, 5 mmol/L KCl, 2.6 mmol/L CaCl\(_2\), 1 mmol/L MgCl\(_2\), 10 mmol/L HEPES-Na, and 5.6 mmol/L glucose adjusted to pH 7.4 with HCl) and added with the culture medium supplemented with 3 µmol/L. Fluo4-AM for 30 min in a humidified 5% CO\(_2\) incubator at 37°C. After washed with the culture medium, cells were added with 1 µmol/L of ouabain or MLB, and Ca\(^{2+}\) fluorescence imaging was monitored at different intervals for 30 min. To examine the possible sources for the elevated cytosolic Ca\(^{2+}\) level in SH-SY5Y cells, 10 µmol/L of KB-R7943 (Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor) or 100 µmol/L of 2-APB (IP\(_3\) receptor antagonist) was added to cells and incubated for 5 or 15 min before the loading of ouabain or MLB in the Ca\(^{2+}\) imaging detection\(^13,14\). KB-R7943 was prepared in ethanol to a concentration of 10 mmol/L, further diluted to 1 mmol/L in Ca\(^{2+}\) free HBSS, and then finally diluted to 10 µmol/L in the culture medium. 2-APB of 100 µmol/L was prepared in the culture medium. For Ca\(^{2+}\) imaging, culture dishes with adherent cells were mounted in the MIU-IBC CO\(_2\) incubation system (Olympus, Tokyo, Japan) and placed on the microscope. Time-lapse images of live cells loaded with Fluo4-AM were taken with an UPlanSapo 60×/1.35 oil immersion objective lens, and collected by the Fluoview 1000 confocal scanning microscopy (Olympus, Tokyo, Japan).

**Digital image processing**

Images collected at different time intervals were processed using the Olympus FV1000 software and NIH ImageJ program (v 1.40) (Bethesda, Maryland, USA). The pictures were acquired at 512×512 pixels, and analyzed frame by frame with a Time Series Analyzer\(^15,16\). This plugin was used to analyze time-lapse image stacks. Each cell was chosen as a region of interest (ROI) through mouse click and its fluorescence intensity of each time point was measured.

**Molecular modeling and docking**

The crystal structure of shark rectal gland Na\(^+\)/K\(^-\)-ATPase
(PDB code 3A3Y) was downloaded from Protein Data Bank. In order to facilitate the docking process, the β and γ subunits of the Na⁺/K⁺-ATPase were removed, as well as the water molecules and counter-ions surrounding the remaining α subunit. After hydrogen saturation, the modified Na⁺/K⁺-ATPase was minimized with CHARMM force field using the Discover Studio 2.1 package. The 2D structures of ouabain and MLB were constructed by using the ChemDraw program, and their corresponding 3D structures were converted by the Chem3D program. The binding pocket of the Na⁺/K⁺-ATPase α subunit was defined as ouabain occupancy site in the structure of Na⁺/K⁺-ATPase-ouabain complex. Docking of MLB was performed in silico by employing the LigandFit module in the Discover Studio 2.1 package. The protein-ligand complexes generated by LigandFit were further minimized with CHARMM force-field by Smart Minimizer algorithm. Among the candidate structures, reported by the docking simulation, the docking structure with the highest Ligscore1 value, as computed by the score ligand pose module, was selected to represent MLB inside the binding pocket.

**Measurement of relative cell volume**

To measure volume of SH-SY5Y cells in different treatments, dishes were mounted in the MIU-IBC CO₂ incubation system and placed on the microscope with a differential interference contrast (DIC) mode. The cell profile was viewed through Nomarski optics with a 60× objective (NA, 1.35) under oil immersion and their images were captured consequently. By using the Olympus FV1000 software, ROI was drawn around the cell body or dendrites of each cell. The selected areas were measured. The fractional change in cell volume within each ROI, was expressed as [volume30 min/volume0 min] % where volume0 min and volume30 min represented the cell volume before and after treatment of ouabain or MLB for 30 min, respectively.

**Statistical analysis**

Data were expressed as mean±SEM. Relative values for data were compared using analysis of variances (ANOVA) and t-test on SigmaPlot 2001 for Windows version 7.0 (SSI, San Jose, USA). Differences were considered statistically significant at P<0.05.

**Results**

**Effects of MLB and ouabain on viability of SH-SY5Y cells**

Viability of SH-SY5Y cells treated with various concentrations (0.01–100 µmol/L) of MLB or ouabain for 5, 10, 30, 60 min, and 24 h was examined (Figure 1). No apparent MLB toxicity to the cells was observed except for a partial reduction of viability when they were treated with 100 µmol/L of this compound for more than 60 min. In contrast, cell viability gradually decreased in a dose-dependent manner when cells were treated with ouabain in the range of 1–100 µmol/L within 60 min, and severe toxicity was observed in cells treated with ouabain of concentration higher than 1 µmol/L for 24 h. On the basis of the above observation, cells were treated with 1 µmol/L of MLB or ouabain for 30 min in the following experiments.

**Effects of MLB and ouabain on intracellular Ca²⁺ levels in SH-SY5Y cells**

To examine the effects of MLB and ouabain on their intracellular Ca²⁺ levels, SH-SY5Y cells were preloaded with Fluo4-AM, incubated with 1 µmol/L of MLB or ouabain, and monitored for their intracellular fluorescence fluctuation at different intervals for 30 min. Compared with cells treated with buffer alone (control), SH-SY5Y cells treated with either MLB or ouabain displayed significantly elevated fluorescence intensity that reached maximum approximately 5-10 min after treatment (Figure 2). These results indicate that MLB and ouabain increased the intracellular Ca²⁺ levels of SH-SY5Y cells in similar patterns.

**Effects of KB-R7943 and 2-APB on the elevation of Ca²⁺ levels of MLB- and ouabain-treated SH-SY5Y cells**

To evaluate the possible calcium sources for the elevation of cytosolic Ca²⁺ levels in SH-SY5Y cells treated with MLB and ouabain, a Na⁺/Ca²⁺ exchanger inhibitor, KB-R7943, or an IP₃ receptor antagonist, 2-APB, was added to cells before loading MLB and ouabain. Significant reduction of fluorescence inten-
In SH-SY5Y cells treated with either MLB or ouabain, an increase in intracellular Ca\(^{2+}\) levels was observed in the presence of KB-R7943 (Figure 3) or 2-APB (Figure 4). These results suggest that the elevated intracellular Ca\(^{2+}\) levels in SH-SY5Y cells treated with either MLB or ouabain were possibly supplied by extracellular Ca\(^{2+}\) influx through the Na\(^+\)/Ca\(^{2+}\) exchanger on the plasma membrane and intracellular Ca\(^{2+}\) release via the IP\(_3\) receptor channel on the membrane of endoplasmic reticulum (ER).

**Docking of MLB to Na\(^+\)/K\(^{-}\)-ATPase**

To compare the inhibitory potency of ouabain and MLB on Na\(^+\)/K\(^{-}\)-ATPase at molecular level, both compounds were subjected to molecular modeling and docking to the extracellular domain of Na\(^+\)/K\(^{-}\)-ATPase \(\alpha\) subunit. The results showed that MLB could be localized in the ouabain binding pocket of Na\(^+\)/K\(^{-}\)-ATPase, and that equivalent interaction with the binding cavity of Na\(^+\)/K\(^{-}\)-ATPase was observed for ouabain and MLB by forming five intermolecular hydrogen bonds (H-bonds), respectively (Figure 5). Detailed analyses showed that three H-bonds are formed between the lactone of ouabain and Ile328 (forming one H-bond) and Ala330 (forming two H-bonds) of Na\(^+\)/K\(^{-}\)-ATPase, and two H-bonds between the hydroxyl group at C-14 of ouabain and Thr804 of Na\(^+\)/K\(^{-}\)-ATPase. In contrast, three H-bonds are formed between the hydroxyl group at C-4’ position of MLB and Lys912 (forming two H-bonds) and Glu915 (forming one H-bond) of Na\(^+\)/K\(^{-}\)-ATPase, one H-bond between the carbonyl group at C-9’ position of MLB and Thr804 of Na\(^+\)/K\(^{-}\)-ATPase, and one H-bond between the hydroxyl group at C-4”’ position of MLB and Leu110 of Na\(^+\)/K\(^{-}\)-ATPase. Similar to the hydrophobic steroidal core of ouabain, the four aromatic rings of MLB form strong hydrophobic interaction with hydrophobic residues (Leu132, Tyr315, Ile322, Phe323, Ile325, Phe793, Ile794, and Leu802) around the binding pocket of Na\(^+\)/K\(^{-}\)-ATPase.

**Effects of MLB or ouabain treatment on cell and dendrite morphology**

Changes of cell body and dendrite morphology after treated with 1 \(\mu\)mol/L of MLB or ouabain for 30 min were calculated.
by measuring the volumes of cell body and dendrites before and after treatment over the same location. Similar to cells of the control group, no significant changes were observed in the volumes of cells treated with MLB or ouabain (Figure 6, left panels). However, a significant shrink of dendrite volume was noticed in cells treated with ouabain, but not in those treated with MLB and those of the control group (Figure 6, right panels). These results suggest that treatment of ouabain, but not MLB, induced dendritic shrink of SH-SY5Y cells under our experimental conditions.

**Discussion**

In a previous study, we demonstrated that MLB could produce potent inhibition of Na⁺/K⁺-ATPase in vitro, and proposed that its cardiac therapeutic effects could have been due to the same molecular mechanism as triggered by cardiac glycosides[9]. In this study, we demonstrated that intracellular Ca²⁺ levels of SH-SY5Y cells treated with MLB were substantially elevated in a manner similar to that observed in cells treated with ouabain, a cardiac glycoside. Molecular modeling showed that equivalent hydrogen bonding and hydrophobic interaction were observed for ouabain and MLB when these two compounds bound to the cavity of Na⁺/K⁺-ATPase, and the results were in agreement with our previous observation that both ouabain and MLB possessed strong inhibitory potency on Na⁺/K⁺-ATPase[9]. Evidently, the results reinforce our previous proposition that MLB, being a potent inhibitor of Na⁺/K⁺-ATPase, acts as the active component responsible for the cardiac therapeutic effect of danshen via the same physiological responses subsequently activated by effective inhibition of cardiac glycosides on Na⁺/K⁺-ATPase.

Calcium signals are mostly delivered as brief transients that are often organized into regulatory oscillations[24]. Fluctuation of cytosolic Ca²⁺ level is generally a coordinated consequence of a number of molecular cascade reactions responsible for Ca²⁺ influx and efflux in exchange with either extracellular space or intracellular ER storage compartment[25]. Free Ca²⁺ enters the cells through either voltage-gated channels or receptor-operated channels located in their plasma membrane[26, 27]. However, much of the signal Ca²⁺ comes from the
intracellular Ca\(^{2+}\) sources, and is primarily released via the IP\(_3\) receptor channel on the ER membrane\[^{28, 29}\]. According to the reduction effects of two inhibitors KB-R7943 and 2-APB on the intracellular Ca\(^{2+}\) levels elevated by the treatment of either MLB or ouabain (Figures 3 and 4), the elevation of cytosolic Ca\(^{2+}\) level was likely supplied by both Ca\(^{2+}\) influx through the reversed mode of the Na\(^+\)/Ca\(^{2+}\) exchanger and intracellular release from ER storage compartment. Whether other sources are also involved in the elevation of intracellular Ca\(^{2+}\) levels in SH-SY5Y cells treated with MLB or ouabain has not been evaluated.

Na\(^+\)/K\(^+\)-ATPase is well known for its role as a maintainer of electrolyte and fluid balance in cells, organs and whole body. More and more findings indicate that Na\(^+\)/K\(^+\)-ATPase can be a drug target for the treatment of several diseases, including congestive heart failure, ischemic stroke, neurodegenerative diseases and even cancer\[^{30–32}\]. These therapeutic effects may be possibly resulted from the inhibition of Na\(^+\)/K\(^+\)-ATPase leading to the fluctuation of Ca\(^{2+}\) level, which in turn activates diverse physiological responses in different cells in variable microenvironments\[^{33, 34}\]. However, the inhibition of Na\(^+\)/K\(^+\)-ATPase may also lead to the fluctuation of other cations presumably via cross-talks held among different ion channels\[^{35, 36}\]. Therefore, the possibility that some pharmacological effects following Na\(^+\)/K\(^+\)-ATPase inhibition may be generated by biological reactions insensitive to Ca\(^{2+}\) concentration should not be ruled out.

Dendrite structure is correlated to neuronal function, and its degeneration is generally regarded as an early indicator of cell damage\[^{37}\]. In the current study, dendritic shrink was observed in SH-SY5Y cells treated with ouabain, but not in cells treated with MLB (Figure 6); and this observation was in agreement with the toxicity of SH-SY5Y cells treated with high dosages of ouabain, but not with MLB in the viability assay (Figure 1). Similar cell toxicity caused by ouabain at high concentrations has also been noticed in other studies, and the toxicity is blamed to ouabain for its putative triggering of several signaling cascade responses that lead to cell death\[^{38}\]. In contrast, MLB is generally regarded as a non-toxic antioxidant, and has also been shown to possess neuroprotective effects against ischemic stroke in a brain slice assay model\[^{39}\]. Moreover, water extraction of Salvia miltiorrhiza containing mainly MLB has no toxicity to neonatal rat cardiomyocytes at dosage of 5–80 µg/mL\[^{39}\]. Taken together, we surmise that MLB has a great potential, after clinical trials, to become a safer drug than cardiac glycosides.

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Author contribution
Ruey-jane FAN and Jason TC TZEN designed research and wrote the paper; Yi-ching CHEN and Tse-yu CHUNG performed research; Tzyy-rong JINN and Feng-yin LI contributed new analytical tools and reagents.

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