Development and characterization of novel molecular probes for Ca^{2+}/calmodulin-dependent protein kinase kinase, derived from STO-609

Satomi Ohtsuka[1], Yui Ozeki[2], Moeno Fujiwara[3], Tomoyuki Miyagawa[2], Naoki Kanayama[1], Masaki Magari[1], Naoya Hatano[1], Futoshi Suizu[4], Teruhiko Ishikawa[2,**], and Hiroshi Tokumitsu[1,**]

[1]Applied Cell Biology, Graduate School of Interdisciplinary Science & Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan; [2]Department of Science Education, Graduate School of Education, and [3]Faculty of Education, Okayama University, Okayama 700-8530, Japan; [4]Division of Cancer Biology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan.

**To whom correspondence should be addressed:

Hiroshi Tokumitsu, Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; tokumit@okayama-u.ac.jp.

Teruhiko Ishikawa, Department of Science Education, Graduate School of Education, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama, Okayama 700-8530 Japan; teruhiko@cc.okayama-u.ac.jp.

ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) activates particular multifunctional kinases including CaMKI, CaMKIV, and 5'AMP-activated protein kinase (AMPK), resulting in the regulation of various Ca^{2+}-dependent cellular processes including neuronal, metabolic, and pathophysiological pathways. Herein we developed and characterized a novel pan-CaMKK inhibitor, TIM-063 (2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one) derived from STO-609 (7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-7-one-3-carboxylic acid), and an inactive analog (TIM-062) as molecular probes for the analysis of CaMKK-mediated cellular responses. Unlike STO-609, TIM-063 had an inhibitory activity against CaMKK isoforms (CaMKKα and β) with a similar potency (Ki = 0.35 μM for CaMKKα and Ki = 0.2 μM for CaMKKβ) *in vitro*. Two TIM-063 analogues lacking a nitro group (TIM-062) or a hydroxy group (TIM-064) completely impaired CaMKK inhibitory activities, indicating that both substituents are necessary for the CaMKK inhibitory activity of TIM-063. Enzymatic analysis revealed that TIM-063 is an ATP-competitive inhibitor that directly targets the catalytic domain of CaMKK, similar to STO-609. TIM-063 suppressed the ionomycin-induced phosphorylation of exogenously expressed CaMKI, CaMKIV and endogenous AMPKα in HeLa cells with an IC_{50} of ~0.3 μM, and it suppressed the CaMKK isoforms-mediated CaMKIV phosphorylation in transfected COS-7 cells. Thus, TIM-063, but not the inactive analogue (TIM-062), displayed cell permeability and the ability to inhibit CaMKK activity in cells. Taken together, these results indicate that TIM-063 could be a useful tool for the precise analysis of CaMKK-mediated signaling pathways and may be a promising lead compound for the development of therapeutic agents for the treatment of CaMKK-related diseases.

INTRODUCTION

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) was originally identified as an activating kinase for CaMKI and CaMKIV, phosphorylating their activation loop threonine residues (Thr177 in CaMKIα and Thr196 in CaMKIV)(1-4). In addition, CaMKK was later found to activate PKB/Akt in various cell lines including NG108 neuroblastoma cells, human embryonic kidney (HEK) 293 cells, LNCaP prostate cancer cells, and ovarian cancer cell lines including OVCAR-3 cells (5-8). Furthermore, 5'AMP-activated protein kinase (AMPK) is activated by CaMKKβ via the phosphorylation of Thr172 in the catalytic subunit (α subunit), which plays an important role in regulating cell energy metabolism (9-11). CaMKK is a member of CaM-dependent kinase family, which is regulated by an intrasteric autoinhibition and activated by Ca^{2+}/CaM binding to the regulatory domain in a similar manner to other CaMKs (12, 13), although it is not genetically classified as a member of the CaMK family (14). CaMKK-mediated Ca^{2+}-signaling plays an important role in various neuronal activities (15-23), metabolic responses (24-26), and pathophysiological responses including cancer cell growth (8, 27) and proliferation (28). CaMKKβ-mediated AMPK activation is involved especially in cell growth of various cancers including prostate (29) and lung cancers (30) as well as metabolically-related hepatic diseases (31). Therefore, CaMKKβ could be a promising molecular target for the treatment of these diseases.

In 2002, the first CaMKK inhibitor STO-609 (7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-7-one-3-carboxylic acid) was developed by our laboratory members (32), and it has been widely used to demonstrate the involvement of CaMKK activity in various Ca^{2+}-dependent physiological responses mediated by CaMKK/CaMKI, CaMKK/CaMKIV, CaMKK/AMPK, and CaMKK/PKB (Akt) pathways (9-11, 15-17, 33-37). Previous studies have reported the *in vivo* use of STO-609 for the inhibition of CaMKK2 (β) in the control of satiety (24) that was

confirmed using a recently developed orally bioavailable CaMKK2 inhibitor, compound 4t (38), as well as to confer protection against prostate cancer (29), liver cancer (27), and non-alcoholic fatty liver disease (31). Recently, STO-609 has been shown to attenuate mammary tumor growth in immunocompetent mice (39). Like other protein kinase inhibitors, STO-609 is a cell-membrane-permeable ATP-competitive inhibitor (32). Therefore, several protein kinases including casein kinase 2, AMPK, and MAPK-interacting kinase 1 are inhibited by STO-609 with a 10-fold lower potency to inhibit CaMKKβ (40). Accordingly, although STO-609 is relatively selective but not specific to CaMKKs, it is still useful for the evaluation of the physiological functions of CaMKK in cells because of the successful development of inhibitor-resistant mutants (16, 41, 42). Because STO-609 preferably inhibits CaMKKβ rather than CaMKKα (32,40,41), the specific role of CaMKKα is difficult to ascertain by pharmacological approaches using an inhibitor. Thus, a distinct type of CaMKK inhibitor must be developed to examine the physiological roles of CaMKK isoforms. To date, the structure–function study of STO-609 to develop an STO-609-related CaMKK inhibitor has not been performed. Therefore, in this study, we attempted to synthesize STO-609 derivatives as CaMKK inhibitors. We discovered a compound that potently suppresses the activities of both CaMKK isoforms equally *in vitro* as well as in cultured cells together with a closely-related inactive compound.

MATERIALS AND METHODS

Materials. Recombinant rat CaMKKα and β (wild type) were expressed in *E. coli* BL21 Star (DE3) and purified by CaM-Sepharose and Q-Sepharose chromatographies (43). GST-rat CaMKKα (126–434) and GST-rat CaMKKβ (162–470) were expressed in *E. coli* BL21 Star (DE3) cells and purified as previously described (41). GST-rat CaMKIα 1–293 Lys49Glu (GST-CaMKI

1–293 KE, kinase dead mutant) was expressed in *E. coli* JM109 and purified as described previously (12). GST-fused enzymes were purified with glutathione-sepharose (GE Healthcare Life Sciences, Buckinghamshire, England) chromatography. Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using the plasmid pET-CaM (kindly provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan) (44). The catalytic subunit of cAMP-dependent protein kinase from bovine cardiac tissue was kindly provided by Dr. Yasuo Watanabe (Showa Pharmaceutical University, Tokyo, Japan). Rat CaMKII holoenzyme was purified from rat forebrain as previously described (32). Recombinant rat CaMKIα and mouse CaMKIV were expressed in *E. coli*., purified, and activated by phosphorylation with CaMKKβ in the presence of Ca^{2+}/CaM as previously described (45). Anti-phospho-CaMKIα at Thr177 (clone 9H8) and anti-phospho-CaMKIV at Thr196 (clone 8D4) monoclonal antibodies were generated as previously described (45). Anti-HA antibody (12CA5) was obtained from Roche Applied Sciences (Indianapolis, IN). Anti-phospho-AMPKα at Thr172 (2535L) and anti-AMPKα (2532) antibodies were purchased from Cell Signaling Technology (Danvers, MA). STO-609 was purchased from Tocris Bioscience (Ellsville, MO). All other chemicals were obtained from standard commercial sources.

Synthesis of STO-609 Analogues. TIM-055 through TIM-061 was prepared by the reported procedure (46). TIM-062, TIM-063, and TIM-064 were synthesized from corresponding 1,8-naphthalenedicaroxylic anhydride derivatives (**1**–**3**) by cyclization reaction with *o*-phenylenediamine in tetrahydrofuran (THF) under reflux (Scheme 1). Derivatives (**1**) and **(3)** are commercially available compounds, and 3-hydroxy-4-nitro-1,8-naphthalenedicaroxylic anhydride (**2)** was prepared by nitration of (**1)** with trifluoroacetic anhydride and ammonium nitrate at room

temperature. Structures of these final products were confirmed by ^{1}H and ^{13}C NMR as well as mass spectrometry (see Supporting Information S1).

NMR Spectroscopy. NMR spectra were recorded on a Varian MERCURY-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C), using the solvent peak as the internal reference ($CDCl_3$: δ H 7.26; δ C 77.0; DMSO-d_6: δ H 2.50; δ C 39.5). Multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants (*J*) are given in Hertz (Hz). Mass spectra were recorded on an Agilent Q-TOF G6520.

***In Vitro* CaMKK Activity Assay.** CaMKK activity was measured at 30°C for 10 min in a solution (25 μL) containing 30 ng CaMKKβ or 3 ng CaMKKα, 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP (795–2294 cpm/pmol, PerkinElmer Life Sciences, Waltham, MA) or indicated concentrations of [γ-^{32}P]ATP and 12.5 μg GST-CaMKI 1–293 Lys49Glu in the presence of 2 mM $CaCl_2$/6 μM CaM. Each reaction was initiated by the addition of [γ-^{32}P]ATP. Reactions were terminated by spotting the samples (20 μL) onto P81 phosphocellulose paper (Whatman) to measure the ^{32}P incorporation into the substrate. Specific activities of CaMKKα and CaMKKβ in the absence of compounds (5% DMSO) were calculated to be 451 ± 78 nmol/min/mg and 126 ± 24 nmol/min/mg, respectively. The autophosphorylation of CaMKKα and β was measured at 30°C for 120 min in 20 μL of a solution containing 1 μg CaMKK isoforms, 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP in the presence of 2 mM $CaCl_2$/6 μM CaM (for CaMKKα) or 2 mM EGTA (for CaMKKβ) with or without compounds. The reaction was terminated by addition of SDS-PAGE sample buffer and subjected to SDS-PAGE, followed by autoradiography. ^{32}P incorporation into

CaMKK isoforms was estimated by densitometric scanning of the autoradiogram with ImageJ software (47).

Protein Kinase Assays. Protein kinase activities of activated CaMKI (2 ng), CaMKII (10 ng), activated CaMKIV (6.3 ng), and PKA (125 ng) were measured at 30°C for 10 min in a solution as described for the CaMKK assay without or with TIM-compounds using either peptide substrate (40 μM Syntide-2 for CaMKI, CaMKII, and CaMKIV; 40 μM Kemptamide for PKA) in the presence of 2 mM $CaCl_2$/6μM CaM (except for PKA) and 100 μM [γ-^{32}P]ATP as previously described [32]. Specific activities of activated CaMKI, CaMKII, activated CaMKIV, and PKA in the absence of TIM-compounds (5% DMSO) were calculated to be 5450 ± 344, 110 ± 5, 263 ± 14, and 9.3 ± 0.8 nmol/min/mg, respectively.

Cell Culture and Transfection. HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. HeLa cells-in 6-well dishes were transfected with 1 μg of HA-rat CaMKIα (pME-HA-CaMKIα) or 0.25 μg of HA-mouse CaMKIV (pME-HA-CaMKIV) expression plasmid, and COS-7 cells were transfected with 1 μg of pME-HA-CaMKIV in the absence or presence of 0.5 μg of rat CaMKKα or β expression plasmid (pcDNA3-FLAG-CaMKKα or pME-CaMKKβ) using polyethylenimine "MAX" (Polysciences, Inc. Warrington, PA, USA) according to the manufacturer's protocol. After 42 h, the cells were cultured in the absence of serum with indicated concentrations of TIM-compounds for 6 h, followed by treatment with 1 μM of ionomycin for 5 min. Then cells were lysed with 1× SDS-PAGE sample buffer (100 μL), followed by immunoblot analyses (5–10 μL cell lysates).

Other Methods. Immunoblot analysis was performed with the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (GE Healthcare Life Sciences) as the secondary antibody. A chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA) was used for signal detection of immunoblots, followed by quantification of the immunoreactivity with ImageJ software (47). Protein concentrations in samples were estimated by Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc. Hercules, CA), using bovine serum albumin as a standard. Student's *t* tests were used to evaluate the statistical significance of two-group comparisons. Probability (p) values < 0.05 were considered statistically significant.

RESULTS

Characterization of STO-609 Derivatives as CaMKK Inhibitors. To develop a novel CaMKK inhibitor, we attempted to synthesize compounds derived from STO-609 and characterize them as CaMKK inhibitors *in vitro* and in cultured cells. First, we synthesized 10 compounds that were analogues of STO-609 with different substitutions at positions R_1, R_2 and R_3 (Fig. 1A) and evaluated their inhibitory potencies at a concentration of 10 μg/mL (27–35 μM) against recombinant CaMKKα and CaMKKβ kinase activities *in vitro* using GST-CaMKI catalytic domain (1-293 Lys49Glu, kinase dead mutant) as a substrate (Fig. 1B).

Among the STO-609 derivatives, only TIM-063 inhibited approximately 90% of both CaMKKα and CaMKKβ activities at a concentration of 10 μg/mL (30 μM), to the same extent as STO-609 (27 μM). Two TIM-063 analogues lacking either a nitro group at the R_2 position (TIM-062) or a hydroxy group at R_1 (TIM-064) showed no CaMKK inhibitory activity, thereby indicating that both substituents are required for the suppression of CaMKK activity by TIM-063. Therefore,

we further analyzed TIM-063 as a novel CaMKK inhibitor and TIM-062 and TIM-064 as inactive analogues.

Inhibition of CaMKKα and CaMKKβ Activities by TIM-063. We evaluated the dose-dependent inhibition of TIM-063 using acetic acid salt of the compound (see Supporting Information S1) against the activities of recombinant rat CaMKKα and β isoforms (Fig. 2A). TIM-063 was capable of inhibiting CaMKKα activity (IC_{50} = 0.63 μM) with a similar potency as STO-609 (IC_{50} = 0.91 μM, Fig. 2A, *left panel*). In contrast to STO-609 that inhibits CaMKKβ activity 5–10-fold more effectively than against CaMKKα activity (Fig. 2A) (32, 40), TIM-063 suppresses CaMKKβ with an IC_{50} of 0.96 μM (Fig. 2A, *right panel*) indistinguishably from its inhibitory potency toward CaMKKα (Fig. 3A, *left panel*). We confirmed that TIM-062 and TIM-064 were incapable of inhibiting the activities of both CaMKK isoforms at least up to 10 μg/mL (29 μM and 27 μM, respectively), which is consistent with the results shown in Fig. 1B. Next, we checked the effect of TIM-063 and inactive analogues (TIM-062 and -064) on the autophosphorylation activities of CaMKK isoforms (Fig. 2B and 2C). Because previous studies have shown that CaMKKβ undergoes Ca^{2+}/CaM-independent intramolecular autophosphorylation (48), we tested the effects of the compounds on the autophosphorylation activity of CaMKKβ in the absence of Ca^{2+}/CaM (Fig. 2B and 2C, *right panel*). As shown in Fig. 2B, *right panel*, TIM-063 suppressed the Ca^{2+}/CaM-independent autophosphorylation activity of CaMKKβ with an IC_{50} value of 0.66 μM, which is a similar inhibitory potency as the substrate -phosphorylation activity of CaMKKβ (Fig. 2A, *right panel*). Furthermore, TIM-063 suppressed Ca^{2+}/CaM-dependent autophosphorylation of CaMKKα (Fig. 2B, *left panel*) as its inhibition of substrate phosphorylation (Fig. 2A, *left panel*). These results indicate that TIM-063 is neither a CaM

antagonist nor a competitor with respect to protein substrates. In addition, we observed that up to 10 μg/mL of TIM-062 and TIM-064 (29 μM and 27 μM, respectively) had no or minor effect on CaMKKα and β autophosphorylation activities (Fig. 2C). When we examined the effect of TIM-063 on the activities of cAMP-dependent protein kinase (PKA) and multifunctional Ca^{2+}/CaM-dependent protein kinases including CaMKI, CaMKII, and CaMKIV using 100 μM [γ-^{32}P]ATP (Table I), the activities of PKA, CaMKI and CaMKIV were slightly affected by the presence of 26 μM TIM-063 (10–30% inhibitory rate). CaMKII activity was suppressed by TIM-063 with 15–22-fold less potency (IC_{50} = 14 μM) than that required for CaMKK isoforms. Both TIM-062 and TIM-064 exhibited no significant inhibition up to 10 μg/mL (29 μM and 27 μM, respectively) against all protein kinases we tested. In addition, TIM-063 was incapable of inhibiting CaMKI and CaMKIV activities in the presence of Ca^{2+}/CaM, also supporting that the compound is unlikely a CaM antagonist.

TIM-063, an ATP-Competitive Inhibitor. To ascertain whether TIM-063 directly targets the catalytic domains of the CaMKK isoforms, we tested the effect of TIM-063 on the activities of the GST-CaMKKα catalytic domain (residues 126–434) and GST-CaMKKβ catalytic domain (residues 162–470) mutants that lack autoinhibitory and CaM-binding domains (Fig. 3A) (16, 41, 42). The inhibitory potencies of TIM-063 against the catalytic domain mutants of the CaMKK isoforms were indistinguishable from the full-length enzymes. These results clearly indicated that TIM-063 is a direct inhibitor targeting the catalytic domain of the CaMKK isoforms. Because STO-609 has shown to be an ATP-competitive inhibitor (32), we expected that TIM-063 also would inhibit CaMKK activity competitively with respect to ATP. To test this, we performed a CaMKK kinase assay with varying concentrations of ATP (25–200 μM) in the absence or presence

of 0.84 μM TIM-063. Lineweaver–Burk plots of CaMKK inhibition by TIM-063 (Fig. 3B) indicated that the apparent *Km* values for ATP of both CaMKK isoforms were increased by the addition of TIM-063 without a significant effect on *Vmax* values. Thus, these results strongly suggest that TIM-063 is an ATP-competitive inhibitor. Based on the kinetic data (Fig. 3B), the *Ki* values of TIM-063 were calculated to be 0.35 μM for CaMKKα, comparable to that of STO-609 (32) and 0.2 μM for CaMKKβ (Supplemental Figure S2).

Suppression of Endogenous CaMKK Activity in HeLa Cells by TIM-063. To test whether TIM-063 inhibits CaMKK activity in intact cells, we monitored phosphorylation of CaMKK targets including endogenous AMPK at Thr172 and exogenously expressed HA-CaMKIα and HA-CaMKIV in HeLa cells, which are induced by an increasing concentration of Ca^{2+} (Fig. 4). After the cells were treated with 1 μM ionomycin for 5 min, phosphorylation of CaMKK-target kinases was monitored by immunoblot analysis using each phospho-specific antibody against Thr residue in the activation loop. We treated HeLa cells with various concentrations of either TIM-063 or its inactive analogues TIM-062 and TIM-064. It is noteworthy that TIM-064 exhibited a cytotoxic effect, indicating the off-target effect of the compound. Thus, we thereafter used TIM-062 as an inactive compound. When we applied various concentrations of TIM-063 to HeLa cells, 1 μM ionomycin-induced Thr172 phosphorylation of endogenous AMPKα was inhibited by TIM-063 in a dose-dependent manner but was not affected by up to 29 μM of TIM-062 (Fig. 4A). To test the inhibitory effect of TIM-063 on the phosphorylation of other CaMKK targets, CaMKI and CaMKIV, we transfected a hemagglutinin (HA)-tagged either CaMKIα or CaMKIV expression vector into HeLa cells and subsequently treated the cells with 1 μM ionomycin for 5 min (Fig. 4B and C). We then analyzed the phosphorylation of Thr177 in HA-CaMKIα (Fig. 4B) and Thr196

in HA-CaMKIV (Fig. 4C). In a similar manner to the inhibition of AMPK phosphorylation, endogenous CaMKK-mediated phosphorylation of exogenously expressed HA-CaMKIα (at Thr177) and HA-CaMKIV (at Thr196) was suppressed by TIM-063 in a dose-dependent manner but not by the inactive analogue, TIM-062 up to 29 μM (Fig. 4B and C). TIM-063 at 2.6 μM significantly suppressed (>80 % inhibition) the phosphorylation of endogenous AMPKα and exogenously expressed CaMKI and CaMKIV in ionomycin-stimulated HeLa cells (IC_{50} = ~0.3 μM) as its inhibition of the CaMKK isoforms *in vitro* (Fig. 2A and Fig. 3B). Thus, these results show that TIM-063 but not an inactive analogue (TIM-062), is capable of inhibiting endogenous CaMKK activity in intact cells as well as *in vitro*.

Inhibition of CaMKK Isoforms-Catalyzed CaMKIV Phosphorylation by TIM-063 in Transfected COS-7 Cells. To test the effect of TIM-063 on CaMKK isoforms-dependent phosphorylation of CaMKIV (at Thr196) in cultured cells, we transfected a HA-CaMKIV expression vector into COS-7 cells together with either FLAG-CaMKKα (Fig. 5A) or CaMKKβ (Fig. 5B)-expression vectors and subsequently stimulated the cells with 1 μM ionomycin for 5 min. We then analyzed the phosphorylation of Thr196 in HA-CaMKIV (Fig. 5). Phosphorylation of HA-CaMKIV was induced by co-transfection with either FLAG-CaMKKα or CaMKKβ. In a similar manner to the inhibition of endogenous CaMKK activity in HeLa cells (Fig. 4A and B), both CaMKKα- and CaMKKβ-induced HA-CaMKIV phosphorylation were significantly inhibited by 26 μM TIM-063 treatment, indicating that TIM-063 suppressed activities of both exogenously expressed CaMKK isoforms in cultured cells. It is noteworthy that up to 29 μM TIM-062 did not affect the phosphorylation of HA-CaMKIV by CaMKK isoforms.

DISCUSSION

We synthesized STO-609 derivatives and found a novel CaMKK inhibitor, TIM-063. In contrast to STO-609 that is approximately 5–10-fold more effective in inhibiting CaMKKβ than CaMKKα (Fig. 1A) (32), TIM-063 suppresses CaMKK isoforms with a~~n~~ similar potency (*Ki* = 0.2–0.35 μM), indicating that TIM-063 is a pan-CaMKK inhibitor (Figs. 1 and 2). Similarly to STO-609, TIM-063 directly inhibited CaMKK in an ATP-competitive manner. This novel CaMKK inhibitor penetrated the cell membrane, resulting in the suppression of ionomycin-induced phosphorylation of endogenous AMPK at Thr172 and exogenously expressed CaMKIα at Thr177 and CaMKIV at Thr196, which were mediated by endogenous CaMKK in cultured cells with an IC_{50} of ~0.3 μM (Fig. 4). This is consistent with the results that TIM-063 inhibited exogenously expressed CaMKKα and CaMKKβ-mediated CaMKIV phosphorylation in transfected cells (Fig. 5) although inhibition of exogenously expressed CaMKKs requires higher concentration of TIM-063 than that of endogenous CaMKKs as shown in Fig. 4. This is probably due to high concentration of transiently expressed CaMKK isoforms in the cells. Other STO-609 derivatives synthesized with replacement of the only carboxylic acid group at position R_2 by various substituents (TIM-055, -057, -059, -060) were inactive possibly due to steric hindrance caused by bulky substituents and only TIM-063 with a hydroxy group at position R_1 and a nitro group at position R_2 possessed CaMKK inhibitory activity (Fig. 1). Both TIM-062 lacking a nitro group at position R_2 and TIM-064 lacking a hydroxy group at position R_1 were unable to suppress CaMKK activity *in vitro* or in cultured cells, indicating that both substituents are required for the CaMKK inhibitory activity of TIM-063. Whereas TIM-064 exhibits a cytotoxic effect, TIM-062 is an inactive analogue *in vitro* as well as in cultured cells without a cytotoxic effect; thus, TIM-062 would be helpful to evaluate the pharmacological effect of TIM-063 as a CaMKK inhibitor.

The 2.4 Å crystal structure of the catalytic domain of human CaMKKβ complexed with STO-609 revealed mostly hydrophobic interactions between STO-609 and CaMKKβ (49). Furthermore, it has been shown that STO-609 ~~forms~~ hydrogen bonds with the backbone~~s~~ atoms of human CaMKKβ Val270 and Asp330, as well as Glu236 in a water-mediated manner (49). These interactions make the carboxylic acid moiety of STO-609 slightly tilted, resulting in the inhibitor fitting within a narrow pocket of the CaMKKβ catalytic domain that adopts a closed conformation (49). In contrast to STO-609, which preferably inhibits CaMKKβ rather than CaMKKα (32), replacement of the carboxylic acid group at position R_2 by a nitro group in TIM-063 may cause a conformational change, resulting in an equal inhibitory potency of TIM-063 against both CaMKK isoforms. Furthermore, the hydroxy group at position R_1 in TIM-063 may form an additional hydrogen bond with CaMKKs or increase the electron density of the nitro group by resonance effects, resulting in enhancement of hydrogen bonds with CaMKK isoforms to stabilize the inhibitor/CaMKK complex formation. Therefore, structural determination of the CaMKK/TIM-063 complex remains to be done for detailed analyses of CaMKK inhibition by TIM-063. This study also indicates that STO-609 and TIM-063 might be promising lead compounds for the development of a new therapeutically useful CaMKK inhibitor. Although a novel CaMKK inhibitor (TIM-063) in combination with structurally related inactive compound (TIM-062) could be helpful to evaluate the physiological significance(s) of CaMKK-mediated signaling cascades *in vivo*, the inhibitor should be used for that purpose carefully and cautiously because the specificity of the inhibitor has not become completely clear.

ASSOCIATED CONTENT

Supporting Information S1

: Synthesis of TIM-063 and its derivatives (MS Word file)

Supplemental Figure S2

: Dixon plot of CaMKK inhibition by TIM-063 (TIF file)

Accession Codes

Rattus norvegicus CaMKKα : UniProtKB - P97756 (KKCC1_RAT)

Rattus norvegicus CaMKKβ : UniProtKB - O88831 (KKCC2_RAT)

Rattus norvegicus calmodulin : UniProtKB - P0DP29 (CALM1_RAT)

Rattus norvegicus CaMKIα : UniProtKB - Q63450 (KCC1A_RAT)

Mus musculus CaMKIV : UniProtKB - P08414 (KCC4_MOUSE)

Rattus norvegicus CaMKIIα : UniProtKB - P11275 (KCC2A_RAT)

Rattus norvegicus CaMKIIβ : UniProtKB - P08413 (KCC2B_RAT)

Rattus norvegicus AMPKα1 : UniProtKB - P54645 (AAPK1_RAT)

Rattus norvegicus AMPKβ1 : UniProtKB - P80386 (AAKB1_RAT)

Rattus norvegicus AMPKγ1 : UniProtKB - P80385 (AAKG1_RAT)

Bos taurus PKA catalytic subunit : UniProtKB - P00517 (KAPCA_BOVIN)

AUTHOR INFORMATION

Corresponding Authors

Hiroshi Tokumitsu – Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; Phone +81-86-251-8197; Email: tokumit@okayama-u.ac.jp

Teruhiko Ishikawa – Department of Science Education, Graduate School of Education, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama, Okayama 700-8530 Japan; Phone +81-86-251-7639; Email: teruhiko@cc.okayama-u.ac.jp

Authors

Satomi Ohtsuka – Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan

Yui Ozeki – Department of Science Education, Graduate School of Education, Okayama University, Okayama 700-8530, Japan

Moeno Fujiwara – Faculty of Education, Okayama University, Okayama 700-8530, Japan

Tomoyuki Miyagawa – Department of Science Education, Graduate School of Education, Okayama University, Okayama 700-8530, Japan

Naoki Kanayama – Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan

Masaki Magari – Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan

Naoya Hatano – Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan

Futoshi Suizu – Division of Cancer Biology, Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan

Funding

This work was supported by Grants-in-aid for Scientific Research (JP18K06113 and JP19H03447 to HT) from Japan Society for the Promotion of Science.

Notes

The authors declare that they have no conflict of interest with the contents of this article.

ABBREVIATIONS

CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; HA, hemagglutinin; CaMKKα, β, Ca^{2+}/CaM-dependent protein kinase kinase α, β; AMPK, 5'AMP-activated protein kinase; TIM-063, 2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one; STO-609, 7*H*-benzimidazo[2,1-*a*]benz[de]isoquinoline-7-one-3-carboxylic acid; PKA, cAMP-dependent protein kinase; PKB, protein kinase B

REFERENCES

1. Soderling, T. R., and Stull, J. T. (2001) Structure and regulation of calcium/calmodulin-dependent protein kinases, *Chem Rev 101*, 2341-2352, DOI: 10.1021/cr0002386
2. Hook, S. S., and Means, A. R. (2001) Ca^{2+}/CaM-dependent kinases: from activation to function, *Annu Rev Pharmacol Toxicol 41*, 471-505, DOI: 10.1146/annurev.pharmtox.41.1.471

3. Wayman, G. A., Lee, Y. S., Tokumitsu, H., Silva, A. J., and Soderling, T. R. (2008) Calmodulin-kinases: modulators of neuronal development and plasticity, *Neuron 59*, 914-931, DOI: 10.1016/j.neuron.2008.08.021

4. Takemoto-Kimura, S., Suzuki, K., Horigane, S. I., Kamijo, S., Inoue, M., Sakamoto, M., Fujii, H., and Bito, H. (2017) Calmodulin kinases: essential regulators in health and disease, *J Neurochem 141*, 808-818, DOI: 10.1111/jnc.14020

5. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway, *Nature 396*, 584-587, DOI: 10.1038/25147

6. Chen, B. C., Wu, W. T., Ho, F. M., and Lin, W. W. (2002) Inhibition of interleukin-1β -induced NF-kappa B activation by calcium/calmodulin-dependent protein kinase kinase occurs through Akt activation associated with interleukin-1 receptor-associated kinase phosphorylation and uncoupling of MyD88, *J Biol Chem 277*, 24169-24179, DOI: 10.1074/jbc.M106014200

7. Schmitt, J. M., Smith, S., Hart, B., and Fletcher, L. (2012) CaM kinase control of AKT and LNCaP cell survival, *J Cell Biochem 113*, 1514-1526, DOI: 10.1002/jcb.24020

8. Gocher, A. M., Azabdaftari, G., Euscher, L. M., Dai, S., Karacosta, L. G., Franke, T. F., and Edelman, A. M. (2017) Akt activation by Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) in ovarian cancer cells, *J Biol Chem 292*, 14188-14204, DOI: 10.1074/jbc.M117.778464

9. Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005) Ca^{2+}/calmodulin-dependent protein kinase kinase-β

acts upstream of AMP-activated protein kinase in mammalian cells, *Cell Metab 2*, 21-33, DOI: 10.1016/j.cmet.2005.06.005

10. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase, *Cell Metab 2*, 9-19, DOI: 10.1016/j.cmet.2005.05.009

11. Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases, *J Biol Chem 280*, 29060-29066, DOI: 10.1074/jbc.M503824200

12. Tokumitsu, H., Muramatsu, M., Ikura, M., and Kobayashi, R. (2000) Regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase, *J Biol Chem 275*, 20090-20095, DOI: 10.1074/jbc.M002193200

13. Osawa, M., Tokumitsu, H., Swindells, M. B., Kurihara, H., Orita, M., Shibanuma, T., Furuya, T., and Ikura, M. (1999) A novel target recognition revealed by calmodulin in complex with Ca^{2+}-calmodulin-dependent kinase kinase, *Nat Struct Biol 6*, 819-824, DOI: 10.1038/12271

14. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome, *Science 298*, 1912-1934, DOI: 10.1126/science.1075762

15. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) CREB phosphorylation and dephosphorylation: a Ca^{2+}- and stimulus duration-dependent switch for hippocampal gene expression, *Cell 87*, 1203-1214, DOI: 10.1016/s0092-8674(00)81816-4

16. Wayman, G. A., Kaech, S., Grant, W. F., Davare, M., Impey, S., Tokumitsu, H., Nozaki, N., Banker, G., and Soderling, T. R. (2004) Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I, *J Neurosci 24*, 3786-3794, DOI: 10.1523/JNEUROSCI.3294-03.2004

17. Wayman, G. A., Impey, S., Marks, D., Saneyoshi, T., Grant, W. F., Derkach, V., and Soderling, T. R. (2006) Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2, *Neuron 50*, 897-909, DOI: 10.1016/j.neuron.2006.05.008

18. Saneyoshi, T., Wayman, G., Fortin, D., Davare, M., Hoshi, N., Nozaki, N., Natsume, T., and Soderling, T. R. (2008) Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase I/betaPIX signaling complex, *Neuron 57*, 94-107, DOI: 10.1016/j.neuron.2007.11.016

19. Ageta-Ishihara, N., Takemoto-Kimura, S., Nonaka, M., Adachi-Morishima, A., Suzuki, K., Kamijo, S., Fujii, H., Mano, T., Blaeser, F., Chatila, T. A., Mizuno, H., Hirano, T., Tagawa, Y., Okuno, H., and Bito, H. (2009) Control of cortical axon elongation by a GABA-driven Ca^{2+}/calmodulin-dependent protein kinase cascade, *J Neurosci 29*, 13720-13729, DOI: 10.1523/JNEUROSCI.3018-09.2009

20. Nakamuta, S., Funahashi, Y., Namba, T., Arimura, N., Picciotto, M. R., Tokumitsu, H., Soderling, T. R., Sakakibara, A., Miyata, T., Kamiguchi, H., and Kaibuchi, K. (2011) Local application of neurotrophins specifies axons through inositol 1,4,5-trisphosphate, calcium, and Ca^{2+}/calmodulin-dependent protein kinases, *Sci Signal 4*, ra76, DOI: 10.1126/scisignal.2002011

21. Horigane, S., Ageta-Ishihara, N., Kamijo, S., Fujii, H., Okamura, M., Kinoshita, M., Takemoto-Kimura, S., and Bito, H. (2016) Facilitation of axon outgrowth via a Wnt5a-CaMKK-CaMKIα pathway during neuronal polarization, *Mol Brain 9*, 8, DOI: 10.1186/s13041-016-0189-3

22. Peters, M., Mizuno, K., Ris, L., Angelo, M., Godaux, E., and Giese, K. P. (2003) Loss of Ca^{2+}/calmodulin kinase kinase beta affects the formation of some, but not all, types of hippocampus-dependent long-term memory, *J Neurosci 23*, 9752-9760, DOI: 10.1523/JNEUROSCI.23-30-09752.2003

23. Mizuno, K., Ris, L., Sanchez-Capelo, A., Godaux, E., and Giese, K. P. (2006) Ca^{2+}/calmodulin kinase kinase α is dispensable for brain development but is required for distinct memories in male, though not in female, mice, *Mol Cell Biol 26*, 9094-9104, DOI: 10.1128/MCB.01221-06

24. Anderson, K. A., Ribar, T. J., Lin, F., Noeldner, P. K., Green, M. F., Muehlbauer, M. J., Witters, L. A., Kemp, B. E., and Means, A. R. (2008) Hypothalamic CaMKK2 contributes to the regulation of energy balance, *Cell Metab 7*, 377-388, DOI: 10.1016/j.cmet.2008.02.011

25. Anderson, K. A., Lin, F., Ribar, T. J., Stevens, R. D., Muehlbauer, M. J., Newgard, C. B., and Means, A. R. (2012) Deletion of CaMKK2 from the liver lowers blood glucose and improves whole-body glucose tolerance in the mouse, *Mol Endocrinol 26*, 281-291, DOI: 10.1210/me.2011-1299

26. Marcelo, K. L., Means, A. R., and York, B. (2016) The Ca^{2+}/Calmodulin/CaMKK2 Axis: Nature's Metabolic CaMshaft, *Trends Endocrinol Metab 27*, 706-718, DOI: 10.1016/j.tem.2016.06.001

27. Lin, F., Marcelo, K. L., Rajapakshe, K., Coarfa, C., Dean, A., Wilganowski, N., Robinson, H., Sevick, E., Bissig, K. D., Goldie, L. C., Means, A. R., and York, B. (2015) The camKK2/camKIV relay is an essential regulator of hepatic cancer, *Hepatology 62*, 505-520, DOI: 10.1002/hep.27832

28. Karacosta, L. G., Foster, B. A., Azabdaftari, G., Feliciano, D. M., and Edelman, A. M. (2012) A regulatory feedback loop between Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and the androgen receptor in prostate cancer progression, *J Biol Chem 287*, 24832-24843, DOI: 10.1074/jbc.M112.370783

29. Massie, C. E., Lynch, A., Ramos-Montoya, A., Boren, J., Stark, R., Fazli, L., Warren, A., Scott, H., Madhu, B., Sharma, N., Bon, H., Zecchini, V., Smith, D. M., Denicola, G. M., Mathews, N., Osborne, M., Hadfield, J., Macarthur, S., Adryan, B., Lyons, S. K., Brindle, K. M., Griffiths, J., Gleave, M. E., Rennie, P. S., Neal, D. E., and Mills, I. G. (2011) The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis, *EMBO J 30*, 2719-2733, DOI: 10.1038/emboj.2011.158

30. Jin, L., Chun, J., Pan, C., Kumar, A., Zhang, G., Ha, Y., Li, D., Alesi, G. N., Kang, Y., Zhou, L., Yu, W. M., Magliocca, K. R., Khuri, F. R., Qu, C. K., Metallo, C., Owonikoko, T. K., and Kang, S. (2018) The PLAG1-GDH1 Axis Promotes Anoikis Resistance and Tumor Metastasis through CamKK2-AMPK Signaling in LKB1-Deficient Lung Cancer, *Mol Cell 69*, 87-99 e87, DOI: 10.1016/j.molcel.2017.11.025

31. York, B., Li, F., Lin, F., Marcelo, K. L., Mao, J., Dean, A., Gonzales, N., Gooden, D., Maity, S., Coarfa, C., Putluri, N., and Means, A. R. (2017) Pharmacological inhibition of CaMKK2 with the selective antagonist STO-609 regresses NAFLD, *Sci Rep 7*, 11793, DOI: 10.1038/s41598-017-12139-3

32. Tokumitsu, H., Inuzuka, H., Ishikawa, Y., Ikeda, M., Saji, I., and Kobayashi, R. (2002) STO-609, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase kinase, *J Biol Chem 277*, 15813-15818, DOI: 10.1074/jbc.M201075200

33. Fortin, D. A., Davare, M. A., Srivastava, T., Brady, J. D., Nygaard, S., Derkach, V. A., and Soderling, T. R. (2010) Long-term potentiation-dependent spine enlargement requires synaptic Ca^{2+}-permeable AMPA receptors recruited by CaM-kinase I, *J Neurosci 30*, 11565-11575, DOI: 10.1523/JNEUROSCI.1746-10.2010

34. Tinsley, C. J., Narduzzo, K. E., Brown, M. W., and Warburton, E. C. (2012) A role for the CAMKK pathway in visual object recognition memory, *Hippocampus 22*, 466-476, DOI: 10.1002/hipo.20913

35. Sato, K., Suematsu, A., Nakashima, T., Takemoto-Kimura, S., Aoki, K., Morishita, Y., Asahara, H., Ohya, K., Yamaguchi, A., Takai, T., Kodama, T., Chatila, T. A., Bito, H., and Takayanagi, H. (2006) Regulation of osteoclast differentiation and function by the CaMK-CREB pathway, *Nat Med 12*, 1410-1416, DOI: 10.1038/nm1515

36. Park, D., Na, M., Kim, J. A., Lee, U., Cho, E., Jang, M., and Chang, S. (2017) Activation of CaMKIV by soluble amyloid-β1-42 impedes trafficking of axonal vesicles and impairs activity-dependent synaptogenesis, *Sci Signal 10*, eaam8661, DOI: 10.1126/scisignal.aam8661

37. Hawley, S. A., Ross, F. A., Chevtzoff, C., Green, K. A., Evans, A., Fogarty, S., Towler, M. C., Brown, L. J., Ogunbayo, O. A., Evans, A. M., and Hardie, D. G. (2010) Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation, *Cell Metab 11*, 554-565, DOI: 10.1016/j.cmet.2010.04.001

38. Price, D. J., Drewry, D. H., Schaller, L. T., Thompson, B. D., Reid, P. R., Maloney, P. R., Liang, X., Banker, P., Buckholz, R. G., Selley, P. K., McDonald, O. B., Smith, J. L., Shearer, T. W., Cox, R. F., Williams, S. P., Reid, R. A., Tacconi, S., Faggioni, F., Piubelli, C., Sartori, I., Tessari, M., and Wang, T. Y. (2018) An orally available, brain-penetrant CAMKK2 inhibitor reduces food intake in rodent model, *Bioorg Med Chem Lett 28*, 1958-1963, DOI: 10.1016/j.bmcl.2018.03.034

39. Racioppi, L., Nelson, E. R., Huang, W., Mukherjee, D., Lawrence, S. A., Lento, W., Masci, A. M., Jiao, Y., Park, S., York, B., Liu, Y., Baek, A. E., Drewry, D. H., Zuercher, W. J., Bertani, F. R., Businaro, L., Geradts, J., Hall, A., Means, A. R., Chao, N., Chang, C. Y., and McDonnell, D. P. (2019) CaMKK2 in myeloid cells is a key regulator of the immune-suppressive microenvironment in breast cancer, *Nat Commun 10*, 2450, DOI: 10.1038/s41467-019-10424-5

40. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R., and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update, *Biochem J 408*, 297-315, DOI: 10.1042/BJ20070797

41. Tokumitsu, H., Inuzuka, H., Ishikawa, Y., and Kobayashi, R. (2003) A single amino acid difference between alpha and beta Ca^{2+}/calmodulin-dependent protein kinase kinase dictates sensitivity to the specific inhibitor, STO-609, *J Biol Chem 278*, 10908-10913, DOI: 10.1074/jbc.M213183200

42. Fujiwara, Y., Hiraoka, Y., Fujimoto, T., Kanayama, N., Magari, M., and Tokumitsu, H. (2015) Analysis of Distinct Roles of CaMKK Isoforms Using STO-609-Resistant Mutants in Living Cells, *Biochemistry 54*, 3969-3977, DOI: 10.1021/acs.biochem.5b00149

43. Tokumitsu, H., Iwabu, M., Ishikawa, Y., and Kobayashi, R. (2001) Differential regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase isoforms, *Biochemistry 40*, 13925-13932, DOI: 10.1021/bi010863k

44. Hayashi, N., Matsubara, M., Takasaki, A., Titani, K., and Taniguchi, H. (1998) An expression system of rat calmodulin using T7 phage promoter in Escherichia coli, *Protein Expr Purif 12*, 25-28, DOI: 10.1006/prep.1997.0807

45. Tokumitsu, H., Hatano, N., Inuzuka, H., Yokokura, S., Nozaki, N., and Kobayashi, R. (2004) Mechanism of the generation of autonomous activity of Ca^{2+}/calmodulin-dependent protein kinase IV, *J Biol Chem 279*, 40296-40302, DOI: 10.1074/jbc.M406534200

46. Verma, M., Luxami, V., and Paul, K. (2013) Synthesis, in vitro evaluation and molecular modelling of naphthalimide analogue as anticancer agents, *Eur J Med Chem 68*, 352-360, DOI: 10.1016/j.ejmech.2013.07.027

47. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nat Methods 9*, 671-675, DOI: 10.1038/nmeth.2089

48. Tokumitsu, H., Hatano, N., Fujimoto, T., Yurimoto, S., and Kobayashi, R. (2011) Generation of autonomous activity of Ca^{2+}/calmodulin-dependent protein kinase kinase beta by autophosphorylation, *Biochemistry 50*, 8193-8201, DOI: 10.1021/bi201005g

49. Kukimoto-Niino, M., Yoshikawa, S., Takagi, T., Ohsawa, N., Tomabechi, Y., Terada, T., Shirouzu, M., Suzuki, A., Lee, S., Yamauchi, T., Okada-Iwabu, M., Iwabu, M., Kadowaki, T., Minokoshi, Y., and Yokoyama, S. (2011) Crystal structure of the Ca^{2+}/calmodulin-dependent protein kinase kinase in complex with the inhibitor STO-609, *J Biol Chem 286*, 22570-22579, DOI: 10.1074/jbc.M111.251710

Table 1. Inhibitory profile of various protein kinases by TIM-compounds

	IC_{50} value (% inhibition at 10 μg/mL)		
Protein kinases	TIM-062	TIM-063	TIM-064
CaMKKα	> 29 μM (18 ± 3 %)	0.63 μM	> 27 μM (4 ± 3 %)
CaMKKβ	> 29 μM (15 ± 5 %)	0.96 μM	> 27 μM (1 ± 4 %)
CaM kinase I	> 29 μM (22 ± 12 %)	> 26 μM (32 ± 6 %)	> 27 μM (9 ± 4 %)
CaM kinase II	> 29 μM (14 ± 12 %)	14 μM	> 27 μM (26 ± 8 %)
CaM kinase IV	> 29 μM (ND) [a]	> 26 μM (8 ± 6 %)	> 27 μM (ND)
PKA[b]	> 29 μM (17 ± 5 %)	> 26 μM (24 ± 2 %)	> 27 μM (29 ± 9 %)

[a]ND, not detected; [b]PKA, cAMP-dependent protein kinase

10 μg/mL compounds (TIM-062: 29 μM, TIM-063: 26 μM, TIM-064: 27 μM)

Figure Legends

Figure 1. Characterization of STO-609 derivatives as CaMKK inhibitors. A. Chemical structure of STO-609 and its derivatives with different substitutions at positions R_1, R_2, and R_3. **B.** Protein kinase activities of CaMKKα (*open column*) and β (*closed column*) were measured at 30°C for 10 min in a solution containing 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 12.5 μg GST-CaMKI 1–293 Lys49Glu, 100 μM [γ-^{32}P]ATP and 2 mM $CaCl_2$/6 μM CaM in the absence (5% DMSO) or presence of 10 μg/mL STO-609 or its derivatives (TIM-055–064) as described in the "**MATERIALS AND METHODS**." CaMKK activities are quantitated and expressed as a percentage of the average value in the absence of compound. Results are represented as the mean ± S.D. from triplicate experiments. Error bars represent S.D. 10 μg/mL compounds (STO-609: 27 μM, TIM-055: 30 μM, TIM-056: 30 μM, TIM-057: 28 μM, TIM-058: 28 μM, TIM-059: 28 μM, TIM-060: 30 μM, TIM-061: 30 μM, TIM-062: 35 μM, TIM-063: 30 μM, TIM-064: 32 μM)

Figure 2. Effect of TIM-063 and its analogues on the activities of CaMKK isoforms. A. Protein kinase activities of CaMKKα (*left panel*) and CaMKKβ (*right panel*) were measured at 30°C for 10 min in a solution containing 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 12.5 μg GST-CaMKI 1–293 Lys49Glu, 100 μM [γ-^{32}P]ATP and 2 mM $CaCl_2$/6 μM CaM-in the absence (5% DMSO) or presence of indicated concentrations of STO-609 (*open circle*), TIM-062 (*closed triangle*), TIM-063 (*closed circle*), and TIM-064 (*closed diamond*). CaMKK activities are quantitated and expressed as a percentage of the average value in the absence of compound. Results are represented as the mean ± S.D. from triplicate experiments. Error bars represent S.D. **B.** Autophosphorylation of CaMKKα (*right panel*) and CaMKKβ (*left panel*) was measured at 30°C

for 120 min in a solution containing 1 μg CaMKK isoforms, 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 100 μM [γ-^{32}P]ATP in the presence of 2 mM $CaCl_2$/6 μM CaM (for CaMKKα) or 2 mM EGTA (for CaMKKβ) as described in the "**MATERIALS AND METHODS**" in the absence (5% DMSO) or presence of indicated concentrations (0.26–26 μM) of TIM-063 (*closed circle*) as duplicate experiments. **C.** CaMKKα (*right panel*) and CaMKKβ (*left panel*) autophosphorylation were measured at 30°C for 120 min as described in *panel B* in the absence (DMSO) or presence of 10 μg/mL of TIM-062 (29 μM) and TIM-064 (27 μM). The autophosphorylation was quantitated and expressed as a percentage of the average value in the absence of compounds. Results are represented as an average ± S.D. from triplicate experiments. Error bars represent S.D. Statistical differences are marked: *$p < 0.05$; n.s., not significant versus autophosphorylation in the absence of compounds (DMSO). *Insert*, autoradiograms of the CaMKKα (B, *left panel*) and CaMKKβ (B, *right panel*) autophosphorylation in the absence or presence of TIM-063 (0.26–26 μM). The molecular mass in kilodaltons (kDa) is indicated on the *left*. An asterisk in each panel indicates CaMKK autophosphorylation.

Figure 3. TIM-063, an ATP-competitive CaMKK inhibitor. A. Activities of CaMKKα full-length enzyme (*left pane*l, *closed circle*), CaMKKα catalytic domain mutant (GST-CaMKKα 126-434, *left panel*, *open circle*), CaMKKβ full-length enzyme (*right panel, closed circle*), CaMKKβ catalytic domain mutant (GST-CaMKKβ 162-470, *right panel*, *open circle*) were measured at 30°C for 10 min in a solution containing 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 12.5 μg GST-CaMKI 1–293 Lys49Glu, 100 μM [γ-^{32}P]ATP and 2 mM $CaCl_2$/6 μM CaM in the absence (5% DMSO) or presence of indicated concentrations of TIM-063 with either 2 mM $CaCl_2$/6 μM CaM (for CaMKK full-length enzymes) or 2 mM EGTA (for CaMKK catalytic

domain mutants). CaMKK activities were quantitated, expressed as a percentage of the average value in the absence of TIM-063, and plotted as duplicate experiments. **B.** Protein kinase activities of CaMKKα (*left panel*) and CaMKKβ (*right panel*) were measured at 30°C for 10 min in a solution containing 50 mM HEPES pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 12.5 μg GST-CaMKI 1–293 Lys49Glu, and 2 mM $CaCl_2$/6 μM CaM with various concentrations (25–200 μM) of [γ-^{32}P]ATP in either the absence (*open circle*) or presence of 0.84 μM TIM-063 (*closed circle*). CaMKK activities were presented as double reciprocal plots (Lineweaver–Burk) from duplicate experiments.

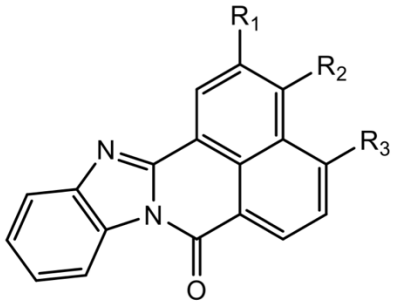
Figure 4. Effect of TIM-063 on ionomycin-induced phosphorylation of endogenous AMPKα, exogenously expressed CaMKIα and CaMKIV in HeLa cells. Either HeLa cells (**A**) , HA-CaMKIα expression vector (1 μg)-transfected HeLa cells (**B**), HA-CaMKIV expression vector (0.25 μg)-transfected HeLa cells (**C**) were cultured in 6-well dishes in the absence of serum for 6 h without (0) or with indicated concentrations (0.26, 2.6, 26 μM) of TIM-063 or 29 μM TIM-062, and then stimulated without (-) or with (+) 1 μM ionomycin (iono) for 5 min as described in the "**MATERIALS AND METHODS**." Subsequently, cell lysates were subjected to immunoblot analysis using either an anti-pAMPKα (at Thr172) antibody (*panel A, inset upper panel, an arrow*), anti-AMPKα antibody (*panel A, inset lower panel, an asterisk*), anti-pCaMKIα (at Thr177) antibody (*panel B, inset upper panel, an arrow*), anti-pCaMKIV (at Thr196) antibody (*panel C, inset upper panel, an arrow*), or anti-HA antibody (*panel B and C, inset lower panel, an asterisk*). The phosphorylation levels of AMPKα at Thr172 (*panel A*), HA-CaMKIα at Thr177 (*panel B*) and HA-CaMKIV at Thr196 (*panel C*) was quantitated, expressed as a percentage of the average

value of stimulated cells with 1 μM ionomycin without compounds, and plotted as duplicate experiments.

Figure 5. Inhibition of CaMKK isoforms-mediated CaMKIV phosphorylation by TIM-063 in transfected COS-7 cells. COS-7 cells in 6-well dishes were transfected with HA-CaMKIV expression vector (1 μg) without (-) or with FLAG-CaMKKα (**A**) or CaMKKβ (**B**) expression vector (0.5 μg) followed by culturing in the absence of serum for 6 h without (-) or with indicated concentrations (0.26, 2.6, 26 μM) of TIM-063 or 29 μM TIM-062, and then treated with 1 μM ionomycin for 5 min as described in the "**MATERIALS AND METHODS**." Subsequently, the cell lysates were subjected to immunoblot analysis using either an anti-pCaMKIV (at Thr196) antibody (*inset upper panels, arrows*) or anti-HA antibody (*inset lower panels, asterisks*). The phosphorylation levels of HA-CaMKIV at Thr196 were quantitated, expressed as a percentage of the average value of stimulated cells with 1 μM ionomycin without compounds, and plotted as duplicate experiments.

Figure 1. Characterization of STO-609 derivatives as CaMKK inhibitors.

A



Compound	R_1	R_2	R_3
STO-609	H	COOH	H
TIM-055	H	$NHCH_2CH_2OH$	H
TIM-056	H	H	$NHCH_2CH_2OH$
TIM-057	H		H
TIM-058	H	H	
TIM-059	H		H
TIM-060	H		H
TIM-061	H	H	
TIM-062	OH	H	H
TIM-063	OH	NO_2	H
TIM-064	H	NO_2	H

B

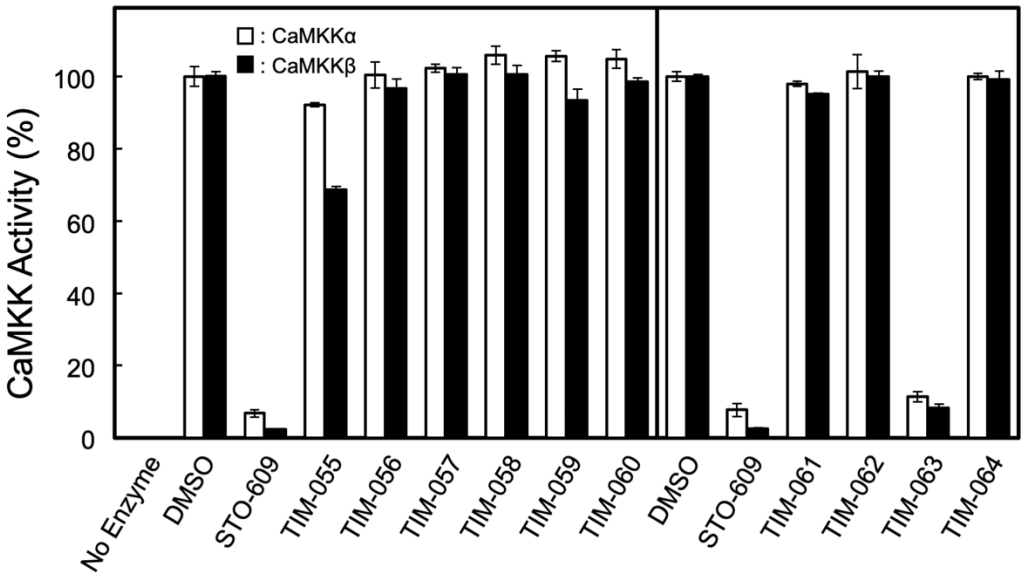


Figure 2. Effect of TIM-063 and its analogues on the activities of CaMKK isoforms.

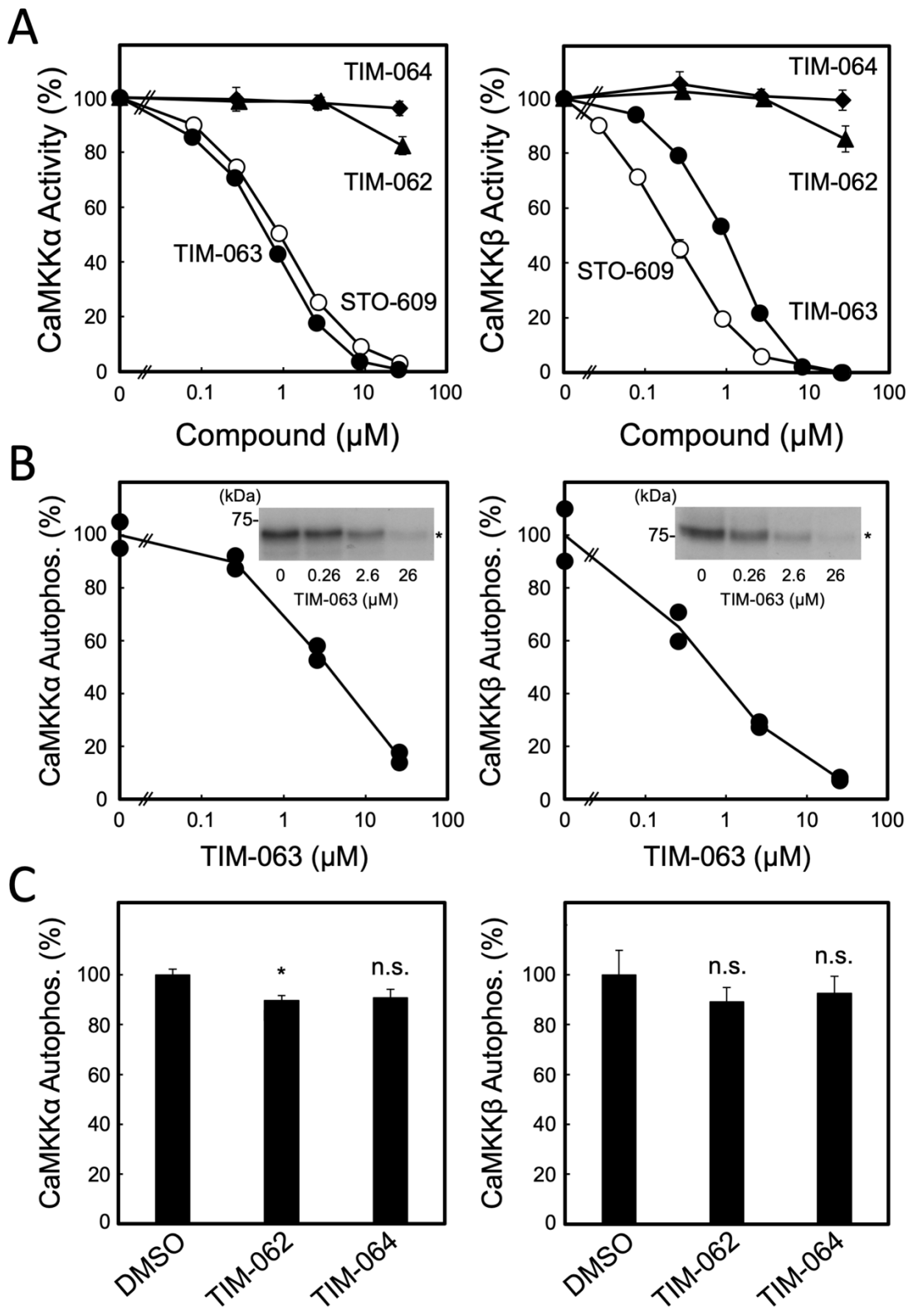


Figure 3. TIM-063, an ATP-competitive CaMKK inhibitor.

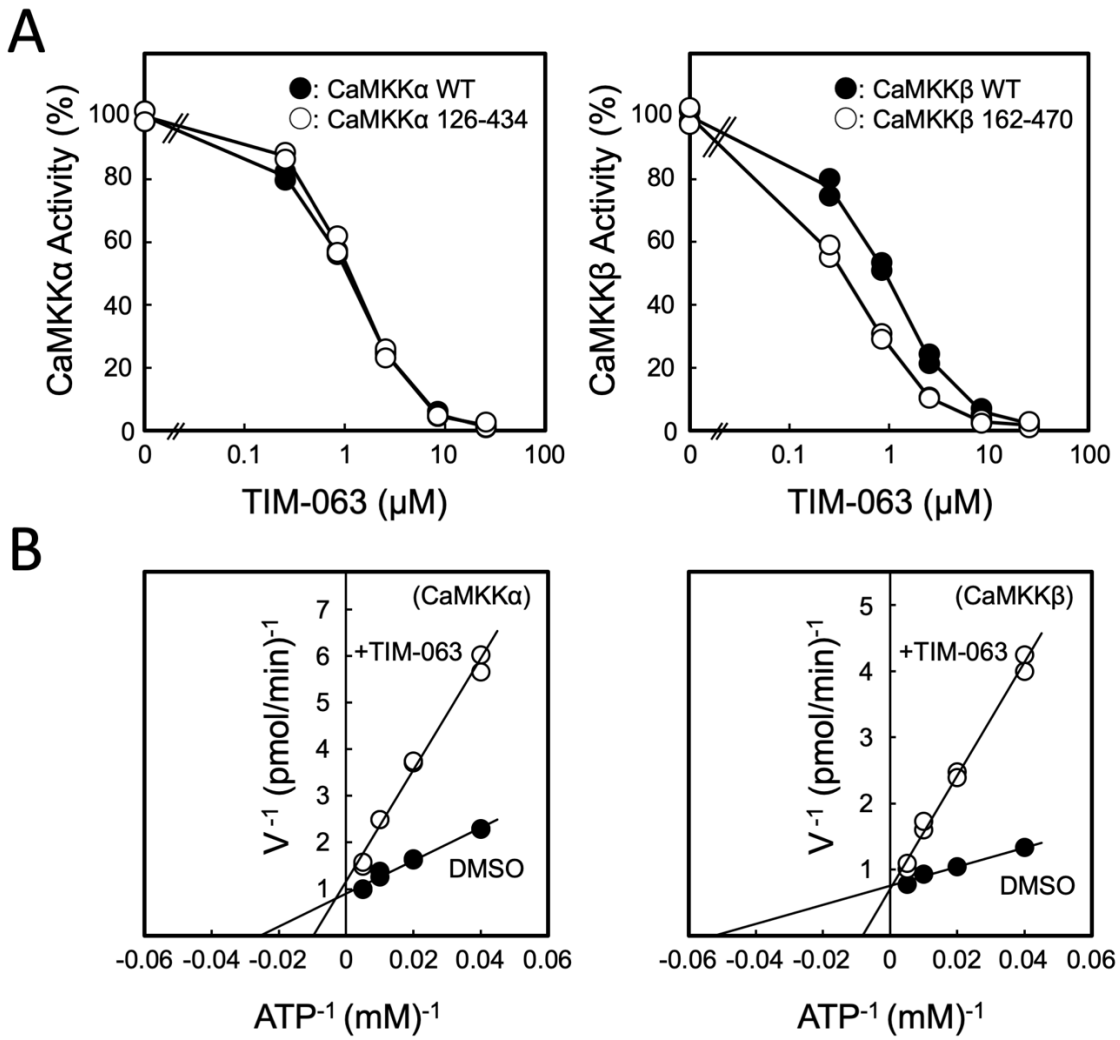


Figure 4. Effect of TIM-063 on ionomycin-induced phosphorylation of endogenous AMPKα, exogenously expressed CaMKIα and CaMKIV in HeLa cells.

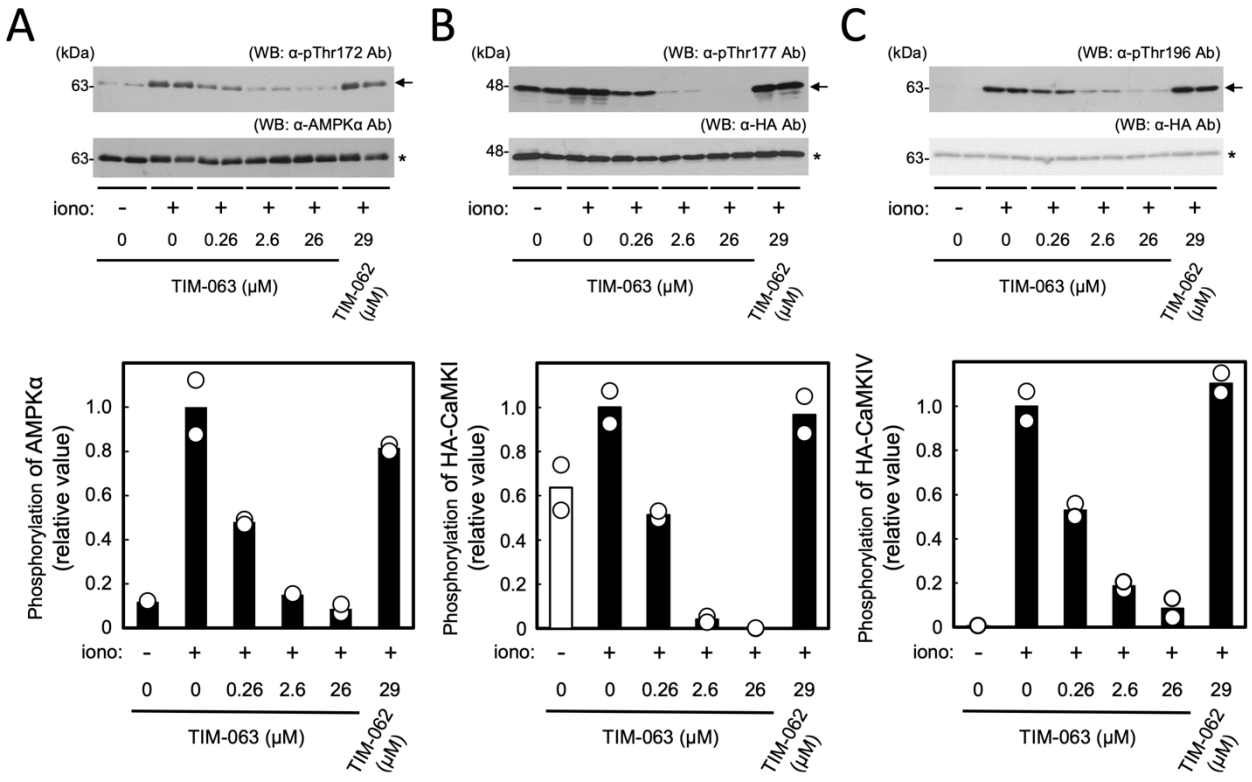
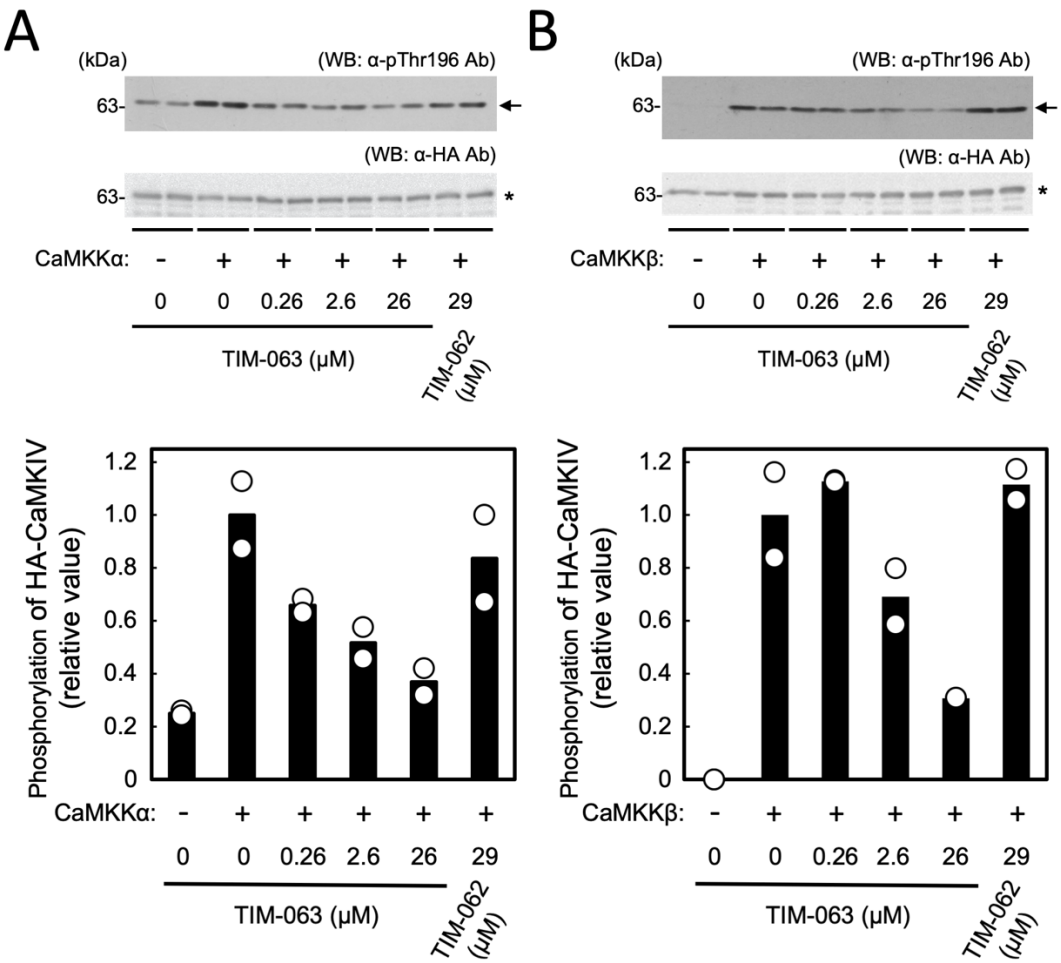
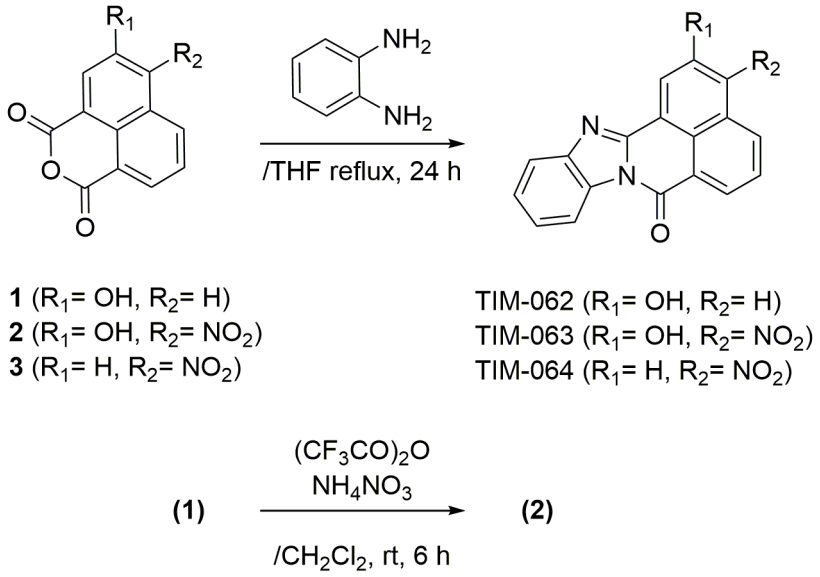


Figure 5. Inhibition of CaMKK isoforms-mediated CaMKIV phosphorylation by TIM-063 in transfected COS-7 cells.





Scheme 1. Synthesis of TIM-063 and its analogues

Supporting Information S1

Synthesis of TIM-063

A mixture of 3-hydroxy-1,8-naphthalenedicaroxylic anhydride **1** (300 mg, 1.40 mmol), ammonium nitrate (1212 mg, 14.0 mmol) in dichloromethane (7.5 mL) was added trifluoroacetic anhydride (0.4 mL, 2.80 mmol) at room temperature. The suspension was stirred at room temperature for 6 h. The mixture was filtered through a celite pad using ethyl acetate and the filtrate was concentrated to give a brown oil, which was purified by column chromatography on silica gel (ethyl acetate/hexane) to yield 3-hydroxy-4-nitro-1,8-naphthalenedicaroxylic anhydride **2** as a yellow solid (176 mg, 0.68 mmol, 49%).

A mixture of **2** (176 mg, 0.68 mmol) and 1,2-phenylenediamine (81 mg, 0.75 mmol) in THF (6 mL) was heated under refluxing for 24 h and cooled to room temperature. The obtained precipitate was filtered and washed with THF to yield pure TIM-063 as a yellow solid (149 mg, 0.45 mmol, 66%).
^{1}H NMR (300 MHz, DMSO-d$_6$) δ 5.15-5.35 (br, 1H), 6.55 (dt, 1H, *J* = 1.5, 7.9 Hz), 6.72 (dd, 1H, *J* = 1.5, 8.2 Hz), 6.98 (dd, 1H, *J* = 1.5, 7.9 Hz), 7.19 (dt, 1H, *J* = 1.5, 8.2 Hz), 7.92 (dd, 1H, *J* = 7.3, 8.5 Hz), 8.02 (dd, 1H, *J* = 1.2, 8.5 Hz), 8.20 (s, 1H), 8.33 (dd, 1H, *J* = 1.2, 7.3 Hz). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 116.1. 116.2, 120.8, 122.8, 123.2, 124.8, 125.3, 126.1, 127.7, 128.8, 129.9, 130.4, 130.8, 137.0, 146.4, 149.0, 163.0, 163.9. ESI-MS, *m/z*: 332 (M^++1).

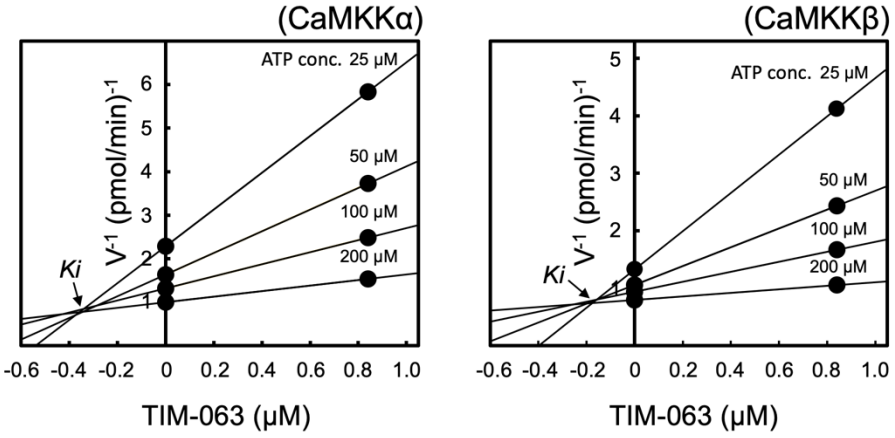
Preparation of acetic acid salt of TIM-063

A mixture of TIM-063 (60 mg, 0.18 mmol) and acetic acid (1 mL) in THF (10 mL) was heated at 60°C for 1h, and concentrated *in vacuo* to yield acetic acid salt of TIM-063 as an orange solid (65 mg). The obtained salt was used without further purification.

TIM-062 and **TIM-064**, and their acetic acid salts were prepared by a similar procedure for the synthesis of **TIM-063**.

TIM-062 (yellow solid, 65%)
^{1}H NMR (300 MHz, DMSO-d$_6$) δ 5.19-5.22 (br, 1H), 6.56 (dt, 1H, *J* = 1.5, 7.9 Hz), 6.73 (dd, 1H, *J* = 1.5, 8.2 Hz), 6.93 (dd, 1H, *J* = 1.5, 7.9 Hz), 7.07 (dt, 1H, *J* = 1.5, 8.2 Hz), 7.62 (d, 1H, *J* = 2.3 Hz), 7.74 (dd, 1H, *J* = 7.3, 8.2 Hz), 7.98 (d, 1H, *J* = 2.3 Hz), 8.20 (d, 1H, *J* = 7.3 Hz), 8.22 (d, 1H, *J* = 8.2, Hz). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 116.1, 116.2, 116.3, 121.4, 122.4, 124.0, 124.2, 125.7, 127.9, 128.0, 129.7, 130.5, 133.0, 134.4, 145.5, 156.9, 164.1, 164.4. ESI-MS, *m/z*: 287 (M^++1).

TIM-064 (orange solid, 68%)
^{1}H NMR (300 MHz, DMSO-d$_6$) δ 5.26-5.36 (br, 1H), 6.56 (dt, 1H, *J* = 1.5, 7.9 Hz), 6.73 (dd, 1H, *J* = 1.5, 8.2 Hz), 7.01 (dd, 1H, *J* = 1.5, 7.9 Hz), 7.10 (dt, 1H, *J* = 1.5, 8.2 Hz), 8.10 (dd, 1H, *J* = 7.2, 8.4 Hz), 8.56 (s, 2H), 8.60 (d, 1H, *J* = 7.2 Hz), 8.72 (d, 1H, *J* = 8.4 Hz). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 116.2, 116.3, 120.7, 123.7, 124.9, 125.1, 129.0, 129.2, 129.9, 130.0, 130.1, 130.4, 130.7, 132.1, 146.5, 149.9, 163.0, 163.8. ESI-MS, *m/z*: 316 (M^++1).



Supplemental Figure S2. Dixon plot analysis for the inhibition of CaMKKα (*left panel*) and CaMKKβ (*right panel*) activities by TIM-063 (0.84 μM) in the presence of various concentrations (25—200 μM) of [γ-^{32}P]ATP as shown in Figure 3B. The *Ki* values of TIM-063 were calculated to be 0.35 μM for CaMKKα and 0.2 μM for CaMKKβ.