

Efficient High-Throughput Screening by Endoplasmic Reticulum Ca^{2+} Measurement to Identify Inhibitors of Ryanodine Receptor Ca^{2+} -Release Channels[§]

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Received December 18, 2017; accepted April 12, 2018

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 of 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 the 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 fluorometer. False positives were effectively excluded by using cells expressing wild-type (WT) RyR1. By screening 1535 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 [^3H]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 (Fill and Copello, 2002; Bers, 2004). Genetic mutations in the skeletal muscle isoform [type 1 RyR (RyR1)] cause several diseases, including malignant hyperthermia (MH) and central core disease (Robinson et al., 2006; Treves et al., 2008), and those in the cardiac isoform [type 2 RyR (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 triggers

This work was supported in part by Japan Society for the Promotion of Science KAKENHI [Grants JP16K08507, JP15K08243, JP16H04748, and JP16K08917]; Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from Japan Agency for Medical Research and Development (AMED) [Grant JP17am0101080j0001]; Practical Research Project for Rare/Intractable Diseases from AMED [Grant JP17ek0109202h0001]; Intramural Research Grant for Neurologic and Psychiatric Disorders of National Center for Neurology and Psychiatry [Grant 29-4]; the Vehicle Racing Commemorative Foundation; the Institute of Seizon and 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 Program for the Strategic Research Foundation at Private Universities supported by Ministry of Education, Culture, Sports, Science and Technology, Japan.

The authors declare that they have no conflict of interest.
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<https://doi.org/10.1124/mol.117.111468>.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: $[\text{Ca}^{2+}]_{\text{ER}}$, Ca^{2+} concentrations in the endoplasmic reticulum; $[\text{Ca}^{2+}]_{\text{i}}$, cytoplasmic Ca^{2+} concentrations; CV, coefficient of variation; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; HTS, high-throughput screening; MH, malignant hyperthermia; RyR, ryanodine receptor; RyR1, type 1 ryanodine receptor; RyR2, type 2 ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; TMDU, Tokyo Medical and Dental University; WT, wild-type.

disease phenotypes. Therefore, drugs inhibiting RyR channels are expected to be a promising treatment of 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 central core disease 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 an RyR-directed HTS platform, it is essential to quantitatively evaluate the channel activity under physiologic 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., 2015, 2016). We found that the Ca^{2+} concentration in the endoplasmic reticulum (ER) ($[\text{Ca}^{2+}]_{\text{ER}}$) of the mutants was inversely correlated with their channel activity and, thus, was expected to be an excellent quantitative index for an 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 $[\text{Ca}^{2+}]_{\text{ER}}$ measurements in HEK293 cells expressing mutant RyR1s. By screening a chemical library of well characterized drugs (1535 compounds), we successfully identified dantrolene and three other compounds (oxolinic acid, 9-aminoacridine, and alexidine) that specifically prevent Ca^{2+} leakage to increase $[\text{Ca}^{2+}]_{\text{ER}}$ 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 wild-type (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 using $[\text{Ca}^{2+}]_{\text{ER}}$ measurement, cDNA for R-CEPIA1er (Suzuki et al., 2014) was transfected in these cells using the Jump-In system (Thermo Fisher Scientific, Waltham, MA), 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 $\mu\text{g}/\text{ml}$ blasticidin, 100 $\mu\text{g}/\text{ml}$ hygromycin, and 400 $\mu\text{g}/\text{ml}$ 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 $[\text{Ca}^{2+}]_{\text{ER}}$ 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 $\mu\text{g}/\text{ml}$) to the culture medium. After 24–28 hours of induction, the culture medium was replaced with 90 μl of HEPES-buffered Krebs solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 11 mM glucose, and 5 mM HEPES, pH 7.4), and the microplates were placed in the FlexStation II fluorometer, which 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) was applied to the cells 100 seconds after starting. The fluorescence change induced by the compounds was expressed as F/F_0 , in which averaged fluorescence intensity of the last 100 seconds (F) was normalized to that of the initial 100 seconds (F_0). We screened 1535 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% dimethylsulfoxide (DMSO)] controls, as indexed by the Z' factor:

$$Z' = 1 - 3 \left(\frac{\sigma_P + \sigma_N}{\mu_P - \mu_N} \right) \quad (1)$$

where σ_P and σ_N are the S.D.s of positive and negative controls, and μ_P and μ_N are the means of positive and negative controls, respectively. A compound was considered a hit if it increased F/F_0 by >3 S.D.s relative to negative control samples. The hit selection threshold of three S.D.s is typical for normally distributed HTS data in which 0.27% of the readings are expected to fall outside of this limit.

$[^3\text{H}]$ Ryanodine Binding. The assay was carried out as described previously (Murayama et al., 2015, 2016) with some modifications. In brief, microsomes prepared from HEK293 cells stably expressing the R2163C mutant of RyR1 or WT RyR2 were incubated with 5 nM $[^3\text{H}]$ ryanodine for 2 hours at 37°C (for RyR1) or 25°C (for RyR2) in medium containing 0.17 M NaCl, 20 mM 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO) (pH 7.0), 2 mM dithiothreitol, 1 mM β , γ -methyleneadenosine 5'-triphosphate, 1 μM calmodulin, and various concentrations of free Ca^{2+} buffered with 10 mM EGTA. Free Ca^{2+} concentrations were calculated using WEBMAXC STANDARD (<http://web.stanford.edu/~cpatton/webmaxcS.htm>) (Bers et al., 2010). Protein-bound $[^3\text{H}]$ ryanodine was separated by filtration through polyethyleneimine-treated glass filters (Filtermat B, PerkinElmer, Waltham, MA) using a Micro 96 Cell Harvester (Skatron Instruments, Lier, Norway). Nonspecific binding was determined in the presence of 20 μM unlabeled ryanodine.

Measurement of ATPase Activity of SERCA. Sarcoplasmic reticulum (SR) vesicles were obtained from white skeletal muscle of rabbit hind legs as described by Eletr and Inesi (Eletr and Inesi, 1972). Ca^{2+} -dependent ATPase activity of SERCA was measured at 25°C by a coupled enzyme assay (Madden et al., 1979) using grating microplate reader SH8100Lab (CORONA). 6.4 μg of the SR vesicles was incubated with the assay buffer (60 mM 3-Morpholinopropanesulfonic acid, pH 7.0, 120 mM KCl, 6 mM MgCl_2 , 1 mM EGTA, 5 mM $\text{Na}_2\text{S}_2\text{O}_8$, 0.2 mM NADH, 0.5 mM phosphoenol pyruvate, 5.2 IU of lactate dehydrogenase, 5.2 IU of pyruvate kinase, 0.01 mM A23187, and CaCl_2 added to set free $[\text{Ca}^{2+}]$ to the desired values) containing 10 μM of each compound for 5 min. After the incubation, the assay was started upon the addition of ATP at a final concentration of 2 mM. The ATPase activity was monitored by following the decrease of NADH absorbance (A_{340}) for 5 min.

Statistics. Data are presented as the mean \pm S.D. Statistical analysis was performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA). One-way analysis of variance, followed by Dunnett's test, was performed to compare multiple groups. Statistical significance was defined as $P < 0.05$ compared with DMSO (negative control).

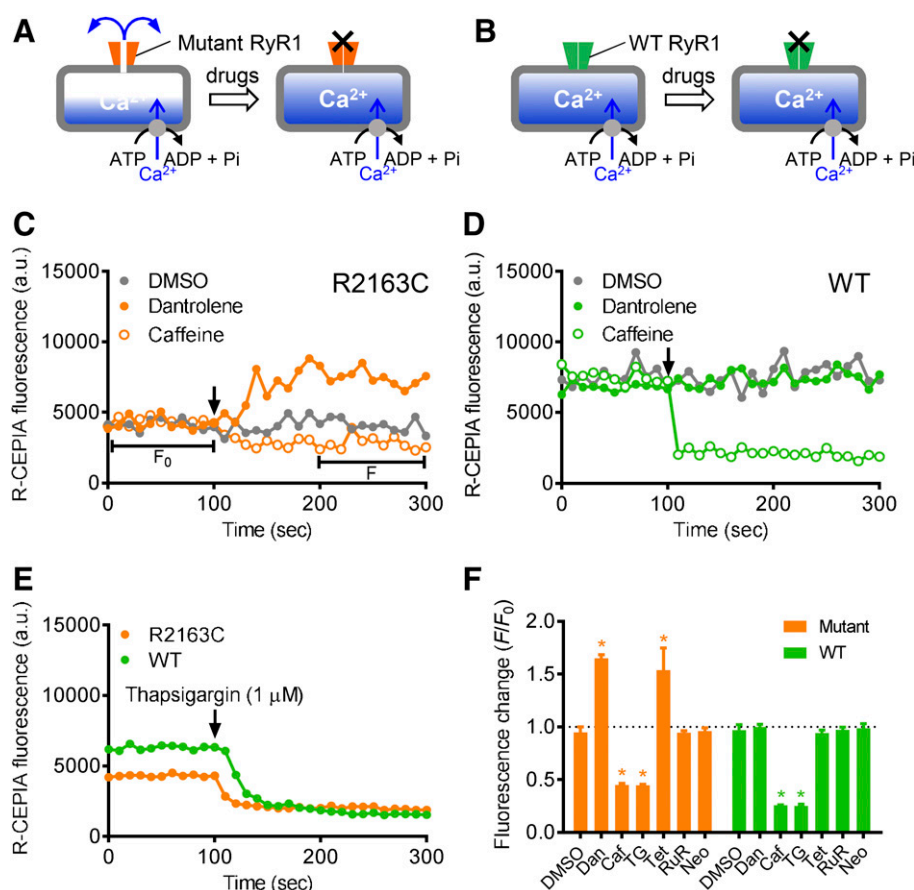


Fig. 1. Strategy and establishment of HTS platform for RyR1 inhibitors using $[Ca^{2+}]_{ER}$ measurement. (A and B) Schematic drawings of strategy of HTS platform using $[Ca^{2+}]_{ER}$ measurement. (A) In the mutant RyR1 cells, $[Ca^{2+}]_{ER}$ is reduced because of Ca^{2+} leakage via the RyR1 channel. 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 gray circles), 10 μ M 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 cells, 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. One micromolar 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 denotes 1 F/F_0 , i.e., no fluorescent change by the drug. Caffeine (Caf; 10 mM) and thapsigargin (TG; 1 μ M) reduced F/F_0 to a similar level. Dantrolene (Dan) and tetracaine (Tet; 1 mM) greatly increased F/F_0 in R2163C RyR1 cells, whereas ruthenium red (RuR; 10 μ M) and neomycin (Neo; 10 μ M) did not. Data are the mean \pm S.D. ($n = 8$). * $P < 0.05$ compared with DMSO (one-way analysis of variance with Dunnett's test).

Results

Development and Validation of the HTS Platform for RyR Inhibitors Using $[Ca^{2+}]_{ER}$ Measurements. The concept of the HTS platform for RyR1 inhibitors by $[Ca^{2+}]_{ER}$ measurements is as follows. $[Ca^{2+}]_{ER}$ is generally determined by the balance between Ca^{2+} release via the Ca^{2+} -release pathways and Ca^{2+} uptake by sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) Ca^{2+} pumps. We found that expression of WT RyR1 in HEK293 cells does not significantly change $[Ca^{2+}]_{ER}$ because of its very low Ca^{2+} -release activity at resting intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Murayama et al., 2015, 2016). Expression of RyR1 carrying disease-associated mutations, in contrast, reduces $[Ca^{2+}]_{ER}$ to inversely correlate with their channel activity (Supplemental Fig. 1). This is explained by Ca^{2+} leakage from the ER at resting $[Ca^{2+}]_i$ via the mutant RyR1 channels that overcomes Ca^{2+} uptake. Thus, $[Ca^{2+}]_{ER}$ is an excellent quantitative indicator of the RyR1 activity. If a drug inhibits RyR1, then Ca^{2+} leakage will be prevented to increase $[Ca^{2+}]_{ER}$ in the

mutant RyR1 cells by means of Ca^{2+} uptake (Fig. 1A). This provides an efficient HTS platform for screening of the RyR1 inhibitors. The drug, in contrast, will not increase $[Ca^{2+}]_{ER}$ in the WT RyR1 cells, because $[Ca^{2+}]_{ER}$ 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 the $[Ca^{2+}]_{ER}$ of R2163C-mutant cells is reduced to one-third of that in WT cells (Murayama et al., 2016) (Supplemental Fig. 1). Then, R-CEPIA1er, a genetically encoded ER-targeted red fluorescent Ca^{2+} indicator (Suzuki et al., 2014), was stably expressed in RyR1-HEK293 cells. Because R-CEPIA1er is a single wavelength excitation 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,

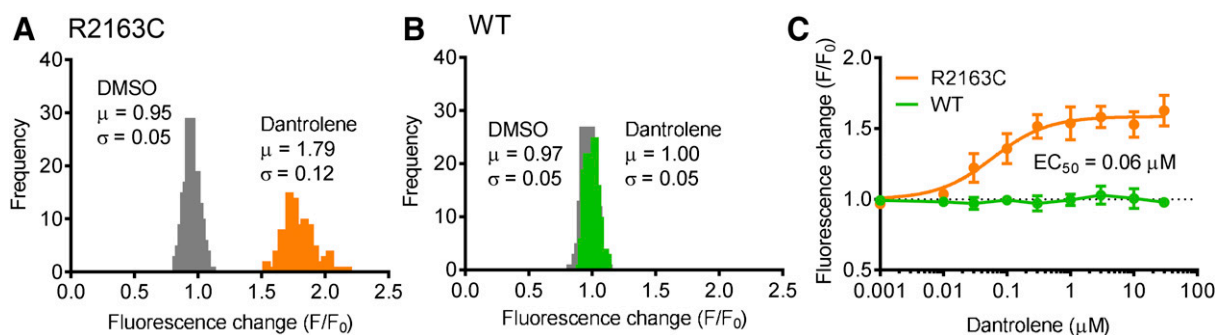


Fig. 2. Validation of the HTS platform using $[Ca^{2+}]_{ER}$ measurement. (A and B) Histograms of F/F_0 induced by DMSO ($n = 144$) and dantrolene ($n = 144$) in R2163C RyR1 cells (A) or WT RyR1 cells (B). (C) Dose-dependent effect of dantrolene on F/F_0 in R2163C (orange, $n = 7$) and WT (green, $n = 4$) RyR1 cells. Data are the mean \pm S.D. Note that dantrolene increased F/F_0 in R2163C RyR1 cells in a dose-dependent manner with an EC_{50} of $0.06 \mu M$.

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 $10 \mu M$ 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 were consistent with the aforementioned idea. In addition, a SERCA inhibitor, thapsigargin ($1 \mu M$), reduced the fluorescence for both cells to a level similar to that by caffeine (Fig. 1E), confirming the importance of SERCA Ca^{2+} pumps in maintaining $[Ca^{2+}]_{ER}$. 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 ($10 \mu M$) and neomycin ($10 \mu M$) 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, $[Ca^{2+}]_{ER}$ 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 the 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 $10 \mu M$ dantrolene ($\mu = 1.00$ and $\sigma = 0.05$) overlapped with that induced by DMSO ($\mu = 0.97$ and $\sigma = 0.05$) (Fig. 2B). Dose-response assay revealed that F/F_0 was increased by dantrolene in a dose-dependent manner, and the

results were well fitted by the Michaelis-Menten equation with an EC_{50} of $0.06 \mu M$ (Fig. 2C). Taken together, these results indicate that our HTS platform has excellent detection sensitivity.

Screening of a Chemical Compound Library by $[Ca^{2+}]_{ER}$ Measurements. Using this HTS platform by $[Ca^{2+}]_{ER}$ measurements, we screened a TMDU chemical compound library of well characterized drugs (1535 compounds) at $10 \mu M$ concentrations. The overall results with the R2163C and WT RyR1 cells in duplicate assays are shown in Fig. 3, A 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/F_0 in the WT RyR1 cells by > 3 S.D.s were excluded as false positives, all of which exhibited autofluorescence excited by 560 nm (Supplemental Table 1). A threshold for hit compounds was set at $+3$ S.D.s for DMSO in the R2163C cells, which was expected to cover $\sim 0.14\%$ of total compounds. We successfully identified four compounds (#1–#4) that greatly increased the fluorescence of the R2163C RyR1 cells ($F/F_0 > 1.5$), but not that of WT RyR1 cells (Fig. 3, A 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 $[Ca^{2+}]_{ER}$ 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 $30 \mu M$ (Fig. 4B).

Because hit compounds were identified using the R2163C mutant, a concern was that the inhibitory effect might be specific to this mutation. Therefore, we 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 were forced to reduce $[Ca^{2+}]_{ER}$ in the WT RyR1 cells by caffeine (Supplemental 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 $30 \mu M$

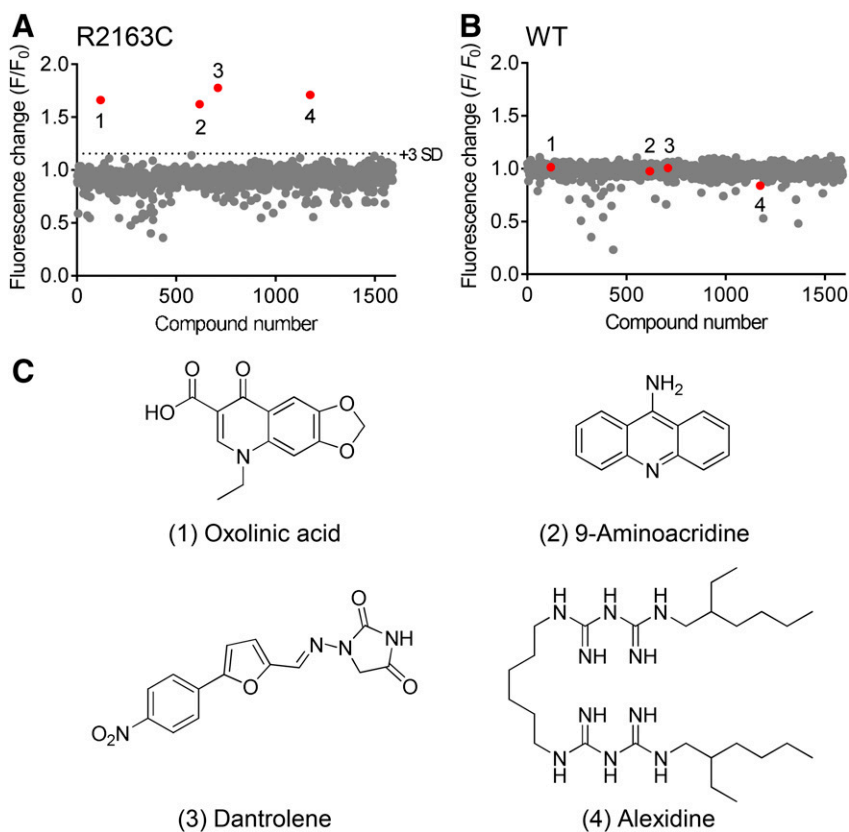


Fig. 3. HTS results. A TMDU chemical compound library of well characterized drugs (1535 compounds, 10 μ M) was screened in HEK293 cells expressing R2163C (A) or WT (B) RyR1. Four compounds (1–4, red) were identified as hits using a threshold of +3 S.D.s ($F/F_0 = 1.15$, dotted line). Data are the mean of duplicate screens. Dotted line denotes 1 F/F_0 . (C) Structures of the hit compounds: 1) oxolinic acid, 2) 9-aminoacridine, 3) dantrolene, and 4) alexidine.

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., 2015, 2016) (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 μ M, suggesting that the two compounds specifically inhibit RyR1. In contrast, 9-aminoacridine and alexidine greatly increased $[Ca^{2+}]_{ER}$ at 30 μ M, an indication 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 $[^3H]$ -ryanodine binding. Since ryanodine binds to only an open RyR channel, binding of ryanodine reflects activity of the RyR channel (Meissner, 1994; Ogawa, 1994). We initially determined Ca^{2+} -dependent $[^3H]$ ryanodine binding with microsomes isolated from the R2163C RyR1 cells (Fig. 5, A and B). Without compounds, the R2163C RyR1 exhibited biphasic Ca^{2+} dependency with a peak at about 10 μ 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 on the $[^3H]$ ryanodine binding. 9-Aminoacridine and alexidine similarly inhibited the binding by reducing the peak value without affecting Ca^{2+} dependency (Fig. 5B). Dose-dependent inhibition at 1 μ M Ca^{2+} 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 $[Ca^{2+}]_{ER}$ measurements (see Fig. 4A). We also examined the effects of hit compounds on $[^3H]$ ryanodine binding of WT RyR1. Because WT RyR1 exhibits much lower $[^3H]$ ryanodine binding than R2163C (Murayama et al., 2015, 2016), 10 mM caffeine was added to accelerate the channel activity. Under this condition, all hit compounds, except for oxolinic acid, effectively inhibited $[^3H]$ ryanodine binding (Fig. 5D). Taken together, these results strongly indicate that the three of the hit compounds (dantrolene, 9-aminoacridine, and alexidine) directly inhibit RyR1. The fact that oxolinic acid had no effects on $[^3H]$ 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 $[^3H]$ ryanodine binding. The effects of hit compounds (30 μ M) were also examined with RyR2 (Fig. 5E). Whereas 9-aminoacridine and alexidine significantly reduced $[^3H]$ ryanodine binding to WT RyR2, dantrolene and oxolinic acid had no effects, which was consistent with the results of $[Ca^{2+}]_{ER}$ measurements (see Fig. 4E).

Effects of Hit Compounds on the Ca^{2+} Pump Activity. As described earlier, $[Ca^{2+}]_{ER}$ 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 $[Ca^{2+}]_{ER}$ will increase. We tested this possibility by measuring Ca^{2+} -ATPase activity using skeletal muscle microsomes. Dantrolene, oxolinic acid, and 9-aminoacridine at 10 μ M had no effects on the Ca^{2+} -ATPase activity (Fig. 6). This result excluded the possibility of Ca^{2+} pump activation by these drugs. To our surprise, alexidine (10 μ M) strongly inhibited the Ca^{2+} -ATPase activity at all examined Ca^{2+} concentrations (Fig. 6).

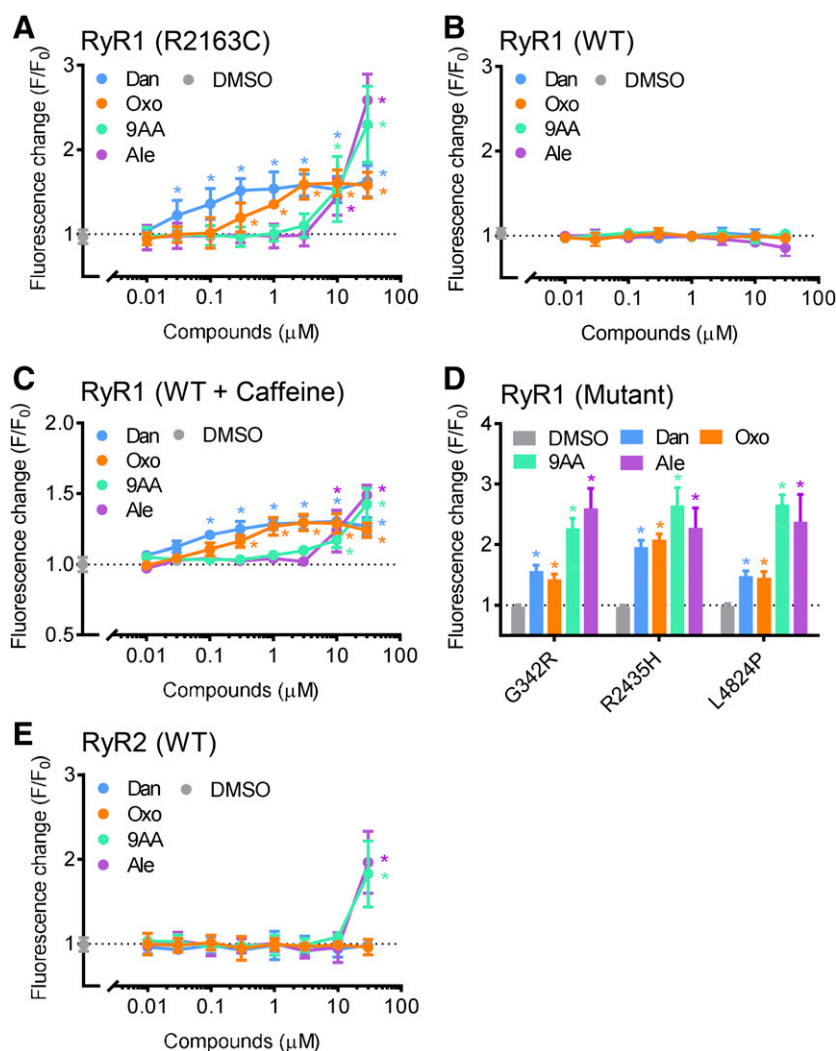


Fig. 4. Dose dependency and isoform specificity of hit compounds. (A–C) Dose-dependent effects of hit compounds (0.01–30 μM) on $[Ca^{2+}]_{ER}$ measurements in HEK293 cells expressing R2163C (A), WT RyR1 (B), or WT RyR1 in the presence of 5 mM caffeine (C). Data are the mean \pm S.D. ($n = 8$ for R2163C, $n = 6$ for WT, and $n = 4$ for WT + caffeine). (D) Effects of hit compounds (30 μM) on the $[Ca^{2+}]_{ER}$ of cells with RyR1 carrying disease-associated mutations (G342R, R2435H, and L4824P). Data are the mean \pm S.D. ($n = 4$). (E) Dose-dependent effects of hit compounds (0.01–30 μM) on $[Ca^{2+}]_{ER}$ measurements in HEK293 cells expressing WT RyR2. Data are the mean \pm S.D. ($n = 4$). Dotted line denotes 1 F/F_0 . * $P < 0.05$ compared with DMSO (one-way analysis of variance with Dunnett's test). 9AA, 9-aminoacridine; Ale, alexidine; Dan, dantrolene; Oxo, oxolinic acid.

Discussion

Hyperactivation of the RyR channel is implicated in the pathology of several muscular and arrhythmogenic heart diseases (Robinson et al., 2006; Treves et al., 2008; Betzenhauser and Marks, 2010; Priori and Chen, 2011). 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 Ca^{2+} -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 $[Ca^{2+}]_{ER}$ is inversely correlated with the channel activity of the RyR1 mutants in HEK293 cells (Murayama et al., 2015, 2016) (Supplemental Fig. 1), we developed a novel HTS platform for RyR1 inhibitors by time-lapse fluorescence measurement of $[Ca^{2+}]_{ER}$ using R-CEPIA1er, a genetically encoded Ca^{2+} 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 a TMDU chemical compound library of 1535 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 of the hit compounds were also effective on WT and other mutant RyR1s (Fig. 4). Three of the 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 because it simply measures $[Ca^{2+}]_{ER}$ in cells using a standard fluorometer without any pretreatment. Second, $[Ca^{2+}]_{ER}$ measurement evaluates the channel activity under the resting $[Ca^{2+}]_i$ that mimics the situation in vivo. Finally, our method measures the steady-state level of $[Ca^{2+}]_{ER}$, which is more stable and reproducible than the method measuring transient $[Ca^{2+}]_i$ changes that are triggered by 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 1535 compounds) (Supplemental Table 1). A long excitation wavelength for R-CEPIA1er (560 nm) may contribute to the low rate of false positives. $[Ca^{2+}]_{ER}$ is generally

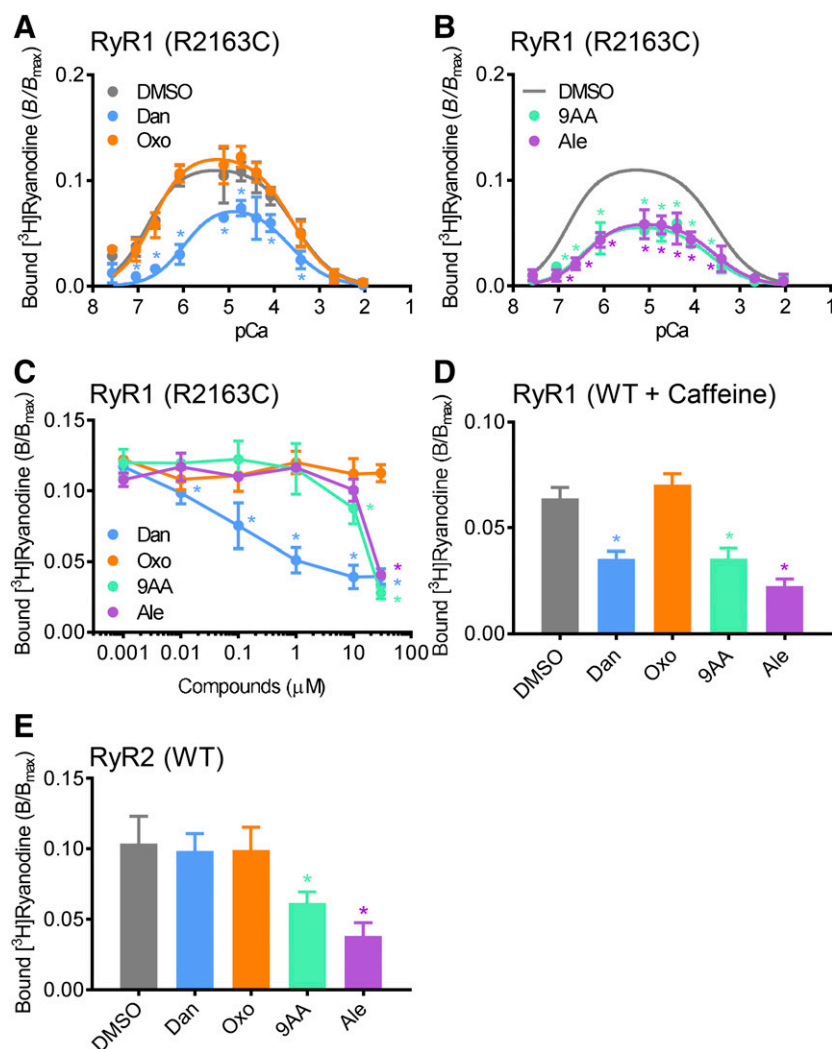


Fig. 5. Effects of hit compounds on [³H]ryanodine binding. (A and B) Ca²⁺-dependent [³H]ryanodine binding to R2163C RyR1 in the presence of hit compounds (30 μ M). Data are the mean \pm S.D. ($n = 4$). (C) Dose-dependent inhibition of R2163C RyR1 by hit compounds at 1 μ M Ca²⁺. Data are the mean \pm S.D. ($n = 4$). (D) Effect of hit compounds (30 μ M) on [³H]ryanodine binding to WT RyR1 in the presence of 10 mM caffeine at 1 μ M Ca²⁺. Data are the mean \pm S.D. ($n = 4$). (E) Effect of hit compounds (30 μ M) on [³H]ryanodine binding to WT RyR2 at 10 μ M Ca²⁺. Data are the mean \pm S.D. ($n = 4$). * $P < 0.05$ compared with DMSO (one-way analysis of variance with Dunnett's test). 9AA, 9-aminoacridine; Ale, alexidine; Dan, dantrolene; Oxo, oxolinic acid.

influenced by both Ca²⁺-uptake pathways (i.e., SERCA Ca²⁺ pumps) and Ca²⁺-release pathways (i.e., RyRs, inositol 1,4,5-trisphosphate receptors, and other ER Ca²⁺-leak channels). Therefore, it seems possible that screening by [Ca²⁺]_{ER} measurements will also identify modulators for these pathways other than RyR1. However, all of the hit compounds targeted to RyR1. This high specificity may be reasonably explained by the fact that ER Ca²⁺-leak activity from the exogenously expressed mutant RyR1 channels is much greater than that via the endogenous Ca²⁺-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 the 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 have a serious impact on the results, although we cannot exclude the possibility of some bias.

Recently, Rebbeck et al. (2017) reported an excellent HTS strategy to identify RyR modulators using time-resolved fluorescence resonance energy transfer. By measuring changes in fluorescence resonance energy transfer signals between calmodulin and FKBP12.6 in the RyR1 channel,

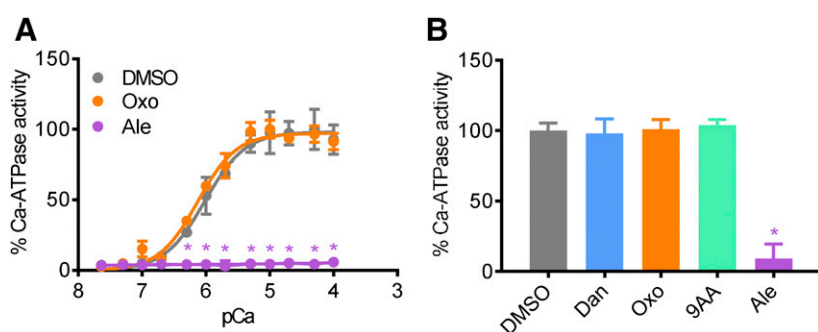


Fig. 6. Effects of hit compounds on Ca²⁺-ATPase activity. (A) Ca²⁺-dependent Ca²⁺-ATPase activity of SERCA was determined using skeletal muscle microsomes in the presence of 10 μ M hit compounds. Data are the mean \pm S.D. ($n = 3$). (B) Ca²⁺-ATPase activity under treatments with hit compounds at 100 μ M Ca²⁺ ($n = 3$). One hundred percent denotes the activity under DMSO treatment. * $P < 0.05$ compared with DMSO (one-way analysis of variance with Dunnett's test). 9AA, 9-aminoacridine; Ale, alexidine; Dan, dantrolene; Oxo, oxolinic acid.

they successfully identified six compounds that activate (cefatrizine, 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 $[Ca^{2+}]_{ER}$ ($F/F_0 = 0.60$ and 0.70 for disulfiram and tacrolimus, respectively) in the mutant RyR1 (Supplemental Table 2). This indicates activation of the RyR1 channel, which is consistent with their conclusion. However, chloroxine exhibited a slight reduction of $[Ca^{2+}]_{ER}$ ($F/F_0 = 0.80$) in our screening (Supplemental Table 2), 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 $[Ca^{2+}]_{ER}$ of RyR1 cells, but not RyR2 cells (Fig. 4). The RyR1-specific inhibition is a great advantage for clinical use. Unexpectedly, oxolinic acid did not affect $[^3H]$ ryanodine binding to RyR1 (Fig. 5). This raises the possibility of indirect action of oxolinic acid that it inhibits RyR1 by binding to some associated molecules, which were removed from RyR1 through the preparation of microsomes used for $[^3H]$ ryanodine binding. Alternatively, oxolinic acid might act as a pore blocker which does not affect $[^3H]$ ryanodine binding. Our chemical compound library includes 18 quinolone antibiotics (first to fourth generations), but no compounds other than oxolinic acid had significant effects on $[Ca^{2+}]_{ER}$ in the screening (Supplemental Table 2). The structure-function relationships will provide useful information for structural expansion of oxolinic acid.

9-Aminoacridine is a green fluorescent dye that has been used as an antimalarial and antimicrobial drug, and is now expected to be an anticancer agent (Sebestík et al., 2007). We found that 9-aminoacridine strongly inhibited $[^3H]$ 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 (Palade, 1987; Brunder et al., 1992). In our compound library, 9-aminoacridine derivatives (amsacrine, ethacridine, and mepacrine) had no effects (Supplemental Table 2). 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 $10 \mu M$ or higher concentrations (Fig. 5). It also strongly inhibited Ca^{2+} pumps in skeletal muscle (Fig. 6). Because the other biguanides (chlorhexidine, metformin, phenformin, phenylbiguanide, and proguanil) included in our library had no effects (Supplemental Table 2), 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 not be included in 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 1535 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 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.

Authorship Contributions

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

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Wrote or contributed to the writing of the manuscript: Murayama, Kurebayashi, Ogawa.

Note Added in Proof—Some descriptions in the Methods section with references were accidentally not included in the Fast Forward version published April 19, 2018. The Methods and references have now been corrected.

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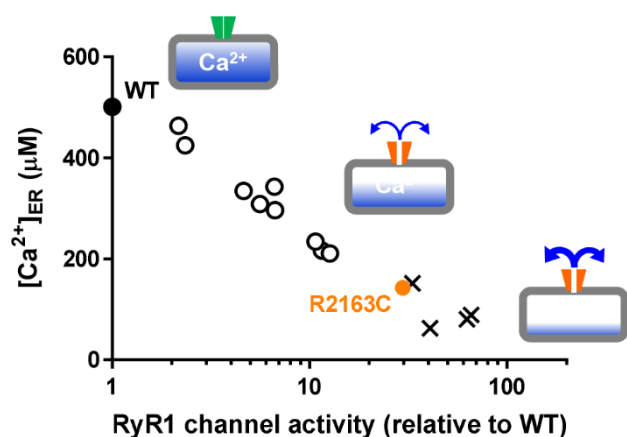
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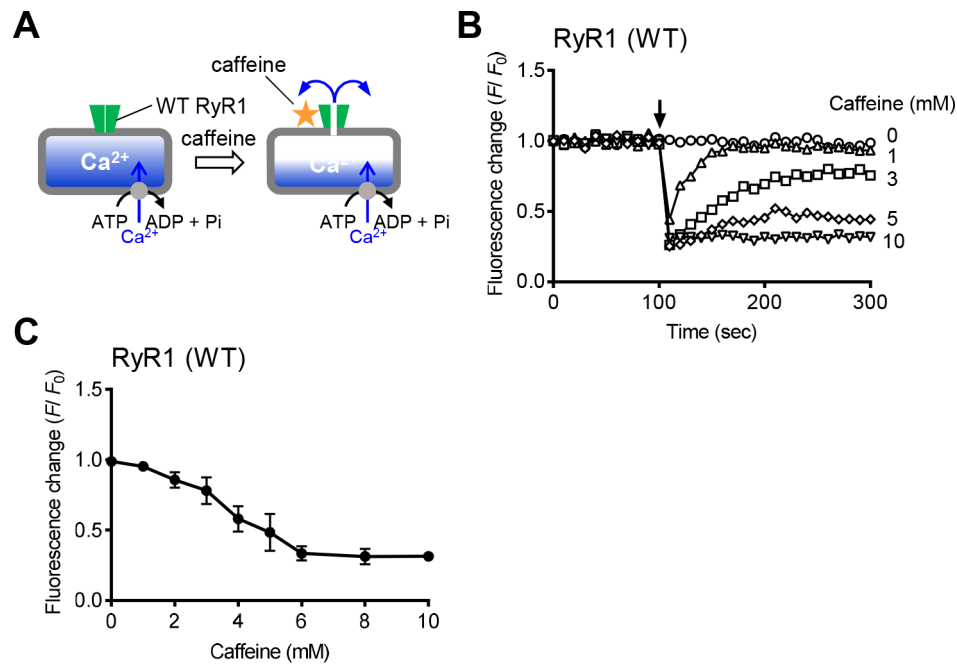
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Efficient high-throughput screening by ER Ca^{2+} measurement to identify inhibitors of ryanodine receptor Ca^{2+} -release channels

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Supplementary Fig. 1. Relationship between RyR1 channel activity and $[\text{Ca}^{2+}]_{\text{ER}}$ in HEK293 cells expressing wild-type (WT, *filled circle*) or mutations associated with MH (*open circles*) and MH/CCD (*crosses*) in the central region. Data are modified from Murayama et al. (Murayama et al., 2016). Schematic illustrations represent $[\text{Ca}^{2+}]_{\text{ER}}$ and Ca^{2+} leakage through RyR1 in the indicated states. Note that $[\text{Ca}^{2+}]_{\text{ER}}$ are reduced to inversely correlate with the channel activity of the mutant RyR1, indicating Ca^{2+} leakage from the ER.



Supplementary Fig. 2. Effect of caffeine on $[\text{Ca}^{2+}]_{\text{ER}}$ in the WT RyR1 cells. (A) In the WT RyR1 cells, caffeine activates the RyR1 channels to cause Ca^{2+} leakage from ER. (B) Effect of caffeine on time-lapse R-CEPIA1er fluorescence measurement of the WT RyR1 cells. Application of caffeine at 1-10 mM (arrows) resulted in a rapid decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ followed by gradual recovery toward their respective steady-state levels. (C) Caffeine dose-dependency of steady-state level of R-CEPIA1er fluorescence of the WT RyR1 cells. The steady-state level was reduced with caffeine in a dose-dependent manner. Data are the mean \pm SD ($n = 3$).

Supplementary TABLE 1. Compounds that increased F/F_0 in WT cells by >3 SDs.

Compounds	Mean F/F_0 (RC)	Mean F/F_0 (WT)	ΔF (w/o cell)	ΔF (w cell)
Daunorubicin	3.88	2.36	18.4 ± 3.0	17.3 ± 0.2
Doxorubicin	4.97	3.79	28.2 ± 2.0	26.9 ± 1.8
Epirubicin	3.75	3.05	20.6 ± 2.0	17.9 ± 0.5
Erythrosine	10.15	6.11	53.0 ± 2.4	48.7 ± 1.5
Homidium Bromide	2.66	1.69	6.5 ± 0.6	7.3 ± 0.7
Oxidopamine	2.93	3.13	13.4 ± 1.1	12.8 ± 2.2
Pararosaniline	1.37	1.2	2.5 ± 0.1	2.4 ± 0.4
Pyrvinium	1.66	1.35	5.3 ± 0.4	7.6 ± 0.8

Mean F/F_0 represents ratio of the averaged R-CEPIA1er fluorescence for the last 100 seconds (F) to that for the first 100 seconds (F_0) in R2163C (RC) and WT RyR1 cells induced by the compounds. ΔF represents fluorescence changes by compounds in the presence (w cell) and absence (w/o cell) of the WT RyR1 cells. All the 8 compounds that increased F/F_0 in WT cells by >3 SDs exhibited autofluorescence.

Supplementary TABLE 2. Effects of hit and related compounds on $[Ca^{2+}]_{ER}$ of mutant and WT RyR1 cells.

Compounds	Mean F/F_0 (Mutant RyR1)	Mean F/F_0 (WT RyR1)
Oxolinic Acid	1.66	1.01
Cinoxacin	1.05	0.98
Ciprofloxacin	1.04	0.98
Enoxacin	0.96	0.97
Enrofloxacin	0.97	1.02
Flumequine	0.98	1.01
Gatifloxacin	1.00	0.99
Gemifloxacin	0.92	1.00
Levofloxacin	0.94	0.99
Lomefloxacin	0.89	0.95
Moxifloxacin	0.87	0.95
Norfloxacin	0.99	0.99
Ofloxacin	0.95	0.96
Orbifloxacin	0.95	0.94
Pefloxacin	0.96	0.93
Pipemidic Acid	0.94	0.90
Piromidic Acid	0.97	0.97
Sarafloxacin	1.01	0.94
9-Aminoacridine	1.62	0.98
Amsacrine	0.88	1.04
Ethacridine	1.06	1.04
Mepacrine	1.00	0.96
Alexidine	1.71	0.84
Chlorhexidine	0.93	0.97
Metformin	0.89	1.01
Phenformin	0.99	1.00
Phenylbiguanide	0.96	0.98
Proguanil	0.96	0.95
Compounds described in Rebbeck et al. (2017)		
Chloroxine	0.80	0.96
Disulfiram	0.60	0.78
Tacrolimus	0.70	1.00

Hit compounds are indicated in *bold* and the related compounds in the library used in this study are listed below. Values indicate F/F_0 of R-CEPIA1er fluorescence in mutant (R2163C) and WT RyR1 cells induced by the compounds (see **Fig. 3**).