

## Original Article

# Synergistic combination therapy with SC79 and sevoflurane reduces ischemia-reperfusion injuries in rat hearts

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**Abstract:** Background and Objective: Activation of PI3K/AKT and ERK1/2 signaling has become a primary goal of therapeutic intervention for myocardial protection against I/R injuries. SC79 is a novel, selective, and highly-efficient Akt activator. Sevoflurane, an inhalation anesthetic, has been reported to activate ERK1/2 signaling during myocardial I/R. This study aimed to investigate the effects of SC79 in combination with sevoflurane against myocardial I/R injuries in a rat model. Methods: Male adult Wistar rats received myocardial ischemia for 30 minutes, followed by reperfusion for 2 hours. The heart I/R injury animal model was treated with SC79 or sevoflurane alone or combined with SC79 and sevoflurane treatment. Myocardial infarct size was determined with triphenyltetrazolium chloride staining. Cardiac function was assessed by echocardiography. Plasma creatine kinase-MB (CK-MB) was measured with colorimetric assays. Cardiomyocyte apoptosis was assessed by TUNEL staining. Myocardial Akt, ERK, Bim, bax, and Bcl-2 were determined by Western blotting. Results: Myocardial ischemia for 30 minutes, followed by reperfusion for 2 hours, significantly increased infarct size and cardiomyocyte apoptosis and reduced heart function, accompanied with significantly increased plasma CK-MB. SC79 or sevoflurane alone did not significantly decrease infarct size and restored heart function. Compared with SC79 or sevoflurane alone, combined SC79 and sevoflurane treatment effectively decreased myocardium infarct size, reduced myocardial apoptosis, diminished CK-MB, and enhanced heart function. Combined SC79 and sevoflurane treatment effectively induced phosphorylation of AKT and ERK, abolished Bax and Bim protein phosphorylation, and induced Bcl-2 protein expression. Conclusion: Enhancing phosphorylation of AKT and ERK by combination of SC79 and sevoflurane treatment provides a novel modality to treat heart I/R injuries.

**Keywords:** Heart ischemia-reperfusion, Akt, ERK, SC79, sevoflurane

## Introduction

Prolonged blockage of coronary ischemia and subsequent coronary blood reperfusion may result in cardiomyocyte apoptosis and necrosis. Ischemia/reperfusion (I/R) is usually associated with myocardial damage, the leading cause of deaths worldwide. Despite progress in the understanding of its underlying molecular mechanisms, myocardial I/R injuries remain an important clinical problem. Numerous studies have confirmed that several biological agents can reduce heart I/R injuries in experimental animal models. Unfortunately, none of these

agents have been developed as cardioprotective modalities for I/R injuries in clinical practice [1, 2].

I/R injury is an intricate process involving numerous mechanisms. PI3K/Akt pathways have important biological functions in cell proliferation, survival, and apoptosis [3]. Activation of PI3K/Akt-dependent signaling has been demonstrated to result in the attenuation of myocardial I/R injuries [4]. SC79, a small molecule Akt activator, specially suppresses Akt membrane translocation while activating Akt in the cytosol [5]. It has shown cytoprotective

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effects in experimental ischemia-elicited neuronal death [5]. It protects against early brain injuries through the dual activities of antioxidation and antiapoptosis [6]. Previous studies have reported that genetic models of cardiac AKT overexpression or cardiac-specific expression of a constitutively active mutant of Akt (myr-Akt) attenuated myocardial I/R injuries [7, 8]. However, SC79 alone has failed to reduce myocardial I/R injuries [9], in contrast to previous studies with genetic models of cardiac AKT overexpression [7, 8]. Whether other molecular signaling induced by SC79 could have blocked the potential cardioprotective effects of the drug remains unknown.

ERK signal pathways are a main component of the MAPK signal family, mainly associated with cells, cell proliferation, differentiation, and apoptosis [10]. Previous studies have indicated that activated ERK1/2 contributes to cardioprotection against I/R injuries via anti-apoptotic mechanisms [11]. Sevoflurane, an inhalation anesthetic, has been reported to protect myocardium from I/R injuries in human and experimental investigations [12-15]. Ma et al. found that sevoflurane reduced rat myocardial I/R injuries by activation of phosphorylation of Akt (p-Akt) and p-ERK1/2 [16]. Inamura et al. found that sevoflurane postconditioning activated p-Akt and p-ERK and blocked caspase-3 and -9, resulting in attenuation of apoptosis in ischemia-reperfusion injuries [17]. The present study investigated the effects of SC79 in combination with sevoflurane on myocardial I/R injuries, exploring the underlying mechanisms.

### Materials and methods

#### *Ethics statement*

The study was conducted in accordance with the Declaration of Helsinki and according national and international guidelines. It was approved by The People's Hospital of Chengyang, Qingdao, Shandong, China.

#### *In vivo myocardial ischemia/reperfusion model and experimental groups*

Male adult Wistar rats (8-10 weeks old) were obtained from Shanghai Lab Animal Research Center, Shanghai, China. The rats were placed under a 12-hour light cycle with food and drinking water available ad libitum. Experimental

procedures were approved by The Affiliated Hospital of Qingdao University. The myocardial ischemia/reperfusion model was induced by anesthesia with ketamine (80 mg/kg i.p) and xylazine (8 mg/kg i.p). The rats were ventilated via a tracheostomy on a rodent respirator. Catheters were inserted via cut downs into the jugular vein (drug delivery) and the right carotid artery (cardiac function). Myocardial ischemia was induced by exteriorizing the heart at the left fourth intercostal space, followed by a slip-knot (5-0 silk) around the left anterior descending coronary artery (LAD). Ischemia was monitored and confirmed by ST segment elevation upon electrocardiogram (ECG). Rats underwent 30 minutes of ischemia followed by coronary reperfusion for 2 hours, following the release of the tube. Rats were randomly divided into 5 groups (n = 20 rats/group) as follows: (1) Sham groups: LAD was encircled by a suture but not occluded; (2) I/R groups: ischemia (30 minutes)/reperfusion (120 minutes); (3) I/R + SC79 groups: Hearts received SC79 (100 nM) for the initial 30 minutes of reperfusion; (4) I/R + sevoflurane groups: 2.4% sevoflurane for 15 minutes at onset of reperfusion, then reperfusion of hearts for 105 minutes; and (5) I/R + sevoflurane + SC79 groups: SC79 (100 nM) for the initial 30 minutes of reperfusion, 2.4% sevoflurane for 15 minutes at onset of reperfusion, then reperfusion of hearts for 105 minutes.

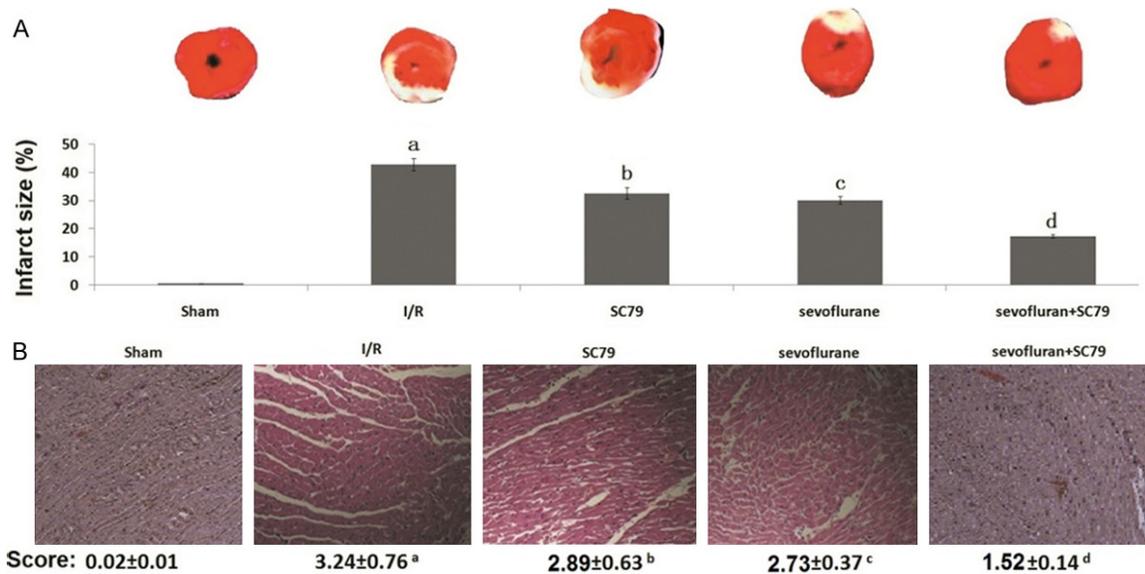
#### *Histopathological analysis*

After reperfusion, the rats were sacrificed with abdominal anesthesia and the hearts were harvested. The ventricular wall was removed and fixed with 10% buffered formalin. Sections, 4  $\mu$ m thick, were stained with hematoxylin and eosin and observed under light microscopy. Histological damage scoring criteria was: 0-no damage; 1-myocytes focal swelling and necrosis; 2-neutrophil infiltration in the myocytes; and 3-necrosis with massive neutrophil infiltration.

#### *Determination of myocardial infarct size*

At the end of the experiment, 1.5 mL of 2% Evans blue dye was perfused into the LV cavity to stain the non-ischemic region (area not at risk). Left ventricles were cut into five 2 mm transverse slices and incubated in 1% TTC in phosphate buffer (pH 7.4, 37°C) for 20 minutes. Non-infarcted myocardium was deep red,

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**Figure 1.** Synergistic combination therapy with SC79 and sevoflurane on cardioprotective effects of rats in cardiac I/R. A. Representative myocardial cross sections of TTC-stained hearts from the rats with different treatments. White area infarcted tissue; red (TTC-positive) area, viable myocardium. B. Representative H&E-stained histological images ( $\times 200$  magnification) of the myocardial sections from different treatment groups. Scales = 100  $\mu\text{m}$ . Vs Sham, <sup>a</sup> $P < 0.01$ ; Vs I/R, <sup>b</sup> $P > 0.05$ , <sup>c</sup> $P > 0.05$ , <sup>d</sup> $P < 0.01$ .

in contrast to the pale white of infarcted myocardium. Infarcted and non-infarcted myocardium were digitally measured using image analysis software (ImageJ, version 1.6, National Institutes of Health). The ratio of infarct size (white)/total area (white plus red) was used to compare differences among groups.

### Determination of myocardial apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed with *in situ* cell-death detection kit to identify the extent of DNA fragmentation, in accordance with manufacturer instructions. TUNEL-positive cells (%) were calculated as the ratio of the number of TUNEL-positive cell nuclei divided by the number of total cell nuclei.

### Determination of cardiac function

Left ventricular function, prior to the I/R protocol, was assessed using an echocardiograph, as previously described [18]. Fractional shortening (FS) was calculated using the formula:  $\%FS = [\text{left ventricular internal dimensions during diastole (LVPWd)} - \text{left ventricular internal dimensions during systole (LVIDs)}] \times 100 / \text{LVIDd}$ . At the end of the reperfusion period, LV fractional shortening (FS), left ventricular (LV)

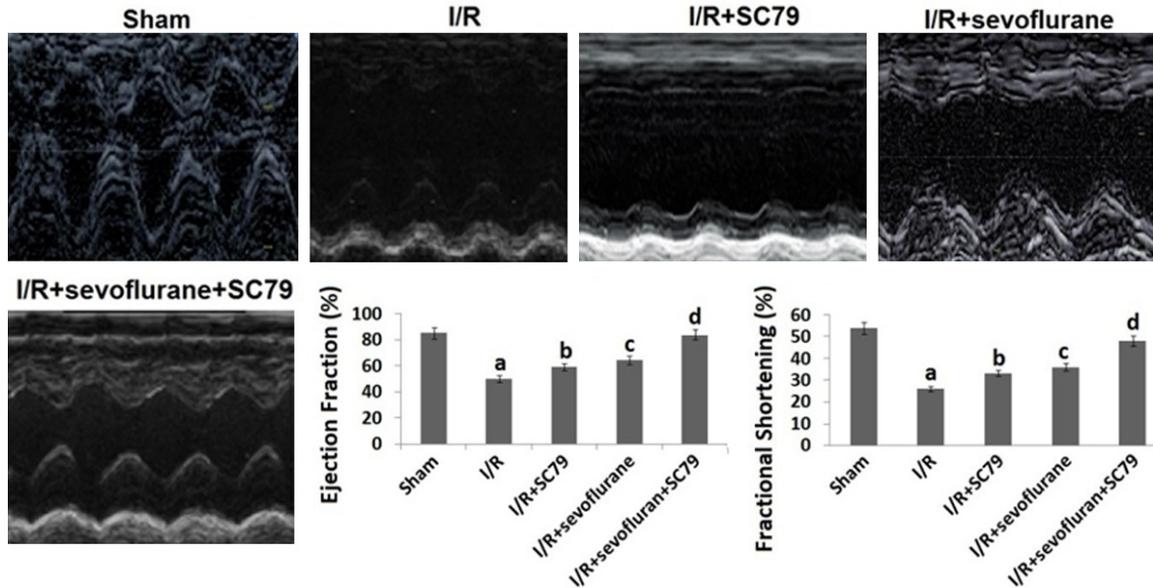
end-diastolic diameter (LVEDD), and end-systolic diameter (LVESD), and ejection fraction (EF) were recorded.

### Measurement of serum creatine kinase

After the 120-minute reperfusion period, 1 mL of blood samples were obtained from the right carotid artery. They were placed at RT for 30 minutes and centrifuged at 1000 rev min<sup>-1</sup> for 10 minutes. They were then stored at -80°C for future use. Supernatants were then obtained. Creatine kinase mb isoenzyme (CK-MB) was measured using CK-MB Assay Kits (Sigma-Aldrich, United Kingdom), according to manufacturer instructions.

### Western blot assay

Myocardial tissue from the infarcted area of the left ventricle downstream of the ligature was isolated from the rats. Tissue samples were homogenized in ice-cold modified RIPA buffer and protein concentrations were determined according to the method of Bradford protein assay. An equal amount of proteins from each sample was separated on 12% SDS-PAGE, then transferred onto PVDF membranes. Membranes were blocked with 5% skim milk for 1 hour at room temperature, then incubated with the



**Figure 2.** Synergistic combination therapy with SC79 and sevoflurane on cardiac function with echocardiographic evaluation. A representative echocardiograph recorded from a mouse after 30-min/2-h I/R in different groups of treatment. Average cardiac EF and left ventricular FS were computed. Data are expressed as mean  $\pm$  standard deviation (SD) (n = 6). Vs Sham, <sup>a</sup>P < 0.01; Vs I/R, <sup>b</sup>P > 0.05, <sup>c</sup>P > 0.05, <sup>d</sup>P < 0.01.

appropriate primary antibodies against pAkt (Ser473), Akt, pERK1/2 (Thr202/Tyr204), ERK1/2, Bcl-2, Bax, Bim [Santa Cruz Biotechnology (Santa Cruz, Shanghai, China)], and  $\beta$ -actin (Sigma). Staining was visualized by ECL detection reagents.

#### Statistical analysis

All data are expressed as mean  $\pm$  SD. Results were analyzed by two-tailed Student's *t*-test or by ANOVA. *P* < 0.05 is considered statistically significant.

#### Results

##### *Synergistic combination therapy with SC79 and sevoflurane reduces infarct size of rat model of heart I/R injury*

To determine the effects of SC79 alone on I/R injuries, the hearts received SC79 (100 nM) during the first 30 minutes of reperfusion. Rat hearts exposed to a 30-minute global ischemia and 120-minute reperfusion developed a  $41 \pm 4\%$  infarct size in the basal portion of ventricle, as evaluated by TCC staining (**Figure 1A**). SC79 administration alone ( $36 \pm 3\%$ , *P* = 0.078) or 2.4% sevoflurane administration alone ( $33 \pm 4\%$ , *P* = 0.057) did not significantly decrease

infarct size after 24 hours of reperfusion, compared with the untreated I/R group (**Figure 1A**). However, synergistic combination therapy with SC79 and sevoflurane reduced infarct size to  $17 \pm 2\%$ , significantly decreased compared to SC79 or sevoflurane alone (**Figure 1A**).

Histological examination showed that untreated I/R hearts demonstrated tissue damage and necrosis with massive neutrophil infiltration (**Figure 1B**). In combination treated groups, tissue damage was significantly decreased, compared to SC79 or sevoflurane alone (**Figure 1B**).

##### *Synergistic combination therapy with SC79 and sevoflurane improves cardiac function following I/R*

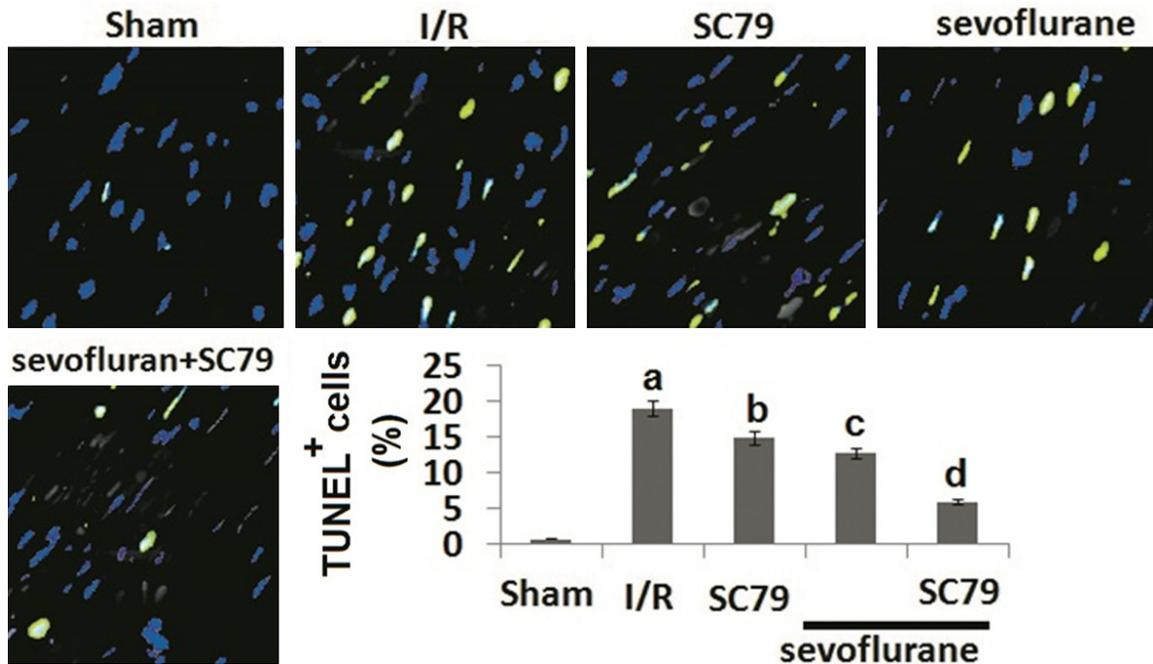
Cardiac function was examined by echocardiography after I/R, as shown in **Figure 2A** and **Table 1**. LV fractional shortening (FS), left ventricular (LV) end-diastolic diameter (LVEDD), and end-systolic diameter (LVESD), and ejection fraction (EF) were decreased significantly, compared with the sham group. Although the increase in hemodynamic parameters above was observed in SC79 or sevoflurane alone treated animals compared to untreated I/R, these differences were not statistically signifi-

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**Table 1.** Circulating levels of CK-MB and echocardiography findings in six study groups of animals

	Control	Sham	I/R	I/R + SC79	I/R + sevoflurane	I/R + SC79 + sevoflurane
CK-MB (ng/ml)	110 ± 12.24	114.8 ± 14.26	208 ± 20.38 <sup>a</sup>	184 ± 13.85 <sup>b</sup>	176 ± 12.89 <sup>c</sup>	132 ± 11.8 <sup>d</sup>
EF (%)	87 ± 3.4	85.2 ± 3.2	51.8 ± 2.7 <sup>a</sup>	55.3 ± 2.9 <sup>b</sup>	61.4 ± 3.3 <sup>c</sup>	77.8 ± 3.6 <sup>d</sup>
FS (%)	55 ± 4.3	54 ± 3.8	26 ± 1.8 <sup>a</sup>	29 ± 1.9 <sup>b</sup>	31 ± 2.6 <sup>c</sup>	44 ± 3.8 <sup>d</sup>
LVEDD (cm)	0.60 ± 0.068	0.59 ± 0.071	0.81 ± 0.042 <sup>a</sup>	0.76 ± 0.053 <sup>b</sup>	0.74 ± 0.064 <sup>c</sup>	0.63 ± 0.049 <sup>d</sup>
LVESD (cm)	0.34 ± 0.042	0.33 ± 0.046	0.68 ± 0.045 <sup>a</sup>	0.61 ± 0.052 <sup>b</sup>	0.60 ± 0.045 <sup>c</sup>	0.42 ± 0.044 <sup>d</sup>

Data are expressed as mean ± standard deviation (SD) (n = 6). Vs Sham, <sup>a</sup>P < 0.01; Vs I/R, <sup>b</sup>P > 0.05, <sup>c</sup>P < 0.05, <sup>d</sup>P < 0.01.



**Figure 3.** Apoptotic cells were examined using TUNEL assay for DNA fragmentation. Cardiomyocytes were photographed by fluorescence microscopy after 30-min/2-h I/R in different groups of treatment. Representative pictures of sections stained with TUNEL (× 400). TUNEL-positive nuclei are shown in green. Blue fluorescence indicated total cardiomyocyte nuclei. Quantitative analysis (percentage of apoptotic cells versus total) is shown in histogram. Vs Sham, <sup>a</sup>P < 0.01; Vs I/R, <sup>b</sup>P > 0.05, <sup>c</sup>P > 0.05, <sup>d</sup>P < 0.01.

cant (**Table 1**). Synergistic combination therapy with SC79 and sevoflurane significantly increased these hemodynamic parameters, compared to SC79 or sevoflurane alone (**Table 1**). Sevoflurane or SC79 alone did not show any effects on the myocardium without I/R injuries (data not shown).

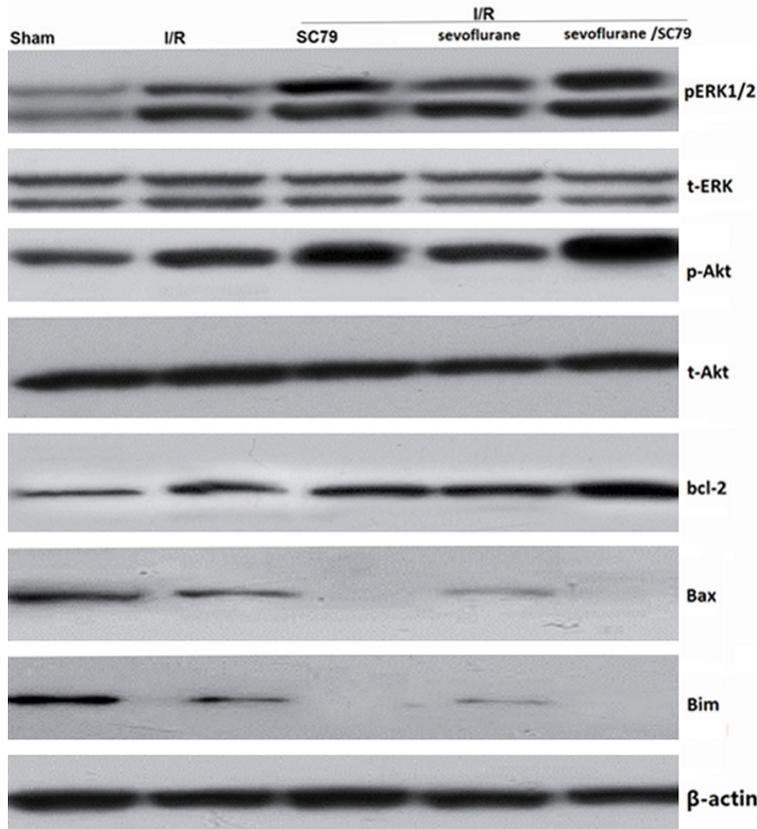
CK-MB isoenzyme is a major biomarker for myocardial cellular injuries. As shown in **Table 1**, CK-MB was significantly increased in untreated I/R groups. SC79 or sevoflurane alone partly decreased CK-MB levels, compared to the untreated I/R groups, but these differences were not statistically significant. Synergistic combination therapy with SC79 and sevoflu-

rane significantly decreased CK-MB levels, compared to SC79 or sevoflurane alone (**Table 1**).

### *Synergistic combination therapy with SC79 and sevoflurane inhibits myocardial apoptosis*

As shown in **Figure 3**, the percentage of TUNEL positive nuclei was significantly increased in untreated I/R myocardial sections (20.4 ± 2.9% vs 0.84 ± 0.3% of Sham group, *P* < 0.01). The number of TUNEL-positive nuclei was decreased by 15.6 ± 2.1% in sevoflurane treated groups and 17.6 ± 2.4% in SC79 treated groups, compared to the untreated I/R group (*P* > 0.05, respectively). However, synergistic combination

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**Figure 4.** Akt and ERK activation during 30-min/2-h I/R. Western blot assay for pAkt (Ser473), Akt, pERK1/2 (Thr202/Tyr204), ERK1/2, Bcl-2, Bax, and Bim in different groups.

therapy with SC79 and sevoflurane significantly decreased TUNEL-positive nuclei, compared to SC79 or sevoflurane alone groups ( $P < 0.01$ , respectively).

### *Synergistic combination therapy with SC79 and sevoflurane activates PI3K/AKT and ERK signaling in vivo*

ERK1/2 and AKT phosphorylation (p-ERK1/2 and pAkt) was increased after 30-minutes/2-hours I/R. Treatment of SC79 or sevoflurane alone also increased p-ERK1/2 and pAkt levels, according to Western blot assay (**Figure 4**). Combined SC79 and sevoflurane treatment led to significantly increased pAKT and pERK levels, compared to them alone (**Figure 4**). Furthermore, Bcl-2 protein was significantly increased after 30-minutes/2-hours I/R, while combined SC79 and sevoflurane treatment led significantly increased pAKT and pERK, compared to SC79 or sevoflurane alone (**Figure 4**). In contrast, expression of Bax and Bim was significantly inhibited when treated with SC79,

sevoflurane alone, or combined treatment.

### **Discussion**

Coronary artery occlusion can cause irreversible damage to cardiac function. Early restoration of coronary flow is an essential prerequisite for restoring myocardial function. Paradoxically, myocardial blood flow recirculation may cause re-injury of myocardial function in the ischemic region through a reperfusion injury mechanism [19]. Currently, no drugs approved by the FDA can directly be used to protect hearts against ischemia-reperfusion (I/R) injuries.

Activation of PI3K/AKT and ERK1/2 signal pathways has been reported to exert cardioprotection at the time of myocardial reperfusion [20]. Thus, activation of PI3K/AKT and ERK1/2 signaling has become a primary goal of therapeutic intervention for myocardial protection against I/R injuries.

SC79 is a novel and safe small molecule compound, which can be used as a selective, highly-efficient, and cell-permeable Akt activator [5]. The inhaled anesthetic sevoflurane has been demonstrated to protect against myocardial I/R injuries. In the present study, pretreatment of SC79 or sevoflurane alone exerted partial cardioprotective effects when given SC79 or sevoflurane before I/R, as evidenced by reduced myocardial apoptosis and decreased myocardial infarct size. Echocardiographies revealed that cardiac function was partly preserved in SC79 or sevoflurane alone treated mice, compared to untreated mice at 2 hours following I/R. However, combined SC79 and sevoflurane treatment significantly alleviated post-ischemic cardiac dysfunction and injury and reduced myocardial infarct size, compared to SC79 or sevoflurane alone groups.

Activation of PI3K/Akt signaling has been reported to protect cells from apoptosis induced by I/R, through which phosphorylates pro-apoptotic molecules Bax or Bim, leading to

dissociation of Bax or Bim from anti-apoptotic molecule Bcl-2 [21]. ERK1/2 signaling pathways are the major pathways involved in Bim ubiquitination and consequent proteasomal degradation. Several studies have shown that activation of ERK1/2 can inhibit Bim expression and prevent cell death [22]. Recently, it has reported that Bcl-2 and Bax played a pivotal role in gastric mucosal I-R injuries and repair by activation of ERK1/2, which also plays an important role in protecting against myocardial I/R injuries [23]. In the present study, SC79 mainly activated AKT, decreased Bax and Bim expression, and increased Bcl-2 expression. Sevoflurane treatment alone mainly activated ERK, decreased Bax and Bim expression, and increased Bcl-2 expression. Combined SC79 and sevoflurane treatment synergistically activated AKT and ERK, followed by increased Bcl-2 expression and decreased Bax and Bim expression, compared to SC79 or sevoflurane alone groups.

In conclusion, the cardioprotective benefits of SC79 or sevoflurane treatment alone were not significantly different, compared to untreated I/R. ERK and AKT activation was a key factor in the cardioprotective benefits of combined SC79 and sevoflurane treatment, following 2 hours of reperfusion, significantly different than SC79 or sevoflurane treatment alone groups.

### Disclosure of conflict of interest

None.

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