European Journal of Pharmacology 814 (2017) 1–8

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

A pyridone derivative activates SERCA2a by attenuating the inhibitory effect of phospholamban

Manami Kaneko[a,*], Hisato Yamamoto[b], Hiroki Sakai[b], Yusuke Kamada[c], Toshiki Tanaka[a], Shuji Fujiwara[a], Syunsuke Yamamoto[d], Hiroki Takahagi[a], Hideyuki Igawa[a], Shizuo Kasai[a], Masakuni Noda[a], Makoto Inui[b], Tomoyuki Nishimoto[a]

[a] Cardiovascular and Metabolic Drug Discovery Unit, Research, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan
[b] Department of Pharmacology, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan
[c] Biomolecular Research Laboratories, Research, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan
[d] Drug Metabolism and Pharmacokinetics Research Laboratories, Research, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan

ARTICLE INFO

Keywords:
Sarco/endoplasmic reticulum Ca^{2+}-dependent ATPase 2a
Phospholamban
Ca^{2+} handling
Inotropic effect
Lusitropic effect

ABSTRACT

The cardiac sarco/endoplasmic reticulum Ca^{2+}-dependent ATPase 2a (SERCA2a) plays a central role in Ca^{2+} handling within cardiomyocytes and is negatively regulated by phospholamban (PLN), a sarcoplasmic reticulum (SR) membrane protein. The activation of SERCA2a, which has been reported to improve cardiac dysfunction in heart failure, is a potential therapeutic approach for heart failure. Therefore, we developed a novel small molecule, compound A and characterized it both *in vitro* and *in vivo*. Compound A activated the Ca^{2+}-dependent ATPase activity of cardiac SR vesicles but not that of skeletal muscle SR vesicles that lack PLN. The surface plasmon resonance assay revealed a direct interaction between compound A and PLN, suggesting that the binding of compound A to PLN attenuates its inhibition of SERCA2a, resulting in SERCA2a activation. This was substantiated by inhibition of the compound A-mediated increase in Ca^{2+} levels within the SR of HL-1 cells by thapsigargin, a SERCA inhibitor. Compound A also increased the Ca^{2+} transients and contraction and relaxation of isolated adult rat cardiomyocytes. In isolated perfused rat hearts, the compound A enhanced systolic and diastolic functions. Further, an infusion of compound A (30 mg/kg, *i.v.* bolus followed by 2 mg/kg/min, *i.v.* infusion) significantly enhanced the diastolic function in anesthetized normal rats. These results indicate that compound A is a novel SERCA2a activator, which attenuates PLN inhibition and enhances the systolic and diastolic functions of the heart *in vitro* and *in vivo*. Therefore, compound A might be a novel therapeutic lead for heart failure.

1. Introduction

Heart failure (HF) is the most important cause of mortality in developed countries, despite advances in pharmacotherapy and resynchronization therapy (Cleland et al., 2005). Impaired Ca^{2+} handling within cardiomyocytes, which is one of the most consistent features of HF at cellular level (del Monte et al., 1999), is responsible for contractile dysfunction, remodeling, abnormal electrical activity, and reduced adenosine triphosphate (ATP) production in the failing heart (Cho et al., 2016; Gorski et al., 2015; Tham et al., 2015). Sarco/endoplasmic reticulum Ca^{2+}-dependent ATPase 2a, (SERCA2a), which is expressed in the cardiac sarcoplasmic reticulum (SR), plays a central role in intracellular Ca^{2+} handling by pumping Ca^{2+} into the SR lumen from the cytosol. SERCA2a is modulated by phospholamban (PLN), another cardiac SR protein that acts as an inhibitory peptide (Fleischer and Inui, 1989). This inhibition can be attenuated by the phosphorylation of PLN by cAMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase II. The expression and activity of SERCA2a significantly decrease in HF, impairing Ca^{2+} uptake into the SR and decreasing subsequent Ca^{2+} release from the SR, thereby causing

* Corresponding author.
E-mail addresses: manami.kaneko@takeda.com (M. Kaneko), hisyama@outlook.jp (H. Yamamoto), hirokis@yamaguchi-u.ac.jp (H. Sakai), yusuke.kamada1@takeda.com (Y. Kamada), toshiki.tanaka@takeda.com (T. Tanaka), shuji.fujiwara@takeda.com (S. Fujiwara), syunsuke.yamamoto@takeda.com (S. Yamamoto), hiroki.takahagi@takeda.com (H. Takahagi), hideyuki.igawa@takeda.com (H. Igawa), shizuo.kasai@takeda.com (S. Kasai), masakuni.noda@takeda.com (M. Noda), minui@yamaguchi-u.ac.jp (M. Inui), tomoyuki.nishimoto@takeda.com (T. Nishimoto).

http://dx.doi.org/10.1016/j.ejphar.2017.07.035
Received 1 May 2017; Received in revised form 18 July 2017; Accepted 18 July 2017
Available online 20 July 2017

systolic and diastolic dysfunction (Marks, 2013). Several evidences indicate that the normalization of SERCA2a function restores the intracellular Ca^{2+} homeostasis and improves the contractility and relaxation of cardiomyocytes and animal hearts (Byrne et al., 2008; Sato et al., 2001). The SERCA2a-PLN system of cardiac SR is thus a potential target for HF therapy. Various therapeutic approaches that activate SERCA2a, such as SERCA2a gene therapy (Byrne et al., 2008), or inhibit PLN, such as the generation of dominant negative mutants (Hoshijima et al., 2002; Iwanaga et al., 2004), shRNA (Suckau et al., 2009), microRNA (Gröβl et al., 2014), or antibody of PLN (Kaye et al., 2007), have been implemented. However, these approaches are characterized by difficulties in drug delivery and dose adjustment, and safety issues in clinical settings. Hence, the development of a small-molecule SERCA2a activator would be advantageous for treating HF. Some compounds that have been reported to activate SERCA2a are currently in the early stages of development (Cornea et al., 2013; Rocchetti et al., 2008; Tilgmann et al., 2013). Of them, istaroxime, which was reported to increase SERCA2a activity (Ferrandi et al., 2013), showed inotropic and lusitropic effects in animal models when administered intravenously (Sabbah et al., 2007) and in a small clinical trial on patients with HF (Shah et al., 2009). As istaroxime was originally discovered as a Na^+/K^+-ATPase inhibitor, its effects on hemodynamic parameters might at least partially be due to Na^+/K^+-ATPase inhibition. Although other compounds also increased SERCA2a activity and cardiomyocyte contractility at the preclinical stage, it was unclear whether they exhibited inotropic and lusitropic effects on the heart *in vivo*.

In this study, we characterized the compound A [5'-benzyl-1'-butyl-N-(naphthalen-2-ylsulfonyl)-6'-oxo-1',6'-dihydro-2,3'-bipyridine-4-carboxamide] (Fig. 1), a novel SERCA activator that was discovered at Takeda Pharmaceutical Company, both *in vitro* in isolated SR vesicles, cardiomyocytes, and isolated perfused hearts and *in vivo* in rat hearts.

2. Materials and methods

2.1. Drugs and Materials

Compound A was synthesized at Takeda Pharmaceutical Company Limited and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies and DMSO: polyethylene glycol 400 (1: 2, v/v) solutions (1.7% NaOH) for *in vivo* study.

2.2. Measurement of ATPase activity

The SR vesicles were prepared from dog hearts as described previously (Tanaka et al., 2009). All animal procedures were performed in accordance with the NIH (U.S. National Institutes of Health) guidelines and approved by the Animal Ethics Committee of Yamaguchi University. The SERCA2a activity of the cardiac SR vesicles was measured as described previously (Sasaki et al., 1992; Tada et al., 1983) with some modifications. The vesicles (final protein concentration: 5–100 μg/ml) and compound A were incubated for 4 min at 37 °C in a final volume of 50 μl containing 20 mM imidazole-HCl (pH 6.9), 100 mM KCl, 2 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM ATP, 0.567 μM free Ca^{2+}, 5 μM ionomycin, and an ATP-regenerating system consisting of 2.5 mM phosphoenolpyruvate and pyruvate kinase (50 IU/ml). The reaction was initiated by adding ATP and stopped by adding 170 μl of a solution containing 0.3 mM 2-dinitrophenylhydrazine and 0.35 M HCl. Free Ca^{2+} concentrations were calculated using the WEBMAXC program (http://maxchelator.stanford.edu). Data were plotted as percentage of vehicle.

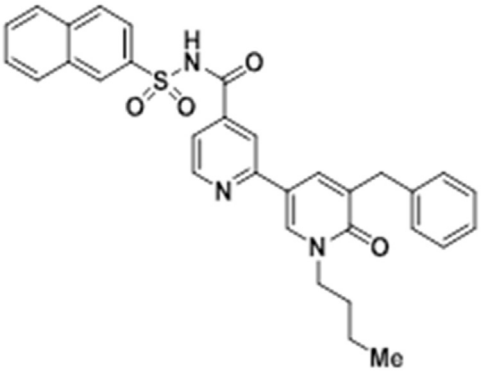

Fig. 1. Chemical structure of the pyridone derivative, compound A.

2.3. Evaluation of the PLN binding

The surface plasmon resonance (SPR) biosensing experiments were performed on a Biacore 3000 instrument equipped with a streptavidin-coated (SA) sensor chip (GE healthcare, Illinois, USA). Phosphate buffered saline (PBS) supplemented with 1 mM dithiothreitol (DTT) was used as the running buffer for the immobilization. A biotinylated synthetic human PLN peptide (MEKVQYLTRSAIRRASTIEMPQQARQNLQNLFINFC-PEG2-Biotin) (PLN (1–36)) was immobilized, via streptavidin-biotin binding, onto a SA sensor chip. The interaction studies were performed in PBS supplemented with 1 mM DTT and 1% DMSO at 22 °C. Compound A was injected for 60 s at a flow rate of 75 ml/min and the dissociation was subsequently followed for up to 150 s. Data processing and analysis were performed using the Scubber 2.0 software (BioLogic Software, Campbell, Australia). The sensorgrams were double referenced prior to curve fitting of the concentration series to a 1:1 binding model for the determination of the binding rate constant (k_{off}). The steady state values were calculated from the sensorgrams and plotted against the concentrations. The data were fitted to a single-site binding model for calculating the K_D.

2.4. Measurement of the SR Ca^{2+} load in HL-1 cells

The mouse cardiac cell line, HL-1, was obtained from Dr. Claycomb (Louisiana State University Health Science Center, Louisiana, USA) and cultured in the Claycomb medium (Sigma–Aldrich, Missouri, USA) as previously described (Claycomb et al., 1998; White et al., 2004). Prior to experiments, HL-1 cells were washed twice with PBS and incubated in the Claycomb medium with the acetoxymethyl ester of fluo-4 (1 mM, Molecular probe, Oregon, USA) without fetal bovine serum. The Ca^{2+}-dependent fluorescence of fluo-4 in HL-1 cells was monitored using a FLIPRtetra® microplate reader (Molecular Devices, California, USA). The SR Ca^{2+} load was estimated from the peak Ca^{2+} release induced by caffeine treatment (10 mM, Wako, Osaka, Japan). The cells were treated with compound A for 60 min prior to the caffeine treatment. In some experiments, thapsigargin was added 5 min before the treatment with compound A.

2.5. Measurement of Ca^{2+} transients and sarcomere shortening in isolated adult rat cardiomyocytes

Cardiomyocytes were enzymatically isolated from the left ventricle of 6- to 8-week-old male Wistar rats, as previously described (Kajstura et al., 1997). All animal procedures were performed in accordance with NIH guidelines and approved by the Animal Ethics Committee of Yamaguchi University. The isolated cardiomyocytes were incubated under 5% CO_2 at 37 °C for 3 h in an incubation buffer consisting of Joklik's modified minimum essential medium (MEM)/ Dulbecco's modified Eagle's medium DMEM (1:1) supplemented with 7.5 mM taurine, 4.3 mM creatine, 1 mM L-carnitine, 15 mM 2,3-butanedione monoxime, 1 mM $CaCl_2$, 2.5% BSA, 1% insulin-transferrin-selenium-sodium pyruvate (Thermo Scientific, Massachusetts, USA), 1 × Glutamax™ (Thermo Scientific), and 1% penicillin-streptomycin at pH 7.3. The cardiomyocytes were then loaded with 1 μM of the acetoxymethyl ester of fura-2 (Thermo Scientific) for 20 min at room temperature, washed twice with HEPES buffer containing 24 mM HEPES, 126 mM NaCl,