



- (51) International Patent Classification:
C07K 14/415 (2006.01) *C07K 14/43* (2006.01)
- (21) International Application Number:
PCT/US2015/065876
- (22) International Filing Date:
15 December 2015 (15.12.2015)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
62/092,185 15 December 2014 (15.12.2014) US
- (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US).
- (72) Inventors: CHEN, Jau-Nian. KWON, Ohyun. HUANG, Jie. SHIMIZU, Hirohito. LU, Kui. SCHREDELSEKER, Johann. FAN, Yi, Chiao.
- (74) Agent: ORSAK, Thomas; Norton Rose Fulbright US LLP, 98 San Jacinto Blvd., Suite 1100, Austin, TX 78701 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

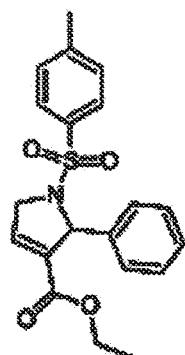
Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

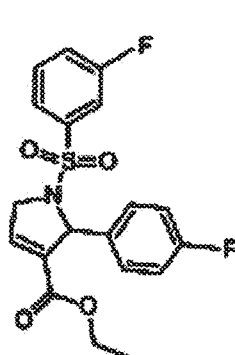
(54) Title: ANTI-ARRHYTHMICITY AGENTS

FIG. 3A

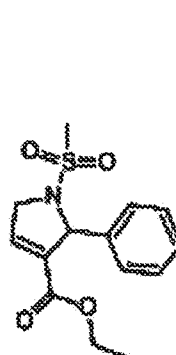
efsevin



OK-C125



OK-C19



(57) Abstract: Agents, compositions, and methods for regulating cardiac rhythmicity are disclosed.

ANTI-ARRHYTHMICITY AGENTS**CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional Application No. 62/092,185 filed December 15, 2014, the contents of which are incorporated into the present application by reference.

FEDERAL GOVERNMENT GRANT INFORMATION

This invention was made with Government support under Grant Nos. HL09698, GM071779, and GM081282 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION**A. Field Of The Invention**

The present invention relates to agents, compositions, and methods for regulating cardiac rhythmicity.

B. Description of Related Art

Cardiac diseases are the leading cause of death in Western countries. Many of these conditions, including hypertrophy, heart failure and arrhythmias, have a root in aberrant Ca^{2+} homeostasis. Identifying clinically relevant targets and pharmacological agents that can effectively modulate cardiac Ca^{2+} homeostasis may lead to the development of new therapeutic strategy for cardiac diseases.

The present invention addresses such needs for targets and agents for regulating cardiac rhythmicity.

SUMMARY OF THE INVENTION

In one embodiment, there are methods of regulating cardiac rhythmicity in a subject, comprising potentiating mitochondrial Ca^{2+} uptake by inducing VDAC2 or VDAC1 overexpression in the subject to restore rhythmic contraction, inducing overexpression of or activating VDAC (VDAC2 or VDAC1) and/or MCU (MCU or MICU1) complex, or administering to the subject in need thereof an agent effective to induce Ca^{2+} transporting activity of VDAC2 or VDAC1.

In some embodiments, optionally in combination with any or all of the various

embodiments disclosed herein, there are methods of regulating cardiac rhythmicity in a subject in need thereof comprising administering to the subject a composition that comprises an agent or compound that increases the activity of VDAC2 or VDAC1. In some embodiments, the agent or compound binds to VDAC2 or VDAC1. In some embodiments, the agent or compound increases Ca²⁺ transporting activity of VDAC2.

The agent or compound disclosed in the embodiments may be any agent or compound of the formulas described herein or compounds described herein. In some embodiments, one or more specific compounds described herein may be excluded.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in a composition. In some embodiments, the composition comprises a pharmaceutically acceptable carrier.

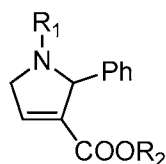
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the subject suffers from a disorder related to cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca²⁺ handling. Such disorder includes, for example, cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), or cardiomyopathy.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, inducing VDAC2 or VDAC1 overexpression in the subject is via gene therapy.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the agent is a VDAC2 or VDAC1 gene product.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the agent is a VDAC2 or VDAC1 protein, or a VDAC2 or VDAC1 RNA.

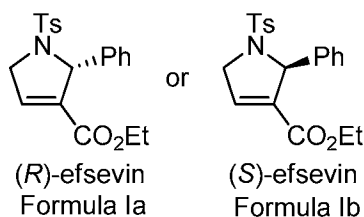
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the agent is the compound of Formula I or a derivative thereof, wherein compound of Formula I comprises



Formula I

wherein: R₁ is tosyl, or mesyl group; and wherein R₂ is a hydrocarbyl group with or without

a heteroatom or wherein the carboxylic ester of R₂ is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine), and wherein the compound is effective to potentiate mitochondrial Ca²⁺ uptake so as to modulate cardiac rhythmicity in a subject, provided that when R₁ is tosyl and R₂ is ethyl, the compound is in an optionally active (e.g., substantially enantiomerically pure) form Formula Ia or Formula Ib:



In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, R₂ is methyl, ethyl, C3-C6 short alkyl, or menthyl group.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, R₂ is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

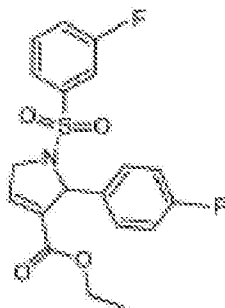
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the agent is efsevin.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the efsevin is an efsevin enantiomer.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound of Formula I is in a composition.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the composition comprises a pharmaceutically acceptable carrier.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the agent is the compound of Formula II, wherein compound of Formula II comprises:



Formula II

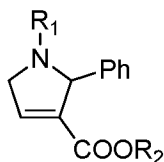
or wherein the carboxylic ester is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine).

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound of Formula II is in a composition.

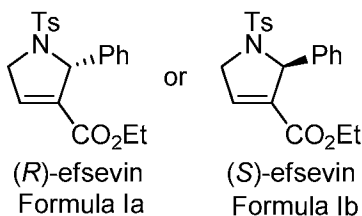
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the composition comprises a pharmaceutically acceptable carrier.

In another aspect, embodiments provide an anti-arrhythmicity compound of structure of Formula I or derivative thereof:



Formula I

wherein: R_1 is tosyl, or mesyl group; and wherein R_2 is a hydrocarbonyl group with or without a heteroatom or wherein the carboxylic ester of R_2 is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine), and wherein the compound is effective to potentiate mitochondrial Ca^{2+} uptake so as to modulate cardiac rhythmicity in a subject, provided that when R_1 is tosyl and R_2 is ethyl, the compound is in an optionally active (e.g., substantially enantiomerically pure) form Formula Ia or Formula Ib:

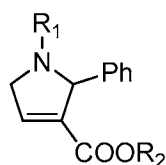


In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is methyl, ethyl, C3-C6 short alkyl, or menthyl group.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.

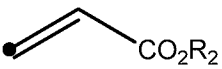
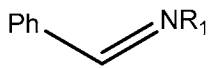
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

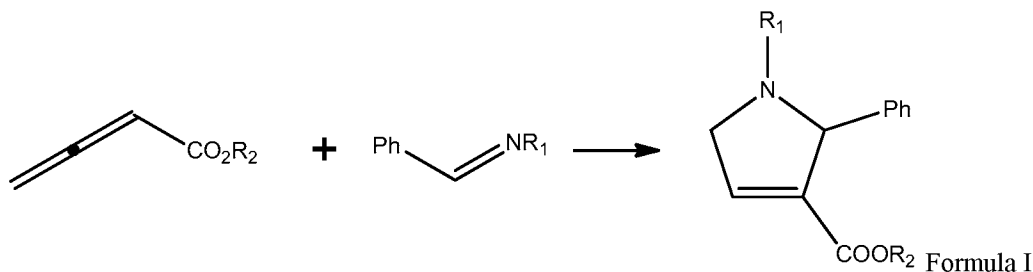
In a further aspect, embodiments involve methods of forming the compound of Formula I:



Formula I

where R_1 is tosyl, or mesyl group; and R_2 is a hydrocarbyl group with or without a heteroatom or wherein the carboxylic ester of R_2 is attached to a mono-N-Boc protected

2,2'-(ethylenedioxy)bis(ethylamine), comprising reacting  with  according to a reaction of Scheme I



(Scheme I)

to form the compound of Formula I.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the reaction of Scheme I is carried out under asymmetric synthesis conditions.

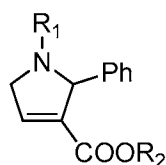
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the method further comprises performing chiral resolution of compound of Formula I to yield *R*- or *S*- enantiomers of the compound in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the chiral resolution is performed on an HPLC chiral stationary phase.

In some embodiments, optionally in combination with any or all of the various
5 embodiments disclosed herein, the chiral resolution is achieved by reacting the compound with a chiral agent.

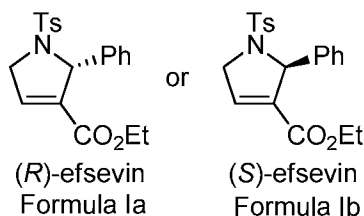
In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the chiral agent is menthol.

In a further aspect, there is provided a composition comprising the compound of
10 Formula I or a derivative thereof:



Formula I

wherein: R_1 is tosyl, or mesyl group; and wherein R_2 is a hydrocarbyl group with or without a heteroatom or wherein the carboxylic ester of R_2 is attached to a mono-N-Boc protected
15 2,2'-(ethylenedioxy)bis(ethylamine), and wherein the compound is effective to potentiate mitochondrial Ca^{2+} uptake so as to modulate cardiac rhythmicity in a subject, provided that when R_1 is tosyl and R_2 is ethyl, the compound is in an optionally active (e.g., substantially enantiomerically pure) form Formula Ia or Formula Ib:



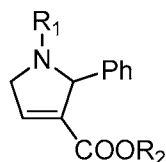
20 In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is methyl, ethyl, C3-C6 short alkyl, or menthyl group.

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is a C1-C10 straight or
25 branched, acyclic or cyclic alkyl group, or aryl group.

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

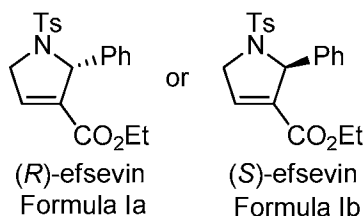
In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation suitable for administration to a subject.

In a further aspect of the present invention, it is provided a method of forming a composition, comprising providing a compound of Formula I or a derivative thereof in an effective amount, and forming the composition, wherein the compound of Formula I comprises:



Formula I

wherein: R_1 is tosyl, or mesyl group; and wherein R_2 is a hydrocarbyl group with or without a heteroatom or wherein the carboxylic ester of R_2 is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine), and wherein the compound is effective to potentiate mitochondrial Ca^{2+} uptake so as to modulate cardiac rhythmicity in a subject, provided that when R_1 is tosyl and R_2 is ethyl, the compound is in an optionally active (e.g., substantially enantiomerically pure) pure form Formula Ia or Formula Ib:



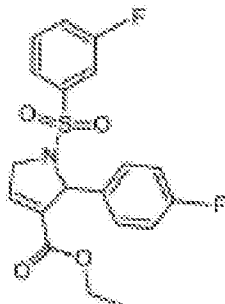
In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is methyl, ethyl, C3-C6 short alkyl, or menthyl group.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, R_2 is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation suitable for administration to a subject.

In another aspect, embodiments provide an anti-arrhythmicity compound of structure of Formula II:

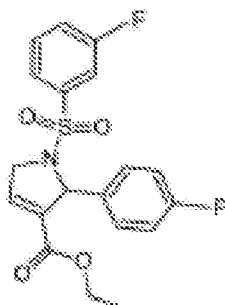


Formula II

5 or wherein the carboxylic ester is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine).

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

10 In a further aspect, there is provided a composition comprising the compound of Formula II:



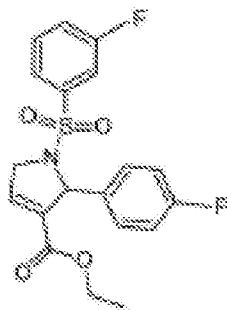
Formula II

15 or wherein the carboxylic ester is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine).

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

20 In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation suitable for administration to a subject.

In a further aspect of the present invention, it is provided a method of forming a composition, comprising providing a compound of Formula II in an effective amount, and forming the composition, wherein the compound of Formula II comprises:



Formula II

or wherein the carboxylic ester is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine).

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation suitable for administration to a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-G show test results demonstrating that efsevin restores rhythmic cardiac contractions in zebrafish tremblor embryos. (A,B) Fractional shortening (FS) deduced from line scans across the atria of *Tg(myf7:GFP)* embryonic hearts at 48 hpf. Rhythmically alternating systoles and diastoles were recorded from vehicle- or efsevin- treated wild type and efsevin-treated *tre* embryos, while only sporadic unsynchronized contractions were recorded from vehicle-treated *tre* embryos. While cardiac contraction was not observed in *tre*, efsevin-treated wild type and *tre* hearts have similar levels of FS to those observed in control hearts. Ventricular FS of wild type v.s. wild type+efsevin vs. *tre*+efsevin: $39 \pm 0.6\%$, $n=8$ vs. $39 \pm 1\%$, $n=10$ vs. $35 \pm 3\%$, $n=6$; and Atrial FS: $37 \pm 1\%$, $n=11$ vs. $35 \pm 2\%$, $n=11$ vs. $33 \pm 2\%$, $n=15$. (C) While efsevin restored a heart rate of 46 ± 2 beats per minute (bpm) in *tre* embryos, same treatment does not affect the heart rate in wild type embryos (126 ± 2 bpm in vehicle-treated embryos vs. 123 ± 3 bpm in efsevin-treated wild-type embryos). ***, $p < 0.001$ by one-way ANOVA. (D) Dose-dependence curve for efsevin. The *tre* embryos were treated with various concentrations of efsevin from 24 hpf and cardiac contractions were analyzed at 48 hpf. (E-G) Representative time traces of local field potentials for wild type (E), *tre* (F) and efsevin-treated *tre* (G) embryos clearly display periods of regular, irregular, and restored

periodic electrical activity.

Figures 2A-F show test results demonstrating efsevin reduces arrhythmogenic events in ES cell-derived cardiomyocytes (A-D). Representative graph of rhythmic Ca^{2+} transients detected in mESC-CMs of line-scan analysis of Ca^{2+} transients in mESC-CMs after 10 days of differentiation (A). After treatment with 10 mM Ca^{2+} for 10 minutes, the EB showed an irregular pattern of Ca^{2+} transients (B). Efsevin treatment restores regular Ca^{2+} transients under Ca^{2+} overload conditions in mESC-CMs (C). (D) Plotted intervals between peaks of Ca^{2+} signals detected in mESC-CMs prior to treatment (control), in 10 mM $\text{Ca}^{2+}_{\text{ext}}$ (Ca^{2+}) and in 10 mM $\text{Ca}^{2+}_{\text{ext}}$ +10 μM efsevin (Ca^{2+} +efsevin). (E,F) Plotted intervals of contractions detected in EBs prior to treatment (control), in 10mM $\text{Ca}^{2+}_{\text{ext}}$ (Ca^{2+}) and in 10 mM $\text{Ca}^{2+}_{\text{ext}}$ + 10 μM efsevin (Ca^{2+} + efsevin) for mouse ESC-CMs (E) and 5 mM $\text{Ca}^{2+}_{\text{ext}}$ (Ca^{2+}) and in 5 mM $\text{Ca}^{2+}_{\text{ext}}$ + 5 μM efsevin (Ca^{2+} + efsevin) for human ESC-CMs (F). ***, $p < 0.001$ by F-test.

Figures 3A-E show test results demonstrating VDAC2 is a protein target of efsevin. (A) Structures of efsevin and two derivatives, OK-C125 and OK-C19. (B) Efsevin and OK-C125 restored rhythmic contractions in the majority of *tremblor* embryos, whereas OK-C19 failed to rescue the *tremblor* phenotype. (C) Structures of linker-attached compounds (indicated by superscript L). (D) Compounds efsevin^L and OK-C125^L retained their ability to restore rhythmic contractions in NCX1hMO injected embryos, while the inactive derivative OK-C19^L was still unable to induce rhythmic contraction. (E) Mass Spectrometry identification of VDAC 2, a 32kD band pulled down with affinity agarose beads covalently linked with efsevin (efsevin^{LB}) or OK-C125 (OK-C125^{LB}) that was sensitive to competition with a 100 fold excess free efsevin^L. The 32 kD band was not detected in proteins eluted from beads capped with ethanolamine alone (beads^C) or beads linked to OK-C19 (OK-C19^{LB}). Peptides identified by mass spectrometry (underlined) account for 30% of the total sequence (SEQ ID NO: 1).

Figures 4A-F show test results demonstrating VDAC2 restores rhythmic cardiac contractions in *tre*. In situ hybridization analysis showed that VDAC2 is expressed in embryonic hearts at 36 hpf and 48 hpf. (A) Injection of 25 pg *in-vitro* synthesized VDAC2 mRNA restored cardiac contractions in $52.9 \pm 12.1\%$ ($n=78$) of one-day-old *tre* embryos, compared to $21.8 \pm 5.1\%$ in uninjected siblings ($n=111$). (B) Schematic diagram of *myl7:VDAC2* construct. (C) While only ~20% of *myl7:VDAC2*;NCX1hMO embryos have coordinated contractions ($n=116$), $52.3 \pm 2.4\%$ of these embryos established persistent,

rhythmic contractions after TBF induction of VDAC2 (n=154). (D) On average, $71.2 \pm 8.8\%$ efsevin treated embryos have coordinated cardiac contractions (n=131). Morpholino antisense oligonucleotide knockdown of VDAC2 (MO^{VDAC2}) attenuates the ability of efsevin to suppress cardiac fibrillation in *tre* embryos ($45.3 \pm 7.4\%$ embryos with coordinated contractions, n=94). (E) Efsevin treatment restores coordinated cardiac contractions in NCX1MO embryos, only $54.1 \pm 3.6\%$ $VDAC2^{zfn/zfn}$;NCX1MO embryos have coordinated contractions (n=250). (F) Diagram of Zinc finger target sites. $VDAC2^{zfn/zfn}$ carries a 34 bp deletion in exon 3 which results in a premature stop codon (asterisk).

Figures 5A-5D show test results demonstrating efsevin enhances mitochondrial Ca^{2+} uptake. HeLa cells were transfected with a flag-tagged zebrafish VDAC2 ($VDAC2^{flag}$), immunostained against the flag epitope and counterstained for mitochondria with MitoTracker Orange and for nuclei with DAPI. (A) Representative traces of mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) detected by Rhod2. Arrows denote the addition of Ca^{2+} . Mitochondrial Ca^{2+} uptake was assessed when VDAC2 was overexpressed (left), cells were treated with 1 μ M efsevin (middle) and combination of both at suboptimal doses (right). Control-traces with ruthenium red (RuRed) show mitochondrial specificity of the signal. (B) Representative traces of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) changes upon the application of 7.5 μ M IP_3 in the presence (+) or absence (-) of RuRed. Mitochondrial Ca^{2+} uptake was assessed by the difference of the - and + RuRed conditions normalized to the total release (n=4; mean $\pm$ SE). (C) MEFs overexpressing zebrafish VDAC2 (polycistronic with mCherry) were stimulated with 1 μ M ATP in a nominally Ca^{2+} free buffer. Changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were imaged using fura2 and mitochondria-targeted inverse pericam, respectively. Black and gray traces show the $[Ca^{2+}]_c$ (in nM) and $[Ca^{2+}]_m$ (F_0/F mtpericam) time courses in the absence (left) or present (right) of efsevin. (D) Bar charts: Cell population averages for the peak $[Ca^{2+}]_c$ (left), the corresponding $[Ca^{2+}]_m$ (middle), and the coupling time (time interval between the maximal $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ responses) in the presence (black, n=24) or absence (gray, n=28) of efsevin.

Figures 6A-C show effects of efsevin on isolated cardiomyocytes. (A) Electrically paced Ca^{2+} transients at 0.5 Hz (top). Normalized quantification of Ca^{2+} transient parameters reveals no difference for transient amplitude (efsevin-treated at $98.6 \pm 4.5\%$ of vehicle-treated) and time to peak ($95 \pm 3.9\%$), but a significant decrease for the rate of decay ($82.8 \pm 4\%$ of vehicle- for efsevin-treated) (lower panel). (B) Representation of typical Ca^{2+} sparks of vehicle- and efsevin treated cardiomyocytes (top). No differences were observed for spark

frequency ($101.1 \pm 7.7\%$ for efsevin- compared to vehicle-treated), maximum spark amplitude ($101.6 \pm 2.5\%$) and Ca^{2+} release flux ($98.7 \pm 2.8\%$). In contrast, the decay phase of the single spark was significantly faster in efsevin treated cells ($82.5 \pm 2.1\%$ of vehicle-treated). Consequently, total duration of the spark was reduced to $85.7 \pm 2\%$ and the total width was reduced to $89.5 \pm 1.4\%$ of vehicle-treated cells. *, $p < 0.05$; ***, $p < 0.001$. (C) Quantitative analysis of spontaneous Ca^{2+} waves spanning more than half of the entire cell. Addition of $1 \mu\text{M}$ efsevin reduced Ca^{2+} waves to approximately half. Increasing the concentration of efsevin to $10 \mu\text{M}$ further reduced the number of spontaneous Ca^{2+} waves and $25 \mu\text{M}$ efsevin almost entirely blocked the formation of Ca^{2+} waves.

Figures 7A-7E show test results demonstrating that mitochondria regulate cardiac rhythmicity through a VDAC2-dependent mechanism. MCU and MICU1 are expressed in the developing zebrafish hearts. (A) Overexpression of MCU is sufficient to restore coordinated cardiac contractions in *tre* embryos ($47.1 \pm 1.6\%$ embryos, $n=112$ as opposed to $18.3 \pm 5.3\%$ of uninjected siblings, $n=64$) while this effect is significantly attenuated when co-injected with morpholino antisense oligonucleotide targeted to VDAC2 ($27.1 \pm 1.9\%$ embryos, $n=135$). (B) Suboptimal overexpression of MCU (MCU^S) and VDAC2 (VDAC2^S) in combination is able to suppress cardiac fibrillation in *tre* embryos ($42.9 \pm 2.6\%$ embryos, $n=129$). (C) The ability of VDAC2 to restore rhythmic contractions in *tre* embryos ($48.5 \pm 3.5\%$ embryos, $n=111$) is significantly attenuated when MCU is knocked down by antisense oligonucleotide (MO^{MCU}) ($25.6 \pm 2.4\%$ embryos, $n=115$). (D) Overexpression of MICU1 is sufficient to restore rhythmic cardiac contractions in *tre* embryos ($49.3 \pm 3.4\%$ embryos, $n=127$ compared to $16.8 \pm 1.4\%$ of uninjected siblings, $n=150$). This effect is abrogated by VDAC2 knockdown (MO^{VDAC2} , $25.3 \pm 5.5\%$ embryos, $n=97$). (E) Suboptimal overexpression of MICU1 (MICU1^S) and VDAC2 (VDAC2^S) in combination is able to restore rhythmic cardiac contractions in *tre* embryos ($48.6 \pm 6.0\%$, $n=106$). Error bars represent s.d.; * $p < 0.05$; *** $p < 0.001$.

Figure 8 shows that local Ca^{2+} delivery between IP3 receptors and VDAC2. V1/V3DKO MEFs were stimulated with $100 \mu\text{M}$ ATP (left) or $2 \mu\text{M}$ thapsigargin (Tg) (right). Changes in $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ were imaged using fura 2 and mitochondria targeted inverse pericam, respectively. Representative traces obtained in 3 cells are shown.

Figures 9A-G show that mitochondria regulate cardiac rhythmicity through a VDAC dependent mechanism.

A) Injection of 25pg in-vitro synthesized VDAC1 and VDAC2 mRNA restored cardiac contractions in $53.0 \pm 10.2\%$ ($n=126$) and $52.9 \pm 12.1\%$ ($n=78$) of one-day-old *tre*

embryos, respectively, compared to $21.8 \pm 5.1\%$ in uninjected siblings ($n=111$).

B) While only $\sim 20\%$ of *myl7:VDAC2;NCX1hMO* embryos have coordinated contractions ($n=116$), $52.3 \pm 2.4\%$ of these embryos established persistent, rhythmic contractions after TBF induction of VDAC2 ($n=154$).

5 C) On average, $71.2 \pm 8.8\%$ efsevin treated embryos have coordinated cardiac contractions ($n=131$). Morpholino antisense oligonucleotide knockdown of VDAC2 (MO^{VDAC2}) or VDAC1 (MO^{VDAC1}) attenuates the ability of efsevin to suppress cardiac fibrillation in *tre* embryos ($45.3 \pm 7.4\%$ and $46.9 \pm 10.7\%$ embryos with coordinated contractions, $n=94$ and 114 , respectively). Knocking down VDAC1/2 simultaneously further
10 suppresses efsevin's effect ($30.3 \pm 6.3\%$, $n=75$).

D) Efsevin treatment restores coordinated cardiac contractions in $76.2 \pm 8.7\%$ NCX1MO embryos, only $54.1 \pm 3.6\%$ VDAC2^{zfn/zfn}; NCX1MO embryos and $35.7 \pm 7.1\%$ VDAC2^{zfn/zfn}; VDAC1MO; NCX1MO embryos have coordinated contractions ($n=250$).

E) Overexpression of MCU is sufficient to restore coordinated cardiac contractions in
15 *tre* embryos ($47.1 \pm 1.6\%$ embryos, $n=112$ as opposed to $18.3 \pm 5.3\%$ of uninjected siblings, $n=64$) while this effect is significantly attenuated when co-injected with morpholino antisense oligonucleotide targeted to VDAC2 ($27.1 \pm 1.9\%$ embryos, $n=135$).

F) Suboptimal overexpression of MCU (MCU^S) and VDAC2 ($VDAC2^S$) in combination is able to suppress cardiac fibrillation in *tre* embryos ($42.9 \pm 2.6\%$ embryos,
20 $n=129$).

G) The ability of VDAC2 to restore rhythmic contractions in *tre* embryos ($48.5 \pm 3.5\%$ embryos, $n=111$) is significantly attenuated when MCU is knocked down by antisense oligonucleotide (MO^{MCU}) ($25.6 \pm 2.4\%$ embryos, $n=115$). Error bars represent s.d.; * $p < 0.05$; *** $p < 0.001$.

25 **Figure 10** shows resolution of (R)- and (S)-efsevin from a mixture of (R)- and (S)-efsevin through HPLC separation.

Figure 11 shows purification of (R)-efsevin through HPLC separation.

Figure 12 shows purification of (S)-efsevin through HPLC separation.

Figure 13 shows resolution of (R)- and (S)-efsevin from a racemic mixture of efsevin
30 through HPLC separation.

Figure 14 shows ^1H NMR (top) and ^{13}C NMR (bottom) of (R)-Efsevin menthol ester.

Figure 15 shows ^1H NMR (top) and ^{13}C NMR (bottom) of (S)-Efsevin menthol ester.

Figure 16A and B show active compounds found through forward chemical genetics.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The term “effective amount”, as used herein, is an amount of an agent that is sufficient to produce a statistically significant, measurable change of a condition in cardiac rhythmicity as compared with the condition in cardiac rhythmicity without using the agent. Such effective amounts can be gauged in clinical trials as well as animal studies. Such a statistically significant, measurable, and positive change of a condition in cardiac rhythmicity using the agent disclosed herein as compared with the condition in the cardiac rhythmicity without using the agent is referred to as being an “improved condition”.

As used herein, the term “agent” refers to an agent that capable of potentiating mitochondrial Ca^{2+} uptake to effect VDAC2 expression in a subject. In some embodiments, the term agent can refer to as “anti-arrhythmicity” drug or compound.

As used herein, the term "subject" as used herein is any vertebrate. Subjects include individuals in need of drug (e.g. an agent disclosed herein such as efsevin) treatment (patients) and individuals not in need of drug treatment (e.g. normal healthy volunteers). Humans are preferred subjects and patients.

"Treat" or "treatment" refers to any treatment of a disorder or disease, such as preventing the disorder or disease from occurring in a subject which may be predisposed to the disorder or disease, but has not yet been diagnosed as having the disorder or disease; inhibiting the disorder or disease, e.g., arresting the development of the disorder or disease, relieving the disorder or disease, causing regression of the disorder or disease, relieving a condition caused by the disease or disorder or reducing the symptoms of the disease or disorder.

As used herein, the term “disorder” generally refers to a condition related to cardiac cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca^{2+} handling. Such disorder includes, for example, cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia, or cardiomyopathy.

As used herein, the term “derivative” refers to a form of the agent or compound disclosed herein, which derivative is capable of generating an active species or moiety in vitro or in vivo having anti-arrhythmicity activities as is the agent or compound disclosed herein. Non-limiting examples of such “derivative” includes prodrug or metabolite.

As used herein, the term “optically active” refers to the compound Formula I disclosed herein that is not a 50/50 R/S racemic mixture of the compound of Formula I.

As used herein, the term “substantially enantiomerically pure” refers to the purity of S- or R- enantiomer of the compound of Formula I of about 60% -- about 100%, of about 70% - about 100%, of about 80% - about 100%, of about 90% - about 100%, of about 95% - about 100%, e.g., of about 75%, of about 80%, of about 85%, or of about 99%.

As used herein, the term “enantiomer” refers to a compound disclosed herein that is optically active or substantially enantiomerically pure.

As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" include one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

B. Mitochondrial Ca²⁺ uptake regulates cardiac rhythmicity

In an aspect of the present invention, it is provided a method of regulating cardiac rhythmicity in a subject, comprising potentiating mitochondrial Ca²⁺ uptake by inducing VDAC2 or VDAC1 overexpression in the subject to restore rhythmic contraction, inducing overexpression of or activating VDAC (VDAC2 or VDAC1) and/or MCU (MCU or MICU1) complex, or administering to the subject in need thereof an agent effective to induce Ca²⁺ transporting activity of VDAC2 or VDAC1.

In some embodiments, optionally in combination with any or all of the various embodiments disclosed herein, the subject suffers from a disorder related to cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca²⁺ handling. Such disorder includes, for example, cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus

syndrome, catecholaminergic polymorphic ventricular tachycardia, or cardiomyopathy.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, inducing VDAC2 or VDAC1 overexpression in the subject is via gene therapy.

5 In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the agent is a VDAC2 or VDAC1 gene product.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the agent is a VDAC2 or VDAC1 protein,
10 or a VDAC2 or VDAC1 RNA.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the agent is the compound of Formula I, which is described in detail below. For concise description of the present invention, the description of the compound of Formula I is not repeated here but is fully incorporated hereto
15 by reference.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the agent is efsevin.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the efsevin is an efsevin enantiomer.

20 In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the compound of Formula I is in a composition.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the composition comprises a
25 pharmaceutically acceptable carrier.

C. Gene therapy

In some embodiments, potentiating mitochondrial Ca^{2+} uptake can be achieved by overexpression of VDAC2 or VDAC1 by delivery to a subject in need thereof a VDAC2- or VDAC1-encoding gene sequence using a viral or non-viral vector. Vectors for
30 transduction of a VDAC2- or VDAC1-encoding sequence are well known in the art. While overexpression using a strong non-specific promoter, such as a CMV promoter, can be used, it can be helpful to include a tissue- or cell-type-specific promoter on the expression construct. Further, treatment can include the administration of viral vectors that

drive the overexpression of VDAC2 or VDAC1 proteins in infected host cells. Viral vectors are well known to those skilled in the art.

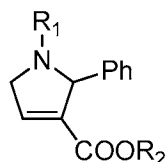
These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked VDAC2 or VDAC1 encoding nucleic acid segment into the selected expression/delivery vector, many equivalent vectors for the practice of the methods described herein can be generated. It will be appreciated by those of skill in the art that cloned genes readily can be manipulated to alter the amino acid sequence of a protein.

The cloned gene for VDAC2 or VDAC1 can be manipulated by a variety of well-known techniques for *in vitro* mutagenesis, among others, to produce variants of the naturally occurring human protein, herein referred to as muteins or variants or mutants of VDAC2 or VDAC1, which may be used in accordance with the methods and compositions described herein. The variation in primary structure of muteins of VDAC2 or VDAC1 protein useful in the invention, for instance, may include deletions, additions and substitutions. The substitutions may be conservative or non-conservative. The differences between the natural protein and the mutein generally conserve desired properties, mitigate or eliminate undesired properties and add desired or new properties. The VDAC2 or VDAC1 protein can also be a fusion polypeptide, fused, for example, to a polypeptide that targets the product to a desired location, or, for example, a tag that facilitates its purification, if so desired. Fusion to a polypeptide sequence that increases the stability of the VDAC2 protein is also contemplated. For example, fusion to a serum protein, e.g., serum albumin, can increase the circulating half-life of a VDAC2 or VDAC1 protein. Tags and fusion partners can be designed to be cleavable, if so desired. Another modification specifically contemplated is attachment, e.g., covalent attachment, to a polymer. In one aspect, polymers such as polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG) can increase the *in vivo* half-life of proteins to which they are conjugated. Methods of PEGylation of polypeptide agents are well known to those skilled in the art, as are considerations of, for example, how large a PEG polymer to use. In another aspect, biodegradable or absorbable polymers can provide extended, often localized, release of polypeptide agents. Such synthetic bioabsorbable, biocompatible polymers, which may release proteins over several weeks or months can include, for example, poly- α -hydroxy acids (e.g. polylactides, polyglycolides and their copolymers), polyanhydrides, polyorthoesters, segmented block copolymers of polyethylene glycol and polybutylene

terephthalate (PolyactiveTM), tyrosine derivative polymers or poly(ester-amides). Suitable bioabsorbable polymers to be used in manufacturing of drug delivery materials and implants are discussed e.g. in U.S. Pat. Nos. 4,968,317; 5,618,563, among others, and in "Biomedical Polymers" edited by S. W. Shalaby, Carl Hanser Verlag, Munich, Vienna, New York, 1994 and in many references cited in the above publications. The particular bioabsorbable polymer that should be selected will depend upon the particular patient that is being treated.

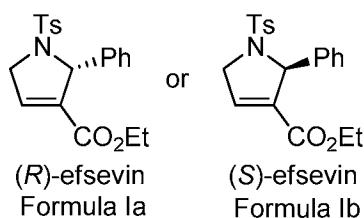
D. Anti-arrhythmicity compound

In another aspect of the present invention, it is provided an anti-arrhythmicity compound of structure of Formula I or a derivative thereof:



Formula I

wherein: R₁ is tosyl, or mesyl group; and wherein R₂ is a hydrocarbonyl group with or without a heteroatom, and wherein the compound is effective to potentiate mitochondrial Ca²⁺ uptake so as to modulate cardiac rhythmicity in a subject, provided that when R₁ is tosyl and R₂ is ethyl, the compound is in an optionally active (e.g., substantially enantiomerically pure) form Formula Ia or Formula Ib:

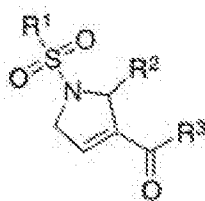


In some embodiments of the invention compound, optionally in combination with any or all of the various embodiments disclosed herein, R₂ is methyl, ethyl, C3-C6 short alkyl, or menthyl group.

In some embodiments of the invention compound, optionally in combination with any or all of the various embodiments disclosed herein, R₂ is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.

In some embodiments of the invention compound, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

Non-limiting examples of derivatives of Formula I include Formula Ic:



Formula Ic

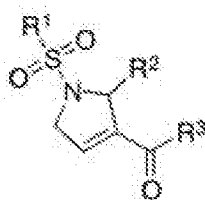
wherein: R₁ is an alkane, phenyl, heteroaryl, or substituted phenyl group; R₂ is a phenyl, heteroaryl, substituted phenyl, or hydrocarbyl group with or without a heteroatom, and R₃ is an alkoxy, amino, or amino ether. Non-limiting examples of a substituted phenyl group is a phenyl group wherein one or more hydrogens are replaced with an alkane, amino, amino ether, or heteroatom.

In some embodiments, R₃ is an alkoxy, amino, amino ether, N-Boc-protected 2-aminoethoxyethoxyethylamino group, or a C1-C10 straight or branched, acyclic or cyclic alkyloxy group, aryloxy, or amino group with or without heteroatom

In some embodiments, the R₁ is para-toyl, R₂ is phenyl, and R₃ is ethoxy.

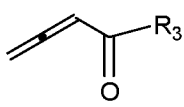
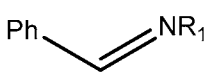
In some embodiments, R₃ is ethoxy, menthyloxy, or N-Boc-protected 2-aminoethoxyethoxyethylamino group.

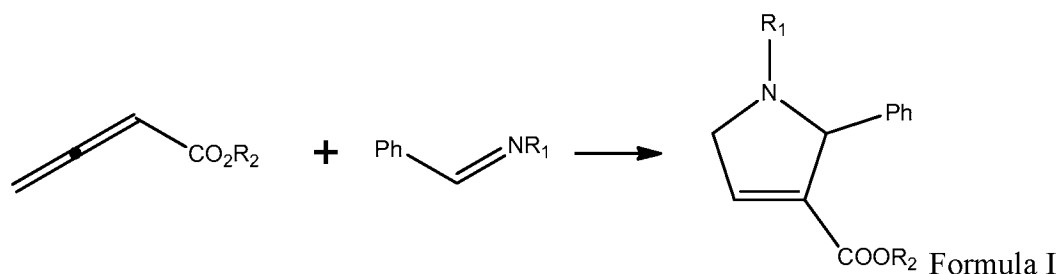
In some embodiments, there is a method of forming the compound of Formula Ic:



Formula Ic

where R₁ is substituted phenyl or substituted or unsubstituted heteroaryl; and R₂ is substituted phenyl or substituted or unsubstituted heteroaryl; and R₃ is an alkoxy, amino, amino ether, N-Boc-protected 2-aminoethoxyethoxyethylamino group, or a C1-C10 straight or branched, acyclic or cyclic alkyloxy group, aryloxy, or amino group with or without heteroatom

comprising reacting  with  according to a reaction of Scheme I



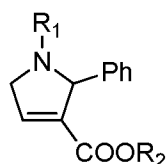
(Scheme I)

to form the compound of Formula Ic.

In some embodiments, the compound is any one of, (Fig. 16A-B)

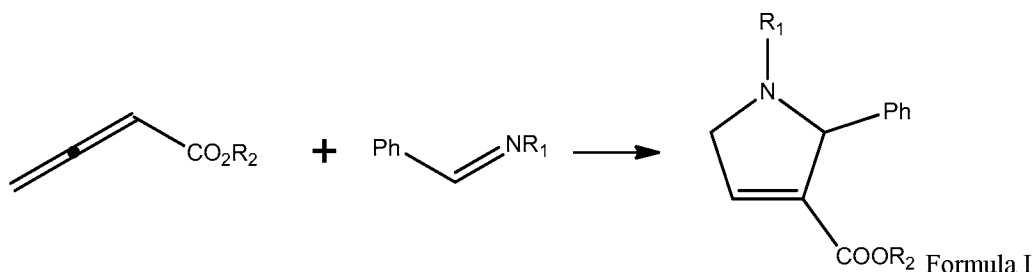
5 E. Method of synthesis

In a further aspect of the present invention, it is provided a method of forming the compound of Formula I:



Formula I

10 where R_1 is tosyl, or mesyl group; and R_2 is a hydrocarbyl group with or without a heteroatom, comprising reacting $\text{CH}_2=\text{CH}-\text{CO}_2\text{R}_2$ with $\text{Ph}-\text{CH}=\text{NR}_1$ according to a reaction of Scheme I



(Scheme I)

15 to form the compound of Formula I.

Description of the compound of Formula I is fully provided above. For concise description of the present invention, the description of the compound of Formula I is not repeated here but is fully incorporated hereto by reference.

20 In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the reaction of Scheme I is carried out under asymmetric synthesis conditions.

In some embodiments of the invention method, optionally in combination with any or

all of the various embodiments disclosed herein, the method further comprises performing chiral resolution of compound of Formula I to yield *R*- or *S*- enantiomers of the compound in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments of the invention method, optionally in combination with any or
5 all of the various embodiments disclosed herein, the chiral resolution is performed on an HPLC chiral stationary phase.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the chiral resolution is achieved by reacting the compound with a chiral agent.

10 In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the chiral agent is menthol.

F. Compositions

In a further aspect of the present invention, it is provided a composition comprising an anti-arrhythmicity compound of Formula I, which is described above. For concise description
15 of the present invention, the description of the compound of Formula I is not repeated here but is fully incorporated hereto by reference.

In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

20 In some embodiments of the invention composition, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation suitable for administration to a subject.

In a further aspect of the present invention, it is provided a method of forming a composition, comprising providing a compound of Formula I in an effective amount, and
25 forming the composition.

The compound of Formula I is fully described above. For concise description of the present invention, the description of the compound of Formula I is not repeated here but is fully incorporated hereto by reference.

In some embodiments of the invention method, optionally in combination with any
30 or all of the various embodiments disclosed herein, the compound is in an optionally active (e.g., substantially enantiomerically pure) form.

In some embodiments of the invention method, optionally in combination with any or all of the various embodiments disclosed herein, the composition is in a formulation

suitable for administration to a subject to treat or ameliorate a disorder related to cardiac arrhythmicity, e.g., cardiac fibrillation.

G. Formulations

The compound of invention or composition of invention can be formulated into any desirable formulation. Such formulations can include a pharmaceutically acceptable carrier, which can be, e.g., salient or can comprise a polymeric material.

In some embodiments, the carrier disclosed herein can be a polymeric material. Exemplary polymeric material that can be used here include but are not limited to a biocompatible or bioabsorbable polymer that is one or more of poly(DL-lactide), poly(L-lactide), poly(L-lactide), poly(L-lactide-co-D,L-lactide), polymandelide, polyglycolide, poly(lactide-co-glycolide), poly(D,L-lactide-co-glycolide), poly(L-lactide-co-glycolide), poly(ester amide), poly(ortho esters), poly(glycolic acid-co-trimethylene carbonate), poly(D,L-lactide-co-trimethylene carbonate), poly(trimethylene carbonate), poly(lactide-co-caprolactone), poly(glycolide-co-caprolactone), poly(tyrosine ester), polyanhydride, derivatives thereof. In some embodiments, the polymeric material comprises a combination of these polymers.

In some embodiments, the polymeric material comprises poly(D,L-lactide-co-glycolide). In some embodiments, the polymeric material comprises poly(D,L-lactide). In some embodiments, the polymeric material comprises poly(L-lactide). [0065] Additional exemplary polymers include but are not limited to poly(D-lactide) (PDLA), polymandelide (PM), polyglycolide (PGA), poly(L-lactide-co-D,L-lactide) (PLDLA), poly(D,L-lactide) (PDLA), poly(D,L-lactide-co-glycolide) (PLGA) and poly(L-lactide-co-glycolide) (PLLGA). With respect to PLLGA, the stent scaffolding can be made from PLLGA with a mole% of GA between 5-15 mol%. The PLLGA can have a mole% of (LA:GA) of 85: 15 (or a range of 82: 18 to 88: 12), 95:5 (or a range of 93:7 to 97:3), or commercially available PLLGA products identified as being 85: 15 or 95:5 PLLGA. The examples provided above are not the only polymers that may be used. Many other examples can be provided, such as those found in Polymeric Biomaterials, second edition, edited by Severian Dumitriu; chapter 4.

In some embodiments, polymers that are more flexible or that have a lower modulus than those mentioned above may also be used. Exemplary lower modulus bioabsorbable polymers include, polycaprolactone (PCL), poly(trimethylene carbonate) (PTMC), polydioxanone (PDO), poly(3-hydroxybutyrate) (PHB), poly(4-hydroxybutyrate) (P4HB), poly(hydroxyalkanoate) (PHA), and poly(butylene succinate), and blends and copolymers

thereof.

In exemplary embodiments, higher modulus polymers such as PLLA or PLLGA may be blended with lower modulus polymers or copolymers with PLLA or PLGA. The blended lower modulus polymers result in a blend that has a higher fracture toughness than the high modulus polymer. Exemplary low modulus copolymers include poly(L-lactide)-b-polycaprolactone (PLLA-b-PCL) or poly(L-lactide)-co-polycaprolactone (PLLA-co-PCL). The composition of a blend can include 1-5 wt% of low modulus polymer.

More exemplary polymers include but are not limited to at least partially alkylated polyethyleneimine (PEI); at least partially alkylated poly(lysine); at least partially alkylated polyornithine; at least partially alkylated poly(amido amine), at least partially alkylated homo- and co-polymers of vinylamine; at least partially alkylated acrylate containing aminogroups, copolymers of vinylamine containing aminogroups with hydrophobic monomers, copolymers of acrylate containing aminogroups with hydrophobic monomers, and amino containing natural and modified polysaccharides, polyacrylates, polymethacryates, polyureas, polyurethanes, polyolefins, polyvinylhalides, polyvinylidenehalides, polyvinylethers, polyvinylaromatics, polyvinylesters, polyacrylonitriles, alkyd resins, polysiloxanes and epoxy resins, and mixtures thereof. Additional examples of biocompatible biodegradable polymers include, without limitation, polycaprolactone, poly(L-lactide), poly(D,L-lactide), poly(D,L-lactide-co-PEG) block copolymers, poly(D,L-lactide-co-trimethylene carbonate), poly(lactide-co-glycolide), polydioxanone (PDS), polyorthoester, polyanhydride, poly(glycolic acid-co-trimethylene carbonate), polyphosphoester, polyphosphoester urethane, poly(amino acids), polycyanoacrylates, poly(trimethylene carbonate), poly(iminocarbonate), polycarbonates, polyurethanes, polyalkylene oxalates, polyphosphazenes, PHA-PEG, and combinations thereof. The PHA may include poly(α -hydroxyacids), poly(β -hydroxyacid) such as poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-co-valerate) (PHBV), poly(3-hydroxypropionate) (PHP), poly(3-hydroxyhexanoate) (PHH), or poly(4-hydroxyacid) such as poly poly(4-hydroxybutyrate), poly(4-hydroxyvalerate), poly(4-hydroxyhexanoate), poly(hydroxyvalerate), poly(tyrosine carbonates), poly(tyrosine arylates), poly(ester amide), polyhydroxyalkanoates (PHA), poly(3-hydroxyalkanoates) such as poly(3-hydroxypropanoate), poly(3-hydroxybutyrate), poly(3-hydroxyvalerate), poly(3-hydroxyhexanoate), poly(3-hydroxyheptanoate) and poly(3-hydroxyoctanoate), poly(4-hydroxyalkanoate) such as poly(4-hydroxybutyrate), poly(4-hydroxyvalerate), poly(4-hydroxyhexanoate), poly(4-hydroxyheptanoate), poly(4-

hydroxyoctanoate) and copolymers including any of the 3-hydroxyalkanoate or 4-hydroxyalkanoate monomers described herein or blends thereof, poly(D,L-lactide), poly(L-lactide), polyglycolide, poly(D,L-lactide-co-glycolide), poly(L-lactide-co-glycolide), polycaprolactone, poly(lactide-co-caprolactone), poly(glycolide-co-caprolactone),
5 poly(dioxanone), poly(ortho esters), poly(anhydrides), poly(tyrosine carbonates) and derivatives thereof, poly(tyrosine ester) and derivatives thereof, poly(imino carbonates), poly(glycolic acid-co-trimethylene carbonate), polyphosphoester, polyphosphoester urethane, poly(amino acids), polycyanoacrylates, poly(trimethylene carbonate), poly(iminocarbonate), polyphosphazenes, silicones, polyesters, polyolefms, polyisobutylene and ethylene-
10 alphaolefin copolymers, acrylic polymers and copolymers, vinyl halide polymers and copolymers, such as polyvinyl chloride, polyvinyl ethers, such as polyvinyl methyl ether, polyvinylidene halides, such as polyvinylidene chloride, polyacrylonitrile, polyvinyl ketones, polyvinyl aromatics, such as polystyrene, polyvinyl esters, such as polyvinyl acetate, copolymers of vinyl monomers with each other and olefins, such as ethylene-methyl
15 methacrylate copolymers, acrylonitrile-styrene copolymers, ABS resins, and ethylene-vinyl acetate copolymers, polyamides, such as Nylon 66 and polycaprolactam, alkyd resins, polycarbonates, polyoxymethylenes, polyimides, polyethers, poly(glyceryl sebacate), poly(propylene fumarate), poly(n-butyl methacrylate), poly(sec-butyl methacrylate), poly(isobutyl methacrylate), poly(tert-butyl methacrylate), poly(n-propyl methacrylate),
20 poly(isopropyl methacrylate), poly(ethyl methacrylate), poly(methyl methacrylate), epoxy resins, polyurethanes, rayon, rayon-triacetate, cellulose acetate, cellulose butyrate, cellulose acetate butyrate, cellophane, cellulose nitrate, cellulose propionate, cellulose ethers, carboxymethyl cellulose, polyethers such as poly(ethylene glycol) (PEG), copoly(ether-
esters) (e.g. poly(ethylene oxide-co-lactic acid) (PEO/PLA)), polyalkylene oxides such as
25 poly(ethylene oxide), poly(propylene oxide), poly(ether ester), polyalkylene oxalates, phosphoryl choline containing polymer, choline, poly(aspirin), polymers and co-polymers of hydroxyl bearing monomers such as 2-hydroxyethyl methacrylate (HEMA), hydroxypropyl methacrylate (HPMA), hydroxypropylmethacrylamide, PEG acrylate (PEGA), PEG methacrylate, methacrylate polymers containing 2-methacryloyloxyethyl- phosphorylcholine
30 (MPC) and n-vinyl pyrrolidone (VP), carboxylic acid bearing monomers such as methacrylic acid (MA), acrylic acid (AA), alkoxymethacrylate, alkoxyacrylate, and 3-trimethylsilylpropyl methacrylate (TMSPMA), poly(styrene-isoprene-styrene)-PEG (SIS-PEG), polystyrene-PEG, polyisobutylene-PEG, polycaprolactone-PEG (PCL-PEG), PLA-PEG, poly(methyl methacrylate), MED610, poly(methyl methacrylate)-PEG (PMMA-PEG),

polydimethylsiloxane-*co*-PEG (PDMS-PEG), poly(vinylidene fluoride)-PEG (PVDF-PEG), PLURONIC™ surfactants (polypropylene oxide-*co*-polyethylene glycol), poly(tetramethylene glycol), hydroxy functional poly(vinyl pyrrolidone), biomolecules such as collagen, chitosan, alginate, fibrin, fibrinogen, cellulose, starch, dextran, dextrin, 5 hyaluronic acid, fragments and derivatives of hyaluronic acid, heparin, fragments and derivatives of heparin, glycosamino glycan (GAG), GAG derivatives, polysaccharide, elastin, elastin protein mimetics, or combinations thereof.

In some embodiments, polyethylene is used to construct at least a portion of the device. For example, polyethylene can be used in an orthopedic implant on a surface that is 10 designed to contact another implant, as such in a joint or hip replacement. Polyethylene is very durable when it comes into contact with other materials. When a metal implant moves on a polyethylene surface, as it does in most joint replacements, the contact is very smooth and the amount of wear is minimal. Patients who are younger or more active may benefit from polyethylene with even more resistance to wear. This can be accomplished through a 15 process called crosslinking, which creates stronger bonds between the elements that make up the polyethylene. The appropriate amount of crosslinking depends on the type of implant. For example, the surface of a hip implant may require a different degree of crosslinking than the surface of a knee implant.

Additional examples of polymeric materials can be found, for example, in US Pat. 20 No. 6,127,448 to Domb, US Pat. Pub. No. 2004/0148016 by Klein and Brazil, US Pat. Pub. No. 2009/0169714 by Burghard et al, US Pat. No. 6,406,792 to Briquet et al, US Pat. Pub. No. 2008/0003256 by Martens et al, each of which is hereby incorporated by reference herein in its entirety.

H. Dosage and Administration

25 The dosage can be determined by one of skill in the art and can also be adjusted by the individual physician in the event of any complication. Typically, the dosage ranges from 0.0005 mg/kg body weight to 1 g/kg body weight. In some embodiments, the dosage range is from 0.001 mg/kg body weight to 0.5 g/kg body weight, from 0.0005 mg/kg body weight to 0.1 g/kg body weight, from 0.001 mg/kg body weight to 0.05 g/kg body weight.

30 As another alternative, dosages are selected for localized delivery and are not necessarily selected for body weight or to achieve a certain serum level, but to achieve a localized effect, e.g., as for a localized injection, implantation or other localized administration to the eye.

Administration of the doses recited above can be repeated for a limited period of time. In some embodiments, the doses are given once a day, or multiple times a day, for example, but not limited to, three times a day. In a preferred embodiment, the doses recited above are administered daily for several weeks or months. The duration of treatment depends upon the subject's clinical progress and responsiveness to therapy. Continuous, relatively low maintenance doses are contemplated after an initial higher therapeutic dose.

Agents useful in the methods and compositions described herein can be administered topically, intravenously (by bolus or continuous infusion), orally, by inhalation, intraperitoneally, intramuscularly, subcutaneously, intracavity, and can be delivered by peristaltic means, if desired, or by other means known by those skilled in the art. It is preferred that the agents for the methods described herein are administered topically to the eye. For the treatment of tumors, the agent can be administered systemically, or alternatively, can be administered directly to the tumor e.g., by intratumor injection or by injection into the tumor's primary blood supply.

Therapeutic compositions containing at least one agent disclosed herein can be conventionally administered in a unit dose. The term "unit dose" when used in reference to a therapeutic composition refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required physiologically acceptable diluent, i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. An agent can be targeted by means of a targeting moiety, such as e.g., an antibody or targeted liposome technology.

Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are particular to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

An agent may be adapted for catheter-based delivery systems including coated

balloons, slow-release drug-eluting stents or other drug-eluting formats, microencapsulated PEG liposomes, or nanobeads for delivery using direct mechanical intervention with or without adjunctive techniques such as ultrasound, together with an active agent as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes. As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. A pharmaceutically acceptable carrier will not promote the raising of an immune response to an agent with which it is admixed, unless so desired. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition. The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient. The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like. Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at a physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous

carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes. Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions. The amount of an active agent used in the methods described herein that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques.

It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

EXAMPLES

The following examples illustrate rather than limit the embodiments of the present invention.

Example 1

(Studies on mitochondrial Ca^{2+} uptake by the voltage-dependent anion channel 2 regulates cardiac rhythmicity)

A. Summary

Tightly regulated Ca^{2+} homeostasis is a prerequisite for proper cardiac function. To dissect the regulatory network of cardiac Ca^{2+} handling, we performed a chemical suppressor screen on zebrafish *tremblor* embryos, which suffer from Ca^{2+} extrusion defects. Efsevin was identified based on its potent activity to restore coordinated contractions in *tremblor*. We

show that efsevin binds to VDAC2, potentiates mitochondrial Ca^{2+} uptake and accelerates the transfer of Ca^{2+} from intracellular stores into mitochondria. In cardiomyocytes, efsevin restricts the temporal and spatial boundaries of Ca^{2+} sparks and thereby inhibits Ca^{2+} overload-induced erratic Ca^{2+} waves and irregular contractions. We further show that overexpression of VDAC2 recapitulates the suppressive effect of efsevin on *tremblor* embryos whereas VDAC2 deficiency attenuates efsevin's rescue effect and that VDAC2 functions synergistically with MCU to suppress cardiac fibrillation in *tremblor*. Together, these findings demonstrate a critical modulatory role for VDAC2-dependent mitochondrial Ca^{2+} uptake in the regulation of cardiac rhythmicity.

B. Introduction

During development, well-orchestrated cellular processes guide cells from diverse lineages to integrate into the primitive heart tube and establish rhythmic and coordinated contractions. While many genes and pathways important for cardiac morphogenesis have been identified, molecular mechanisms governing embryonic cardiac rhythmicity are poorly understood. The findings that Ca^{2+} waves traveling across the heart soon after the formation of the primitive heart tube (Chi et al., 2008, PLoS Biol 6, e109) and that loss of function of key Ca^{2+} regulatory proteins, such as the L-type Ca^{2+} channel, Na/K-ATPase and sodium-calcium exchanger 1 (NCX1), severely impairs normal cardiac function (Rottbauer et al., 2001, Dev Cell 1, 265-275; Shu et al., 2003, Development 130, 6165-6173; Ebert et al., 2005, Proc Natl Acad Sci U S A 102, 17705-17710; Langenbacher et al., 2005, Proc Natl Acad Sci U S A 102, 17699-17704), indicate an essential role for Ca^{2+} handling in the regulation of embryonic cardiac function.

Ca^{2+} homeostasis in cardiac muscle cells is tightly regulated at the temporal and spatial level by a subcellular network involving multiple proteins, pathways, and organelles. The release and reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR), the largest Ca^{2+} store in cardiomyocytes, constitutes the primary mechanism governing the contraction and relaxation of the heart. Ca^{2+} influx after activation of the L-type Ca^{2+} channel in the plasma membrane induces the release of Ca^{2+} from the SR via ryanodine receptor (RyR) channels, which leads to an increase of the intracellular Ca^{2+} concentration and cardiac contraction. During diastolic relaxation, Ca^{2+} is transferred back into the SR by the SR Ca^{2+} pump or extruded from the cell through NCX1. Defects in cardiac Ca^{2+} handling and Ca^{2+} overload, for example during cardiac ischemia/reperfusion or in long QT syndrome, are well known causes of contractile

dysfunction and many types of arrhythmias including early and delayed afterdepolarizations and Torsade des pointes (Bers, 2002, *Nature* 415, 198-205; Choi et al., 2002, *J Physiol* 543, 615-631; Yano et al., 2008, *Circ J* 72, A22-30; Greiser et al., 2011, *Cardiovasc Res* 89, 722-733).

5 Ca²⁺ crosstalk between mitochondria and ER/SR has been noted in many cell types and the voltage-dependent anion channel (VDAC) and the mitochondrial Ca²⁺ uniporter (MCU) serve as primary routes for Ca²⁺ entry through the outer and inner mitochondrial membranes, respectively (Rapizzi et al., 2002, *J Cell Biol* 159, 613-624; Bathori et al., *J Biol Chem* 2006, 281, 17347-17358; Shoshan-Barmatz et al., 2010, *Mol Aspects Med* 31, 227-10 285; Baughman et al., 2011, *Nature* 476, 341-345; De Stefani et al., 2011, *Nature* 476, 336-340). In the heart, mitochondria are tethered to the SR and are located in close proximity to Ca²⁺ release sites (Garcia-Perez et al., 2008, *J Biol Chem* 283, 32771-32780; Boncompagni et al., 2009, *Mol Biol Cell* 20, 1058-1067; Hayashi et al., 2009, *J Cell Sci* 122, 1005-1013). This subcellular architecture exposes the mitochondria near the Ca²⁺ release sites to a high 15 local Ca²⁺ concentration that is sufficient to overcome the low Ca²⁺ affinity of MCU and facilitates Ca²⁺ crosstalk between SR and mitochondria (Garcia-Perez et al., 2008; Dorn et al., 2010, *Circ Res* 107, 689-699; Kohlhaas et al., 2013, *Cardiovasc Res* 98, 259-268). Increase of the mitochondrial Ca²⁺ concentration enhances energy production during higher workload and dysregulation of SR-mitochondrial Ca²⁺ signaling results in energetic deficits 20 and oxidative stress in the heart and may trigger programmed cell death (Brandes et al., 1997, *Circ Res* 80, 82-87; Maack et al., 2006, *Circ Res* 99, 172-182; Kohlhaas et al., 2013). However, whether SR-mitochondrial Ca²⁺ crosstalk also contributes significantly to cardiac Ca²⁺ signaling during excitation-contraction coupling requires further investigation.

In zebrafish, the *tremblor* (*tre*) locus encodes a cardiac-specific isoform of the 25 Na⁺/Ca²⁺ exchanger 1, NCX1h (also known as slc8a1a) (Ebert et al., 2005; Langenbacher et al., 2005). The *tre* mutant hearts lack rhythmic Ca²⁺ transients and display chaotic Ca²⁺ signals in the myocardium leading to unsynchronized contractions resembling cardiac fibrillation (Langenbacher et al., 2005). In this study, we used *tre* as an animal model for aberrant Ca²⁺ handling-induced cardiac dysfunction and took a chemical genetic approach to 30 dissect the Ca²⁺ regulatory network important for maintaining cardiac rhythmicity. A synthetic compound named efsevin was identified from a suppressor screen due to its potent ability to restore coordinated contractions in *tre*. Using biochemical and genetic approaches we show that efsevin interacts with VDAC2 and potentiates its mitochondrial Ca²⁺ transporting activity and spatially and temporally modulates cytosolic Ca²⁺ signals in

cardiomyocytes. The important role of mitochondrial Ca^{2+} uptake in regulating cardiac rhythmicity is further supported by the suppressive effect of VDAC2 and MCU overexpression on cardiac fibrillation in *tre*.

C. Materials and Methods

5 *Zebrafish husbandry and transgenic lines.*

Zebrafish of the mutant line *tremblor* (*tre^{tc318}*) were maintained and bred as described previously (Langenbacher et al., 2005). Transgenic lines, *myl7:gCaMP4.1^{LA2124}* and *myl7:VDAC2^{LA2309}* were created using the Tol2kit (Esengil et al., 2007, Nat Chem Biol 3, 154-155; Kwan et al., 2007, Dev Dyn 236, 3088-3099; Shindo et al., 2010, PLoS One 5, e8897). The *VDAC2^{LA2256}* was created using the zinc finger array OZ523 and OZ524 generated by the zebrafish Zinc Finger Consortium (Foley et al., 2009a, Nature protocols 4, 1855-1867; Foley et al., 2009b, PLoS One 4, e4348).

Molecular Biology.

Full length VDAC2 cDNA was purchased from Open Biosystems and cloned into pCS2+ or pCS2+3XFLAG. Full length cDNA fragments of zebrafish MCU (Accession number: JX424822) and MICU1 (JX42823) were amplified from 2 dpf embryos and cloned into pCS2+. For mRNA synthesis, plasmids were linearized and mRNA was synthesized using the SP6 mMESSAGE mMachin kit according to the manufacturers manual (Ambion).

Zebrafish injections.

VDAC2 mRNA and morpholino antisense oligos (5'-GGGAACGGCCATTTTATCTGTAAA-3') (Genetools) (SEQ ID NO: 9) were injected into one-cell stage embryos collected from crosses of *tre^{tc318}* heterozygotes. Cardiac performance was analyzed by visual inspection on 1 dpf. The *tre* mutant embryos were identified either by observing the fibrillation phenotype at 2-3 dpf or by genotyping as previously described (Langenbacher et al., 2005).

Chemical Screen.

Chemicals from a synthetic library (Castellano et al., 2007, J Am Chem Soc 129, 5843-5845; Choi et al., 2011, Development 138, 1173-1181; Cruz et al., 2011, Proc Natl Acad Sci U S A 108, 6769-6774) and from Biomol International LP were screened for their ability to partially or completely restore persistent heartbeat in *tre* embryos. 12 embryos collected from crosses of *tre^{tc318}* heterozygotes were raised in the presence of individual compounds at a concentration of 10 μM from 4 hpf (Choi et al., 2011). Cardiac function was

analyzed by visual inspection at 1 and 2 dpf. The hearts of *tre*^{tc318} embryos manifest a chaotic movement resembling cardiac fibrillation with intermittent contractions in rare occasion (Ebert et al., 2005; Langenbacher et al., 2005). Compounds that elicit persistent coordinated cardiac contractions were validated on large number of *tre* mutant embryos and NCX1h morphants (>500 embryos).

Zebrafish cardiac imaging.

Movies of GFP-labelled *myl7:GFP* hearts were taken at 30 frames per second. Line-scan analysis was performed along a line through the atria or the ventricles of these hearts (Nguyen et al., 2009, Drug Discovery Today: Disease Models 5). Fraction of shortening was deduced from the ratio of diastolic and systolic width and heart rate was determined by beats per minute. Cardiac parameters were analyzed in *tremblor*^{tc318} and *VDAC2*^{LA2256} at 2 dpf.

Zebrafish optical mapping.

36 hpf *myl7:gCaMP4.1* embryos were imaged at a frame rate of 30 ms/frame. Electromechanical isolation was achieved by tnnt2MO (Milan et al., 2006, Development 133, 1125-1132). The fluorescence intensity of each pixel in a 2D map was normalize to generate heat maps and isochronal lines at 33 ms intervals were obtained by identifying the maximal spatial gradient for a given time point (Chi et al., 2008).

Mouse and human embryonic stem cells.

The mouse E14Tg2a ESC and human H9 ESC line were cultured and differentiated as previously described (Blin et al., 2010, J Clin Invest 120, 1125-1139; Arshi et al., 2013, Sci Technol Adv Mater 2013, 025003). At day 10 of differentiation, beating mouse EBs were exposed to external solution containing 10 mM CaCl₂ for 10 minutes before DMSO or efsevin (10 μM) treatment. Human EBs were differentiated for 15 days and treated with 5 mM CaCl₂ for 10 minutes before DMSO or efsevin (5 μM) treatment. Images of beating EBs were acquired at a rate of 30 frames/sec and analyzed by motion-detection software. For calcium recording, the EBs were loaded with 10 μM fluo-4 AM in culture media for 30 minutes at 37°C. Line-scan analysis was performed and fluorescent signals were acquired by a Zeiss LSM510 confocal microscope.

Microelectrode array measurements.

Two-day-old wild type, *tre*, and efsevin-treated *tre* embryos were placed on uncoated, microelectrode arrays (MEAs) containing 120 integrated TiN electrodes (30 μm diameter, 200 μm interelectrode spacing). Local field potentials (LFPs) at each electrode were collected

for three trials per embryo type over a period of three minutes at a sampling rate of 1 kHz using the MEA2100-HS120 system (Multichannel Systems, Reutlingen, Germany). Raw data was low-pass filtered at a cutoff frequency of 10 Hz using a third-order Butterworth filter.

Data analysis was carried out using the MC_DataTool (Multichannel Systems) and Matlab (MathWorks).

Ca²⁺ imaging.

Murine ventricular cardiomyocytes were isolated as previously described (Reuter et al., 2004, J Physiol 554, 779-789). Cells were loaded with 5 μ M fluo-4 AM in external solution containing: 138.2 mM NaCl, 4.6 mM KCl, 1.2 mM MgCl, 15 mM glucose, 20 mM HEPES for 1 hr and imaged in external solution supplemented with 2, 5 or 10 mM CaCl₂. For the recording of Ca²⁺ sparks and transients, the external solution contained 2 mM CaCl₂. For Ca²⁺ transients, cells were field stimulated at 0.5Hz with a 5 ms pulse at a voltage of 20% above contraction threshold. For all measurements, efsevin was added 2 hours prior to the actual experiment. Images were recorded on a Zeiss LSM 5 Pascal confocal microscope. Data analysis was carried out using the Zeiss LSM Image Browser and ImageJ with the SparkMaster plugin (Picht et al., 2007, Am J Physiol Cell Physiol 293, C1073-1081). Cells were visually inspected prior to and after each recording. Only those recordings from healthy looking cells with distinct borders, uniform striations and no membrane blebs or granularity were included in the analysis.

Biochemistry.

For pull down assays mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine) was attached to the carboxylic ester of efsevin and its derivatives through the amide bond. After removal of the Boc group using TFA, the primary amine was coupled to the carboxylic acid of Affi-Gel 10 Gel (Biorad). Two-day-old zebrafish embryos were deyolked by centrifugation before being lysed with Rubinfeld's lysis buffer (Rubinfeld et al., 1993, Science 262, 1731-1734). The lysate was precleaned by incubation with Affi-Gel 10 Gel to eliminate non-specific binding. Precleaned lysate was incubated with affinity beads overnight. Proteins were eluted from the affinity beads and separated on SDS-PAGE. Protein bands of interest were excised. Gel plugs were dehydrated in acetonitrile (ACN) and dried completely in a Speedvac. Samples were reduced and alkylated with 10 mM dithiotreitol and 10 mM TCEP solution in 50 mM NH₄HCO₃ (30 min at 56°C) and 100 mM iodoacetamide (45 min in dark), respectively. Gel plugs were washed with 50 mM NH₄HCO₃, dehydrated with ACN, and dried down in a Speedvac. Gel pieces were then swollen in digestion buffer

containing 50 mM NH_4HCO_3 , and 20.0 ng/ μL of chymotrypsin (25°C, overnight). Peptides were extracted with 0.1% TFA in 50% ACN solution, dried down and resuspended in LC buffer A (0.1% formic acid, 2% ACN).

Mass spectrometry analyses and database searching.

5 Extracted peptides were analyzed by nano-flow LC/MS/MS on a Thermo Orbitrap with dedicated Eksigent nanopump using a reversed phase column (New Objective). The flow rate was 200 nL/min for separation: mobile phase A contained 0.1% formic acid, 2% ACN in water, and mobile phase B contained 0.1% formic acid, 20% water in ACN. The gradient used for analyses was linear from 5% B to 50% B over 60 min, then to 95% B over
10 15 min, and finally keeping constant 95% B for 10 min. Spectra were acquired in data-dependent mode with dynamic exclusion where the instrument selects the top six most abundant ions in the parent spectra for fragmentation. Data were searched against the *Danio rerio* IPI database v3.45 using the SEQUEST algorithm in the BioWorks software program version 3.3.1 SP1. All spectra used for identification had $\Delta\text{CN} > 0.1$ and met the following
15 Xcorr criteria: >2 (+1), >3 (+2), >4 (+3), and >5 (+4). Searches required full cleavage with the enzyme, ≤ 4 missed cleavages and were performed with the differential modifications of carbamidomethylation on cysteine and methionine oxidation.

In situ hybridization.

 In situ hybridization was performed as previously described (Chen et al., 1996,
20 Development 122, 3809-3816). DIG-labeled RNA probe was synthesized using the DIG RNA labeling kit (Roche).

Immunostaining.

 HeLa cells were transfected with a C-terminally flag-tagged zebrafish VDAC1 or VDAC2 in plasmid pCS2+ using Lipofectamine™ 2000 (Invitrogen). After staining with
25 MitoTracker® Orange (Invitrogen) cells were fixed in 3.7% formaldehyde and permeabilized with acetone. Immunostaining was performed using primary antibody ANTI-FLAG® M2 (Sigma Aldrich) at 1:100 and secondary antibody Anti-Mouse IgG1-FITC (Southern Biotechnology Associates) at 1:200. Cells were mounted and counterstained using Vectashield® Hard Set™ with DAPI (Vector Laboratories).

30 *Mitochondria Ca^{2+} uptake assay in HeLa cells.*

 HeLa cells were transfected with zebrafish VDAC2 using Lipofectamine™ 2000 (Invitrogen). 36 hrs after transfection, cells were loaded with 5 μM Rhod2-AM (Invitrogen),

a Ca^{2+} indicator preferentially localized in mitochondria, for 1 hour at 15°C followed by a 30 min de-esterification period at 37°C. Subsequently, cells were permeabilized with 100 μM digitonin for 1 min at room temperature. Fluorescence changes in Rhod2 (ex: 544 nm, em: 590 nm) immediately after the addition of Ca^{2+} (final free Ca^{2+} concentration is calculated to be approximately 10 μM using WEBMAXC at <http://web.stanford.edu/~cpatton/webmaxcS.htm>) were monitored in internal buffer (5 mM K-EGTA, 20 mM HEPES, 100 mM K-aspartate, 40 mM KCl, 1 mM MgCl_2 , 2 mM maleic acid, 2 mM glutamic acid, 5 mM pyruvic acid, 0.5 mM KH_2PO_4 , 5 mM MgATP, pH adjusted to 7.2 with Trizma base) using a FLUOSTAR plate reader (BMG Labtech).

10 *Mitochondria Ca^{2+} uptake assay in VDAC1/VDAC3 double knockout (V1/V3 DKO) MEFs.*

V1/V3 DKO MEFs were cultured as previously described (Roy et al., 2009a, EMBO Rep 10, 1341-1347). Efsevin-treated (15 μM for 30 min) or mock-treated MEFs were used for measurements of $[\text{Ca}^{2+}]_c$ in suspensions of permeabilized cells or imaging of $[\text{Ca}^{2+}]_m$ simultaneously with $[\text{Ca}^{2+}]_c$ in intact single cells. Permeabilization of the plasma membrane was performed by digitonin (40 $\mu\text{M}/\text{ml}$). Changes in $[\text{Ca}^{2+}]$ in the cytoplasmic buffer upon IP_3 (7.5 μM) addition in the presence or absence of ruthenium red (3 μM) was measured by fura2 in a fluorometer (Csordas et al., 2006, J Cell Biol 174, 915-921; Roy et al., 2009b, Mol Cell 33, 377-388). To avoid endoplasmic reticulum Ca^{2+} uptake 2 μM thapsigargin was added before IP_3 . For imaging of $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_c$, MEFs were co-transfected with plasmids encoding polycistronic zebrafish VDAC2 with mCherry and mitochondria-targeted inverse pericam for 40 hours. Cells were sorted to enrich the transfected cells and attached to glass coverslips. In the final 10 min, of the efsevin or mock-treatment, the cells were also loaded with fura2AM (2.5 μM) and subsequently transferred to the microscope stage. Stimulation with 1 μM ATP was carried out in a norminally Ca^{2+} free buffer. Changes in $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ were imaged using fura2 (ratio of ex:340 nm to 380 nm) and mitochondria-targeted inverse pericam (ex: 495 nm), respectively (Csordas et al., 2010, Mol Cell 39, 121-132).

Statistics.

All values are expressed as mean $\pm$ SEM. Significance values are calculated by unpaired student's t-test unless noted otherwise.

Test data not shown include the following information:

- 1) a heart of a wild-type zebrafish embryo at 2 dpf. Robust rhythmic contractions can be observed in atrium and ventricle.

- 2) a heart of a *tremblor* embryo at 2 dpf. Embryos of the mutant line *tremblor* display only local, unsynchronized contractions, comparable to cardiac fibrillation.
- 3) a heart of a *tremblor* embryo at 2 dpf treated with efsevin. Treatment of *tremblor* embryos with efsevin restores rhythmic contractions with comparable atrial fractional shortening compared to wild-type embryos and approximately 40% of wild-type heart rate.
- 4) a heart of a wild-type zebrafish embryo at 2 dpf treated with efsevin. Treatment of wild-type embryos with efsevin did not affect cardiac performance, indicated by robust, rhythmic contractions comparable to untreated wild-type embryos.
- 5) heat map of Ca^{2+} transients recorded in one day old wild type heart.
- 6) heat map of Ca^{2+} transients recorded in one day old *tremblor* heart.
- 7) heat map of Ca^{2+} transients recorded in one day old efsevin treated *tremblor* heart.
- 8) a heart of a wild-type zebrafish embryo at 1 dpf. Robust rhythmic contractions can be observed in atrium and ventricle.
- 9) a heart of a wild-type zebrafish embryo injected with zebrafish VDAC2 mRNA at 1 dpf. Robust rhythmic contractions can be observed in atrium and ventricle.
- 10) a heart of a *tremblor* embryo at 1 dpf. *Tremblor* embryos display only local, unsynchronized contractions, comparable to cardiac fibrillation.
- 11) a heart of a *tremblor* embryo injected with zebrafish VDAC2 mRNA at 1 dpf. Overexpression of zebrafish VDAC2 mRNA restores rhythmic contractions in *tremblor* embryos.
- 12) a heart of a 2 dpf Tg-VDAC2 embryo injected with a morpholino targeting NCX1h. Morpholino knock-down of NCX1h results in a fibrillating heart.
- 13) a heart of a 2 dpf NCX1h morphant in the Tg-VDAC2 genetic background. TBF treatment induces VDAC2 expression and restores coordinated cardiac contractions.
- 14) a heart of a 2 dpf wild type zebrafish embryo injected with a morpholino targeting VDAC2. Morpholino knockdown of VDAC2 did not have obvious effects on cardiac performance.
- 15) a heart of a 2 dpf *tremblor* mutant embryo injected with a morpholino targeting VDAC2.
- 16) a heart of a 2 dpf *tremblor* mutant embryo injected with a morpholino targeting VDAC2. Efsevin treatment cannot restore coordinated cardiac contractions in the absence of VDAC2.

D. Results and Discussion

*Identification of a chemical suppressor of *tre* cardiac dysfunction*

Homozygous *tre* mutant embryos suffer from Ca^{2+} extrusion defects and manifest chaotic cardiac contractions resembling fibrillation (Ebert et al., 2005; Langenbacher et al., 2005). To dissect the regulatory network of Ca^{2+} handling in cardiomyocytes and to identify mechanisms controlling embryonic cardiac rhythmicity, we screened the BioMol library and a collection of synthetic compounds for chemicals that are capable of restoring heartbeat either completely or partially in *tre* embryos. A dihydropyrrole carboxylic ester compound named efsevin was identified based on its ability to restore persistent and rhythmic cardiac contractions in *tre* mutant embryos in a dose-dependent manner (**FIG. 1D**). To validate the effect of efsevin, we assessed cardiac performance of wild type, *tre* and efsevin-treated *tre* embryos (Nguyen et al., 2009). Line scans across the atria of Tg(myl7:GFP) embryonic hearts at 48 hpf showed rhythmically alternating systoles and diastoles from vehicle- or efsevin- treated wild type and efsevin-treated *tre* embryos, while only sporadic unsynchronized contractions were seen from vehicle-treated *tre* embryos. Fractional shortening of efsevin treated *tre* mutant hearts was comparable to that of their wild type siblings and heart rate was restored to approximately 40% of that observed in controls (**FIG. 1A-C**). Periodic local field potentials accompanying each heartbeat were detected in wild type and efsevin-treated *tre* embryos using a microelectrode array (**FIG. 1E-G**). Furthermore, while only sporadic Ca^{2+} signals were detected in *tre* hearts, *in vivo* Ca^{2+} imaging revealed steady Ca^{2+} waves propagating through efsevin-treated *tre* hearts, demonstrating that cardiomyocytes are functionally coupled and that efsevin treatment restores regular Ca^{2+} transients in *tre* hearts.

Efsevin suppresses Ca^{2+} overload-induced irregular contraction

We next examined whether efsevin could suppress aberrant Ca^{2+} homeostasis-induced arrhythmic responses in mammalian cardiomyocytes. Mouse embryonic stem cell-derived cardiomyocytes (mESC-CMs) establish a regular contraction pattern with rhythmic Ca^{2+} transients (**FIG. 2A, D, E**). Mimicking Ca^{2+} overload by increasing extracellular Ca^{2+} levels was sufficient to disrupt normal Ca^{2+} cycling and induce irregular contractions in mESC-CMs (**FIG. 2B, D, E**). Remarkably, efsevin treatment restored rhythmic Ca^{2+} transients and cardiac contractions in these cells (**FIG. 2C-E**). Similar effect was observed in human embryonic stem cell-derived cardiomyocytes (hESC-CMs) (**FIG. 2F**). Together, these findings suggest that efsevin targets a conserved Ca^{2+} regulatory mechanism critical

for maintaining rhythmic cardiac contraction in fish, mice and humans.

*VDAC2 mediates the suppressive effect of efsevin on *tre**

To identify the protein target of efsevin, we generated a N-Boc-protected 2-aminoethoxyethoxyethylamine linker-attached efsevine^L (**FIG. 3A and C**). This modified compound retained the activity of efsevin to restore cardiac contractions in *ncx1h* deficient embryos (**FIG. 3B and D**) and was used to create efsevin-conjugated agarose beads (efsevin^{LB}). A 32kD protein species was detected from zebrafish lysate due to its binding ability to efsevin^{LB} and OK-C125^{LB}, an active efsevin derivative conjugated to beads, but not to beads capped with ethanolamine alone or beads conjugated with an inactive efsevin analog (OK-C19^{LB}) (**FIG. 3A-D**). Furthermore, preincubation of zebrafish lysate with excess efsevin prevented the 32kD protein from binding to efsevin^{LB} or OK-C125^{LB}. Mass spectrometry analysis revealed that this 32kD band represents a zebrafish homologue of the mitochondrial voltage-dependent anion channel 2 (VDAC2) (**FIG. 3E**) by identification of VDAC2 peptide LTFTDTTSPNTGK by b- and y-series ions analysis (SEQ ID NO: 8).

VDAC2 is expressed in the developing zebrafish heart, as confirmed by in situ hybridization analysis of embryonic hearts at 36 hpf and 48 hpf, making it a good candidate for mediating efsevin's effect on cardiac Ca²⁺ handling. To examine this possibility, we injected in vitro synthesized VDAC2 RNA into *tre* embryos and found that the majority of these embryos had coordinated cardiac contractions similar to those subjected to efsevin treatment (**FIG. 4A**). In addition, we generated *myl7:VDAC2* transgenic fish in which VDAC2 expression can be induced in the heart by tebufenozide (TBF) (**FIG. 4B**). In situ hybridization analysis showed that TBF treatment induces VDAC2 expression in the heart. Knocking down NCX1h in *myl7:VDAC2* embryos results in chaotic cardiac movement similar to *tre*. Like efsevin treatment, induction of VDAC2 expression by TBF treatment restored coordinated and rhythmic contractions in *myl7:VDAC2*;NCX1h MO hearts (**FIG. 4C**). Conversely, knocking down VDAC2 in *tre* hearts attenuated the suppressive effect of efsevin (**FIG. 4D**). Furthermore, we generated VDAC2 null embryos by the Zinc Finger Nuclease gene targeting approach (**FIG. 4F**). In situ hybridization analysis showed loss of VDAC2 transcripts in *VDAC2*^{zfn/zfn} embryos (SEQ ID NO: 2-7). Similar to that observed in morpholino knockdown embryos, homozygous *VDAC2*^{LA2256} embryos do not exhibit noticeable morphological defects, but the suppressive effect of efsevin was attenuated in homozygous *VDAC2*^{LA2256}; NCX1MO embryos (**FIG. 4E**). These findings demonstrate that VDAC2 is a major mediator for efsevin's effect on *ncx1h* deficient hearts.

VDAC2-dependent effect of efsevin on mitochondrial Ca^{2+} uptake

VDAC is an abundant channel located on the outer mitochondrial membrane serving as a primary passageway for metabolites and ions (Rapizzi et al., 2002; Bathori et al., 2006; Shoshan-Barmatz et al., 2010). At its close state, VDAC favours Ca^{2+} flux (Tan et al., 2007, Biochim Biophys Acta 1768, 2510-2515). To examine whether efsevin would modulate mitochondrial Ca^{2+} uptake via VDAC2, we transfected HeLa cells with VDAC2. HeLa cells transfected with a flag-tagged zebrafish VDAC2 (VDAC2^{flag}) were immunostained against the flag epitope and counterstained for mitochondria with MitoTracker Orange and for nuclei with DAPI to confirm transfection. We noted increased mitochondrial Ca^{2+} uptake in permeabilized VDAC2 transfected and efsevin-treated cells after the addition of Ca^{2+} and the combined treatment further enhanced mitochondrial Ca^{2+} levels (**FIG. 5A**).

Mitochondria are located in close proximity to Ca^{2+} release sites of the ER/SR and an extensive crosstalk between the two organelles exists (Garcia-Perez et al., 2008; Hayashi et al., 2009; Brown et al., 2010, Cardiovasc Res 88, 241-249; Dorn et al., 2010; Kohlhaas et al., 2013). We examined whether Ca^{2+} released from intracellular stores could be locally transported into mitochondria through VDAC2 in VDAC1/VDAC3 double knockout (V1/V3DKO) MEFs where VDAC2 is the only VDAC isoform being expressed (Roy et al., 2009a). While treatments with ATP, an IP₃-linked agonist, and Thapsigargin, a SERCA inhibitor, stimulated similar global cytoplasmic [Ca^{2+}] elevation in intact cells, only ATP induced a rapid mitochondrial matrix [Ca^{2+}] rise (**FIG. 8**). This finding is consistent with observations obtained in other cell types (Rizzuto et al., 1994, J Cell Biol 126, 1183-1194; Hajnoczky et al., 1995, Cell 82, 415-424) and suggests that Ca^{2+} was locally transferred from IP₃ receptors to mitochondria through VDAC2 at the close ER-mitochondrial associations. We next investigated whether this process could be modulated by efsevin. In permeabilized V1/V3DKO MEFs, treatment with efsevin increased the amount of Ca^{2+} transferred into mitochondria during IP₃-induced Ca^{2+} release (**FIG. 5B**). Also, in intact V1/V3 DKO MEFs, efsevin accelerated the transfer of Ca^{2+} released from intracellular stores into mitochondria during stimulation with ATP (**FIG. 5C and D**).

Efsevin modulates Ca^{2+} sparks and suppresses erratic Ca^{2+} waves in cardiomyocytes

We next examined the effect of efsevin on cytosolic Ca^{2+} signals in isolated adult murine cardiomyocytes. We found that efsevin treatment induced faster inactivation kinetics without affecting the amplitude or time to peak of paced Ca^{2+} transients (**FIG. 6A**). Similarly, efsevin treatment did not significantly alter the frequency, amplitude or Ca^{2+} release flux of

spontaneous Ca^{2+} sparks, local Ca^{2+} release events, but accelerated the decay phase resulting in sparks with a shorter duration and a narrower width (**FIG. 6B**). These results indicate that by activating mitochondrial Ca^{2+} uptake, efsevin accelerates Ca^{2+} removal from the cytosol in cardiomyocytes and thereby restricts local cytosolic Ca^{2+} sparks to a narrower domain for a shorter period of time without affecting SR Ca^{2+} load or RyR Ca^{2+} release. Under conditions of Ca^{2+} overload, single Ca^{2+} sparks can trigger opening of neighbouring Ca^{2+} release units and thus induce the formation of erratic Ca^{2+} waves. Efsevin treatment significantly reduced the number of propagating Ca^{2+} waves in a dosage-dependent manner (**FIG. 6C**), demonstrating a potent suppressive effect of efsevin on the propagation of Ca^{2+} overload-induced Ca^{2+} waves and suggesting that efsevin could serve as a pharmacological tool to manipulate local Ca^{2+} signals.

Mitochondrial Ca^{2+} uptake modulates embryonic cardiac rhythmicity

We believe that efsevin treatment/VDAC2 overexpression suppresses aberrant Ca^{2+} handling-associated arrhythmic cardiac contractions by buffering excess Ca^{2+} into mitochondria. Therefore we predict that activating other mitochondrial Ca^{2+} uptake molecules would likewise restore coordinated contractions in *tre*. To test this model, we cloned zebrafish MCU and MICU1, an inner mitochondrial membrane Ca^{2+} transporter and its regulator (Perocchi et al., 2010, Nature 467, 291-296; Baughman et al., 2011; De Stefani et al., 2011; Mallilankaraman et al., 2012, Cell 151, 630-644; Csordas et al., 2013, Cell Metab 17, 976-987). In situ hybridization showed that MCU and MICU1 were expressed in the developing zebrafish heart and their expression levels were comparable between the wild type and *tre* hearts and embryos with and without efsevin treatment. Overexpression of MCU restored coordinated contractions in *tre*, akin to what was observed with VDAC2 (**FIG. 7A**). In addition, *tre* embryos injected with suboptimal concentrations of MCU or VDAC2 had a fibrillating heart, but embryos receiving both VDAC2 and MCU at the suboptimal concentration manifested coordinated contractions (**FIG. 7B**), demonstrating a synergistic effect of these proteins. Furthermore, overexpression of MCU failed to suppress the *tre* phenotype in the absence of VDAC2 activity and VDAC2 could not restore coordinated contractions in *tre* without functional MCU (**FIG. 7A,C**). Similar results were observed by manipulating MICU1 activity (**FIG. 7D and E**). Together, these findings indicate that mitochondrial Ca^{2+} uptake mechanisms on outer and inner mitochondrial membranes act cooperatively to regulate cardiac rhythmicity.

Mitochondria regulate cardiac rhythmicity through a VDAC dependent mechanism

Affinity agarose beads covalently linked with efsevin (efsevin^{LB}) pulled down 2 protein species from zebrafish embryonic lysate, whereof one, the 32 kD upper band, was sensitive to competition with a 100 fold excess free efsevin^L. The 32 kD band was not detected in proteins eluted from beads capped with ethanolamine alone (beads^C) or beads
 5 linked to an inactive derivative of efsevin, OK-C19^{LB}, but was detected in samples eluted from beads attached with a biologically active derivative, OK-C125^{LB}. Also for the OK-C125^{LB} pull-down the 32kD band was again sensitive to competition with free efsevin. In situ hybridization analysis showed that VDAC1, VDAC2 and MCU are expressed in embryonic hearts at 36 hpf and 48 hpf.

10 **FIG. 9 A)-G)** show that mitochondria regulate cardiac rhythmicity through a VDAC dependent mechanism.

A) Injection of 25pg in-vitro synthesized VDAC1 and VDAC2 mRNA restored cardiac contractions in 53.0±10.2% (n=126) and 52.9±12.1% (n=78) of one-day-old *tre* embryos, respectively, compared to 21.8±5.1% in uninjected siblings (n=111).

15 B) While only ~20% of *myl7:VDAC2;NCX1hMO* embryos have coordinated contractions (n=116), 52.3±2.4% of these embryos established persistent, rhythmic contractions after TBF induction of VDAC2 (n=154).

C) On average, 71.2±8.8% efsevin treated embryos have coordinated cardiac contractions (n=131). Morpholino antisense oligonucleotide knockdown of VDAC2 (MO^{VDAC2}) or VDAC1 (MO^{VDAC1}) attenuates the ability of efsevin to suppress cardiac
 20 fibrillation in *tre* embryos (45.3±7.4% and 46.9±10.7% embryos with coordinated contractions, n=94 and 114, respectively). Knocking down VDAC1/2 simultaneously further suppresses efsevin's effect (30.3±6.3%, n=75).

D) Efsevin treatment restores coordinated cardiac contractions in 76.2±8.7%
 25 NCX1MO embryos, only 54.1±3.6% VDAC2^{zfn/zfn}; NCX1MO embryos and 35.7±7.1% VDAC2^{zfn/zfn};VDAC1MO; NCX1MO embryos have coordinated contractions (n=250).

E) Overexpression of MCU is sufficient to restore coordinated cardiac contractions in *tre* embryos (47.1±1.6% embryos, n=112 as opposed to 18.3±5.3% of uninjected siblings, n=64) while this effect is significantly attenuated when co-injected with morpholino antisense
 30 oligonucleotide targeted to VDAC2 (27.1±1.9% embryos, n=135).

F) Suboptimal overexpression of MCU (MCU^S) and VDAC2 (VDAC2^S) in combination is able to suppress cardiac fibrillation in *tre* embryos (42.9±2.6% embryos, n=129).

G) The ability of VDAC2 to restore rhythmic contractions in *tre* embryos (48.5±3.5%

embryos, n=111) is significantly attenuated when MCU is knocked down by antisense oligonucleotide (MO^{MCU}) ($25.6 \pm 2.4\%$ embryos, n=115). Error bars represent s.d.; * $p < 0.05$; *** $p < 0.001$.

E. Conclusion

5 In summary, we conducted a chemical suppressor screen in zebrafish to dissect the regulatory network critical for maintaining rhythmic cardiac contractions and to identify mechanisms underlying aberrant Ca^{2+} handling-induced cardiac dysfunction. We show that activation of VDAC2 through overexpression or efsevin treatment potently restores rhythmic contractions in NCX1h deficient zebrafish hearts and effectively suppresses Ca^{2+} overload-
 10 induced arrhythmogenic Ca^{2+} events and irregular contractions in mouse and human cardiomyocytes. We provide evidence that potentiating VDAC2 activity enhances mitochondrial Ca^{2+} uptake, accelerates Ca^{2+} transfer from intracellular stores into mitochondria and spatially and temporally restricts single Ca^{2+} sparks in cardiomyocytes. The crucial role of mitochondria in the regulation of cardiac rhythmicity is further supported by
 15 the findings that VDAC2 functions in concert with MCU; these genes have a strong synergistic effect on suppressing cardiac fibrillation and loss of function of either gene abrogates the rescue effect of the other in *tre*.

The regulatory roles of mitochondrial Ca^{2+} in cardiac metabolism, cell survival and fate have been studied extensively (Brown et al., 2010; Dorn et al., 2010; Doenst et al., 2013,
 20 *Circ Res* 113, 709-724; Kasahara et al., 2013, *Science* 342, 734-737; Kohlhaas et al., 2013; Luo et al., 2013, *Circ Res* 113, 690-708). Our study provides genetic and physiologic evidence supporting an additional role for mitochondria in regulating cardiac rhythmicity and reveals VDAC2 as a modulator of Ca^{2+} handling in cardiomyocytes. Our findings, together with recent reports of the physical interaction between VDAC2 and RyR2 (Min et al., 2012,
 25 *Biochem J* 447, 371-379) and the close proximity of outer and inner mitochondrial membranes at the contact sites between the mitochondria and the SR (Garcia-Perez et al., 2011, *Am J Physiol Heart Circ Physiol* 301, H1907-1915), suggest an intriguing model. We propose that mitochondria facilitate an efficient clearance mechanism in the Ca^{2+} microdomain, which modulates Ca^{2+} handling without affecting global Ca^{2+} signals in
 30 cardiomyocytes. In this model, VDAC facilitates mitochondrial Ca^{2+} uptake via MCU complex and thereby controls the duration and the diffusion of cytosolic Ca^{2+} near the Ca^{2+} release sites to ensure rhythmic cardiac contractions. This model is consistent with our observation that efsevin treatment induces faster inactivation kinetics of cytosolic Ca^{2+}

transients without affecting the amplitude or the time to peak in cardiomyocytes and the reports that blocking mitochondrial Ca^{2+} uptake has little impact on cytosolic Ca^{2+} transients (Maack et al., 2006; Kohlhaas et al., 2010, *Circulation* 121, 1606-1613). Further support for this model comes from the observation of the Ca^{2+} peaks on the OMM (Drago et al., 2012, Proc Natl Acad Sci U S A 109, 12986-12991) and the finding that downregulating VDAC2 extends Ca^{2+} sparks (Subedi et al., 2011, *Cell Calcium* 49, 136-143; Min et al., 2012) and that blocking mitochondrial Ca^{2+} uptake by Ru360 leads to an increased number of spontaneous propagating Ca^{2+} waves (Seguchi et al., 2005, *Cell Calcium* 38, 1-9). Future studies on the kinetics of VDAC2-dependent mitochondrial Ca^{2+} uptake and exploring potential regulatory molecules for VDAC2 activity will provide insights into how the crosstalk between SR and mitochondria contributes to Ca^{2+} handling and cardiac rhythmicity.

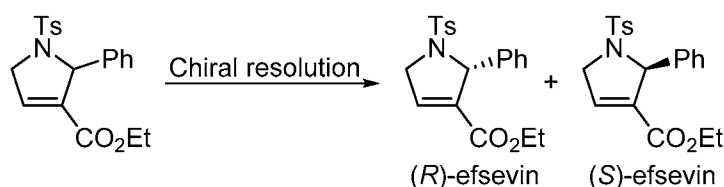
Aberrant Ca^{2+} handling is associated with many cardiac dysfunctions including arrhythmia. Establishing animal models to study molecular mechanisms and develop new therapeutic strategies are therefore major preclinical needs. Our chemical suppressor screen identified a potent effect of efsevin and its biological target VDAC2 on manipulating cardiac Ca^{2+} handling and restoring regular cardiac contractions in fish and mouse and human cardiomyocytes. This success indicates that fundamental mechanisms regulating cardiac function are conserved among vertebrates despite the existence of species-specific features and suggests a new paradigm of using zebrafish cardiac disease models for the dissection of critical genetic pathways and the discovery of new therapeutic approaches. Future studies examining the effects of efsevin on other arrhythmia models would further elucidate the potential for efsevin as a pharmacological tool to treat cardiac arrhythmia associated with aberrant Ca^{2+} handling.

Examples 2-4

Synthesis of Efsevin: synthesis of (*R*)-Efsevin and (*S*)-Efsevin & identification of (*R*)-efsevin as the active antipode for the previously reported defibrillator activity of Efsevin

Example 2

(Resolution of (*R*)- and (*S*)-efsevin through HPLC separation on chiral stationary phase)

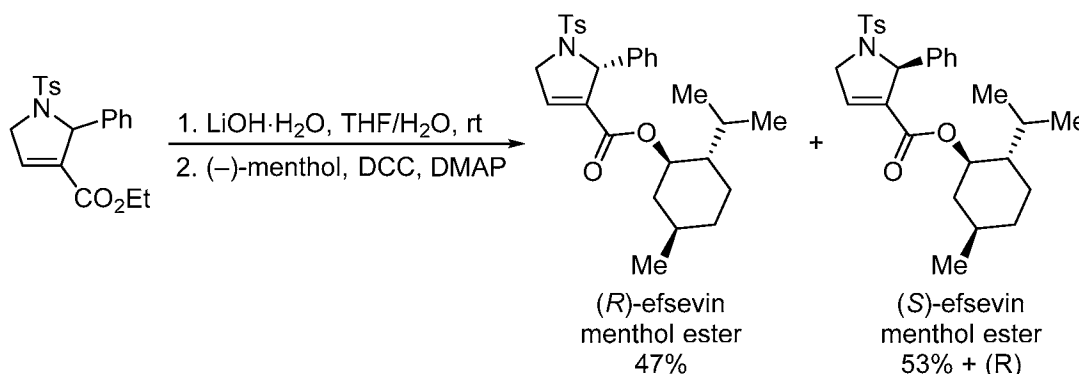


Racemic-efsevin (50 mg, 0.13 mmol) was dissolved in DCM (0.2 mL) and injected into a Shimadzu CBM Lite system using a REGIS (*R, R*)-DACH DNB 5/100 preparatory column (25 cm x 30 mm) with DCM/hexanes (70:30) as eluent at a flow rate of 10.0 mL/min. (*R*)-efsevin eluted at 75.78 min and (*S*)-efsevin at 102.72 min (**FIGs. 10-13**). Fractions were collected and concentrated in vacuo. The protocol was repeated six more times to give (*R*)-efsevin (158 mg) which was recrystallized with hexanes/EtOAc to give (*R*)-efsevin crystals (112 mg, >99% ee).

Enantiomeric excess were determined by a REGIS (*R, R*)-DACH DNB 5/100 analytical column (25 cm x 4.6 mm) with DCM/hexanes (60:40) as eluent at a flow rate of 2.0 mL/min.

Example 3

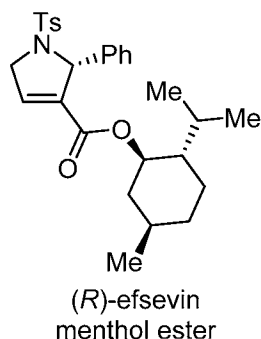
(Resolution of (*R*)-efsevin and (*S*)-efsevin through derivatization using menthol)



Racemic-efsevin (500 mg, 1.35 mmol) was dissolved in 1:1 H₂O/THF (34.0 mL) at room temperature. Lithium hydroxide monohydrate (141.2 mg, 3.37 mmol) was added to the reaction mixture. The reaction was allowed to stir overnight at room temperature. The reaction was monitored by TLC. Upon completion, mixture was cooled to 0 °C using an ice bath and acidified to pH 1 with aq. 1N HCl. The mixture was extracted with DCM (30 mL x 3). The combined organic layer was dried with Na₂SO₄ and concentrated in vacuo. The resulting efsevin carboxylic acid was used in the next step without further purification.

Racemic-efsevin carboxylic acid (1.35 mmol) and (-)-menthol (317.0 mg, 2.0 mmol) were dissolved in DCM (1.0 mL) and cooled to 0 °C using an ice bath. DCC (279.0 mg, 1.35 mmol) and DMAP (1.6 mg, 0.014 mmol) were dissolved in DCM (1.0 mL) and added to the reaction mixture over 1 h using a syringe pump. After addition, the reaction mixture was allowed to warm to room temperature by removing the cooling bath. Upon completion, the mixture was filtered through a short pad of celite and concentrated in vacuo. The crude product was purified using FCC on silica gel (20% EtOAc in hexanes) to yield two diastereoisomeric efsevin menthol esters (385.0 mg, 58%). Selective crystallization in 9:1

hexanes/EtOAc yielded (*R*)-efsevin menthol ester (180 mg) and (*S*)-efsevin menthol ester [200 mg with trace (*R*)-ester]. Both (*R*)- and (*S*)-efsevin menthol esters can be hydrolyzed and esterified to give enantiomerically pure (*R*)- and (*S*)-efsevin. See (a) Jonas, R.; Wurziger, H. *Tetrahedron* **1987**, *43*, 4539–4547. (b) Ito, Y.; Miyake, T.; Hatano, S.; Shima, R.; Ohara, T.; Sugimoto, M. *J. Org. Chem.* **1998**, *120*, 11880–11893. (d) Yang, D.; Ye, X.-Y.; Xu, M. *J. Org. Chem.* **2000**, *65*, 2208–2217. (e) Holý, R.; Kováč, M.; Tichý, M.; Závada, J.; Buděšínský, M.; Císařová, I. *Tetrahedron: Asymmetry* **2005**, *16*, 2031–2038.



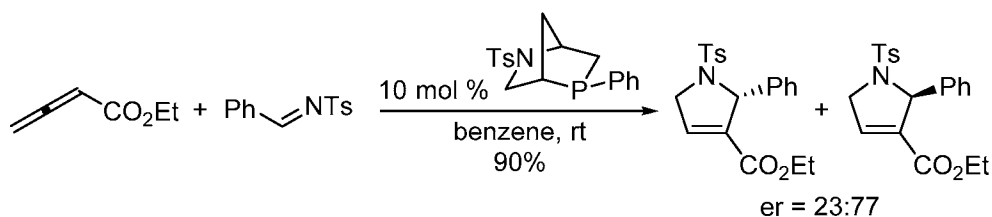
(R)-Efsevin menthol ester

¹H NMR (500 MHz, CDCl₃) (**FIG. 14 top**) δ 7.34 (d, *J* = 8.3 Hz, 2H), 7.22–7.16 (m, 5H), 7.09 (d, *J* = 8.0 Hz, 2H), 6.86 (q, *J* = 2.0 Hz, 1H), 5.74 (dt, *J* = 5.9, 1.9 Hz, 1H), 4.55 (td, *J* = 10.5, 3.9 Hz, 1H), 4.51 (dt, *J* = 16.9, 2.4 Hz, 2H), 2.35 (s, 3H), 1.87–1.82 (m, 1H), 1.63–1.59 (m, 1H), 1.54–1.51 (m, 2H), 1.41–1.35 (m, 1H), 1.05 (tt, *J* = 5.4, 3.1 Hz, 1H), 0.93–0.84 (m, 5H), 0.82–0.71 (m, 2H), 0.54 (d, *J* = 7.0 Hz, 3H), 0.40 (d, *J* = 7.0 Hz, 3H)

¹³C NMR (125 MHz, CDCl₃) (**FIG. 14 bottom**) δ 161.6, 143.1, 139.2, 136.2, 136.1, 135.9, 129.4, 128.2, 128.01, 127.96, 127.0, 74.8, 68.8, 54.6, 46.8, 40.8, 34.1, 31.4, 25.0, 22.7, 22.0, 21.5, 21.0, 15.4.

Example 4

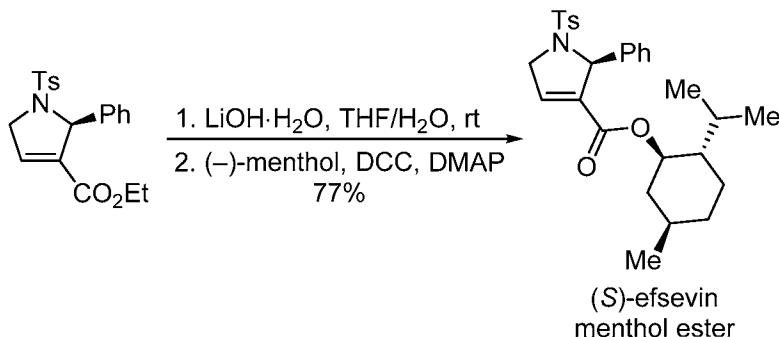
(Catalytic asymmetric synthesis of (*S*)-efsevin)



N-Tosyl benzaldimine (5.67 g, 21.9 mmol) was dissolved in benzene (175.0 mL) at room temperature. Exo-phenyl Kwonphos (756.0 mg, 2.19 mmol) was added to the reaction mixture. Ethyl allenoate (2.94 g, 26.3 mmol) was then added into the reaction mixture dropwise. The reaction was allowed to stir at room temperature and monitored by TLC. Upon

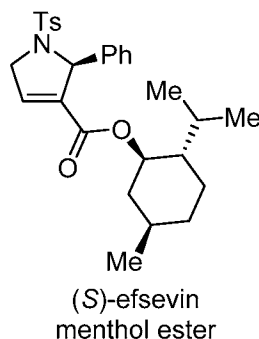
completion, the mixture was concentrated in vacuo. The resulting mixture was purified using FCC on silica gel (20% EtOAc in hexanes) to yield a 23:77 mixture of (*R*)- and (*S*)-efsevin (6.78 g, 90%). Selective crystallization in 4:1 hexanes/EtOAc gave racemic-efsevin (3.12 g), leaving (*S*)-efsevin (3.66 g) in the mother liquor. The mother liquor was recrystallized in 4:1
 5 hexanes/EtOAc to give (*S*)-efsevin (2.1 g, >99% ee)

A. Synthesis of (*S*)-efsevin menthol ester



(*S*)-Efsevin (50 mg, 0.135 mmol) was dissolved in 1:1 H₂O/THF (3.4 mL) at room
 10 temperature. Lithium hydroxide monohydrate (14.1 mg, 0.34 mmol) was added into the reaction mixture. The reaction was allowed to stir overnight at room temperature. The reaction was monitored by TLC. Upon completion, mixture was cooled to 0 °C using an ice bath and acidified to pH 1 with aq. 1N HCl. The mixture was extracted with DCM (10 mL x 3). The combined organic layer was dried with Na₂SO₄ and concentrated in vacuo. The
 15 resulting carboxylic acid was used in the next step without further purification.

(*S*)-Efsevin carboxylic acid (0.135 mmol) and (–)-menthol (31.7 mg, 0.2 mmol) were dissolved in DCM (1.0 mL) and cooled to 0 °C using an ice bath. DCC (27.9 mg, 0.135 mmol) and DMAP (0.2 mg, 0.0014 mmol) were dissolved in DCM (1.0 mL) and added to the reaction mixture over 1 h using a syringe pump. After addition, the reaction mixture was
 20 allowed to warm to room temperature by removing the cooling bath. Upon completion, the mixture was filtered through a short pad of celite and concentrated in vacuo. The crude product was purified using FCC on silica gel (20% EtOAc in hexanes) to yield (*S*)-efsevin menthol ester (50.0 mg, 77%).



(S)-Efsevin menthol ester

¹H NMR (500 MHz, CDCl₃) (**FIG. 15 top**) δ 7.39 (d, *J* = 8.3 Hz, 2H), 7.22–7.17 (m, 5H), 7.12 (d, *J* = 8.0 Hz, 2H), 6.74 (q, *J* = 2.0 Hz, 1H), 5.72 (dt, *J* = 5.8, 2.0 Hz, 1H), 4.54–4.49 (m, 2H), 4.35 (ddd, *J* = 16.9, 5.9, 2.0 Hz, 1H), 2.36 (s, 3H), 1.69–1.63 (m, 1H), 1.61–1.58 (m, 3H), 1.35–1.20 (m, 3H), 0.99–0.90 (m, 1H), 0.83 (d, *J* = 7.1 Hz, 3H), 0.80–0.71 (m, 4H), 0.61 (d, *J* = 7.0 Hz, 3H), 0.51 (q, *J* = 11.8 Hz, 1H)

¹³C NMR (125 MHz, CDCl₃) (**FIG. 15 bottom**) δ 161.5, 143.2, 139.4, 136.5, 135.7, 134.9, 129.4, 128.2, 127.93, 127.86, 127.1, 75.0, 69.1, 54.9, 46.9, 40.2, 34.1, 31.2, 26.5, 23.6, 21.9, 21.5, 20.6, 16.5.

Examples 5-7

Synthesis of screening library & Zebrafish screening

A. Introduction

We generation a large number of diverse small molecules to screen zebrafish *tremblor* mutants, defective in a cardiac-specific sodium calcium exchanger gene, NCX1h, to study cardiac arrhythmia induced by abnormal calcium ion handling. To achieve this, our group build up a diverse library by constructing diverse scaffolds such as dihydropyrroles (Zhu, *et al.*, 2005, *Tetrahedron*, 61, 6276-6282), tetrahydropyridines (Zhu, *et al.*, 2003, *J. Am. Chem. Soc.*, 125, 4716-4717), cyclohexenes (Tran, *et al.*, 2007, *J. Am. Chem. Soc.*, 129, 12632-12633), bicyclic succinimides, coumarins (Henry, *et al.*, 2007, *Org. Lett.*, 9, 3069-3072), dioxanyidenes (Zhu, *et al.*, 2005, *Org. Lett.*, 7, 1387-1390.), dihydropyrones (Creech, *et al.*, 2008, *Org. Lett.*, 10, 429-432), and pyrenes (Zhu, *et al.*, 2005, *Org. Lett.*, 7, 2977-2980) using phosphine-catalyzed reactions, phosphine-catalyzed reactions combined with Michael addition, or the sequence of phosphine-catalyzed reactions/Tebbe reactions/Diels–Alder reactions (see non-limiting examples in **Fig. 18A-E**). The library included a large number of efsevin analogs, including: analogs with ethyl ester motif by using different imines as one of the annulation coupling partners; and analogs with *meta*-, *para*-methyl substitutes, halogen

substitutes, and unsubstituted benzaldehyde to react with *ortho*-, *para*-methyl substitutes and halogen substituted benzenesulfonamide for imine synthesis.

B. General Information

Benzene and dichloromethane were distilled fresh from CaH₂. THF was distilled fresh from sodium. All other reagents were used as received from commercial sources. Reactions were monitored using thin layer chromatography (TLC) performed on 0.25-mm E. Merck silica gel plates (60F-254) and visualized under UV light or through permanganate staining. Flash column chromatography was performed using E. Merck silica gel 60 (230–400 mesh) and compressed air. NMR spectra were obtained on Bruker ARX-400, or Bruker AXR-300 instruments (as indicated), calibrated using residual undeuterated chloroform as an internal reference (7.26 and 77.0 ppm for ¹H and ¹³C NMR spectra, respectively). ¹H NMR spectral data are reported as follows: chemical shift (δ, ppm), multiplicity, coupling constant (Hz), and integration. ¹³C spectral data are reported in terms of the chemical shift. The following abbreviations are used to indicate multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. Affi-gel-10 was purchased from BioRad. EDTA, EGTA, Glycine, NaF, phenylmethylsulfonylfluoride (PMSF) and Trizma base were purchased from Sigma Chemicals. Silver Stain Kit and pre-casted tris-glycine gel were obtained from Invitrogen. Protein inhibitor cocktail was purchased from Roche. Nonidet P-40 was from Fluka. Resin filtration procedures were carried out using a 70μ PE frit cartridges from Applied Separations (cat. # 2449).

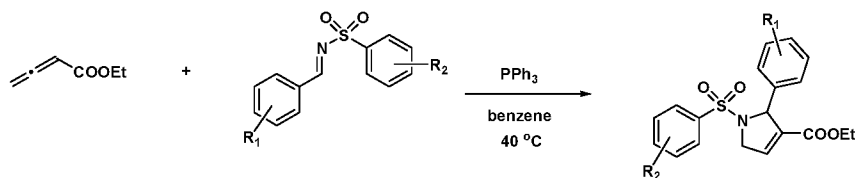
Example 5

(Synthesis of N- Sulfonyimines and allenates)

N-sulfonylimines for the screening library were synthesized from corresponding aldehydes and sulfonamides through the use of TiCl₄, according to the procedure in McKay, *et al.* (McKay, *et al.*, 1981, *J. Chem. Soc., Perkin Trans. 1*, 2435). The rest of the imine were synthesized through the condensation of the corresponding aldehydes with the sulfonamides catalyzed by BF₃·OEt₂ with azeotropic water removal (Dean–Stark), according to the procedure in Jennings, *et al.* (Jennings, *et al.*, 1991, *Tetrahedron*, 47, 5561). Ethyl buta-2,3-dienoate was synthesis according to the procedure in Lang, *et al.* (Lang, *et al.*, 1984, *Organic Syntheses.*, 62, 202).

Example 6

(Synthesis of dihydropyrroles)



Scheme II

Dihydropyrroles for the screening library were synthesized according to the following general procedure. PPh₃ (1 mmol) and imine (1 mmol) were dissolved in dry benzene. Ethyl buta-2,3-dienoate (1.2 mmol) was added dropwise. The mixture was heated to 40 °C and stirred overnight (Scheme II). Solvent was removed under vacuum and the residue was purified by flash chromatography. (Ethyl acetate: Hexane=1 : 6-1 : 4).

Example 7

(Zebrafish screening)

We screened our above mention library by using *tremblor* mutants Zebrafish embryos. The heart cells of *tremblor* mutant embryos do not establish rhythmic synchronized contraction but rather contract independently and create a chaotic contraction pattern. Compounds were screened for their ability to suppress cardiac fibrillation and/or restore rhythmic synchronized contraction. Briefly, we treated the embryo by soaking in 20 μM solution or injecting compounds after 24 hours fertilization and observed the phenotype after 48 hours. Further, we determined the structure-activity relationship (SAR) between the active compound's structures and the ability to suppress cardiac fibrillation and/or restore rhythmic synchronized contraction.

C. Methods

Soaking

Zebrafish *tremblor* mutant eggs were collected after fertilization, and arrayed in 24-well plates (20 twenty embryos/well) in 500 μL buffer. After 24 hour fertilization in 29 °C, 20 μM testing compound was added and using E3 buffer as background control. After 48 hour fertilization in 29 °C, phenotypic changes were observed using a high magnification dissecting microscope.

Injection with Morpholino

Zebrafish eggs were collected at 1 cell stages. 1 nL of compound in Daneu's buffer and 1 nL of morpholino (1mM) to NCX1h was injected into the embryo. After 24 hours the embryo was arrayed in 24-well plates (20 twenty embryos/well) in 500 μ L buffer. After 48 hour fertilization in 29 °C, phenotypic changes were observed using a high magnification dissecting microscope.

Injection into tremblor Mutants

Zebrafish *tremblor* mutant eggs were collected at 1 cell stages. 1 nL of compound in Daneu's buffer were injected into the embryo. After 24 hours the embryo was arrayed in 24-well plates (20 twenty embryos/well) in 500 μ L buffer. After 48 hour fertilization in 29 °C, phenotypic changes were observed using a high magnification dissecting microscope.

D. Results

Activity analysis

We identified multiple active compounds from the library with similar or greater activity than efseven (see non-limiting examples in Fig. 0A and 0B). Table 1 shows some of the most active compounds found in the screens.

TABLE 1

Chemical	Soaking: Number of <i>tremblor</i> Mutants / % Rescued	Injection with Morpholino: Number of Fish / % Rescued	Injection into <i>tremblor</i> Mutants: Number of fish / % Rescued
Formula Id	18 / 80%		
Formula Ie	8 / 75%		
Formula If	19 / 78%		
Formula Ig	17 / 88%		
Formula Ih		14 / 50%	10 / 80%
Formula Ii		8 / 75%	15 / 87%
Formula Ij		8 / 88%	28 / 79%
Formula Ik		8 / 75%	26 / 85%
Formula Il		8 / 63%	12 / 83%
Formula Im		8 / 88%	
Formula In		8 / 88%	

Formula Io		8 / 75%	
Formula Ip		8 / 38%	27 / 74%
Formula Iq		6 / 67%	21 / 81%
Formula Ir			21 / 76%
Formula Is		6 / 67%	23 / 87%
Formula It		6 / 0%	30 / 73%
Formula Iu		8 / 88%	43 / 74%

Tables 2, 3, and 4 show additional screening results (see Fig. 18A-D for structures). Table 2 shows screening results using *tremblor* mutant soaked in 20 μ M test compounds.

5

TABLE 2

Chemical	Number of fish	Rescue percent	Chemical	Number of fish	Rescue percent
F7	89	72%	F7-I-B10	15	63%
F7-I-A02	12	33%	F7-I-B11	19	30%
F7-I-A03	21	38%	F7-I-B12	19	78%
F7-I-A04	27	31%	F7-I-C01	15	33%
F7-I-A05	10	40%	F7-I-C02	14	86% *
F7-I-A06	17	88%	F7-I-C03	17	29%
F7-I-A07	0	Dead	F7-I-C04	26	62%
F7-I-A08	14	57%	F7-I-C05	26	23%
F7-I-A09	19	42%	F7-I-C06	15	11%
F7-I-A10	14	50%	F7-I-C07	16	25%
F7-I-A11	17	50%	F7-I-C08	9	11%
F7-I-A12	14	60%	F7-I-C09	0	Dead
F7-I-B01	20	11%	F7-I-C10	0	Dead
F7-I-B02	15	40%	F7-I-C11	0	Dead
F7-I-B03	23	0%	F7-I-C12	15	100% **
F7-I-B04	8	75%	F7-I-D01	1	Dead
F7-I-B05	15	29%	F7-I-D02	9	0.0%
F7-I-B06	13	0%	F7-I-D03	0	Dead

F7-I-B07	18	80%	F7-I-D04	19	15.8%
F7-I-B08	13	43%	F7 acid	47	21%
F7-I-B09	16	67%	F7 methyl ester	46	44%
F7 ketone	51	26%	F7 isopropyl ester	42	41%

Table 3 shows screening results using morpholino mutants.

TABLE 3

Chemical	Number of fish	Rescue percent	Chemical	Number of fish	Rescue percent
F7-I-D05	8	0%	F7-I-G04	8	75%
F7-I-D06	8	38%	F7-I-G05	8	100%
F7-I-D07	8	38%	F7-I-G06	8	75%
F7-I-D08	15	73%	F7-I-G07	8	88%
F7-I-D09	8	13%	F7-I-G08	8	38%
F7-I-D10	8	0%	F7-I-G09	6	67%
F7-I-D11	8	0%	F7-I-G10	6	67%
F7-I-D12	8	50%	F7-I-G11	7	86%
F7-I-E01	16	63%	F7-I-G12	8	38%
F7-I-E02	8	13%	F7-I-H01	8	63%
F7-I-E03	8	0%	F7-I-H02	8	88%
F7-I-E04	8	0%	F7-I-H03	8	88%
F7-I-E05	16	75%	F7-I-H04	8	75%
F7-I-E06	8	13%	F7-I-H05	8	88%
F7-I-E07	16	50%	F7-I-H06	8	63%
F7-I-E08	14	71%	F7-I-H07	8	63%
F7-I-E09	8	50%	F7-I-H08	7	100%
F7-I-E10	8	25%	F7-I-H09	10	10%
F7-I-E11	8	0%	F7-I-H10	8	25%
F7-I-E12	8	25%	F7-I-H11	8	88%
F7-I-F01	16	81%	F7-I-H12	8	100%
F7-I-F02	14	79%	F7-II-A01	8	38%

F7-I-F03	8	0%	F7-II-A02	8	0%
F7-I-F04	8	0%	F7-II-A03	8	88%
F7-I-F05	14	50%	F7-II-A04	8	100%
F7-I-F06	8	100%	F7-II-A05	8	13%
F7-I-F07	8	0%	F7-II-A06	7	86%
F7-I-F08	8	75%	F7-II-A07	6	17%
F7-I-F09	8	75%	F7-II-A08	7	86%
F7-I-F10	8	88%	F7-II-A09	6	17%
F7-I-F11	8	88%	F7-II-A10	5	80%
F7-I-F12	8	63%	F7-II-A11	6	0%
F7-I-G01	8	100%	F7-II-A12	8	0%
F7-I-G02	8	75%	F7-II-B01	6	67%
F7-I-G03	8	100%	F7-II-B02	8	38%

Table 4 shows screening results using injection into *tremblor* mutants.

TABLE 4

Chemical	Screen 1		Screen 2		Screen 3		Total number of fish	Average rescue ratio
	Number of fish	Rescue percent	Number of fish	Rescue percent	Number of fish	Rescue percent		
F7-I-D08	13	23%					13	23%
F7-I-E01	8	0%					8	0%
F7-I-E05	8	50%					8	50%
F7-I-E07	8	50%					8	50%
F7-I-E08	12	42%					12	42%
F7-I-F01	7	14%					7	14%
F7-I-F02	12	58%					12	58%
F7-I-F05	10	80%					10	80%
F7-I-F06	10	40%					10	40%
F7-I-F08	10	20%					10	20%
F7-I-F09	15	87%					15	87%
F7-I-F10	8	50%					8	50%
F7-I-F11	10	80%	18	78%			28	79%
F7-I-F12	5	60%					5	60%
F7-I-G01	11	73%	12	50%			23	61%
F7-I-G02	7	57%					7	57%
F7-I-G03	10	20%					10	20%

F7-I-G04	9	78%	17	88%			26	85%
F7-I-G05	11	73%	14	50%			25	60%
F7-I-G06	11	55%					11	55%
F7-I-G07	10	40%					10	40%
F7-I-G09	23	78%					23	78%
F7-I-G10	28	66%					28	66%
F7-I-G11	31	62%					31	62%
F7-I-G12	42	61%					42	61%
F7-I-H01	12	83%					12	83%
F7-I-H02	5	100%	19	74%			24	79%
F7-I-H03	7	86%	9	78%			16	81%
F7-I-H04	6	100%	6	67%			12	83%
F7-I-H05	7	86%	16	69%	20	75%	43	74%
F7-I-H06	9	44%					9	44%
F7-I-H07	10	90%	21	52%			31	65%
F7-I-H08	9	44%					9	44%
F7-I-H09	8	88%	15	46%			23	61%
F7-I-H10	5	60%					5	60%
F7-I-H11	9	56%					9	56%
F7-I-H12	13	54%					13	54%
F7-II-A01	27	74%					27	74%
F7-II-A02	18	62%					18	62%
F7-II-A03	17	60%					17	60%
F7-II-A04	22	48%					22	48%
F7-II-A05	7	29%					7	29%
F7-II-A06	36	64%					36	64%
F7-II-A07	15	33%					15	33%
F7-II-A08	20	26%					20	26%
F7-II-A09	16	22%					16	22%
F7-II-A10	33	40%					33	40%
F7-II-A11	30	73%					30	73%
F7-II-A12	14	5%					14	5%
F7-II-B01	7	91%	14	79%			21	81%
F7-II-B02	13	7%					13	7%
F7-II-B10	33	58%					33	58%
F7-II-B11	35	37%					35	37%
F7-II-B12	21	76%					21	76%
F7-II-C01	25	60%					25	60%
F7-II-C02	17	65%					17	65%
F7-II-C03	22	55%					22	55%

E. SAR analysis

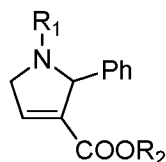
Further, SAR analysis showed that *para*-halogen, especially *para*-fluoro substituted benzaldehyde has good structure-function correlation. For
benznensulfonamide the *orth*-fluoro and *para*-fluro substituted groups also showed
5 good structure-function correlation.

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

10

CLAIMS

1. A compound of structure of Formula I or a derivative thereof:



Formula I

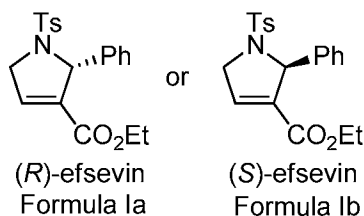
wherein:

R₁ is tosyl, or mesyl group; and

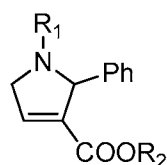
wherein R₂ is a hydrocarbyl group with or without a heteroatom or wherein the carboxylic ester of R₂ is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine), and

wherein the compound is effective to potentiate mitochondrial Ca²⁺ uptake so as to modulate cardiac rhythmicity in a subject.

2. The compound of claim 1, wherein R₁ is tosyl and R₂ is ethyl.
3. The compound of claim 2, wherein the compound is in an enantiomerically pure form Formula Ia or Formula Ib:



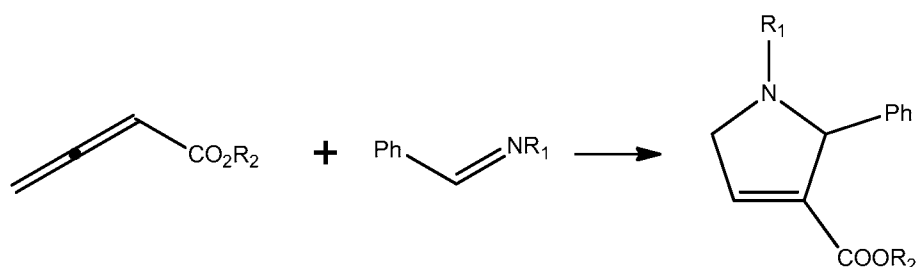
4. The compound of claim 1, wherein R₂ is methyl, ethyl, C3-C6 short alkyl, or menthyl group.
5. The compound of claim 1, wherein R₂ is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.
6. The compound of claim 1, which is in an optionally active (e.g., substantially enantiomerically pure) form.
7. A method of forming the compound of Formula I:



Formula I

where R_1 is tosyl, or mesyl group; and R_2 is a hydrocarbyl group with or without a heteroatom or wherein the carboxylic ester of R_2 is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine),

comprising reacting with according to a reaction of Scheme I



Formula I (Scheme I)

to form the compound of Formula I.

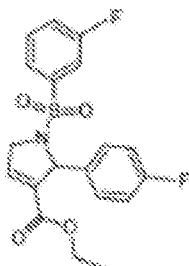
8. The method of claim 7, wherein the reaction of Scheme I is carried out under asymmetric synthesis conditions.
9. The method of claim 7, further comprising performing chiral resolution of compound of Formula I to yield *R*- or *S*- enantiomers of the compound in an optionally active form.
10. The method of claim 9, wherein the chiral resolution is performed on an HPLC chiral stationary phase.
11. The method of claim 9, wherein the chiral resolution is achieved by reacting the compound with a chiral agent.
12. The method of claim 11, wherein the chiral agent is menthol.
13. A method of regulating cardiac rhythmicity in a subject, comprising potentiating mitochondrial Ca^{2+} uptake by inducing VDAC2 or VDAC1 overexpression in the subject to restore rhythmic

contraction,
inducing overexpression of or activating VDAC (VDAC2 or VDAC1) and/or MCU (MCU or MICU1) complex, or
administering to the subject in need thereof an agent effective to induce Ca^{2+} transporting activity of VDAC2 or VDAC1.

14. The method of claim 13, wherein inducing VDAC2 or VDAC1 overexpression in the subject is via gene therapy.
15. The method of claim 13, wherein the agent is a VDAC2 or VDAC1 gene product.
16. The method of claim 15, wherein the VDAC2 or VDAC1 gene product is a VDAC2 or VDAC1 protein, or a VDAC2 or VDAC1 RNA.
17. The method of claim 13, wherein the agent is the compound of Formula I and/or Formula II.
18. The method of claim 17, wherein the agent is efsevin.
19. The method of claim 18, wherein the efsevin is an efsevin enantiomer.
20. The method of claim 17, wherein the compound of Formula I and/or Formula II is in a composition.
21. The method of claim 20, wherein the composition comprises a pharmaceutically acceptable carrier.
22. The method of claim 20, wherein the subject suffers from a disorder related to cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca^{2+} handling.
23. The method of claim 22, wherein the disorder is cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia, or cardiomyopathy.
24. A composition comprising the compound of claim 1.
25. The composition of claim 24, in a formulation suitable for administration to a subject.
26. The composition of claim 24, wherein the composition further comprises a

pharmaceutically acceptable carrier.

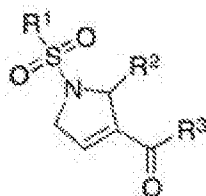
27. A method of forming the composition of claim 25, comprising providing the compound of claim 1 in an effective amount, and forming the composition.
28. A compound of structure of Formula II:



Formula II

or wherein the carboxylic ester is attached to a mono-N-Boc protected 2,2'-(ethylenedioxy)bis(ethylamine).

29. A composition comprising the compound of claim 28.
30. The composition of claim 29, in a formulation suitable for administration to a subject.
31. The composition of claim 29, wherein the composition further comprises a pharmaceutically acceptable carrier.
32. A method of forming the composition of claim 30, comprising providing the compound of claim 1 in an effective amount, and forming the composition.
33. A compound of structure of Formula Ic:



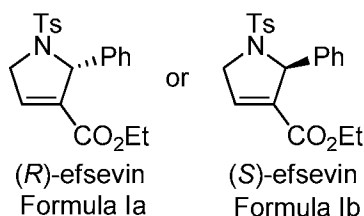
Formula Ic

wherein:

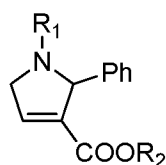
R₁ is an alkane, phenyl, heteroaryl, or substituted phenyl group; R₂ is a phenyl, heteroaryl, substituted phenyl, or hydrocarbyl group with or without a heteroatom; and R₃ is an alkoxy, amino, amino ether, N-Boc-protected 2-aminoethoxyethoxyethylamino group, or a C1-C10 straight or branched, acyclic or cyclic alkyloxy group, aryloxy, or amino group with or without heteroatom; and

wherein the compound is effective to potentiate mitochondrial Ca^{2+} uptake so as to modulate cardiac rhythmicity in a subject.

34. The compound of claim 33, wherein R_1 is phenyl and R_2 is ethyl.
35. The compound of claim 33, wherein R_1 is para-tolyl, R_2 is phenyl, and R_3 is ethoxy.
36. The compound of claim 34, wherein the compound is in an enantiomerically pure form Formula Ia or Formula Ib:



37. The compound of claim 33, wherein R_1 is orth-fluoro substituted phenyl or para-fluoro substituted phenyl.
38. The compound of claim 33, wherein R_3 is ethoxy, menthyloxy, or N-Box-protected 2-aminoethoxyethoxyethylamino group.
39. The compound of claim 33, wherein R_2 is para-fluoro substituted phenyl.
40. The compound of claim 33, wherein R_2 is methyl, ethyl, C3-C6 short alkyl, or menthyl group.
41. The compound of claim 33, wherein R_2 is a C1-C10 straight or branched, acyclic or cyclic alkyl group, or aryl group.
42. The compound of claim 33, which is in an optionally active (e.g., substantially enantiomerically pure) form.
43. A method of forming the compound of Formula I:

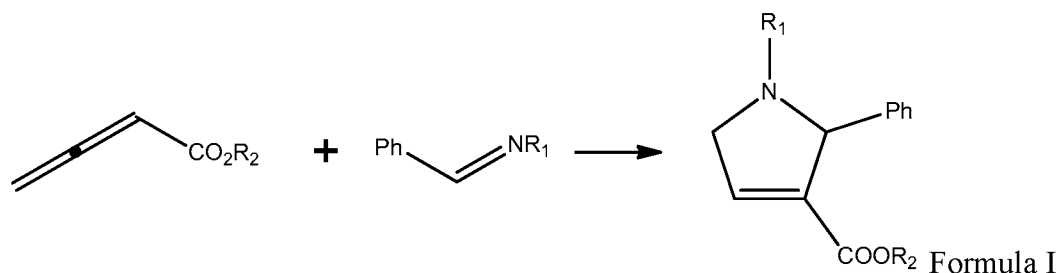


Formula I

where R_1 is tosyl, or mesyl group; and R_2 is a hydrocarbyl group with or without a

heteroatom,

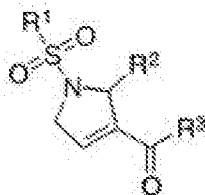
comprising reacting $\text{CH}_2=\text{CHCO}_2\text{R}_2$ with Ph-CH=NR_1 according to a reaction of Scheme I



(Scheme I)

to form the compound of Formula I.

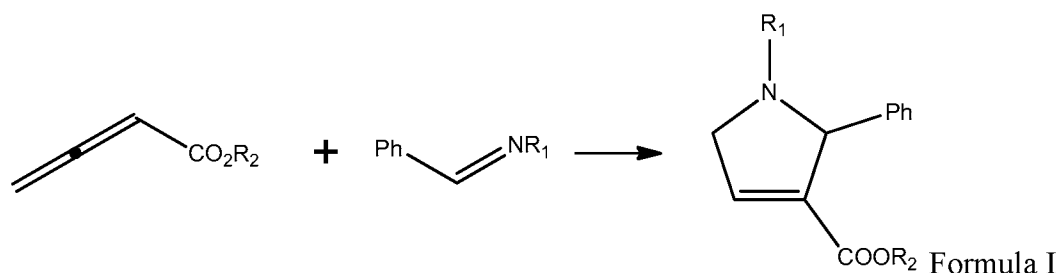
44. A method of forming the compound of Formula Ic:



Formula Ic

where R_1 is substituted phenyl or substituted or unsubstituted heteroaryl; and R_2 is substituted phenyl or substituted or unsubstituted heteroaryl; and R_3 is an alkoxy, amino, amino ether, N-Boc-protected 2-aminoethoxyethoxyethylamino group, or a C1-C10 straight or branched, acyclic or cyclic alkyloxy group, aryloxy, or amino group with or without heteroatom

comprising reacting $\text{CH}_2=\text{CHC(=O)R}_3$ with Ph-CH=NR_1 according to a reaction of Scheme I



(Scheme I)

to form the compound of Formula Ic.

45. The method of claim 43, wherein the reaction of Scheme I is carried out under asymmetric synthesis conditions.
46. The method of claim 43, further comprising performing chiral resolution of compound of Formula I to yield *R*- or *S*- enantiomers of the compound in an optionally active form.
47. The method of claim 46, wherein the chiral resolution is performed on an HPLC chiral stationary phase.
48. The method of claim 46, wherein the chiral resolution is achieved by reacting the compound with a chiral agent.
49. The method of claim 48, wherein the chiral agent is menthol.
50. A method of regulating cardiac rhythmicity in a subject, comprising potentiating mitochondrial Ca^{2+} uptake by inducing VDAC2 or VDAC1 overexpression in the subject to restore rhythmic contraction, inducing overexpression of or activating VDAC (VDAC2 or VDAC1) and/or MCU (MCU or MICU1) complex, or administering to the subject in need thereof an agent effective to induce Ca^{2+} transporting activity of VDAC2 or VDAC1.
51. The method of claim 50, wherein inducing VDAC2 or VDAC1 overexpression in the subject is via gene therapy.
52. The method of claim 50, wherein the agent is a VDAC2 or VDAC1 gene product.
53. The method of claim 52, wherein the VDAC2 or VDAC1 gene product is a VDAC2 or VDAC1 protein, or a VDAC2 or VDAC1 RNA.
54. The method of claim 50, wherein the agent is the compound of Formula I.
55. The method of claim 54, wherein the agent is efsevin.
56. The method of claim 55, wherein the efsevin is an efsevin enantiomer.
57. The method of claim 54, wherein the compound of Formula I is in a composition.

58. The method of claim 57, wherein the composition comprises a pharmaceutically acceptable carrier.
59. The method of claim 57, wherein the subject suffers from a disorder related to cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca²⁺ handling.
60. The method of claim 59, wherein the disorder is cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia, or cardiomyopathy.
61. A composition comprising the compound of claim 33.
62. The composition of claim 61, in a formulation suitable for administration to a subject.
63. The composition of claim 61, wherein the composition further comprises a pharmaceutically acceptable carrier.
64. A method of forming the composition of claim 62, comprising providing the compound of claim 33 in an effective amount, and forming the composition.
65. A method of regulating cardiac rhythmicity in a subject in need thereof comprising administering to the subject a composition that comprises a compound that increases the activity of VDAC2 or VDAC1.
66. The method of claim 65, wherein the compound binds to VDAC2 or VDAC1.
67. The method of claim 65 or 66, wherein the compound increases Ca²⁺ transporting activity of VDAC2.
68. The method of any of claim 65-67, wherein the compound is the compound of claim 1, 28, or 33.
69. The method of claim 68, wherein the compound is the compound of claim 1.
70. The method of claim 68, wherein the compound is the compound of claim 28.
71. The method of claim 68, wherein the compound is efsevin.
72. The method of claim 71, wherein the efsevin is an efsevin enantiomer.

73. The method of claim 68, wherein the compound is in a composition.
74. The method of claim 73, wherein the composition comprises a pharmaceutically acceptable carrier.
75. The method of claim 65, wherein the subject suffers from a disorder related to cardiac arrhythmicity or cardiac disorder with a root in aberrant Ca²⁺ handling.
76. The method of claim 75, wherein the disorder is cardiac fibrillation, arrhythmia, atrial fibrillation, sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia, or cardiomyopathy.

FIG. 1A

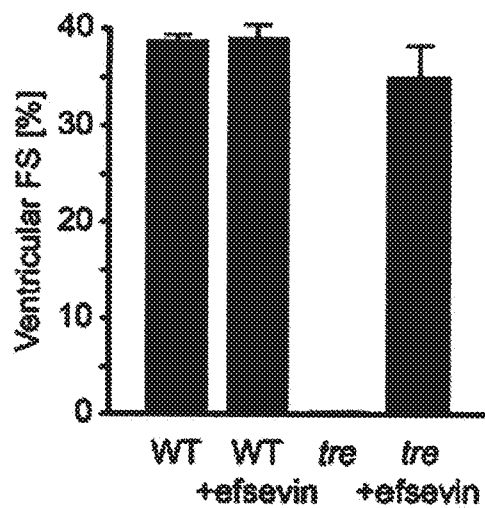


FIG. 1B

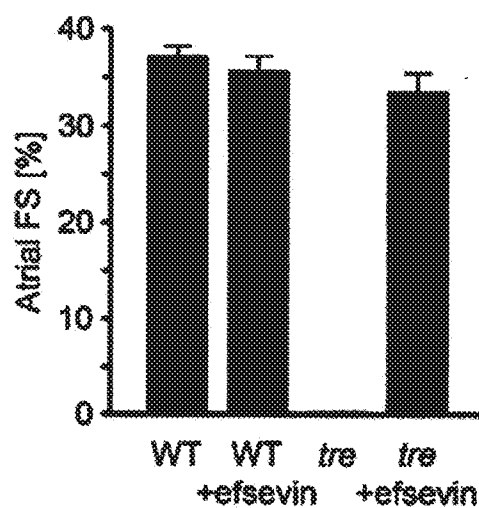


FIG. 1C

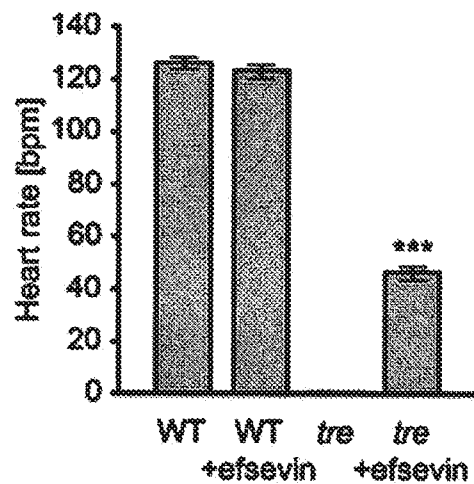


FIG. 1D

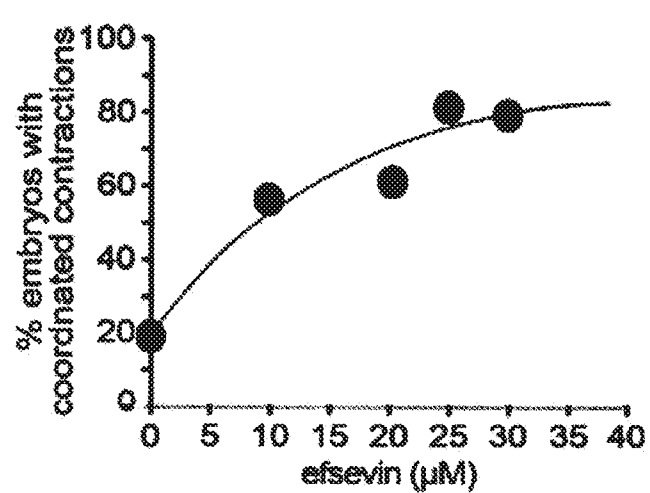


FIG. 1E

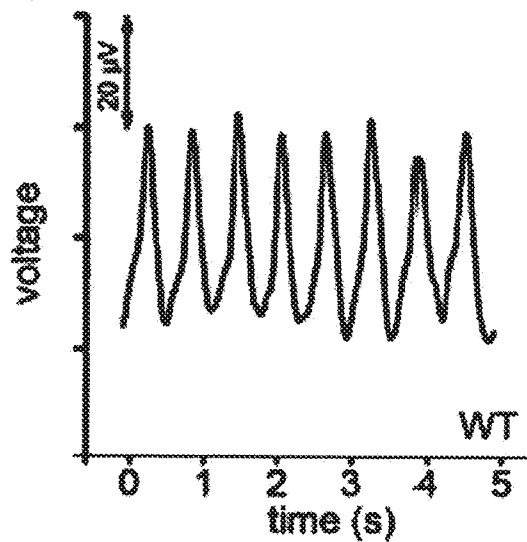


FIG. 1F

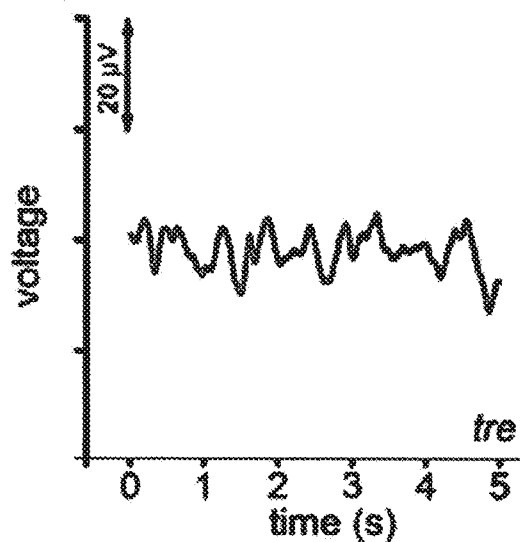


FIG. 1G

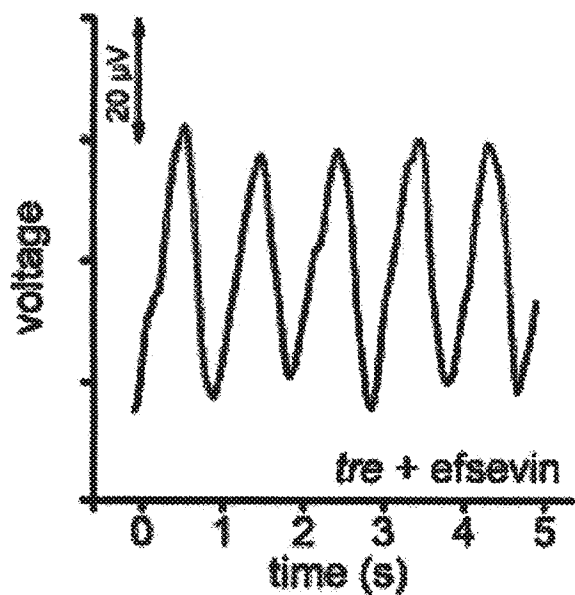


FIG. 2A

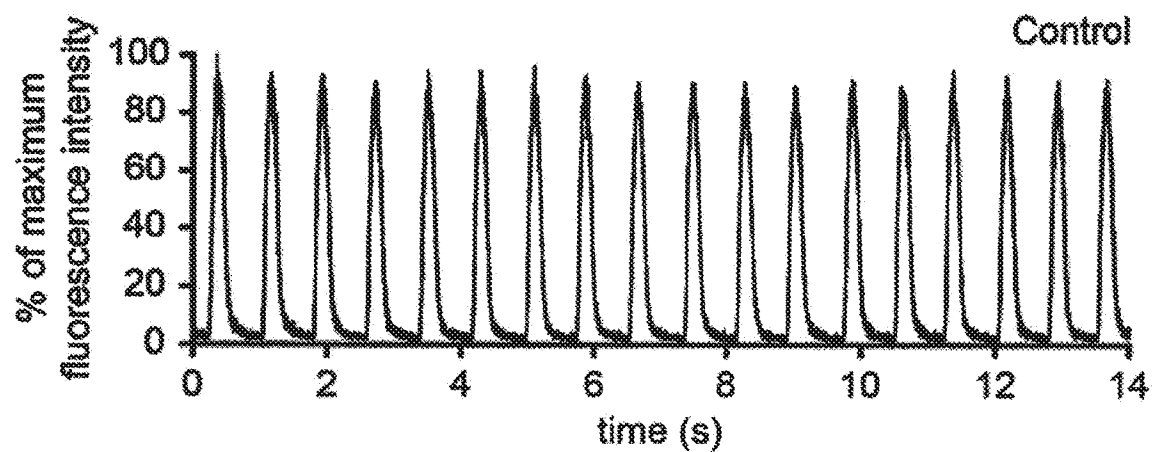


FIG. 2B

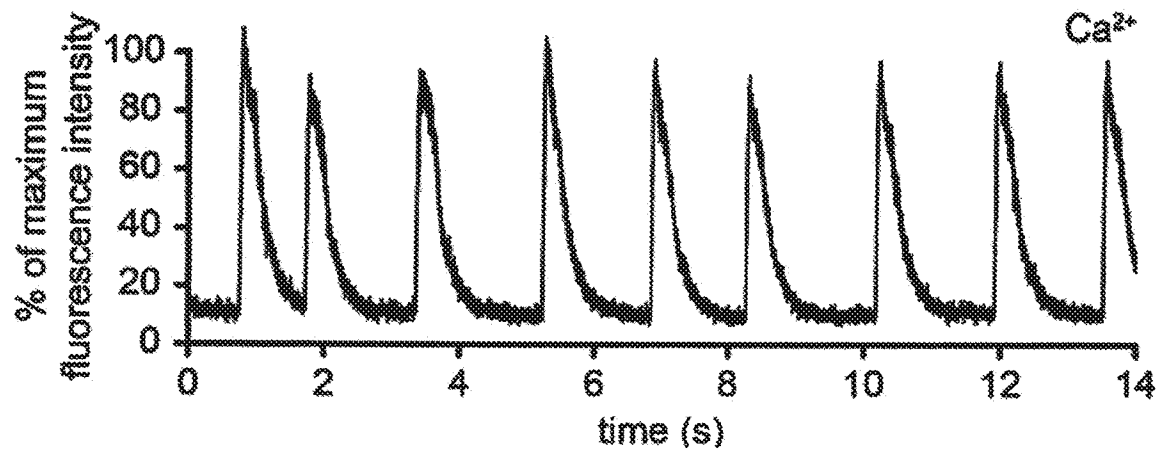


FIG. 2C

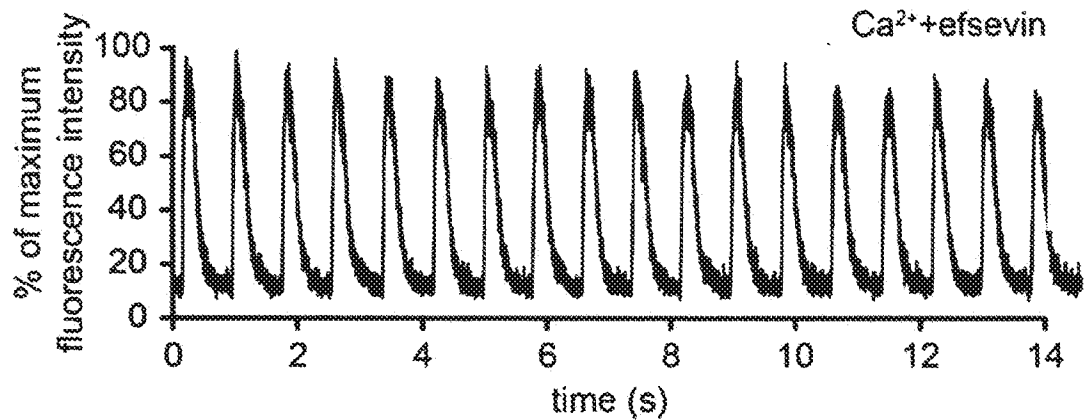


FIG. 2D

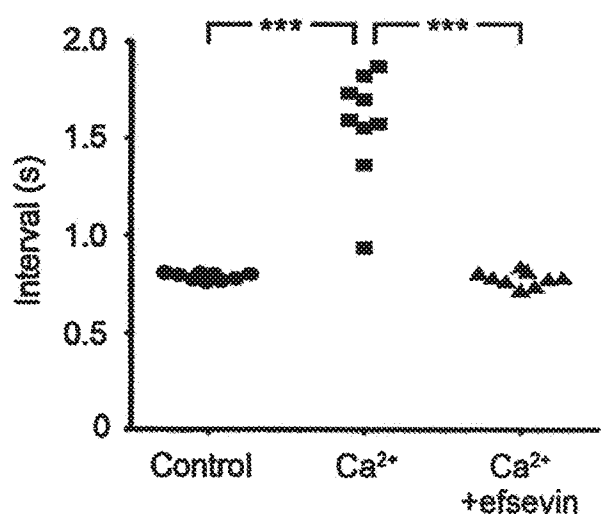


FIG. 2E

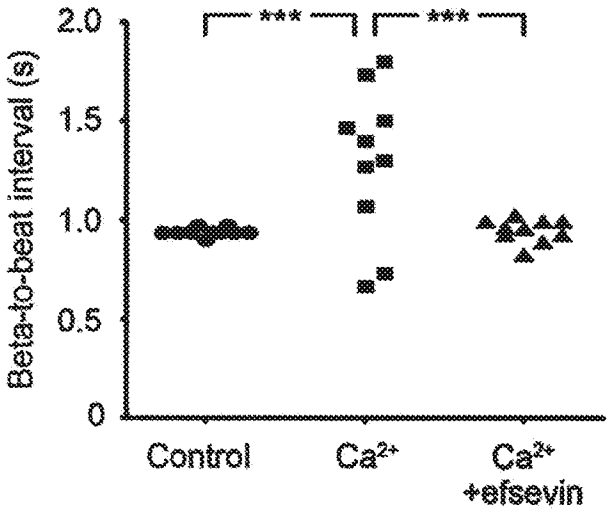


FIG. 2F

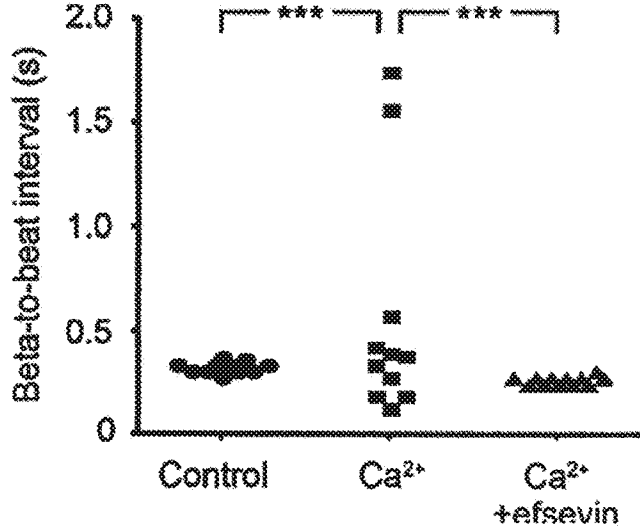


FIG. 3A

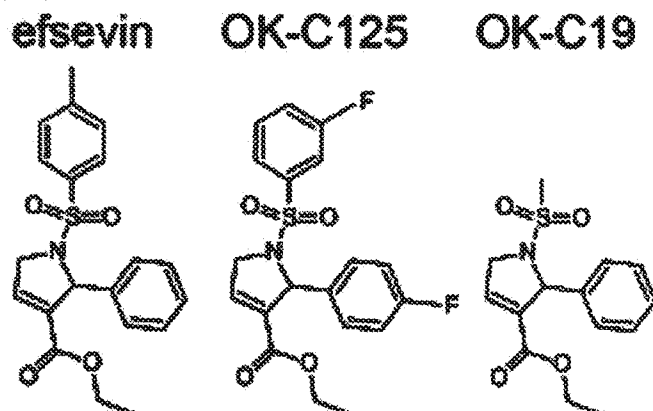


FIG. 3B

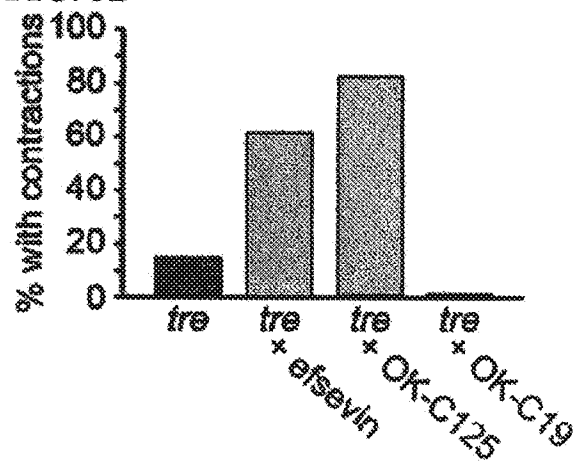


FIG. 3C

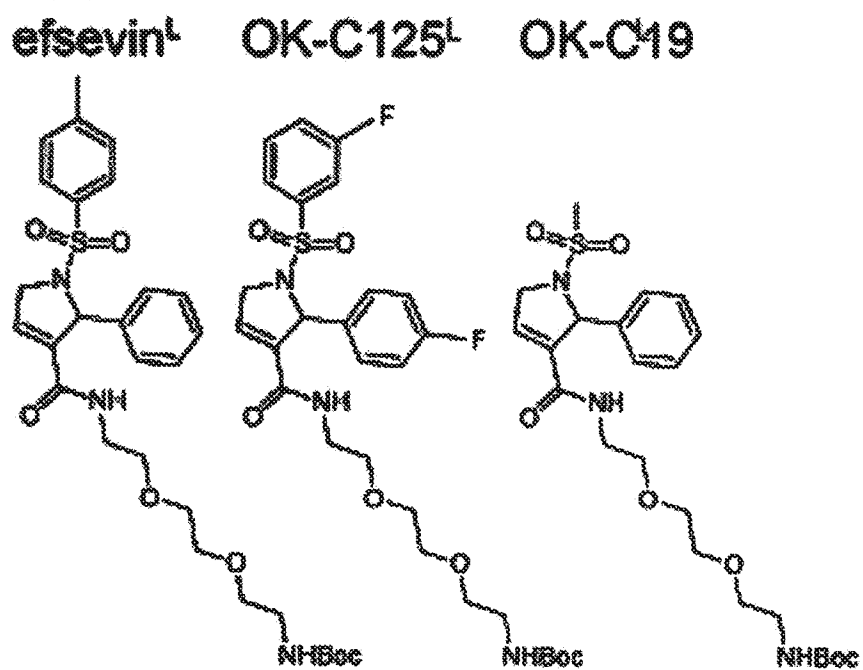


FIG. 3D

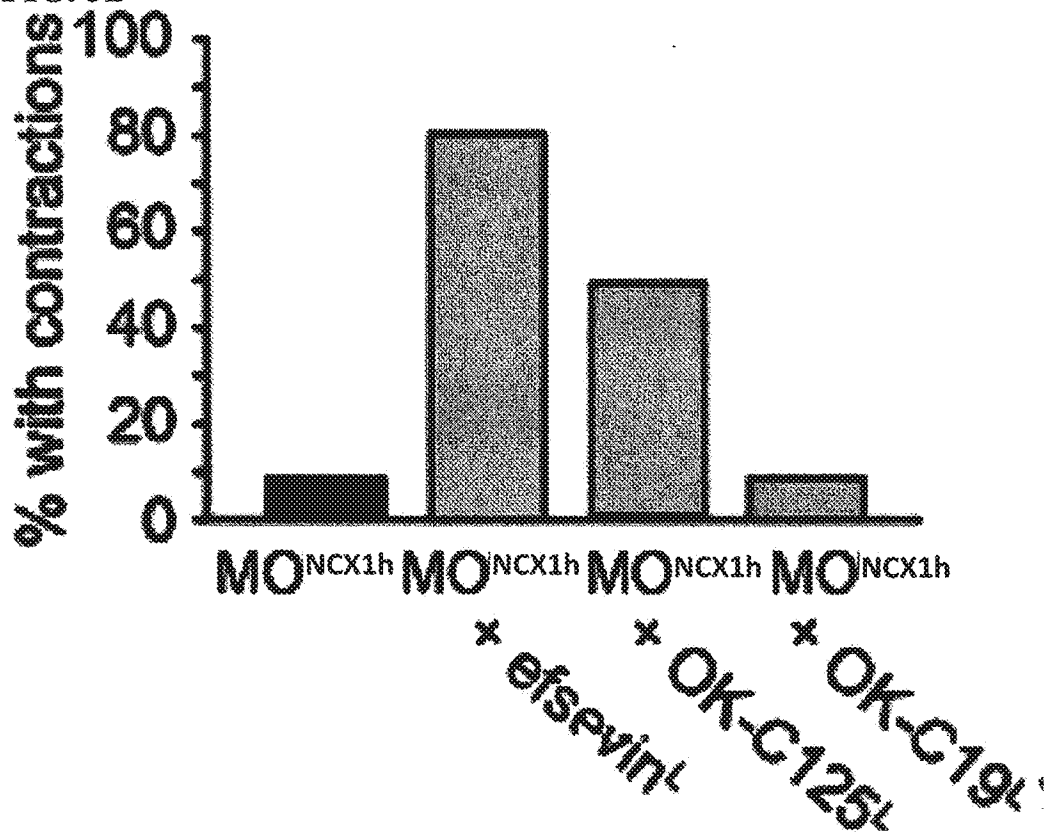


FIG. 3E

VDAC2 - 30% Sequence Coverage

MAVPPAYADLGKSAKDIFN
 KGYGFGMVKLDVKTKSASG
 VEFKTSOSSNTDTSKVVG
 LETKYKRSEYGLTFTEKWN
 TDNTLGTEINIEDQIAKGL
 KLTFDTTFSPNTGKKSGKV
 KTAYKREFVNLGCDVDFDF
 AGPTIHGAADVGYEGWLAG
 YQMSFDTAKSKMTQNNFAV
 GYKTGDFQLHTNVNDGSEF
 GGSIIYQKVSDKLETAVNLA
 WTAGSNSTRFGIAAKYQLD
 KDASISAKVNNTSLVGVGY
 TQSLRPGIKLTLSALVDGK
 SINSGGHLGLGLELEA

FIG. 4A

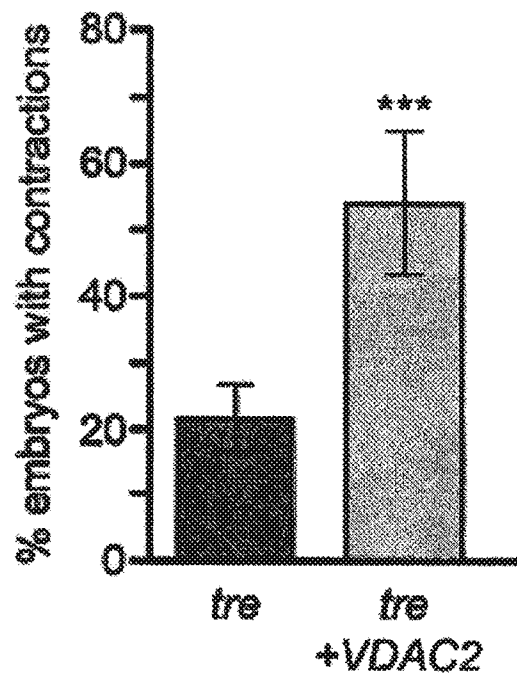


FIG. 4B

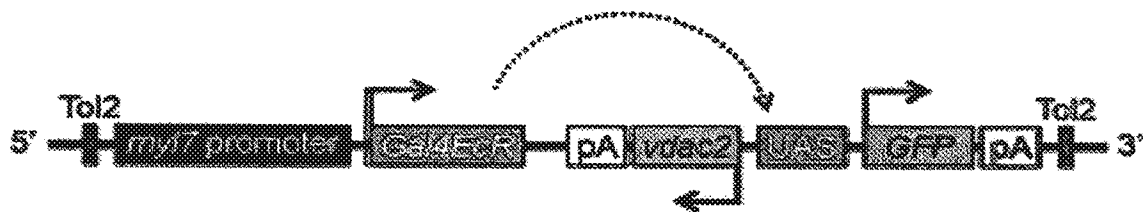


FIG. 4C

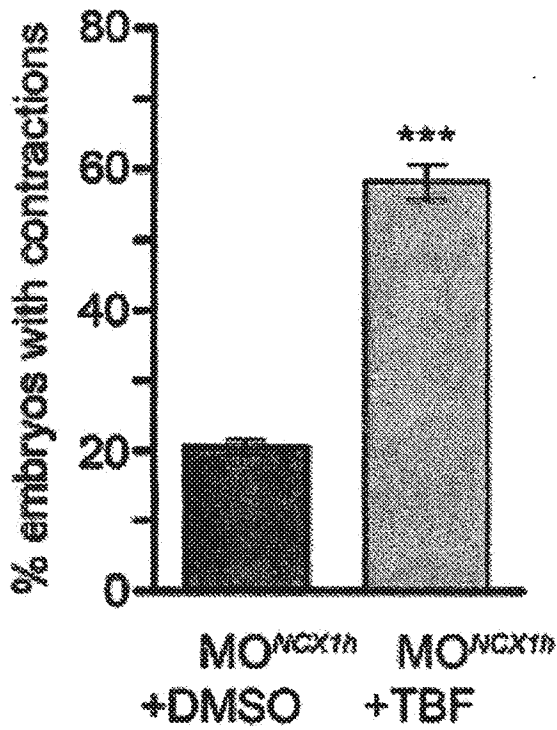


FIG. 4D

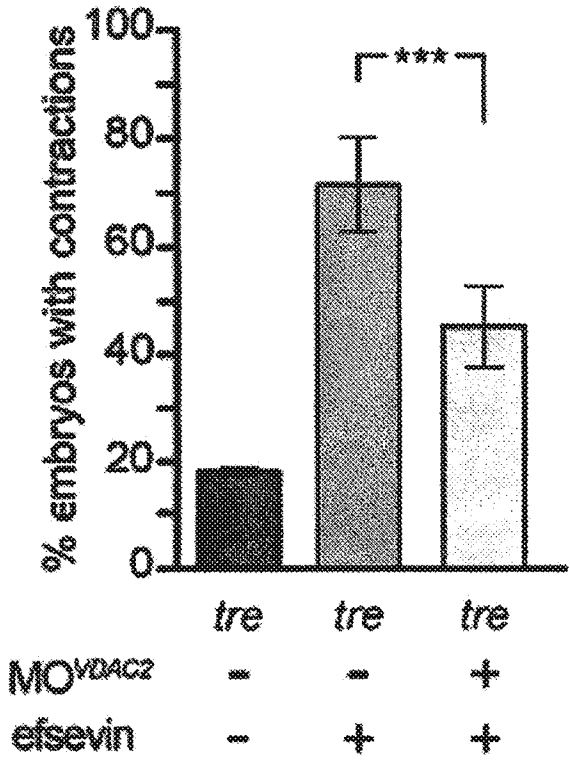


FIG. 4E

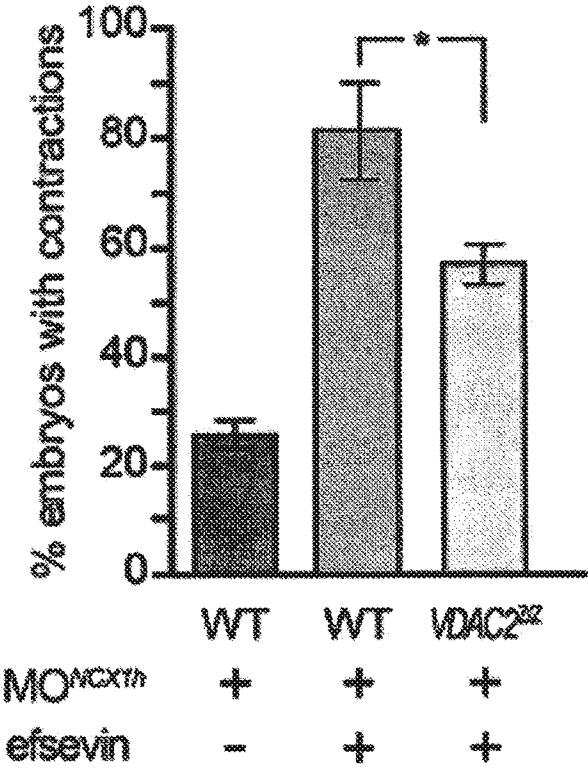


FIG. 4F

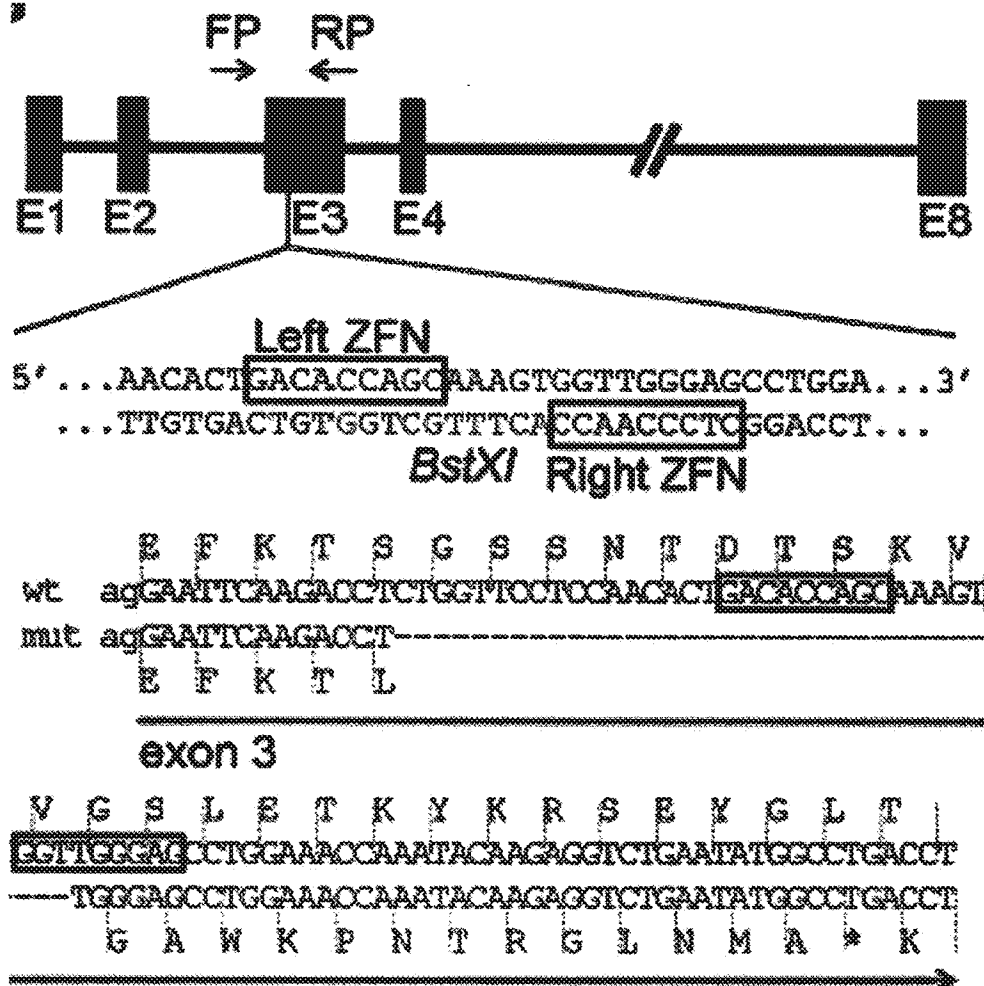


FIG. 5A

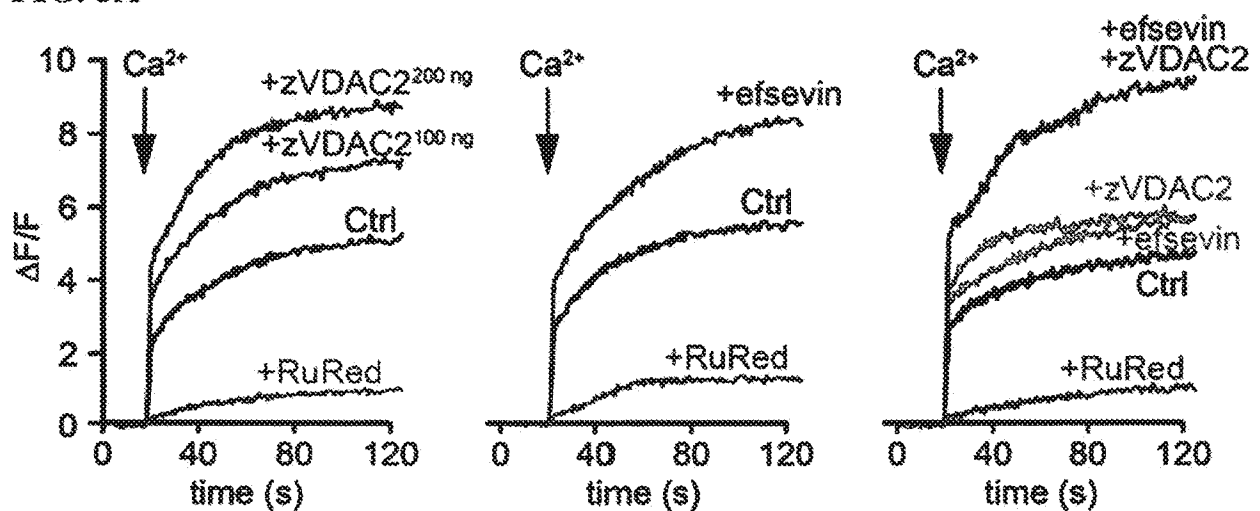


FIG. 5B

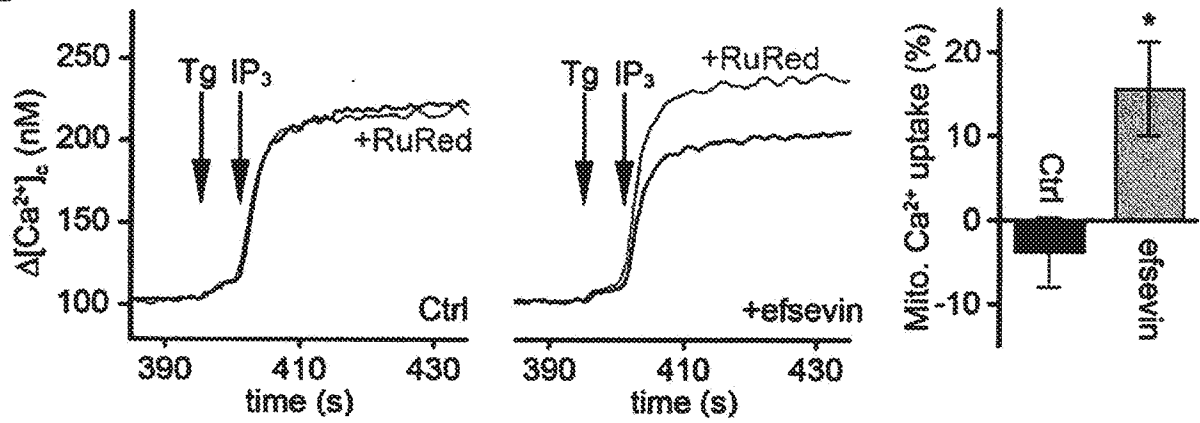


FIG. 5C

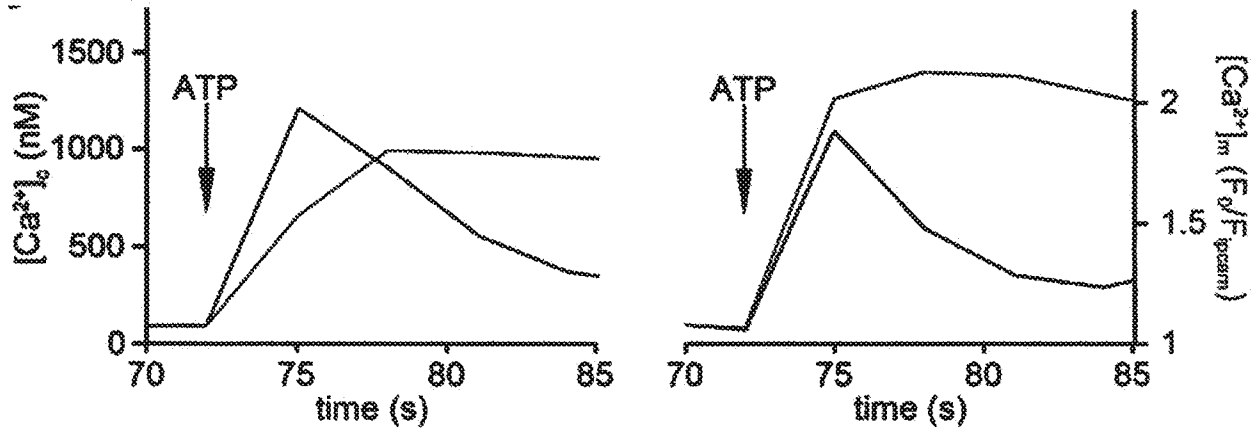


FIG. 5D

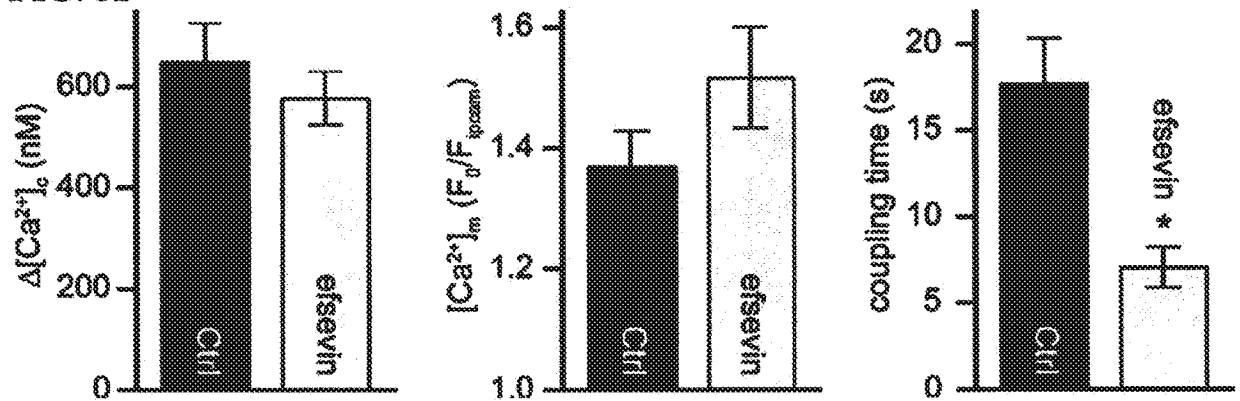


FIG. 6A

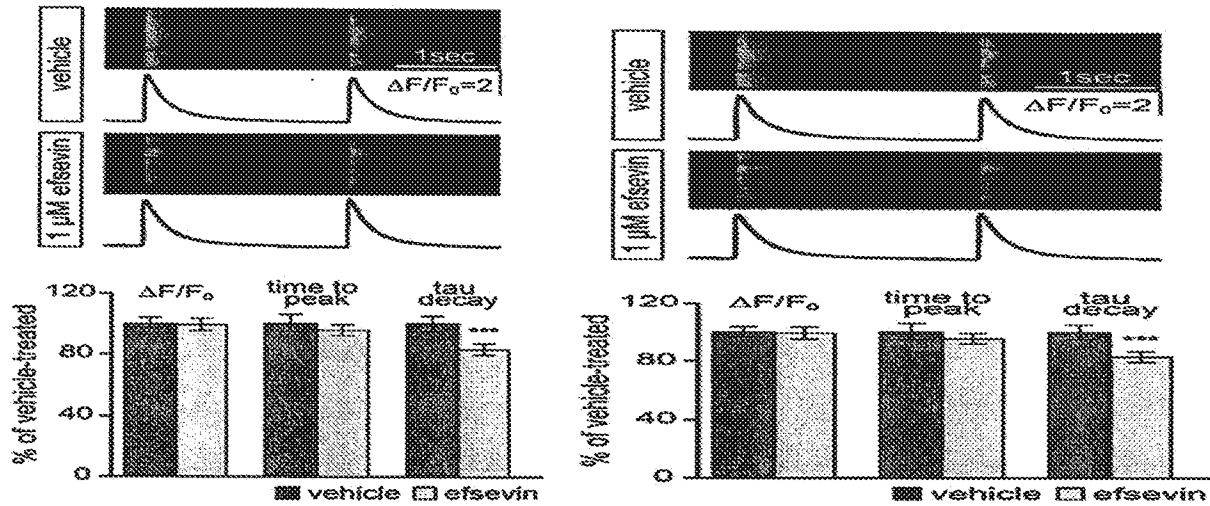


FIG. 6B

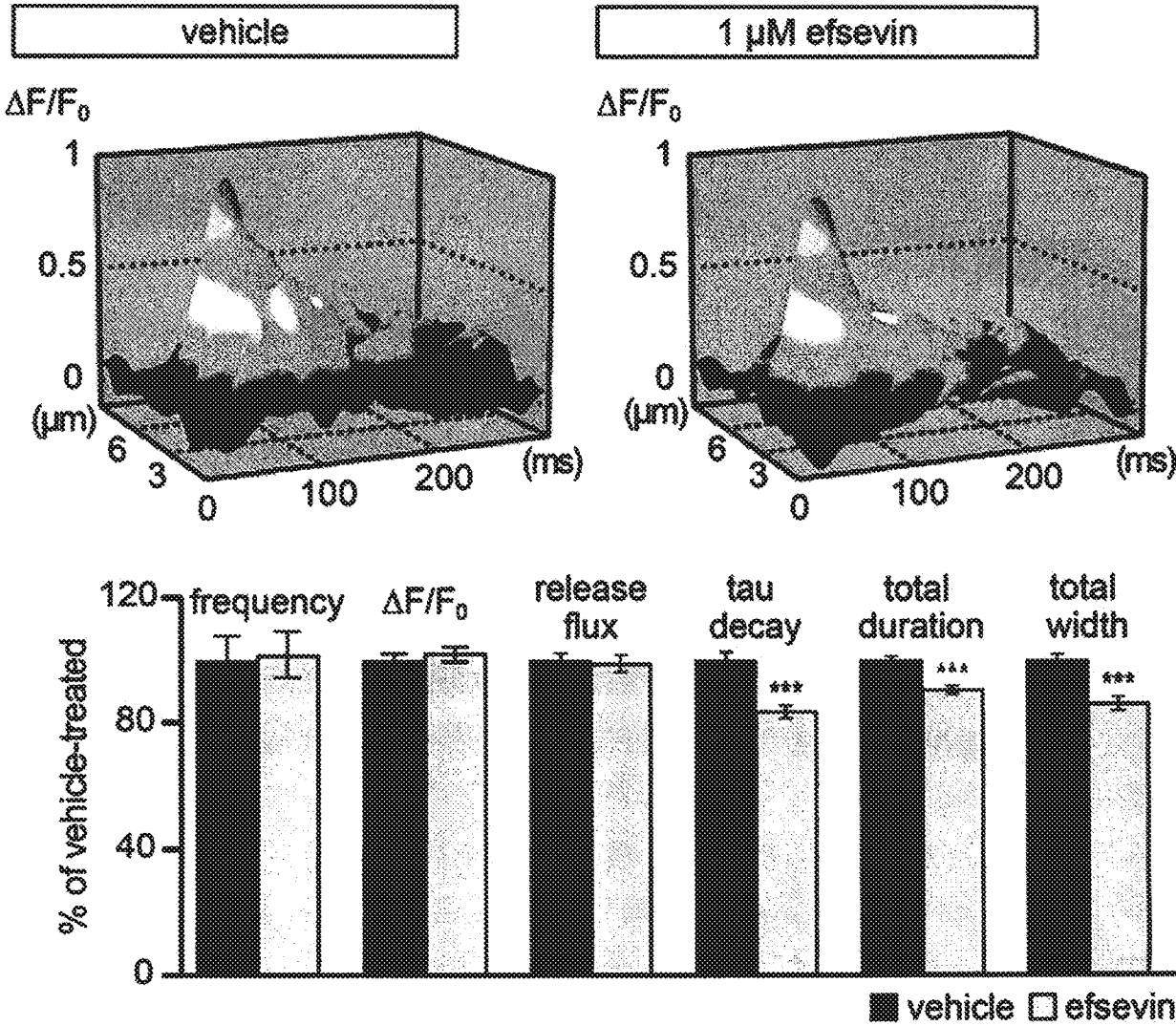


FIG. 6C

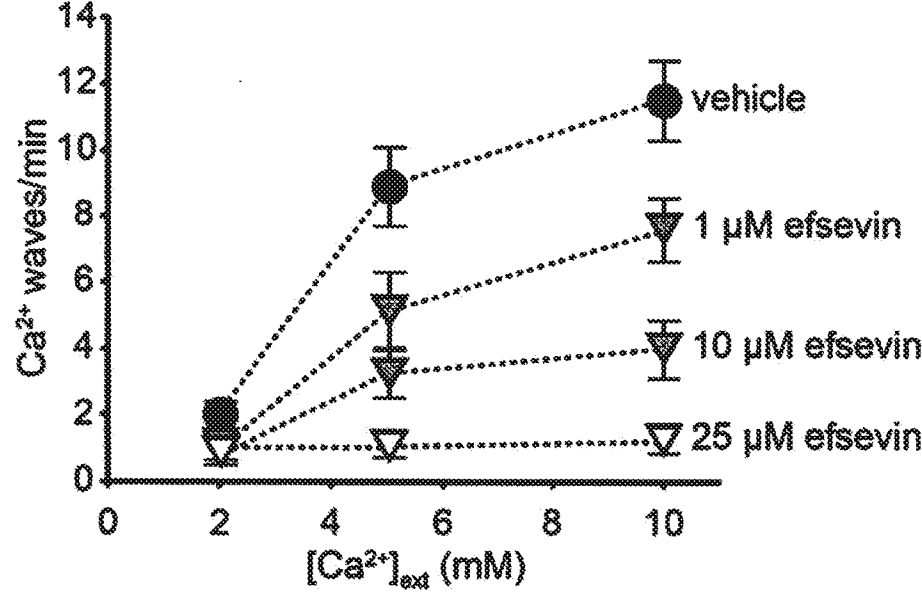


FIG. 7A

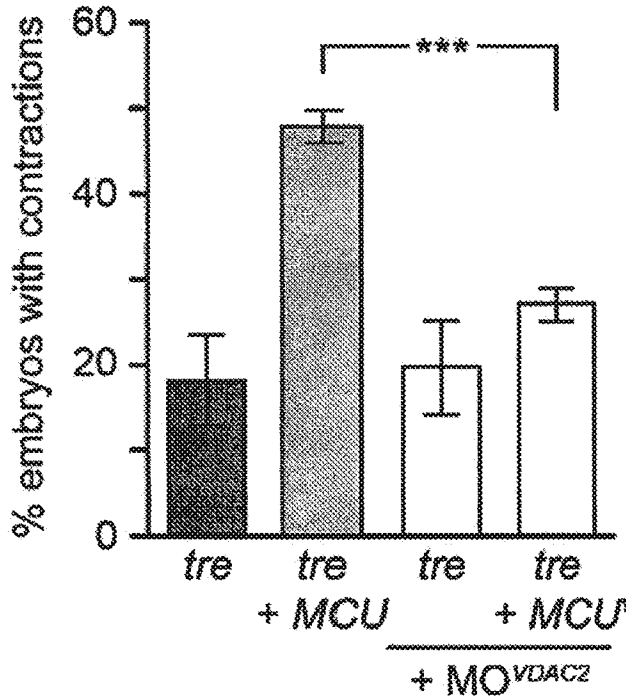


FIG. 7B

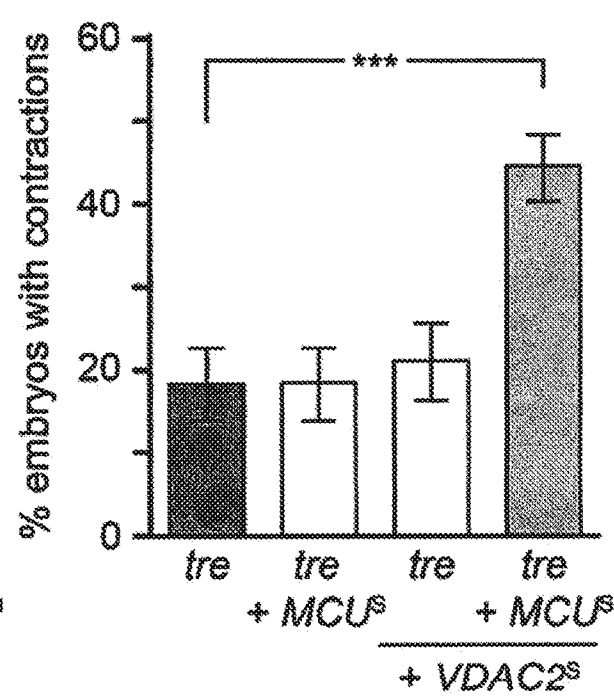


FIG. 7C

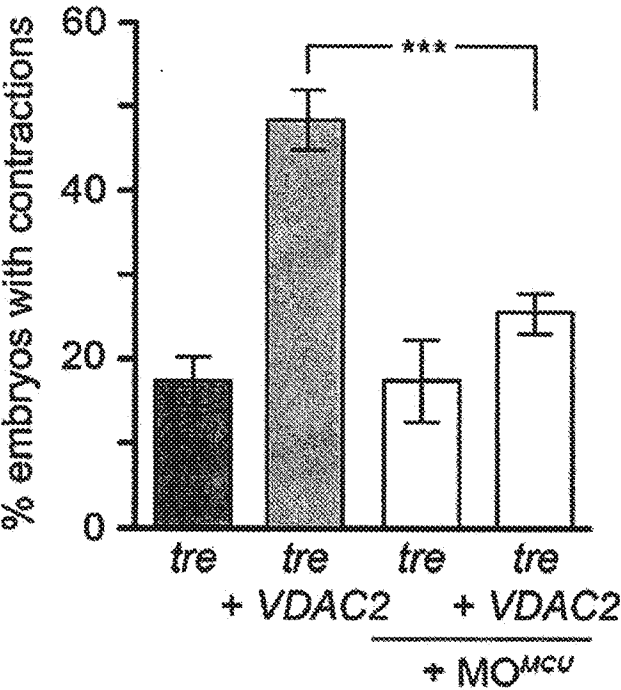


FIG. 7D

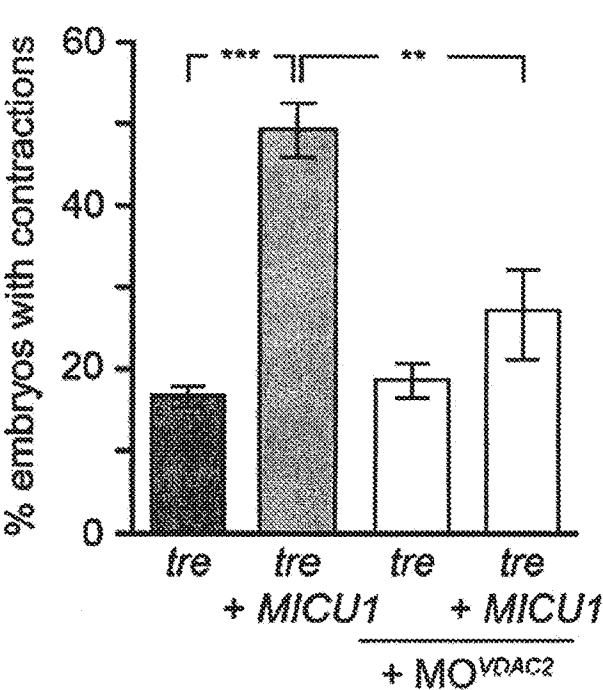


FIG. 7E

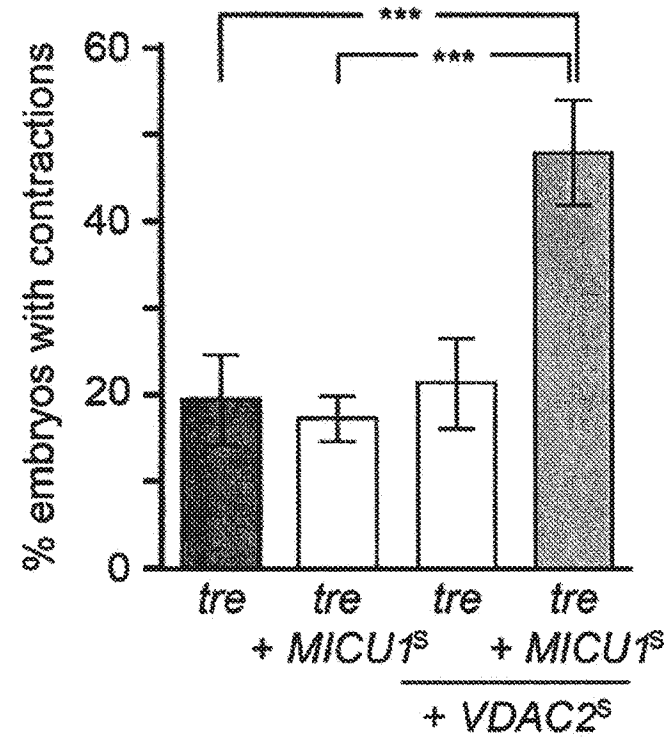


FIG. 8

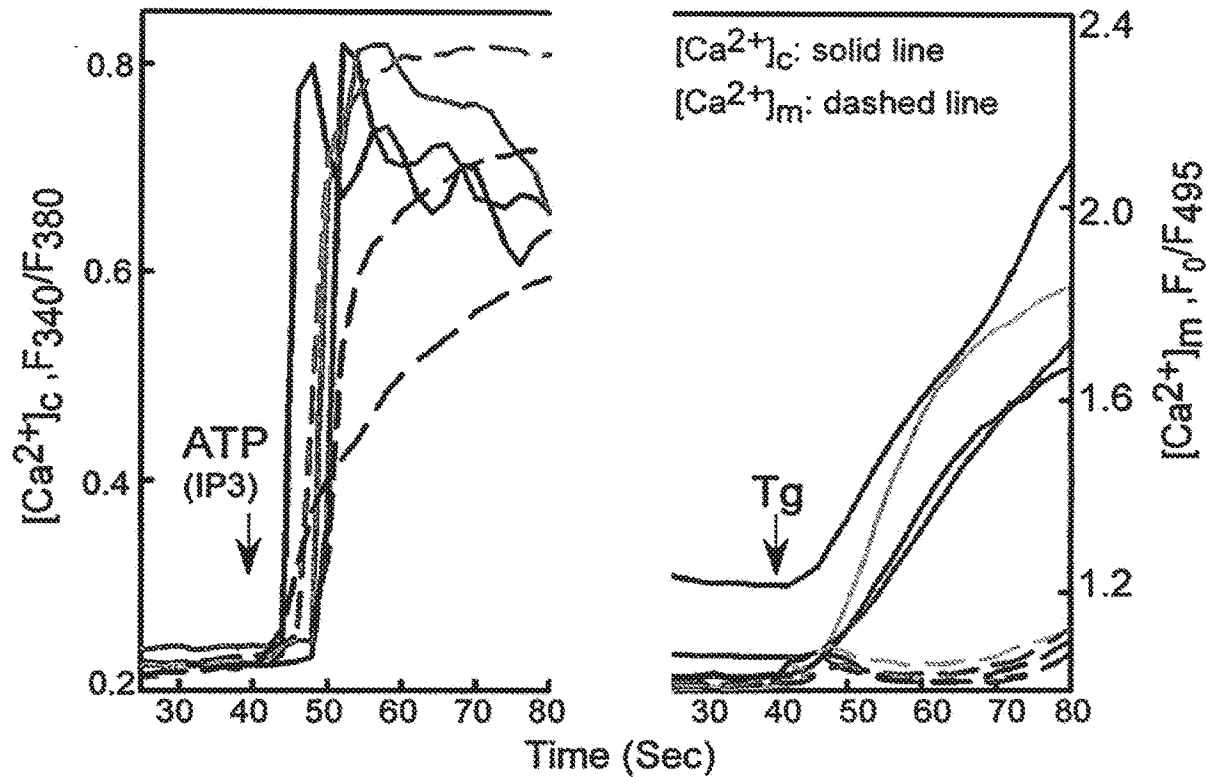


FIG. 9A

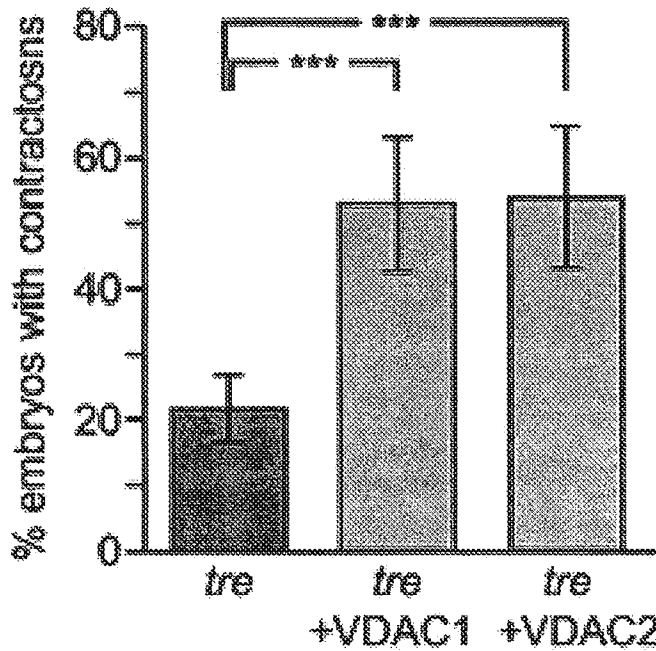


FIG. 9B

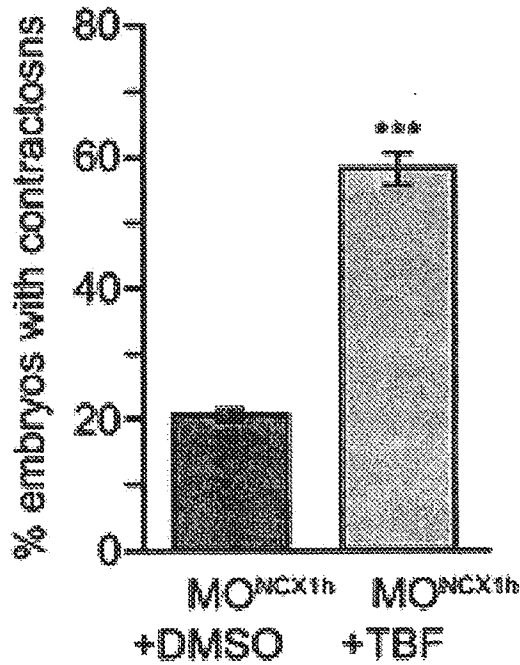


FIG. 9C

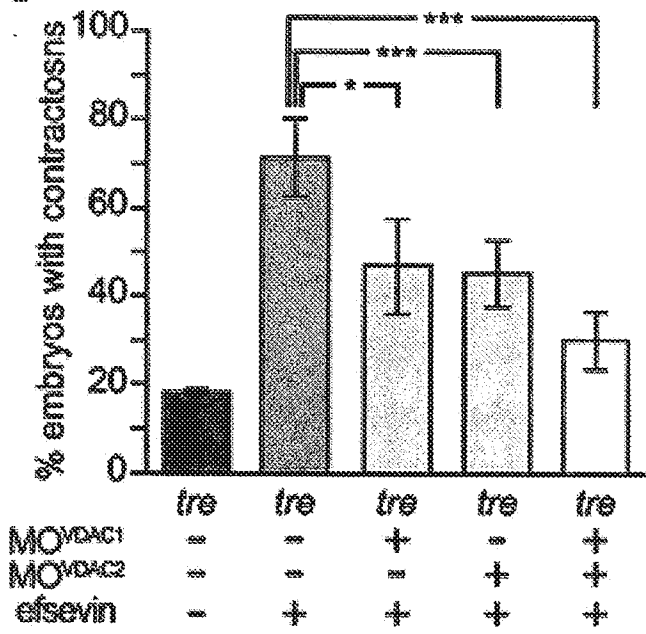


FIG. 9D

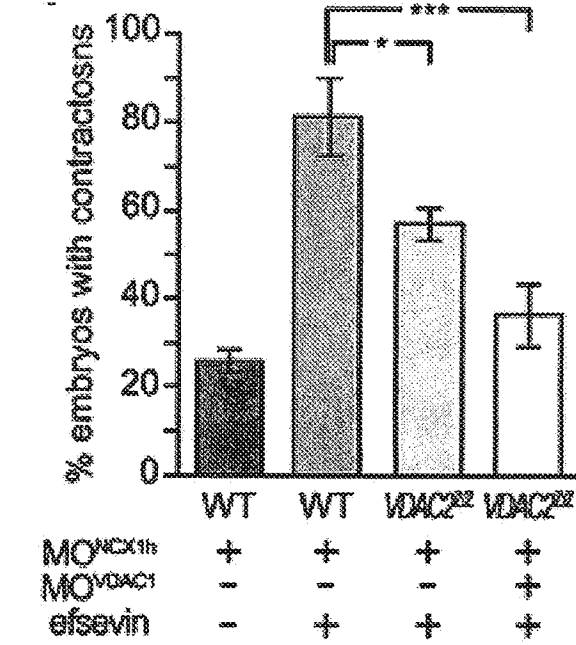


FIG. 9E

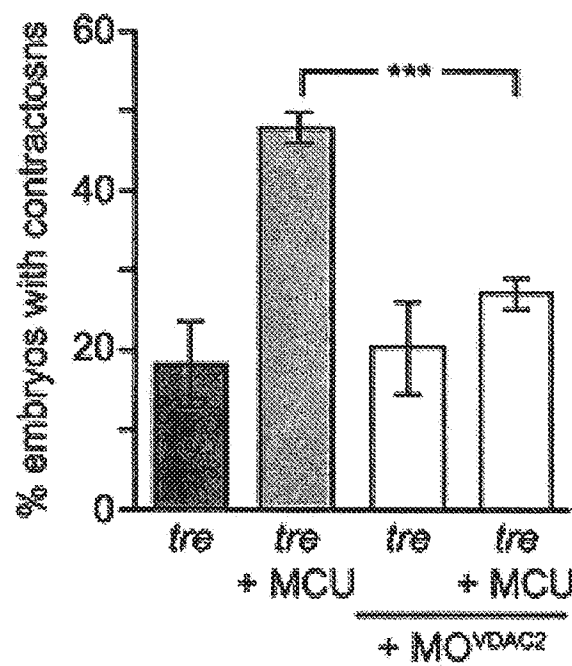


FIG. 9F

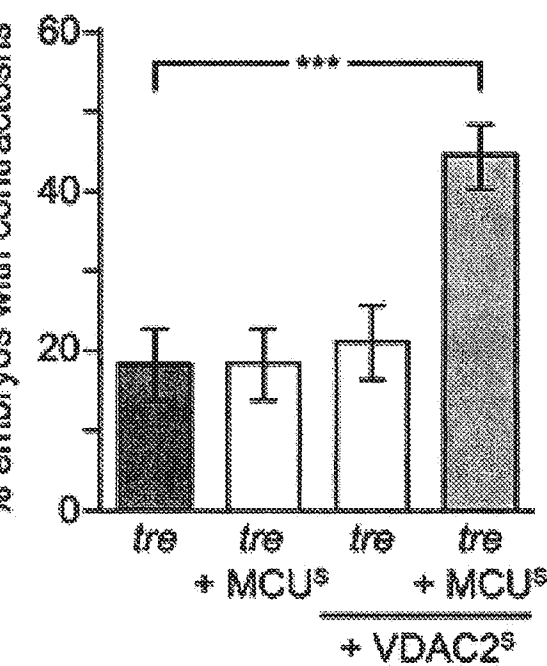


FIG. 9G

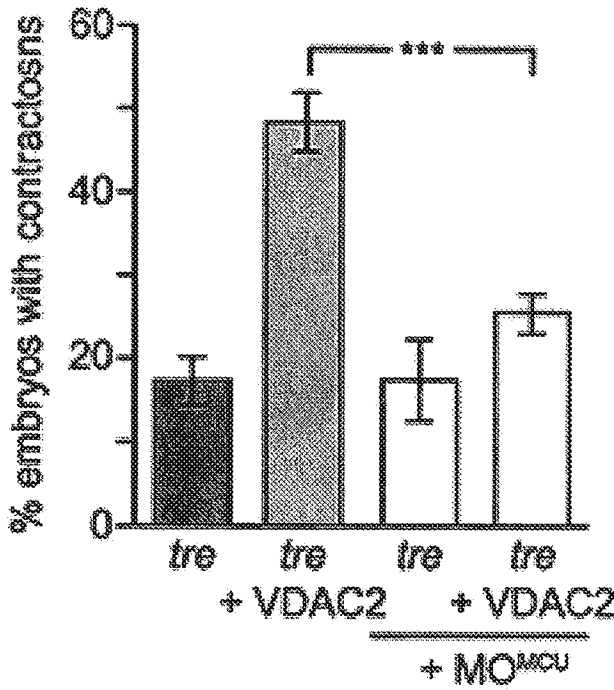


FIG. 10

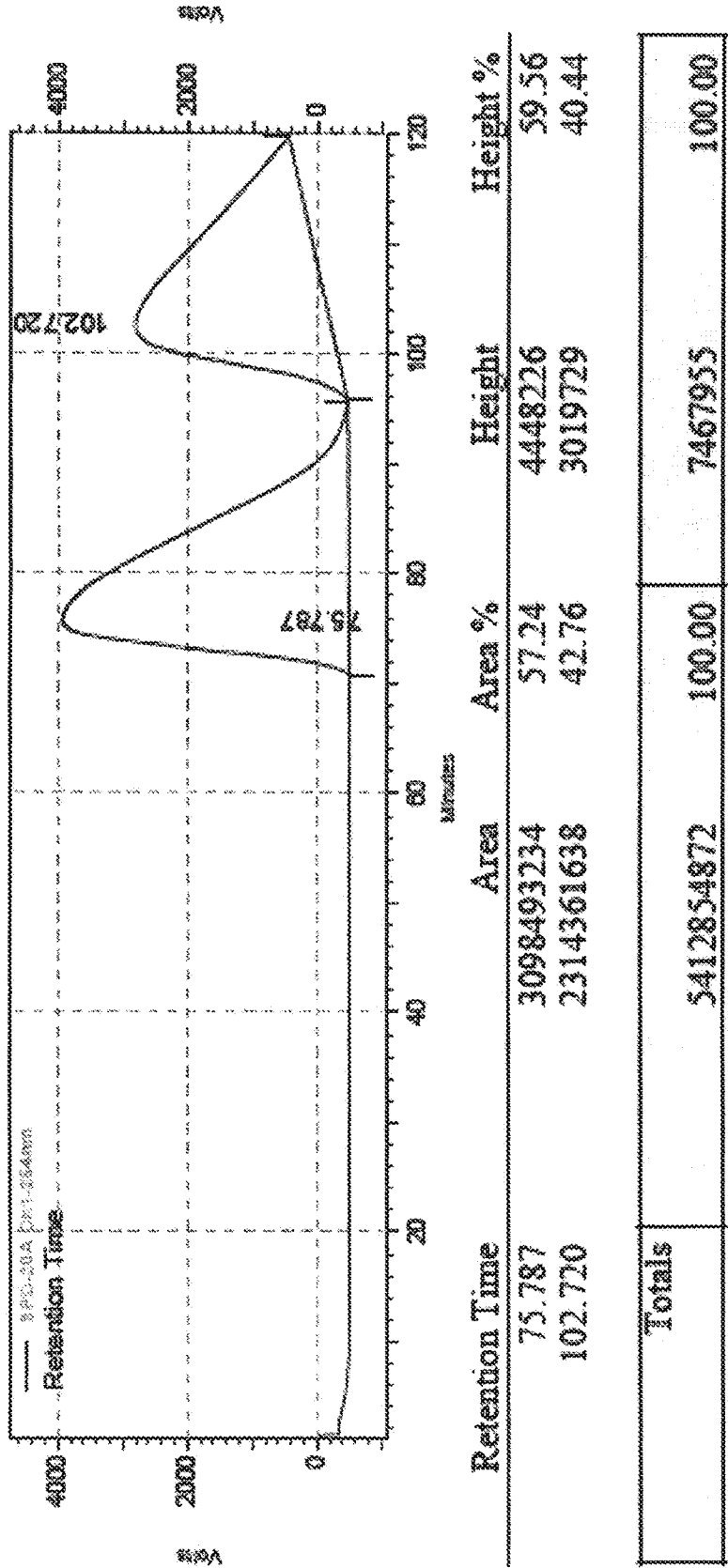


FIG. 11

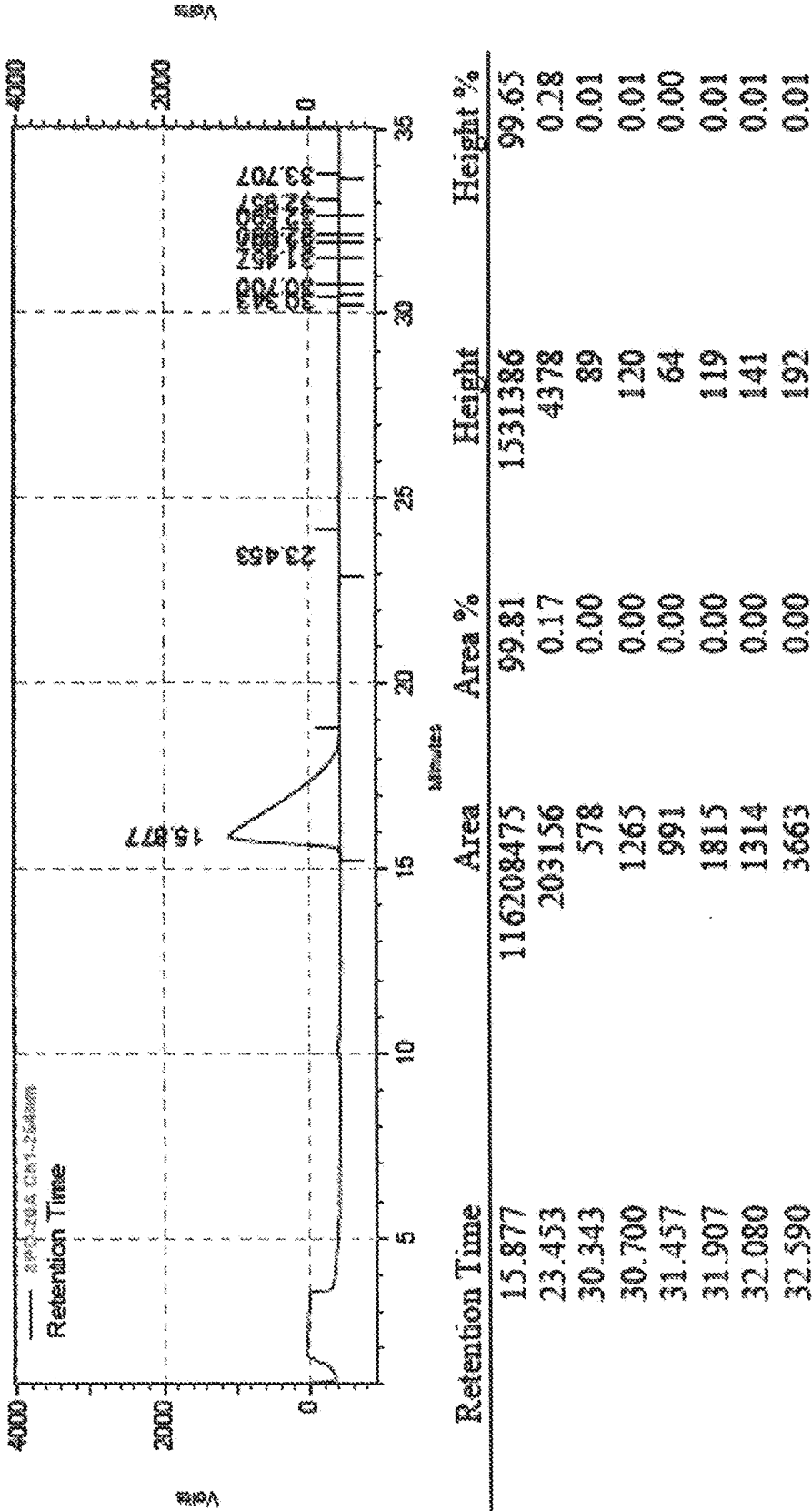


FIG. 12

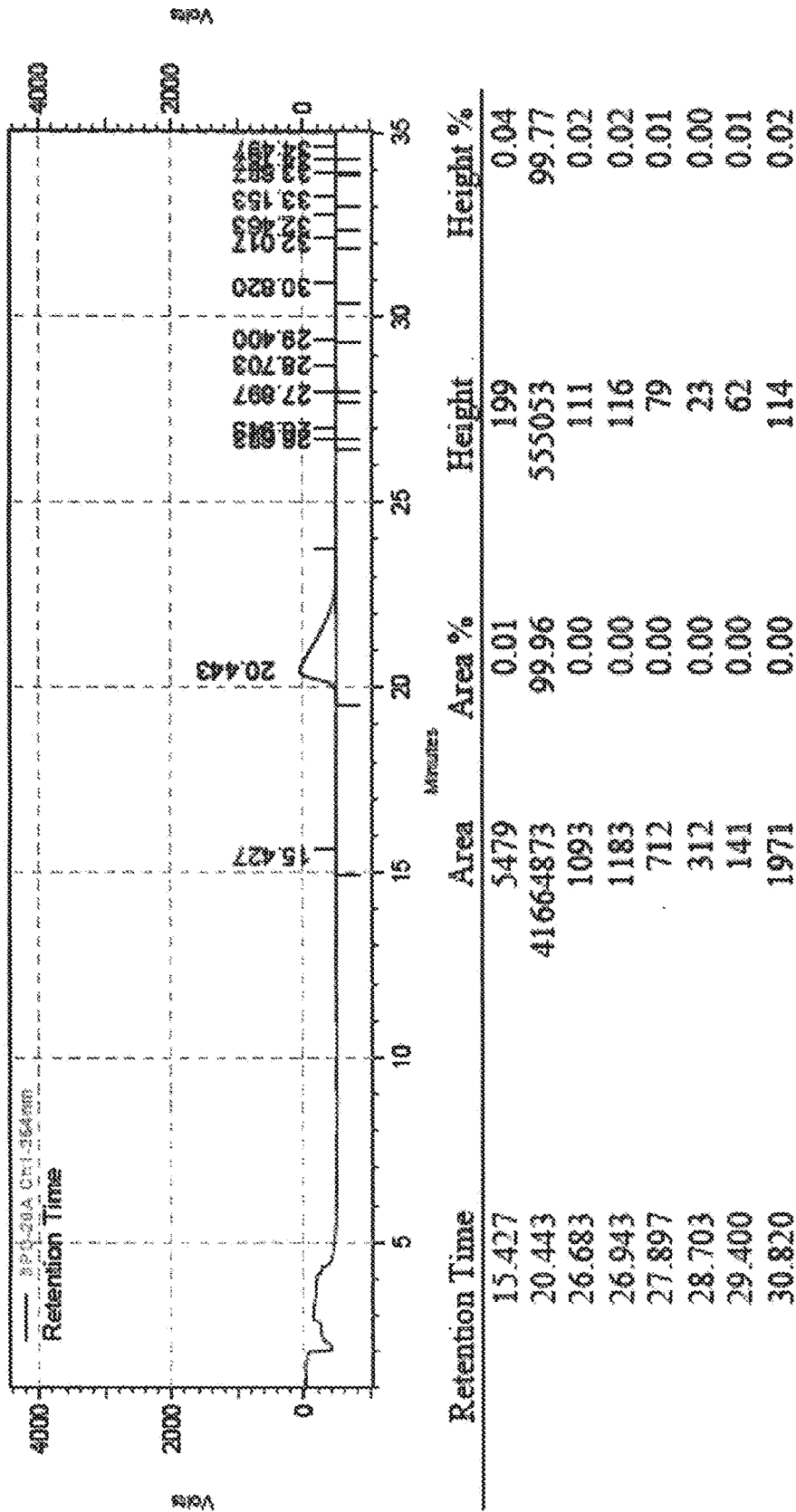


FIG. 13

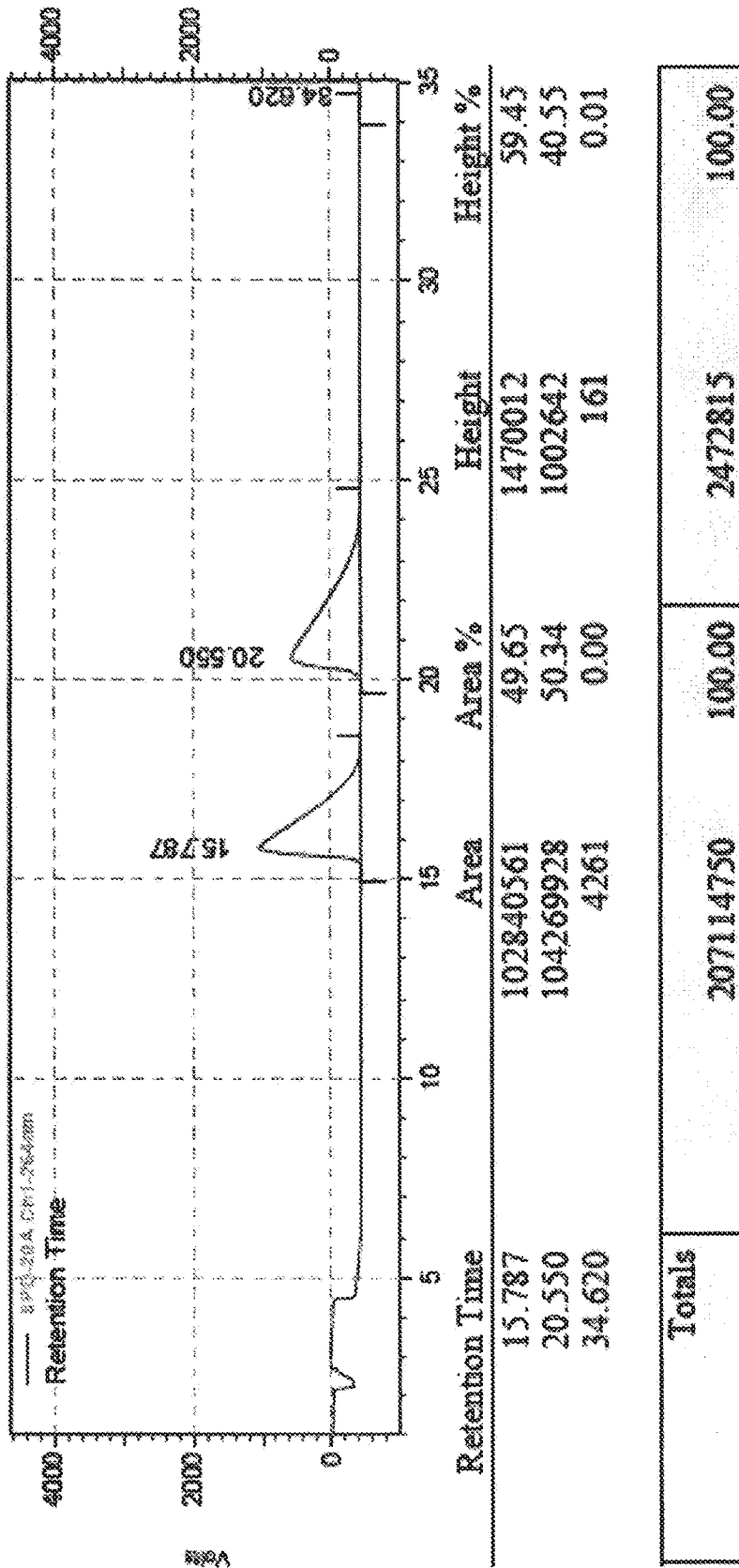


FIG. 14

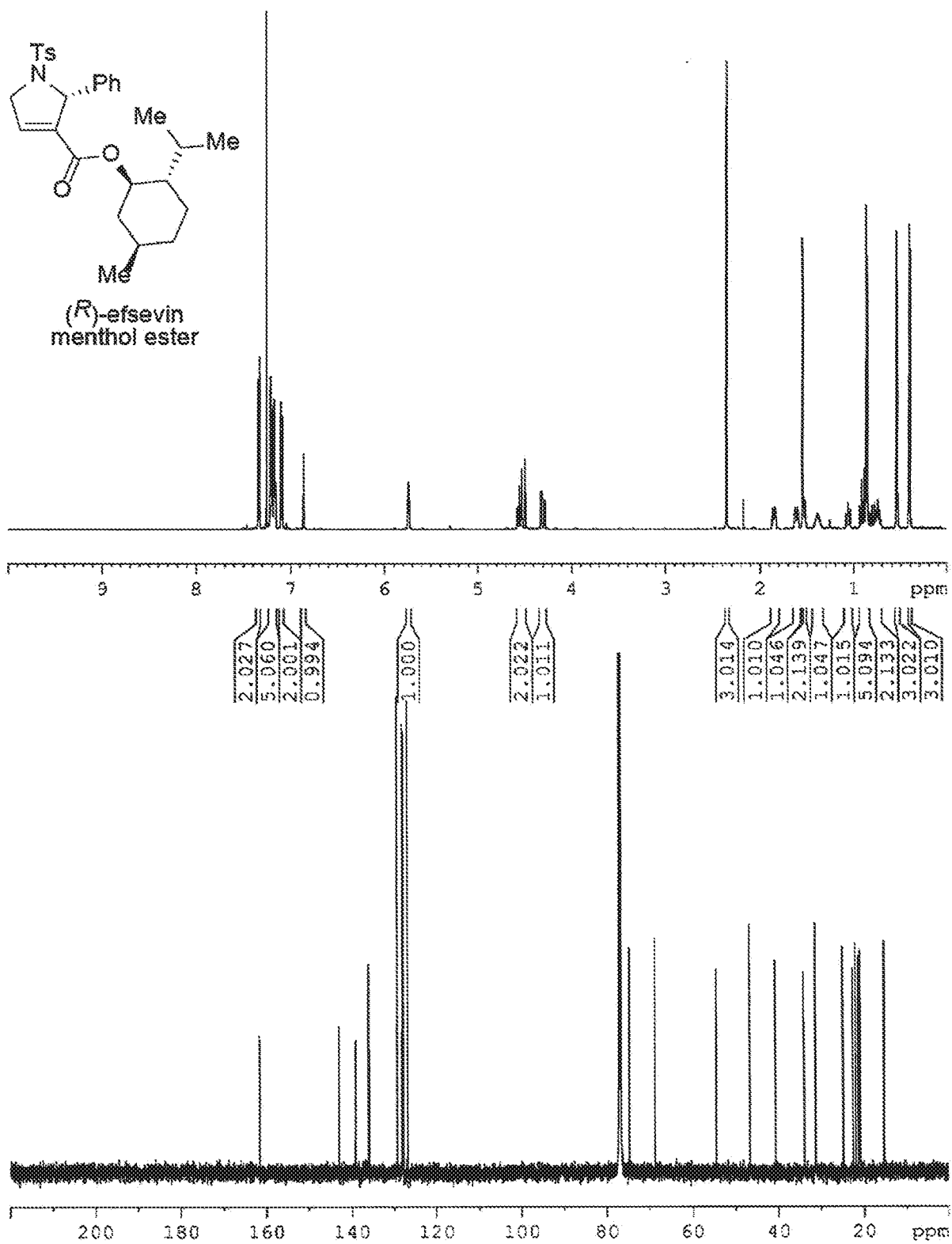


FIG. 15

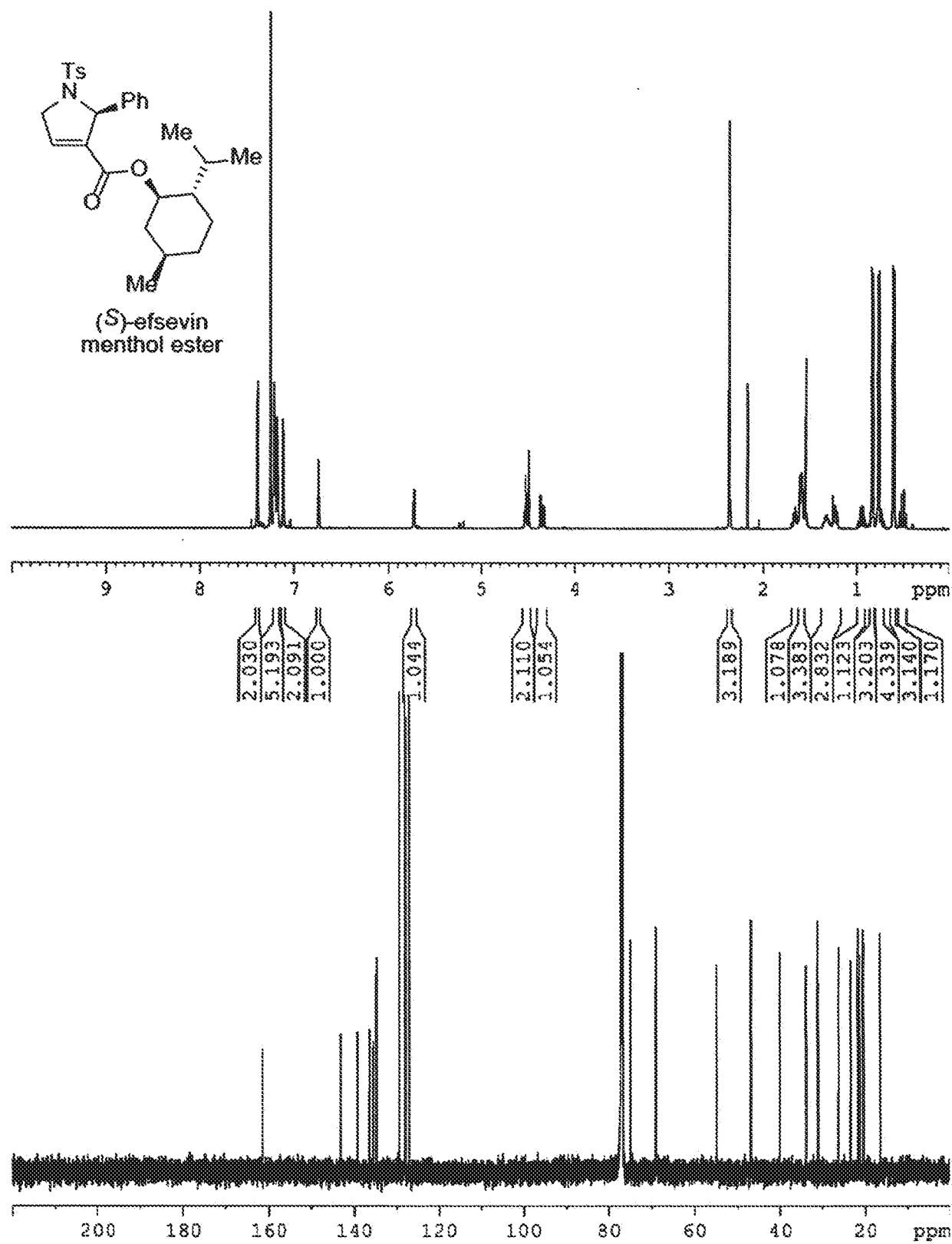


FIG. 16A

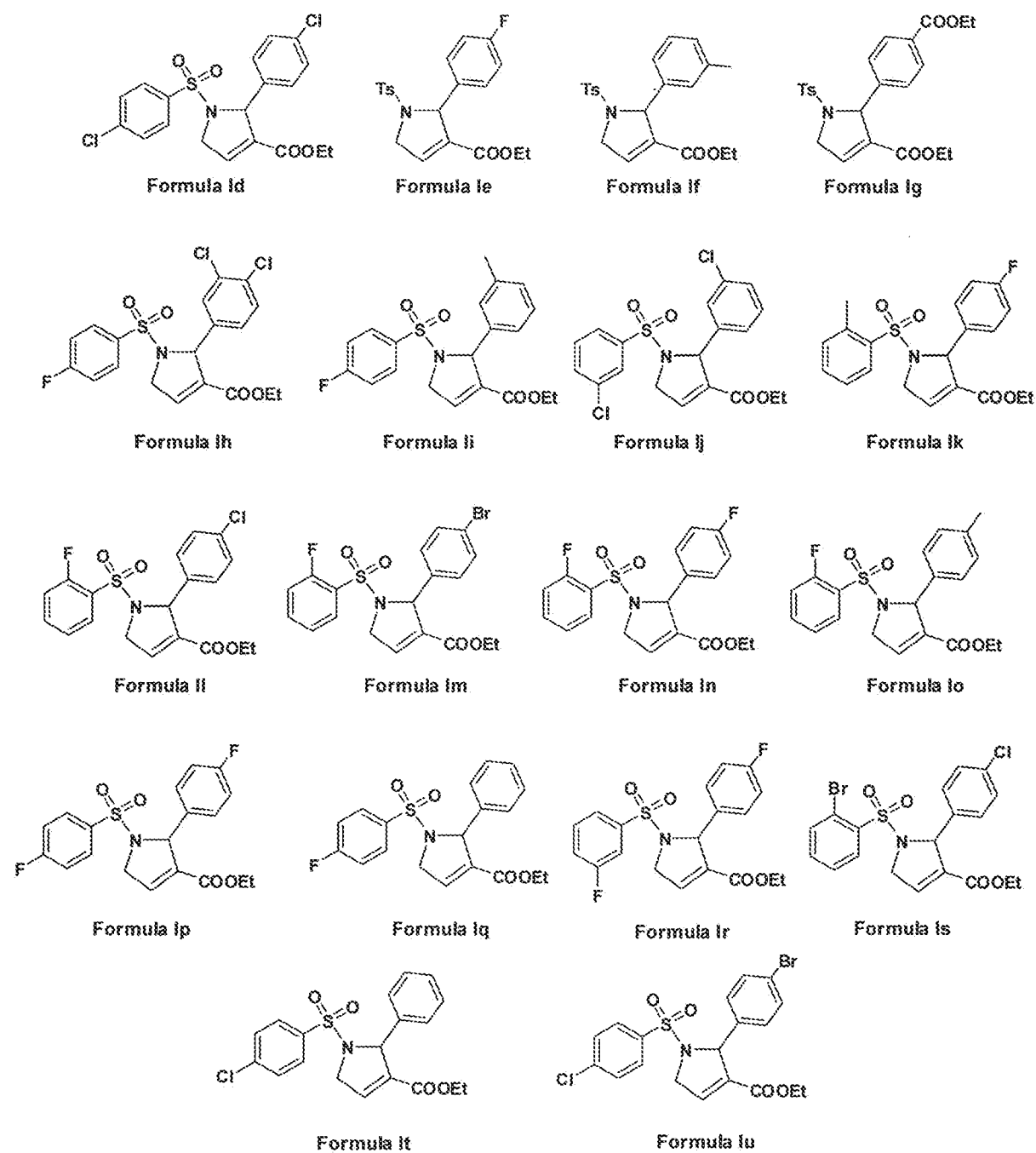
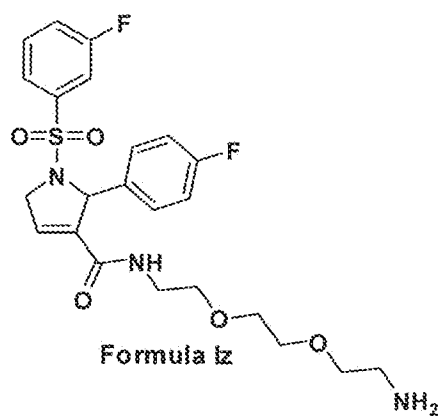
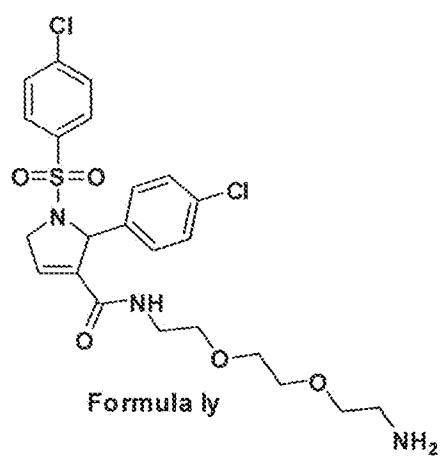
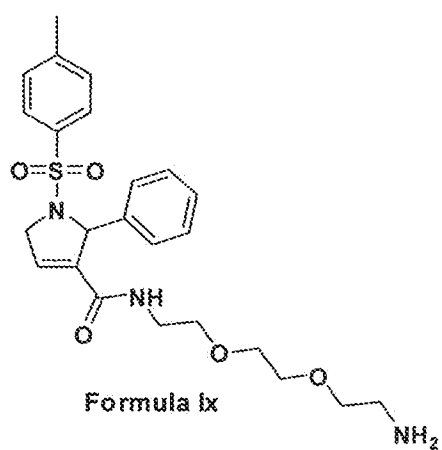
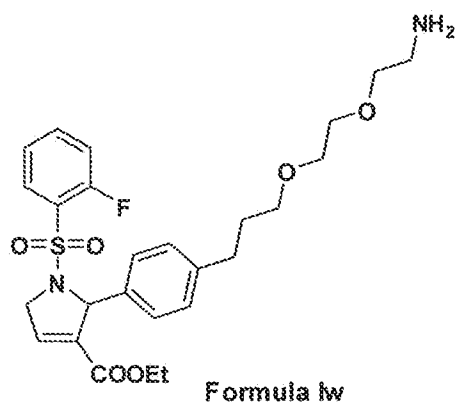
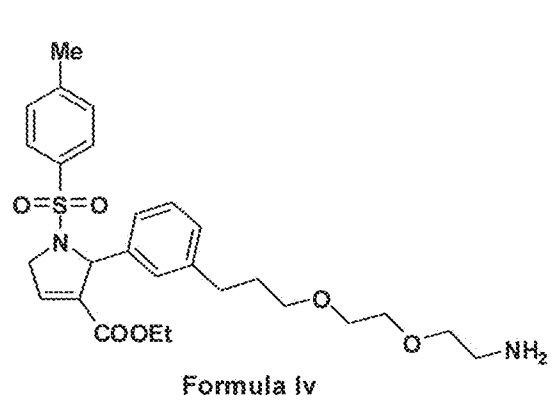


FIG. 16B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/65876

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/415, 14/43 (2016.01)

CPC - C07K 14/4747, 14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C07K 14/415, 14/43 (2016.01)

CPC: C07K 14/4747, 14/705

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); ProQuest; Scifinder; Google/Google Scholar;
 KEYWORDS: efsevin, mitochondrial, calcium, Ca²⁺, cardiac, rhythmicity, VDAC1, VDAC2, expression, gene, therapy, protein, cardiomyopathy, chiral

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	✓ HENRY, CE et al. Hydroxyproline-Derived Pseudoenantiomeric [2.2.1] Bicyclic Phosphines: Asymmetric Synthesis of (+)- and (-)-Pyrrolines. Journal of the American Chemistry Society, 06 August 2014, pp. 11890–11893; page 11891, paragraphs [1]-[2], table 2; page 11892, paragraph [3], table 3	7-10, 43, 45-47, 61 ----- 1-6, 11-12, 17-26, 48-49, 54-60, 62-63
X -- Y	US 2010/0075898 A1 (SHOSHAN-BARMATZ, V et al.) 25 March 2010; paragraphs [0018], [0020], [0040]-[0041], [0034], [0038], [0048], [0053]-[0055], [0145]-[0146]	65-66, 67/65-66 ----- 1-6, 13-26, 50-60, 62-63, 75-76
Y	✓ SCHREDELSEKER, J et al. High Resolution Structure and Double Electron-Electron Resonance of the Zebrafish Voltage-dependent Anion Channel 2 Reveal an Oligomeric Population. The Journal of Biological Chemistry, 02 May 2014, pp. 12566–12577; page 12567, paragraph [2]	1-6, 13-16, 24-26, 50-53, 65-66, 67/65-66
Y	US 6,838,460 B2 (HIMMELSBACH, F et al.) 04 January 2005; column 4, lines 24-41	11-12, 48-49
Y	US 7,605,185 B2 (GRAUPNER, G) 20 October 2009; column 17, lines 12-23; claim 1	22-23, 59-60, 75-76
Y	WO 2005/079155 A2 (BEN GURION UNIVERSITY OF THE NEGEV RESEARCH AND DEVELOPMENT AUTHORITY LTD) 01 September 2005; claim 11	13-23, 50-60

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 March 2016 (25.03.2016)

Date of mailing of the international search report

25 APR 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
 P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/65876

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 27, 32, 64, 68-74
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Group I: Claims 1-27, 43, 45-63, 64 (in-part), 65-67, 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula I or a derivative thereof; a methods forming a compound of Formula I and methods of regulating cardiac rhythmicity therewith.

Group II: Claims 28-32, 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula II.

Group III: Claims 33-42, 44, 64 (in-part), 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula Ic; and a method of making the compound.

Continued Within the Next Supplemental Box.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-26, 43, 45-63, 65-66, 67/65-66, 75-76
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US15/65876

-Continued from Box III: Observations where unity of invention is lacking -

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-27, 43, 45-63, 64 (in-part), 65-67, 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula I or a derivative thereof; a methods forming a compound of Formula I and methods of regulating cardiac rhythmicity therewith.

Group II: Claims 28-32, 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula II.

Group III: Claims 33-42, 44, 64 (in-part), 68 (in-part), 69 (in-part), 70 (in-part), 71 (in-part), 72 (in-part), 73 (in-part), 74 (in-part), 75 (in-part) and 76 (in-part) are directed toward a compound of structure of Formula Ic; and a method of making the compound.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include an agent that increases the activity of VDAC2 or VDAC1, which is not present in either of Groups II or III; the special technical features of Group II include a compound of Formula II, which is not present in either of Groups I or III; the special technical features of Group III include a compound of Formula Ic, which is not present in either of Groups I or II.

No technical features are shared between the compounds of Groups I, II and III and, accordingly, these groups lack unity a priori.

Groups I-III share the technical features including compounds including a phenyl-substituted pyrrolidiny ring linked to a sulfur via the ring amine, and a 4-carboxyl substituent. Groups I and III share the technical features including a method of making the compound (the language 'wherein the compound is effective to potentiate mitochondrial Ca²⁺ uptake, so as to modulate cardiac rhythmicity in a subject,' present in Claims 1 and 33, constitutes functional language, and, accordingly, has not been afforded any patentable weight).

However, these shared technical features are previously disclosed by US 5,296,494 A to Lavielle, et al. (hereinafter 'Lavielle').

Lavielle discloses a compound comprising a phenyl-substituted pyrrolidiny ring linked to a sulfur via the ring amine, and a 4-carboxyl substituent (Ethyl (2a,3a)-1-(4-fluorophenylsulfonyl)-2-(3-methoxyphenyl)-3-pyrrolidinecarboxylate (a compound comprising a phenyl-substituted pyrrolidiny ring linked to a sulfur via the ring amine, and a 4-carboxyl substituent); column 6, lines 35-52); and a method of forming the compound (a method of forming the compound; column 6, lines 30-67 to column 8, lines 1-3).

Since none of the special technical features of the Groups I-III inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Lavielle reference, unity of invention is lacking.