Efficient high-throughput screening by ER Ca\(^{2+}\) measurement to identify inhibitors of ryanodine receptor Ca\(^{2+}\)-release channels

Takashi Murayama, Nagomi Kurebayashi, Mari Ishigami-Yuasa, Shuichi Mori, Yukina Suzuki, Ryunosuke Akima, Haruo Ogawa, Junji Suzuki, Kazunori Kanemaru, Hideto Oyamada, Yuji Kiuchi, Masamitsu Iino, Hiroyuki Kagechika, Takashi Sakurai

Department of Pharmacology, Juntendo University School of Medicine, Tokyo, Japan (T.M., N.K., Y.S., R.A., T.S.); Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan (M.I.-Y., S.M., H.K); Institute for Quantitative Biosciences, The University of Tokyo, Tokyo, Japan (H.Og.); Department of Cellular and Molecular Pharmacology, Graduate School of Medicine, The University of Tokyo, Tokyo Japan (J.S., K.K., M.I.); Division of Cellular and Molecular Pharmacology, Nihon University School of Medicine, Tokyo, Japan (K.K., M.I.); and Department of Pharmacology, School of Medicine, Showa University, Tokyo, Japan (H.Oy., Y.K.).
Running title: High-throughput screening for ryanodine receptor inhibitors

Corresponding author: Takashi Murayama, Ph.D. Department of Pharmacology, Juntendo University School of Medicine, Tokyo 113-8421, Japan. Tel: +81-3-5802-1035. Fax: +81-3-5802-0419. E-mail: takashim@juntendo.ac.jp.

Number of text pages: 26
Number of tables: 0
Number of figures: 6
Number of references: 33
Number of words in Abstract: 240
Number of words in Introduction: 464
Number of words in Discussion: 1287

Abbreviations: [Ca^{2+}]_i, cytoplasmic Ca^{2+} concentrations; [Ca^{2+}]_{ER}, Ca^{2+} concentrations in the endoplasmic reticulum; CCD, central core disease; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; HTS, high-throughput screening; MH, malignant hyperthermia; RyR, ryanodine receptor; RyR1, type 1 ryanodine receptor; RyR2, type 2 ryanodine receptor.
Abstract

Genetic mutations in ryanodine receptors (RyRs), Ca\(^{2+}\)-release channels in the sarcoplasmic reticulum essential for muscle contractions, cause various skeletal muscle and cardiac diseases. Because the main underlying mechanism of the pathogenesis is overactive Ca\(^{2+}\) release by gain-of-function of the RyR channel, inhibition of RyRs is expected to be a promising treatment for these diseases. Here, to identify inhibitors specific to skeletal muscle type 1 RyR (RyR1), we developed a novel high-throughput screening (HTS) platform using time-lapse fluorescence measurement of Ca\(^{2+}\) concentrations in the endoplasmic reticulum (ER) (\([\text{Ca}^{2+}]_{\text{ER}}\)). Because expression of RyR1 carrying disease-associated mutation reduces \([\text{Ca}^{2+}]_{\text{ER}}\) in HEK293 cells through Ca\(^{2+}\) leakage from RyR1 channels, specific drugs that inhibit RyR1 will increase \([\text{Ca}^{2+}]_{\text{ER}}\) by preventing such Ca\(^{2+}\) leakage. RyR1 carrying R2163C mutation and R-CEPIA1er, a genetically-encoded ER Ca\(^{2+}\) indicator, were stably expressed in HEK293 cells and time-lapse fluorescence was measured using a FlexStation II fluorometer. False positives were effectively excluded by using cells expressing wild-type (WT) RyR1. By screening 1,535 compounds in a library of well-characterized drugs, we successfully identified four compounds that significantly increased \([\text{Ca}^{2+}]_{\text{ER}}\). They include dantrolene, a known RyR1 inhibitor, and three structurally-different compounds; oxolinic acid, 9-aminoacridine and alexidine. All the hit compounds, except for oxolinic acid, inhibited \(^{3}\text{H}\)ryanodine binding of WT and mutant RyR1. Interestingly, they showed different dose-dependencies and isoform specificities. The highly quantitative nature and good correlation with the channel activity validated this HTS platform by \([\text{Ca}^{2+}]_{\text{ER}}\) measurement to explore drugs for RyR-related diseases.
Introduction

Ryanodine receptors (RyRs) are Ca^{2+}-release channels in the sarcoplasmic reticulum (SR) of skeletal and cardiac muscles that play a central role in muscle contractions (Bers, 2004; Fill and Copello, 2002). Genetic mutations in the skeletal muscle isoform (RyR1) cause several diseases including malignant hyperthermia (MH) and central core disease (CCD) (Robinson et al., 2006; Treves et al., 2008), and those in the cardiac isoform (RyR2) are associated with various arrhythmogenic heart diseases (Betzenhauser and Marks, 2010; Priori and Chen, 2011). The underlying mechanism of most of these diseases is a gain-of-function of the channel. Hyperactivation of RyR channels may cause dysregulation of Ca^{2+} release or Ca^{2+} leakage that trigger disease phenotypes. Therefore, drugs inhibiting RyR channels are expected to be a promising treatment for such diseases.

In terms of RyR1-related diseases, dantrolene is the only approved drug for MH, which prevents large amounts of Ca^{2+} release caused by volatile anesthetics (Kolb et al., 1982). However, dantrolene is not approved for CCD due to its side effects in chronic administration. For arrhythmogenic diseases associated with RyR2, several drugs such as carvedilol (Zhou et al., 2011), flecainide (Watanabe et al., 2009), and S107 (Lehnart et al., 2008) have been proposed to prevent the hyperactivity of mutated RyR2. However, most of these drugs also interact with molecules other than RyR causing undesired effects. Therefore, there is an urgent need to develop or identify specific RyR inhibitors.

High-throughput screening (HTS) is a powerful method for rapid evaluation of thousands to millions of chemical compounds, which greatly accelerates drug discovery. To effectively identify true hit compounds, establishment of an appropriate HTS platform for the target molecule is critically important. For the success of a RyR-directed HTS platform, it is essential to quantitatively evaluate the channel activity under physiological conditions. We have recently investigated genotype-phenotype correlations of the channel activity of RyR1 carrying...
various disease-associated mutations that were expressed in HEK293 cells (Murayama et al., 2016; Murayama et al., 2015). We found that the Ca\(^{2+}\) concentration in the endoplasmic reticulum (ER) ([Ca\(^{2+}\)]\(_{ER}\)) of the mutants is inversely correlated with their channel activity, and thus expected to be an excellent quantitative index for a HTS platform to explore RyR inhibitors.

In this study, we developed and validated a novel HTS platform to identify RyR1 inhibitors using time-lapse fluorescence [Ca\(^{2+}\)]\(_{ER}\) measurements in HEK293 cells expressing mutant RyR1s. By screening a chemical library of well-characterized drugs (1,535 compounds), we successfully identified dantrolene and three other compounds (oxolinic acid, 9-aminoacridine, and alexidine) that specifically prevent Ca\(^{2+}\) leakage to increase [Ca\(^{2+}\)]\(_{ER}\) in cells expressing mutant RyR1s. The hit compounds exhibited different dose-dependencies and isoform specificities, suggesting that they inhibit RyR1 by different mechanisms. The highly quantitative nature and good correlation with the channel activity validate this novel HTS platform to explore drugs for RyR-related diseases.
Materials and Methods

**Generation of Stable and Inducible HEK293 Cell Lines.** HEK293 cells stably and inducibly expressing WT and the R2163C mutant of RyR1 (Murayama et al., 2016) or WT RyR2 (Uehara et al., 2017) were generated as described previously. For the HTS assay employing \([\text{Ca}^{2+}]_{\text{ER}}\) measurement, cDNA for R-CEPIA1er (Suzuki et al., 2014) was transfected in these cells using the Jump-In system (Invitrogen), and clones with suitable fluorescence were selected and used for further experiments. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 15 µg/ml blasticidin, 100 µg/ml hygromycin, and 400 µg/ml G418. In the case of other disease-associated RyR1 mutants (G342R, R2435H, and L4824P), R-CEPIA1er was transiently expressed using a baculovirus vector (Uehara et al., 2017).

**Time lapse [Ca\(^{2+}\)]_{ER} Measurements.** Time lapse \([\text{Ca}^{2+}]_{\text{ER}}\) measurements were performed using the FlexStation II fluorometer (Molecular Devices, San Jose, CA). HEK293 cells were seeded on 96-well flat clear bottom black microplates (#3603, Corning, New York, NY) at a density of 2×10^4 cells/well. One day after seeding, expression of RyR1 was induced by addition of doxycycline (2 µg/ml) to the culture medium. After 24–28 h of induction, the culture medium was replaced with 90 µl of HEPES-buffered Krebs solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 11 mM glucose, and 5 mM HEPES, pH 7.4), and the microplates were placed in the FlexStation II fluorometer that was preincubated at 37 °C. R-CEPIA1er signals, which were excited at 560 nm and emitted at 610 nm, were captured every 10 seconds for 300 seconds. Sixty microliters of the compounds dissolved in HEPES-Krebs solution (25 µM) were applied to the cells at 100 seconds after starting. The fluorescence change induced by the compounds was expressed as F/F₀ in which averaged fluorescence...
intensity of the last 100 seconds (F) was normalized to that of the initial 100 seconds (F₀). We screened 1,535 well-characterized drugs owned by Tokyo Medical and Dental University (TMDU) chemical compound library at 10 µM (Yoshizaki et al., 2017).

**HTS Data Analysis.** Assay quality was determined based on positive (10 µM dantrolene) and negative (0.1% DMSO) controls, as indexed by the Z’ factor:

\[
Z' = 1 - 3 \left( \frac{\sigma_P + \sigma_N}{\mu_P - \mu_N} \right) \tag{Eq. 1}
\]

where \(\sigma_P\) and \(\sigma_N\) are the SDs of positive and negative controls, and \(\mu_P\) and \(\mu_N\) are the means of positive and negative controls, respectively. A compound was considered a hit if it increased \(F/F_0\) by >3 SDs relative to negative control samples. The hit selection threshold of 3 SDs is typical for normally distributed HTS data in which 0.27% of the readings are expected to fall outside of this limit.

**[³H]Ryanodine Binding.** The assay was carried out as described previously (Murayama et al., 2016; Murayama et al., 2015) with some modifications. Briefly, microsomes prepared from HEK293 cells stably expressing the R2163C mutant of RyR1 or WT RyR2 were incubated with 5 nM [³H]ryanodine for 2 hours at 37 °C (for RyR1) or 25 °C (for RyR2) in medium containing 0.17 M NaCl, 20 mM MOPS (pH 7.0), 2 mM dithiothreitol, 1 mM β,γ-methyleneadenosine 5’-triphosphate, 1 µM calmodulin, and various concentrations of free Ca²⁺ buffered with 10 mM EGTA. Free Ca²⁺ concentrations were calculated using WEBMAXC STANDARD (http://web.stanford.edu/~cpatton/webmaxcS.htm) (Bers et al., 2010). Protein-bound [³H]ryanodine was separated by filtration through polyethyleneimine-treated glass filters (Whatman GF/B) using a Micro 96 Cell Harvester (Skatron Instruments, Lier, Norway). Nonspecific binding was determined in the presence of 20 µM unlabeled ryanodine.
Statistics. Data are presented as the mean ± SD. Statistical analysis was performed using Prism 6 (GraphPad Software, Inc., La Jolla, USA). One-way ANOVA, followed by Dunnett’s test, was performed to compare multiple groups. Statistical significance was defined as p < 0.05 compared with DMSO (negative control).
Results

Development and Validation of the HTS Platform for RyR inhibitors employing \([\text{Ca}^{2+}]_{\text{ER}}\) Measurements. The concept of the HTS platform for RyR1 inhibitors by \([\text{Ca}^{2+}]_{\text{ER}}\) measurements is as follows. \([\text{Ca}^{2+}]_{\text{ER}}\) is generally determined by the balance between \(\text{Ca}^{2+}\) release via the \(\text{Ca}^{2+}\) release pathways and \(\text{Ca}^{2+}\) uptake by sarco/endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA) \(\text{Ca}^{2+}\) pumps. We found that expression of wild-type (WT) RyR1 in HEK293 cells does not significantly change \([\text{Ca}^{2+}]_{\text{ER}}\) because of its very low \(\text{Ca}^{2+}\) release activity at resting intracellular \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) (Murayama et al., 2016; Murayama et al., 2015). Expression of RyR1 carrying disease-associated mutations, in contrast, reduces \([\text{Ca}^{2+}]_{\text{ER}}\) to inversely correlate with their channel activity (Supplementary Fig. 1). This is explained by \(\text{Ca}^{2+}\) leakage from ER at resting \([\text{Ca}^{2+}]_i\) via the mutant RyR1 channels that overcomes \(\text{Ca}^{2+}\) uptake. Thus, \([\text{Ca}^{2+}]_{\text{ER}}\) is an excellent quantitative indicator for the activity of RyR1. If a drug inhibits RyR1, then \(\text{Ca}^{2+}\) leakage will be prevented to increase \([\text{Ca}^{2+}]_{\text{ER}}\) in the mutant RyR1 cells by means of \(\text{Ca}^{2+}\) uptake (Fig. 1A). This provides an efficient HTS platform for screening of the RyR1 inhibitors. The drug, in contrast, will not increase \([\text{Ca}^{2+}]_{\text{ER}}\) in the WT RyR1 cells, because \([\text{Ca}^{2+}]_{\text{ER}}\) is not reduced (Fig. 1B). In this respect, WT RyR1 cells should be used as a negative control to effectively exclude false positives.

To establish the HTS platform, we generated HEK293 cell lines that stably expressed WT or mutant RyR1s. R2163C was chosen as a typical mutation, because \([\text{Ca}^{2+}]_{\text{ER}}\) of R2163C-mutant cells is reduced to one third of that in WT cells (Murayama et al., 2016) (Supplementary Fig. 1). Then, R-CEPIA1er, a genetically encoded ER-targeted red fluorescent \(\text{Ca}^{2+}\) indicator (Suzuki et al., 2014), was stably expressed in RyR1-HEK293 cells. Because R-CEPIA1er is a single wavelength excitation \(\text{Ca}^{2+}\) indicator, it is compatible with standard fluorometers. Time-lapse fluorescence measurements were performed using a FlexStation II fluorometer with 96-well plates. We initially examined the effects of dantrolene
and caffeine, known RyR1 inhibitor and activator, respectively, on the R-CEPIA1er fluorescence of the R2163C and WT RyR1 cells. R-CEPIA1er was excited at 560 nm, and emission at 610 nm was captured every 10 seconds for 300 seconds. Compounds were applied at 100 seconds after starting. In the R2163C RyR1 cells, application of 10 µM dantrolene gradually increased the fluorescence intensity over time and reached a plateau at 200 seconds or later (Fig. 1C). Conversely, 10 mM caffeine decreased the fluorescence. In WT RyR1 cells, dantrolene did not change the fluorescence intensity, but 10 mM caffeine rapidly and markedly decreased the fluorescence (Fig. 1D). Quantitative results are summarized in Fig. 1F, in which the fluorescence change ($F/F_0$) induced by compounds was determined by normalizing the averaged fluorescence intensity for the last 100 seconds ($F$) to that for the initial 100 seconds ($F_0$). The different responses of the R2163C and WT RyR1 cells to dantrolene and caffeine are consistent with the above idea. In addition, a SERCA inhibitor thapsigargin (1 µM) reduced the fluorescence for both the cells to the level similar to that by caffeine (Fig. 1E), confirming the importance of SERCA Ca$^{2+}$ pumps in maintaining $[\text{Ca}^{2+}]_{\text{ER}}$. We also investigated other known RyR1 inhibitors; tetracaine (Laver and van Helden, 2011), ruthenium red (Ma, 1993), and neomycin (Mead and Williams, 2004) (Fig. 1F). Tetracaine (1 mM) increased $F/F_0$ in the R2163C, but not WT, RyR1 cells. In contrast, ruthenium red (10 µM) and neomycin (10 µM) had no effects. These results are reasonably explained by membrane permeability of the inhibitors. Ruthenium red and neomycin are least permeable through the cell membrane (Brodie and Reed, 1991). Thus, $[\text{Ca}^{2+}]_{\text{ER}}$ measurement successfully detected known membrane-permeable RyR1 inhibitors, which strongly supports usefulness of our HTS platform.

To quantitatively validate the assay system, we then determined coefficient of variation (CV) and $Z'$-factor. Histograms of $F/F_0$ for the R2163C RyR1 cells showed that fluorescence changed by dantrolene ($\mu = 1.79$ and $\sigma = 0.12$) was perfectly separated from that changed by DMSO ($\mu = 0.95$ and $\sigma = 0.05$) (Fig. 2A). CV values for dantrolene and DMSO
were 0.07 and 0.05, respectively, which satisfied the criteria for HTS (CV < 0.1). The Z'-factor was calculated by Eq. 1 as 0.40 (see Materials and Methods) which was within the acceptable range. In the WT RyR1 cells, in contrast, the fluorescence change induced by 10 µM dantrolene (µ = 1.00 and σ = 0.05) overlapped with that induced by DMSO (µ = 0.97 and σ = 0.05) (Fig. 2B). Dose-response assay revealed that F/F₀ was increased by dantrolene in a dose-dependent manner, and the results were well fitted by the Michaelis-Menten equation with an EC₅₀ of 0.06 µM (Fig. 2C). Taken together, these results indicate that our HTS platform have an excellent detection sensitivity.

**Screening of a Chemical Compound Library by [Ca²⁺]ER Measurements.** Using this HTS platform by [Ca²⁺]ER measurements, we screened a Tokyo Medical and Dental University (TMDU) chemical compound library of well-characterized drugs (1,535 compounds) at 10 µM concentrations. The overall results with the R2163C and WT RyR1 cells in duplicate assays are shown in Fig. 3A and B, respectively. Based on the finding that RyR1 inhibitors did not increase the fluorescence intensity of the WT RyR1 cells, eight compounds that increased F/F₀ in the WT RyR1 cells by >3 standard deviations (SDs) were excluded as false positives, all of which exhibited autofluorescence excited by 560 nm (Supplementary TABLE 1). A threshold for hit compounds was set at +3 SDs for DMSO in the R2163C cells, which was expected to cover ~0.14% of total compounds. We successfully identified four compounds (#1–#4) that greatly increased the fluorescence of the R2163C RyR1 cells (F/F₀ > 1.5), but not that of WT RyR1 cells (Fig. 3A and B). They were oxolinic acid (#1), 9-aminoacridine (#2), dantrolene (#3), and alexidine (#4) (Fig. 3C). The fact that dantrolene was yielded as a hit strongly validated our HTS platform using [Ca²⁺]ER measurements.

**Dose-dependency and subtype specificity of hit compounds.** To further characterize the hit
compounds, we investigated dose effects on $[Ca^{2+}]_{ER}$ using the same assay conditions as the HTS. The hit compounds exhibited different dose effects on the R2163C RyR1 cells (Fig. 4A). Dantrolene showed the highest potency ($EC_{50} \sim 0.05 \mu M$), followed by oxolinic acid ($EC_{50} \sim 0.5 \mu M$). They both exhibited apparent saturation of $[Ca^{2+}]_{ER}$. 9-Aminoacridine and alexidine were much less potent ($EC_{50} > 10 \mu M$), but their maximum activities (i.e., efficacy) were 2-fold higher than those of dantrolene or oxolinic acid. All compounds had no apparent effects on the WT RyR1 cells up to 30 µM (Fig. 4B).

Because hit compounds had been identified using the R2163C mutant, a concern was that the inhibitory effect might be specific to this mutation. We therefore examined whether hit compounds are effective on WT and other mutant RyR1s. Because WT RyR1 does not reduce $[Ca^{2+}]_{ER}$ in the resting state (see Fig. 1), we forced to reduce $[Ca^{2+}]_{ER}$ in the WT RyR1 cells by caffeine (Supplementary Fig. 2). In the presence of 5 mM caffeine, four hit compounds successfully increased $[Ca^{2+}]_{ER}$ of the WT RyR cells in a dose-dependent manner (Fig. 4C), as in case of the R2163C RyR1 cells (Fig. 4A). In addition, all four hit compounds at 30 µM significantly increased $[Ca^{2+}]_{ER}$ of cells expressing RyR1 carrying MH mutations in different regions; G342R, R2435H, and L4824P, all of which exhibit gain-of-function phenotype (Murayama et al., 2016; Murayama et al., 2015) (Fig. 4D). These results confirmed that the hit compounds are also effective on WT and the other mutant RyR1s. We also tested isoform specificity of the hit compounds using HEK293 cells stably expressing RyR2, an RyR isoform in the heart (Fig. 4E). Dantrolene and oxolinic acid had no effects on RyR2 up to 30 µM, suggesting that the two compounds specifically inhibit RyR1. In contrast, 9-aminoacridine and alexidine greatly increased $[Ca^{2+}]_{ER}$ at 30 µM, an indicative of inhibition of both RyR1 and RyR2.

Effects of Hit Compounds on $[^3H]$Ryanodine Binding. To validate whether hit compounds
directly inhibit RyR1, we examined the effects of hit compounds on \[^3\text{H}\]ryanodine binding that reflects activity of the RyR channel (Meissner, 1994; Ogawa, 1994). We initially determined Ca\(^{2+}\)-dependent \[^3\text{H}\]ryanodine binding with microsomes isolated from the R2163C RyR1 cells (Fig. 5A and B). Without compounds, the R2163C RyR1 exhibited biphasic Ca\(^{2+}\) dependency with a peak at about 10 \(\mu\text{M}\) Ca\(^{2+}\) (Fig. 5A). Dantrolene reduced the peak binding with a slight rightward shift of the curve for activation. Unexpectedly, oxolinic acid had no effects at all examined Ca\(^{2+}\) concentrations. 9-Aminoacridine and alexidine similarly inhibited the binding by reducing the peak value without affecting Ca\(^{2+}\) dependency (Fig. 5B). Dose-dependent inhibition at pCa 6 revealed different potencies of the hit compounds (Fig. 5C). The dose-dependencies of the hit compounds, except for oxolinic acid, were consistent with those determined by \([\text{Ca}^{2+}]_{\text{ER}}\) measurements (see Fig. 4A). We also examined the effects of hit compounds on \[^3\text{H}\]ryanodine binding of WT RyR1. Because WT RyR1 exhibits much lower \[^3\text{H}\]ryanodine binding than R2163C (Murayama et al., 2016; Murayama et al., 2015), 10 mM caffeine was added to accelerate the channel activity. Under the condition, all the hit compounds, except for oxolinic acid, effectively inhibited \[^3\text{H}\]ryanodine binding (Fig. 5D). Taken together, these results strongly indicate that the three hit compounds (dantrolene, 9-aminoacridine and alexidine) directly inhibit RyR1. The fact that oxolinic acid had no effects on \[^3\text{H}\]ryanodine binding raises the possibility that the drug might inhibit RyR1 through target molecules other than RyR1 or act as a pore blocker that does not affect \[^3\text{H}\]ryanodine binding. The effects of hit compounds (30 \(\mu\text{M}\)) were also examined with RyR2 (Fig. 5E). Whereas 9-aminoacridine and alexidine significantly reduced \[^3\text{H}\]ryanodine binding to WT RyR2, dantrolene and oxolinic acid had no effects, which was consistent with the results of \([\text{Ca}^{2+}]_{\text{ER}}\) measurements (see Fig. 4E).

**Effects of Hit Compounds on the Ca\(^{2+}\) Pump Activity.** As described above, \([\text{Ca}^{2+}]_{\text{ER}}\) is
determined by the balance between Ca\(^{2+}\) release pathways and Ca\(^{2+}\) uptake by SERCA Ca\(^{2+}\) pumps. Therefore, if a drug activates Ca\(^{2+}\) pumps, then [Ca\(^{2+}\)]\(_{\text{ER}}\) will increase. We tested this possibility by measuring Ca-ATPase activity using skeletal muscle microsomes. Dantrolene, oxolinic acid, and 9-aminoacridine at 10 µM had no effects on the Ca-ATPase activity (Fig. 6A and B). This result excluded the possibility of Ca\(^{2+}\) pump activation by these drugs. To our surprise, alexidine (10 µM) strongly inhibited the Ca-ATPase activity at all examined Ca\(^{2+}\) concentrations (Fig. 6A and B).
Discussion

Hyperactivation of the RyR channel is implicated in the pathology of several muscular and arrhythmogenic heart diseases (Betzenhauser and Marks, 2010; Priori and Chen, 2011; Robinson et al., 2006; Treves et al., 2008). Therefore, chemical compounds that inhibit the RyR channel are expected to be potentially therapeutic for these diseases. Several methods are used to evaluate RyR1 channel activity, such as single channel current recordings in lipid bilayers, [³H]ryanodine binding to isolated SR vesicles, and Ca²⁺ release measurements in isolated SR or skinned fibers (Meissner, 1994; Ogawa, 1994). However, these methods are difficult to apply to HTS for RyR-directed drugs because of their complicated procedures or requirement of a specific apparatus.

Based on the finding that [Ca²⁺]ER is inversely correlated with the channel activity of the RyR1 mutants in HEK293 cells (Murayama et al., 2016; Murayama et al., 2015) (Supplementary Fig. 1), we developed a novel HTS platform for RyR1 inhibitors by time-lapse fluorescence measurement of [Ca²⁺]ER using R-CEPIA1er, a genetically-encoded Ca²⁺ indicator (Fig. 1). Indeed, our HTS platform perfectly separated negative (DMSO) and positive (10 μM dantrolene) controls with excellent CV values (Fig. 2). By screening of a TMDU chemical compound library of 1,535 well-characterized drugs, we identified four hit compounds (dantrolene, oxolinic acid, 9-aminoacridine, and alexidine) (Fig. 3). The fact that dantrolene was yielded as a hit strongly validated our HTS platform. All the hit compounds were also effective on WT and other mutant RyR1s (Fig. 4). The three hit compounds, except for oxolinic acid, inhibited [³H]ryanodine binding of RyR1, indicating that they directly inhibit RyR1 (Fig. 5). Our method is superior to existing methods as follows. First, it is quite easy by simply measuring [Ca²⁺]ER in cells using a standard fluorometer without any pretreatment. Second, [Ca²⁺]ER measurement evaluates the channel activity under the resting [Ca²⁺], that mimics the situation in vivo. Finally, our method measures steady-state level of [Ca²⁺]ER, which
is more stable and reproducible than the method measuring transient \([\text{Ca}^{2+}]\), changes that are triggered by \(\text{Ca}^{2+}\) release via RyR1.

Fluorescence measurements are generally affected by compounds that emit or absorb fluorescence. In our method, the rate of false positives with autofluorescence was as low as 0.5% (8 out of 1,535 compounds) (Supplementary TABLE 1). Long excitation wavelength for R-CEPIA1er (560 nm) may contribute to low rate of false positives. \([\text{Ca}^{2+}]_{\text{ER}}\) is generally influenced both by \(\text{Ca}^{2+}\) uptake pathways, i.e. SERCA \(\text{Ca}^{2+}\) pumps and \(\text{Ca}^{2+}\) release pathways, i.e., RyRs, inositol 1,4,5-trisphosphate (IP\(_3\)) receptors and other ER \(\text{Ca}^{2+}\) leak channels. Therefore, it seems possible that screening by \([\text{Ca}^{2+}]_{\text{ER}}\) measurements will also identify modulators for these pathways other than RyR1. However, all the hit compounds targeted to RyR1. This high specificity may be reasonably explained by the fact that ER \(\text{Ca}^{2+}\) leak activity from the exogenously expressed mutant RyR1 channels is much greater than that via the endogenous \(\text{Ca}^{2+}\) leak pathways in HEK293 cells (Fig. 1). A general concern for drug screening by the mutant protein is a biased result, e.g., hit compounds affect only the mutant but not WT protein. However, all four hit compounds were effective not only on R2163C RyR1 but also on WT and the other mutant RyR1s (Fig. 4). Thus, use of the mutant RyR1 may not give serious impact on the results, although we cannot exclude the possibility of some bias.

Recently, Rebbeck et al. reported an excellent HTS strategy to identify RyR modulators using time-resolved fluorescence resonance energy transfer (FRET) (Rebbeck et al., 2017). By measuring changes in FRET signals between calmodulin and FKB12.6 in the RyR1 channel, they successfully identified six compounds that activate (cefartrizine, cefixime, disulfiram, ebselen, and tacrolimus) or inhibit (chloroxine) RyR1. Among three compounds (chloroxine, disulfiram, and tacrolimus) in our library, disulfiram and tacrolimus exhibited significant reductions of \([\text{Ca}^{2+}]_{\text{ER}}\) \((F/F_0 = 0.60\) and 0.70 for disulfiram and tacrolimus, respectively\) in the mutant RyR1 (Supplementary TABLE2). This indicates activation of the
RyR1 channel, which is consistent with their conclusion. However, chloroxine exhibited a slight reduction of $[\text{Ca}^{2+}]_{\text{ER}}$ ($F/F_0 = 0.80$) in our screening (Supplementary TABLE2), which is indicative of a weak activating effect. So far, the reason for this difference remains unclear. One plausible explanation is the method adopted. A comparison of the HTS results between the two strategies will provide useful information for RyR1 modulators.

Oxolinic acid is a first generation quinolone antibiotic that has been used for urinary tract infections (Gleckman et al., 1979) and now mainly for aquaculture (Martinsen et al., 1992). It inhibits bacterial DNA gyrase (Cozzarelli, 1977). Oxolinic acid is also reported to act as a dopamine reuptake inhibitor and has stimulant effects in mice (Garcia de Mateos-Verchere et al., 1998). We found that oxolinic acid increased $[\text{Ca}^{2+}]_{\text{ER}}$ of RyR1, but not RyR2, cells (Fig. 4). The RyR1-specific inhibition is a great advantage for clinical use. Unexpectedly, oxolinic acid did not affect $[^3\text{H}]$ryanodine binding to RyR1 (Fig. 5). This raises a possibility that oxolinic acid indirectly inhibits RyR1 via some associated molecules, which was removed from RyR1 through the preparation of microsomes used for $[^3\text{H}]$ryanodine binding. Alternatively, oxolinic acid might act as a pore blocker which does not affect $[^3\text{H}]$ryanodine binding. Our chemical compound library includes 18 quinolone antibiotics (first to forth generations), but no compounds other than oxolinic acid had significant effects on $[\text{Ca}^{2+}]_{\text{ER}}$ in the screening (Supplementary TABLE2). The structure-function relationships will provide useful information for structural expansion of oxolinic acid.

9-Aminoacridine is a green fluorescent dye that have been used as an anti-malarial and anti-microbial drug, and is now expected to be an anti-cancer agent (Sebestik et al., 2007). We found that 9-aminoacridine strongly inhibited $[^3\text{H}]$ryanodine binding to RyR1 and RyR2 (Fig. 5). This result corresponds to previous reports showing that 9-aminoacridine inhibits Ca$^{2+}$ release from skeletal muscle SR (Brunder et al., 1992; Palade, 1987). In our compound library, 9-aminoacridine derivatives (amsacrine, ethacridine, and mepacrine) had
no effects (Supplementary TABLE2). Thus, introduction of substituents of the amino group or acridine ring may decrease its activity. Alexidine is an alkyl bisbiguanide antiseptic that is used in mouthwashes to eliminate plaque-forming microorganisms (Coburn et al., 1978). It also inhibits the mitochondrial phosphatase PTPMT1 (protein tyrosine phosphatase, mitochondrial 1) (Doughty-Shenton et al., 2010), and induces apoptosis in cancer cells (Yip et al., 2006). Alexidine inhibited [³H]ryanodine binding to RyR1 and RyR2 at 10 µM or higher concentrations (Fig. 5). It also strongly inhibited Ca²⁺ pumps in skeletal muscle (Fig. 6). Because the other biguanides (chlorhexidine, metformin, phenformin, phenylbiguanide, and proguanil) included in our library had no effects (Supplementary TABLE2), the biguanide moiety is not responsible for the inhibitory effect of alexidine.

In the current method, compounds impermeable to the cell membrane were recognized as false negatives (Fig. 1). Although the false negative rate is unclear, this might be a potential limitation of our HTS platform. In skeletal muscle, many proteins interact with RyR1 to regulate the channel activity (Fill and Copello, 2002). Because HEK293 cells do not express such associated proteins, compounds targeted to certain factors to indirectly inhibit the RyR1 channel might be escaped from our HTS platform. Coexpression of regulatory factors in our HTS platform will identify compounds more specific to "skeletal muscle RyR1". In the current HTS of 1,535 compounds, we identified four RyR1 inhibitors. By screening a large library of ~150,000 compounds, we anticipate detection of ~390 novel RyR1 inhibitors that may include several viable candidates for novel drugs. This would greatly expand our understanding of the pharmacology of RyR1.
Acknowledgments

We are grateful for the Laboratory of Proteomics and Biomolecular Science, Research Support Center, Juntendo University Graduate School of Medicine, for their technical support. We thank Prof. Paul D. Allen (University of Leeds) for careful reading of the manuscript. We also thank Mitchell Arico from Edanz Group (www.edanzediting.com/ac) for editing a draft of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

Participated in research design: Murayama, Kurebayashi, Ishigami-Yuasa, Mori, Kagechika, Sakurai.

Conducted experiments: Murayama, Kurebayashi, Y. Suzuki, Akima, Ogawa.

Contributed new reagents or analytic tools: J. Suzuki, Kanemaru, Oyamada, Kiuchi, Iino

Performed data analysis: Murayama, Kurebayashi, Ishigami-Yuasa, Mori, Ogawa, Kagechika, Sakurai.

Wrote the manuscript: Murayama, Kurebayashi, Ogawa.
References


Palade P (1987) Drug-induced Ca\textsuperscript{2+} release from isolated sarcoplasmic reticulum. II. Releases involving a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release channel. *J Biol Chem* **262**(13): 6142-6148.


Footnotes

This work was supported in part by JSPS KAKENHI [Grants JP16K08507, JP15K08243, JP16H04748, JP16K08917]; Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)) from AMED [Grant JP17am0101080j0001]; Practical Research Project for Rare/Intractable Diseases from AMED [Grant JP17ek0109202h0001]; Intramural Research Grant for Neurological and Psychiatric Disorders of NCNP [Grant 29-4]; the Vehicle Racing Commemorative Foundation; the Institute of Seizon & Life Sciences; the Cooperative Research Project of Research Center for Biomedical Engineering; a grant from the Institute for Environmental & Gender-specific Medicine, Juntendo University; and MEXT-supported Program for the Strategic Research Foundation at Private Universities. ¹T.M. and N.K. contributed equally to this work. ²Present address of J.S.: Department of Physiology, University of California, San Francisco, CA 94158, USA.
Figure legends

**Fig. 1.** Strategy and establishment of HTS platform for RyR1 inhibitors using [Ca\(^{2+}\)]\(_{ER}\) measurement. *(A and B)* Schematic drawings of strategy of HTS platform using [Ca\(^{2+}\)]\(_{ER}\) measurement. *(A)* In the mutant RyR1 cells, [Ca\(^{2+}\)]\(_{ER}\) is reduced because of Ca\(^{2+}\) leakage via the RyR1 channel. Drugs inhibiting RyR1 will prevent Ca\(^{2+}\) leakage and increase [Ca\(^{2+}\)]\(_{ER}\). *(B)* In the WT RyR1 cells, drugs inhibiting the RyR1 channel will not change [Ca\(^{2+}\)]\(_{ER}\), because [Ca\(^{2+}\)]\(_{ER}\) in the WT RyR1 cells is not reduced because of very small Ca\(^{2+}\) leakage. *(C and D)* Time-lapse R-CEPIA1er fluorescence measurement using a FlexStation II fluorometer with HEK293 cells expressing R2163C *(C)* or WT *(D)* RyR1. DMSO *(filled grey circles)*, 10 µM dantrolene *(filled color circles)* or 10 mM caffeine *(open color circles)* was applied at 100 seconds *(arrows)*. In R2163C RyR1 cells, dantrolene increased the R-CEPIA1er fluorescence, whereas caffeine decreased it. In the WT RyR1 cell, dantrolene did not change the R-CEPIA1er fluorescence, whereas caffeine markedly decreased it. *(E)* Effect of thapsigargin, a SERCA Ca\(^{2+}\) pump inhibitor, on the fluorescence change *(F/F\(_0\))* of R2163C *(orange)* and WT *(green)* RyR1 cells. 1 µM thapsigargin was applied at 100 seconds *(arrows)*. *(F)* Summary of fluorescence change *(F/F\(_0\))* of R2163C *(orange)* and WT *(green)* RyR1 cells by various drugs. F/F\(_0\) was obtained by normalizing the averaged fluorescence for the last 100 seconds *(F)* to that for the first 100 seconds *(F\(_0\))* . Dotted line demotes 1 F/F\(_0\), i.e., no fluorescent change by the drug. Caffeine *(10 mM)* and thapsigargin *(1 µM)* reduced F/F\(_0\) to similar level. Dantrolene and tetracaine *(1 mM)* greatly increased F/F\(_0\) in R2163C RyR1 cells, whereas ruthenium red *(10 µM)* and neomycin *(10 µM)* did not. Data are the mean ± SD *(n = 8)*. *, p<0.05 compared with DMSO *(one-way ANOVA with Dunnett's test)*.

**Fig. 2.** Validation of the HTS platform using [Ca\(^{2+}\)]\(_{ER}\) measurement. *(A and B)* Histograms of
F/F₀ induced by DMSO (n = 144) and dantrolene (n = 144) in R2163C RyR1 cells (A) or WT RyR1 cells (B). (C) Dose-dependent effect of dantrolene on F/F₀ in R2163C (orange, n = 7) and WT (green, n = 4) RyR1 cells. Data are the mean ± SD. Note that dantrolene increased F/F₀ in R2163C RyR1 cells in a dose-dependent manner with an EC₅₀ of 0.06 µM.

Fig. 3. HTS results. A TMDU chemical compound library of well-characterized drugs (1,535 compounds, 10 µM) was screened in HEK293 cells expressing R2163C (A) or WT (B) RyR1. Four compounds (1–4, red) were identified as hits using a threshold of +3 SDs (F/F₀ = 1.15, dotted line). Data are the mean of duplicate screens. Dotted line demotes 1 F/F₀. (C) Structures of the hit compounds: (1) oxolinic acid, (2) 9-aminoacridine, (3) dantrolene, and (4) alexidine.

Fig. 4. Dose-dependency and isoform specificity of hit compounds. (A–C) Dose-dependent effects of hit compounds (0.01–30 µM) on [Ca²⁺]₅ER measurements in HEK293 cells expressing R2163C (A), WT RyR1 (B), or WT RyR1 in the presence of 5 mM caffeine (C). Data are the mean ± SD (n = 8 for R2163C, 6 for WT, and 4 for WT + caffeine). (D) Effects of hit compounds (30 µM) on the [Ca²⁺]₅ER of cells with RyR1 carrying disease-associated mutations (G342R, R2435H, and L4824P). Data are the mean ± SD (n = 4). (E) Dose-dependent effects of hit compounds (0.01–30 µM) on [Ca²⁺]₅ER measurements in HEK293 cells expressing WT RyR2. Data are the mean ± SD (n = 4). Dotted line demotes 1 F/F₀. *, p<0.05 compared with DMSO (one-way ANOVA with Dunnett's test).

Fig. 5. Effects of hit compounds on [³H]ryanodine binding. (A and B) Ca²⁺-dependent [³H]ryanodine binding to R2163C RyR1 in the presence of hit compounds (30 µM). Data are the mean ± SD (n = 4). (C) Dose-dependent inhibition of R2163C RyR1 by hit compounds at 1 µM Ca²⁺. Data are the mean ± SD (n = 4). (D) Effect of hit compounds (30 µM) on
[3H]ryanodine binding to WT RyR1 in the presence of 10 mM caffeine at 1 µM Ca^{2+}. Data are the mean ± SD (n = 4). (E) Effect of hit compounds (30 μM) on [3H]ryanodine binding to WT RyR2 at 10 μM Ca^{2+}. Data are the mean ± SD (n = 4). *, p<0.05 compared with DMSO (one-way ANOVA with Dunnett's test).

**Fig. 6.** Effects of hit compounds on Ca-ATPase activity. (A) Ca^{2+}-dependent Ca-ATPase activity of SERCA was determined using skeletal muscle microsomes in the presence of 10 μM hit compounds. Data are the mean ± SD (n = 3). (B) Ca-ATPase activity under treatments with hit compounds at 100 μM Ca^{2+} (n = 3). 100% denotes the activity under DMSO treatment. *, p<0.05 compared with DMSO (one-way ANOVA with Dunnett's test).
Fig. 1.
Fig. 2.
Fig. 3.

A

R2163C

Fluorescence change (F/F₀)

Compounds number

B

WT

Fluorescence change (F/F₀)

Compounds number

C

(1) Oxolinic acid

(2) 9-Aminoacridine

(3) Dantrolene

(4) Alexidine
Fig. 4.

Fluorescence change (F/F₀) vs. Compounds (µM)

A. RyR1 (R2163C)

B. RyR1 (WT)

C. RyR1 (WT + Caffeine)

D. RyR1 (Mutant)

E. RyR2 (WT)

Compounds:
- Dan
- DMSO
- Oxo
- 9AA
- Ale

This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 5.

(A) RyR1 (R2163C) 

(B) RyR1 (R2163C) 

(C) RyR1 (R2163C) 

(D) RyR1 (WT + Caffeine) 

(E) RyR2 (WT)

Bound [3H]Ryanodine (B/B<sub>max</sub>) 

Bound [3H]Ryanodine (B/B<sub>max</sub>) 

Bound [3H]Ryanodine (B/B<sub>max</sub>) 

Bound [3H]Ryanodine (B/B<sub>max</sub>) 

Bound [3H]Ryanodine (B/B<sub>max</sub>)
Fig. 6.