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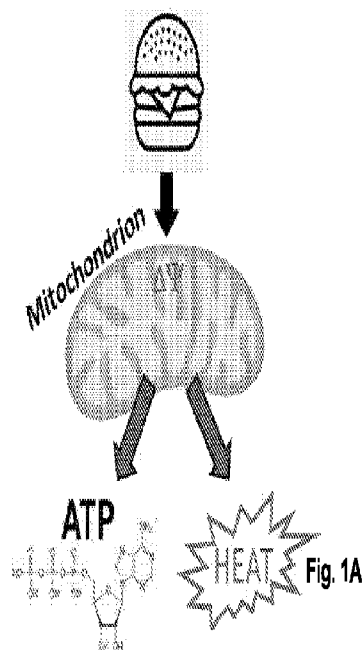
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(54) Title: NONHORMONAL UNISEX CONTRACEPTIVES

(57) Abstract: Nonhormonal unisex contraceptive products, compositions, formulations and methods of use comprise an effective amount of a targeted mild mitochondria uncoupler.



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NONHORMONAL UNISEX CONTRACEPTIVES

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims the priority benefit of United States Provisional Application No. 62/808,861, filed February 22, 2019, the contents of which are incorporated herein in their entirety.

INCORPORATION OF THE SEQUENCE LISTING

[02] The contents of the file named B19-097_ST25.txt, which was created on Feb 20 2020, and is 11KB in size are hereby incorporated by reference in their entirety.

GOVERNMENT SUPPORT

[03] This invention was made with government support under Grant Numbers GM111802, GM118939, and HD081403 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[04] The urgent need for contraception worldwide concerns billions of people. According to the National Health Statistics Report, 62 percent of women of reproductive age that use contraception are relying mostly on hormonal pills and intrauterine devices (IUDs)²¹. However, numerous reports indicate that despite being highly effective, undesirable side effects associated with hormonal contraceptives often result in discontinuation of their use^{6,8,9}. Steroid hormones are associated with powerful side effects, such as depression, weight gains, ectopic pregnancy and others because of their pleiotropic mode of action on various cell types. Almost a third of American women discontinue using hormonal contraceptives within the first year of use, due to the side effects. Additionally, the only modern options for male contraception are condoms, which have a high real-world failure rate, and vasectomies, which are surgically invasive. Therefore, there is a large unmet need for novel non-hormonal unisex contraceptives. Such a contraceptive could potentially be used by billions of people across the globe.

SUMMARY

[05] The disclosure demonstrates that targeted mild mitochondrial uncouplers, DNP, niclosamide, and BAM15, uncouple sperm mitochondria. Niclosamide, the most potent of the compounds, also decreased sperm beat frequency from 14Hz to 6Hz (Fig. 6A), inhibited

hyperactivation (Fig. 6C) at concentrations as low as 1 μ M.

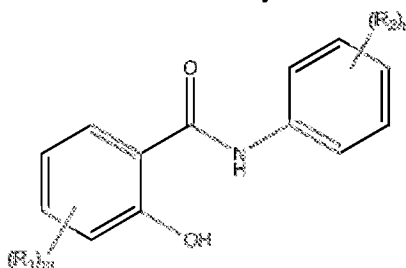
[06] The disclosure provides nonhormonal unisex contraceptive products, compositions, formulations and methods of use, which comprise an effective amount of a targeted mild mitochondria uncoupler.

[07] The disclosure provides a method of promoting contraception, comprising administering to a person in need thereof a composition comprising an effective amount of a targeted mild mitochondria uncoupler.

[08] In some embodiments of the methods of the disclosure, the uncoupler comprises or is a salicylanilide or salt thereof, such as an ethanolamine salt thereof, such as niclosamide ethanolamine (NEN)

[09] In some embodiments of the methods of the disclosure, the uncoupler comprises or is a salicylanilide compound or salt thereof, and the salicylanilide compound is selected from: niclosamide, bromochlorosalicylanilide, oxyclozanide, rafoxanide, 3-tert-butyl-5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxy-6-methylbenzamide, dibromsalan, metabromsalan, tribromsalan, and 2-iodo-N-phenylbenzamide (benodanil).

[010] In some embodiments of the methods of the disclosure, the uncoupler comprises or is a salicylanilide compound or salt thereof, and the salicylanilide compound has a structure:



wherein R_1 and R_2 are independently halide, such as F, Cl, Br or I, substituted heteroatom selected from O and N, such as -OH, -NO₂ or O-Ph-Cl, or lower (C1-C4) alkyl, such as Me, Et, or t-butyl, m is an integer 1, 2, 3 or 4, and n is an integer 1, 2, 3, 4 or 5.

[011] In some embodiments of the methods of the disclosure, the uncoupler comprises or is BAM15 (N5,N6-bis(2-Fluorophenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazine- 5,6-diamine), or salt thereof.

[012] In some embodiments of the methods of the disclosure, the composition is administered via an oral route, a topical route, a rectal route, or a vaginal route.

[013] In some embodiments of the methods of the disclosure, the composition is administered as a pill, a cream, a vaginal ring, a vaginal film or a patch.

[014] In some embodiments of the methods of the disclosure, the composition is administered orally and the composition is administered as a pill

[015] In some embodiments of the methods of the disclosure, the composition is

administered topically and the composition is administered as a cream or a patch.

[016] In some embodiments of the methods of the disclosure, the composition is administered intravaginally and the composition is administered as a cream, a patch, a vaginal ring or a vaginal film.

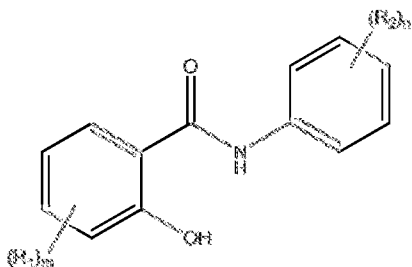
[017] In some embodiments of the methods of the disclosure, the composition is administered intrarectally and the composition is administered as a cream or a patch.

[018] The disclosure provides a use of a targeted mild mitochondria uncoupler of the disclosure as a contraceptive.

[019] In some embodiments of the uses of the disclosure, the uncoupler comprises or is a salicylanilide or salt thereof, such as an ethanolamine salt thereof, such as niclosamide ethanolamine (NEN).

[020] In some embodiments of the uses of the disclosure, the uncoupler comprises or is a salicylanilide compound or salt thereof, and the salicylanilide compound is selected from: niclosamide, bromochlorosalicylanilide, oxyclozanide, rafoxanide, 3-tert-butyl-5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxy-6-methylbenzamide, dibromsalan, metabromsalan, tribromsalan, and 2-iodo-N-phenylbenzamide (benodanil).

[021] In some embodiments of the uses of the disclosure, the uncoupler comprises or is a salicylanilide compound or salt thereof, and the salicylanilide compound has a structure:



wherein R1 and R2 are independently halide, such as F, Cl, Br or I, substituted heteroatom selected from O and N, such as -OH, -NO₂ or O-Ph-Cl, or lower (C1-C4) alkyl, such as Me, Et, or t-butyl, m is an integer 1, 2, 3 or 4, and n is an integer 1, 2, 3, 4 or 5.

[022] In some embodiments of the uses of the disclosure, the uncoupler comprises or is BAM15 (N5,N6-bis(2-Fluorophenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine), or salt thereof.

[023] In some embodiments of the uses of the disclosure, the composition is administered via an oral route, a topical route, a rectal route, or a vaginal route.

[024] In some embodiments of the uses of the disclosure, the composition is administered as a pill, a cream, a vaginal ring, a vaginal film or a patch.

[025] In some embodiments of the uses of the disclosure, the composition is administered

orally and wherein the composition is administered as a pill.

[026] In some embodiments of the uses of the disclosure, the composition is administered topically and wherein the composition is administered as a cream or a patch.

[027] In some embodiments of the uses of the disclosure, the composition is administered intravaginally and wherein the composition is administered as a cream, a patch, a vaginal ring or a vaginal film.

[028] In some embodiments of the uses of the disclosure, the composition is administered intrarectally and wherein the composition is administered as a cream or a patch.

[029] The disclosure provides a contraceptive formulation comprising a targeted mild mitochondria uncoupler. In some embodiments, the contraceptive formulation comprises an effective amount of the targeted mild mitochondria uncoupler. In some embodiments, the contraceptive formulation is in the form of a pill, capsule, suppository, cream, vaginal ring, vaginal film or patch.

[030] The disclosure encompasses all combinations of the particular embodiments of the disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[031] **Figures 1A-C** is a series of schematic diagrams showing that the sperm cell relies on its mitochondria to generate energy. **(A)** General diagram of energy conversion within mitochondria. **(B)** The mechanism of mitochondrial ATP and heat production.

Mitochondria have two membranes, the outer mitochondrial membrane (OMM, freely permeable to ions and small molecules <1500 Da) and the IMM (which has much more tightly controlled permeability and contains the apparatus for ATP and heat production).

The electron transport chain (ETC) generates potential ($\Delta\Psi$) across the IMM, which is used by ATP synthase (AS) to produce ATP and H^+ leak pathways to generate heat.

Modified from¹. **(C)** Spermatozoa rely on energy produced by their mitochondria that are tightly packed in the midpiece region of the tail. A proton gradient is established by the ETC to power the synthesis of ATP. In active swimming sperm cells, cellular ATP consumption causes active APT/ADP exchange via ANT that inhibits the ANT-mediated H^+ leak, and $\Delta\Psi$ is used to produce ATP only: **(#1)**. When cellular activity is reduced, less ATP is consumed, leading to increases in $\Delta\Psi$ and reactive oxygen species (ROS) production: **(#2)**^{2,3}. To prevent this from happening, proton leak through ANT increases, because now it is not inhibited by active ADP/ATP exchange **(#3)**. This proton leak converts $\Delta\Psi$ into heat, returning $\Delta\Psi$ back to the normal value and decreasing ROS production. However, when $\Delta\Psi$ is reduced by the proton leak, which is carried in sperm

by ANT2 and ANT4, the mitochondria dissipate the energy in the form of heat and cannot produce enough ATP to power sperm motility¹².

[032] **Figure 2** is a series of chemical structures of mild mitochondrial uncouplers: Niclosamide, BAM15 and 2,4-dinitrophenol.

[033] **Figure 3A-B** is a pair of schematic diagrams depicting a mitochondrial patch-clamp. **(A)** Preparation of mitoplasts (vesicles of the whole IMM): (1) Isolation of mitochondria from cell lysate by centrifugation; (2) OMM removal using a low-pressure French press; (3) isolated mitoplasts in a KCl solution. Remnants of the OMM (arrow) are attached to the IMM. **(B)** Patch-clamp recording from mitoplasts. After formation of the gigaohm seal (mitoplast- attached configuration), the membrane patch under the pipette can be destroyed by high-amplitude voltage pulses to form a whole-mitoplast configuration for recording currents (I) across the whole IMM. Alternatively, the patch pipette can be withdrawn from the mitoplast to form an inside-out mode for recording single-channel activity.

[034] **Figure 4** is a series of graphs (upper panels) and a schematic diagram (lower panel) showing regulators of ANT-mediated H^+ current (I_H). Left panel: I_H activated by 2 μ M arachidonic acid (AA) followed by a transient inhibition by 1 mM of bath ADP (2), and subsequent recovery (3). Control current is shown in black. Middle panel: I_H time course of the left panel. Skeletal muscle mitoplast. I_H amplitudes were measured upon stepping from 0 to -160 mV. Right panel: ADP inhibition of I_H in points 2 and 3. Remaining I_H measured at -160 mV is shown as a percentage of control, $n=8$. Explanation of transient I_H inhibition by cytosolic adenine nucleotides. ANT in c-state, with fatty acid (FA) anion in the translocation pathway, mediates I_H (1). Cytosolic ADP³⁻ binds in c-state and expels FA anion/blocks translocation pathway, leading to I_H inhibition (2). Upon ANT conformation change, ADP dissociates into matrix (pipette) solution (2 and 3). FA anion re-associates with ANT in m-state, restoring I_H (4 and 5). Cytosolic ADP cannot inhibit I_H while AAC is in m-state (5). Preliminary data from (Bertholet, A. M. Chouchani., E.T.; Kazak, L.; Angelin, A.; Fedorenko, A.; Long, J.Z.; Vidoni, S.; Garrity, R.; Cho, J.; Terada, N.; Wallace, D.C.; Spiegelman, B.M.; and Kirichok, Y. Proton Transport is an Integral Function of the Mitochondrial ADP/ATP Carrier Nature Jul;571(7766):515-520 (2019)).

[035] **Figures 5A-B** is a series of graphs demonstrating that ANT is required for H^+ leak activated by FA and DNP. **a**, Representative currents induced by 2 μ M AA in WT and ANT1^{-/-} mitoplasts of heart. Right panel: I_H current densities at -160 mV for WT and

ANT1^{-/-} mitoplasts. **b**, Representative currents induced by 50 μ M DNP in WT and ANT1^{-/-} mitoplasts of heart. Right panel: I_H current densities induced by 50 and 200 μ M DNP at -160 mV in WT and ANT1^{-/-} mitoplasts.

[036] Figures 6A-C is a series of graphs demonstrating the impact of mitochondrial uncouplers (MU) on sperm physiology and fertilizing capacity. Niclosamide significantly decreases sperm motility, uncouples mitochondria and prevents fertilization. **A)** Human sperm were purified by swim-up method and their basal beat frequency (BF, in Hz) was measured as described in (Mannowetz, N., Naidoo, N. M., Choo, S. A., Smith, J. F. & Lishko, P. V. Slo1 is the principal potassium channel of human spermatozoa. eLife 2, e01009, doi:10.7554/eLife.01009 (2013)) in control HS solution ((1); in the presence of vehicle control ((2); DMSO), and in the presence of MU: 1 μ M Niclosamide ethanolamine (NEN), 0.76 μ M BAM15, and 5 μ M DNP. Data are standard boxplots, with middle line representing median, upper and lower bounds of the box representing first and third quartiles, and whiskers representing maximum and minimum values, not including outliers. X's represent the mean of the sample, and dots represent individual data points. **B)** Human sperm mitochondrial membrane potential (MMP) assessment. Human sperm were incubated for 15 minutes in the presence of 0.1% DMSO (vehicle control), in the presence of 1 μ M NEN or 0.75 μ M BAM15. Samples from each condition (at least 100 of sperm midpieces per condition) were then assessed for fluorescence intensity measured with 45 nM MitoRed, which is a cell membrane permeable rhodamine-based dye. MitoRed localizes to mitochondria, and emits red fluorescence. The interaction of MitoRed with mitochondria depends on the membrane potential of the mitochondria. Uncoupled mitochondria emit less fluorescence. Data are standard boxplots, as in (A). **C)** Effect of sMU on human capacitated sperm hyperactivated motility. Cells were capacitated as described (11) for 4 hours. 4 μ M Progesterone was added 2 minutes before imaging to trigger hyperactivation (HA). Cells were assessed as either Hyperactivated or Not Hyperactivated. N>45 cells. Error bars represent 95% CI calculated through the Wilson/Brown method.

DETAILED DESCRIPTION

[037] Mitochondria generate ATP by coupling the H⁺ transport activities of the mitochondrial electron transport chain (ETC) and ATP synthase²², two gigantic transport protein complexes located in the inner mitochondrial membrane (IMM). Specifically, the ETC, fueled by high-energy electron donors provided by the Krebs cycle, pumps H⁺ out of the mitochondrial matrix to generate an electrochemical H⁺ gradient ($\Delta\Psi$) across the IMM.

ATP synthase then returns H^+ back into the mitochondrial matrix down the $\Delta\Psi$ and uses the released energy to synthesize ATP from ADP and inorganic phosphate. To maximize the energy transfer between ETC and ATP synthase, the chemiosmotic theory postulated that the IMM conductance for H^+ (and other ions) must be close to zero. However, it is now well-established that the IMM of any tissue is “leaky” for H^+ . The H^+ leak (I_H) across the IMM is mediated by uncoupling proteins (UCPs). Similar to ATP synthase, UCPs return H^+ back into the mitochondrial matrix down the $\Delta\Psi$, but do not generate ATP and instead dissipate the released energy as heat. This phenomenon, known as mitochondrial uncoupling, is crucial for mitochondrial function and integrity (Figure 1). Free fatty acids (FA) are physiological activators of H^+ leak via UCPs. Mitochondrial uncoupling in the specialized thermogenic tissues, brown and beige fat, helps to maintain core body temperature and control body weight^{3,23-25}, and is happening via uncoupling protein 1 (UCP1) that is brown and beige fat-specific. In contrast to brown fat, mitochondria of regular somatic and reproductive tissues are only “mildly” uncoupled, which implies a smaller I_H . However, because this mild uncoupling occurs in the majority of tissues, it may have a significant impact on thermogenesis, body weight, healthy metabolism and reproduction potential^{3,26}.

[038] In addition, the mild uncoupling reduces mitochondrial reactive oxygen species (ROS) production to preserve mitochondrial integrity¹². The uncontrolled escape of electrons from the mitochondrial ETC to oxygen is the primary source of ROS in cells. The mild mitochondrial uncoupling that slightly reduces the potential across the IMM is a major mechanism that prevents ROS generation by ETC. Indeed, mild uncoupling was shown to be potentiated/activated by FA, ROS, and by hyperpolarized $\Delta\Psi$ ^{12,27}. Despite the importance of mild mitochondrial uncoupling, the molecular identity of UCP(s) in all tissues except for brown fat remained elusive.

[039] Therefore, chemical protonophores, such as 2,4-dinitrophenol (DNP), have been widely used to induce I_H and mitochondrial uncoupling for research and therapeutic purposes. DNP is hydrophobic, membrane soluble weak acids that can carry H^+ across biological membranes without the help of membrane transport proteins. What distinguishes them from FA (also hydrophobic weak acids, but poor protonophores) is the ability to diffuse through the membrane not only in the protonated form but also in the H^+ -free, negatively charged form. In accordance with the classic model, DNP, in the protonated form, carry H^+ through the lipid bilayer to release it on the opposite side. Then, in the anionic form, they diffuse back across the membrane, bind another H^+ , and repeat the cycle. DNP was used to demonstrate that $\Delta\Psi$ is essential for mitochondrial ATP production^{22,28}, providing key evidence for the chemiosmotic theory. Later, DNP was shown to increase body energy

expenditure and thermogenesis, while dramatically reducing fat deposition and body weight²⁹. However, DNP has significant side effects in humans, and because it was considered a simple chemical protonophore without a protein target, efficiency and safety of DNP could not be improved²⁹.

[040] However, recent reports challenge the classic dogma that known mitochondrial uncouplers such as DNP act as a membrane protonophore^{2,30}. These data strongly demonstrate that mild mitochondrial uncouplers induce the H⁺ leak by primarily interacting with Adenosine Nucleotide Transporter (ANT) proteins.

[041] In humans and mice, the ANT protein has several isoforms that have a tissue-specific expression patterns with ANT4 being specifically expressed in the testis and sperm cells while completely repressed in other organs. It has been shown that ANT4-deficient male mice exhibit impaired spermatogenesis and are completely infertile¹³. It is important to note that these mice are viable and exhibit otherwise normal development and physiology¹³. ANT4 was previously proposed as a contraceptive target, and a drug screen¹⁴ was executed to look for compounds that inhibit ATP/ADP exchanger activity of ANT4; however, the lead compounds were found nonspecific as they have also inhibited other human ANT isoforms and therefore exhibited broad cytotoxicity.

[042] The disclosure focuses on a completely different ANT transport modality - the ability to conduct protons and hence uncouple mitochondria in response to certain pharmacological intervention. Mild mitochondrial uncoupling is now recognized as a way to achieve weight loss, treat diabetes and even as an anticancer therapy¹⁵. Therefore, this ANT function is not expected to impose the cytotoxic effect on the targeted cells, but rather decrease their efficiency of ATP production.

[043] In some embodiments of the disclosure, an ANT4 protein may comprise or consist of the amino acid sequence of ADP/APT translocase 4 (ANT4) (UniProtKB-Q9H0C2; also known as solute carrier family 25 member 31 (SLC25A31); transcript variant 1):

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1 MHREPAKKKA EKRLFDASSF GKDLLAGGVA AAVSKTAVAP IERVKLLQV QASSKQISPE
61 ARYKGMVDCL VRIPREQGFF SFWRGNLANV IRYFPTQALN FAFKDKYKQL FMSGVNKEKQ
121 FWRWFLANLA SGGAAGATSL CVVYPLDFAR TRLGVDIGKG PEERQFKGLG DCIMKIAKSD
181 GIAGLYQGFG VSVQGIIVYR ASYFGAYDTV KGLLPKPKKT PFLVSEFFIAQ VVTTCSGILS
241 YPFDTVRRRM MMQSGEAKRQ YKGTLDCEVK IYQHEGISSF FRGAFSNVLR GTGGALVVLV
301 YDKIKEFFHI DIGGR (SEQ ID NO: 1); or be encoded by the nucleic acid comprising
or consisting of the sequence of:

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1 gcagcttttc cgcacgcgcc tcgccggcgc gcggtctctc cagcgtccca agagccactt
61 tctcgccagt acgatgctgc agcggttttc cggttttccg cttcccttca tcgtagctcc
121 cgtactcatt tttagccact gctgccggtt tttatatact tctccatcat gcatcgtgag

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 1741 aatcttgcta gtgtgaatat atcttagaac aaaaggatc ctcttgaaaa ttagtttgta
 1801 tattttgttg acaataaagg aagcttaact gttataaagg aaaaaaaaaa aaaaaa
 (SEQ ID NO: 2).

[044] In some embodiments of the disclosure, an ANT4 protein may comprise or consist of the amino acid sequence of ADP/APT translocase 4 (ANT4) (UniProtKB-Q9H0C2; also known as solute carrier family 25 member 31 (SLC25A31); transcript variant 2):

1 MHREPAKKKA EKRLFDASSF GKDLLAGGVA AAVSKTAVAP IERVKLLQV QASSKQISPE
 61 ARYKGMVDCL VRIPREQGFF SFWRGNLNV IRYFPTQALN FAFKDKYKQL FMSGVNKEKQ
 121 FWRWFLANLA SGGAAGATSL CVVYPLDFAR TRLGVDIGKG PEERQFKGLG DCIMKIAKSD
 181 GIAGLYQGFG VSVQGIIVYR ASYFGAYDTV KGLLPKPKKT PFLVSFFIAQ VVTTCSGILS
 241 YPFDTVRRRM MMQSGEAKRQ YKGTLDCEVK IQHEGISSF FRGAFSNVLR GTGGALVVLV
 301 YDKIKEFFHI DIGGR (SEQ ID NO: 3); or be encoded by the nucleic acid comprising
 or consisting of the sequence of:

1 gcagcttttc cgcacgcgcc tcgccggcgc gcggctctct cagcgtccca agagccactt
 61 tctcgccagt acgatgctgc agcggttttc cggttttccg cttcccttca tcgtagctcc

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121 cgtactcatt tttagccact gctgccgggt tttatatacct tctccatcat gcatcgtgag
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241 ctggccggcg gagtcgcggc agctgtgtcc aagacagcgg tggcgcccat cgagcgggtg
301 aagctgctgc tgcaggtgca ggcgtcgtcg aagcagatca gccccgaggc gcggtacaaa
361 ggcattggtg actgcctggt gcggttcct cgcgagcagg gtttcttcag tttttggcgt
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[045] We have identified a class of compounds, such as niclosamide ethanolamine (NEN) and BAM15 (N5,N6-bis(2-Fluorophenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine), that act as targeted mild mitochondrial uncouplers in sperm and can be utilized as sperm incapacitating agents. This rationale is that sperm mitochondrial uncoupling (sMU) drains sperm of energy and makes sperm unable to find and fertilize an egg. The search for specific activators of H⁺ leak via sperm-expressing ANT, is a novel strategy to develop nonhormonal contraceptives. Indeed, according to our data, NEN uncouples human sperm mitochondria, significantly decreases sperm beat frequency and hyperactivation. NEN is an oral salicylanilide derivative that has been approved by the US Food and Drug Administration (FDA) since 1958 for human use in the treatment of parasite tapeworm

infections^{16,28}. It has been shown to act as a mitochondrial uncoupler by translocating protons across the inner mitochondrial membrane resulting in futile cycles of glucose and fatty acid oxidation^{2, 15, 31, 32}. While its mitochondrial uncoupling is sufficient to kill gastrointestinal tapeworms, NEN has shown an excellent safety profile in humans²⁰, unlike DNP which is mildly toxic and has been associated with an unacceptably high rate of significant adverse effects²⁹. According to drug repurposing screening studies, niclosamide has strong in vivo and in vitro activity against antibiotic-resistant bacteria³³, and according to another study, may also inhibit Zika virus replication¹⁹. Moreover, niclosamide is recently characterized for uses in diabetes³², and human glioblastoma tumors³⁴, colon and ovarian cancers^{15,18,35}. While the exact molecular mechanism of niclosamide uncoupling action is not fully understood, similar to FA and DNP it should uncouple mitochondria by activating H⁺ leak via ANT, and according to our data it does so to sperm mitochondria. Therefore, our data, and the excellent safety profile of niclosamide, and its antimicrobial and antiviral properties indicate that NEN-based products exhibit contraceptive properties, in addition to their antimicrobial and antiviral properties.

[046] Unless contraindicated or noted otherwise, in these descriptions and throughout this specification, the terms “a” and “an” mean one or more, the term “or” means and/or. The examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein, including citations therein, are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

[047] EXAMPLE 1: Identification of ANT as the principal mitochondrial uncoupling protein in non-fat tissues.

[048] We employed direct patch-clamp recording from vesicles of the whole intact inner mitochondrial membrane (IMM; so-called mitoplasts, Figure 3), which allow high-resolution functional analysis of mitochondrial ion channels and transporters in their native membrane environment^{10, 36, 37}. We recently used this method to characterize the mechanism by which fatty acids (FA) induce the H⁺ leak via uncoupling protein 1 (UCP1), the protein responsible for mitochondrial thermogenesis in brown fat³⁷. However, UCP1 is fat-specific, and the mechanisms of thermogenesis in non-adipose tissues that constitute the majority of the body, are still poorly understood. Here we demonstrate that the whole-IMM patch clamp can be used to directly measure the UCP1-independent FA-induced H⁺ leak in non-adipose tissues

[049] ANT is responsible for H^+ leak across the IMM of non-adipose tissues and conducts H^+ only in the presence of fatty acids (FA) (Figure 4 and 5)¹⁰. Additionally, using whole-IMM patch-clamp this group showed that mitochondrial uncoupler DNP strongly and specifically activates proton leak via ANT and can serve as pharmacological regulators of ANT-dependent mitochondrial uncoupling by mimicking ANT endogenous activator- FA. I_H mediated by ANT is blocked by a classical ANT inhibitor carboxyatractyloside (CATR, Figure 5).

[050] We recorded DNP-induced I_H in heart of ANT1-deficient mice (ANT1 is the dominant isoform of ANT in this tissue). These experiments demonstrate the I_H induced by DNP primarily depends on ANT (Fig. 5B-D). These results indicate that, in contrast to the previously proposed model, DNP does not uncouple mitochondria as simple chemical protonophores but target ANT. Thus, data demonstrate that the mitochondrial uncoupler-activated I_H can be recorded across the whole IMM using the patch-clamp method, and that this current is mediated by ANT.

EXAMPLE 2: Uncoupling of human sperm mitochondria reduces sperm motility and prevents fertilization.

[051] Here we have studied the effect of DNP, niclosamide, and BAM15, three known mitochondrial uncouplers, on their ability to uncouple sperm mitochondria^{13, 38, 39}. As shown in Figure 6B, both BAM15 and NEN elicit strong sMU as assessed by mitochondrial depolarization. Importantly, niclosamide, the most potent of the two compounds, also decreased sperm beat frequency from 14Hz to 6Hz (Fig. 6A), inhibited hyperactivation (Fig. 6C) at concentrations as low as 1 μ M.

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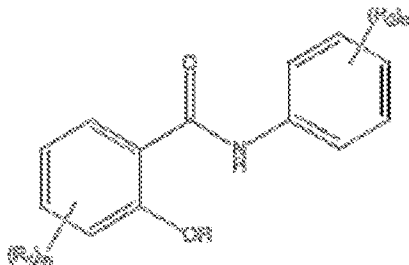
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CLAIMS:

1. A method of promoting contraception, comprising administering to a person in need thereof a composition comprising an effective amount of a targeted mild mitochondria uncoupler.
2. The method of claim 1 wherein the uncoupler comprises a salicylanilide or salt thereof, such as an ethanolamine salt thereof, such as niclosamide ethanolamine (NEN).
3. The method of claim 1 wherein the uncoupler comprises a salicylanilide compound or salt thereof, and the salicylanilide compound is selected from: niclosamide, bromochlorosalicylanilide, oxyclozanide, rafoxanide, 3-tert-butyl-5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxy-6-methylbenzamide, dibromsalan, metabromsalan, tribromsalan, and 2-iodo-N-phenylbenzamide (benodanil).
4. The method of claim 1 wherein the uncoupler comprises a salicylanilide compound or salt thereof, and the salicylanilide compound has a structure:



wherein R1 and R2 are independently halide, such as F, Cl, Br or I, substituted heteroatom selected from O and N, such as -OH, -NO₂ or O-Ph-Cl, or lower (C1-C4) alkyl, such as Me, Et, or t-butyl, m is an integer 1, 2, 3 or 4, and n is an integer 1, 2, 3, 4 or 5.

5. The method of claim 1 wherein the uncoupler comprises BAM15 (N5,N6-bis(2-Fluorophenyl)-[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine), or salt thereof.
6. The method of claim 1, 2, 3, 4 or 5 wherein the composition is administered via an oral route, a topical route, a rectal route, or a vaginal route.
7. The method of any one of claims 1-5, wherein the composition is administered as a pill, a cream, a vaginal ring, a vaginal film or a patch.
8. The method of claim 6, wherein the composition is administered orally and wherein the

composition is administered as a pill.

9. The method of claim 6, wherein the composition is administered topically and wherein the composition is administered as a cream or a patch.

10. The method of claim 6 wherein the composition is administered intravaginally and wherein the composition is administered as a cream, a patch, a vaginal ring or a vaginal film.

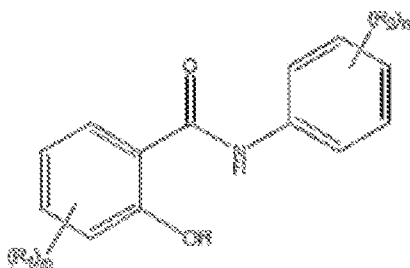
11. The method of claim 6 wherein the composition is administered intrarectally and wherein the composition is administered as a cream or a patch.

12. Use of a targeted mild mitochondria uncoupler as a contraceptive.

13. The use of claim 12, wherein the uncoupler comprises a salicylanilide or salt thereof, such as an ethanolamine salt thereof, such as niclosamide ethanolamine (NEN).

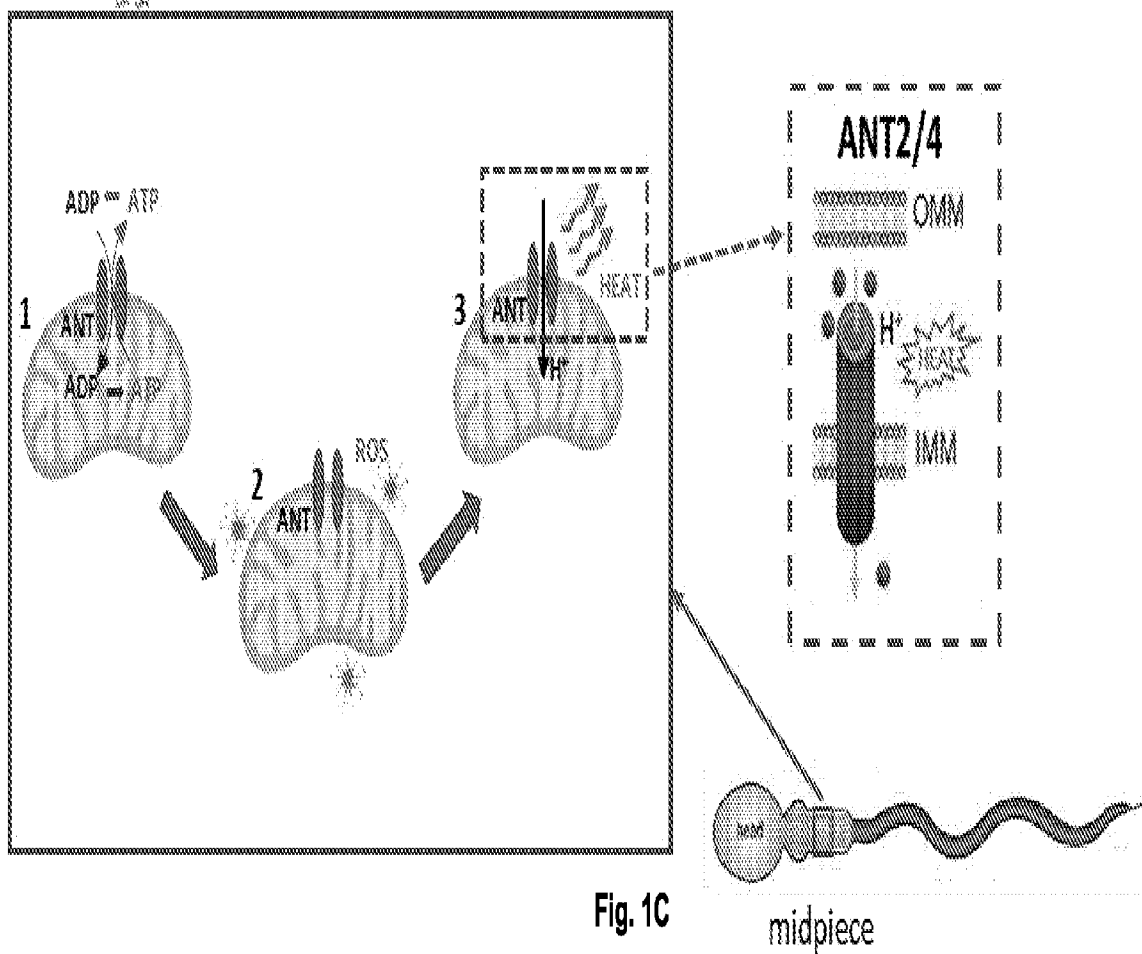
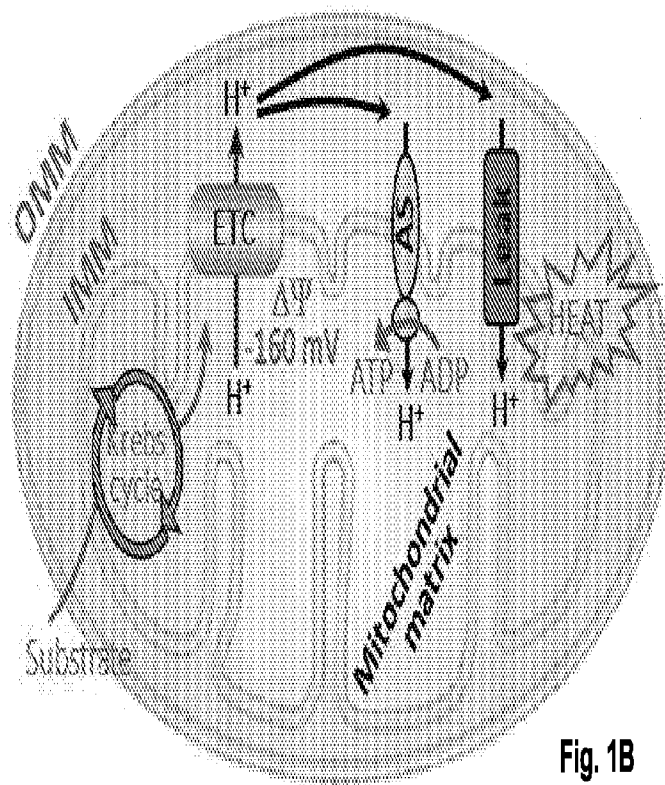
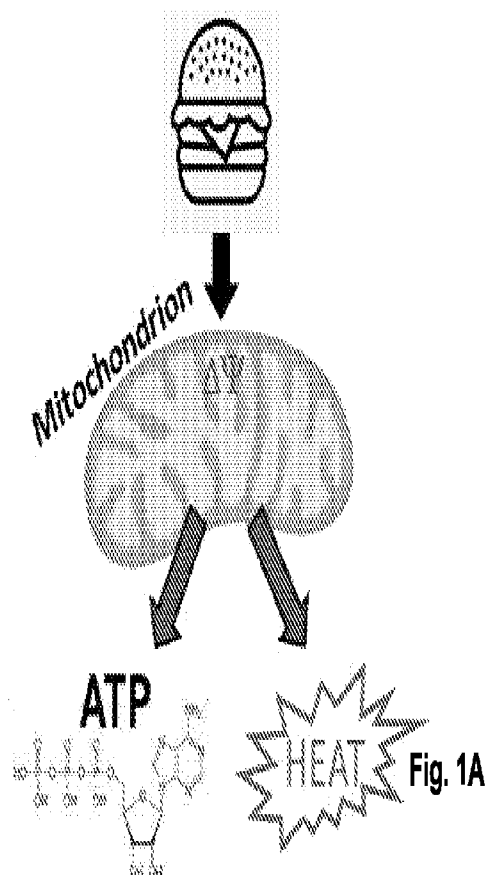
14. The use of claim 12 wherein the uncoupler comprises a salicylanilide compound or salt thereof, and the salicylanilide compound is selected from: niclosamide, bromochlorosalicylanilide, oxyclozanide, rafoxanide, 3-tert-butyl-5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxy-6-methylbenzamide, dibromsalan, metabromsalan, tribromsalan, and 2-iodo-N-phenylbenzamide (benodanil).

15. The use of claim 12 wherein the uncoupler comprises a salicylanilide compound or salt thereof, and the salicylanilide compound has a structure:



wherein R1 and R2 are independently halide, such as F, Cl, Br or I, substituted heteroatom selected from O and N, such as -OH, -NO₂ or O-Ph-Cl, or lower (C1-C4) alkyl, such as Me, Et, or t-butyl, m is an integer 1, 2, 3 or 4, and n is an integer 1, 2, 3, 4 or 5.

16. The use of claim 12 wherein the uncoupler comprises BAM15 (N5,N6-bis(2-Fluorophenyl)- [1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine), or salt thereof.
17. The use of claim 12, 13, 14, 15, or 16 wherein the composition is administered via an oral route, a topical route, a rectal route, or a vaginal route.
18. The use of any one of claims 12-16, wherein the composition is administered as a pill, a cream, a vaginal ring, a vaginal film or a patch.
19. The use of claim 17, wherein the composition is administered orally and wherein the composition is administered as a pill.
20. The use of claim 17, wherein the composition is administered topically and wherein the composition is administered as a cream or a patch.
21. The use of claim 17, wherein the composition is administered intravaginally and wherein the composition is administered as a cream, a patch, a vaginal ring or a vaginal film.
22. The use of claim 17, wherein the composition is administered intrarectally and wherein the composition is administered as a cream or a patch.
23. A contraceptive formulation comprising an effective amount of a targeted mild mitochondria uncoupler.
24. The formulation of claim 23 in the form of a pill, capsule, suppository, cream, vaginal ring, vaginal film or patch.



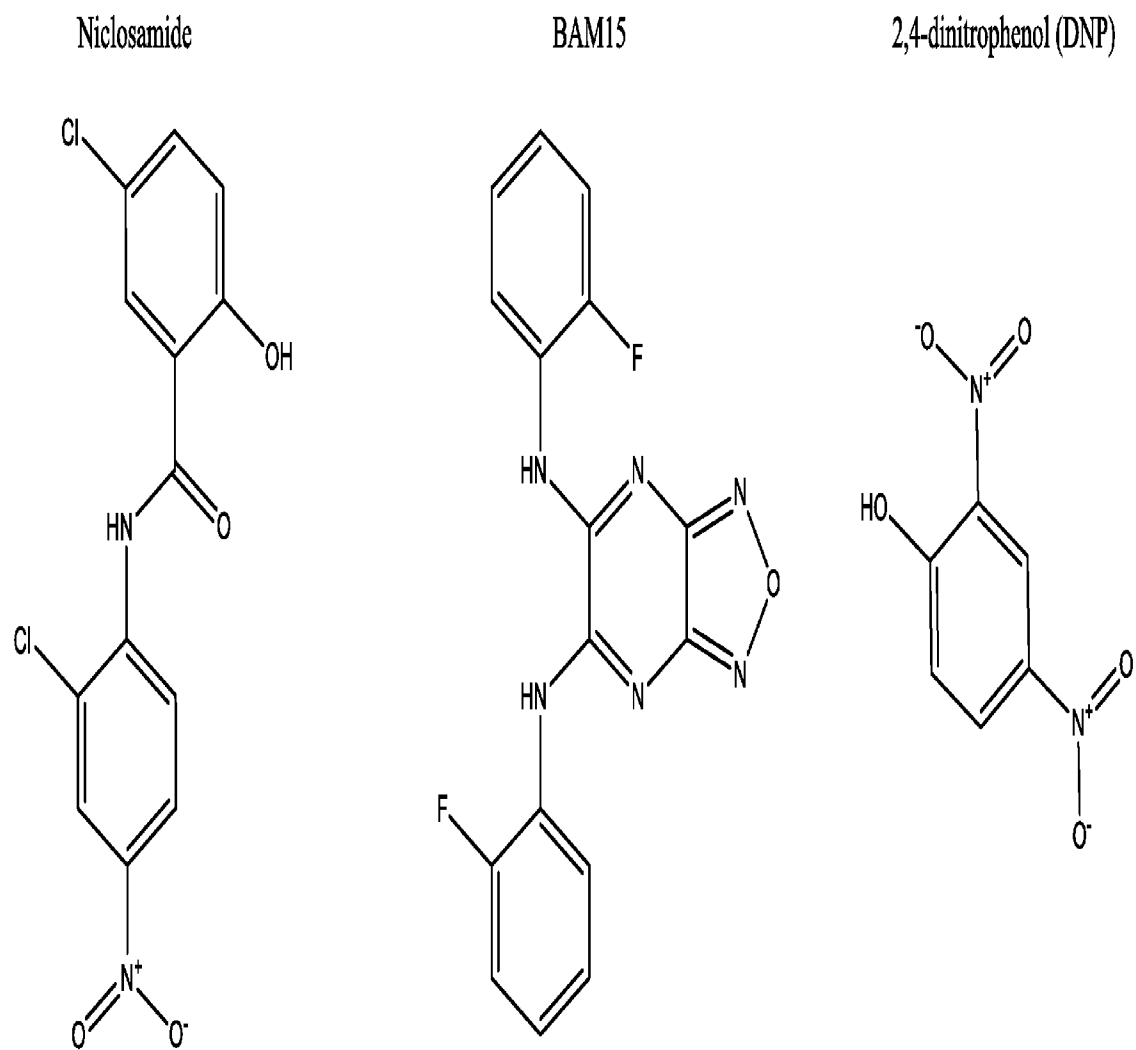


Fig. 2

Fig. 3A

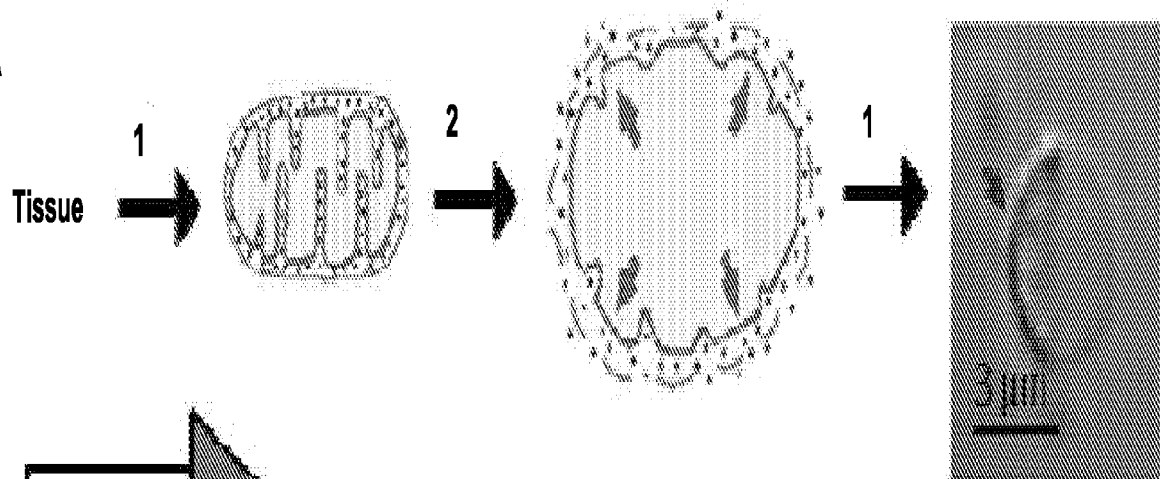
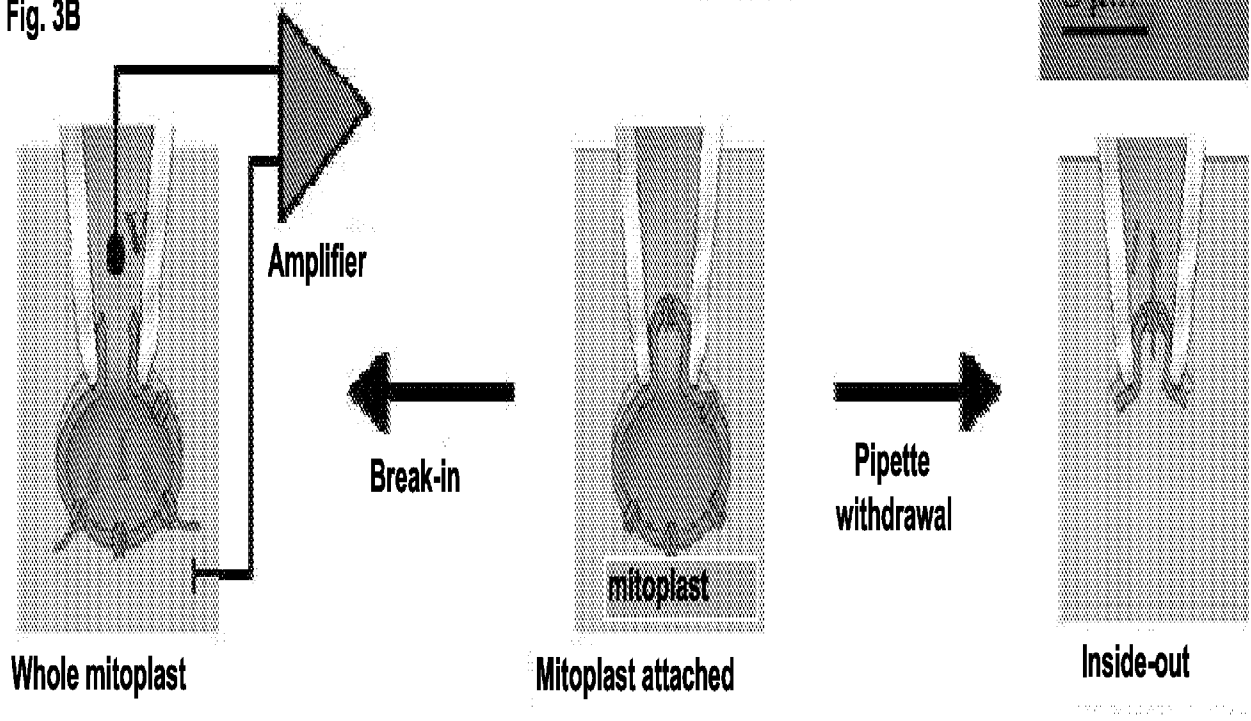


Fig. 3B



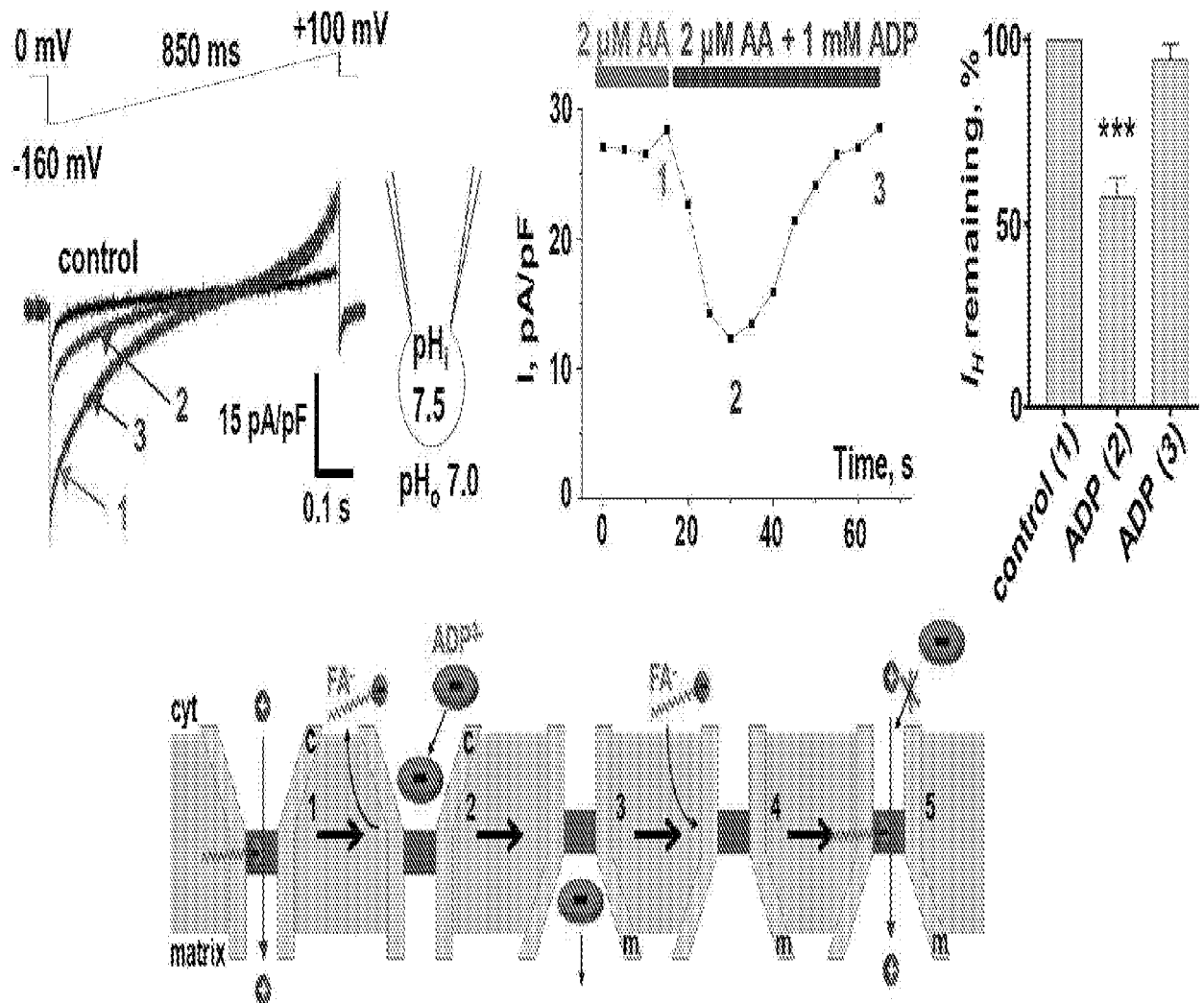


Fig. 4

Fig. 5A

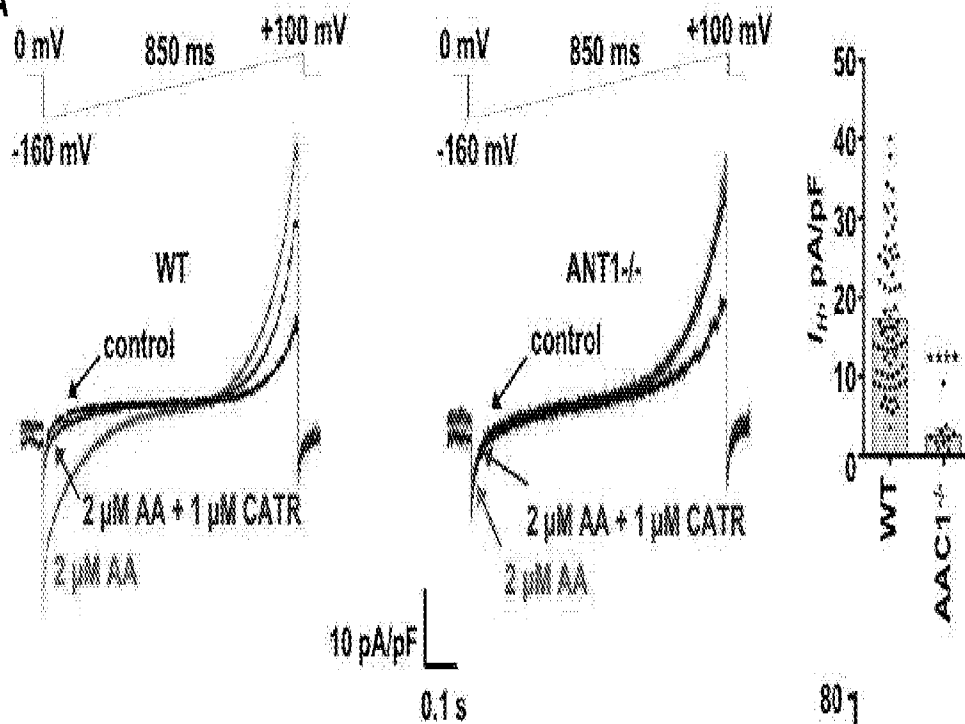


Fig. 5B

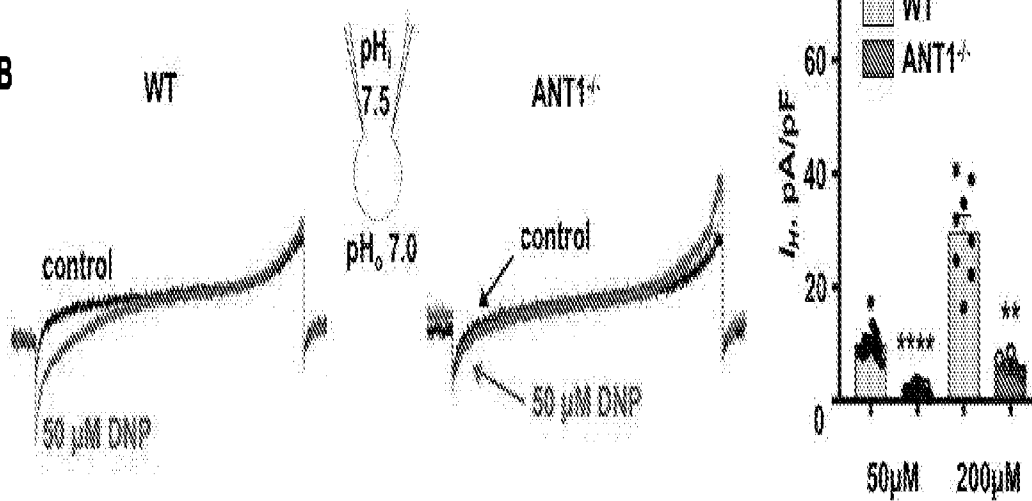


Fig. 6A

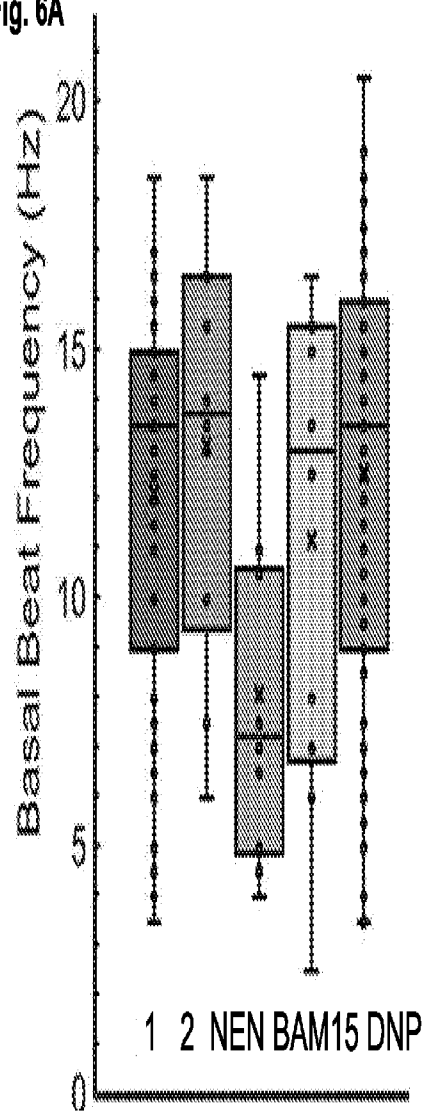


Fig. 6B

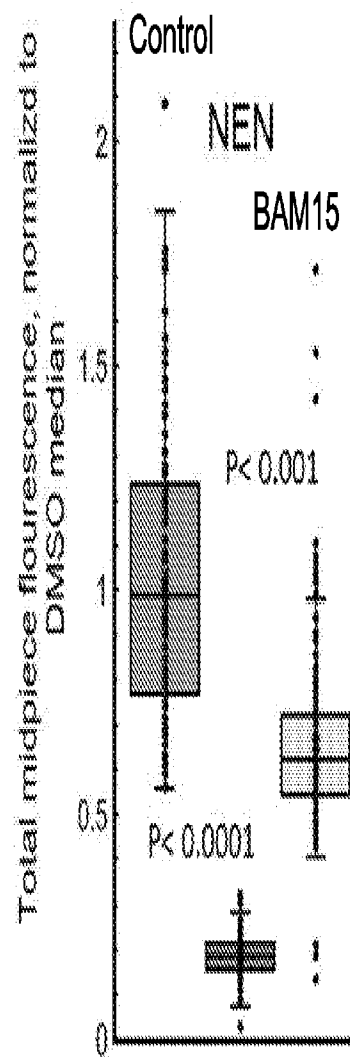
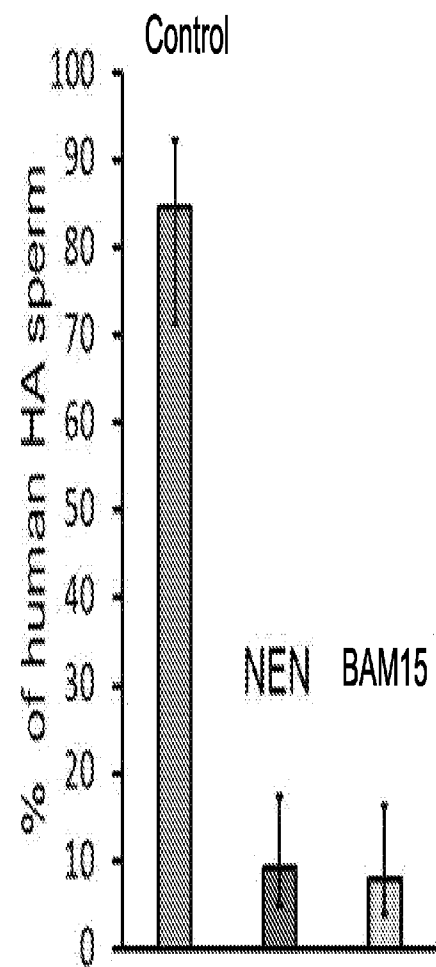


Fig. 6C



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/19373

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/166; A61K 31/609; C07C 235/64; A61P 5/24 (2020.01)

CPC - A61K 31/609; C07C 235/64; A61K 31/4985; A61K 31/166; A61P 15/16; A61P 15/18

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	~ BREITBART et al. 'Molecular Mechanisms of Gossypol Action on Sperm Motility', Int. J. Biochem. 1989, Vol. 21(10), pp. 1097-1102. abstract; pg. 1097, Col 1, para 1 ; pg. 1099, Figure 2; pg. 1100, Col 1, para 4 to Col 2, para 2	1; 12; 23 ----- 2-11; 13-22; 24
Y	✓ CHILDRESS et al. 'Small Molecule Mitochondrial Uncouplers and Their Therapeutic potential', Journal of Medicinal Chemistry, 2018, Vol.61, pp. 4641-4655. pg. 4643-4645, Table 1;pg 4647, Col 1, para 3	2-11; 13-22; 24
Y	WO 2017/201313 A1 (JIN, Shengkan et al.) 23 November 2017 (23.11.2017) para [0027];[0029];[0042]; [0104];[0105]	9-11; 20-22
A	US 2003/0036646 A1 (NI et al.) 20 February 2003 (20.02.2003) ENTIRE DOCUMENT	1-24
A	~ JAN et al. 'Novel Effects of Gossypol, a Chemical Contraceptive in Man: Mobilization of Internal Ca ²⁺ and activation of Extracellular Ca ²⁺ Entry in Intact Cells', Biochimica et Biophysica Acta 2000, Vol 1496, pp. 270-276. ENTIRE DOCUMENT	1-24

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