

Original Article

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

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**Aim:** To examine if magnesium lithospermate B (MLB), a potent inhibitor of  $\text{Na}^+/\text{K}^+$ -ATPase, leads to the elevation of intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels were visualized using Fluo4-AM (fluorescent dye) when cells were treated with ouabain or MLB in the presence or absence of KB-R7943 ( $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor) and 2-APB ( $\text{IP}_3$  receptor antagonist). Molecular modeling was conducted for the docking of ouabain or MLB to  $\text{Na}^+/\text{K}^+$ -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  $1 \mu\text{mol/L}$  for 24 h while no apparent toxicity was observed in those treated with MLB. Intracellular  $\text{Ca}^{2+}$  levels were substantially elevated by MLB ( $1 \mu\text{mol/L}$ ) and ouabain ( $1 \mu\text{mol/L}$ ) in similar patterns, and significantly reduced in the presence of KB-R7943 ( $10 \mu\text{mol/L}$ ) or 2-APB ( $100 \mu\text{mol/L}$ ). Equivalent interaction with the binding cavity of  $\text{Na}^+/\text{K}^+$ -ATPase was simulated for ouabain and MLB by forming five hydrogen bonds, respectively. Treatment of ouabain ( $1 \mu\text{mol/L}$ ), but not MLB ( $1 \mu\text{mol/L}$ ), induced dendritic shrink of SH-SY5Y cells.

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

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

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## Introduction

Gradients of  $\text{Na}^+$  and  $\text{K}^+$  across the plasma membrane of animal cells are important for maintaining membrane potentials, cell volume, and active transport of other solutes<sup>[1]</sup>.  $\text{Na}^+/\text{K}^+$ -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  $\alpha$ - and  $\beta$ -subunits<sup>[2]</sup>. It pumps 3  $\text{Na}^+$  ions out of and 2  $\text{K}^+$  ions into the cells at the expense of hydrolyzing one ATP, and thus maintains the gradients of  $\text{Na}^+$  and  $\text{K}^+$  ions across the cell membrane. For the continuous exchange of  $\text{Na}^+$  and  $\text{K}^+$  across the membrane,  $\text{Na}^+/\text{K}^+$ -ATPase actively consumes 20%–30% of

ATP energy generated in animal cells at rest<sup>[3]</sup>.

Cardiac glycosides, such as ouabain and digoxin, are steroid-like compounds and have been used in the treatment of congestive heart failure<sup>[4]</sup>. The therapeutic effect of cardiac glycosides lies in their reversible inhibition on the  $\text{Na}^+/\text{K}^+$ -ATPase of myocardium<sup>[5]</sup>. An inhibition on  $\text{Na}^+/\text{K}^+$ -ATPase leads to the elevation of intracellular  $\text{Na}^+$  concentration, which in turn activates a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger resulting in an increase of intracellular  $\text{Ca}^{2+}$  level. The elevated intracellular  $\text{Ca}^{2+}$  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<sup>[6]</sup>. Magnesium lithospermate B (MLB), a derivative of caffeic

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acid tetramer and the major soluble ingredient in danshen, has been demonstrated to possess several medicinal effects, such as vasodilating, antihypertensive, antioxidative, and free radical scavenging activities<sup>[7, 8]</sup>. Recently, MLB was suggested to be responsible for the cardiac therapeutic effect of danshen by its effective inhibition on  $\text{Na}^+/\text{K}^+$ -ATPase via the same molecular mechanism triggered by cardiac glycosides<sup>[9]</sup>. Whether MLB leads to the elevation of intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  level. Moreover, a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor, KB-R7943, and an  $\text{IP}_3$  receptor antagonist, 2-APB, were utilized to assess possible intracellular and extracellular sources for the fluctuating cytosolic  $\text{Ca}^{2+}$  level in SH-SY5Y cells treated with MLB and ouabain. Molecular modeling and docking of ouabain and MLB to  $\text{Na}^+/\text{K}^+$ -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-aminorthyl 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 acetoxymethyl ester (Fluo4-AM), and 2-[2-[4-(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 lithospermate B (MLB) was extracted from roots of danshen (*Salvia multiorrhiza*) and purified as described previously<sup>[9]</sup>. Glass bottom culture dishes (35 mm) were obtained from MatTek (Ashland, MA, USA).

### Cell cultures

The human adrenergic neuroblastoma cell line, SH-SY5Y<sup>[10]</sup> was kindly provided by Dr Tin-yn 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%  $\text{CO}_2$ ; and passages were performed every other day by trypsinization. For MTT staining, cells were plated into 24-well culture plates at a density of  $1 \times 10^5$  cells. For fluorescence imaging of  $\text{Ca}^{2+}$  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  $\mu\text{mol/L}$  for 5, 10, 30, 60 min, and 24 h were subjected to cell viability assay by MTT staining<sup>[11]</sup>. Cells were added with MTT to a final concentration of 500  $\mu\text{g/mL}$ , 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 $\text{Ca}^{2+}$ imaging

Fluctuation of the intracellular  $\text{Ca}^{2+}$  level of SH-SY5Y cells was tracked and visualized by a preloaded fluorescent  $\text{Ca}^{2+}$ -sensitive dye, Fluo4-AM<sup>[12]</sup>. Cell-permeable Fluo4-AM was dissolved in DMSO to a concentration of 3 mmol/L, and then further diluted to 3  $\mu\text{mol/L}$  in cell media. The cells were washed once with the culture medium (145 mmol/L NaCl, 5 mmol/L KCl, 2.6 mmol/L  $\text{CaCl}_2$ , 1 mmol/L  $\text{MgCl}_2$ , 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  $\mu\text{mol/L}$  Fluo4-AM for 30 min in a humidified 5%  $\text{CO}_2$  incubator at 37°C. After washed with the culture medium, cells were added with 1  $\mu\text{mol/L}$  of ouabain or MLB, and  $\text{Ca}^{2+}$  fluorescence imaging was monitored at different intervals for 30 min. To examine the possible sources for the elevated cytosolic  $\text{Ca}^{2+}$  level in SH-SY5Y cells, 10  $\mu\text{mol/L}$  of KB-R7943 ( $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor) or 100  $\mu\text{mol/L}$  of 2-APB ( $\text{IP}_3$  receptor antagonist) was added to cells and incubated for 5 or 15 min before the loading of ouabain or MLB in the  $\text{Ca}^{2+}$  imaging detection<sup>[13, 14]</sup>. KB-R7943 was prepared in ethanol to a concentration of 10 mmol/L, further diluted to 1 mmol/L in  $\text{Ca}^{2+}$  free HBSS, and then finally diluted to 10  $\mu\text{mol/L}$  in the culture medium. 2-APB of 100  $\mu\text{mol/L}$  was prepared in the culture medium. For  $\text{Ca}^{2+}$  imaging, culture dishes with adherent cells were mounted in the MIU-IBC  $\text{CO}_2$  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 $\times$ /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 $\times$ 512 pixels, and analyzed frame by frame with a Time Series Analyzer<sup>[15, 16]</sup>. 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  $\text{Na}^+/\text{K}^+$ -ATPase

(PDB code 3A3Y) was downloaded from Protein Data Bank<sup>[17]</sup>. In order to facilitate the docking process, the  $\beta$  and  $\gamma$  subunits of the  $\text{Na}^+/\text{K}^+$ -ATPase were removed, as well as the water molecules and counter-ions surrounding the remaining  $\alpha$  subunit<sup>[18]</sup>. After hydrogen saturation, the modified  $\text{Na}^+/\text{K}^+$ -ATPase was minimized with CHARMM force field<sup>[19]</sup> using the Discover Studio 2.1 package (<http://accelrys.com/products/discovery-studio/>). 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 (<http://www.cambridgesoft.com/>). The binding pocket of the  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$  subunit was defined as ouabain occupancy site in the structure of  $\text{Na}^+/\text{K}^+$ -ATPase-ouabain complex<sup>[17]</sup>. Docking of MLB was performed *in silico* by employing the LigandFit module<sup>[20]</sup> 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<sup>[21]</sup>, 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  $\text{CO}_2$  incubation system and placed on the microscope with a differential interference contrast (DIC) mode. The cell profile was viewed through Nomarski optics with a  $60\times$  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  $[\text{volume}_{30\text{ min}}/\text{volume}_{0\text{ min}}]\%$  where  $\text{volume}_{0\text{ min}}$  and  $\text{volume}_{30\text{ min}}$  represented the cell volume before and after treatment of ouabain or MLB for 30 min, respectively<sup>[22, 23]</sup>.

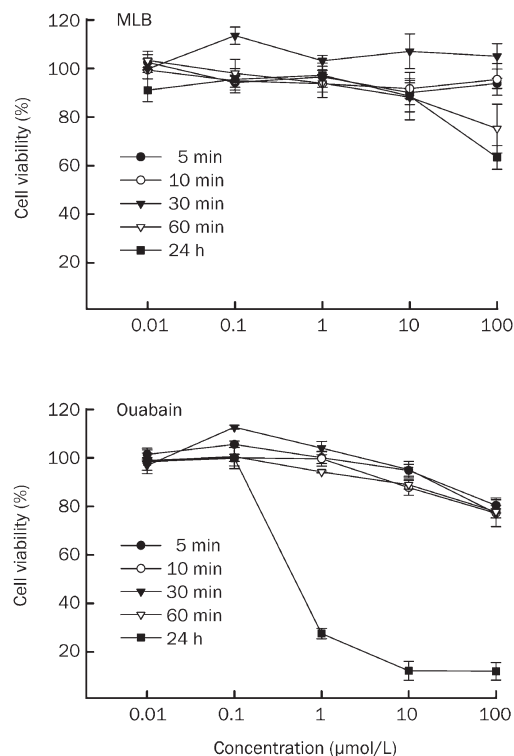
#### Statistical analysis

Data were expressed as mean $\pm$ 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  $\mu\text{mol/L}$ ) 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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$  within 60 min, and severe toxicity was observed in cells treated with



**Figure 1.** Effects of MLB and ouabain on viability of SH-SY5Y cells. SH-SY5Y cells were treated with various concentrations of MLB and ouabain for 5, 10, 30, 60 min, and 24 h. Viability was measured by MTT assay. Data are mean $\pm$ SEM ( $n=3$ ).

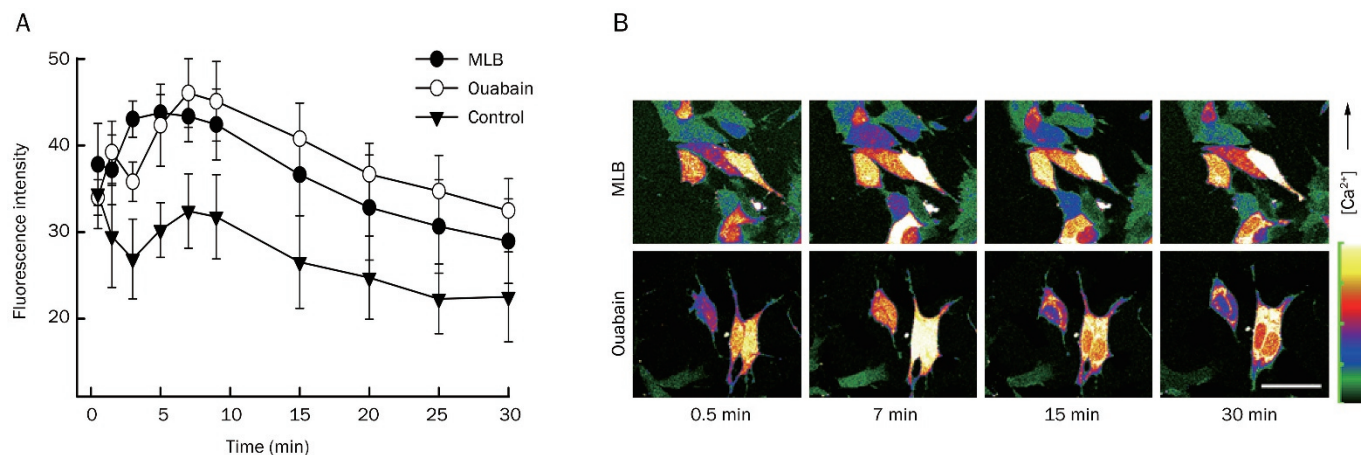
ouabain of concentration higher than 1  $\mu\text{mol/L}$  for 24 h. On the basis of the above observation, cells were treated with 1  $\mu\text{mol/L}$  of MLB or ouabain for 30 min in the following experiments.

#### Effects of MLB and ouabain on intracellular $\text{Ca}^{2+}$ levels in SH-SY5Y cells

To examine the effects of MLB and ouabain on their intracellular  $\text{Ca}^{2+}$  levels, SH-SY5Y cells were preloaded with Fluo4-AM, incubated with 1  $\mu\text{mol/L}$  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  $\text{Ca}^{2+}$  levels of SH-SY5Y cells in similar patterns.

#### Effects of KB-R7943 and 2-APB on the elevation of $\text{Ca}^{2+}$ levels of MLB- and ouabain-treated SH-SY5Y cells

To evaluate the possible calcium sources for the elevation of cytosolic  $\text{Ca}^{2+}$  levels in SH-SY5Y cells treated with MLB and ouabain, a  $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor, KB-R7943, or an  $\text{IP}_3$  receptor antagonist, 2-APB, was added to cells before loading MLB and ouabain. Significant reduction of fluorescence inten-

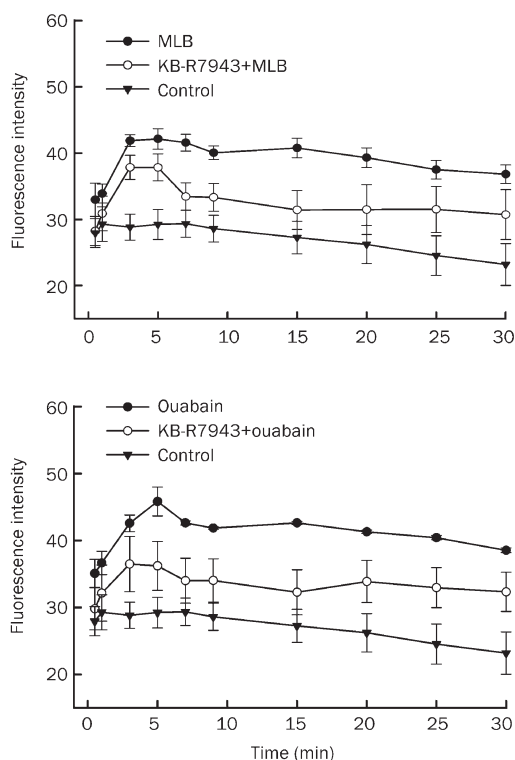


**Figure 2.** Fluctuation of intracellular  $\text{Ca}^{2+}$  levels of SH-SY5Y cells treated with MLB and ouabain. SH-SY5Y cells were loaded with Fluo4-AM prior to incubation with 1  $\mu\text{mol/L}$  of MLB or ouabain. Intensity of fluorescence was collected and calculated at different time intervals for 30 min (A). Each point is representative for 40 ROIs of time-lapse images in 5 independent experiments. Serial images of cells treated with MLB and ouabain for 0.5, 7, 15, and 30 min were captured to display the fluctuation of intracellular  $\text{Ca}^{2+}$  levels (B). Scale bar represents 20  $\mu\text{m}$ .

sity in SH-SY5Y cells by either MLB or ouabain was observed in the presence of KB-R7943 (Figure 3) or 2-APB (Figure 4). These results suggest that the elevated intracellular  $\text{Ca}^{2+}$  levels of SH-SY5Y cells treated with either MLB or ouabain were possibly supplied by extracellular  $\text{Ca}^{2+}$  influx through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger on the plasma membrane and intracellular  $\text{Ca}^{2+}$  release via the  $\text{IP}_3$  receptor channel on the membrane of endoplasmic reticulum (ER).

#### Docking of MLB to $\text{Na}^+/\text{K}^+\text{-ATPase}$

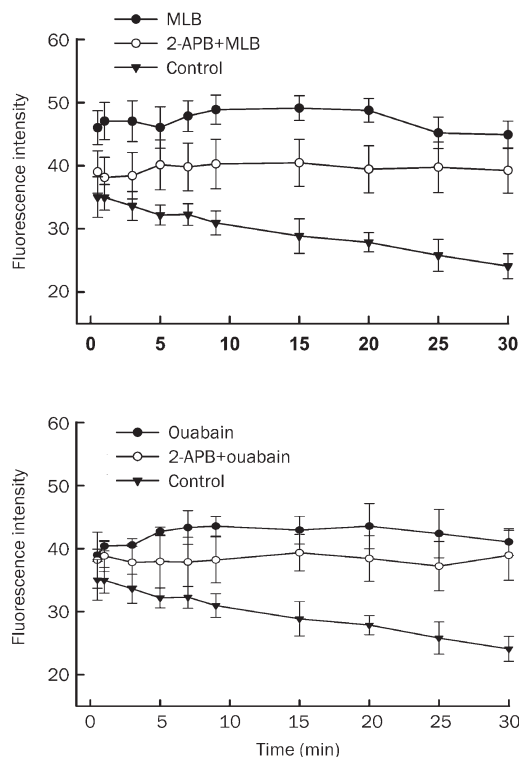
To compare the inhibitory potency of ouabain and MLB on  $\text{Na}^+/\text{K}^+\text{-ATPase}$  at molecular level, both compounds were subjected to molecular modeling and docking to the extracellular domain of  $\text{Na}^+/\text{K}^+\text{-ATPase}$   $\alpha$  subunit. The results showed that MLB could be localized in the ouabain binding pocket of  $\text{Na}^+/\text{K}^+\text{-ATPase}$ , and that equivalent interaction with the binding cavity of  $\text{Na}^+/\text{K}^+\text{-ATPase}$  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  $\text{Na}^+/\text{K}^+\text{-ATPase}$ , and two H-bonds between the hydroxyl group at C-14 of ouabain and Thr804 of  $\text{Na}^+/\text{K}^+\text{-ATPase}$ . 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  $\text{Na}^+/\text{K}^+\text{-ATPase}$ , one H-bond between the carbonyl group at C-9' position of MLB and Thr804 of  $\text{Na}^+/\text{K}^+\text{-ATPase}$ , and one H-bond between the hydroxyl group at C-4''' position of MLB and Leu110 of  $\text{Na}^+/\text{K}^+\text{-ATPase}$ . 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  $\text{Na}^+/\text{K}^+\text{-ATPase}$ .



**Figure 3.** Effects of KB-R7943 on intracellular  $\text{Ca}^{2+}$  levels of SH-SY5Y cells elevated by MLB and ouabain. Similar  $\text{Ca}^{2+}$  fluorescence imaging was executed as described in Figure 2 except that cells were treated with KB-R7943 for 5 min before the loading of MLB and ouabain. Data represent mean  $\pm$  SEM ( $n=3$ ).

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

Changes of cell body and dendrite morphology after treated with 1  $\mu\text{mol/L}$  of MLB or ouabain for 30 min were calculated

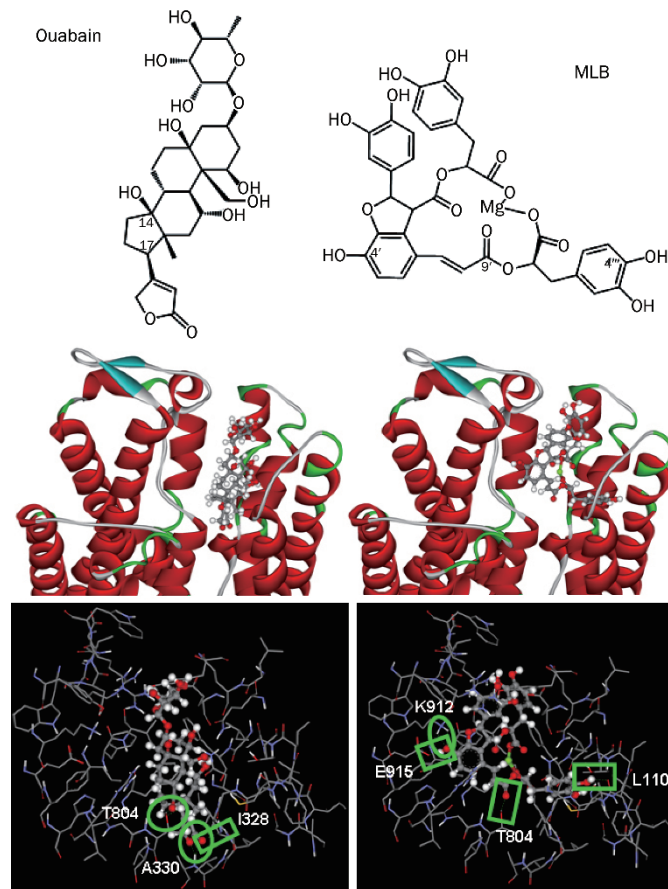


**Figure 4.** Effect of 2-APB on intracellular  $\text{Ca}^{2+}$  levels of SH-SY5Y cells elevated by MLB and ouabain. Similar  $\text{Ca}^{2+}$  fluorescence imaging was executed as described in Figure 2 except that cells were treated with 2-APB for 15 min before the loading of MLB and ouabain. Data represent mean $\pm$ SEM ( $n=3$ ).

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

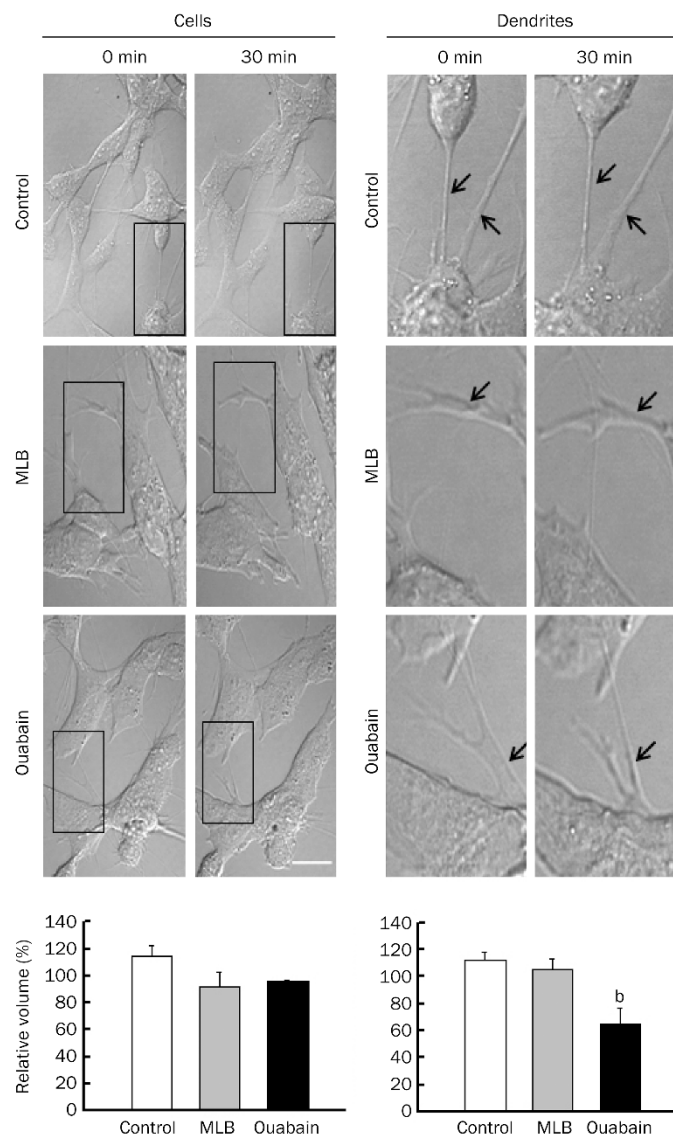
In a previous study, we demonstrated that MLB could produce potent inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase *in vitro*, and proposed that its cardiac therapeutic effects could have been due to the same molecular mechanism as triggered by cardiac glycosides<sup>[9]</sup>. In this study, we demonstrated that intracellular  $\text{Ca}^{2+}$  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  $\text{Na}^+/\text{K}^+$ -ATPase, and the results were in agreement with our previous observation that both ouabain and MLB possessed strong inhibitory



**Figure 5.** Detailed molecular interactions between the extracellular binding pocket of  $\text{Na}^+/\text{K}^+$ -ATPase and ouabain or MLB. (Upper panels) Chemical structures of ouabain and MLB. (Middle panels) Modeling of ouabain and MLB binding to the extracellular pocket of  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$  subunit. The amino acid residues around the binding pocket of  $\text{Na}^+/\text{K}^+$ -ATPase are shown in ribbon structure, and ouabain and MLB in scaled ball and stick. (Lower panels) The amino acid residues of  $\text{Na}^+/\text{K}^+$ -ATPase close to ouabain or MLB are shown in wireframe, and the structures of ouabain and MLB in scaled ball and stick. Green box or oval represents one or two hydrogen bonds formed between  $\text{Na}^+/\text{K}^+$ -ATPase and ouabain or MLB.

potency on  $\text{Na}^+/\text{K}^+$ -ATPase<sup>[9]</sup>. Evidently, the results reinforce our previous proposition that MLB, being a potent inhibitor of  $\text{Na}^+/\text{K}^+$ -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  $\text{Na}^+/\text{K}^+$ -ATPase.

Calcium signals are mostly delivered as brief transients that are often organized into regulatory oscillations<sup>[24]</sup>. Fluctuation of cytosolic  $\text{Ca}^{2+}$  level is generally a coordinated consequence of a number of molecular cascade reactions responsible for  $\text{Ca}^{2+}$  influx and efflux in exchange with either extracellular space or intracellular ER storage compartment<sup>[25]</sup>. Free  $\text{Ca}^{2+}$  enters the cells through either voltage-gated channels or receptor-operated channels located in their plasma membrane<sup>[26, 27]</sup>. However, much of the signal  $\text{Ca}^{2+}$  comes from the



**Figure 6.** Examination of cell and dendrite volumes after treatment of MLB and ouabain. Changes of cell and dendrite volumes were measured after treated with 1  $\mu\text{mol/L}$  of MLB and ouabain for 30 min. Dendrites indicated by arrows were selected from the examined cells (box areas in left panels) and shown in enlarged photos (right panels). Relative volume (cell or dendrite volume at 30 min over its volume at 0 min) was calculated and shown at the bottom figure. Data represent mean  $\pm$  SEM ( $n=3$ ). <sup>b</sup> $P<0.05$  vs control group. Scale bar represents 20  $\mu\text{m}$ .

intracellular  $\text{Ca}^{2+}$  sources, and is primarily released via the  $\text{IP}_3$  receptor channel on the ER membrane<sup>[28, 29]</sup>. According to the reduction effects of two inhibitors KB-R7943 and 2-APB on the intracellular  $\text{Ca}^{2+}$  levels elevated by the treatment of either MLB or ouabain (Figures 3 and 4), the elevation of cytosolic  $\text{Ca}^{2+}$  level was likely supplied by both  $\text{Ca}^{2+}$  influx through the reversed mode of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and intracellular release from ER storage compartment. Whether other sources are also involved in the elevation of intracellular  $\text{Ca}^{2+}$  levels in SH-SY5Y cells treated with MLB or ouabain has not been

evaluated.

$\text{Na}^+/\text{K}^+$ -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  $\text{Na}^+/\text{K}^+$ -ATPase can be a drug target for the treatment of several diseases, including congestive heart failure, ischemic stroke, neurodegenerative diseases and even cancer<sup>[30–32]</sup>. These therapeutic effects may be possibly resulted from the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase leading to the fluctuation of  $\text{Ca}^{2+}$  level, which in turn activates diverse physiological responses in different cells in variable microenvironments<sup>[33, 34]</sup>. However, the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase may also lead to the fluctuation of other cations presumably via cross-talks held among different ion channels<sup>[35, 36]</sup>. Therefore, the possibility that some pharmacological effects following  $\text{Na}^+/\text{K}^+$ -ATPase inhibition may be generated by biological reactions insensitive to  $\text{Ca}^{2+}$  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<sup>[37]</sup>. 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<sup>[38]</sup>. 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<sup>[9]</sup>. Moreover, water extraction of *Salvia miltiorrhiza* containing mainly MLB has no toxicity to neonatal rat cardiomyocytes at dosage of 5–80  $\mu\text{g/mL}$ <sup>[39]</sup>. 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.

### References

- 1 Jorgensen PL, Hakansson KO, Karlsh SJ. Structure and mechanism

- of Na,K-ATPase: functional sites and their interactions. *Annu Rev Physiol* 2003; 65: 817–49.
- 2 Axelsen KB, Palmgren MG. Evolution of substrate specificities in the P-type ATPase superfamily. *J Mol Evol* 1998; 46: 84–101.
  - 3 Skou JC, Esmann M. The Na,K-ATPase. *J Bioenerg Biomembr* 1992; 24: 249–61.
  - 4 Li-Saw-Hee FL, Lip GY. Digoxin revisited. *QJM* 1998; 91: 259–64.
  - 5 Lin SC, Way EL. A high affinity Ca<sup>2+</sup>-ATPase enriched nerve-ending plasma membranes. *Brain Res* 1982; 235: 387–92.
  - 6 Ji XY, Tan BK, Zhu YZ. *Salvia miltiorrhiza* and ischemic diseases. *Acta Pharmacol Sin* 2000; 21: 1089–94.
  - 7 Jiang RW, Lau KM, Hon PM, Mak TC, Woo KS, Fung KP. Chemistry and biological activities of caffeic acid derivatives from *Salvia miltiorrhiza*. *Curr Med Chem* 2005; 12: 237–46.
  - 8 Zhao GR, Zhang HM, Ye TX, Xiang ZJ, Yuan YJ, Guo ZX, *et al*. Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B. *Food Chem Toxicol* 2008; 46: 73–81.
  - 9 Tzen JTC, Jinn TR, Chen YC, Li FY, Cheng FC, Shi LS, *et al*. Magnesium lithospermate B possesses inhibitory activity on Na<sup>+</sup>,K<sup>+</sup>-ATPase and neuroprotective effects against ischemic stroke. *Acta Pharmacol Sin* 2007; 28: 609–15.
  - 10 Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* 1978; 38: 3751–7.
  - 11 Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; 47: 936–42.
  - 12 Aoshima H, Satoh T, Sakai N, Yamada M, Enokido Y, Ikeuchi T, *et al*. Generation of free radicals during lipid hydroperoxide-triggered apoptosis in PC12h cells. *Biochim Biophys Acta* 1997; 1345: 35–42.
  - 13 Nakamura H, Kawasaki Y, Arakawa N, Saeki M, Maeda S, Koyama Y, *et al*. The Na<sup>+</sup>-Ca<sup>2+</sup> exchange inhibitor KB-R7943 inhibits high K<sup>+</sup>-induced increases in intracellular Ca<sup>2+</sup> concentration and [<sup>3</sup>H] noradrenaline release in the human neuroblastoma SH-SY5Y. *Neurochem Res* 2000; 25: 385–7.
  - 14 Li XH, Wu YJ. Characteristics of lysophosphatidylcholine-induced Ca<sup>2+</sup> response in human neuroblastoma SH-SY5Y cells. *Life Sci* 2007; 80: 886–92.
  - 15 Balaji J. <http://rsbweb.nih.gov/ij/plugins/time-series.html> 2007.
  - 16 Vicencio JM, Ibarra C, Estrada M, Chiong M, Soto D, Parra V, *et al*. Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes. *Endocrinology* 2006; 147: 1386–95.
  - 17 Ogawa H, Shinoda T, Cornelius F, Toyoshima C. Crystal structure of the sodium-potassium pump (Na<sup>+</sup>,K<sup>+</sup>-ATPase) with bound potassium and ouabain. *Proc Natl Acad Sci USA* 2009; 106: 13742–7.
  - 18 Chen RJY, Chung TY, Li FY, Yang WH, Jinn TR, Tzen JTC. Steroid-like compounds in Chinese medicines promote blood circulation via inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase. *Acta Pharmacol Sin* 2010; 31: 696–702.
  - 19 Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. CHARMM: A program for macromolecular energy minimization and dynamics calculations. *J Comp Chem* 1983; 4: 187–217.
  - 20 Dixon SL, Merz KM Jr. One-dimensional molecular representations and similarity calculations: methodology and validation. *J Med Chem* 2001; 44: 3795–809.
  - 21 Krammer A, Kirchoff PD, Jiang X, Venkatachalam CM, Waldman M. LigScore: a novel scoring function for predicting binding affinities. *J Mol Graph Model* 2005; 23: 395–407.
  - 22 Takeuchi S, Ando M, Irimajiri A. Changes in the volume of marginal cells induced by isotonic 'Cl<sup>-</sup> depletion/restoration': involvement of the Cl<sup>-</sup> channel and Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporter. *Hear Res* 1997; 113: 99–109.
  - 23 Clough AV, Haworth ST, Ma W, Dawson CA. Effects of hypoxia on pulmonary microvascular volume. *Am J Physiol Heart Circ Physiol* 2000; 279: H1274–82.
  - 24 Berridge MJ. Calcium microdomains: organization and function. *Cell Calcium* 2006; 40: 405–12.
  - 25 Verkhratsky A, Orkand RK, Kettenmann H. Glial calcium: homeostasis and signaling function. *Physiol Rev* 1998; 78: 99–141.
  - 26 Pearce B, Albrecht J, Morrow C, Murphy S. Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. *Neurosci Lett* 1986; 72: 335–40.
  - 27 MacVicar BA, Hochman D, Delay MJ, Weiss S. Modulation of intracellular Ca<sup>++</sup> in cultured astrocytes by influx through voltage-activated Ca<sup>++</sup> channels. *Glia* 1991; 4: 448–55.
  - 28 Kostyuk P, Verkhratsky A. Calcium stores in neurons and glia. *Neuroscience* 1994; 63: 381–404.
  - 29 Deitmer JW, Verkhratsky AJ, Lohr C. Calcium signalling in glial cells. *Cell Calcium* 1998; 24: 405–16.
  - 30 Xie Z, Xie J. The Na<sup>+</sup>/K<sup>+</sup>-ATPase-mediated signal transduction as a target for new drug development. *Front Biosci* 2005; 10: 3100–9.
  - 31 Mijatovic T, Ingrassia L, Facchini V, Kiss R. Na<sup>+</sup>/K<sup>+</sup>-ATPase alpha subunits as new targets in anticancer therapy. *Expert Opin Ther Targets* 2008; 12: 1403–17.
  - 32 Prassas I, Diamandis EP. Novel therapeutic applications of cardiac glycosides. *Nat Rev Drug Discov* 2008; 7: 926–35.
  - 33 Wang JK, Portbury S, Thomas MB, Barney S, Ricca DJ, Morris DL, *et al*. Cardiac glycosides provide neuroprotection against ischemic stroke: discovery by a brain slice-based compound screening platform. *Proc Natl Acad Sci USA* 2006; 103: 10461–6.
  - 34 Tzen JTC, Chen RJY, Chung TY, Chen YC, Lin NH. Active compounds in Chinese herbs and medicinal animal products for promoting blood circulation via inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase. *Chang Gung Med J* 2010; 33: 126–36.
  - 35 Hasegawa H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. *J Pharmacol Sci* 2004; 95: 153–7.
  - 36 Chen RJY, Chung TY, Li FY, Lin NH, Tzen JTC. Effect of sugar positions in ginsenosides and their inhibitory potency on Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. *Acta Pharmacol Sin* 2009; 30: 61–9.
  - 37 Bywood PT, Johnson SM. Dendrite loss is a characteristic early indicator of toxin-induced neurodegeneration in rat midbrain slices. *Exp Neurol* 2000; 161: 306–16.
  - 38 Kulikov A, Eva A, Kirch U, Boldyrev A, Scheiner-Bobis G. Ouabain activates signaling pathways associated with cell death in human neuroblastoma. *Biochim Biophys Acta* 2007; 1768: 1691–702.
  - 39 Ou-yang X, Takahashi K, Komatsu K, Nakamura N, Hattori M, Baba A, *et al*. Protective effect of *Salvia miltiorrhiza* on angiotensin II-induced hypertrophic responses in neonatal rat cardiac cells. *Jpn J Pharmacol* 2001; 87: 289–96.