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Protective effect of novel pyridoindole derivatives on ischemia/reperfusion injury of the isolated rat heart

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Abstract:
Generation of reactive oxygen species is a major, well-known cause of heart injury induced by ischemia-reperfusion. This injury is manifested through myocardial stunning, reperfusion and lethal reperfusion injury of cardiocytes. The pyridoindole stobadine has been shown to exhibit significant antioxidant, free-radical scavenging and hypoxic-tissue-protecting properties. The present study examined the effects of stobadine and two novel derivatives, SMe1 and SMe1EC2, which exhibit improved pharmacodynamic and toxicity profiles, on the functional properties and reperfusion dysrhythmias of the isolated rat heart in ischemia-reperfusion conditions. All experiments were performed on isolated Langendorff-perfused hearts isolated from 3-month-old male Wistar rats. After 15 min of stabilization, the hearts were subjected to a 30-minute period of global no-flow ischemia, followed by a 30-minute reperfusion period. Stobadine, SMe1 and SMe1EC2 were applied at a concentration of 1×10^{-5} M 10 min before the onset of ischemia, and during reperfusion through the perfusion medium. As compared to the untreated group, addition of SMe1EC2 during reperfusion significantly increased left ventricular developed pressure, decreased pathologically elevated left ventricular end-diastolic pressure and enhanced recovery of the stunned myocardium after ischemia. Both SMe1 and stobadine failed to influence these parameters; however, all derivatives tested inhibited serious life-threatening reperfusion dysrhythmias such as ventricular tachycardia and ventricular fibrillation. Our findings suggest that SMe1EC2 promotes an improved recovery of the left ventricular function following ischemia compared to either stobadine or SMe1. However, both SMe1EC2 and SMe1 manifested a significant anti-dysrhythmic effect comparable with that of stobadine and partially reduced myocardial ischemia-reperfusion-induced injury.

Key words:
pyridoindole, stobadine, isolated heart, ischemia-reperfusion, dysrhythmias

Abbreviations: ECG – electrocardiogram, HR – heart rate, I/R – ischemia and reperfusion, LVDP – left ventricular developed pressure, LVEDP – left ventricular end-diastolic pressure, VF – ventricular fibrillation, VT – ventricular tachycardia

Introduction

Ischemic heart disease arising secondary to acute myocardial infarction is among the most prevalent health problems in the world and is the leading cause of morbidity and mortality. In the ischemic heart, initial cardiac damage is prompted by a diminished blood supply to the heart tissue. Coronary reperfusion has been proven to be the only method of limiting infarct size. The sooner is the reperfusion, the better should be the outcome. Unfortunately, reperfusion itself can lead to additional injury in the form of myocyte death, reperfusion arrhythmias, myocardial stunning, endothelial and microvascular dysfunction and exacerbated myocardial infarction [4, 10, 17, 18]. Oxidative stress appears to play a major role in organ injury during ischemia and reperfusion (I/R) [4, 16,

26]. Indeed, the major causative factors of I/R injury are: (1) re-energization, (2) rapid normalization of tissue pH (causing accumulation of calcium in the cytosol and mitochondria and subsequent hypercontracture development), (3) rapid normalization of tissue osmolality leading to cell swelling and, in particular, (4) increased production of reactive oxygen species (ROS). Thus drugs with antioxidant properties are predicted to be useful in protecting the heart against I/R injury [14–18].

The pyridoindole derivative stobadine ((–)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole) [25], which was synthesized as a potential new anti-dysrhythmic drug, was found to decrease the rate of $CaCl_2$-evoked ventricular fibrillation in rats [4] and to reduce the severity of reperfusion-induced dysrhythmias, ventricular premature beats, ventricular tachycardia and fibrillation in isolated rat hearts [9]. Stobadine was recognized to have significant antioxidant, free-radical scavenging and hypoxic-tissue-protecting properties [23, 24]. Multiple studies have shown that stobadine is effective in oxidative stress-mediated pathologies. For example, in an experimental model of cerebral ischemia-reperfusion injury, stobadine treatment induced neuroprotection with a significant benefit in terms of functional deficit [1, 19]. In an experimental model of diabetes mellitus of rats, stobadine exhibited a positive effect on pathologies such as diabetic neuropathy [22] and diabetic cataracts [11] (for review see [6]). In some diabetic models the effect of stobadine was comparable to the effects seen with vitamin E [22] or α-lipoic acid [21]. Due to good toxicity and teratogenicity profiles, stobadine proved to be applicable to *in vivo* studies [28].

Novel stobadine derivatives with improved pharmacodynamic and toxicity profiles and increased selectivity have been developed [28]. 8-Methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indolinium dichloride (SMe1) and 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indolinium dichloride (SMe1EC2) – structural analogs of stobadine, were selected from more than 70 new derivatives as the most potent antioxidants. Recent studies have shown that SMe1EC2 restores reduced endothelium-mediated relaxation in an experimental diabetic rat model [30] and protected rat hippocampal slices against hypoxia at levels comparable to stobadine, melatonin, 21-aminosteroids, and other antioxidants [27]. In ischemia-reperfused diabetic rat hearts, SMe1EC2 also reduced the incidence of spontaneous and evoked dysrhythmias [7]. The aim of this work is to evaluate the potential cardioprotective and anti-dysrhythmic effect of SMe1 and SMe1EC2 in comparison with stobadine in a model of I/R induced injury in isolated rat hearts.

Materials and Methods

Animals and chemicals

Three-month-old male Wistar rats weighing 360–410 g (Breeding Facility of the Institute of Experimental Pharmacology and Toxicology SASc, Dobra Voda, Slovakia) were used. Experimental groups of 6 rats and control groups of 8 rats were formed by random assignment. The rats were maintained under a 12 h light/dark cycle with free access to water and a standard laboratory diet. Animal housing, care and experimental procedures were conducted under the guidelines of the Animal Ethics Committee and were approved by the State Veterinary and Food Administration of the Slovak Republic.

All experimental substances, (–)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3-b]indolinium dichloride (stobadine), 2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3-b]indolinium chloride (SMe1EC2) and 8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indolinium dichloride (SMe1), were synthesized at the Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences [25, 26].

Langendorff-isolated heart preparation and perfusion protocol

Experimental animals were anesthetized with diethyl ether and then the hearts were removed and mounted on a perfusion apparatus *via* the aorta. Aortic perfusion was then started at a constant pressure (85 mm Hg) according to the Langendorff method. Krebs-Henseleit perfusion solution was prepared as follows (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), NaH_2PO_4 (1.18), $NaHCO_3$ (25), and glucose (11.1) equilibrated with a gas mixture of 95% O_2 and 5% CO_2 at 37°C, pH 7.4. Following 20 min of stabilization, hearts were subjected to 30 min global no-flow ischemia, followed by 30 min reperfusion (see Fig. 1). Stobadine. 2HCl, SMe1.2HCl and SMe1EC2.2HCl were applied in the

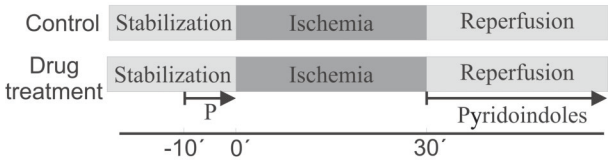

Fig. 1. Experimental protocol (P = treatment with pyridoindoles)

concentration of 1×10^{-5} M 10 min before the onset of ischemia and during reperfusion through the perfusion medium.

Analysis of heart function

The effects of SMe1 and SMe1EC2 on functional recovery during reperfusion was compared with both control (untreated) hearts and stobadine-treated hearts. Left ventricular developed pressure (LVDP) was measured iso-volumetrically using a water-filled latex balloon connected to a pressure transducer with tubing inserted into the left ventricle through the left atrium. The balloon volume was adjusted to give a left ventricular end-diastolic pressure (LVEDP) of 0–10 mm Hg at the beginning of the experiment. LVDP was calculated as the difference between the left ventricular systolic pressure and LVEDP.

Two contact electrodes for ECG recording were placed on the right atrium and left ventricle. Heart electrical activity (ECG as well as LVDP and LVEDP) was continuously recorded by a BioLabF system. During reperfusion, myocardial stunning, ventricular dysrhythmias and dysrhythmia scores [3, 12] were evaluated.

Statistical analysis

The results are presented as the mean ± SEM. An unpaired Student's *t*-test was used to compare untreated and pyridoindole-treated groups. A value of $p < 0.05$ was considered as statistically significant.

Results

Functional parameters

The initial values of the contractile parameters of all experimental groups were not significantly different, indicating a similar baseline myocardial function of all hearts. LVDP in the control group before the onset of ischemia was 105.2 ± 7.7 mm Hg. As shown in Fig. 2, ischemia initiated by clamping the perfusion line resulted in rapid decrease of LVDP in all experimental groups. In untreated hearts, 30 min reperfusion achieved by opening the perfusion line resulted in a recovered LVDP of 27.3 ± 5.3% of the pre-ischemic value of this group. In stobadine-treated hearts, recovery of LVDP reached 28.7 ± 9.2% of the pre-ischemic value of untreated hearts. In comparison to the untreated and stobadine-treated groups, SMe1EC2 significantly increased LVDP beginning at the third minute of reperfusion and exhibited a recovery of 36.1 ± 7.7% at the end of reperfusion. In contrast, the LVDP in the SMe1-treated group did not differ from that of the control group during reperfusion.

The LVEDP in untreated ischemic hearts increased from 5.3 ± 2.2 mm Hg to a maximum 33.0 ± 5.6 mm

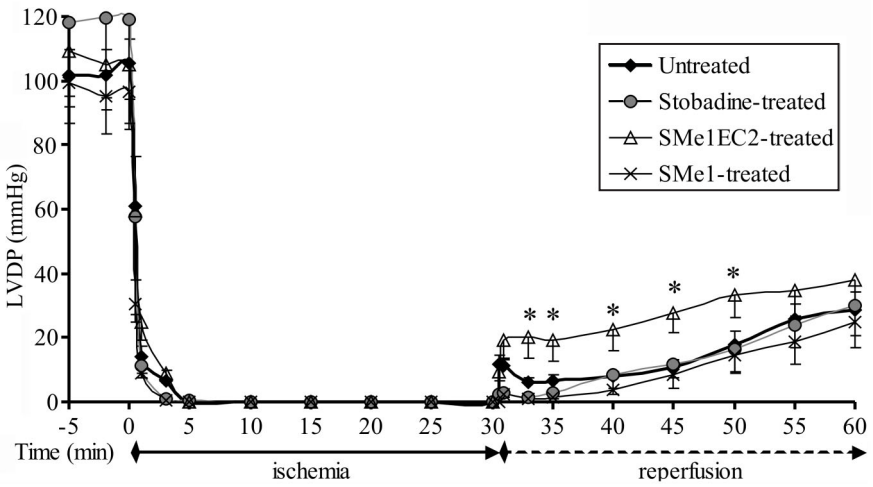

Fig. 2. Left ventricular developed pressure (LVDP) of untreated, stobadine-, SMe1- and SMe1EC2-treated hearts during ischemia-reperfusion. * $p < 0.05$ compared to untreated group

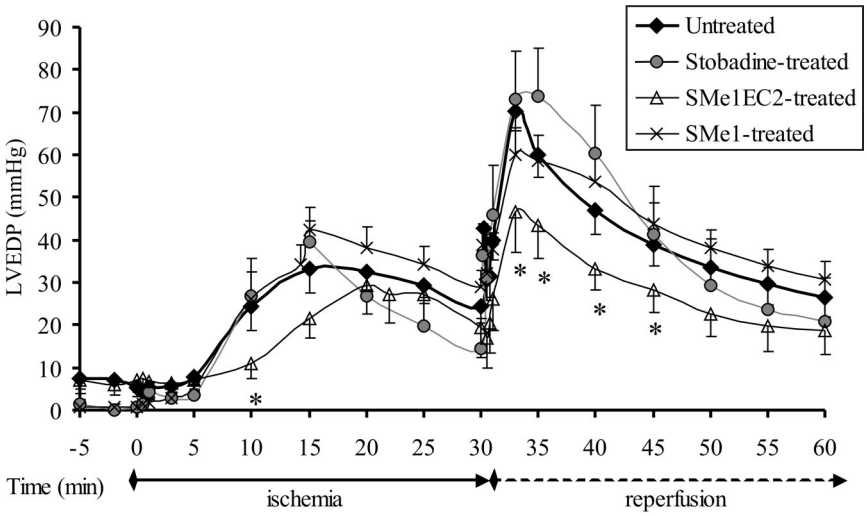

Fig. 3. Left ventricular end-diastolic pressure (LVEDP) of untreated, stobadine-, SMe1- and SMe1EC2-treated hearts during ischemia-reperfusion. * $p < 0.05$ compared to untreated group

Hg during the 15 min ischemic period (see Fig. 3). During reperfusion, LVEDP was further increased and reached the maximum of 70.3 ± 4.8 mm Hg in the third minute of reperfusion. Subsequent recovery reached 497 ± 82% of the initial values at the end of the reperfusion period. Administration of stobadine or SMe1 did not influence the course of LVEDP during ischemia-reperfusion. However, SMe1EC2 significantly decreased the pathologically elevated LVEDP during the first 10 min of ischemia and in a similar manner during reperfusion. In this experimental group, recovery of LVEDP at the end of reperfusion reached 351 ± 103% of baseline values in the untreated group.

The heart rate (HR) of untreated hearts reached 248 ± 13 beats/min before the onset of ischemia, and treatment with pyridoindoles caused a non-significant reduction in this value (data not shown). At the end of reperfusion, the HR in the untreated group reached 95% of pre-ischemic values. All experimental substrates significantly reduced HR in comparison with the untreated group.

Reperfusion dysrhythmias and myocardial stunning

An average of 248 ± 51 ventricular premature beats (VPB) was found in untreated rat hearts during the reperfusion period. As shown in Figure 4, none of the experimental substances altered the number of VPB. Ventricular tachycardia (VT) was sustained for 160 ± 40 s in untreated hearts. Administration of stobadine or SMe1EC2 significantly shortened the duration of VT compared to the control group (79 ± 22 s and 50 ± 31 s, respectively). SMe1 also evoked a reduction in VT analogous to stobadine, but this effect was non-significant compared to the untreated group. Another life-threatening dysrhythmia, ventricular fibrillation (VF), was sustained for an average of 76 ± 39 s in the untreated group. The incidence of VF was completely suppressed in the presence of stobadine as well as with treatment of SMe1EC2 and SMe1 during the reperfusion period.

To create a summary analysis of the reperfusion dysrhythmias, three dysrhythmia scores regarding the incidence and the duration of VT and VF have been previously described. Score A and score C were selected from a number of scores developed by Curtis and Walker [3] as the most suitable for evaluation of the reperfusion dysrhythmias in an isolated rat heart model. Score A accounts for the number of episodes and the duration of VT and VF; whereas score C accounts for the number of episodes of VT and VF. Score L according to [12] considers the duration of VT and VF. As shown in Table 1, all experimental substrates partially decreased the three dysrhythmia scores during 30 min of reperfusion. Administration of stobadine had a significant effect only on score C, SMe1EC2 reduced both score C and A, and all three scores were significantly reduced by SMe1.

Along with the electric dysfunction, ischemia impairs also cardiac contractility. Myocardial stunning was evaluated as the period measured from the onset of reperfusion, to the point where LVDP reached 20 mmHg. In the untreated group, myocardial stunning persisted for an average of 21.1 ± 2.4 min (see

Tab. 1. Dysrhythmia score of untreated, stobadine-treated, SMe1EC2-treated and SMe1-treated rat hearts during 30-minute reperfusion.

	Score A [(a)]	Score C [(a)]	Score L [(b)]
Untreated	4.2 ± 0.3	3.5 ± 0.3	4.0 ± 0.3
Stobadine-treated	3.3 ± 0.6	2.2 ± 0.3*	3.2 ± 0.6
SMe1EC2-treated	3.0 ± 0.4*	2.5 ± 0.3*	3.2 ± 0.5
SMe1-treated	3.0 ± 0.6*	2.2 ± 0.3*	2.7 ± 0.7*

Notes: [(a)] according to Curtis and Walker [3], [(b)] according to Lepran and Szekeres [12]. * $p < 0.05$ *vs.* untreated group

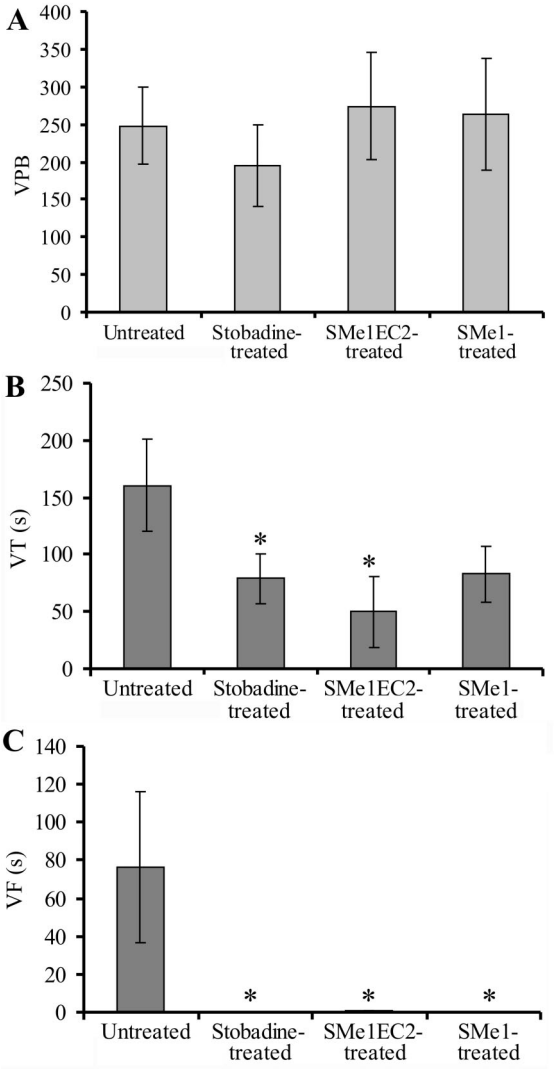

Fig. 4. Effects of stobadine, SMe1EC2 and SMe1 on reperfusion-induced ventricular dysrhythmias during 30 min of reperfusion. (**A**) Number of ventricular premature beats (VPB). (**B**) Duration of ventricular tachycardia (VT) in s. (**C**) Duration of ventricular fibrillation (VF) in s. * $p < 0.05$ compared to untreated group

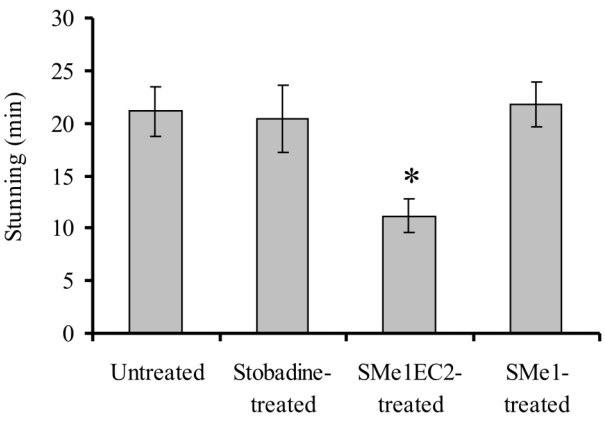

Fig. 5. Duration of myocardial stunning in untreated, stobadine-, SMe1EC2- and SMe1-treated hearts in minutes during reperfusion. * $p < 0.05$ compared to untreated group

Fig. 5). Stobadine and SMe1 did not influence this parameter. However, SMe1EC2 significantly reduced the persistence of this myocardial contractile dysfunction to 11.2 ± 1.6 min, thereby enhancing myocardial recovery after stunning resulting from global ischemia.

Discussion

Reperfusion of the ischemic myocardium results in irreversible tissue injury and cell necrosis, leading to decreased cardiac performance. Considerable evidence suggests that ROS, particularly when produced during the early phase of reperfusion, are involved in cardiac injury caused by I/R. The mechanism of cellular damage involves the stepwise reduction of molecular oxygen to the relatively inactive superoxide and hydrogen peroxide, with subsequent metal ion-catalyzed formation of the highly reactive hydroxyl radical. These radicals may exceed the capacity of the cellular intrinsic free radical scavenging systems and lead to cellular dysfunction and death. Activated oxygen species can then induce direct cell membrane damage *via* peroxidation of polyunsaturated fatty acids, further contributing to cell death. Most importantly, ROS can also cause damage to proteins, which may lead to destruction of critical enzymes and the interference of ion pumps, followed by dysregulation of intracellular and mitochondrial Ca^{2+} levels. In contrast, levels of ROS seen in cardioprotection are esti-

mated to be 10-fold lower than that of ischemic injury. Thus, targeting ROS should prevent direct deleterious effects on membranes and protect the heart against reperfusion injury [13–16, 31].

A number of pharmacological approaches designed to prevent or limit oxygen free-radical mediated damage have been investigated, including the administration of various ROS scavengers or antioxidants such as stobadine. Stobadine, considered a leading pyridoindole compound, was repeatedly shown to be an efficient antioxidant both in a membranous lipid environment and also in non-lipid systems of a pure protein. This effect is primarily due to its ability to scavenge deleterious ROS such as hydroxyl-, peroxyl- and alkoxyl- radicals [6, 23]. Thus, in studies of indole compounds, stobadine has been proposed as an acceptable alternative to the structurally diverse trolox, a popular reference antioxidant [6]. Previous studies have demonstrated that stobadine possessed, among other effects, an effective anti-dysrhythmic and cardioprotective ability. In a rat heart model of global ischemia-reperfusion, Knezl et al. [8, 9] applied stobadine in the concentration of 1×10^{-6} M three minutes before the onset of a 30-min period of ischemia and during the 30-min reperfusion period. In this model, stobadine prevented the deleterious effect of I/R injury by increasing LVDP and decreasing LVEDP during reperfusion and by reducing the number of reperfusion dysrhythmias. In the present study, stobadine was used as the reference substance due to its beneficial properties and structural similarity to the experimental substrates SMe1 and SMe1EC2. The concentration of 1×10^{-5} M for all substances was determined based on previous concentration dependency tests [8].

As previously mentioned, ischemia and reperfusion are both associated with an increase in intracellular calcium. This effect may be related to increased sarcolemmal calcium entry through L-type calcium channels as a consequence of sodium overload, or it may be secondary to alterations in sarcoplasmic reticulum calcium cycling. ROS have been shown to interfere with this process. Reperfusion injury results in significant desensitization of the myofibrils to calcium. Subsequently, the myocardium can be damaged by contracture development, causing mechanical stiffness, tissue necrosis, and the "stone heart" phenomenon [15, 16, 18]. In the experiments presented herein, reperfusion resulted in a rapid decrease of LVDP and an increase in LVEDP in the untreated group. At the end of a 30-min reperfusion period, the hearts treated with SMe1EC2 exhibited a better functional recovery during reperfusion *via* a significant improvement of LVDP and a reduction of the pathologically elevated LVEDP. This effect was only evident in hearts treated with SMe1EC2. In contrast to previous studies by Knezl et al. [8, 9], in our experiments stobadine failed to influence these functional parameters. This discrepancy might be explained by the application of different concentrations of stobadine. It is conceivable that stobadine at the higher concentration of 1×10^{-5} M used in our study might have a stabilizing effect on myocyte membranes.

Reperfusion dysrhythmias, one of the most serious manifestations of I/R injury, were also evaluated in this study. The clinical importance of reperfusion-induced arrhythmias, particularly VT and VF, is based on evidence of their role in sudden cardiac death [15–18]. The pathophysiological mechanism responsible for the initiation of VT and VF include the overproduction of oxygen-derived free radicals and calcium overload during the first minutes of reflow [15]. In our experiments, SMe1EC2 as well as SMe1 reduced severe life-threatening dysrhythmias (VT and VF), as evidenced by decreasing dysrhythmia scores. These anti-dysrhythmic effects of both experimental substrates were comparable with that of stobadine and were likely due to antioxidant and free radical scavenging properties.

Post-ischemic dysfunction, or myocardial stunning, is the mechanical dysfunction persisting after reperfusion, despite the absence of irreversible damage and the restoration of normal or near-normal coronary flow. This dysfunction, even if severe or prolonged, is fully reversible and is not caused by a primary deficit of myocardial perfusion. The generation of oxygen radicals is one of the likely contributing factors [2]. Our findings suggest that the pyridoindole SMe1EC2 significantly enhanced recovery of the stunned myocardium after ischemia. The protective action of this substrate can be attributed to the reduction of free-radical mediated cell damage after ischemia-reperfusion. Neither stobadine nor SMe1 influenced this parameter.

The enhanced effect of SMe1EC2 likely originates from differences in chemical structure compared to the analogs tested. This substrate possesses a higher lipophilicity, represented by the value of the calculated partition coefficient (logP = 2.21; [29]), compared to SMe1 (logP = 1.165; [29]), a compound with

an aromatic substitution ensuring comparable intrinsic anti-radical efficacy [20]. Thus, an ethoxycarbonyl- substitution can be associated with the improved bioavailability of this derivative. The aromatic methoxy group of SMe1EC2 may ensure its improved free radical scavenging properties compared to stobadine, a compound with a comparable lipophilicity (logP = 1.95). However, the high degree of protonation of stobadine and SMe1 at the piperidine nitrogens at physiological pH can considerably contribute to an additional decrease in bioavailabity compared to SMe1EC2 [20].

Conclusion

Our study shows a protective effect of the pyridoindole derivative SMe1EC2 against I/R injury of the rat heart in terms of improved functional recovery. Both SMe1EC2 and SMe1 demonstrated anti-dysrhythmic properties against reperfusion dysrhythmias, comparable to the effect of the reference substrate stobadine. The beneficial properties of pyridoindoles appear to be mediated by the antioxidant and free-radical scavenging protection of cardiomyocyte membranes; however, additional studies will be necessary to determine other potential mechanisms of action.

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