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Letter

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Indolylmaleimide Derivative IM-17 Shows Cardioprotective Effects in Ischemia-Reperfusion Injury

Kosuke Dodo,*[†,‡,§,∥] Tadashi Shimizu,[†,§,#] Jun Sasamori,[⊥] Kazuyuki Aihara,[⊥] Naoki Terayama,[†,∥] Shuhei Nakao,[†,∥] Katsuya Iuchi,[†,‡,∇] Masahiro Takahashi,[§] and Mikiko Sodeoka*[†,‡,∥]

[†]RIKEN, Synthetic Organic Chemistry Laboratory, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan

[‡]Sodeoka Live Cell Chemistry Project, ERATO, JST, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan

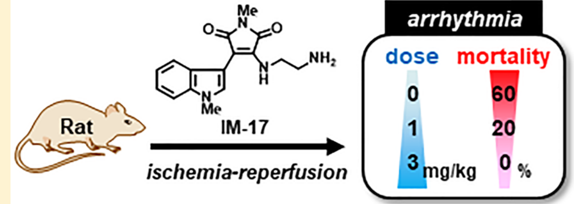
[§]Institute of Multidisciplinary Research for Advanced Materials (IMRAM), Tohoku University, 2-1-1, Katahira, Aoba, Sendai, Miyagi 980-8577, Japan

[∥]AMED-CREST, AMED, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

[⊥]Drug Research Department, Fukushima Research Laboratories, Toa Eiyo Ltd., 1,Yuno-tanaka, Iizaka-machi, Fukushima-shi, Fukushima 960-0280, Japan

Supporting Information

ABSTRACT: We previously developed **IM-54** as a novel type of inhibitor of hydrogen-peroxide-induced necrotic cell death. Here, we examined its cell death inhibition profile. **IM-54** was found to selectively inhibit oxidative stress-induced necrosis, but it did not inhibit apoptosis induced by various anticancer drugs or Fas ligand, or necroptosis. **IM-17**, an IM derivative having improved water-solubility and metabolic stability, was developed and confirmed to retain necrosis-inhibitory activity. **IM-17** showed cardioprotective effects in an isolated rat heart model and an *in vivo* arrhythmia model, suggesting that IM derivatives may have therapeutic potential.



KEYWORDS: *Indolylmaleimide, necrosis, ischemia-reperfusion, cell death, arrhythmia*

In recent decades, cell death has been recognized to play an important role in the development and maintenance of multicellular organisms. Originally, cell death was divided into two major categories, apoptosis and necrosis according to the morphological features.[1] Apoptosis involves characteristic and regulated changes, such as membrane blebbing, nuclear condensation, and formation of apoptotic bodies. On the other hand, necrosis is associated with cellular swelling and rupture of the cellular membrane, which could be induced by physical damage. Therefore, necrosis is thought to be accidental and unregulated cell death, and only apoptosis is considered as naturally occurring cell suicide, so-called "programmed cell death". Most cell death research has focused on apoptosis, and the molecular mechanisms and physiological importance of apoptosis have been well characterized.[2] In particular, a family of proteases, called caspase, was identified as mediators and executors of apoptotic signals, and caspase-mediated apoptosis was found to play an important role in the selection of lymphocytes[3,4] and in cellular immunity.[5] Inhibition of apoptosis is thought to be involved in the pathogenesis of various diseases, such as cancer, autoimmune disease, and AIDS.

On the other hand, abnormal acceleration of cell death is known to cause various diseases.[2] In some cases, such as neurodegenerative diseases[6] or infarction,[7,8] necrosis was found to contribute to critical damage leading to lethality. Rupture of the cell membrane, a typical hallmark of necrosis, causes the release of various factors from cells, including Ca^{2+}, glutamate, and proteases, which cause damage to surrounding cells and expand the damaged area. Therefore, the reduction of necrosis-induced damage may be a potent therapeutic approach for diseases involving acceleration of cell death.

We previously developed a bisindolylmaleimide derivative **MS-1** (Figure 1)[9] from a well-known PKC inhibitor, BM I, and reported that it inhibited necrotic cell death induced by H_2O_2 through PKC-independent mechanisms. This molecule was also found to reduce the area of myocardial infarction in an *in vivo* rat ischemia-reperfusion injury model,[10] in which oxidative-stress-induced necrosis is thought to be involved.[11,12] This fact

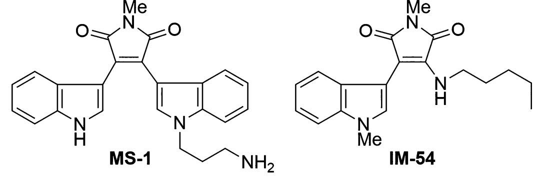


Figure 1. Structures of **MS-1** and **IM-54**.

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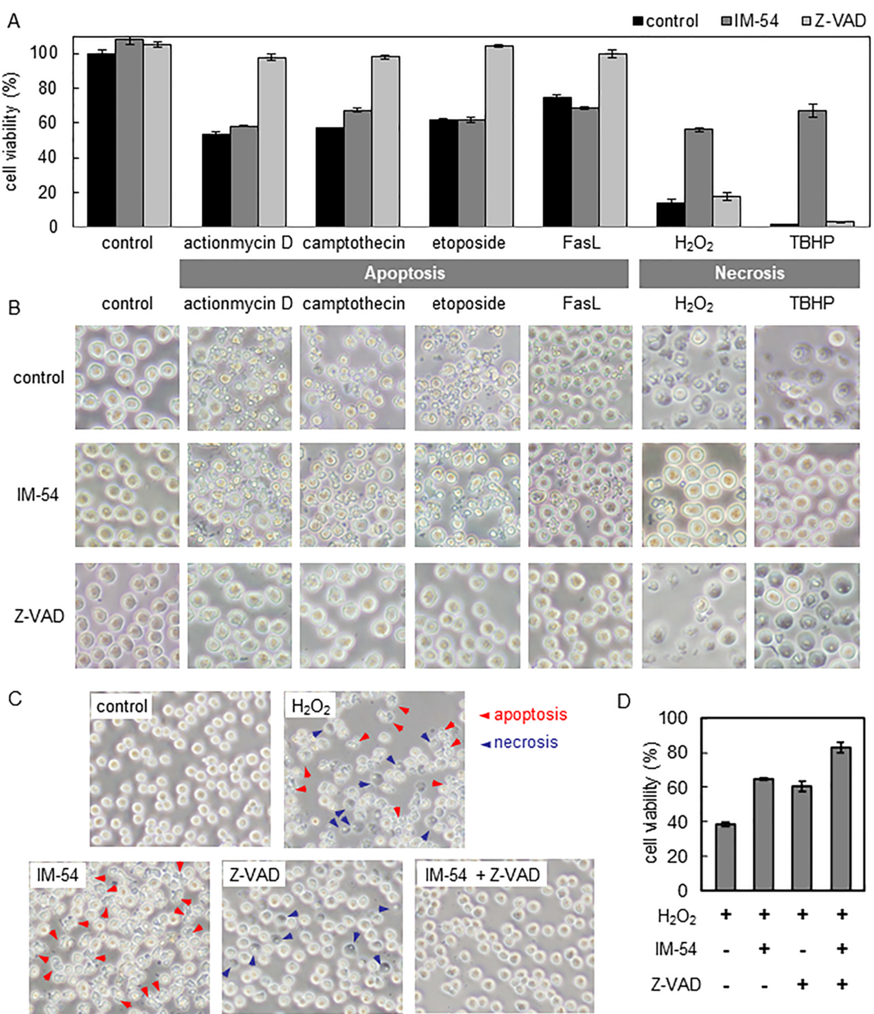

Figure 2. Cell death inhibition profile of **IM-54**. (A, B) Effects of **IM-54** and Z-VAD on various types of cell death. HL-60 cells were treated with various stimuli, actinomycin D (1 μM, 6 h), camptothecin (1 μM, 6 h), etoposide (100 μM, 4 h), Fas ligand (FasL) (100 ng/mL, 18 h), H_2O_2 (100 μM, 3 h), or *tert*-butyl hydroperoxide (TBHP) (30 μM, 3 h) in the presence or absence of **IM-54** (10 μM) or Z-VAD (100 μM). Cell viability was determined by AlamarBlue assay (A), and morphological changes were observed by means of phase-contrast imaging (B). (C, D) HL-60 cells were exposed to a low concentration of H_2O_2 (30 μM, 4 h) in the presence or absence of **IM-54** (10 μM) or Z-VAD (100 μM). Cell viability was determined by AlamarBlue assay (C), and morphological changes were observed by means of phase-contrast imaging (D). Apoptotic (red arrows) and necrotic (blue arrows) morphologies were observed.

suggested that **MS-1** could be a novel therapeutic lead. However, **MS-1** showed cytotoxicity and inhibitory activities toward several kinases at high concentrations.[9] Further work led to the development of **IM-54**,[13−15] which shows strong inhibition of H_2O_2-induced necrosis (comparable to **MS-1**), with greatly reduced cytotoxicity.[13] In addition **IM-54** did not show significant inhibitory activities against a panel of 467 kinases (Tables S1 and S2). Therefore, **IM-54** is also expected to have a therapeutic effect on ischemia-reperfusion injury. Here, we report the cell death inhibition profile of **IM-54**, as well as the protective effect of a new water-soluble IM derivative against ischemia-reperfusion injury in rat heart.

First, we examined the effects of **IM-54** on various types of cell death (Figure 2). HL-60 cells were treated with various cell death inducers in the presence or absence of **IM-54** or Z-VAD, a general caspase inhibitor. Cell viability was determined by AlamarBlue assay (Figure 2A), and morphological changes were observed by phase-contrast imaging (Figure 2B). As shown in Figure 2B, HL-60 cells showed typical morphological changes of apoptosis (blebbing and formation of apoptotic bodies) and necrosis (swelling and rupture of the cell membrane). We found that **IM-54** inhibited necrosis induced by oxidative stress (TBHP and H_2O_2), whereas Z-VAD did not. On the other hand, **IM-54** did not inhibit apoptosis induced by anticancer drugs (actinomycin D, camptothecin, and etoposide) or physiological death ligand (Fas ligand), which was strongly inhibited by Z-VAD in each case. Interestingly, at a low concentration, H_2O_2 was found to induce both apoptotic and necrotic cell death (Figures 2C). In this case, apoptotic cell death was inhibited by Z-VAD, and necrotic cell death was inhibited by **IM-54**, and cotreatment with Z-VAD and **IM-54** completely inhibited both apoptotic and necrotic cell death (Figures 2C and 2D). These results imply a complementary character of **IM-54** and Z-VAD as cell death inhibitors. In our previous study, **IM-54** itself did not react directly with H_2O_2, and the data in Figure 2C also support the idea that **IM-54** is not a sacrificial antioxidant, which could inhibit both apoptotic and necrotic cell death.

In recent studies, nonapoptotic molecular mechanisms were proposed for some types of necrosis;[16] thus, it may be possible

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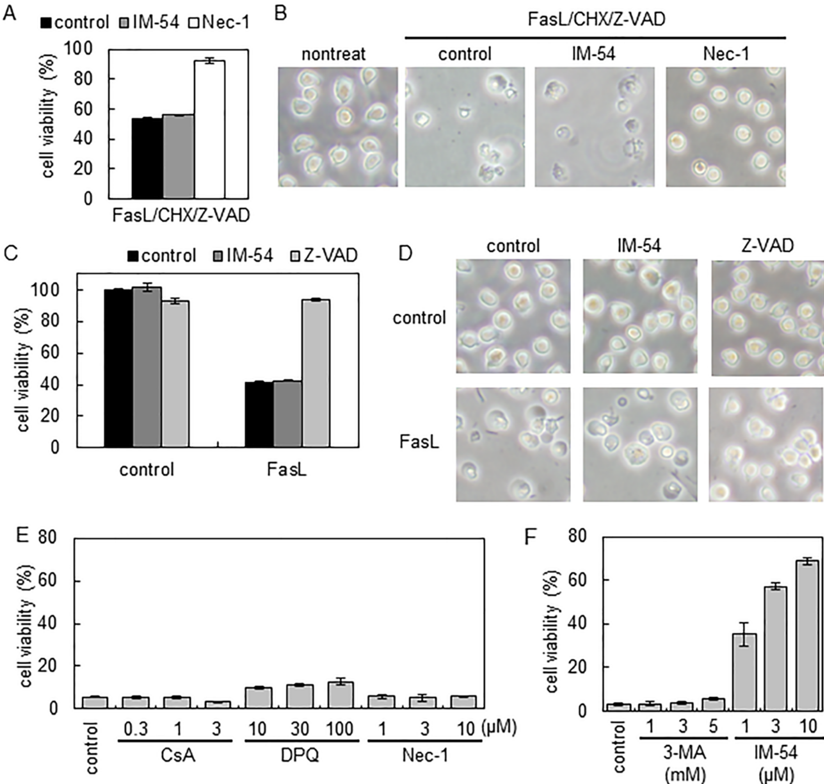

Figure 3. Comparison of **IM-54** with well-known cell death inhibitors. (A, B) Necroptosis was induced in Jurkat cells. Jurkat cells were treated with FasL (100 ng/mL, 20 h) in the presence of Z-VAD (100 μM) or cycloheximide (CHX) (5 μM). The effects of **IM-54** (10 μM) or Nec-1 (30 μM) on necroptosis were evaluated by examination of cell viability (A) and morphology (B). (C, D) Jurkat cells were treated with FasL (100 ng/mL, 18 h) in the presence or absence of **IM-54** (10 μM) or Z-VAD (100 μM). Cell viability (C) and morphological changes (D) were examined. (E, F) HL-60 cells were treated with H_2O_2 (100 μM, 3 h) in the presence or absence of various cell death inhibitors, cyclosporine A (a cyclophilin D inhibitor), DPQ (a PARP-1 inhibitor), Nec-1, and 3-methyladenine (3-MA, an autophagy inhibitor). Cell viability was determined by AlamarBlue assay (A, C, E, F), and morphological changes were observed by means of phase-contrast imaging (B, D).

to reduce necrosis by employing inhibitors of necrosis signaling. Indeed, inhibitors of necroptosis, which was defined as regulated necrosis induced by physiological death ligand, showed therapeutic activity in various disease models, including an ischemia-reperfusion injury model.[17,18] Therefore, we also examined the effects of **IM-54** on necroptosis induced by Fas ligand, cycloheximide, and Z-VAD in Jurkat cells, using a reported procedure.[17] We found that **IM-54** had no effect on necroptosis, which was inhibited by Nec-1,[17] a necroptosis inhibitor (Figures 3A and 3B). We also examined the effects on cell death induced by Fas ligand alone (Figure 3C and 3D). As in the case of HL-60 cells, Z-VAD inhibited the Fas-ligand-induced Jurkat cell death, whereas **IM-54** did not (Figure 3C). Interestingly, the morphological changes of Jurkat cells induced by Fas ligand were not the same as in the case of HL-60. They seemed rather similar to the necrotic morphology of H_2O_2-treated HL-60 cells, the appearance of which was inhibited by Z-VAD (Figure 3D). These results imply that **IM-54** has no effect on either apoptotic or necrotic cell death induced by death ligand.

In addition to Nec-1, we examined the effects of well-known cell death inhibitors, cyclosporine A (CsA, a cyclophilin D inhibitor), DPQ (a PARP-1 inhibitor), and 3-methyladenine (3-MA, an autophagy inhibitor) on oxidative stress-induced necrosis. Although CsA[19] and DPQ[20] have been reported to show therapeutic effects on myocardial infarction, none of the tested molecules inhibited H_2O_2-induced necrosis of HL-60 cells (Figure 3E and 3F). These results imply the involvement of a unique mechanism in cell death inhibition by IM derivatives.

We next planned an *in vivo* study of **IM-54**, but its water-solubility was too low. To overcome this problem, we designed and synthesized more water-soluble IM derivatives. Using a procedure similar to the one we reported before,[13,15] we introduced various hydroxyl or amino groups into IM derivatives (Scheme S1) and examined the necrosis-inhibitory activity of the obtained compounds. For quantitative estimation of the effect of each compound on necrosis, we used the lactate dehydrogenase (LDH) assay (Table 1). In this assay, rupture of the cellular membrane, a typical hallmark of necrosis, is quantified in terms of LDH release from the cytosol. By using this method, we determined the IC_{50} values for necrotic cell death induced by H_2O_2. As previously reported,[13] the effect of alkyl chain length was also examined in this assay system with **IM-20**, **IM-12**, **IM-13**, **IM-54**, and **IM-25**. **IM-54** having the C_5 alkyl chain showed the greatest activity among the aminoalkyl derivatives. Introduction of a hydroxyl or amino group into the side chain generally decreased the activity (**IM-17**, **IM-18**, **IM-19**, **IM-27**, **IM-90**, and **IM-91**), regardless of the length of the alkyl chain. These results indicate that the hydrophobicity of the aminoalkyl chain is important for the cell death-inhibitory activity. However, among several hydrophilic-chain-containing derivatives, **IM-17** showed reasonably good activity and was easily converted into the water-soluble HCl salt by treatment with an ethereal solution of HCl (Scheme S2). Moreover, **IM-17** showed the higher stability to metabolism in the rat liver S9

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Table 1. Cell Death-Inhibitory Activities of IM Derivatives against HL-60 Cells Treated with H_2O_2

compound	R	cell death inhibition IC_{50} (μM)
IM-20		0.91 ± 0.16
IM-12		0.44 ± 0.04
IM-13		0.42 ± 0.05
IM-54		0.25 ± 0.05
IM-25		0.38 ± 0.03
IM-17	NH_2	1.0 ± 0.23
IM-18	NH_2	1.3 ± 0.18
IM-19	NH_2	1.3 ± 0.28
IM-27	OH	1.3 ± 0.27
IM-90	OH	1.9 ± 0.03
IM-91	OH	1.1 ± 0.06

fraction than **IM-12** and **IM-54** (Figure S1). Therefore, **IM-17** was selected for further investigation.

Since HL-60 is a leukemia cell line, we next examined the cytoprotective activity of IM derivatives using a cardiac cell line before moving on to study the effect in a rat heart *in vivo* model. Rat cardiomyoblast H9c2 cells were reported to show necrotic cell death induced by TBHP, which is thought to mimic the oxidative stress in ischemia-reperfusion injury.[21] Therefore, the effects on TBHP-induced necrotic cell death might provide a measure of the therapeutic potential of compounds in ischemia-reperfusion injury. In the same manner as described for HL-60 cells, H9c2 cells were treated with TBHP in the absence or presence of a test compound, and necrotic cells were quantified by LDH assay (Figure 4A). All compounds showed cytoprotective activity, suggesting that they would have cardioprotective activity. **MS-1** showed the strongest activity at low concentration, but it also showed cytotoxicity at high concentration, as observed in HL-60 cells. Among the IM derivatives, **IM54**, **IM-12**, and **IM-17** also showed strong cytoprotective effects, and no cytotoxicity was observed even at 30 μM.

With these promising results in hand, we next tested the potency of **IM-17** in a Langendorff isolated rat heart model (Figure 4B, C). According to the reported method,[22] rat hearts were isolated and perfused in the Langendorff mode with Krebs-Henseleit buffer. After **IM-17** treatment (3 μM) for 10 min, isolated rat heart was subjected to 30 min of global ischemia followed by 60 min of reperfusion. The left ventricular developed pressure (LVDP) was measured as a marker of heart function (Figure 4B). Creatine kinase (CK) released from myocardial cells was monitored as a marker of cell membrane integrity, which is rapidly impaired in necrosis (Figure 4C). The results were obtained from three independent experiments. As shown in Figures 4B and 4C, **IM-17** clearly improved the recovery of LVDP and suppressed the release of CK, indicating that it can ameliorate the damage to rat heart cells caused by ischemia-reperfusion. Moreover, another IM derivative **IM-12** was also effective in this model (Figure S2). These results suggest that IM derivatives have therapeutic potential.

Encouraged by the results in the Langendorff model, we finally examined the *in vivo* cardioprotective effects of **IM-17**. In some cases, ischemia-reperfusion injury induces ventricular arrhythmia leading to a sudden death within minutes to

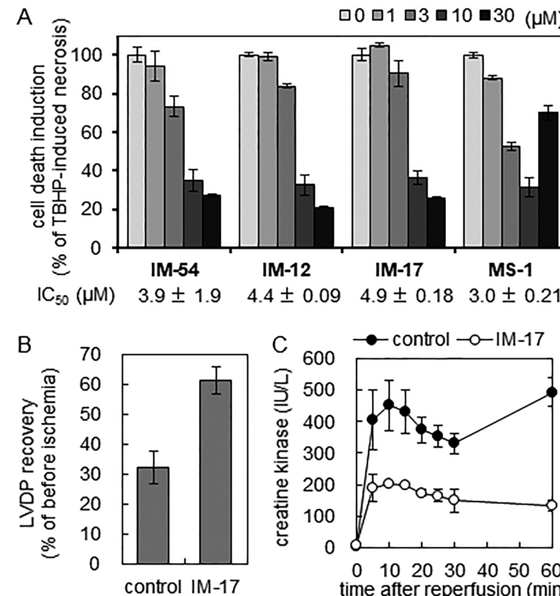

Figure 4. Cardioprotective effects of IM derivatives. (A) Cell death-inhibitory activities of IM derivatives against H9c2 cells treated with TBHP. Rat cardiomyoblast H9c2 cells were treated with TBHP (300 μM) in the presence or absence of IM derivatives. Cell death-inhibitory activity was determined by using an LDH assay. (B, C) Cardioprotective effects of **IM-17** in a Langendorff rat heart ischemia-reperfusion injury model. **IM-17** HCl salt (3 μM) was added to the perfusion buffer 10 min before ischemia. No-flow ischemia was maintained for 30 min, and reperfusion was accomplished by restoring flow for 60 min. Cardioprotective effects of **IM-17** were examined based on recovery of LVDP (left ventricular developed pressure) (B) and release of CK (creatine kinase) (C).

hours.[23−25] Thus, it is important to overcome ischemia-reperfusion-injury-induced arrhythmia as well as myocardial infarction. Although the precise mechanism is not fully understood, reactive oxygen species (ROS) are thought to be involved,[26,27] as in the case of myocardial infarction. Therefore, we examined the protective effects of **IM-17** against ischemia-reperfusion-induced arrhythmia in a rat model according to reported methods.[28,29] In this model, myocardial ischemia was achieved by tightening the coronary snare, and at 5 min after ischemia, reperfusion was started by releasing the snare. **IM-17** was intravenously injected at 5 min before ischemia (preischemia treatment) or at 1 min before reperfusion (postischemia treatment). The total duration of ventricular fibrillation (Vf) was calculated as the sum of the duration of episodes occurring within 10 min after reperfusion. As shown in Table 2, 60−75% of rats of the control group died of ischemia-reperfusion-induced arrhythmia within 10 min after reperfusion. In the case of preischemia treatment experiments, the incidence and the total duration of ischemia-reperfusion-induced Vf after reperfusion was reduced from 100% to 60% and from ca. 95.3 s to ca. 38.2 s, respectively, by treatment with 1 mg/kg of **IM-17**. Furthermore, treatment with 3 mg/kg **IM-17** completely abolished Vf and reduced mortality to 0%. Even in the case of postischemia treatment, 3 mg/kg of **IM-17** significantly reduced the mortality (to 20%) and the Vf duration (to ca. 57.0 s). These results suggest that **IM-17** reduced the death rate by inhibiting ischemia-reperfusion-induced arrhythmia. In industrialized countries, ischemic disorders, such as heart attack or cerebral ischemia, are major causes of death, and better therapeutic strategies are still

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Table 2. Effects of IM-17 on the Ischemia/Reperfusion-Induced Ventricular Fibrillation and Mortality in Rats[a]

	dose (mg/kg)	mortality (%)	ventricular fibrillation incidence (%)	ventricular fibrillation duration (sec)	n
preischemia treatment	0	60	100	95.3 ± 13.0	5
	1	20	60	38.2 ± 21.7	5
	3	0	0	0	5
postischemia treatmen	0	75	75	96.3 ± 24.1	8
	3	20	100	57.0 ± 25.5	5

[a]**IM-17** HCl salt was injected i.v. at 5 min before ischemia (preischemia treatment) or 1 min before reperfusion (postischemia treatment) over 1 min. N indicates the number of rats used in each experiment.

needed.[30] Due to the severe damage induced by reperfusion, simple recanalization is not so effective. In this study, **IM-17** was found to show cardioprotective effects in ischemia-reperfusion injury *in vivo* as well as at the organ level. Therefore, IM derivatives could be promising leads for innovative therapeutic agents to treat ischemic diseases. Further structural development studies are in progress, together with studies of possible therapeutic applications and examination of the molecular mechanism of action of IM derivatives.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00454.

Details for synthetic procedures, analytical data, and biological studies for IM derivatives (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: dodo@riken.jp.

*E-mail: sodeoka@riken.jp.

ORCID

Mikiko Sodeoka: 0000-0002-1344-364X

Present Addresses

[#](T.S.) Advanced Medical Research Center, Hyogo University of Health Sciences, 1-3-6 Minatojima, Kobe 650-8530, Japan.

[∇](K.I.) Department of Materials and Life Science, Seikei University, Musashino, Tokyo 180-8633, Japan.

Author Contributions

K.D. and M.S. designed the study. Synthesis of IM derivatives was conducted by T.S., M.T., and K.D. Biological study using cultured cells was designed and performed by K.D. and K.I. Experiments on the Langendorff isolated rat heart model were performed by J.S., and ischemia-reperfusion-induced arrhythmia model experiments were performed by K.A. Stabilities of compounds to liver metabolism were analyzed by T.S., N.T., and S.N. The manuscript was written through contributions of all authors and edited by K.D. and M.S.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

IM, indolylmaleimide; LDH, lactate dehydrogenase; CK, creatine kinase; LVDP, left ventricular developed pressure; Vf, ventricular fibrillation

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