The Molecular Mechanisms of Action of Two Drug Candidates for the Treatment of Myocardial Infarction and Sepsis

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Tomohiro KAWAMOTO

Abbreviations

BCECF:	2', 7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein
CCD:	charge coupled device
CD:	cluster of differentiation
CHO:	Chinese hamster ovary
CHP:	calcineurin B homologous proteins
CMV:	cytomegalovirus
DMSO:	dimethyl sulfoxide
ds:	double-stranded
EMS:	ethylmethane sulfonate
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
HEK:	human embryonic kidney
HMGB:	high-mobility group box
HSP:	heat-shock protein
IBD:	inflammatory bowel diseases
IFN:	interferon
IL:	interleukin
IND:	investigational new drug
IRAK:	IL-1 receptor-associated kinase 1
LBP:	lipopolysaccaride binding protein
LRR:	leucine-rich-repeat
LPS:	lipopolysaccharide
MD-2:	myeloid differentiation protein-2
MI:	myocardial infarction
mPTP:	mitochondrial permeability transition pore
MyD88:	myeloid differentiation factor 88
NBC:	Na^+:HCO_3^- cotransporter
NCX:	Na^+/Ca^{2+} exchanger
NF-κB:	nuclear factor-kappa B
NHE:	Na^+/H^+ exchanger
NO:	nitric oxide
ODN:	oligodeoxynucleotides
OxLDL:	oxidized low-density lipoprotein
PBS:	phosphate-buffered saline

PCR:	polymerase chain reaction
PMA:	phorbol myristate acetate
Poly (I:C):	polyinosinic-polycytidylic acid
PSS:	physiological salt solution
pHi:	intracellular pH
SDS:	sodium dodecyl sulfate
S.E.M.:	standard error of the mean
SLC:	solute carrier
ss:	single-stranded
TBS:	tris buffered saline
TICAM1:	TRIF/TIR-domain-containing molecule 1
TIR:	toll/interleukin-1 receptor homology
TIRAP:	TIR-associated protein
TLR:	toll-like receptor
TNF:	tumor necrosis factor
TRAM:	TRIF-related adaptor molecule
TRIF:	TIR-domain-containing adaptor protein-inducing IFN-β

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Abstract

Drug discovery strategies are divided into two types of approaches, target-oriented screens and phenotypic screens. The phenotypic approach is a strategy whereby pharmaceutical research focuses on compounds that demonstrate phenotypic efficacy against a disease model. However, clarification of the molecular mechanism and targets of bioactive compounds can be a bottleneck for drug development using this approach. The objectives of this study were to elucidate the molecular mechanisms of action and the selectivity profiles of two drug candidates, T-162559 and TAK-242, which were optimized or discovered by phenotypic assays in myocardial infarction (MI) and sepsis.

T-162559 was identified in an *in vitro* assay monitoring platelet swelling induced by acidosis as a Na^+/H^+ exchanger (NHE) inhibitor and demonstrated cardioprotective effects *in vivo* in ischemia-reperfusion injury models. The hypothesis for the mechanism of action for T-162559 is that the main molecular target is an NHE1 isoform that plays a role in intracellular pH (pHi) regulation after acidosis. To establish an assay system for the human NHE1 isoform, NHE-deficient Chinese hamster ovary (CHO)-K1 cells were isolated by a proton suicide method of selection, since most cells express NHEs. An NHE assay system was then developed to monitor pH recovery of cells from acidosis after NH_4Cl prepulse treatment. This assay system demonstrated that T-162559 is a selective and potent inhibitor of NHE1.

TAK-242 was identified *in vitro* in a cell-based assay monitoring nitric oxide (NO) and cytokine production from lipopolysaccharide (LPS)-treated macrophages (RAW264.7). This compound had a therapeutic effect *in vivo* in endotoxin shock models. Initial studies on effects of TAK-242 on the production of inflammatory mediators

induced with various Toll-like receptor (TLR) ligands were carried out *in vitro* with the RAW264.7 macrophage cell line. These studies showed that TAK-242 selectively suppressed TLR4 ligand-induced production of NO, while little effect was noted for other TLR ligand activities. TAK-242 inhibited LPS-mediated nuclear factor-kappaB (NF-κB) activation in HEK293 cells transiently expressing TLR4 and its co-receptors, myeloid differentiation protein-2 (MD2) and cluster of differentiation (CD) 14. TAK-242 also inhibited ligand-independent NF-κB activation mediated by TLR4 overexpression or chimeric TLR4 receptors consisting of the extracellular domain of CD4 and intracellular domain of TLR4. These results indicated that TAK-242 suppresses TLR4-signaling mediated by the intracellular domain.

In conclusion, T-162559 and TAK-242 were shown to specifically target NHE1 and TLR4, respectively, and these data suggest that these compounds have promise as treatments for NHE1 and TLR4-dependent diseases.

General Introduction

There are two different approaches for drug discovery, target-oriented screens and phenotypic screens. Target-oriented drug discovery, called reverse pharmacology, begins with identification of a target relevant to a disease of interest. After identification and validation of the target, a biochemical assay system is established based on the function of the target. Drug screening is then conducted using a diverse compound library. After the selection of compounds with positive activity, lead optimization is carried out, typically utilizing medicinal chemistry methodologies to improve potency, efficacy, selectivity and bioavailability. Lead compounds with potent activity are then evaluated further in *in vitro* cell-based assays and *in vivo* disease models. Compounds with acceptable preclinical potency and efficacy are then moved into investigational new drug (IND)-enabling studies, including toxicology and safety pharmacology studies to enable initiation of clinical trials. Phenotypic drug discovery, termed forward pharmacology, screens test compounds by monitoring phenotypic changes in systems that are related to disease processes. This approach has been used historically for drug discovery, with subsequent identification of the molecular targets being affected by the compounds. Recent scientific discoveries and technological advancements, such as high-throughput screening, structure-based drug design, combinatorial chemistry, and the studies of the human genome and the role of gene products in various diseases, have shifted the focus of drug discovery and development to target-oriented approaches in biotechnology and pharmaceutical industries.

An advantage of target-oriented drug discovery is that the molecular target is defined at the initial stage of drug discovery. This enables the development of

biochemical or biophysical assays with recombinant proteins or cells that allow informed medicinal chemistry efforts to develop active compounds utilizing techniques such as molecular modeling and structure-based drug design. Pharmacological or pharmacodynamic experiments can be designed based on the profile of the target molecule. Despite these advantages, in many cases, selected compounds do not show sufficient efficacy in disease models or in patients. This can occur for two general reasons. First, the molecular target, while correlated with presence of the disease, may not play a primary role in its pathogenesis. Second, the drug candidate that is supposed to affect the target lacks adequate potency for a variety of reasons, such as pharmacokinetic behavior, toxicity, poor affinity, or lack of selectivity. Despite technological advancements in drug and target discovery, the productivity of drug discovery has been disappointing, particularly for the discovery and successful development of first in class drugs for a given target or indication. Consequently, phenotypic approaches have been reimplemented to contribute to the discovery of new targets and first in class drugs. For example, the discovery of peroxisome proliferator-activated receptor gamma ligands was facilitated by studies with thiazolidinediones, which were identified in phenotypic screens targeting diabetes (Karak et al., 2013). A key advantage of the phenotypic approach is that pharmacological research can be initiated using compounds that have shown efficacy in relevant cell-based systems *in vitro* or animal models *in vivo*. This approach is straightforward for the identification of compounds with promising phenotypic activity. A key requirement for a sound phenotypic-based discovery approach is the establishment of physiologically relevant assay systems that can serve as a translational bridge to clinical efficacy and safety. The phenotypic approach holds the promise of

identifying new therapeutic modalities and molecular pathways for untreatable diseases (Eder et al., 2014). However, a caveat is that the molecular mechanisms for compounds discovered by phenotypic screens may be poorly characterized. An understanding of the molecular target can facilitate utilization of advantages of the target-oriented approach. Furthermore, insights on the potential efficacy and toxicity of compounds that affect the target protein can be assessed using transgenic or knockout animals before initiating clinical trials. However, identification of the molecular target is still a bottleneck for this approach.

In this study, the molecular mechanisms of action and the selectivity profiles are characterized for T-162559 and TAK-242 which were optimized or discovered by phenotypic assays using native cells. These small-molecule compounds are being developed for the treatment of myocardial infarction and sepsis, respectively, which are life-threatening diseases with unmet medical needs. This study demonstrates that T-162559 and TAK-242 are selective inhibitors of NHE1 and TLR4, respectively.

Chapter I: Molecular Mechanism of Action of T-162559

1. Introduction

Myocardial infarction (MI) is a life-threatening disease caused by the loss of blood perfusion to a specific region of the heart. Acute MI occurs when plaque deposits that have developed in patients with coronary artery disease break up and fragments lodge in arteries blocking blood flow to the heart. Prompt emergency medical attention is crucial for the treatment of acute MI, including treatment with antithrombotics and angioplasty to restore and protect blood flow. However, MI remains a leading cause of morbidity and mortality worldwide, with approximately 600,000 deaths in the United States every year (Murphy et al., 2013). Furthermore, reperfusion therapy has the risk of additional myocardial damage by causing acute ischemia-reperfusion injury. Thus, there is still a high medical unmet need for more effective treatments for MI. The pathological consequences of MI arise from the detrimental effects of acute ischemia-reperfusion injury, which leads to cardiomyocyte cell death, cardiac failure, arrhythmias and patient death. Thus, more effective therapeutic approaches are needed to protect the heart from acute ischemia-reperfusion injury to reduce myocardial infarct size (Sluijter et al., 2014). During ischemia, accumulation of lactate in cells results in acidosis and cellular swelling with plasma membrane blebbing. Subsequently, the swollen cells develop physical defects in their cell membranes and rupture. These features of cell injury with cell swelling have been shown to involve cardiomyocyte cells subjected to hypoxia *in vitro* and cardiac muscle during MI *in vivo*. The underlying membrane damage to ischemic myocyte cells has served as the basis for the diagnosis of MI (Buja et al., 1998).

T-162559 is being developed as a small molecule NHE inhibitor for the treatment of MI (Kusumoto et al., 2002, Fukumoto et al., 2002). To generate an NHE inhibitor, a cell-based assay was established by monitoring an increase in light transmission that is associated with acidosis-induced swelling in platelets. Lead optimization was initiated with an aminoguanidine derivative having an IC_{50} value of 2.3 μM. After optimization, a potent inhibitor, T-162559 was identified. This compound inhibited platelet cell swelling with an IC_{50} value of 13 nM, and had a protective effect on perfused rat hearts after global ischemia and reperfusion. Intravenous administration of T-162559 (0.03 and 0.1 mg/kg) significantly inhibited MI progression induced by left coronary artery occlusion and reperfusion in rabbits (Kusumoto et al., 2002). These results demonstrated that T-162559 is a promising drug candidate for the treatment of MI. Intracellular pH is regulated by Na^+/H^+ exchangers (NHEs), which are ion transporters that mediate Na^+ and H^+ exchange across lipid bilayers in prokaryotes and eukaryotes. NHEs participate in various cellular functions such as the maintenance of pHi and cell volume. The Na^+ electrochemical gradient is utilized by NHEs to remove H^+ from cells with a stoichiometric coupling ratio of 1 Na^+:1 H^+. Hydropathy analysis of NHE predicts that they have similar membrane topologies, with an N-terminal membrane domain consisting of twelve transmembrane segments and a divergent C-terminal cytoplasmic domain (Pullikuth et al., 2003, Orlowski et al., 2004) (Fig. 1-1).

Currently, thirteen conserved NHE isoforms are known in mammals. Human NHEs are encoded by the solute carrier (SLC) classification of the transporter 9 family. NHE isoforms are divided into two classes, plasma membrane NHEs (NHE1–NHE5) and organelle NHEs (NHE6–NHE9) (Fuster et al., 2014). Although NHE1 is ubiquitously expressed, other plasma membrane NHEs show tissue specific expression profiles

(Table1-1). NHE2–NHE4 isoforms are predominantly expressed in epithelial cells of the kidney, small intestine, and stomach, and NHE5 is primarily expressed in the brain (Wakabayashi et al., 2013). In contrast, NHE6–NHE9 are expressed in intracellular organelles, including the trans-Golgi network, endosomes, and mitochondria in various tissues where they function to control organelle luminal pH by K^+/H^+ exchange (Hill et al., 2006).

The NHE1 isoform is the most well-characterized isoform of the NHE family. The amino acid identity of NHE1 among human plasma NHE isoforms ranges from 36% to 49% (Table 1-1). Human NHE1 consists of 815 amino acids, with residues 1 to 500 comprising the membrane-associated domain and residues 501 to 815 comprising the cytoplasmic domain (Fliegel et al., 1993). The membrane domain of NHE1 is important for ion transport, whereas the cytoplasmic domain is implicated in regulation of Na^+/H^+ exchange activity. The cytoplasmic domain of NHE1 regulates NHE activity by interacting with Ca^{2+}-binding proteins, protein kinases, phosphatases, and cytoskeletal proteins (Wakabayashi et al., 2013).

NHE1 plays a critical role in controlling intracellular acidosis. After myocardial ischemia, tissue pH significantly declines and returns to normal after reperfusion. During ischemia-reperfusion, excessive activation of NHE1 by a decrease in pHi causes a significant elevation in intracellular Na^+ (MacLeod, et al., 1991, Karmazyn et al., 1999). The increase in intracellular Na^+ leads to Ca^{2+} overload through reverse-mode activation of the Na^+/Ca^{2+} exchanger (NCX) (Lazdunski et al., 1985, Guarnieri, 1987, Wier, 1990) and Ca^{2+} overload is thought to be a major mechanism involved in the development of irreversible damage during ischemia-reperfusion. A variety of NHE inhibitors have been developed, or are currently being developed, for the treatment of

ischemia-reperfusion injury. Acylguanidine derivatives such as cariporide and eniporide have been reported to be selective inhibitors of NHE1, and these compounds have shown cardioprotective effects in ischemia-reperfusion models (Counillon et al., 1993a, Scholz et al., 1995, Gumina et al., 1998, Yamamoto et al., 2000).

Na^+/H^+ exchanger 1 (NHE1) inhibitors, including the acylguanidine derivatives, caliporide and eniporide, inhibit platelet swelling and have a protective effect in ischemic injury (Kusumoto et al., 2002). T-162559 has a protonated guanidine structure and mimics a sodium cation hydrated with three water molecules. While cell-based discovery approach using platelets was used to identify T-162559, this is also chemistry-based approach since structurally related compounds with known NHE1 activity can serve as a starting point for lead optimization. On the basis of the effects of related compounds, molecular target of this compound was expected to be NHE1. To characterize the molecular mechanism of action of T-162559, its effect on human NHE isoforms were investigated. An assay to measure Na^+/H^+ exchange activity was established by monitoring intracellular pH alterations with cells stably expressing human NHE isoforms (hNHE1, hNHE2, and hNHE3). We investigated the effects of a novel aminoguanidine derivative, T-162559, on human NHE isoforms and demonstrated that T-162559 is a selective inhibitor of the hNHE1 isoform with greater potency than cariporide and eniporide.

2. Materials and Methods

2.1. Compounds

T-162559 ((5E,7S)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6H)-quinolinylideneamino] guanidine dimethanesulfonate), T-162561 ((5E,7R)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6H)-quinolinylideneamino] guanidine dimethanesulfonate), cariporide, and eniporide were synthesized at Takeda Pharmaceutical Company (Fig.1-2). These compounds were dissolved in dimethyl sulfoxide (DMSO).

2.2. Plasmids

cDNAs encoding hNHE1, hNHE3 and human Na^+:HCO_3^- cotransporter 1 (hNBC1) were isolated by polymerase chain reaction (PCR) and subcloned into the mammalian expression vector pMSRα-neo, which contained the SRα promoter. cDNAs encoding hNHE2 and human NCX1 were isolated by PCR and subcloned into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen), which contained the cytomegalovirus (CMV) promoter.

2.3. Cell culture

CHO-K1 cells and their NHE-deficient cells were maintained in Ham's F-12 medium (Life Technologies, Inc., USA.) containing 10% fetal calf serum and 50 μg/ml gentamicin (Life Technologies, Inc., USA.) in a humidified 5% CO_2 incubator at 37 °C.

2.4. Isolation of NHE-deficient mutant cells

The NHE-deficient mutant was isolated by the proton suicide technique (Pouyssegur

et al., 1984). CHO-K1 cells were initially mutagenized with 150 μg/ml ethylmethane sulfonate (EMS) (Aldrich) in the culture medium for 16 h. Then, cells were trypsinized and incubated in Li^+ saline solution (130 mM LiCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES, pH7.4) for 2 h at 37 °C. After Li^+ loading, Li^+ saline solution was removed by centrifugation. Then, the cell pellet was washed with and incubated in choline-Cl saline solution (130 mM choline-Cl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM 2-(N-morpholino) ethanesulfonic acid, pH5.5) for 60 min at 37 °C. Cells were centrifuged, suspended and transferred to the culture medium. After 4 days of culture, resistant cells were trypsinized and subjected to two cycles of selection. NHE-deficient clones were selected and a single clone was isolated by limited dilution.

2.5. Transfection

Transfection was performed with NHE-deficient CHO-K1 cells ($8x10^6$ cells per 800 μl) for hNHE1, hNHE2, hNHE3, and hNBC1, and with wild-type CHO-K1 cells ($8x10^6$ cells per 800 μl) for hNCX1 by electroporation using a Gene Pulser (Bio-Rad Laboratories) at 250 mV and 960 μF. Stable transformants of hNHE1, hNHE3, and hNBC1 were selected in the presence of 500 μg/ml geneticin (Life Technologies, Inc., USA). Stable transformants of hNHE2 and hNCX1 were selected in the presence of 250 μg/ml zeocin (Life Technologies, Inc., USA).

2.6. Measurement of Na^+/H^+ exchange activity

Na^+/H^+ exchange activity was determined by using the membrane-permeable acetoxymethyl (AM) ester form of the pH-sensitive fluorescent indicator

2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure the Na^+-dependent recovery of pHi from NH_4Cl prepulse acidification (Nakanishi et al., 1991). Aliquots of $5x10^4$ cells were seeded in WhiteClini Plates (white 96-well plates, Labsystems, Finland) and cultured at 37 °C overnight. After removal of the medium, the cells were loaded with 5 μM BCECF-AM (Wako Pure Chemical Ind., Ltd., Japan) for 30 min at 37 °C. Cells were washed with Na^+ solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES, pH7.4) and subsequently incubated in Na^+ solution containing 25 mM NH_4Cl for 10 min at 37°C. Acid loading was performed by washing cells with Na^+-free solution (140 mM N-methyl-D(-)-glucamine, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES, pH7.4). Then, 47.5 μl of Na^+-free solution and 2.5 μl of test compounds were added to the cells, followed by incubation for 5 min. Fluorescence was measured by using a fluorescence drug screening system, FDSS-2000 (Hamamatsu Photonics, Ltd., Japan). Aliquots of 200 μl of Na^+ solution were added to the cells and the fluorescence ratio (450 nm/490 nm) was monitored. The initial velocity of pHi recovery via NHE was calculated using linear regression of the initial data points (10 s for NHE1, 40 s for NHE2 and NHE3). An inhibitory activity of NHE inhibitors was evaluated by a reduction of the velocity. For calculation of the inhibition, pHi recovery with DMSO in cells expressing NHE isoforms was equated with 100% activity and pHi recovery with DMSO in NHE-deficient cells was equated with 0% activity.

2.7. Measurement of Na^+:HCO_3^- cotransport activity

Na^+:HCO_3^- cotransport activity was determined using BCECF-AM to measure the Na^+-dependent recovery of pHi from NH_4Cl prepulse acidification (Burnham et al.,

1997). Cells were seeded at 5 x 10^4 cells in WhiteClini Plates (white 96-well plates, Labsystems, Finland) and cultured at 37 °C overnight. After removal of the medium, cells were loaded with 5 μM BCECF-AM (Wako, Japan) for 30 min at 37 °C. Cells were washed with solution A (140 mM tetramethyl ammonium chloride, 25 mM $KHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH7.4) and then incubated in solution B (100 mM tetramethyl ammonium chloride, 40 mM NH_4Cl, 25 mM $KHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH7.4) for 10 min at 37°C. Acid loading was performed by washing cells with solution A. Then, 47.5 μl of solution A and 2.5 μl of test compounds were added to the cells, followed by incubation for 5 min. Fluorescence was measured with FDSS-2000. Aliquots of 200 μl of solution C (115 mM NaCl, 25 mM KCl, 25 mM $NaHCO_3$, 0.8 mM K_2HPO_4, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH7.4) were added to the cells and fluorescence ratio (450 nm/490 nm) was monitored for 5 min.

2.8. Measurement of Na^+/Ca^{2+} exchange activity

Na^+/Ca^{2+} exchange activity was determined using the membrane-permeable AM ester form of the Ca^{2+}-sensitive fluorescent indicator FuraPE3 to measure cytoplasmic Ca^{2+} alterations (Komuro et al, 1992, Fang et al., 1998). Cells were seeded at 5x10^4 cells in Opaque Plates (white 96-well plates, Corning Coster) and cultured at 37°C overnight. After removal of the medium, cells were loaded with 20 μM FuraPE3-AM (Wako, Japan) for 60 min at 37 °C. Cells were washed three times with physiological salt solution (PSS) (120 mM NaCl, 20 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH7.4). Then, 188 μl of PSS and 2.0 μl of test compounds

were added to the cells, followed by incubation for 5 min. Fluorescence was measured with FDSS-2000. Aliquots of 10 μl of phosphate-buffered saline (PBS) containing 200 μM gramicidin (Sigma, USA) were added to the cells and the fluorescence ratio (340 nm/380 nm) was monitored for 15 min.

3. Results

3.1. Functional expression of human NHE (hNHE) isoforms in NHE-deficient CHO-K1 cells

Na^+/H^+ exchangers are endogenously expressed in most mammalian cells. We isolated an NHE-deficient Chinese hamster ovary (CHO)-K1 mutant using the proton suicide selection method (Poussegur et al., 1984). The cell growth of the NHE-deficient mutant was not significantly different from that of the wild-type in regular medium buffered at pH 7.4 with CO_2/HCO_3^- (data not shown).

NHE-deficient CHO-K1 cells that were transfected with human NHE isoform-expressing plasmids and stable transformants were isolated. Na^+/H^+ exchange activity was determined using BCECF-AM to measure Na^+-dependent recovery of the pHi after NH_4Cl prepulse acidification. pHi was measured with a fluorescent screening system, FDSS-2000 (Hamamatsu Photonics), which was able to measure fluorescence alterations in real time in a 96-well microplate using a charge-coupled device (CCD) camera.

Human NHE isoforms, hNHE1, hNHE2, and hNHE3, expressed in NHE-deficient CHO-K1 cells had functional Na^+/H^+ exchange activity, whereas NHE-deficient cells showed no change in pHi after the addition of external Na^+ (Fig. 1-3). Especially, NHE1 expressing cells showed a rapid recovery of pHi after NH_4Cl prepulse acidification as compared with hNHE2- and hNHE3-expressing cells. These results support the conclusion that NHE1 is the primary isoform involved in the recovery of pHi and the entry of Na^+ during cellular acidosis.

3.2. Selective inhibition of the hNHE1 isoform by cariporide, eniporide, and T-162559

Selectivity of the NHE inhibitors cariporide and eniporide was assessed on the

various human NHE isoforms. In the Na^+-dependent pHi recovery assay, cariporide and eniporide inhibited hNHE1 in a concentration-dependent manner (Fig. 1-4). The NHE1 IC_{50} values for cariporide and eniporide were 30 nM and 4.5 nM, respectively (Table 1-2). Cariporide and eniporide inhibited hNHE2 with low potencies (IC_{50} values of 4.3 μM and 2.0 μM, respectively), and did not inhibit hNHE3 at concentrations ranging up to 100 μM.

The potency of a novel synthetic NHE inhibitor, the aminoguanidine derivative T-162559, was evaluated in the assay for effects on human NHE isoforms. In the Na^+-dependent pHi recovery assay, T-162559 inhibited hNHE1 in a concentration-dependent manner (Fig. 1-4) with an IC_{50} of 0.96 nM, which was 30-fold and 4.7-fold lower than the IC_{50} values for cariporide and eniporide, respectively (Table 1-2). T-162559 had weak inhibitory effects on hNHE2 and hNHE3 (IC_{50} values of 430 nM and 11 μM, respectively). The IC_{50} values of this compound for hNHE2 and hNHE3 were 450-fold and 11000-fold higher, respectively, than for hNHE1. We also evaluated the potency of T-162561, the R enantiomer of T-162559 (Fig. 1-4). The inhibitory effect of T-162561 on hNHE1 was decreased 36-fold relative to the S enantiomer, T-162559 (35 nM IC_{50} value of T-162561 for hNHE1), whereas the IC_{50} value of T-162561 for hNHE2 was similar to that of T-162559 (Table 1-2).

3.3. Effects of T-162559 on ion transporters hNBC1 and hNCX1

To investigate the effects of T-162559 on other ion transporters, we isolated cells stably expressing the human Na^+:HCO_3^- cotransporter (hNBC1) and the human cardiac Na^+/Ca^{2+} exchanger (hNCX1).

hNBC1 cotransporter activity was measured by monitoring Na^+-dependent pH

recovery after NH_4Cl prepulse acidification in the presence of HCO_3^-. After the addition of external Na^+, a significant increase in pHi was observed in cells expressing hNBC1, whereas non-transfected NHE-deficient CHO-K1 cells only showed a slight increase in pHi (Fig.1-5A). In this assay system, T-162559 did not significantly affect the initial rate of pHi recovery mediated by hNBC1 at 100 μM (Fig. 1-5A).

Na^+/Ca^{2+} exchange activity of hNCX1 expressed in CHO-K1 cells was quantified by assaying Ca^{2+} influx caused by an increase in intracellular Na^+ in the presence of the Na^+ ionophore gramicidin. After reverse-mode activation of hNCX1 by an increase in intracellular Na^+, a slow and sustained increase in intracellular Ca^{2+} was observed in cells transfected with hNCX1, whereas non-transfected CHO-K1 cells showed no change in intracellular Ca^{2+} (Fig. 1-5B). In this assay system, 100 μM T-162559 did not affect hNCX1 (Fig. 1-5B).

4. Discussion

Myocardial infarction is defined as myocardial cell death due to prolonged ischemia. Na^+/H^+ exchange plays an important role in the development of irreversible cellular damage during ischemia-reperfusion. The primary mechanism of myocardial protection by NHE1 inhibitors in ischemia-reperfusion is likely by delaying the progression of ischemic injury, resulting in a decrease in infarct size even after long periods (>4 hrs) of ischemia (Avkiran, 1999). Pharmaceutical companies have been developing NHE inhibitors for the treatment of ischemia-reperfusion injury for the past several years (Scholz et al., 1995; Gumina et al., 1998; Counillon et al., 1993a; Yamamoto et al., 2000). Cariporide is the first NHE inhibitor that was evaluated in clinical trials. Clinical studies with cariporide showed cardioprotective effects in patients with acute anterior MI who were also treated with direct percutaneous transluminal coronary angioplasty (Rupprecht et al., 2000).

Several ion transporters and channels are involved in the regulation of platelet cell volume (Sarkadi et al., 1991). Activation of NHE1 results in platelet swelling associated with a reversal in the transport of cytoplasmic H^+ and extracellular Na^+ under acidified intracellular conditions (Livne et al., 1987). NHE1 constitutes a major pathway for Na^+ influx into the cell, and is coupled to Cl^- and H_2O uptake to ensure restoration of cell volume following cell shrinkage (Fuster et al., 2014). Among NHE isoforms, NHE1 is expressed in human platelets (Aharonovitz et al., 1996), and cariporide inhibits human platelet NHE activity (Weichert et al., 1997, Kusumoto et al., 2002). These results suggest that acidosis-induced cell swelling of platelets is mediated through NHE1.

To clarify the hypothesis that T-162559 targets NHE1, a fluorescence-based screening system was developed. Cariporide and eniporide showed selective inhibition

of hNHE1, with IC_{50} values similar to those previously reported for rat NHE isoforms (Scholz et al., 1995, Gumina et al., 1998). These results are consistent with previous reports that the amiloride binding domain in the fourth transmembrane segment of NHE isoforms is conserved between human and rat sequences (Wakabayashi et al., 1997, Counillon et al., 1993b, Counillon et al., 1997). Taken together, these results validated the assay system used for evaluation of NHE isoforms and their inhibitors.

T-162559 showed selective inhibition of hNHE1 with greater potency than cariporide and eniporide (IC_{50} value of 0.96 nM). Although NHE inhibitors, such as S3226 (Schwark et al., 1998), SL 59.1227 (Lorrain et al., 2000), TY-12533 (Aihara et al., 2000), and SM-20550 (Yamamoto et al., 2000) have been developed, the reported NHE1 IC_{50} values of these compounds are 3.6 μM (S3226), 3.3 nM (SL 59.1227), 17 nM (TY-12533), and 10 nM (SM-20550). T-162559 is a more potent NHE1 inhibitor when compared to these compounds. This potent activity was observed not only *in vitro*, but also *in vivo*. In a rat model of MI induced by coronary occlusion and reperfusion, intravenous administration of T-162559 resulted in a greater degree of reduction in infarct size, than seen for cariporide and eniporide (Kusumoto et al., 2002). The inhibitory effect of the R enantiomer T-162561 on hNHE1 was 36-fold less potent than that of the S enantiomer, T-162559, whereas the IC_{50} value of the R enantiomer for hNHE2 was similar to that of the S enantiomer. This result suggests that the configuration of the asymmetric carbon of T-162559 is important for tight binding to hNHE1. Previously reported NHE inhibitors, including amiloride, cariporide, and eniporide, contain the same basic acylguanidine moiety. The aminoguanidine derivative T-162559 is a potent non-acylguanidine class of NHE1 inhibitor with cardioprotective activity. The activity of T-162559 based on its unique structure may contribute to the

efficacy noted under prolonged ischemia-reperfusion insult.

NHE1-expressing cells recovered rapidly from cellular acidosis when compared to hNHE2- and hNHE3-expressing cells. Additionally, NHE1 is the dominant isoform in cardiac tissue and its mRNA expression is elevated in response to cardiac injury (Humphreys et al., 1999, Dyck et al., 1995, Gan et al., 1999, Piper et al., 1996). The sarcolemmal NHE activity of human ventricular myocytes is due to the NHE1 isoform and is inhibited by cariporide (Yokoyama et al., 2000). Sarcolemmal NHE activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in non-diseased donor hearts (Yokoyama et al., 2000). Other plasma membrane-expressed human NHE isoforms have tissue-specific expression patterns. Therefore, selective inhibition of the NHE1 isoform may offer a novel specific approach for the treatment of cardiac injury during ischemia-reperfusion. Further evidence supporting the target potential of NHE1 for the treatment of MI was demonstrated by genetic ablation of NHE1 in mice, which protected their hearts from ischemia-reperfusion injury (Wang et al., 2003).

Amiloride and its derivatives are non-specific NHE inhibitors because these compounds have been shown to inhibit Ca^{2+} current, Na^{+} current, K^{+} current, and Na^{+}/Ca^{2+} exchangers (Pierce et al., 1993, Lai et al., 1994). Na^{+}:HCO_3^{-} cotransport and Na^{+}/Ca^{2+} exchange are thought to play important roles in ischemia-reperfusion injury (MacLeod, 1991, Lazdunski et al., 1985, Guarnieri, 1987, Wier, 1990, Schafer et al., 2000). However, T-162559 did not affect Na^{+}:HCO_3^{-} cotransport and Na^{+}/Ca^{2+} exchange, demonstrating that T-162559 is a potent and specific inhibitor of hNHE1.

In conclusion, we demonstrated that the molecular target of T-162559 is the NHE1 isoform. Our findings characterized the therapeutic activity of T-162559 on cardiac

injury in experimental ischemia-reperfusion injury models. Myocardial ischemia induces cellular acidosis by accumulation of lactate in cardiomyocytes. NHE is subsequently activated during ischemia and elevates the intracellular Na^+ concentration by removing H^+ from the cell with a 1 Na^+:1 H^+ coupling stoichiometry. After reperfusion, the extracellular protons are washed away. Hence, the resulting pH gradient across the cell membrane leads to activation of NHE1. As a result, concomitant increases in Ca^{2+} influx occur by reverse mode activation of NCX, which is functionally coupled with NHE1. Ca^{2+} overload then results in irreversible cellular damage by activation of Ca^{2+}-dependent proteases such as calpain and caspases. Furthermore, mitochondrial Ca^{2+} accumulation leads to adverse cellular effects, facilitating mitochondrial membrane permeabilization and energetic collapse (Garcia-Dorado et al., 2012). Therefore, inhibition of Na^+/H^+ exchange by T-162559 protects ischemic cells from Ca^{2+} overload.

NHE1 forms a complex with NCX1 through integrin αIIbβ3 (Yi et al., 2009). This interaction on the plasma membrane enables functional coupling between NHE1 and NCX1. Thus, NHE1 can generate local high intracellular sodium concentrations, which activate the reverse mode of NCX1, driving calcium influx. The ischemic condition leads to decreased intracellular concentrations of ATP, resulting in a decrease in ATP-dependent ion transport systems, including the Na^+/K^+-ATPase pump that transports K^+ into the cell and Na^+ out of the cell. This causes cell membrane depolarization due to loss of hyperpolarizing currents (Ikenouchi et al., 1993). Thus, the reverse mode of NCX is activated under conditions such as intracellular Na^+ accumulation via NHE1 and membrane depolarization during ischemia-reperfusion injury. The observed therapeutic effects of T-1625592 in cardiac injury models during

ischemia-reperfusion are believed to be due to suppression of NHE1 in the ischemic heart. Previous studies using animals models of MI indicate that NHE1 is involved in the elevation of intracellular Na^+ and Ca^{2+} during prolonged myocardial ischemia. In fact, cariporide has been reported to suppress intracellular Ca^{2+} transients in an ischemia-reperfusion rat heart model (Strömer et al., 2000). Pharmacological studies with an NCX inhibitor (Takahashi et al., 2003), a calpain inhibitor (Cai et al., 2012) and a mitochondrial permeability transition pore (mPTP) inhibitor, Cyclosporin A (Hausenloy et al., 2012) demonstrated its cardioprotective effects on ischemia-reperfusion injury. These results support our hypothesis regarding the molecular mechanism of action of T-162559.

Clinical trials with NHE1 inhibitors have been carried out previously, but did not show sufficient clinical benefit despite promising preclinical results in animal models (Karmazyn et al., 2013). To achieve a favorable outcome in humans, translational studies are required to understand the molecular pathogenesis of MI patients. T-162559 is a significantly more potent NHE1 inhibitor than other NHE1 inhibitors, and may have sufficient potency to be efficacious in the clinic. Since T-162559 is a selective inhibitor of human NHE1, further research with this compound should be carried out to evaluate its therapeutic potential, possibly in combination with other approved therapies.

Recent studies suggest that NHE1 is also involved in cancer progression (Amith et al., 2013). Extracellular acidification around tumor cells can induce apoptosis of nearby normal cells, resulting in selective survival of cancer cells. NHE1 causes a reversal of the pH gradient in many types of transformed and/or malignant cells and enhances survival of cancer cells. Therefore, NHE1 inhibitors may be potential therapies for the treatment of cancer. Hence, T-162559 would be a drug for the treatment of not only

ischemia-reperfusion injury and other cardiac diseases, but also other NHE1-mediated diseases.

5. Tables

Table 1-1. Plasma membrane NHE isoforms

Protein	*Gene*	Tissue distribution	Amino acid sequence identities with NHE1 (%)
NHE1	*SLC9A1*	Ubiquitous (incl. heart and platelets)	100
NHE2	*SLC9A2*	Stomach, intestinal tract, skeletal muscle, kidney, brain, uterus, testis, heart, lung	49
NHE3	*SLC9A3*	Intestinal tract, stomach, kidney, gall bladder, epididymis, brain	36
NHE4	*SLC9A4*	Stomach, Kidney, brain	43
NHE5	*SLC9A5*	Brain	40

(Revised from Fuster et al., 2014)

Table 1-2. Inhibitory activities of small molecule NHE inhibitors against human NHE isoforms

	IC_{50} values (nM)		
Compound	hNHE1	hNHE2	hNHE3
T-162559 *S* (-)	0.96	430	11000
T-162561 *R* (+)	35	310	>30000
Cariporide	30	4300	>100000
Eniporide	4.5	2000	>100000

6. **Figures**

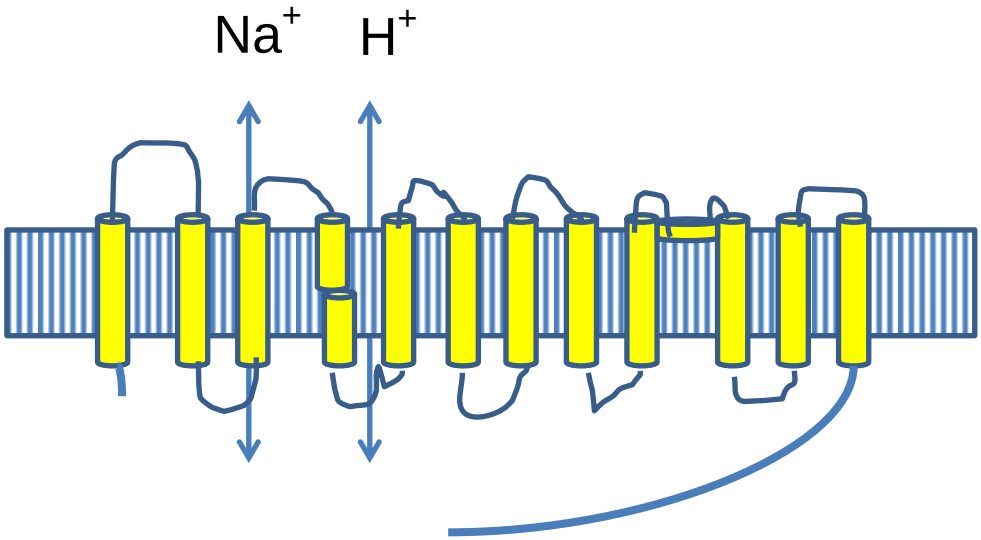
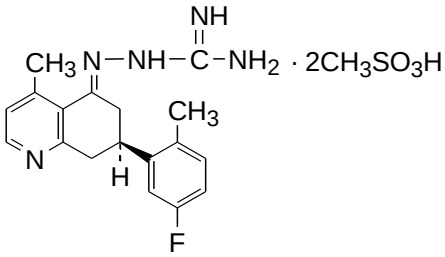
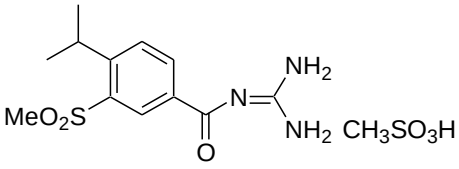


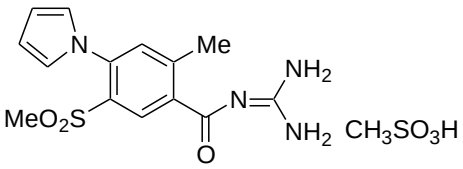
Figure 1-1. Structure and function of NHE (Revised from Pullikuth et al., 2003)



T-162559



Cariporide



Eniporide

Figure 1-2. Chemical structures of T-162559, cariporide, and eniporide.

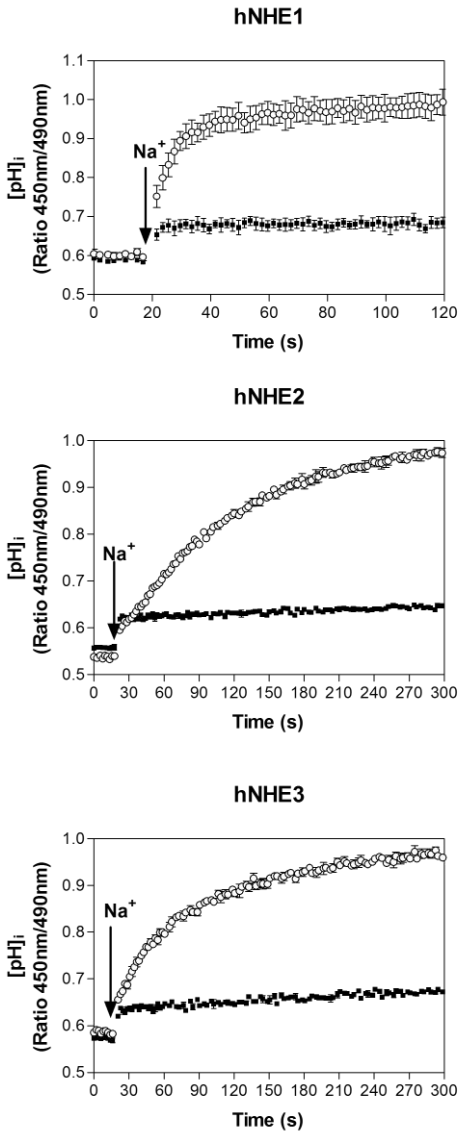


Fig. 1-3. Na^+-dependent recovery of the intracellular pH in cells stably expressing human NHE isoforms.

NHE-deficient CHO-K1 cells were transfected with human NHE isoform expression plasmids and stable transformants were isolated. Cells were loaded with BCECF-AM and then acidified by NH_4Cl prepulse. Recovery of pHi was initiated by the addition of external Na^+ at the time indicated by the arrow. The results shown are for cells expressing hNHE isoforms (○) and non-transfected cells (■). Each data point represents the mean±S.E.(n=3).

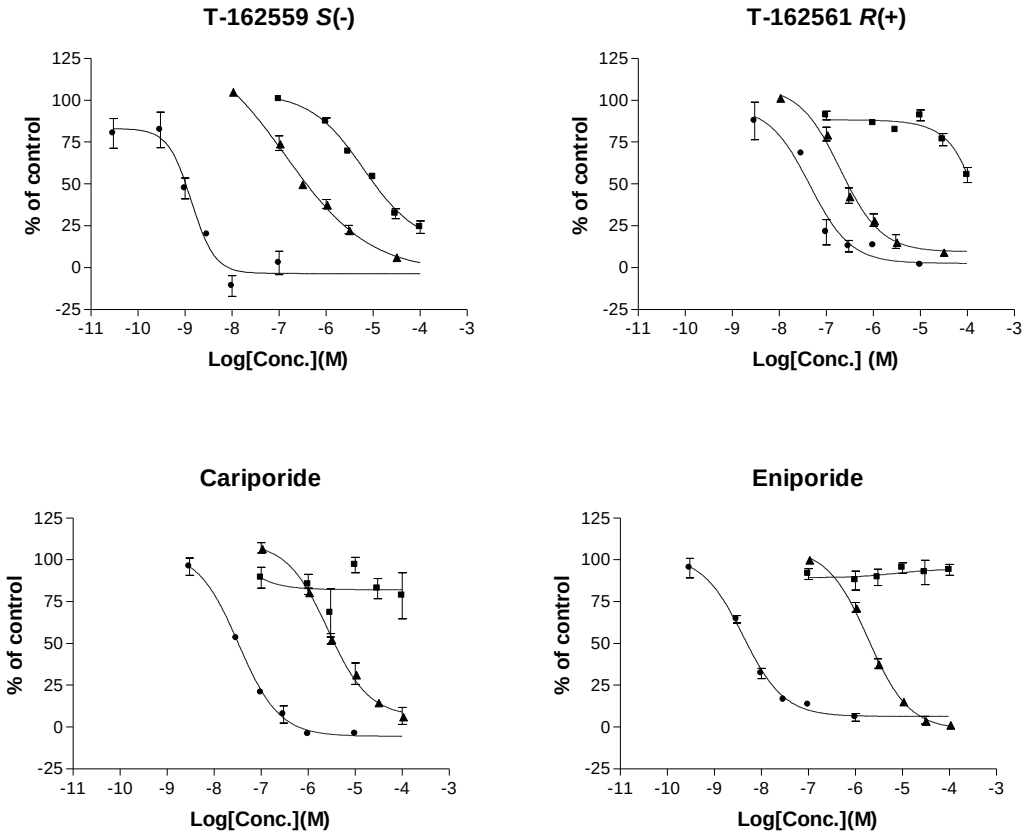


Fig. 1-4. Dose-response curves for inhibition of hNHE1, hNHE2, and hNHE3 by NHE inhibitors.

NHE-deficient CHO-K1 cells expressing hNHE isoforms were loaded with BCECF-AM and then acidified by NH_4Cl prepulse, followed by treatment with increasing concentrations of test compounds for 5 min. Initial velocity of pHi recovery was calculated in the initial data points (10 s for NHE1, 40 s for NHE2 and NHE3). An inhibitory effect of NHE inhibitor was evaluated by a reduction of the velocity of pHi recovery in the presence of test compounds. The results are expressed as percentages of the initial velocity of pHi recovery in cells expressing the human NHE isoforms, hNHE1 (●), hNHE2 (▲), and hNHE3 (■). All data are means±S.E.(n=3).

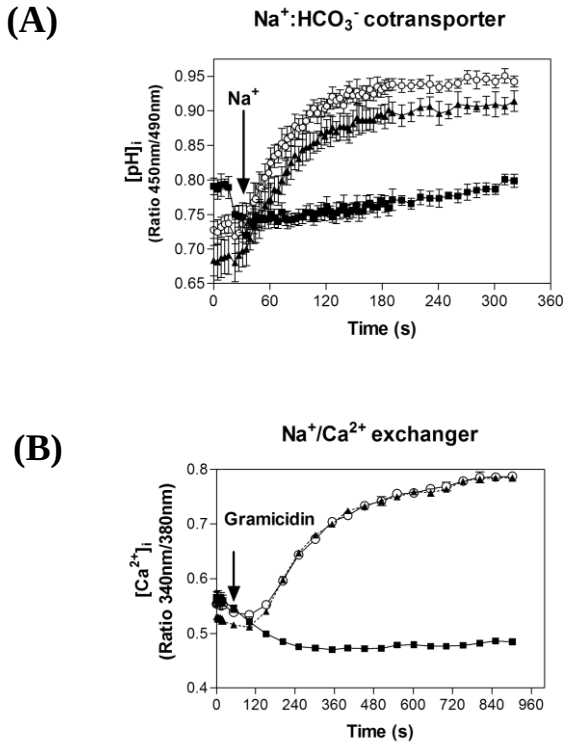


Fig.1-5. Effects of T-162559 on human Na^+:HCO_3^- cotransporter (A) and human cardiac Na^+/Ca^{2+} exchanger (B).

(A) NHE-deficient CHO-K1 cells were transfected with hNBC1 expression plasmid and a stable transformant was isolated. Cells were loaded with BCECF-AM and acidified by NH_4Cl prepulse, followed by incubation with 100 μM T-162559 or DMSO for 5min. Recovery of pHi was initiated by the addition of external Na^+ at the time indicated by the arrow in the presence of HCO_3^-. The results shown are for cells expressing hNBC1 in the presence (○) or absence (▲) of 100 μM T-162559 in DMSO, and for non-transfected cells (■). (B) CHO-K1 cells were transfected with hNCX1 expression plasmid and a stable transformant was isolated. Cells were loaded with FuraPE3-AM, followed by incubation with 100 μM T-162559 or DMSO for 5 min. The reverse-mode of Na^+/Ca^{2+} exchange was activated by the addition of gramicidin at the time indicated by the arrow and the increase in intracellular Ca^{2+} was monitored for 15min. The results shown are for cells expressing hNCX1 in the presence (○) or absence (▲) of 100 μM T-162559 in DMSO, and for non-transfected cells (■). Each data point represents the mean ± S.E. (n=3).

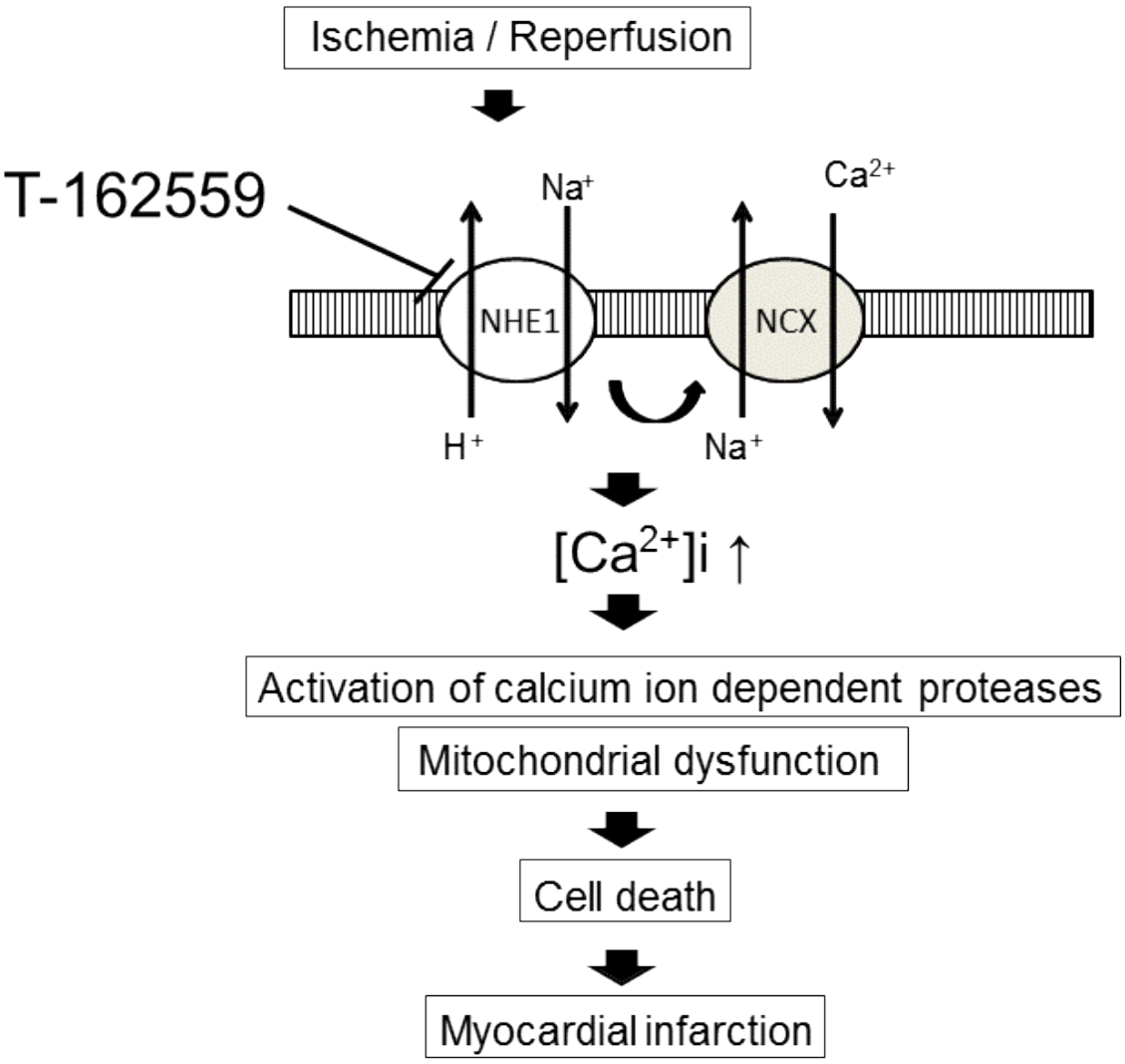


Fig.1-6. Working hypothesis of therapeutic action of T-162559

Chapter II: Molecular Mechanism of Action of TAK-242

1. Introduction

Sepsis occurs when the host mounts a potent immune response against a life-threatening infection. Frequently, the immune response itself causes greater injury to the host than the actual infectious agent. Typically, this excessive immune reaction is triggered by bacterial infections; however, fungi, viruses, and parasites in the blood, urinary tract, lungs, or other tissues can also cause sepsis. Common symptoms of sepsis include fever, rapid heart rate, rapid breathing, flushed skin, hyperventilation, altered mental status, edema, hypotension, and shock. Severe sepsis is a medical emergency affecting up to 19 million individuals worldwide (Adhikari et al., 2011). Although sepsis is the most frequent cause of mortality in many intensive care units, no effective therapy is available, and the overall mortality rate still ranges from 30% to 50% in patients that go into septic shock. Hence, there is a high unmet medical need for managing this disorder (Martin et al, 2003, Angus et al, 2013). Although the systemic pathogenesis of sepsis is complicated, macrophages are thought to represent the first line of defense against infection in early inflammatory responses. Macrophages release a diverse range of inflammatory mediators, cytokines, chemokines, lipid mediators, and oxygen radicals. One of these mediators, nitric oxide (NO) modifies vascular tone and enhances hypotension (Cohen et al., 2002).

TAK-242 is being developed as a small molecule-based anti-sepsis drug. TAK-242 was identified in a cell-based screening assay that measures lipopolysaccharide (LPS) induced NO and cytokine production using the RAW264.7 mouse macrophage cell line. The initial lead compound, a cyclohexene derivative, was identified in a chemical

library screen and lead optimization was carried out using the RAW264.7 cell-based assay. Among the various leads synthesized, TAK-242 had the greatest inhibitory activity, blocking the production of NO and inflammatory cytokines, such as TNF-α and interleukin-6 (IL-6). Intravenous administration of TAK-242 at doses of 0.1 mg/kg or more suppressed the production of NO and various cytokines, including TNF-α, IL-6, and interleukin-1beta (IL-1β) in a mouse endotoxin shock model. TAK-242 also protected mice from LPS-induced death in a dose-dependent manner and all mice survived at a dose of 3 mg/kg. The minimum effective dose to protect mice from lethality in this model was 0.3 mg/kg, which was consistent with the observed inhibitory effects on the production of NO and cytokines (Yamada et al., 2005, Sha et al., 2007). Thus, TAK-242 is a promising therapeutic agent for the treatment of sepsis. Because TAK-242 was identified using a phenotypic assay, its molecular mechanism of action was unclear.

The discovery of Toll-like receptors (TLRs) as primary sensors of microbial infections has led to significant advances in understanding the mechanisms of innate immunity and their relationship to sepsis. Ten TLR members have been identified in humans (Akira et al., 2006). Among human TLRs, ligands for TLR1-TLR9 have been identified through *in vitro* systems or knockout mice (Fig. 2-1). TLR2 dimerizes with TLR1 or TLR6 and recognizes the peptidoglycans and lipopeptides of gram-positive bacteria (Takeuchi et al., 2002). TLR4 detects gram-negative bacteria through recognition of the lipid A moiety of LPS (Poltorak et al., 1998; Hoshino et al., 1999). LPS recognition is mediated by four molecules, LPS binding protein (LBP), CD14, myeloid differentiation protein-2 (MD2), and TLR4 (Guha et al., 2001). LBP is thought to be a shuttle protein catalyzing LPS transfer from the outer membrane of

gram-negative bacteria to CD14 (Tobias et al., 1995). CD14 plays a role in docking LPS with the TLR4/MD2 complex. MD-2, a glycoprotein essential for the innate response to LPS, binds to LPS and the extracellular domain of TLR4 (Shimazu et al., 1999). TLR 3, 5, 7, and 9 recognize viral double-stranded (ds) and single stranded (ss) RNA (Alexopoulou et al., 2001), bacterial flagellin (Hayashi et al., 2001), viral single-stranded (ss) RNA (Heil et al., 2004; Diebold et al., 2004) and viral and bacterial CpG DNA (Hemmi et al., 2001). TLR10 is an orphan receptor involved in induction of innate immune responses to influenza virus infection (Lee et al., 2014).

The recognition of microbial pathogens and their components by TLRs triggers the activation of intracellular signaling pathways that result in the production of inflammatory mediators such as nitric oxide (NO), prostaglandins, cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, interferon-gamma (IFN-γ), and chemokines. TLRs are type I integral membrane glycoproteins characterized by extracellular domains containing varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R) termed the Toll/IL-1R homology (TIR) domain. After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain adaptor molecules to the TIR domain of the TLR (Akira et al., 2006). There are four adaptor molecules, myeloid differentiation factor 88 (MyD88) (Medzhitov et al., 1998), TIR-associated protein (TIRAP) (Yamamoto et al., 2002a; Fitzgerald et al., 2001), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-domain-containing molecule 1 (TICAM1) (Hoebe et al., 2003; Yamamoto et al., 2003; Oshiumi et al, 2003; Sugiyama et al, 2003), and TRIF-related adaptor molecule (TRAM) (Yamamoto et al., 2003). MyD88 is critical for signaling by all TLRs except

TLR3. After stimulation, MyD88 associates with the cytoplasmic domain of TLRs and recruits IL-1 receptor-associated kinase 1 (IRAK-1) and IRAK-4 (Medzhitov et al., 1998; Suzuki et al., 2002; Thomas et al., 1999). These mediate activation of NF-κB, resulting in the induction of inflammatory mediators. TIRAP mediates MyD88-dependent signaling (Yamamoto et al., 2002a; Fitzgerald et al., 2001), and TRIF is required for MyD88-independent signaling and involved in both TLR3 and TLR4 signaling (Yamamoto et al., 2003; Oshiumi et al., 2003). TRIF stimulation results in the activation of NF-κB and IFN regulatory factor 3 (IRF3), and induces the expression of inflammatory cytokines and type I IFN (Yamamoto et al., 2002b). TRAM, which bridges TLR4 and TRIF, is a specific adaptor for TLR4 and transmits its signaling to TRIF (Yamamoto et al., 2003). Thus, TLRs stimulated with microbial components activate intracellular signaling by interacting with adaptors that induce the production of inflammatory mediators.

The initiation of the innate immune response through TLR4 triggers an inflammatory cascade that is the principal cause of the adverse conditions that occur in sepsis. Consequently, TLR4 is a promising therapeutic target for the treatment of sepsis (Opal et al., 2002, Lynn et al., 2004). To clarify the molecular mechanism of action of TAK-242, the effects of TAK-242 on TLR signaling were investigated in this study. It was demonstrated that TAK-242 is a potent and selective inhibitor of TLR4 signaling.

2. Materials and Methods

2.1. Materials

TAK-242 (Ethyl (*6R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate)) was synthesized at Takeda Pharmaceutical Company (Fig. 2-2.). Ultra-pure LPS (from *Escherichia coli* serotype O111:B4) was purchased from Invivogen (SanDiego, CA). Peptidoglycan (from *Staphylococcus aureus*) was from Fluka (Buchs, Switzerland). Phosphorothioate-stabilized CpG oligodeoxynucleotides (ODN) (TCC-ATG-ACG-TTC-CTG-ATG-CT as a ligand for mouse TLR9, TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT as a ligand for human TLR9) were synthesized at Hokkaido System Science (Sapporo, Japan). Flagellin was from Invivogen. Pam_3CSK_4 was from Bachem AG (Bubendorf, Switzerland). Polyinosinic-polycytidylic acid (poly(I:C)) was from Sigma. R-848 was from GLSynthesis Inc. (Worcester, MA). Recombinant mouse IFN-γ was from Genzyme (Minneapollis, MN). Phorbol myristate acetate (PMA) was from Wako (Osaka, Japan).

2.2. Cells

Mouse RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Human U-937 cells were purchased from Dainippon Sumitomo Pharmaceutical (Osaka, Japan). Human P31/FUJ cells were purchased from Japanese Collection of Research Bioresources (Osaka, Japan). These cells were maintained in RPMI-1640 medium supplemented with 10 % inactivated fetal calf serum and 50 μg/ml gentamycin (Invitrogen, USA). Human embryonic kidney (HEK) 293 cells were purchased from Dainippon Sumitomo Pharmaceutical (Osaka, Japan) and maintained in Dulbecco's modified Eagle medium supplemented with 10% inactivated fetal calf serum

and 50 μg/ml gentamycin.

2.3. Plasmids

cDNAs encoding human TLR1, human TLR2, human TLR3, human TLR4, mouse TLR4, human TLR6, human TLR7, human TLR9, human CD14, human MD2, mouse MD2, human MyD88, human TIRAP, human TRIF, and human TRAM were isolated by polymerase chain reaction (PCR) and subcloned into a mammalian expression vector, pcDNA3.1 (Invitrogen, USA). Human TLR5 expression vector, pUNO- human TLR5 was purchased from Invivogen. The transmembrane and the intracellular domain of mouse TLR4 (amino acid residue 623 to 835) or human TLR4 (amino acid residue 632 to 839) were fused to the extracellular domain of mouse CD4 (amino acid residue 1 to 384). The chimera receptors were ligated into a mammalian expression vector pMSRαneo, which contained SRα promoter. All constructions identified by PCR were verified by sequencing.

2.4. Measurement of nitrite

RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells per well. After 24 h culture, cells were incubated with TAK-242 for 1 h, subsequently stimulated with each ligands for TLRs, 1 or 10 ng/ml LPS for TLR4, 10 or 100 ng/ml Pam_3CSK_4 for TLR2/TLR1, 1 or 10 μg/ml Peptidoglycan for TLR2/TLR6, 1 or 10 μg/ml poly(I:C) for TLR3, 1 or 10 μM R-848 for TLR7, or 0.1 or 1 μM CpG ODN for TLR9 in the presence of 0.1 ng/ml mouse IFN-γ for 24 h. The concentrations of nitrite, a stable metabolite of nitric oxide (NO), in culture medium were determined with a fluorescent reagent, 2,3-diaminonaphthalene (Dojindo, Japan). The fluorescence (Excitation 355nm,

Emission 460nm) was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentration (IC_{50} value) of the test compound necessary for 50% inhibition of NO production was calculated using the least-squares linear regression.

2.5. Measurement of TNF-α

RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells per well. After culture for 24 h, cells were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultra-pure LPS for 24 h. U-937 or P31/FUJ cells were seeded in 96-well plates at 4×10^4 cells per well. After culture for 24 h, cells were added 100 nM PMA (Wako, Japan) for differentiation into macrophages. After culture for 48 h, cells were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultra-pure LPS for 24 h. The concentrations of TNF-α in culture medium were determined with human or mouse TNF-α ELISA kits. The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of TNF-α production were calculated using the least-squares linear regression.

2.6.1. Reporter gene assay for ligand-dependent signaling by TLRs

HEK293 cells were seeded in 96-well opaque plates at 2×10^4 cells per well. After culture for 24 h, cells were transiently transfected with 30 ng of TLR expression vectors or empty vector (pcDNA3.1), 10 ng of pNF-κB-luc (Stratagene, USA) and 10 ng of an internal control plasmid phRL-TK (Promega) using FuGENE 6 reagent (Roche, USA). For expression of TLR4/MD2/CD14 complex, cells were transiently transfected with 5 ng of TLR4 expression vector or empty vector, 10 ng of pNF-κB-luc, 10 ng of an internal control plasmid phRL-TK, 12.5 ng of MD-2 and 12.5 ng of CD14 expression

vectors using FuGENE 6 reagent. For expression of TLR1/TLR2 and TLR2/TLR6, cells were transiently transfected with each 15 ng of TLR expression vectors, 10 ng of pNF-κB-luc, 10 ng of phRL-TK using FuGENE 6 reagent. After transfection, cells were cultured for 24 h. Then, cells were incubated in serum-free medium and various concentrations of TAK-242 were added to the wells. After incubation for 30 min, cells were stimulated by each TLR ligands, 1 or 10 ng/ml ultra-pure LPS for TLR4, 10 or 100 ng/ml Pam_3CSK_4 for TLR2/TLR1, 100 or 1000 ng/ml Peptidoglycan for TLR2/TLR6, 1 or 10 μg/ml poly(I:C) for TLR3, 10 or 100 ng/ml flagellin for TLR5, 1 or 10 μM R-848 for TLR7, or 0.1 or 1 μM CpG ODN for TLR9 and then incubated for 4 h. The luciferase activities were measured using Dual-Glo luciferase assay system (Promega). Transfection efficiencies were normalized to luciferase activity derived from *Renilla reniformis*. The luminescence was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of NF-κB activity were calculated using the least-squares linear regression.

2.6.2. Reporter gene assay for ligand-independent signaling by TLR4, CD4-TLR or adaptors

HEK293 cells were seeded in 96-well opaque plates at 2×10^4 cells per well. After culture for 24 h, cells were transiently transfected with 30 ng of TLR4, CD4-TLR4 or adaptors (MyD88, TRIF, TRAM and TIRAP) expression vector or empty vector, 10 ng of pNF-κB-luc and 10 ng of an internal control plasmid phRL-TK per well using FuGENE 6 reagent. After transfection, various concentrations of TAK-242 were added to the wells and further incubated for 24 h. The luciferase activities were measured

using Dual-Glo luciferase assay system. Transfection efficiencies were normalized to luciferase activity derived from *Renilla reniformis*. The luminescence was detected with Arvo 1420 multilabel counter (Perkinelmer, USA). The concentrations (IC_{50} values) of the test compound necessary for 50% inhibition of NF-κB activity were calculated using the least-squares linear regression.

2.7. Western blot analysis

Transfected cells were harvested in cell lysis buffer (Cell signaling technology, Inc, USA). After incubation at 4 °C for 15 min, the lysate was mixed with an equal volume of a sample buffer (Daiichi Pure Chemicals Co., Ltd., Japan) and heated at 60 °C for 5 min. Proteins were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels with Tris-glycine running buffer and electrically transferred onto Clear Blot Membrane P (Atto, Japan), which was followed by incubating with 5% (w/v) bovine serum albumin in TBS (tris buffered saline)/Tween20. After incubation at 4 °C overnight, the membrane was washed and then incubated at room temperature with anti-CD4 (Santa Cruz biotechnology, Inc. USA). After incubation for 1 h, the membrane was incubated at room temperature with horseradish peroxidase-conjugated secondary antibody for 1 h and then visualized by using ECL Plus western blotting detection reagents (GE Healthcare, USA). Horseradish peroxidase-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz biotechnology, Inc.) was used as an internal control.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Differences

between means were analyzed using t-test. IC_{50} values for TAK-242 were determined as the concentration showing 50% of control using the least-squares linear regression. All calculations were carried out with the SAS system.

3. Results

3.1. Selectivity of TAK-242 against TLR ligands in mouse RAW264.7cells

To evaluate if the prevention of LPS-induced inflammation by TAK-242 is caused by inhibition of TLR4 signaling, we examined the effects of TAK-242 on signaling mediated by ultra-pure LPS as a TLR4-specific ligand in this study. The selectivity of TAK-242 against TLR ligands was determined by measuring NO production from mouse RAW264.7 cells. The induction of NO production was studied with various TLR ligands, including ultra-pure LPS (TLR4-specific ligand), Pam_3CSK_4 (TLR2/1 ligand), peptidoglycan (TLR2/6 ligand), poly(I:C) (TLR3 ligand), R-848 (TLR7 ligand), and CpG DNA (TLR9 ligand) with 0.1 ng/ml IFN-γ. TAK-242 selectively inhibited ultra-pure LPS-induced NO production (Fig. 2-3A). In contrast, TAK-242 showed little effect on NO production induced by TLR2/1, TLR2/6, TLR3, TLR7 or TLR9 ligands at doses as high as 1 μM (Fig. 2-3B-F). As shown in Fig. 2-3G, TAK-242 potently inhibited NO production from RAW264.7 cells stimulated with 10 ng/ml ultra-pure LPS in a dose-dependent manner with an IC_{50} value of 5.5 nM.

3.2. Effect of TAK-242 on TNF-α production from human and mouse macrophages stimulated with a TLR4-specific ligand

To examine the effects of TAK-242 on TNF-α production from human and mouse macrophages stimulated with ultra-pure LPS, the inhibitory activity for TAK-242 was evaluated using human U-937, human P31/FUJ, or mouse RAW264.7 cells. The concentration of TNF-α released from LPS-stimulated (10 ng/ml) U-937, P31/FUJ, and RAW264.7 cells was 7.0 ng/ml, 3.5 ng/ml, and 3.0 ng/ml, respectively (data not shown). In human U-937 and P31/FUJ cells, TAK-242 inhibited LPS-mediated TNF-α production in a dose-dependent manner, with IC_{50} values of 52 nM and 37 nM,

respectively (Fig. 2-4). In mouse RAW264.7 cells, the IC_{50} value for inhibition of TNF-α production by TAK-242 treatment was 4.8 nM. TAK-242 showed potent inhibition of inflammatory cytokine production from both mouse and human macrophages stimulated with a TLR4-specific ligand, although the inhibitory activity in the mouse macrophage cell line was 7.7 to 11-fold greater than observed in human cell lines.

3.3. Selectivity of TAK-242 against recombinant TLRs

To confirm the results obtained in macrophages, we constructed an NF-κB reporter gene assay system with HEK293 cells transiently expressing the following recombinant TLRs, TLR2/l, TLR2/6, TLR3, TLR4, TLR5, TLR7 or TLR9. The expressed TLRs activated NF-κB by the addition of their cognate ligands (Fig. 2-5A-H). To detect the activation of TLR4 by LPS, its co-receptors, CD14 and MD2, were co-expressed with TLR4. TAK-242 (1 μM) inhibited NF-κB activation induced by LPS in HEK293 cells expressing human TLR4, human MD2 and human CD14 (Fig. 2-5A) and in cells expressing mouse TLR4, mouse MD2 and human CD14 (Fig. 2-5B). In the absence of co-expression of CD14 and MD2, TLR4 was not activated by LPS (data not shown). In contrast, TAK-242 did not inhibit other TLRs such as TLR2/l, TLR2/6, TLR3, TLR5, TLR7, or TLR9 at doses as high as 1 μM (Fig. 2-5C-H). The IC_{50} values of LPS-induced NF-κB activation for TAK-242 in HEK293 cells expressing human TLR4, human MD2, and human CD14, or mouse TLR4, mouse MD2, and human CD14 were 110 nM and 15 nM, respectively (Fig. 2-5I). This result is consistent with the observation that TAK-242 had a greater inhibitory effect on mediator production in LPS-stimulated mouse cells than human cells.

3.4. Effect of TAK-242 on ligand-independent signaling of TLR4

TLR4 has been shown to activate ligand-independent signaling (Lee et al, 2004). As shown in Fig. 2-6A, both human and mouse TLR4 showed constitutive activation of NF-κB in the absence of MD2 and CD14. The effect of TAK-242 on ligand-independent TLR4 signaling was examined with this assay system. TAK-242 (1 μM) inhibited ligand-independent activation of NF-κB mediated by over-expression of human or mouse TLR4. This result indicated that TAK-242 does not affect the function of MD2 or CD14. The IC_{50} values of ligand-independent NF-κB activation by human TLR4 or mouse TLR4 for TAK-242 were 310 nM and 35 nM, respectively (Fig. 2-6B). Thus, TAK-242 potently suppressed ligand-independent signaling by mouse and human TLR4.

3.5. Effect of TAK-242 on NF-κB activation mediated by adaptors for TLR4.

After ligand binding, intracellular signaling of TLR4 is mediated by adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM (Akira et al., 2006). Expression of these adaptors in cells results in the activation of their downstream signaling cascades. To examine effects of TAK-242 on MyD88, TIRAP, TRIF, and TRAM, signaling activation by transient expression of adaptors for TLR4 in HEK293 cells in the presence of TAK-242 was measured using the NF-κB reporter gene assay. Transfection of expression plasmids for MyD88, TIRAP, TRIF, or TRAM into HEK293 cells led to marked activation of NF-κB compared to the empty vector control. However, TAK-242 did not affect signaling from those adaptors (Fig. 2-7).

3.6. Effect of TAK-242 on NF-κB activation mediated by a CD4-TLR4 chimera

receptor

To determine the region of TLR4 that interacts with TAK-242, the effect of TAK-242 on a CD4-TLR4 chimera receptor comprising the extracellular domain of CD4 and the intracellular domain of TLR4 was examined with the NF-κB reporter gene assay. The chimeric CD4-human TLR4 or CD4-mouse TLR4 receptors activated NF-κB in transiently expressed HEK293 cells (Fig. 2-8A). NF-κB activation mediated by CD4-human TLR4 or CD4-mouse TLR4 was inhibited by TAK-242 in a dose-dependent manner. The TAK-242 IC_{50} values for the CD4-human TLR4 and CD4-mouse TLR4 chimeras were 240 nM and 62 nM, respectively (Fig. 2-8B). To examine whether TAK-242 affected expression of chimera receptors in transfected HEK293 cells, western blot analysis was performed with antibodies that recognize the extracellular domain of CD4. As shown in Fig.2-8C, TAK-242 did not affect the expression levels of CD4-human TLR4 or CD4-mouse TLR4 at 1 μM. Thus, TAK-242 showed potent inhibition of signaling mediated by the intracellular domain of TLR4.

4. Discussion

The pathogenesis of sepsis involves a progressive expansion of the systemic inflammatory response of the host to a microbial infection (Glauser, 2000). A major factor contributing to sepsis is the shedding of LPS from the cell wall of gram-negative bacteria into the circulation. LPS interacts with TLR4 expressed on a variety of cell types and induces the overproduction of inflammatory cytokines. Systemic release of these cytokines induces vascular permeability and disseminated intravascular coagulation, which can trigger shock (Annane et al., 2005). TAK-242 is a promising drug candidate for the suppression of excessive inflammatory responses in sepsis by selective inhibition of TLR4 signaling. Most LPS preparations on the market are contaminated with other bacterial components, such as lipoproteins, thus activating TLR2 signaling as well as TLR4 signaling. To clarify the effect of TAK-242 on TLR4 signaling, we used ultra-pure LPS, which was extracted by enzymatic hydrolysis and purified by phenol re-extraction (Hirschfeld et al., 2000), thus generating a ligand that selectively activates the TLR4 pathway and expression system. Our findings show that TAK-242 is a selective inhibitor of TLR4 signaling, which potentiated its efficacy against LPS-induced inflammation.

LPS induces the dimerization of TLR4, and this can trigger ligand-independent activation of the receptor. Receptor dimerization is required to activate downstream signaling pathways (Lee et al., 2004). In this study, we demonstrated that TAK-242 suppresses ligand-dependent and -independent signaling by TLR4. These results were consistent with our previous study showing that TAK-242 does not inhibit interactions between LPS and its receptor in macrophages (Ii et al., 2006). Additionally, results from the NF-κB reporter gene assay indicated that TLR4 has more constitutive activity than

other TLRs. Expression of TLR4 is induced by treatment with various stimulants such as IFN-γ (Faure et al., 2001), IL-10 (Petit-Bertron et al., 2003), IL-2 (Mita et al., 2002), or oxidized low-density lipoprotein (Xu et al., 2001). Additionally, TLR4 is expressed at high levels in atherosclerosis (Edfeldt et al., 2002) and inflammatory bowel disease (IBD) (Cario et al., 2000). Over-expression of TLR4 has been found to activate NF-κB in the absence of TLR4 ligands (Fitzgerald et al., 2001). Thus, individuals who exhibit enhanced TLR4 expression may be more susceptible to chronic inflammatory conditions, and TAK-242 may have potential as a therapy against various types of TLR4-mediated chronic inflammation disorders.

After ligand binding, TLR4 recruits TIR-domain adaptor molecules MyD88, TIRAP, TRIF, and TRAM to the TIR domain of TLR4 (Akira et al., 2006). Although TAK-242 inhibited the production of various mediators from LPS-stimulated macrophages and TLR4-mediated NF-κB activation, it did not affect signaling mediated by the over-expression of these TLR4 adaptors, suggesting that TAK-242 targets molecules upstream of the adaptors, including TLR4 itself.

TLR signaling is conferred by the TIR domain. Signaling mediated by CD4-TLR4 TIR domain chimeric receptors was inhibited by TAK-242. These results suggest that TAK-242 inhibits TLR4 signaling by targeting the intracellular domain of TLR4 and not the extracellular domain. TAK-242 also showed greater inhibition of mediator production from LPS-stimulated mouse macrophages than human macrophages. This species difference in the inhibitory activity for TAK-242 is consistent with reporter gene assays using recombinant TLR4, and our previous study with primary cultured cells (Ii, et al., 2006). The inhibitory activity for TAK-242 was changed by simple replacement of the TIR for TLR4 under the same cellular conditions, whereas the induction of

NF-κB activity by mouse TLR4 was at same level as seen with human TLR4. This result raises the possibility that TAK-242 targets TIR of TLR4. Although the amino acid sequence of TIR between human and mouse TLR4 is conserved, the affinity of TAK-242 for TLR4 may be affected by a subtle difference in the amino acid sequences of TIR. This proposed mechanism was confirmed by analysis showing that TAK-242 covalently binds to Cys747 in the intracellular domain of TLR4 (Takashima et al., 2009) and disrupts the interaction of TLR4 with adaptor molecules (Matsunaga et al., 2011).

A TLR4 antagonist, E5531, preferentially blocks LPS interaction with TLR4-MD-2, but has a weak effect on blocking the interaction of LPS with CD14 (Akashi et al., 2003). TAK-242, which can also inhibit TLR4-mediated signaling in the absence of MD2 and CD14, should have more potential to suppress inflammation induced by various TLR4 ligands than other TLR4 antagonists. Sepsis is a leading killer in the noncoronary intensive care unit, and remains worldwide health concern (Martin et al., 2003, Strehlow et al., 2006, Angus et al, 2013). These data suggest that TAK-242 could be a novel therapy for the treatment of sepsis.

In conclusion, we demonstrated that TAK-242 selectively suppresses TLR4-signaling by targeting the intracellular domain of TLR4. The hypothesis for the mechanism of action of TAK-242 as a treatment for sepsis is described in Fig. 2-9. Although ligands from bacteria such as LPS are recognized by the TLR4/CD14/MD2 complex, which is expressed on immune cells such as macrophages, TAK-242 suppresses the initiation of signal transduction by targeting the intracellular domain of TLR4. The intracellular domain of TLR4 plays an important role in signaling via adaptors, MyD88, TIRAP, TRIF, and TRAM. Indeed, a single-point mutation in the TIR domain of mouse TLR4 (Pro712His, the Lps(d) mutation) abolishes the host immune response to LPS (Xu et al.,

2000). TAK-242 blocks the activation of NF-κB, which is a master regulator of inflammation, mediated by the TIR domain of TLR4. This inhibitory action results in the suppression of inflammatory mediators such as NO, cytokines such as TNFα, and prostaglandins. These data show that the therapeutic effect of TAK-242 observed in LPS-induced sepsis models is due to specific suppression of TLR4-mediated signaling.

TLR4 signaling pathways are related to human diseases involving innate immunity, adaptive immunity, and both innate and adaptive immunity (Cook et al, 2004). TLR4 has been shown to respond to various endogenous ligands such as heat-shock proteins (HSP60, HSP70, gp96, and HSP22), extracellular matrix degradation products (Biglycan, hyaluronan, fibronectin extradomain A, and surfactant protein-A), high-mobility group box 1 (HMGB-1) protein, oxidized low-density lipoprotein (OxLDL), β-defensin (Miyake, 2007), and saturated free fatty acids (Lee et al., 2001). The interaction of TLR4 with endogenous ligands is involved in the development and progression of various human diseases, including inflammation such as arthritis and asthma, atherosclerosis, diabetes, and MI (Cook et al., 2004, Shi et al., 2006, Edfeldt et al., 2004). Pharmacological manipulation of TLR4 signaling is thought to have great therapeutic potential for a variety of disorders. TAK-242 has been reported to show a therapeutic effect in diabetes, acute chest syndrome, and scleroderma by other groups (Kuno et al., 2009, Ghosh et al., 2013, Bhattacharyya et al., 2014.), and TAK-242 represents a novel therapeutic approach for the treatment of TLR4-mediated diseases.

5. Figures

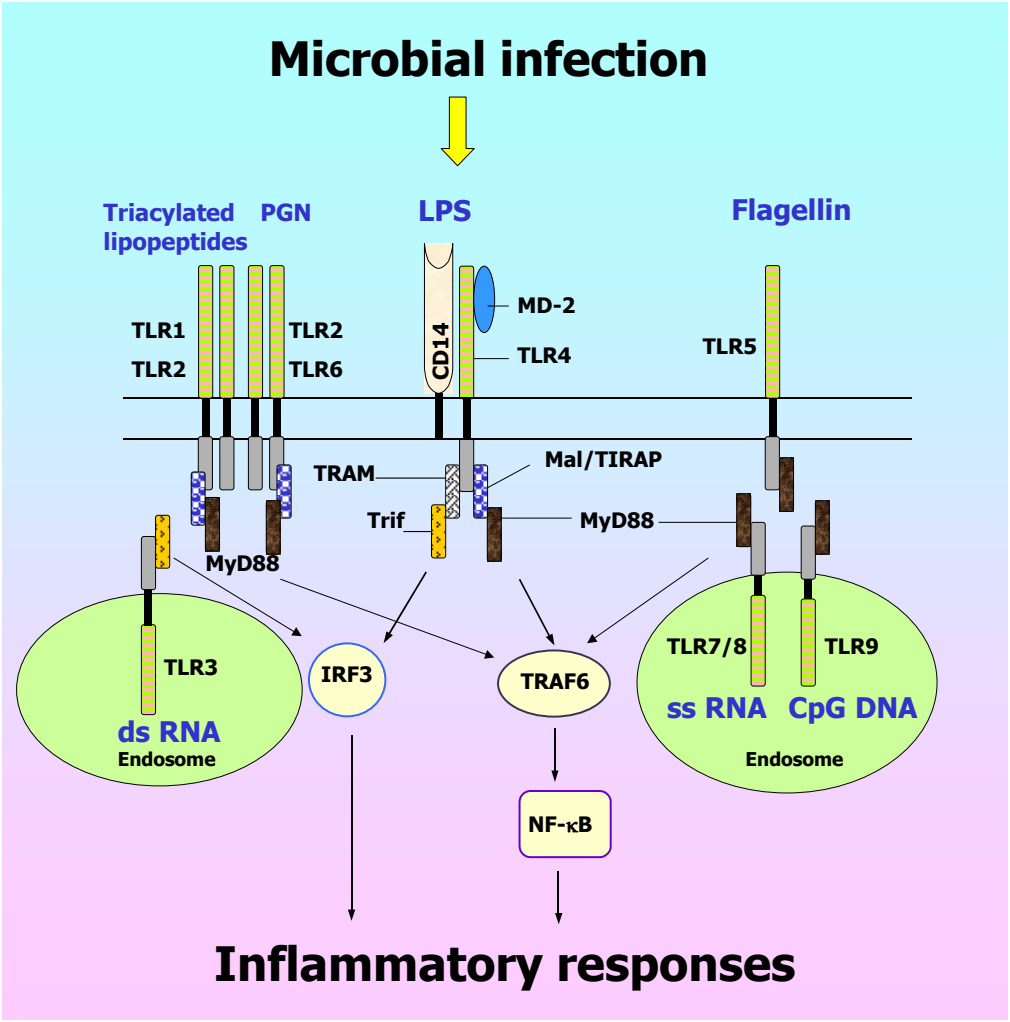


Figure 2-1. TLRs and their signaling pathways

(Revised from Trinchieri G et al., 2007)

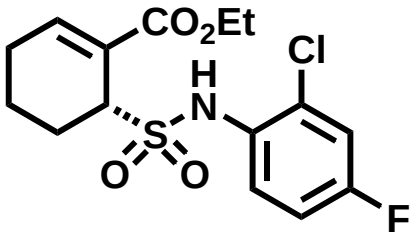


Fig. 2-2. Chemical structure of TAK-242

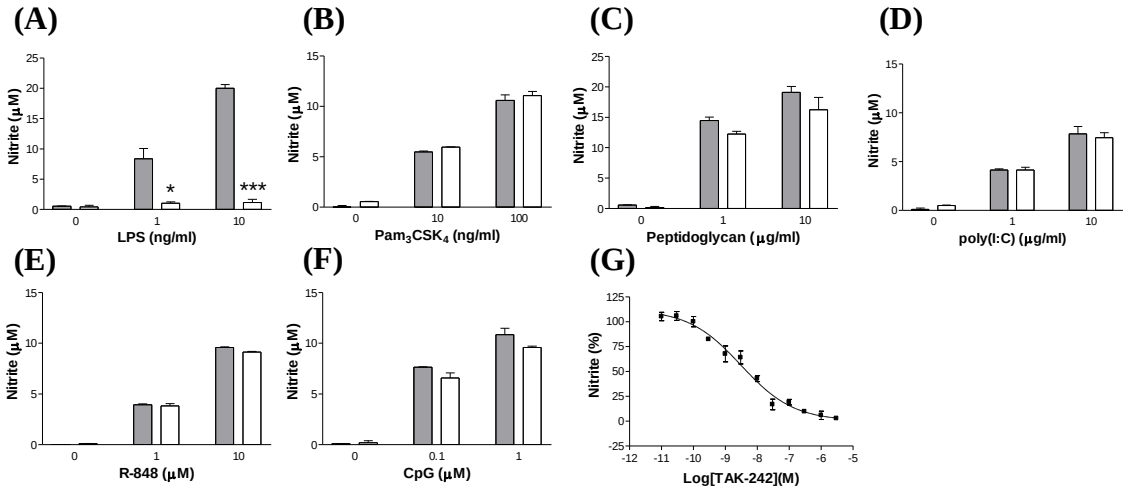


Fig. 2-3. Effect of TAK-242 on TLR ligands-induced nitric oxide production from mouse RAW264.7 cells

Mouse RAW264.7 cells were stimulated by various TLR ligands, LPS (TLR4 ligand) (A) Pam_3CSK_4 (TLR2/1 ligand) (B) peptidoglycan (TLR2/6 ligand) (C) poly(I:C) (TLR3 ligand) (D) R-848 (TLR7 ligand) (E) or CpG ODN (TLR9 ligand) (F) in the absence or presence of 1 μM TAK-242 (gray bars: vehicle, white bars: 1μM TAK-242) with 0.1 ng/ml mouse IFN-γ for 24 h. After incubation, the concentrations of released NO in the medium were measured with 2,3-diaminonaphthalene. (G) Dose-dependent inhibition of NO production by TAK-242 was measured with RAW264.7 cells stimulated with 10 ng/ml ultra-pure LPS. Results are the means ± S.E.M (n=3). *P<0.05 and ***P<0.001 vs. vehicle.

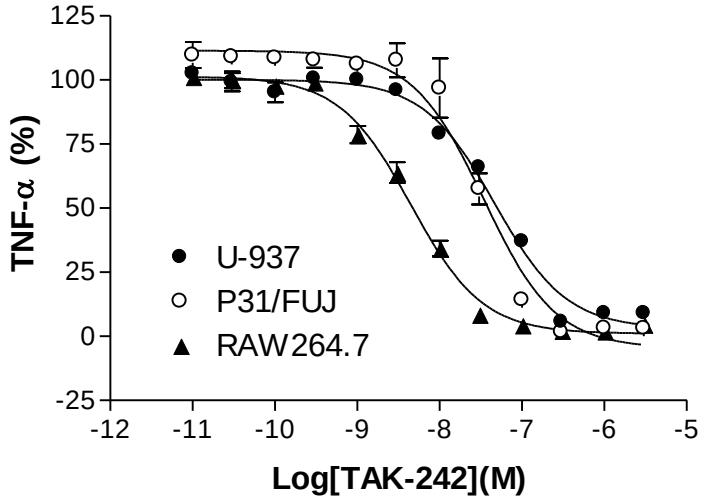


Fig. 2-4. Effect of TAK242 on LPS-induced TNF-α production from human U-937, P31/FUJ cells and mouse RAW264.7 cells

Monocytic cell lines, human U-937 (closed circle), human P31/FUJ cells (opened circle), or mouse RAW264.7 cells (closed triangle) were incubated with TAK-242 for 1 h, subsequently stimulated with 10 ng/ml ultra-pure LPS for 24 h. The concentrations of TNF-α in the culture medium were determined by ELISA. Results are the means ± S.E.M (n=3).

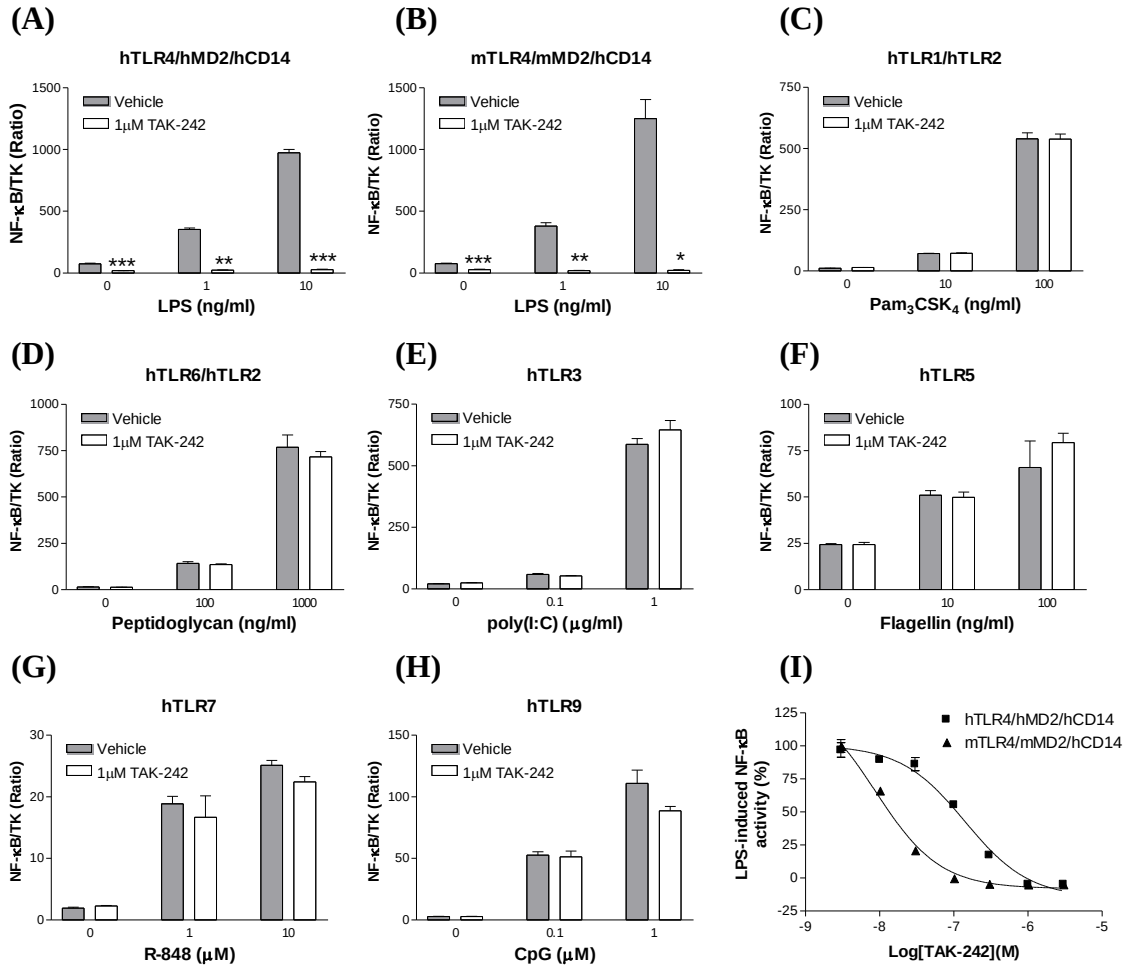


Fig. 2-5. Effect of TAK-242 on NF-κB activation mediated by recombinant TLRs stimulated with their ligands

HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmids for TLRs, and then cultured for 24 h. Cells were incubated with DMSO or 1 μM TAK-242 for 30 min, followed by adding their ligands, in the absence or presence of TAK-242 (gray bars: vehicle, white bars: 1μM TAK-242). After 4 h culture, cells were lysed and the NF-κB reporter activities were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. (I) Dose-dependent inhibition of NF-κB activation stimulated with 10 ng/ml LPS by TAK-242 was examined in HEK293 cells transiently expressing hTLR4/hMD2/hCD14 (closed square) or mTLR4/mMD2/hCD14 (closed triangle). Results are the means ± S.E.M (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle.

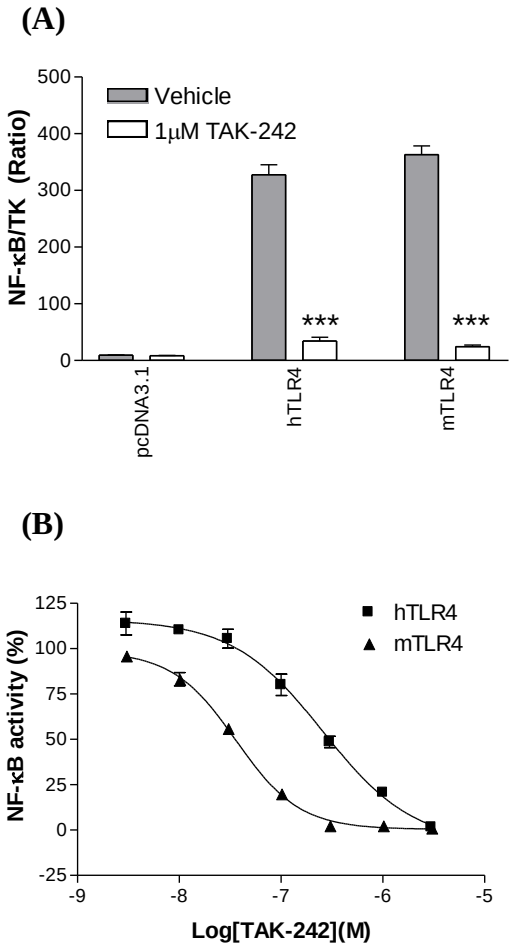


Fig. 2-6. Effect of TAK-242 on ligand-independent signaling of TLR4

(A) HEK293 cells were transiently co-transfected with an NF−κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmid for human TLR4 or mouse TLR4, or the empty plasmid pcDNA3.1 and further cultured for 24 h. (B) After transfection, cells were cultured in the presence of various concentrations of TAK-242. After 24 h culture, cells were lysed and the NF-κB reporter activities induced by human TLR4 (closed square) or mouse TLR4 (closed triangle) were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. Results are the means ± S.E.M (n=3). ***P<0.001 vs. vehicle.

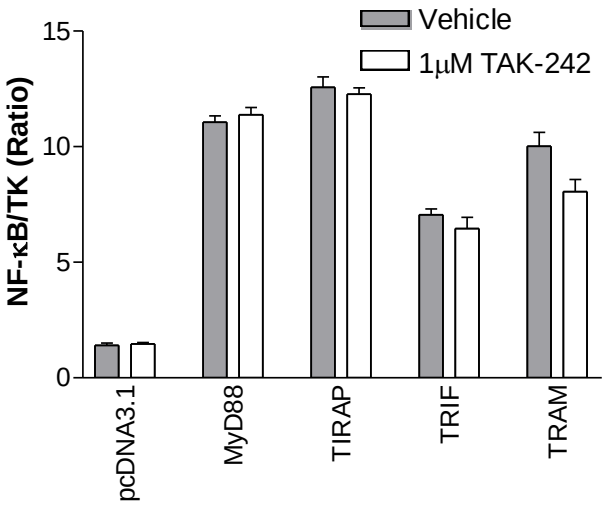


Fig. 2-7. Effect of TAK-242 on NF-κB activation mediated by adaptors for TLR4
HEK293 cells were transiently co-transfected with an NF−κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmids for adaptors, human MyD88, human TIRAP, human TRIF or human TRAM, or empty vector. After transfection, cells were cultured in the absence or presence of TAK-242 (gray bars: vehicle, white bars: 1μM TAK-242) for 24 h. After culture, the NF-κB reporter activities were measured using Dual-Glo luciferase assay system. Promoter activities were normalized to Renilla luciferase activities. Results are the means ± S.E.M (n=3).

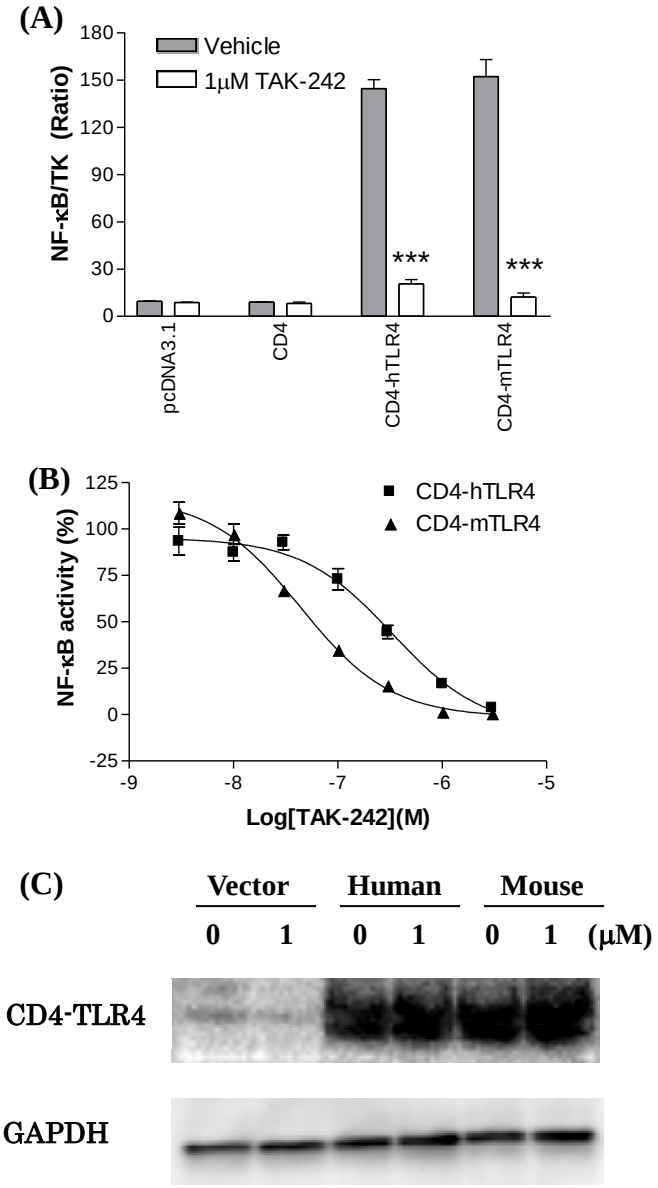


Fig. 2-8. Effect of TAK-242 on ligand-independent signaling by a CD4-TLR4 chimera receptor

(A) HEK293 cells were transiently co-transfected with an NF-κB reporter plasmid, an internal control reporter plasmid phRL-TK and the expression plasmid for CD4-human TLR4, CD4-mouse TLR4, or CD4 and further cultured for 24 h. (B) After transfection, cells were cultured in the presence of various concentrations of TAK-242. After 24 h culture, cells were lysed and the NF-κB reporter activities induced by CD4-human TLR4 (closed square) or CD4-mouse TLR4 (closed triangle) were measured using Dual-Glo luciferase assay system. Results are the means ± S.E.M (n=3). ***P<0.0001 vs. vehicle. (C) Expression of CD4-human or mouse TLR4 in transfected HEK293 cells in the absence or presence of 1 μM TAK-242 was analyzed with western blotting.

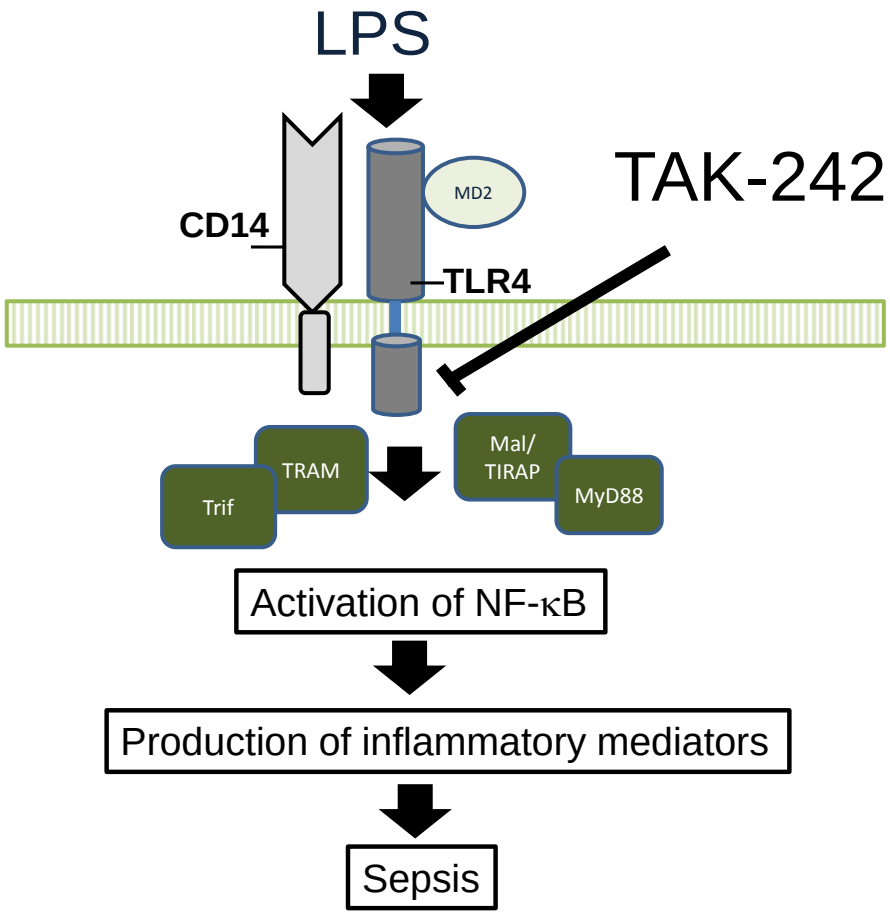


Fig. 2-9. Working hypothesis of therapeutic action of TAK-242

General Conclusion

We have characterized the molecular mechanisms of action of two different drug candidates, which were identified or optimized using phenotypic assays focusing on the development of treatments for myocardial infarction and sepsis. A potential MI drug, T-162559, was generated in a cell-based assay that monitors acidosis-induced platelet swelling as an NHE inhibitor. It was also shown to have a cardioprotective effect in ischemia-reperfusion models (Kusumoto et al., 2002). A potential anti-sepsis drug, TAK-242, was identified in a cell-based assay measuring NO and cytokine production from LPS-treated macrophage cell lines and also showed a therapeutic effect in septic shock models (Sha et al., 2007). Elucidation of the molecular targets and the selectivity profiles of these drug candidates is needed for a better understanding of their efficacy, particularly in relation to clinical applications and potential side effects.

The molecular mechanism of action of T-162559 was described in chapter I. It was found that the mechanism of action of T-162559 for the treatment of MI is by inhibition of NHE1 isoform. The molecular mechanism of action of TAK-242 was described in chapter II. It was shown that the mechanism of action of TAK-242 for the treatment of sepsis was by inhibition of TLR4 at its cytoplasmic domain. The phenotypic efficacy and the selectivity of both compounds were high, suggesting that their molecular targets, NHE1 and TLR4, are centrally responsible for each observed phenotype. By clarifying the molecular mechanisms of actions of T-162559 and TAK-242, the results have identified druggable targets relevant to the development and pathology of each disease.

Phenotypic screening strategies offer ways to identify compounds or targets that modulate the key characteristics of specific diseases. Advanced technologies, such as

chemical proteomics and label-free methods, have been developed for target deconvolution of bioactive compounds (Futamura et al., 2013, Salcius et al., 2014). However, elucidation of the molecular mechanism of a bioactive compound and the target is a challenging issue. Lee et al. suggests that this problem is largely due to the fact that most small molecules do not bind to a single target (Lee et al., 2013). In this study, elucidation of the molecular mechanism for each compound was achieved by applying molecular biological methods based on an understanding of the molecular mechanisms of the phenotype and characteristics of the target cells. This was also facilitated by the generation of selective compounds with potent activity by medicinal chemistry based on data from the phenotypic assays. The generation of potent compounds, including covalent binders like TAK-242, can contribute to an increased success rate in robust target identification.

With regard to T-162559, previous pharmacological and chemical information enabled an informed prediction of the main target protein. Since T-162559 showed more potent and selective inhibition of the NHE1 than caniporide and eniporide, this compound could offer a superior therapeutic approach for the treatment of MI and other NHE1-mediated diseases. In contrast, TLR4-selective inhibitors that act via the intracellular domain, like TAK-242, have not been reported yet. In general, one-transmembrane receptors, including TLRs, are not accepted as chemical therapeutic targets due to low druggability. However, discovery of TAK-242 was achieved because a phenotypic screening approach was used, which enabled comprehensive screening of drugs targeting molecules involved in the phenotype, resulting in the discovery of TAK-242. TAK-242 should open new opportunities for TLR-targeted drug discovery.

We believe that improving the productivity of drug and target discovery requires a

more balanced or combined strategy that utilizes both target-oriented and phenotypic approaches. Information on the molecular mechanism of action can facilitate a target-oriented high-throughput screening campaign to identify other chemotypes with more desirable properties and different mechanisms of inhibition. The established methods that were described and used in these studies are applicable for screening of drugs targeting ion transporters or TLRs. Furthermore, the identified chemical probes for NHE1 and TLR4 are useful not only for the development of drugs, but also for better characterization of their biological roles.

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