Potent block of potassium channels by MEK inhibitor U0126 in primary cultures and brain slices

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U0126 (1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene), a widely used mitogen-activated protein kinase kinase (MEK) inhibitor, was found to accelerate voltage-gated K⁺ channel (Kᵥ) inactivation in heterologous cells expressing several types of Kᵥ. The goal of this study was to examine whether U0126 at a concentration thought to specifically inhibit MEK signaling also inhibits Kᵥ in native neurons of primary cultures or brain slices. U0126 caused a dose-dependent inhibition of both the transient (Iₐ) and sustained (Iₒ) components of K⁺ currents in hippocampal neurons. U0126 also exhibited much higher potency on the Iₐ and Iₒ than the classical Kᵥ blockers 4-aminopyridine (4-AP) and tetraethylammonium (TEA). Consistent with its inhibitory effect on Kᵥ, U0126 broadened action potential duration, profoundly affected the repolarizing phase, and dramatically reduced firing frequency in response to current pulse injections. Despite the potent and reversible action of U0126 on Kᵥ channels, PD98059, a structurally-unrelated MEK inhibitor, did not induce such an effect, suggesting U0126 may act independently of MEK inhibition. Together, these results raise cautions for using U0126 as a specific inhibitor for studying MEK signaling in neurons; on the other hand, further studies on the blocking mechanisms of U0126 as a potent inhibitor of Kᵥ may provide useful insights into the structure-function relationship of Kᵥ in general.

The mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated kinase (ERK1/2) is activated by the dual phosphorylation catalyzed by MAPK kinase (MAPKK, also known as MEK). The MAPK cascade, one of the major intracellular signaling pathways, plays a key role in proliferation, differentiation, survival of various cell types¹⁻³, and in several plasticity-related processes in the nervous system⁴. U0126 (1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene) is widely used as a potent and selective non-competitive inhibitor of MEK⁶, therefore, the activation of its downstream target, MAPK/ERK. U0126 has been a valuable pharmacological tool for studying the ERK signaling pathway. The concentration of U0126 used to block the MAPK/ERK signaling pathway is typically 10μM in cultured neurons⁷,⁸, and 20μM in brain slices⁹⁻¹¹. It has been demonstrated in various cell types that the ERK signaling pathway plays important roles in modulating Kᵥ¹²⁻¹⁴, synaptic plasticity¹⁸,¹⁹⁻²⁰, and learning and memory²¹⁻²³ (reviewed by¹⁹,²⁴,²⁵). On the other hand, U0126 is found to accelerate Kᵥ inactivation in heterologous cells expressing several types of Kᵥ.²⁶ Therefore, it is of interest and importance to determine whether U0126 at a concentration thought to specifically inhibit MEK-MAPK signaling can have a significant effect on Kᵥ in primary neuronal cultures and brain slices.

Voltage-gated potassium channels (Kᵥ) are key regulator of membrane excitability. Mammalian neurons express various types of Kᵥ that exhibit different voltage- and time-dependent channels kinetics. Kᵥ are multimeric proteins assembled from pore-forming α subunits and auxiliary β subunits. The α subunits of Kᵥ channels are encoded by 12 subfamilies of genes (Kᵥ1−12).²⁷ Previous studies have shown that the CA1 pyramidal neurons²⁸,²⁹, like many other types of neurons found in various brain regions³⁰,³¹, express at least three major types of Kᵥ currents; the transient fast-inactivating Kᵥ current (Iₐ), the delayed rectifier Kᵥ current (Iₒ), composed of a non-inactivating, fast delayed rectifier Kᵥ current (Iₒ₉₉), and the slowly inactivating delayed rectifier Kᵥ current (Iₒ).¹³,¹⁴ Kᵥ channels underlying those currents possess distinct biophysical properties, pharmacology, and molecular identity³². The Iₒ, mainly assembled from Kᵥ4.2 and Kᵥ4.3 of Kᵥ4 subfamily, are blocked by

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4-AP but insensitive to TEA. I$_A$ is rapidly activated upon depolarization and quickly recovers from inactivation, and therefore can influence action potential onset time, threshold, and inter-spike intervals as well as dendritic backpropagation action potentials$^{34}$. I$_D$, likely composed of K$_V$3.1 and K$_V$3.2 channels, exhibit rapid activation and therefore can influence action potential onset time, threshold, and inter-spike intervals as well as dendritic effects of U0126 on I$_A$ and I$_{DR}$ with classical KV inhibitors, 4-AP and TEA. As shown in Fig. 3, 3 mM 4-AP produced significant inhibition of the I$_{DR}$ (65 ± 0.0% (n = 20; p < 0.05)) (Fig. 3D) and did not significantly inhibit the I$_{DR}$ (Fig. 3B); whereas 30 mM TEA produced inhibition of the I$_{DR}$ 45 ± 8% (n = 9, p < 0.05) (Fig. 3B) and did not significantly change I$_A$ (Fig. 3A). These results are consistent with well-established observations that 4-AP preferentially blocks I$_A$ and low levels of TEA have no effect on I$_A$ but partially inhibit the residual sustained current$^{43}$. These results

**Results**

**Dose-dependent blockade of K$^+$ currents by U0126 in primary hippocampal neurons.** We first used primary culture of hippocampal neurons prepared from postnatal day 0–3 rats to test U0126 effects on K$^+$ currents (Fig. 1). In voltage-clamp mode, two kinetically distinct K$^+$ current components, the transient fast-inactivating K$^+$ current, I$_A$ and sustained, delayed rectifier K$^+$ current, I$_{DR}$ were identified$^{39}$. A prepulse voltage protocol was used to isolate the I$_A$ and I$_{DR}$ (Fig. 1A1,A2) to selectively activate the I$_{DR}$ (Fig. 1B1,B2), neurons were held at −40 mV and voltage was stepped to 0 mV with a prepulse to −40 mV in order to inactivate the I$_A$. Subtracting I$_{DR}$ from the total K$^+$ current elicited by a voltage step yielded the I$_A$ (Fig. 1C1,C2). Examining the dose-response relationship of I$_A$ and I$_{DR}$ showed a half maximal inhibitory concentration (IC$_{50}$) at 9.5 ± 0.1 μM (Fig. 1D) and 19.3 ± 0.4 μM (Fig. 1E), respectively. At 10 μM, a concentration commonly used in neuronal culture studies, U0126 produced a significant inhibition of the I$_A$ (65 ± 11%, n = 20; p < 0.05) (Fig. 1D) but had no significant inhibition of the I$_{DR}$ (6 ± 3%, n = 20; p > 0.05) (Fig. 1E). At 20 μM, U0126 significantly reduced both the I$_A$ (82 ± 4%, n = 33; p < 0.05) (Fig. 1D) and I$_{DR}$ (38 ± 6%, n = 33; p < 0.05) (Fig. 1E). Therefore, both I$_A$ and I$_{DR}$ can be inhibited by U0126, with the I$_A$ being more sensitive to the inhibition of U0126. In contrast, PD98059, a structurally-unrelated MEK inhibitor, had no effects on K$^+$ currents (Fig. 2). These results raise a possibility that U0126 could directly affect K$_V$ at the concentration that is commonly used as a specific MEK inhibitor.

**U0126 blocked K$^+$ channels with greater potency than 4-AP and TEA.** We then compared the effects of U0126 on I$_A$ and I$_{DR}$ with classical K$_V$ inhibitors, 4-AP and TEA. As shown in Fig. 3, 3 mM 4-AP produced 33 ± 10% (n = 9, p < 0.05) inhibition of the I$_A$ (Fig. 3A) and 6 + 7% (n = 9, p > 0.05) inhibition of the I$_{DR}$ (Fig. 3B); whereas 30 mM TEA produced inhibition of the I$_{DR}$ 45 ± 8% (n = 9, p < 0.05) (Fig. 3B) and did not significantly change I$_A$ (Fig. 3A). These results are consistent with well-established observations that 4-AP preferentially blocks I$_A$ and low levels of TEA have no effect on I$_A$ but partially inhibit the residual sustained current$^{43}$. These results
suggest that U0126 inhibits IA and IDR in the micromolar range of concentrations, exhibiting 100- to 1000-fold higher potency than the classical Kv blockers 4-AP and TEA, which work in millimolar range of concentrations.

**Prolongation of action potentials by U0126.** We next studied the effects of U0126 on action potentials under whole-cell current-clamp recording mode. To elicit action potentials, a series of 20 ms depolarizing current pulses in 10 pA steps from resting membrane potential were injected into cultured hippocampal neurons. To inhibit synaptic activity, 20 µM CNQX (an AMPAR antagonist) and 100 µM APV (a potent and selective antagonist for NMDA receptors) were added to the bath. Consistent with its inhibitory effects on Kv currents, bath perfusion of U0126 increased the half-width and decay time of action potential in a dose-dependent manner.
With exposure to 1 and 3 μM U0126, half-width and decay time of action potentials showed no significant difference to that of controls. At 10 μM, action potential half-width was increased by 392 ± 82% (n = 16, p < 0.001) and decay time increased by 298 ± 153% (n = 16, p < 0.05); At 20 μM, U0126 had a dramatic effect, increasing the action potential half-width by 1573 ± 205% (n = 18, p < 0.001) and decay time by 2023 ± 573% (n = 18, p < 0.001) (Fig. 4B1,C1). In comparison, 3 mM 4-AP or 30 mM TEA increased the action potential decay time by 710 ± 155% (n = 14, p < 0.001) or 296 ± 35% (n = 16, p < 0.001), respectively. Below 10 mM, TEA had no significant effect on decay time of action potentials (Fig. 4C3). These results are consistent with several previously reported effects of 4-AP and TEA on action potentials. Therefore, we conclude that U0126 prolongs the action potential half-width and decay time much more efficiently than 4-AP and TEA, possibly through its high potency on K⁺ current inhibition.

Suppression of action potential firing frequency by U0126. We further examined the potential effect of U0126 on the action potential firing pattern by analyzing the current-frequency (I-F) relationship. Under control conditions, the number of action potentials increased progressively with the increase of current injection steps over the course of 400 ms depolarizations from 0 to +120 pA (Fig. 5A1,B1,C1 and E). In contrast, in the presence of 20 μM U0126, regardless of the strength of current injections, only a single action potential was evoked, followed by a prolonged membrane depolarization (Fig. 5A2,B2 and C2). After washout of U0126 for 5–10 minutes, the repetitive firing pattern of action potentials was restored, similar to that of the control condition (Fig. 5A3,B3 and C3). We conclude that U0126 exerts profound influence on membrane excitability and firing pattern of cultured hippocampal neurons.

Suppression of K⁺ currents by U0126 in acute hippocampal slices. Based on the above results, we further examined the effects of bath perfusion of U0126 on K⁺ currents in CA1 pyramidal neurons of acute
Both $I_A$ and $I_{DR}$ are highly expressed in CA1 pyramidal neurons and are important determinants of their membrane excitability. Consistent with the effects of U0126 on $K^+$ currents in primary hippocampal cultures, U0126 similarly inhibited the $K^+$ currents of CA1 pyramidal neurons in a dose-dependent manner in acute slices. As shown in Fig. 6, bath application of 20 $\mu$M U0126 produced a small but significant reduction of the current density of the early $K^+$ currents (measured at peak of the currents, presumably largely composed by $I_A$: $15.4 \pm 0.76$ pA/pF in control; $n = 9$, at $+50$ mV vs $13.6 \pm 0.68$ pA/pF in U0126; $n = 7$, $p < 0.05$), whereas the late, sustained $K^+$ currents (measured indicated by filled squares, Fig. 6A) showed significant reduction compared to control. These results demonstrate that U0126 also non-selectively blocks native $K^+$ currents in brain slices.

Effects of U0126 on action potential waveform and firing patterns in hippocampal neurons.

Lastly, we examined the functional consequence of U0126 blockade of $K^+$ channels, specifically on action potential (AP) waveform and firing patterns of hippocampal neurons in acute brain slices. As shown in Fig. 6, bath application of 20 $\mu$M U0126 produced a small but significant reduction of the current density of the early $K^+$ currents (measured at peak of the currents, presumably largely composed by $I_A$: $15.4 \pm 0.76$ pA/pF in control; $n = 9$, at $+50$ mV vs $13.6 \pm 0.68$ pA/pF in U0126; $n = 7$, $p < 0.05$), whereas the late, sustained $K^+$ currents (measured indicated by filled squares, Fig. 6A) showed significant reduction compared to control. These results demonstrate that U0126 also non-selectively blocks native $K^+$ currents in brain slices.

Discussion

Our major finding is that U0126, a widely-used MEK inhibitor acts to serve as a highly potent non-selective $K_\text{v}$ blocker. Consequently, it changes neuronal excitability and firing pattern at the concentrations commonly
used in many previous studies. Our results thus raise cautions for using U0126 as specific inhibitor for studying MEK-MAPK signaling in neurons.

We demonstrated that bath perfusion of U0126 significantly inhibits the I_A and I_DR in hippocampal neurons in a dose-dependent manner in primary neuronal cultures as well as acute brain slices. Remarkably, U0126 (in micromolar range of concentrations) inhibits both the I_A and I_DR with much higher potency (100- to 1000-fold) than the classical KV blockers 4-AP and TEA, which preferentially inhibits the I_A and I_DR in the millimolar range of concentrations, respectively. Consistently, U0126 showed clear effects on action potential waveform and the ability of firing repetitive action potentials. Bath perfusion of U0126 not only increased the half-width and decay time of individual action potentials in a dose-dependent manner (Fig. 4), but also dramatically reduced firing frequency in response to long current pulse injections (Figs 5 and 7A). Interestingly, U0126’s reversible channel inhibiting effect was not mimicked by PD98059, a structurally-unrelated MEK inhibitor (Fig. 2), indicating the effect of U0126 was independent of its inhibition on MEK.

In our study, we did not differentiate I_DR into I_D, I_K components. It would be interesting to further evaluate if U0126 exhibits similar or different inhibitory effects on I_D, I_K. Currently, the mechanism of U0126 blocking KV is not clear. In heterologous cells expressing the A-type KV channels, KV4.2 and KV4.3 as well as KV1.1, a typical non-inactivating KV, U0126 exhibits its inhibition on KV by accelerating the inactivation of these different channels26. The authors further demonstrated that the action of U0126 is likely a result from a combination of open channel blocking and modulation of channel-gating but independent on its inhibitory effect on ERK or the phosphorylation of the channels. Together with our current observation that U0126 exhibits non-selective inhibitory effects on I_A and I_DR, we postulate that U0126 may be able to directly bind and block the channel pore of various native KV or alter channel gating by interacting with membrane lipids26.

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![Figure 6. Suppression of K+ currents by U0126 in acute hippocampal slices. (A) Representative traces of whole-cell voltage-gated K+ currents recorded from control pyramidal neurons, neurons treated by 20 μM U0126, 30 μM U0126 and 100 μM U0126, as indicated. The schematic diagram of the voltage protocol used to evoke K+ currents is shown at the bottom of the traces. We recorded K+ currents in CA1 neurons in response to a voltage step from −40 mV to +50 mV with 10 mV increments. (B) Quantitative comparison of the current density of early K+ currents (measured at peak amplitude, indicated by filled circles) in CA1 neurons at different conditions as indicated. (C) Quantitative comparison of the current density of the late, sustained K+ currents (measured indicated by filled square) in CA1 neurons at different concentrations after bath application of U0126. P indicates statistical significance using Two way-ANOVA followed by the post hoc Scheffe’s test, *P < 0.05.]
Several studies have reported that direct phosphorylation of Kv4.2 potassium channel by ERK/MAPK can inhibit IA in different subtypes of neurons including the hippocampal CA1 pyramidal neurons\textsuperscript{13,47–49}. In particular, Watanabe\textsuperscript{49} showed that application of 20μM U0126 to the outside of the neurons or included in the whole cell patch pipette produced a small but significant leftward shift in the activation voltage and an overall reduction in IA. In our study, we found that 20μM U0126 produced a small but significant reduction of the current density of peak K\textsuperscript{+} currents (Fig. 6B). At higher concentrations, U0126 exhibited a greater inhibition on the late, sustained K\textsuperscript{+} currents in a concentration-dependent manner (Fig. 6C). There is no clear explanation for this discrepancy. Differences in species, age (5–8 week-old mature rats used by Watanabe et al.\textsuperscript{49} vs 2–3 week-old young mice used in our study), methodology such as perfusion speed, internal solution or different subtypes of pyramidal neurons recorded, are all plausible factors for the discrepancy.

Kv blockers have been considered as potential drugs for many diseases (Tian et al., 2014) associated with low excitability or compromised motor functions such as multiple sclerosis\textsuperscript{50}, spinal cord injury\textsuperscript{51} or Parkinson's Disease\textsuperscript{52}. For example, 4-AP has been used clinically in treating Lambert-Eaton myasthenic syndrome and multiple sclerosis\textsuperscript{53,54}; and TEA was the first "ganglionic blocker" drug to be introduced into clinical practice\textsuperscript{56} but their uses have been limited due to toxicity\textsuperscript{57,58}, and were soon replaced by other drugs. Because of significant adverse side effects and a narrow range for a safe clinical dose, 4-AP failed in clinical trials in treating patients with spinal cord injuries\textsuperscript{59,60}. As discussed above, 4-AP or TEA typically acts on Kv in the millimolar range of concentrations (Fig. 3), whereas U0126 inhibits both the transient A-type and the residual sustained K\textsuperscript{+} currents in the micromolar range of concentrations as shown in this study (Fig. 1). Such a high potency (100- to 1000-fold) and broad-spectrum action may prove to be favorable properties as a potential new class of Kv blockers in treating those aforementioned diseases. Indeed, our ongoing study indicates that, beside a powerful enhancement on neuronal excitability, U0126 also greatly potentiates presynaptic release, hence evoking synchronized firing in immature cultured hippocampal neurons (unpublished data). We believe that a further understanding of the structural basis and blocking mechanisms of U0126 and its analogs as potent, non-selective inhibitors of Kv, and the removal of its unwanted action on MEK should not only provide useful insights into the structure-function of Kv in general, but also may prove valuable in developing more efficacious and safe new Kv blockers in treating various relevant diseases due to conduction deficits and low excitability in the excitable organs including brain, heart and muscle.

In conclusion, we have observed dramatic effects of U0126 on Kv in primary hippocampal cultures and brain slices. The data reported here suggest that cautions should be taken when interpreting experimental results using U0126. Furthermore, our finding that U0126 exhibits much higher potency on Kv than 4-AP and TEA raises the possibility that U0126 and its derivatives can be further developed as a potent, new class of Kv blockers and may provide a new perspective in the development new treatments for various neuromuscular and neurodegenerative diseases.

**Figure 7.** Effects of U0126 on action potential waveforms and firing patterns in hippocampal neurons. (A) Representative double whole-cell current clamp recordings from two regular-spiking pyramidal neurons from control, neurons treated by 40μM U0126 and washout, as indicated. These tracings were obtained from the same neuron. (B) Representative double whole-cell current clamp recordings from two regular-spiking pyramidal neurons from control, neurons treated by 20mM TEA, washout and treated by 3mM 4-AP, as indicated. These tracings were obtained from the same neuron. Recordings were made from P20 mouse hippocampus. The current step is 20 pA (from −40 pA to 140 pA).
Methods
Hippocampal primary culture. All animals were housed and maintained in accordance with procedures approved by the Ethics Committee for animal research at South China Normal University, in line with the Guidelines for Animal Care established by the National Institute of Health. All experimental procedures were approved by the Ethics Committee for animal research at South China Normal University. Hippocampal cell cultures were prepared as described previously. Briefly, hippocampal CA1/CA3 regions were dissected from 0- to 3-days-old C57BL6/J mouse, dissociated by trypsin XI treatment followed by trituration with a siliconized Pasteur pipette, and then plated onto coverslips coated with Matrigel (Bd Biosciences, USA). Culture medium consisted of minimal essential medium (Invitrogen, CA), 0.6% glucose, 0.1 mg/ml bovine transferrin (Calbiochem, CA), 0.25 mg/l insulin (Sigma-Aldrich, USA), 0.3 mg/l glutamine, 5–10% fetal bovine serum (Sigma-Aldrich, USA), 2% B-27 supplement (Invitrogen, USA), and 2μM cytosine β-D-arabinofuranoside (Sigma-Aldrich, USA). Cultures were maintained at 37 °C in a 95% air, 5% CO₂-humidified incubator. Cultures of 7–9 DIV (days in vitro) were used for whole-cell patch clamp recordings.

Patch-clamp recording. Whole-cell patch-clamp recording in hippocampal cultures. Traditional whole-cell patch-clamp recordings were performed from cultured hippocampal neurons plated on coverslips, which were placed in a recording chamber mounted on a fixed-stage inverted phase-contrast microscope (Nikon, Japan). Patch electrodes (3–5 MΩ) were made from borosilicate glass (WPI, USA). Whole-cell capacitance and series resistances were recorded and compensated to >80%, and in addition, series resistances were less than two times the tip resistance. The Tyrode's bath solution contained (in mM): 129 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 0.01 glycine, 30 D-glucose and 25 HEPES, pH 7.2–7.4. The pipette solution contained (in mM): 110 K-glucuronate, 40 HEPES, 10 EGTA, 2 Na₂-ATP, 2 Mg-ATP and 0.3 GTP, pH 7.35 (adjusted with KOH). To isolate K⁺ currents in pyramidal neurons, 1μM tetrodotoxin (TTX) and 2 mM MnCl₂ were added to the bath to block Na⁺ currents, Ca²⁺ currents, and Ca²⁺-activated K⁺ currents. To isolate the fast inactivating transient current (Iₚ), cells were held at −80 mV, and the voltage was stepped to 0 mV with or without a prepulse to −40 mV. The difference between the currents elicited with and without the prepulse was measured as Iₚ. Leakage and capacitive currents were digitally subtracted on-line with P4 protocol.

Whole-cell patch-clamp recording in acute hippocampal slices. Coronal brain slices (350 μm thick) were prepared from 15- to 21-days-old C57BL6/J mice in cutting solution with a vibratome (Lecia VT1000S, Germany). The cutting solution contained (in mM): 210 Sucrose, 26 NaHCO₃, 3 MgSO₄·7H₂O, 0.75 CaCl₂, 1 NaH₂PO₄·2H₂O, 3 KCl and 10 D-glucose. Slices were incubated at room temperature for 1 hour in the artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄·2H₂O, 1.3 MgSO₄·7H₂O, 26 NaHCO₃, 10 D-glucose and 2.5 CaCl₂. The pipette solution contained (in mM): 110 K-glucuronate, 40 HEPES, 10 EGTA, 2 Na₂-ATP, 2 Mg-ATP and 0.3 GTP, pH 7.35 (adjusted with KOH). At a holding potential of −60 mV, the K⁺ currents were evoked by voltage steps (from −40 mV to +50 mV in 10 mV increments, 400 ms) in the presence of TTX (1μM) and CdCl₂ (2 mM) to block voltage-activated Na⁺ and Ca²⁺ currents, as well as Ca²⁺-activated K⁺ currents.

Drugs. U0126 (Sigma-Aldrich, USA and Calbiochem, CA), PD98059 (Calbiochem, CA), 4-AP and TEA (Sigma-Aldrich, USA) were dispersed in ultrapure water or DMSO as stock solution. Stock solutions were dissolved directly in the Tyrode or ACSF at the desired concentration. Neurons were exposed to different drug solutions until the steady-state effects were obtained, using a perfusion system.

Data analysis. Patch-clamp data were processed by using Clampfit 10.2 (Molecular Devices, USA) and then analyzed in Origin 8 (OriginLab, USA). Dose–response curves were fitted by non-linear curve fitting of the Boltzmann equation to the data using the Origin software. The values were presented as means ± SE. Analysis of variance (ANOVA) followed by the post hoc Scheffe's test was used for statistical analysis. Changes were considered significant when P < 0.05.

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**Author Contributions**

J.Z.W., C.L., K.Y.L. and H.T.X. performed the experiments. C.L., L.L.Y. and G.Y.W. designed and supervised the study. J.Z.W., C.L., K.Y.L., H.T.X. and G.Y.W. analyzed the data. J.Z.W., C.L., L.L.Y. and G.Y.W. wrote the paper. All authors read and approved the final version of the manuscript.

**Additional Information**

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