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THESIS

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Pharmacological evaluation of new *meta*- and *para*-hydroxyl substituted C-4-aryl-1,4-dihydropyridines in cardiomyocytes

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To my beloved mother

"…if you are going through hell, keep going."
Sir Winston Churchill

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium concentration
AP	Action potential
ATP	Adenosin triphosphate
BDM	2,3-butanedione monoxime
BTZ	Benzothiazepine
C	Carbon cation
C4(3,5-diOH-phenyl)-1,4-dihydropyridine	3,5-OH-DHP
C4(3-OH-phenyl)-1,4-dihydropyridine	3-OH-DHP
C4(4-isovanillin)-1,4-dihydropyridine	IDHP
C4(4-OH-phenyl)-1,4-dihydropyridine	4-OH—DHP
C4(4-phenyl)-1,4-dihydropyridine	PDHP
C4(4-vanillin)-1,4-dihydropyridine	VDHP
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cDNA	Deoxyribonucleic acid
CICR	Calcium induced calcium release
Cs-OH	Cesium hydroxide
DHP	Dihydropyridines
DMEM	Dulbecco's modified Eagle´s medium
DMSO	Dimethyl sulfoxide
ECC	Excitation contraction Coupling
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene-glycol tetra-acetic acid
EP	Electrophysiology
Fe_2SO_4	Ferric sulfate
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HVA	High voltage activated
I	Current
IUPHAR	International Union of Pharmacology
jSR	Junctional portion of sarcoplasmic reticulum
KCl	Potassium chloride
kDa	kilo Dalton
L	Long lasting
lSR	Longitudinal portion of sarcoplasmic reticulum
LTCC	L-type calcium channel
LVA	Low voltage activated
M199	Medium 199
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
N	Neuronal
Na^+	Sodium ion
NaCl	Sodium chloride
NIT	Nitrendipine
P	Purkinje
P/Q	Purkinje
PAA	Phenylalkylamines

PHAR	Pharmacology
PI	Propidium iodide
QSAR	Quantitative structure activity relationship
R	Resistant
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SAR	Structure activity relationship
SR	Sarcoplasmic reticulum
T	Transient
TEA-Cl	Tetraethylammonium chloride
TEA-OH	Tetraethylammonium hydroxyde
V	Voltage
VDC	Voltage dependent channel
VDCC	Voltage dependent calcium channel
ω-Aga IVA	ω-Agatoxin IVA
ω-CTX GVIA	ω-conotoxin GVIA

SUMMARY

Voltage-dependent calcium channels are widely expressed throughout the different tissues, including those from the cardiovascular system. They constitute the main route for calcium entry. Voltage gated calcium channels are multimeric proteins that consist of a principal pore forming α_1 subunit in association with auxiliary subunits. There are various types of voltage dependent calcium channels but in cardiomyocytes predominantly the L-type calcium channels is found. Due to the relevance of the L-type calcium channel in cardiac diseases; these channels are the primary target of the calcium channel blockers.

Commonly used calcium channels blockers (CCBs) are classified onto three major classes; they vary chemically, and exhibit different functional effects depending on their biophysical, conformation-dependent interactions with the L-type channel in the α_1. Even though at the beginning of their pharmacological use the phenylalkylamines (diltiazem), the benzothiazepines (verapamil) and the dihydropyridines (nitrendipine), exhibited a somehow similar cardiodepressant activity, the development of new calcium channel blockers seems to have centered in the DHPs which have evolved from a first until a third generation, being the members of this last generation equally potent blockers, and some of them with a reported antioxidant activity, which offers therapeutic benefits based upon the acquiring relevance of oxidative stress in cardiovascular diseases such as hypertension; where reactive oxygen species have deleterious effects due to their interaction with ion transport systems in the plasma membrane.

The aim of this thesis was to explore the calcium channel blocking as well as antioxidant properties, and cell toxicity of novel DHPs. These presented unreported substitutions in the phenyl ring, such as a hydroxyl group, mono substituted, in *meta* and *para*, respectively; then disubstituted in *meta*; a vanillin with and hydroxyl group in meta and a carboxy group in para and its isomer; besides an unsubstituted aryl ring. These unreported molecules were all compared to nitrendipine.

We used confocal microscopy in the line scan and patch clamp in the whole cell mode to test Ca^{2+} antagonism. Antioxidant activity was also measured through confocal microscopy; and flow cytometry, the latter was also applied to assess cell toxicity and antioxidant properties. None of the compounds exhibited significant toxic effects after a 24 hour incubation period, tested on neonatal ventricular myocytes. Our results indicated that these effects were not significantly different from the drug control, nitrendipine (NIT). In consequence, the pharmacological effects here found are the outcome of the DHPs themselves and not because of a toxic side effect.

Doing a quick recap, only one of the novel DHPs exhibited a blocking effect on LTCCs on adult rat cardiomyocytes, the hydroxyl meta substituted (3-OH-DHP), at all three concentrations tested whilst the one with the unsubstituted aryl had a blocking effect only at its highest concentration. Patch clamp results for 3-OH-DHP at concentrations lower than 0.1 µM also confirm this antagonizing effect.

None of the compounds seemed to possess an antioxidant activity in the tests performed on cultured rat cardiomyocytes. Concerning the studies on ROS, it can be inferred, that a bulky substituent in C2 or C3 on the dihydropyridine ring, is relevant to achieve an antioxidant effect.

Our research led to several accomplishments, first, and in agreement with the literature, that any para substitution in the aryl ring abolishes or nullifies the LTCC blocking effect of DHPs. Second, the unsubsituted aryl ring it is a pharmacophore, but it requires an adequate substitution to expect calcium channel blocking effect. Third, despite the structural similarity between a meta-hydroxy-substituted and a meta-dihydroxy- substituted aryl ring, only the former had an effect on the channels; probably this difference can be attributed to a steric effects.

RESUMEN

Los canales de calcio dependientes de voltaje son expresados ampliamente en todos los tejidos, incluyendo los del tejido cardiovascular. Ellos son la principal ruta para la entrada de calcio dentro de la célula. Estos canales son proteínas multiméricas que son compuestos por una subunidad principal, α_1, que es la que forma el poro, y se asocia con otras subunidades denominadas auxiliares: $\alpha_2\delta$, β and γ. Hay varios tipos de canales de calcio pero en las células ventriculares cardíacas se encuentran primordialmente los canales de calcio tipo L. Debido a su relevancia en enfermedades cardíacas; estos canales son el blanco de los bloqueadores de los canales de calcio.

Los bloqueadores de los canales de calcio (CCBs) más utilizados, se clasifican en tres grandes clases; varían químicamente y exhiben diferentes efectos funcionales que dependen de sus interacciones tanto conformacionales como biofísicas con la subunidad α_1 del canal. Aunque al inicio de su uso farmacológico fenilalquilaminas (verapamilo); benzotiazepinas (diltiazem) y dihidropiridinas (nitrendipino), mostraban una actividad cardiodepresora parecida, el desarrollo de nuevos bloqueadores parece haberse centrado en las dihidropiridinas (DHPs), las cuales han evolucionado desde una primera hasta una tercera generación, siendo los miembros de ésta última bloqueadores igualmente potentes además de que tienen reportada actividad antioxidante, lo cual ofrece un mayor beneficio terapéutico, máxime con la relevancia que está adquiriendo el estrés oxidativo en enfermedades cardiovasculares como la hipertensión; donde las especies reactivas de oxígeno tienen efectos deletéreos debido a su interacción con sistemas de transporte iónico en la membrana plasmática.

El objetivo de esta tesis fue investigar la potencia bloqueadora del canal de calcio como también propiedades antioxidantes de nuevas dihidropiridinas. Estas presentaban sustituciones que no han sido reportadas en el anillo fenil, como un grupo hidroxilo; primero en una mono-sustitución en *meta* y en *para*, respectivamente, y una di-sustituida en posición meta; segundo, una vainillina con un grupo hidroxilo en posición *meta* y un grupo carboxi en posición *para*, junto con su isómero; además de arilo sin sustituciones. Estas moléculas no reportadas se compararon frente a nitrendipino.

Se empleó microscopía confocal en su modo line scan y patch clamp, también en este mismo modo para investigar su antagonismo de Ca^{2+}; para medir su capacidad antioxidante se usaron además de la microscopía confocal, la citometría de flujo, ésta última también fue utilizada para evaluar tanto la toxicidad celular como sus propiedades antioxidantes.

Ninguno de los compuestos demostró tener efectos tóxicos relevantes luego de un periodo de incubación de 24 horas en cardiomiocitos. Nuestros resultados indican que estos efectos no fueron significativamente diferentes de aquellos del compuesto control, nitrendipino (NIT). Entonces los efectos farmacológicos observados son el resultado de los mismos compuestos y no de un efecto tóxico.

Sólo una de las nuevas DHPs exhibió un efecto bloqueador sobre los canales de calcio tipo L en cardiomiocitos, meta sustituido con un hidroxilo (3-OH-DHP), en todas las concentraciones en que fue probada, mientras que la que presentaba el arilo sin sustituciones, solo tuvo este efecto cuando se ensayó en su concentración más alta (10 µM). Los resultados obtenidos con patch clamp para 3-OH-DHP a concentraciones inferiores a 0.1 µM, confirman este efecto antagonista.

Ninguno de los compuestos pareció tener actividad antioxidante en los experimentos realizados en cardiomiocitos de rata neonata.

Nuestra investigación llevó a varios logros, primero, y de acuerdo con lo que establece la literatura cualquier sustitución en para del arilo disminuye ostensiblemente o anula el efecto bloqueador de las DHPs sobre los CCTL. Segundo, el anillo arilo sin sustituciones es farmacóforo pero requiere de un sustituyente adecuado para esperar un efecto bloqueador. Tercero, a pesar de las similitudes estructurales entre el compuesto sustituido con un hidroxilo en meta y el bisustituido en la misma posición, solo el primero tuvo un efecto sobre los canales; probablemente esta diferencia se deba a un impedimento estérico. En lo referente a los estudios sobre ROS, se puede inferir que se requiere un sustituyente voluminoso en C2 ó C3 del anillo dihidropiridinico para lograr actividad antioxidante.

1. INTRODUCTION

1.1. Ion channels

They are ubiquitously expressed structures throughout cellular life. Ion channels constitute integral membrane proteins that both produce and transduce electrical signals which are crucial to the maintenance of vital functions in a plethora of cellular types. Ion channels gate or regulate the ion flow through biological membranes as it occurs between the cytoplasmic compartment and the extracellular space as well as between subcellular compartments. Ion channels are water-filled, biological "sub-nanotubes" formed by large peptidic structures and they constitute a class of membrane proteins that serve as conduits for rapid and regulated ion movement across cellular membranes. Thereby, ion channels provide the molecular substrate for fast, electrical signaling in excitable tissues.[1] In addition to playing this important role, ion channels regulate the release of hormones and neurotransmitters and they control cell and body electrolyte and volume homeostasis. They are also involved in the signaling transduction of external stimuli to sensory signals.[1,3]

Opening and closing of ion channels occurs in response to changes in membrane potential, changes in ion concentrations on either side of the membrane, and agonist binding to the channel or closely associated regulatory proteins. Under pathological conditions, ion channels contribute to a variety of diseases among which are different cardiovascular pathologies.

Historically ion channels have been classified in several ways, according to the state of the art knowledge that characterized each of the last decades. For instance, as early as in the 1940s, their first classification refers to the ions for which they are selective. Later on the 50's they were classified according to their primary mode of stimulus namely ligand-gated and voltage-gated channels a classification that is maintained nowadays. With the development of the patch clamp technique in the 70's, ion channels were alternatively classified by their electrophysiological properties. From 50's to 80's, a combination of electrophysiological and biochemical studies led to a classification based on their pharmacological sensitivity to toxins and synthetic drugs. The 21st century was heralded the molecular era in ion channel research that begun at the end of the 70's and early beginning of the 80's. The identification of their genetic sequences demonstrates that ion channels exist as super-families with considerable structural homology between members, despite very different electrophysiological and pharmacological properties.

The first naming of ion channels is typically not systematic,[3] early biophysical work attempted to distinguish different components of membrane permeability by their kinetics, pharmacology, and response to ion substitution. A kinetic model is often made expressing each of

the apparent components mathematically. Finally it is implicitly assumed that each model of the component corresponds to a type of channel, and the putative channels are given the same names as the permeability components in the original analysis. Thus in the cornerstone analysis of ionic currents performed by Hodgkin & Huxley [2] three different components of currents were identified, which they called sodium, potassium and leakage. Today the names of sodium channel and potassium channel are accepted by the scientific community for the corresponding classes of ion channels.

Long after those initial experiments and subsequent naming, there was a new beginning in the identification of ion channels. Molecular genetics tools made possible the cloning of individual channels with this leading to sequencing entire genomes. Nowadays, a large number of channel genes have been discovered and there are more findings to come. There are more channels than the electrophysiological tools have been able to detect. Just for the most extensively studied channels, Ca^{2+}, Na^{+} and K^{+}, more than a hundred genes have been identified in mammals such as rats. The problem then lies in how to name them in order to classify them. As discussing channels nomenclature and names is a very complex and extensive topic and go beyond the scope of this thesis, for the sake of clarity, the classification of ion channels that will be referred in this project shall be the one that divides them into two main families, which will be addressed and discussed briefly.[3]

According to International Union of Pharmacology (IUPHAR), there are two main ion channel families: ***Voltage gated*** and ***Ligand gated ion channels*** (also called voltage dependent or ligand gated channels, the name we will use herein after is voltage dependent channel).[4] This classification is based on the early work of biophysists, who remarked that voltage gated Ca^{2+}, Na^{+} and K^{+} channels have many functional similarities. This was also the case for channels gated by acetylcholine, glycine and γ-aminobutyric acid. The predicted sequences of amino acids for channels reveals strong structural similarities among these groups of channels, currently considered as the result of successive gene duplication, mutation and selection from common ancestral channels.[3] A functionally defined type of channel is not a single structure entity. All channels can be expressed in various isoforms coded by different genes or as different splice variants that may be selectively expressed in certain cell types and in specific time windows during development and growth. Current evidences indicate that in the never-ending process of evolution new classes of channels appear when parts of old ones are recombined with functional domains of enzymes and signaling proteins to create new functions.

Ligand-dependent ion channels recognize and react to specific molecules in their surrounding environment. Upon ligand binding, the ion channel changes its conformation and starts

(or stops) conducting ions. Examples of ligand-gated ion channels include receptors for taste and odors, receptors for different hormones and neurotransmitters such as dopamine and acetylcholine.

Voltage dependent channels (VDCs) switch their conductivity in response to a change of the voltage across the membrane. These channels make it possible for impulses to travel along nerve and muscle fibers; it is on these *voltage-sensitive ion channels or voltage dependent channels or voltage gated channels* that we focused our interest here, and constitute the cornerstones for this thesis.[4]

In turn, VDCs are divided into the following subfamilies:

- Calcium activated Potassium channels
- CatSper and two-pore channels
- Cyclic-nucleotide channels
- Inwardly rectifying potassium channels
- Transient receptor channels
- Two-pore Potassium channels
- Voltage dependent Potassium channels
- Voltage dependent Sodium channels
- **Voltage dependent Calcium channels**

In the work presented here, is the last subfamily: **Voltage dependent Calcium channels,** to which from now on we will refer as **VDCC.** In the paragraph below: **VDCC** will be presented and discussed generally, first describing the subfamily as a whole, then emphasizing in a subclassification, highlighting **L-type calcium channels**, and finally describing their structure, in which the binding sites for calcium channel blockers will be pointed out.

1.2. Voltage dependent Ca^{2+} channels

VDCCs were first identified in crustacean muscle by Fatt & Katz [5] and subsequently studied. These muscles showed action potentials in the absence of external Na^{+}, which were dependent Ca^{2+} entry. Ca^{2+} is generally present at a concentration of a few mM in the extracellular space, but inside the cell, the cytoplasmic concentration is near 0.1μM, kept low by a number of different pumps and buffering systems, as well as the general impermeability of the plasma membrane to the entry of Ca^{2+}. **VDCCs** have subsequently been found in all types of excitable cell in vertebrates and invertebrates, even plants. They fulfill numerous functions depending on the

tissue, and it is thus not surprising that a number of subclasses of **VDCC** have been identified, as well as their role in different diseases.[6]

VDCCs are normally closed at resting membrane potentials, and open upon depolarization, because part of the channel structure senses the change in transmembrane voltage. The resultant current through the cell membrane can be characterized by a number of properties, including the membrane potential range over which the channel reaches its maximum open probability, and as well as the kinetics or time-dependent properties of the current. Different single channel currents can also be identified, with varying properties. The task of matching these single channel types with the currents observed in entire cells is a difficult one, but has been made easier by the cloning of the cDNAs for a number of **VDCCs** and the use of selective drugs and toxins to identify specific current components, that correspond to particular channel types.[3,6]

According to the membrane potential range at which voltage dependent calcium channels open, they can then be divided into two main groups, ***Low threshold and high threshold voltage gated***. In a number of tissues, including cardiac muscle cells, neurons and other excitable cells, it became apparent that there are two types of calcium currents. One is activated by small depolarizations and shows rapid voltage-dependent inactivation; this is termed low voltage-activated (**LVA**), or **T** for **transient**. The second is activated by large depolarizations and is termed high voltage-activated (**HVA**). The single calcium channels underlying these currents are clearly distinct, T type channels being of small conductance (5-9 pS) and showing rapid inactivation during a voltage step, whereas HVA channels are of larger conductance (13-24 pS).[7]

High threshold voltage gated currents have been further divided into smaller subgroups. This has been done in great part to pharmacological tools that have allowed distinguishing these subgroups.

1.2.1. L-type calcium channel

One of this HVA groups was defined as **L** current; for long lasting (ergo the name L-type calcium channels). It was found that this current seemed to be blocked by certain type of groups known as calcium channel blockers amongst which are dihydropyridines (DHPs). Furthermore other drugs had the opposite effect on this current and did not block it but enhance it, they were also in the dihydropyridine group, such as BAYK 8644. Dr Roger Tsien has contributed most to the characterization of these currents (and the channel). His group further found that in sensory neurons, besides exhibiting the L current, another component termed N, for neuronal, was present,[8] of intermediate conductance, was insensitive to DHPs but was selectively and irreversibly inhibited

by ω-conotoxin GVIA (ω-CTX GVIA), a peptide toxin from the cone shell mollusk called *Conus geographus*. Another sub-group of calcium currents, insensitive to both ω-CTX GVIA and DHPs, has now been reported in many tissues, indicating the presence of further current components. For instance in the cerebellar Purkinje cells in which a small proportion of the calcium current corresponds to N and L current, the major calcium current has been termed P type. A selective blocker for the Purkinje cell Ca^{2+} current has been found in a peptide toxin from the venom of the american funnel web spider *Agelenopsis aperta,* called ω-Agatoxin IVA (ω-Aga IVA). At higher concentrations this toxin also blocks a current component that has been termed Q (the letter after P), although the distinction between P and Q current is not always clear[6]. In many neurons, despite the application of all three blockers, there often remains a substantial proportion that cannot be classified as L, N or P/Q. This residual current has been termed R (for resistant). See Figure1.

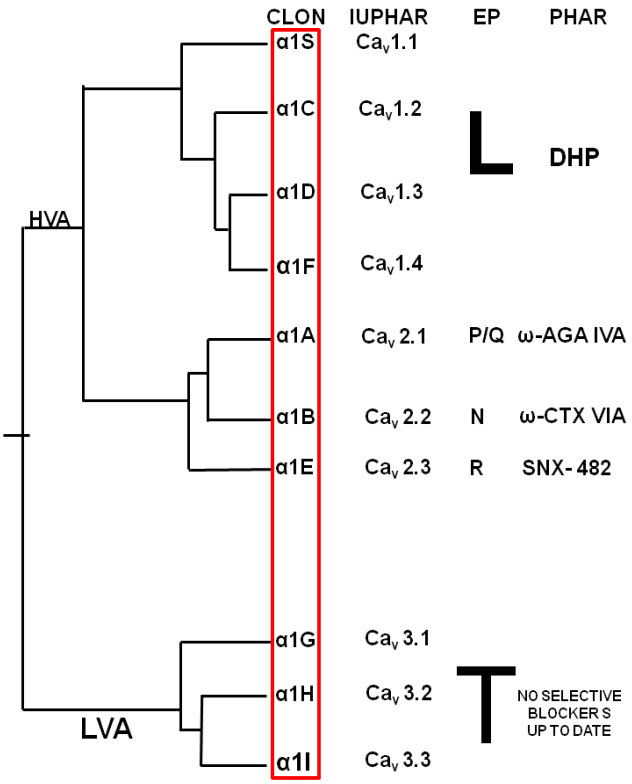
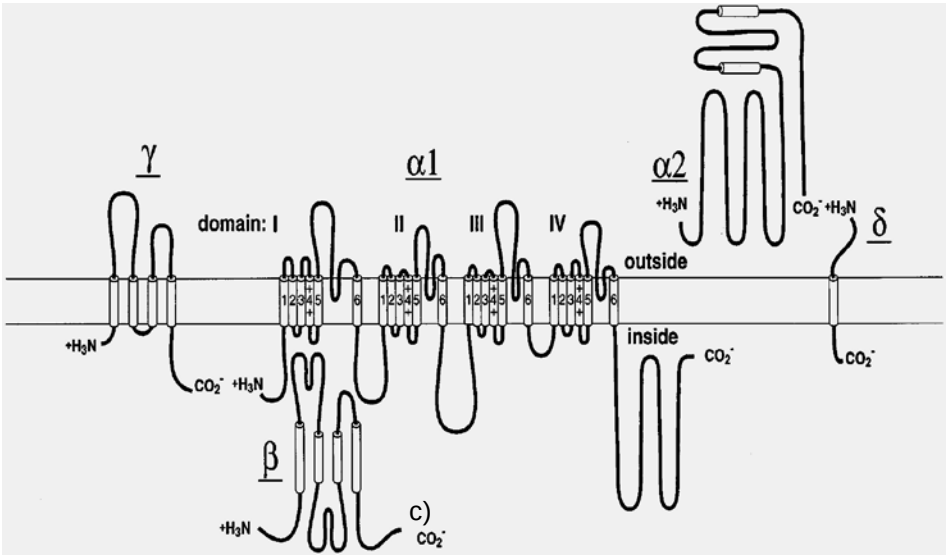


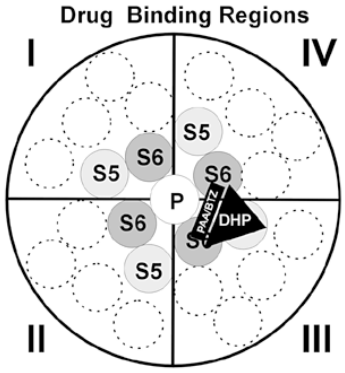
Figure 1. Voltage dependent calcium channels classification.
This figure describes briefly, the nomenclature that calcium channels have undertaken along the years according to the discipline that has studied them. In the case of the clon names, the name comes from the most important subunit the channel has, that is the one that forms the ion pore. As it can be seen the role of pharmacology in identifying and classifying calcium channels and currents has proven to be critical and determinant. EP: electrophysiology; Phar: pharmacology.

Understanding the molecular basis for the physiological subtypes of calcium channel first required the identification of voltage-gated calcium channels as large heteromeric proteins. This era started with purification of the skeletal muscle calcium channel complex, also termed the DHP receptor, which is highly abundant in T-tubules. The purified DHP receptor complex was found to contain five components, which were termed **α1** (170 kDa), **α2** (150 kDa), **β** (52 kDa), **δ** (17–25 kDa) and **σ** (32 kDa), in an approximate stoichiometry.[9-11] The **α1** protein was identified as the component that bound **DHPs**, and was therefore provisionally established as the pore-forming subunit. After individual subunits were identified, the cDNA for the DHP receptor from skeletal muscle was cloned[12], and subsequently from **heart.**[13] Analysis indicated that the **α1** subunits have about 2,000 amino acid residues with an amino acid sequence and predicted transmembrane structure like the previously characterized, pore-forming **α1** subunit of Na^+ channels.[12] The amino acid sequence is organized in four repeated domains (I to IV), each of which contains six transmembrane segments (S1 to S6), and a membrane-associated loop between transmembrane segments S5 and S6, with intracellular linkers and N- and C-termini (figure 2). In each domain S4 constitutes the voltage sensor, besides this voltage sensor, domains III and IV of this pore forming subunit, contain the specific binding sites for calcium channel blockers found so far; these are located in **III S5** and **S6**, and **IV S6** for DHP and III S6 and IV S6 for the other members of the calcium channel blockers family. In all, 10 **α1** subunits have now been cloned, and they all have specialized functions and distributions.[14] The four members of the **Ca_V1** family are all L-type channels, with Ca_V1.1 being the skeletal muscle isoform and **Ca_V1.2** being particularly prevalent in cardiac muscle;[15] whereas the more recently cloned Ca_V1.3 and 1.4 are activated at lower voltage thresholds, and have a more restricted distribution. Ca_V2.1 is the molecular counterpart of P/Q-type calcium channels,[16] Ca_V2.2 or a1B, is the molecular counterpart of the neuronal N-type calcium channels. Ca_V2.3 or a1E was initially thought to be a low voltage-activated channel, and it is certainly more inactivating than the other HVA channels cloned.[17] However, it is now thought to contribute to the molecular counterpart of the R-type calcium current. The Ca_V3 group of channels was the last to be cloned.[18]

a)

b)





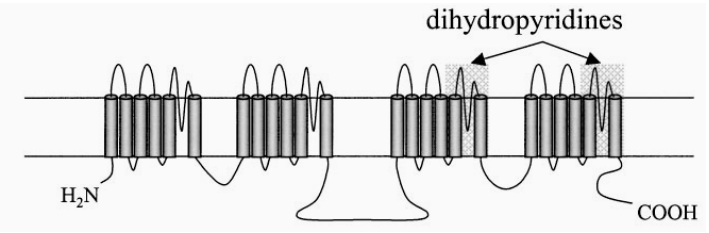


Figure 2. Voltage dependent Ca^{2+} channels topology.

a) Transmembrane organization of voltage-gated Ca^{2+} channels. The primary structures of the subunits of voltage-gated Ca^{2+} are illustrated. Cylinders represent probable alpha helical transmembrane segments. Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues. b) Calcium channel blockers binding residues in LTCCs. Top view along the axis of the ion pore (P). The transmembrane helixes in each repeat and portion of their connecting linkers (not shown) line the pore region (P). The location of drug-binding domains for DHPs, PAAs and BTZs is drawn according to the distribution of aminoacid residues important for drug sensitivity. c) DHPs appear to interact with S5–S6 regions in domain III and S6 in domain IV.

1.3. Calcium

Ca^{2+} is a very versatile second messenger. This ion, is involved in the regulation of diverse functions such as fertilization, electrical signaling, contraction, secretion, memory, gene transcription, and cell death. It has a broad time of action, from microseconds (e.g. exocytosis) to months or even years (e.g. memory processes). At the same time, the actual molecular systems responsible for producing Ca^{2+} signaling events are limited to very few protein families (Ca^{2+} channels and transporters), and these systems appear to be very well conserved and ubiquitously expressed within all kinds of cells. Most importantly, all of these systems are regulated by Ca^{2+} itself, thus making it a very robust, albeit versatile and adaptable messenger.[19]

In the cardiovascular system, most specifically in cardiomyocytes, Ca^{2+} is essential in a variety of processes, of which the most relevant to this thesis are: a) electrophysiology [via ion currents and the mediation of action potential (AP) shape, arrhythmogenic mechanisms; *b*) excitation-contraction coupling (ECC), which governs the Ca^{2+} transient that drives contraction; *c*) contraction itself, in which Ca^{2+} is the activating switch of the myofilaments and a modulator of key contractile properties (e.g., cooperativity, length-dependent activation, and frequency dependent acceleration of relaxation) and *d*) energy consumption (by contraction and Ca^{2+} transport) and production (via the regulation of mitochondrial ATP production).[20]

Excitation-contraction coupling (ECC) in cardiomyocytes is the process by which depolarization of the cell (action potentials) leads to contraction. LTCC localized in transverse-tubule (T-tubule) membranes are opened in response to depolarization, allowing the influx of extracellular Ca^{2+}. This incoming Ca^{2+} ("trigger Ca^{2+}") binds to ryanodine receptors (RyRs) of the SR and causes Ca^{2+} release, giving rise to calcium induced calcium release (CICR).[21,22] In more detail, CICR starts following Ca^{2+} diffusion across a small sub-membrane dyadic space, this influx activates clusters of RyRs controlling ryanodine-sensitive Ca^{2+} release channels in the junctional portion of the sarcoplasmic reticulum (jSR).[23,24] Ca^{2+} ions released from RyR s diffuse through the cytosolic space to the contractile proteins, constitute the peak of Ca^{2+} transients, and bind to troponin C. Ca^{2+} binding to troponin C releases the troponin I-induced inhibition of thin (actin-troponin C-tropomyosin) and thick (myosin) filament interactions,[25] leading to sliding of the filaments (ie,, cardiac contraction). Ultimately, intracellular Ca^{2+} concentration returned to resting levels by combination of: (a) Ca^{2+} buffering in the dyadic space and cytosol; (b) sequestration of Ca^{2+} by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-type calcium pumps lining the longitudinal portion of the sarcoplasmic reticulum (LSR); and (c) Ca^{2+} extrusion from the cytosol by Na^{+}/ Ca^{2+} exchangers and Ca^{2+}-ATPase pumps on the sarcolemmal membrane. CICR in cardiac muscle exhibits both graded behavior and a high gain. Graded behavior refers to the observation

that SR Ca^{2+} release is proportional to the influx of trigger Ca^{2+}, whereas high gain indicates that the SL trigger current elicits a high SR Ca^{2+} release flux.[26]

1.4. Calcium channel blockers

Calcium channel blockers (**CCBs**) are a class of drugs that disrupt calcium (Ca^{2+}) entry through calcium channels. They have effects on many excitable cells, such as cardiac muscle, skeletal muscle and vascular smooth muscle among others.[27] The most widespread clinical usage of calcium channel blockers is to decrease blood pressure in patients with hypertension, with particular efficacy in treating elderly patients. Also, **CCBs** frequently are used to control heart rate and reduce chest pain due to angina pectoris.[28]

The majority of **CCBs** decrease the force of contraction of the myocardium. This is known as the **negative inotropic** effect[29] plus they also slow down the conduction of electrical activity within the heart, by blocking the calcium channel during the plateau phase of the action potential of the heart. This results in a **negative chronotropic**[30] effect ending up in a lowering of the heart rate and the potential for heart block. The **negative chronotropic** effects of **CCBs** make them commonly used drugs in patients with atrial fibrillation or flutter. Negative chronotropy can be beneficial in that elevated heart rate can result in significantly higher 'cardiac work', which can result in anginal symptoms: lower heart rates represent lower cardiac oxygen requirements.[31]

The concept of Ca^{2+} antagonism was first developed by Fleckenstein who, demonstrated that verapamil and prenylamine produced effective electromechanical uncoupling in the heart and this effect was mimicked by Ca^{2+} removal and this uncoupling could be overcome by increasing extracellular Ca^{2} concentrations. Almost simultaneously, Godfraind & Polsteri noted the effects of two others **CCBs** on excitation– contraction coupling. Fleckenstein continued using verapamil as a tool and these initial pharmacologic studies led to the discovery of the “calcium antagonists” in 1968. Fleckenstein confirmed that nifedipine was indeed not only a Ca^{2+} antagonist and led to the introduction of dihydropyridines.[32] The novel CCBs were not only able to block LTCCs but also possess some other pharmacological properties such as antioxidant.

One of the most puzzling characteristics of the Ca^{2+} antagonists is their chemical heterogeneity.[33,35] The currently available CCB drugs are subdivided into three groups, on the basis of their chemistry. These are the phenylalkylamines (PAA) where verapamil is the prototype, containing two benzene cycles connected by long carbohydrate chain with one nitrogen atom; the dihydropyridines (DHP), including nitrendipine, which contains one DHP and one benzene and the benzothiazepines (BTZ), including diltiazem, that contains two benzenes linked by nitrogen and sulfur atoms in thiazepine structure.[34, 35] See **Fig 3** for details

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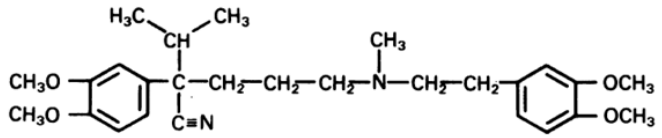
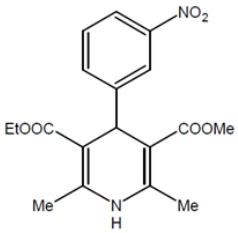
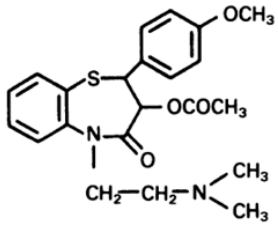


VERAPAMIL



NITRENDIPINE



DILTIAZEM

Figure 3. Chemical formulae of three main calcium channel antagonists.

1.4.1. Dihydropyridines

DHPs are still the most potent group of calcium channel blocker and many efforts have been done to prepare additional cardioselective compounds. DHPs have a broad range of pharmacological actions such as vasodilation, bronchodilation, antiatherosclerosis, antioxidant and anticonvulsant effects. DHP analogs have been synthesized and several second generation commercial products have appeared on the market with superior bioavailability and a slower onset and longer duration of action. Such compounds include nimodipine, nisoldipine and a third generation which not only encompasses calcium channel blocking activity but also antioxidant, among third generation DHPs are amlodipine, felodipine, isradipine, manidipine, nicardipine and nilvadipine.[36] DHPs of the nifedipine type are flexible molecules, in which the C-4-aryl moiety and the C-3 and C-5 ester substituents can rotate and the conformation of the 1,4-DHP ring can change.[37-41] SAR studies of DHP derivatives show that the following structural features are important for their Ca^{2+}-channel-blocking activity:

- The nature and position of C-4-aryl ring substituents optimizes activity. Phenyl is preferred, owing to animal toxicity observed with heteroaromatic rings. The pseudoaxial conformation of C-4 aryl ring is also important.[42,43]

- The 1,4-DHP ring is essential for activity because it is necessary for hydrogen bonding. Substitution at the N-1 position or the use of oxidized (piperidine) or reduced (pyridine) ring systems greatly decrease or abolish activity. Nifedipine and related analogs exist in a boat conformation.

- C-3 and C-5 substituents modulate activity and tissue selectivity.[44,45,46] Asymmetrical substituents in C-3 and C-5 alter the activity.[47,48] In fact, the electronic features of the oxygen of the carboxyl ester group influenced biological activity. Carbonyl oxygen participates in hydrogen bonding with the receptor.[49] Molecular orbital conformational calculations suggest that in DHPs, both carboxy groups are preferentially oriented in a plane that intersects the plane of the DHP ring with an angle of between 30º and 60º.[49,50] In addition, it has been proposed that based on the orientation of the individual carbonyl groups of the C-3 and C-5 ester substituents with respect to the DHP ring double bond, three different conformations are, in principle, possible for the ester groups: trans/trans, in which the carbonyl groups of the both esters are trans to the double bond of DHP (Fig. 4a); cis/cis, with a cis arrangement of both carbonyl groups (Fig 4c); and enantiomeric cis/trans and trans/cis arrangements (Fig. 4c); and enantiomeric cis/trans and trans/cis arrangements (Fig. 4b).[40,47] X-ray structural investigations, theoretical calculations and in

vitro analyses of fused 1,4-DHPs (compounds with an immobilized ester group) indicate that at least one ester must be in the cis arrangement, which is necessary for hydrogen bonding to the receptor.[40,47] It is also suggested that synperiplanar carbonyl groups might be a common feature of DHP calcium-channel antagonists and that an antiperiplanar carbonyl group, such as the lactone group in the rigid compounds, might be a requirement for calcium-channel agonist activity.[43,47]

- When the esters at C-3 and C-5 are nonidentical, C-4 carbon becomes chiral and stereoselectivity between enantiomers is observed.[49,50] The DHP-class of compounds has been the aim of many quantitative structure–activity relationship (QSAR) studies to find the most important quantitative parameter for optimal activity of these compounds. Hansch analysis method has been applied to a series of 4-phenyl-substituted DHPs and, according to this method, the biological activity of DHPs is dependent on the lipophilic, as well as the electronic and steric properties, of the substituents on 4-phenyl DHP analogs of nifedipine.[51] The same group also found that aryl ring substituents exert significant effects both on binding and on pharmacological activity. *Para* substitutions in the 4-phenyl ring lead to activity loss regardless of substituent type. 3D QSAR study (comparative molecular field analysis and comparative molecular similarity studies) of 4-phenyl-substituted DHPs indicates unfavorable steric interactions for any moiety in the para-position of the phenyl ring and that electron withdrawing substituents are favorable in *ortho*- and *meta*-positions.[51,52,53]

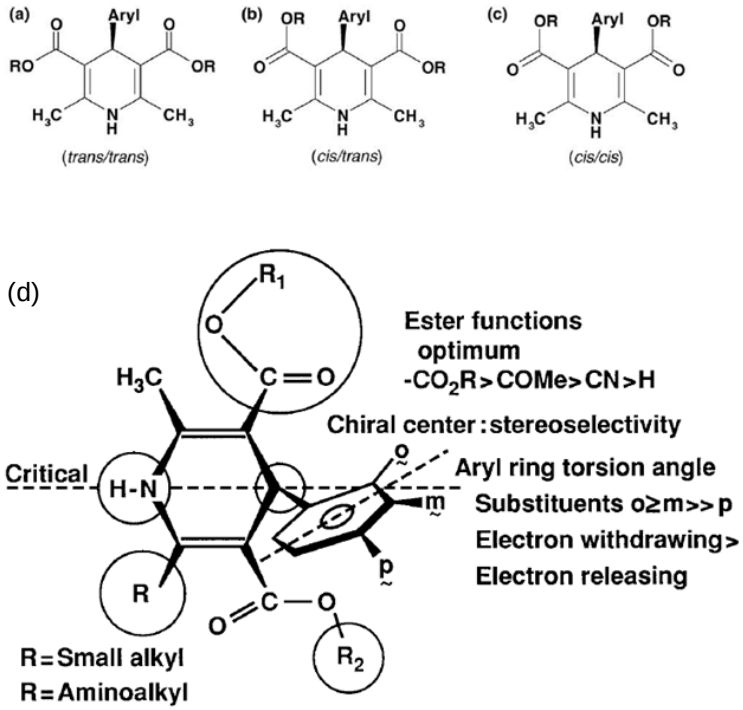


Figure 4. Structure activity relationship aspects of DHPs.
A-C: representation of different conformation of esters groups as determined by X ray analysis; D, SAR for LTCC blocking 1,4 dihydropyridines.

1.5. Reactive oxygen species (ROS)

Changes in the redox state of proteins play an important role in many cell functions including immunological host defense, gene transcription, cell metabolism, ionic homeostasis and cell signaling. Intracellular concentrations of redox-active molecules can significantly increase in the heart as a result of activation of specific signaling pathways or the development of certain pathophysiological conditions. Changes in the intracellular redox environment can affect many cellular processes, including the gating properties of ion channels and the activity of ion transporters. Because cardiac contraction is highly dependent on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and $[Ca^{2+}]_i$ regulation, redox modification of Ca^{2+} channels and transporters has a profound effect on cardiac function.[54,55] Key components of cardiac EC coupling such as the SR Ca^{2+} ATPase and LTCC are subject to redox modulation. Redox-dependent activity of ion channels and pumps is directly involved in cardiac pathologies. Significant bursts of reactive oxygen species (ROS) generation occur during reperfusion of the ischemic heart, and changes in the activity of the major components of $[Ca^{2+}]_i$ regulation, such as RyR, Na^+–Ca^{2+} exchange and Ca^{2+} ATPases, are likely to play an important role in ischemia-related Ca^{2+} overload.[54,55] Oxidative stress refers to an imbalance between the production of ROS (including free radicals such as superoxide and non-radicals such as hydrogen peroxide) and endogenous antioxidant defense mechanisms.

Oxygen is the substrate for the generation of reactive species. ROS are produced as a result of the reduction of oxygen or compounds of oxygen with hydrogen or nitrogen. The term ROS is a generalized description for a number of reactive oxygen molecules (e.g. superoxide, hydroxyl radical, hydrogen peroxide) of biological significance.[56,57] Superoxide is produced as a result of a single electron donation to oxygen. Since superoxide does not easily cross lipid membranes, its role may be limited to oxidation of proteins in the organelle in which it is produced. Hydrogen peroxide is also able to oxidise the thiol groups of cysteine residues to form disulphide bonds with either glutathione, nearby cysteine residues or small protein thiols such as thioredoxin.[57]

Cysteine residues are generally thought to be the most likely target of redox or nitrosylation modification in proteins, as free thiols can easily react with oxygen or reactive nitrogen species and can be assisted in forming intramolecular disulfide bonds.[58] For this reason, the location of the cysteine residues is important when considering the mechanism of redox regulation of calcium channels. Voltage gated calcium channels contain many cysteines. LTCCs' α_1 subunit for example, has 48, not all of these will be susceptible to oxidation or reactions, however, as many will already be involved in disulfide bonds. Oxygen could influence ion channels through many pathways, but it has been shown that Ca^{2+} currents are altered by thiol reducing and oxidizing agents [59-62]

demonstrating that free thiols in the protein are likely to be sensitive to the oxidation, and when oxidation does occur, it augments the probability of an increase in LTCC current.

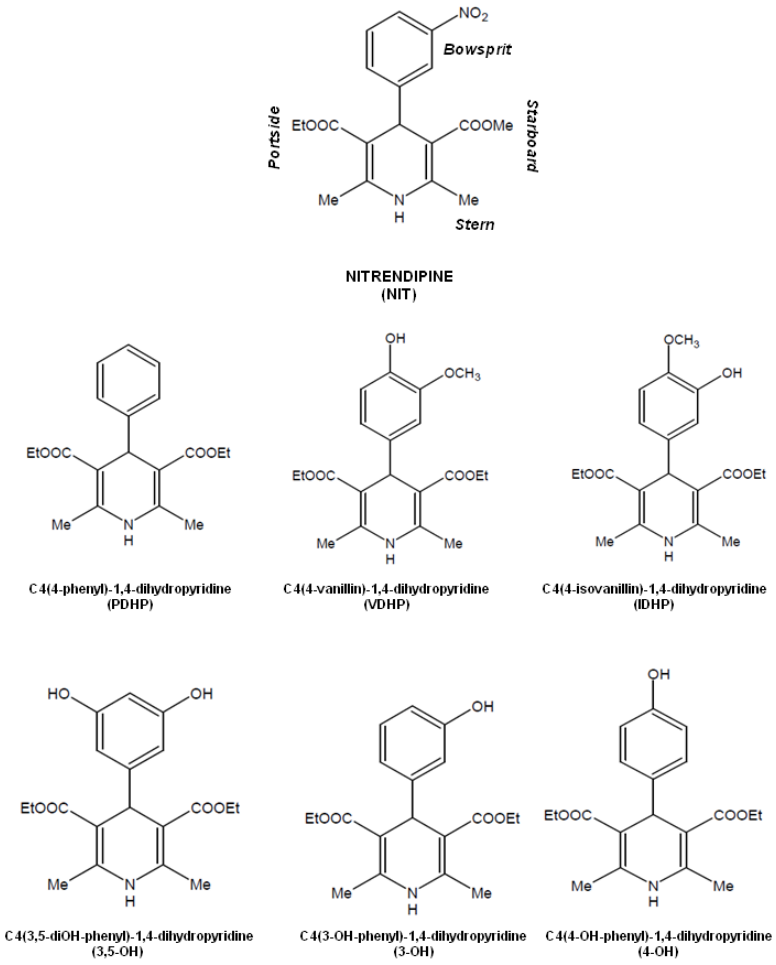


Figure 5. Chemical structures of 1,4-dihydropyridines studied in this thesis.
a) DHP control, with docking studies orientations; b) new synthesized DHPs, product of research done in cell free assays at the Facultad Ciencias Químicas y Farmacéuticas, Universidad de Chile by Dr Núñez-Vergara's group.

Considering the above-discussed antecedents, the following questions appear as fundamental:

- How do these novel substituents (see Fig. 5) affect the impingement on LTCCs?
- Would they allow better interaction with α1C?
- Would they cause these new DHP molecules to be toxic?
- Would they confer antioxidant activity?
- Could they grant the pleiotropic action so much needed for drugs used in cardiovascular pharmacotherapy?

2. WORKING HYPOTHESIS

In order to assess the questions raised above, we propose the following hypothesis:

"The new synthetic 1,4-dihydropyridine molecules are more potent in antioxidant activity than first generation nitrendipine and have similar effects in their calcium channel blocking activity".

3. AIMS

- To investigate the L-type calcium channel blocking activity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 3-OH-DHP; 4-OH-DHP
- To study *in vitro* the antioxidant activity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 4-OH-DHP, 3-OH-DHP.
- To evaluate the toxicity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 4-OH-DHP, 3-OH-DHP

4. MATERIAL AND METHODS

4.1. Materials

From Sigma Chemical Co (St Louis, MO, USA): EDTA; EGTA; butane 2,3 monoxime (BDM), M199 medium, Dulbecco's modified Eagle's medium (DMEM); dihydrorhodamine 1,2,3 (DHR 1,2,3); besides some other biochemical reagents.

From Biological Industries (Kibbutz, Beit Haemek, Israel): fetal bovine serum (FBS), fetal calf serum (FCS), penicillin and streptomycin.

From Invitrogen (Eugene, Oregon, USA): 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), laminin, fluo3-acetoxymethyl ester (Fluo3-AM).

4.2. Animals

Both neonatal and adult rats were bred in the Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). We performed all studies with the approval of the Institutional Bioethical Committee at the Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago. This investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health

4.3. Cell culture

a) Neonatal cardiomyocytes were prepared from hearts of 1–3-day-old Sprague Dawley rats. Cardiomyocytes were isolated and cultured according Foncea et al. Cardiomyocytes were dissociated from the ventricles of 1-2-day-old rat hearts using 0.4 mg/mL collagenase and 0.6 mg/mL pancreatin in 116 mM NaCl, 20 mM HEPES, 0.8 mM Na,HPO, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, (pH 7.35). The cells were resuspended in Dulbecco's modified Eagle's medium 199 (4:1 v/v) supplemented with 10% horse serum, 5% fetal calf serum, and 100 units/mL of both penicillin and streptomycin. Cells were preplated for 2 h on

100-mm culture dishes to deplete fibroblasts. After preplating, cardiomyocytes were centrifuged, and were resuspended in the previously mentioned medium. Cell viability was assessed by the Trypan Blue method. Then cardiomyocytes were plated at the final density required; for the experiments done for antioxidant activity with dihydrorhodamine, they were used in suspension. After 18 h, cardiomyocytes were confluent and beating spontaneously.

b) Adult cardiomyocytes were prepared from hearts of male Sprague Dawley rats (2-3 months, 250-300 g). Animals were anaesthetised by an intraperitoneal injection (i.p.) ketamine:xilazine 2:1. The heart was removed via thoracotomy and transferred to a Gerard ice cold solution. The aorta was cannulated and the heart mounted into a Laggendorf apparatus then successively perfused with the following oxygenated solutions: Gerard buffer (Ca^{2+} 2.6 mM), to allow recovery of spontaneous activity; nominally EGTA (2,5 mM) until contraction ceased and then digestion solution supplemented with collagenase type A and 2,3-butanedione monoxime (BDM), for 30 min. Once flaccid, the heart was rinsed for 2 min without collagenase. Ventricles were removed and finely minced and gently triturated. Then placed in 15 mL of digestion solution, at 37ºC under constant and soft agitation for 10 min; after which the supernatant was transferred to a Falcon tube and centrifuged at 500 rpm for 2 min. The pellet was gently resuspended in a Gerard buffer supplemented with BDM. The cells were then seeded in plates pre-treated with laminin (5 µg/mL). After 20-30 min the buffer was changed and replaced with M199/HEPES/Ca^{2+} 2 mM, supplemented or not, according to the need, with BDM.

4.4. Confocal microscopy

All tests were performed on an inverted confocal microscope (Carl Zeiss Axiovert 200 M-LSM Microsystems).Two parameters were evaluated through this technique, and in accordance to previously reported protocols, cells were labeled with the following fluorochromes: For Ca^{2+}: Fluo 3-AM and for ROS**:** CM-H_2DCFDA

4.4.1. Dynamic *in vivo* Ca^{2+} measurements

Adult rat cardiomyocytes were cultured as described earlier. The cells were used on the same day that the culture was done and only spontaneously beating adult cardiomyocytes were analyzed. Cardiomyocytes were washed three times with Ca^{2+}-containing resting media (Krebs buffer: 145 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose,

pH 7.4) to remove M199 culture medium, and loaded with 5.4 µM Fluo 3-AM (coming from a stock in 20% pluronic acid, DMSO) for 30 min at room temperature. After loading, cardiomyocytes were washed with the same buffer and used immediately thereafter. The cell-containing coverslips were mounted in a 1 mL capacity plastic chamber and placed in the microscope for fluorescence measurements after excitation with a 488-nm wavelength argon laser beam or filter system. Fluorescence measurements were performed on individual cardiomyocytes. Line-scan images were acquired at a sampling rate of 15.8 ms per line, along the longitudinal axis of the cell, always taking care of not crossing the nucleus. An objective lens Plan Apo 63x (numerical aperture 1.4) and a pinhole of 1 Airy unit disc were used. In all acquisitions, the image dimension was 512 x 120 pixels. Intracellular calcium was expressed as a percentage of fluorescence intensity. All line scan images of Ca^{2+} transients were background subtracted. Compounds were added after 30 sec of initiated the recordings. Time zero (0 s) was defined at the point in which the compound was added and the action peak at 180 s.

4.4.2. ROS measurements

Cultured neonatal cardiomyocytes were loaded with ROS-sensitive fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was used to monitor ROS formation. Cells were washed three times with Ca^{2+}-containing resting media (Krebs buffer:145 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose, pH 7.4), to remove culture medium, cells were then loaded with 10 µM CM-H_2DCFDA (coming from a stock in 20% pluronic acid, DMSO), for 10 min at 37°C together with the compounds being assayed, all the compounds were tested at a 10 µM concentration. After this incubation period, cells were washed three times with the same buffer, then placed on a chamber, upon which we took a picture, then added H_2O_2 50 µM and 3 min later a picture was taken again. Fluorescence images were recorded with a 63x oil immersion lens (numerical aperture 1.4). Frame imaging modes were used for measuring ROS; the image dimension was 512 x 120 pixels. CM-H_2DCFDA was excited by 488 nm laser and the fluorescence was collected at 515 nm.

4.5. Docking studies

4.5.1. Homology model

We used for this study the LTCC channel model, a kind gift of Dr. Zhorov. This model contains transmembrane segments S5 and S6 and P-loops from the four repeats. All segments far from the DHP binding site, were not included in the model. Among available x-ray structures that could serve as templates for modeling the open state CaV1.2, we have chosen KvAP because both channels are voltage-gated. The closed state of CaV1.2 was modeled using the KcsA structure as a template. The selectivity filter region was built using the $Na_V1.4$ P-loop domain model as a template. We designate residues using labels, which are universal for P-loop channels. A residue label includes the domain (repeat) number (1 to 4), segment type (*p*, P-loop; *i*, the inner helix; *o*, the outer helix), and relative number of the residue in the segment. The energy expression included van der Waals, electrostatic, H-bonding, hydration, and torsion components, as well as the energy of deformation of bond angles in DHPs. The bond angles of the protein were kept rigid. The hydration energy was calculated by the implicit solvent method. Nonbonded interactions were calculated using the AMBER force field version, which is consistent with the implicit solvent approach. The energy was minimized in the space of generalized coordinates, which include all torsion angles, bond angles of the ligand, positions of ions, and positions and orientations of root atoms of ligands. The channel models and their complexes with ligands were optimized by the Monte Carlo minimization (MCM) method. To ensure similar backbone geometry with the x-ray template, C^{α} atoms of the model were pinned. The proposed specific interactions between ligands and DHP-sensing residues were imposed by distance constraints. Thus, DHP-LTCC H-bonds were imposed with distance constraints that allow penalty-free separation of the H-bonding atoms up to 2.2 Å. The complexes MC minimized with the distance constraints were refined in unconstrained MCM trajectories to ensure that the proposed models are energetically stable. All calculations were performed with the ZMM program.

4.5.2. Docking analysis

Molecular docking of DHPs at the LTCC homology model was investigated using the Lamarckian genetic algorithm search method as implemented in AutoDock. The receptor was kept rigid, while full flexibility was allowed for the ligands so as to translate/rotate. Polar hydrogens were added to the receptors and Kollman-united atom partial charges along with atomic solvation parameters were assigned to the individual protein atoms. The three-dimensional structures of each ligand were generated using the GaussView 98 program. Ligands were then energy minimized

using Gaussian98 software. For each ligand, a rigid root and rotatable bonds were assigned automatically. The non-polar hydrogens were removed and the partial charges from these were added to the carbon (Gasteiger charges). The atom type for aromatic carbons was reassigned in order to use the AutoDock 4.0 aromatic carbon grid map. Docking was carried out using 60 × 60 × 60 grid points with a default spacing of 0.375 Å. The grid was positioned to include the full ligand binding site in the central part of the α1 subunit so as to allow extensive sampling around residue Y^{216}. Within this grid, the Lamarckian genetic search algorithm was used with a population size of 150 individuals, calculated using 200 different runs (*i.e.* 200 dockings). Each run had two stop criteria, a maximum of 1.5×10^6 energy evaluations or a maximum of 50,000 generations, starting from a random position and conformation; default parameters were used for the Lamarckian genetic algorithm search.

4.6. Patch clamp

Patch clamp experiments were performed on the whole cell mode and on isolated adult rat cardiomyocytes. Holding potential was set at -80 *mV*. Single cell voltage clamp recordings were obtained with an Axopatch 1D patch clamp amplifier. Glass micropipettes (G85150T-3 Warner Instruments) with a tip resistance in the range of 1 to 3 MOhms were obtained with a pipette puller and fire polished with a microforge. Once a seal was formed between the pipette tip and the cell membrane, the whole-cell patch configuration was obtained by gentle suction applied to the inside of the pipette. The rupture of the section of membrane sealed against the tip was monitored by a 2 mV test pulse and verified by the increase in the transient capacitive current trace. The membrane capacity was obtained from the integration of the current transient obtained for a -10 mV pulse. The pipette ionic composition was (in mM): Cs-methane sulphonate 150, EGTA 5, $MgCl_2$ 5, Hepes 10 adjusted to pH 7.3 with CsOH. The external saline composition was (in mM): TEA-Cl 140, $MgCl_2$ 2, $CaCl_2$ 1.5, glucose 10 and HEPES 10. Intracellular solution (in mM): Cs-methanesulfonate 150, EGTA 5, HEPES 10 and $MgCl_2$ 5 adjusted to pH 7.3 with TEA-OH.

Voltage clamp stimulus protocol: The pClamp suite of programs was used. Clampex 5.5 was used to generate the command voltage protocol as family of 16 pulses of 50 ms duration and stepped from 10 mV above the holding potential (usually -80 mV) with an 8 mV increment between them. In this manner, the protocol voltage range covered from -70 to +58 mV. The net current at each voltage step was obtained after the cancellation of the linear components of the capacity and conductance by subtraction of 6 pulses of the inverse polarity and 1/6 of the amplitude (P/6 protocol).

Data acquisition: the family of the net current signals for a protocol run was filtered at 5 KHz and digitized at 15 KHz. Data generated was stored for off-line analysis.

Analysis of membrane current from voltage clamp recordings: The net inward current obtained during the pulse protocol was considered to be carried mostly by calcium ions due to ionic composition of the extra and intracellular solutions. With the Clampfit program, the peak magnitude of this current was tabulated to generate I-V curves after being normalized for membrane capacity (nA/nF). The value of the peak current obtained at 20 mV showed throughout the records to be the largest. Because each cell studied has control and test values, all the data can be normalized and grouped. The grouped data was adjusted to a single site dose-response curve.

4.7. Flow cytometry

All tests were performed on a flow cytometer: FACSCanto 2 Laser, 488nm y 633nm; six colors. Two parameters were evaluated through this technique, and in accordance to previously reported protocols, cells were labeled with the following fluorochromes: for cell viability: PI and for ROS: DHR 1,2,3

4.7.1. Cell toxicity

To study toxic effects of the compounds, the number of viable cells was assessed by the PI test. Neonatal rat cardiomyocytes were exposed to 0.1; 1 and 10 µM of each one of the compounds for 24 h; this assay also included hydrogen peroxide (H_2O_2) as a positive control and the vehicle (DMSO). After that time, cells were trypsinized and resuspended in PBS, 10-20 sec before data acquisition PI was added. Levels of PI incorporation were quantified. Cell size was evaluated by forward-angle light scattering (FSC). PI negative cells of normal size were considered alive.

4.7.2. ROS measurements

To study the antioxidant capacity of our different DHPs, we used DHR 1,2,3, (25 µM); a probe to study various species of ROS. Neonatal rat cardiomyocytes were used in suspension, in a density of 3 x 10^5/ mL, then they were centrifuged at 1000 rpm for 5 min, the cultured medium was withdrawn and replaced with either a control (Krebs-Ringer:145 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose, pH 7.4) or Krebs plus the compounds. After 30 min, the cells were centrifuged again and the supernatant medium replaced with Krebs

containing H_2O_2 (100 µM) for 1 h, 30 min after these stimuli, the probe was added and the whole mixture was incubated for the remaining time.

5. RESULTS

5.1. AIM 1. *To investigate the L-type calcium channel blocking activity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 3-OH-DHP; 4-OH-DHP*

We had six new DHP compounds available to be tested. Their novelty was that new substitutions never described before were introduced in the aryl ring in C4 (see Fig. 5). In our Faculty, Dr. Luis Núñez Vergara's group has been working extensively in the last decade in synthesizing new DHPs that would have two pharmacological activities: Ca^{2+} channel blocking and antioxidant, but the main goal of this research group was their antioxidant capacity.[70-72]

Some of these new DHPs (PDHP, VDHP, IDHP and 3,5-OH-DHP) have shown *in vitro* an antioxidant capacity in chemical assays.[70.90] However both their antioxidant and LTCC blocking activities *in vivo* have not already been tested. So the next step of this research was to study these DHPs using cultured cardiomyocytes. This model was chosen because of its critical involvement in the development of cardiovascular diseases and cardiomyocytes, especially ventricles, have a rich population of LTCC channels; which are the target of DHPs. This and the fact that DHPs also have a high affinity for cardiac tissue[89] made our model a reasonable choice for the proposed research. The first step was to test the new DHP's Ca^{2+} antagonism, the activity DHP are recognized for in therapeutics.

We approached this aim in three different ways: two practical and another theoretical. First we assayed the new compounds and NIT on adult rat cardiomyocytes through a calcium-fluorescent dye sensor and confocal microscopy, second through patch clamp and the theoretical approach was docking studies to help understand our results *in vivo*.

The LTCC blocking efficacy was evaluated through visualization of Ca^{2+} dynamics by confocal microscopy and patch clamp. Pharmacology parameters of new DHPs were measured through two different actions, first the effect on inotropy (fluorescence intensity) and second, chronotropy (frequency of contraction) in microscopy and for patch clamp through current peak inhibition.

The vehicle (DMSO) was assayed to rule out any possible interference with analysis of the Ca^{2+} blocking activity. We found no interference at all with the outcome of the evaluation performed on the compounds (Figure 6). All compounds were evaluated at three different concentrations, 10, 1 and 0.1 µM.

3-OH-DHP was the only new DHP that shows an inotropic effect at all concentrations (0.1; 1 and 10 µM) as well as being the only to reduce the inotropy and chronotropy at 0.1 µM, the lower concentration; this all comes as a consequence of LTCC blocking. 3-OH-DHP mimicked NIT in all aspects, they share that they did not have a negative chronotropic effect at 1 and 10 µM and the difference between these two compounds was that 3-OH-DHP took a longer time to achieve the inotropic effect, Fig 7 and Fig 9. PDHP, when was tested at a higher concentration blocked the channel, which proved to be effective at 10 µM, but this was only a negative inotropic effect, at this same concentration, the chronotropy remained unaffected, being equal to the control, Fig. 6. Aryl para substituted compounds were not Ca^{2+} antagonists at all, they had no inotropic or chronotropic effect, Figs 11-13, at any concentration tested; these findings are all in agreement with what has already been stated in literature, that, any para-substitution the in aryl ring in C4, regardless of its nature, either abolishes or diminishes to the minimum the Ca^{2+} blocking activity. This also further substantiates our docking studies in which they were not able to reach the binding pocket of the channel, thus having no Ca^{2+} antagonism.

However the most interesting finding was that for the disubstituted homologue of 3-OH-DHP, the 3,5–OH-DHP compound. This molecule was not effective blocking LTCC; not even at the highest concentration there was the slightest hint that this new DHP affected any of the parameters being evaluated. As for the lower concentrations, there was no effect either (Fig 10).

In patch clamp, only 3-OH-DHP was managed to be tested and at concentrations lower than 0.1 µM: This preliminary result shows LTCC blockage below the mentioned concentration, which can be associated with a powerful Ca^{2+} antagonist.

To clarify matters further, docking studies were performed in collaboration with Dr. Gerald Zapata from the Departamento de Química Inorganica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile. These studies were also supported by Dr. Boris Zhorov, Department of Biochemistry and Biomedical Sciences, Mc Master University, Hamilton, Ontario, Canada, whom kindly shared his LTCC model with us. This model includes only the segments of DHPs binding site and those far from it are all excluded. We first had to set our system with that one of Dr. Zhorov, who proposed an orientation for DHPs in his LTCC model. This was done with nimodipine. We set our system with this molecule then we ran NIT and they followed the same orientation inside the binding pocket and having the same critical aminoacid interactions (M4i12, Y4i11 and Y3i10); meaning that our system was fine and ready to use for the rest of the compounds.

As it was described above, DHPs are the most potent CCB of the calcium antagonist families. The SAR questions related to the new DHPs were addressed through theoretical chemistry using docking studies for all molecules. These results showed that *para* substituted DHPs do not get inside the binding pocket thus not reaching its target, which would explain their lack of Ca^{2+} antagonism. However, PDHP and 3,5-OH-DHP manage to get inside the binding pocket (Figure 14), the former had blocking effect only at 10 µM (Figure 8), the latter had no antagonism in our confocal microscopy experiments (Figure 10). This was a very interesting finding, which raised these two questions:

- If 3,5-OH-DHP reaches the binding pocket why does not it exert a LTCC blocking effect and

- PDHP proved to be effective blocking the channel at its highest concentration but not at 1 and 0.1 µM, would this be related to some kind of interaction with the binding pocket.

As general overlook on our results we found out that only two of our compounds, had an effect on reducing inotropy; and that one of these two DHPs reduced the chronotropy. The remaining compounds lacked this blocking activity (See Figures 7-13).

The results showed that:

- Only 3-OH-DHP had channel blocking activity at all concentrations.

- PDHP, which is called the skeleton for not having any substituent in the aryl ring in C4, exhibited an inotropic effect but not a chronotropic effect when assayed at a 10 µM, the highest concentration; when used at lower concentrations this effect disappeared.

- A DHP disubstituted in both sides of the aryl ring in C4 in 3,5-OH-DHP; the disubstituted version of 3-OH-DHP, did not show a blocking effect, contrarily to expected.

- NIT evidenced faster and perhaps stronger blocking activity than either PDHP or 3-OH-DHP.

- NIT had a chronotropic effect at 0.1 µM, from our compounds only 3-OH-DHP reduced the frequency of contraction at the same concentration of NIT.

- NIT and 3-OH-DHP did not exhibit this negative chronotropic effect at concentrations above the one described earlier.

- All DHPs with a substituent on para position of the aryl ring in C4 had no blocking activity at any of the concentrations tested, as it was predicted by our docking studies.

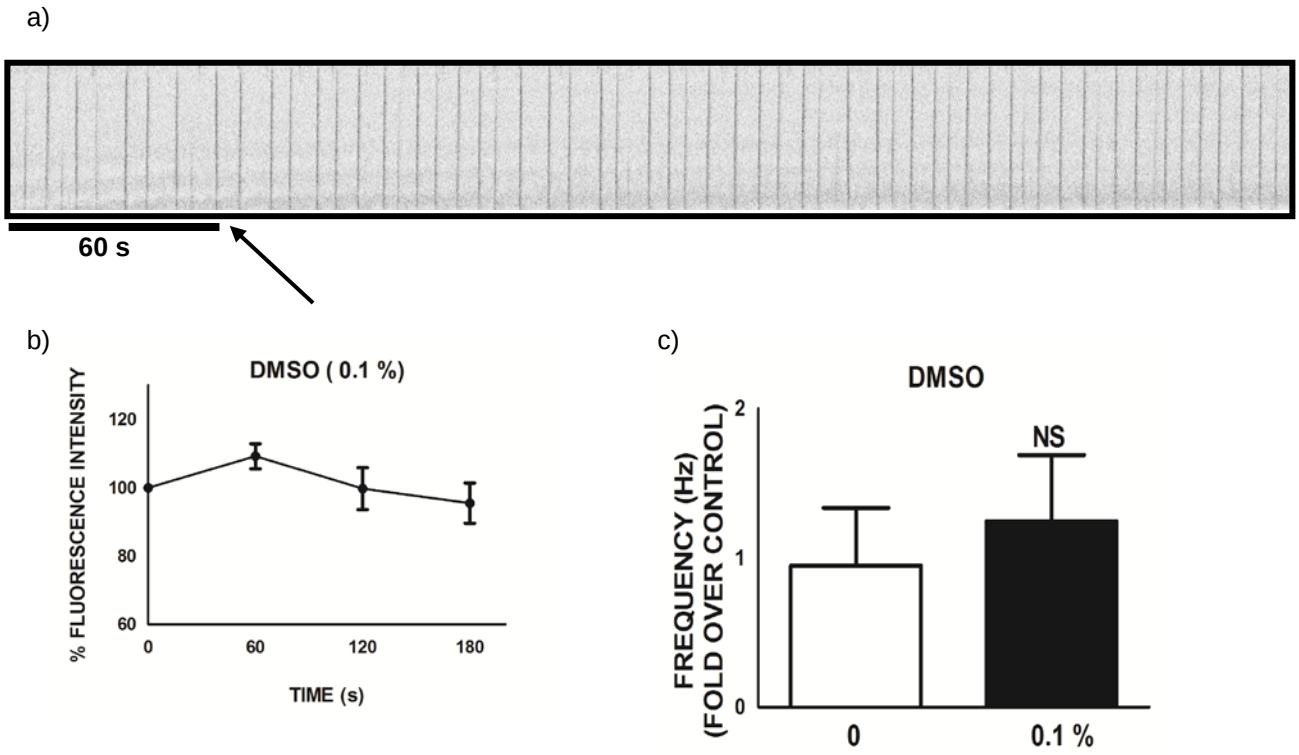
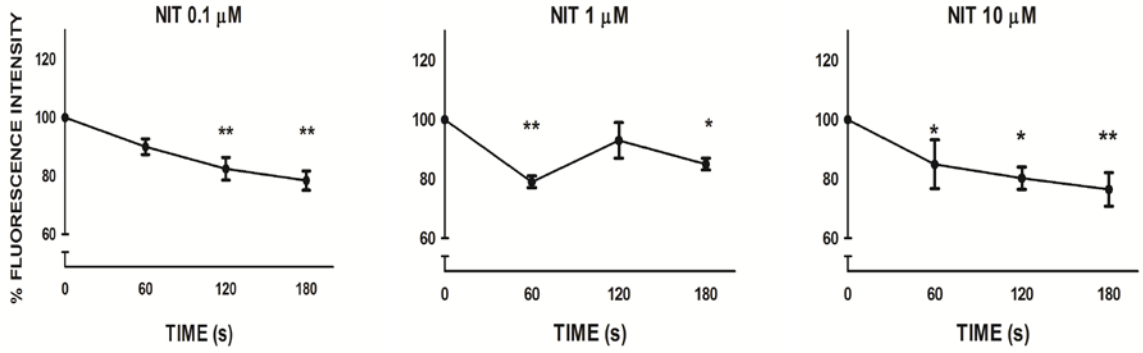


Figure 6. Effect of DMSO on basal calcium oscillations in isolated adult cardiomyocytes. Graphical statistical representation of the experiments conducted on line scan mode for the vehicle, DMSO. All results are presented as means ± SEM (n=3). Treatment was analyzed by one-way ANOVA with *post-hoc* examination by Bonferroni and it was considered statistically significant if $P < 0.05$. For the frequency chart t-student test was performed. NS: no statistical significant. a) Representative line scan. b) Percentage of fluorescence intensity. c) Frequency chart. 0: time in which the compound is added and 180 s compound's action peak.

A. Representative line scan images

B. Fluorescence intensity



C. Frequency

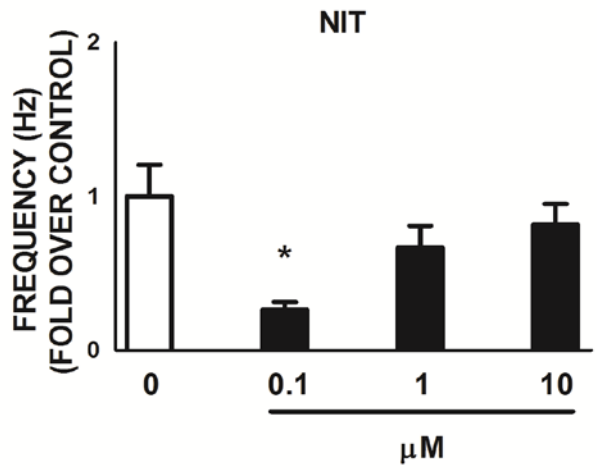
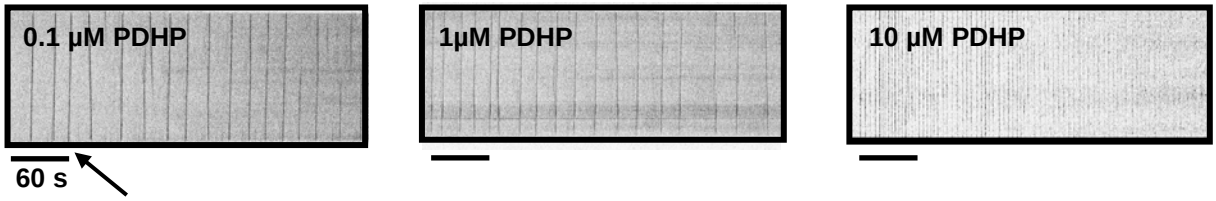
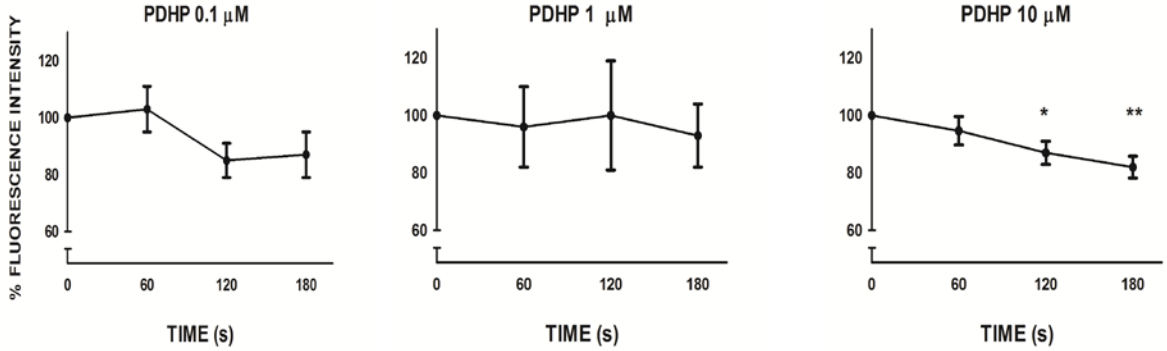


Figure 7. Effect of NIT on basal calcium oscillations in isolated adult cardiomyocytes.
Graphical statistical representation of the experiments conducted on line scan mode for NIT. All results are presented as means ± SEM. (0.1 µM, n=5, 1 µM, n=3; 10 µM, n=5). Treatment was analyzed by one-way ANOVA with post-hoc examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan images



B. Fluorescence intensity



C. Frequency

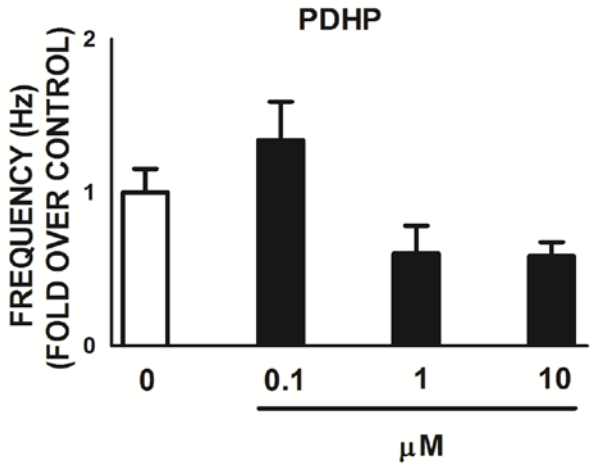
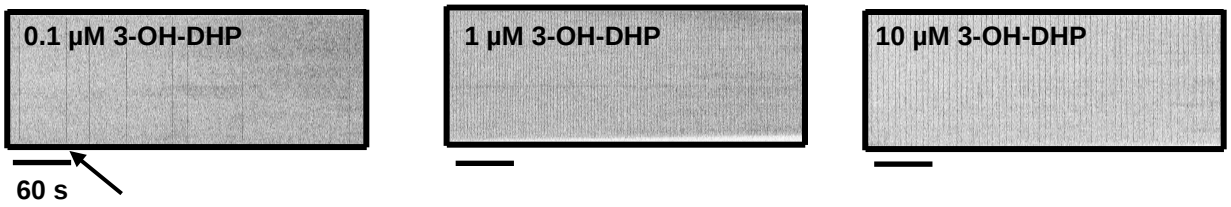
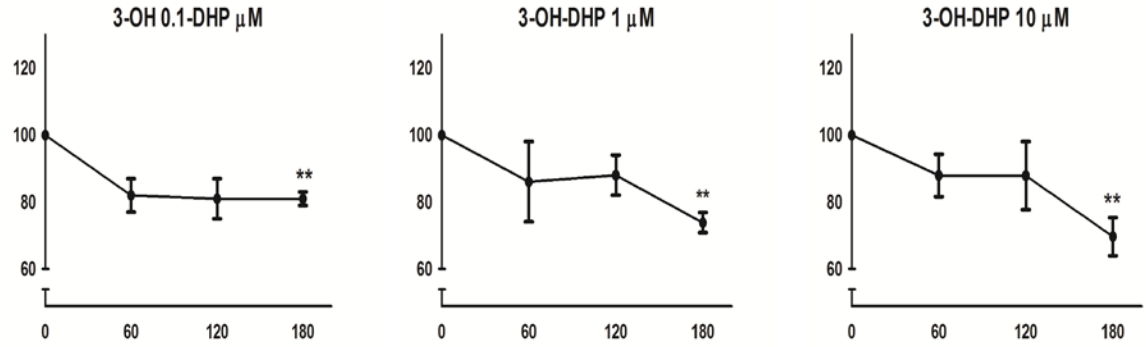


Figure 8. Effect of PDHP on basal calcium oscillations in isolated adult cardiomyocytes.
Graphical statistical representation of the experiments conducted on line scan mode for PDHP. All results are presented as means ± SEM. (n=3). Treatment was analyzed by one-way ANOVA with *post-hoc* examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan images



B. Fluorescence intensity



C. Frequency

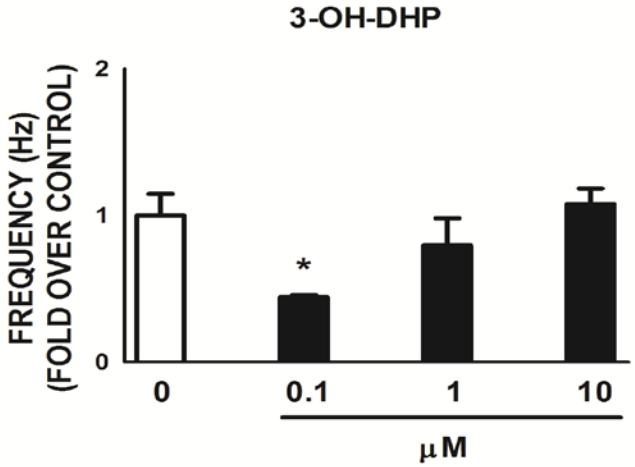
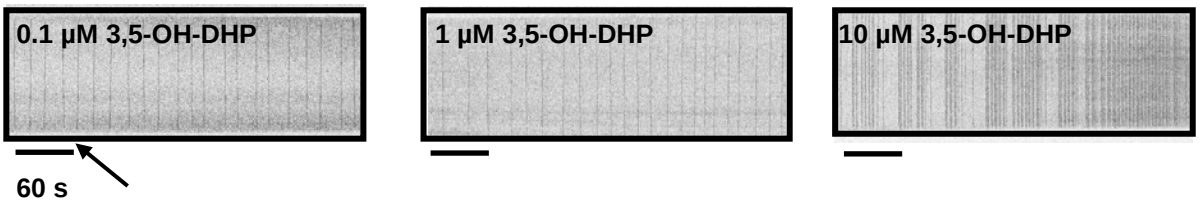
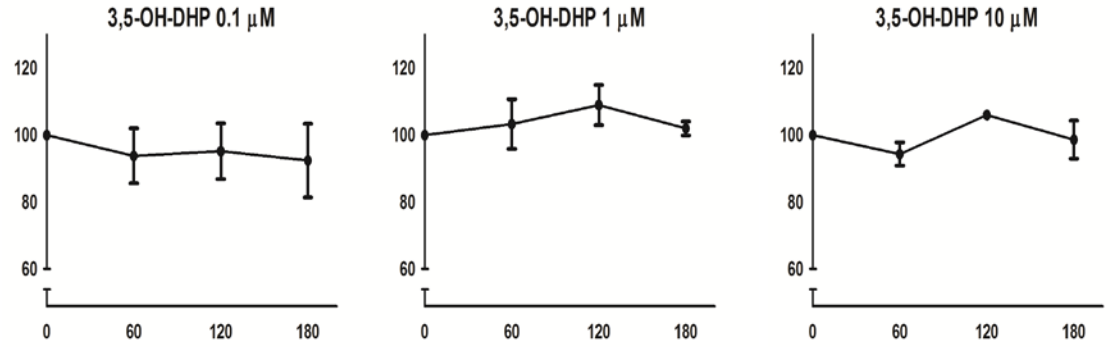


Figure 9. Effect of 3-OH-DHP on basal calcium oscillations in isolated adult cardiomyocytes. Graphical statistical representation of the experiments conducted on line scan mode for 3-OH. All results are presented as means ± SEM. (n=4). Treatment was analyzed by one-way ANOVA with *post-hoc* examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan images



B. Fluorescence intensity



C. Frequency

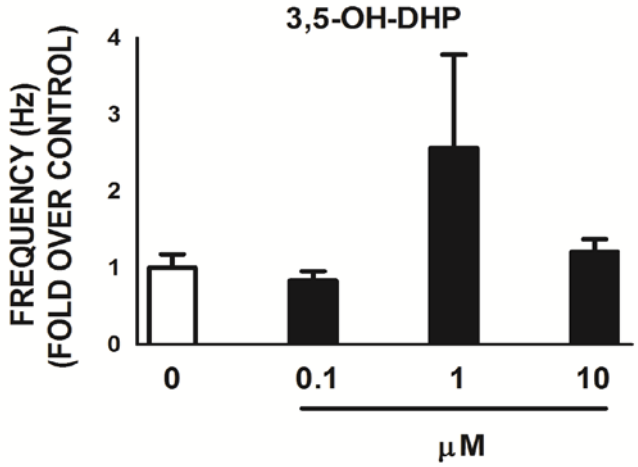
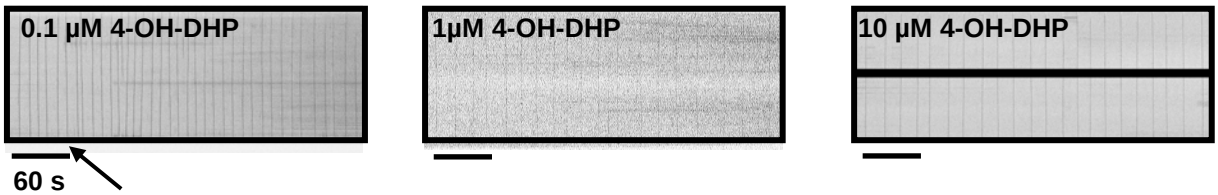


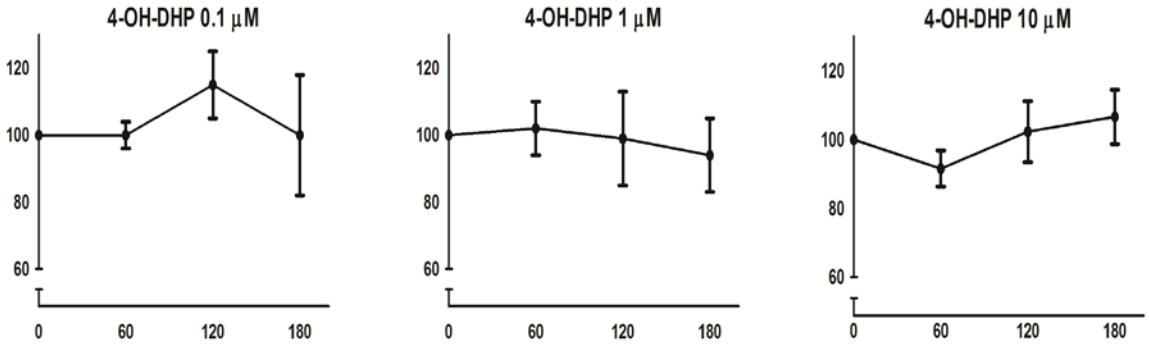
Figure 10. Effect of 3,5-OH-DHP on basal calcium oscillations in isolated adult cardiomyocytes.

Graphical statistical representation of the experiments conducted on line scan mode for 3,5-OH-DHP. All results are presented as means ± SEM. (0.1 µM, n=4; 1-10 µM, n=3). Treatment was analyzed by one-way ANOVA with post-hoc examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan



B. Fluorescence intensity



C. Frequency

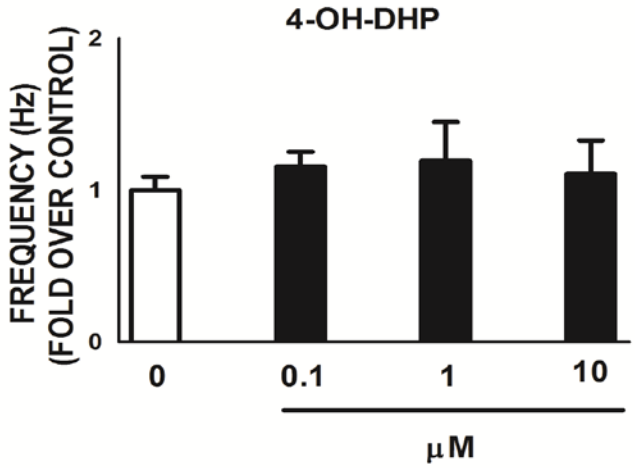
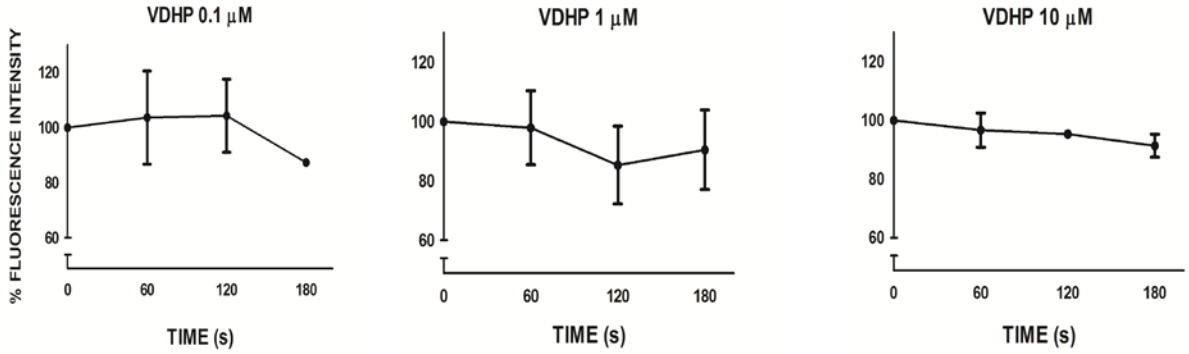


Figure 11. Effect of 4-OH-DHP on basal calcium oscillations in isolated adult cardiomyocytes.

Graphical statistical representation of the experiments conducted on line scan mode for 4-OH-DHP. All results are presented as means ± SEM. (n=4). Treatment was analyzed by one-way ANOVA with post-hoc examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan images

B. Fluorescence



C. Frequency

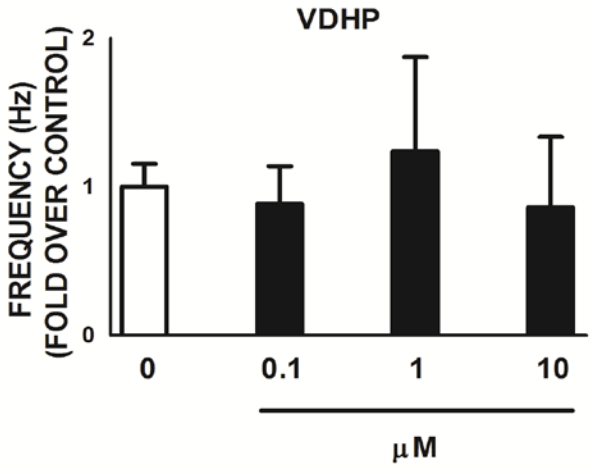
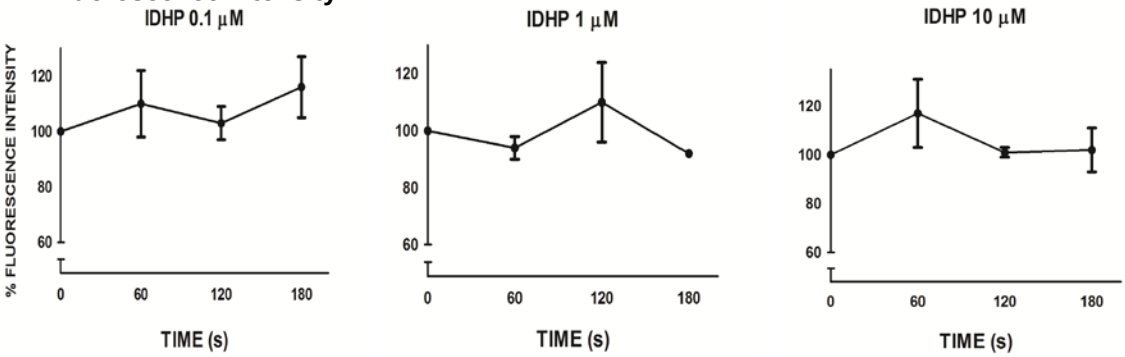


Figure 12. Effect of VDHP on basal calcium oscillations in isolated adult cardiomyocytes. Graphical statistical representation of the experiments conducted on line scan mode for VDHP. All results are presented as means ± SEM. (n=3). Treatment was analyzed by one-way ANOVA with post-hoc examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

A. Representative line scan images

B. Fluorescence intensity



C. Frequency

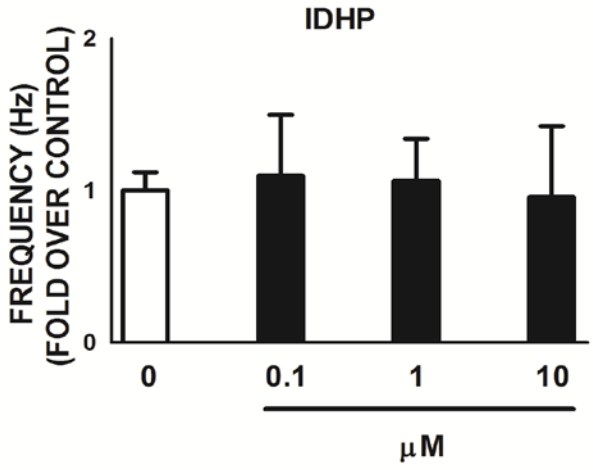


Figure 13. Effect of IDHP on basal calcium oscillations in isolated adult cardiomyocytes.
Graphical statistical representation of the experiments conducted on line scan mode for IDHP. All results are presented as means ± SEM (n=3). Treatment was analyzed by one-way ANOVA with *post-hoc* examination by Bonferroni and it was considered statistically significant if $P < 0.05$. A) Representative line scan. B. Percentage of fluorescence intensity. C. Frequency chart. 0: time in which the compound is added and 180 s compound's action peak. Arrow points the moment when the stimulus was added.

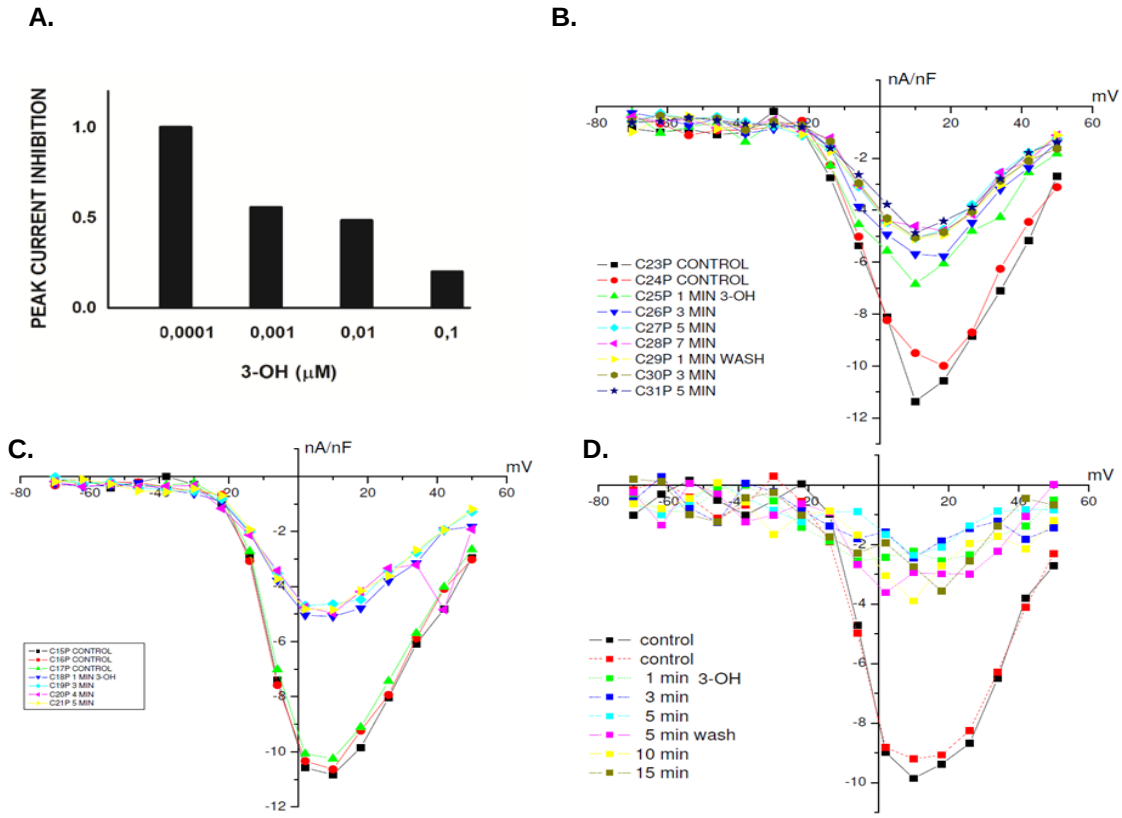
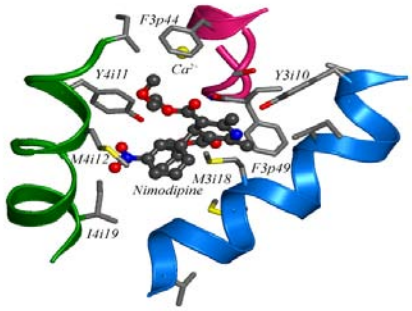
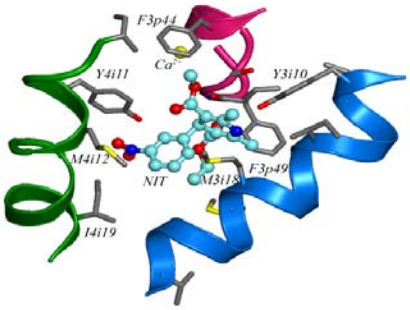


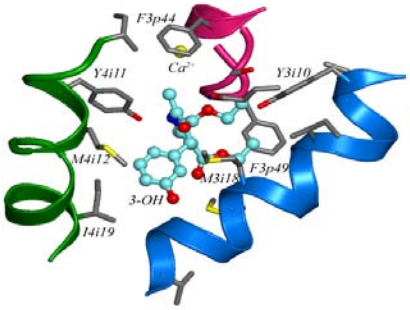
Figure 14. Patch clamp experiment for 3-OH-DHP in cultured cardiac myocytes.
Whole cell patch clamp experiment. A. Quantification of B-D expressed as peak current inhibition. B-D; IV graphs for 0.001; 0.01 and 0.1 µM, respectively.



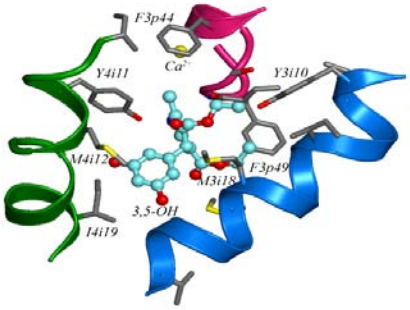
Nimodipine



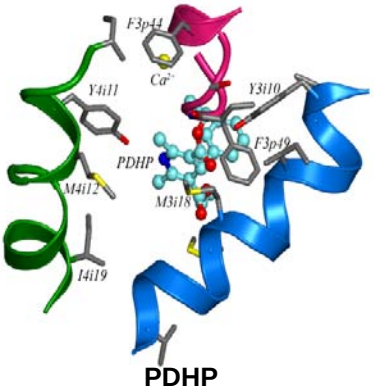
NIT



3-OH-DHP



3,5-OH-DHP



PDHP

Figure 15. Orientation of DHP ligands in the homology model of LTCC.

S6, P, and S5 helices are shown, respectively, as by smooth ribbons, sharp ribbons, and strands. Repeat III is green, repeat IV is purple, and repeats I and II are cyan. Ca^{2+} ions are shown as yellow spheres. Cytoplasmatic and side views of the MC minimized complex. DHP-sensing residues are shown by thin sticks and ligands by thick sticks. Cytoplasmic views with nitrendipine, the stern of nimodipine interacts with Y4i11 so do NIT and 3-OH-DHP and 3,5-OH-DHP and this is not for PDHP. The portside group does not approach the Ca^{2+} ion chelated by the selectivity filter glutamates in repeats III and IV.

5.2. AIM 2. *To study in vitro the antioxidant activity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 4-OH-DHP, 3-OH-DHP*

The increasing relevance of free radicals in the progression of cardiovascular diseases has stimulated the search for a molecule that can conjugate various pharmacological activities. As it was described previously in our Faculty, Dr Núñez Vergara´ group has conducted a very extensive research in developing new DHP molecules, in which the priority was having an antioxidant capacity; this was done successfully in cell free assays. Now, it was necessary to test this capacity in cultured cardiomyocytes.

As cited earlier, three of these new DHPs were tested for antioxidant activity in chemical assays; the compounds tested were: PDHP, VDHP, IDHP and 3,5-OH-DHP[70,90]. Briefly, the reactivity of the compounds towards free radicals was tested using two techniques, voltametry and UV-visible spectrophotometry. These DHPs were reactive to free radicals and were antioxidants, the most active compounds being VDHP and IDHP.

Taking into account these promising findings we set out to test if these results would be the same in cultured neonate rat cardiomyocytes. For this purpose we used two different techniques with different probes and different oxidant stimuli concentration.

Our first assay was done through confocal microscopy, in here we used, CM-H_2DCFDA, a probe that also reacts when there is lipid peroxidation, H_2O_2 (50 µM) and TRO (100 µM) as a positive control. These results are shown in Figure 16. We first evaluated how these new DHPs would act in the absence of oxidant stimuli. We incubated the probe together with the compound for 10 min after washed the coverslips. They were placed into the chamber and measure the fluorescence. As shown in Figure 16A, the results depict that only NIT seem to have an effect on the basal level of ROS, reducing the fluorescence, and VDHP increased the fluorescence.

After the first image was taken, we added H_2O_2 into the chamber, incubated for 3 min, taking the second image then. As depicted in Figure 16B (right panel), in this analysis we included the control in basal conditions and all data were standardized respect control values. This graph does not only show that there is an increase in the fluorescence in the control, it also shows that there is an increment in fluorescence for all DHPs, even for NIT and that the levels of ROS remain always the same

An alternative assay was done by flow cytometry, using the probe DHR 1,2,3. In this assay; H_2O_2 concentration was increased to 100 µM and NAC (3 mM) was used as positive control, instead of TRO. All compounds were tested at 10 µM in both assays. The compounds were added to the cells for 30 min and incubated for 1 h with H_2O_2. During the last 30 min, the DHR probe was added and then the fluorescence was registered.

Our findings shown in Figure 17 correlate with those described in Figure 16. This graph shows that none of the new DHPs had an antioxidant effect in presence of H_2O_2, and that in some cases, such as PDHP, ROS levels raised. Only IDHP slightly decreased the levels of ROS in presence of H_2O_2, but this effect was not significant as compared to NAC.

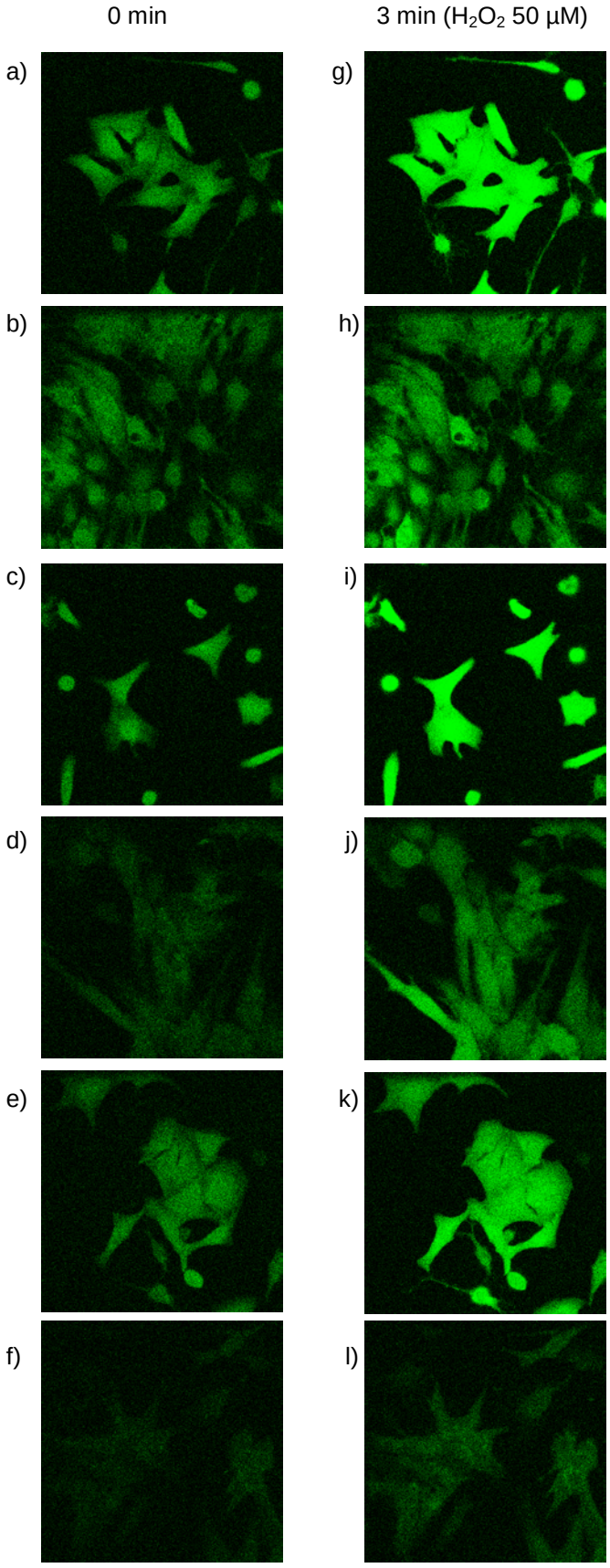


Figure 16**: **Antioxidant activity of some DHPs.

Left: representative confocal fluorescence images of CM-H_2DCFDA (DCF) at baseline and 3 min after exposure to 50 µM H_2O_2; next page (below) fluorescence normalization of the images.

a-g) Control

b-h) NIT

c-i) PDHP

d-j) 3,5-OH-DHP

e-k) VDHP

f-l) TRO

All compounds tested were assayed at 10 µM.

Trolox concentration used 100 µM.

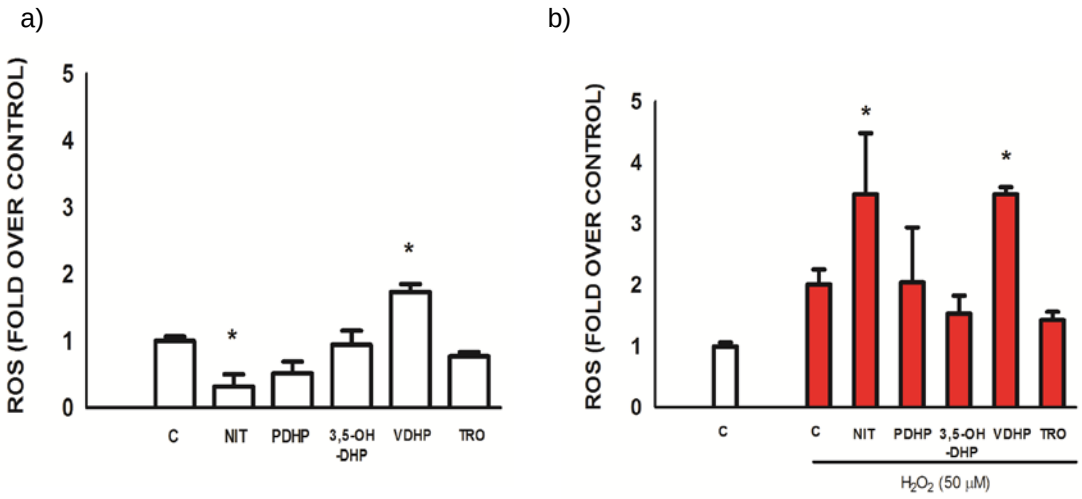


Figure 17*. Assessment of antioxidant activity of DHPs in cultured cardiomyocytes.*
a) ROS basal levels. b) The graph was standardized against the white bar, basal control. Red bars represent same samples in the left panel but H_2O_2 treated. Comparison among different treatment compounds vs control (vehicle) was analyzed by one-way ANOVA with post-hoc examination by Dunnet test. Difference was considered statistically significant if $P < 0.05$. Test was performed separately for baseline and H_2O_2 three min incubation.

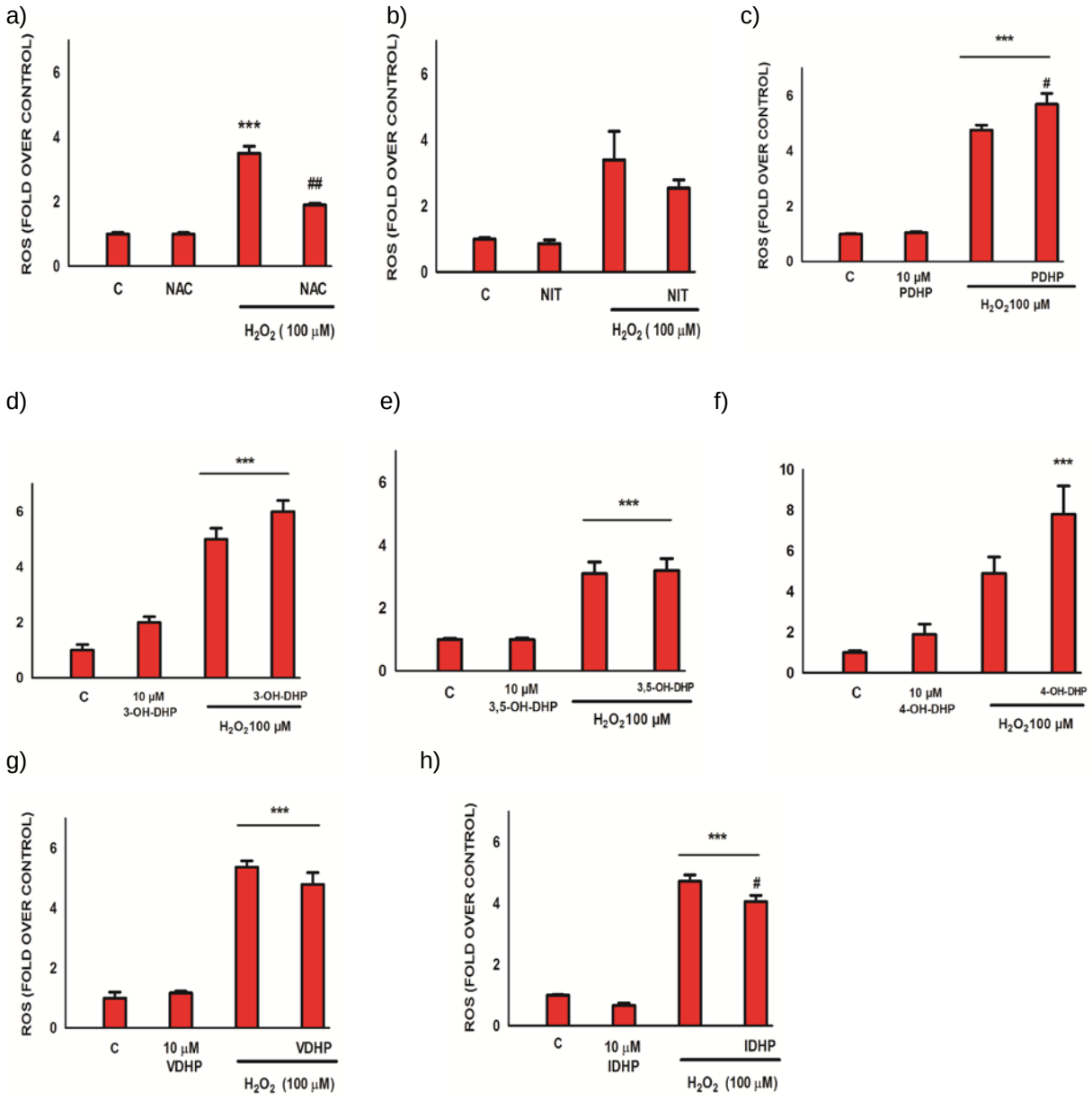


Figure 18. Measurement of antioxidant activity for all DHPs by flow cytometry in cultured cardiomyocytes. NAC was used as a positive control, at 3 mM. Comparison among different treatment compounds was analyzed by one-way ANOVA with post-hoc examination by Bonferroni test, comparing selected pairs of columns, first all points were compared vs. control, but priority was given to the comparison between the oxidant stimulus H2O2 vs. the compound being assayed the compound being assayed plus the oxidant stimuli. Difference was considered statistically significant if $P < 0.05$. * vs control; # vs H_2O_2

5.3. AIM 3. *To evaluate the toxicity of PDHP; VDHP; IDHP, 3,5-OH-DHP; 4-OH-DHP, 3-OH-DHP*

Our last aim was to investigate the toxicities of these new DHP compounds in cultured neonate rat cardiomyocytes. Cell viability was measured as end point. To this end, cells were treated with the compounds (0.1, 1 and 10 µM) for 24 h. These concentrations were the same to those assayed when we tested their channel blocking activity. H_2O_2 (300 mM) was used as a positive cell death control. The cells were incubated with the compounds for the time described above and cell viability was assayed using PI and flow cytometry.

This technique and this probe are widely used and accepted as determinants of cell viability; based on PI exclusion staining for drug toxicity determination: PI is a DNA intercalating dye that is excluded by cells that have their plasma membrane integrity preserved but it enters and stains DNA in cells that have damaged membranes and these are considered dead cells.

Figure 18 shows percentage of live cells, as ones that excluded PI.

Our results show that none of the compounds exhibited relevant toxic effects during the time period tested (24 h). Among these new DHPs tested for cell toxicity, 4-OH-DHP proved to be the safest, because the percentage of death cells in all concentrations was similar to the control, DMSO. NIT and most of the new DHPs have slightly toxic effect at higher concentrations (1 and 10 µM). At 0.1 µM, none of the new DHPs differ from the control except for 3,5-OH--DHP.

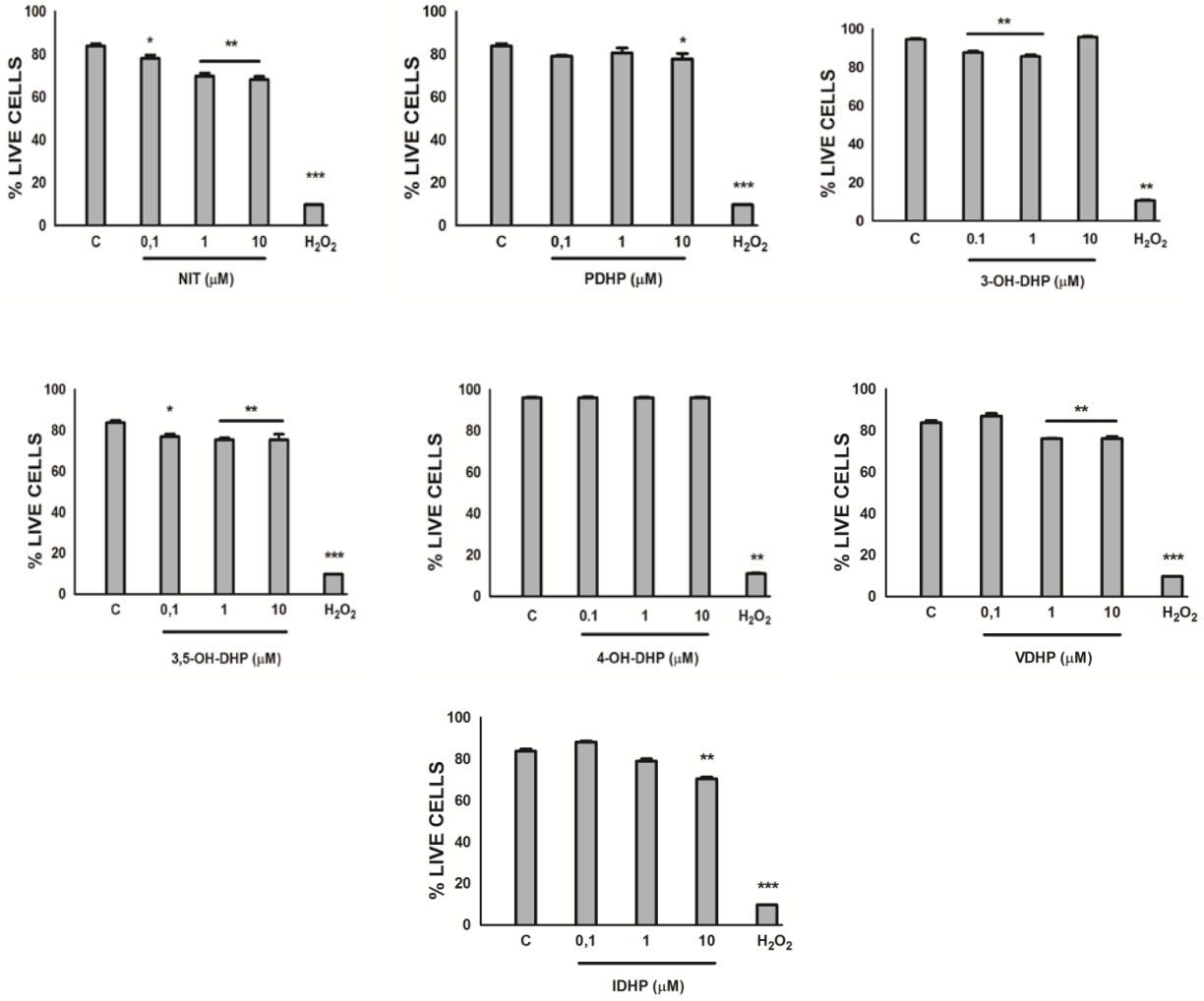


Figure 19. Effect of new synthetic DHPs on cardiomyocyte viability.

A. viability tested for µM range concentration, 24 h incubation; flow cytometry, PI. C: DMSO. Data are mean ± SEM. Comparison among different treatment compounds vs control (vehicle) was analyzed by one-way ANOVA with post-hoc examination by Dunnet test. Difference was considered statistically significant if $P < 0.05$.

6. DISCUSSION

As cited previously in this work DHPs are known for their powerful effect blocking LTCCs; given this effect they are used in the treatment of a wide range of cardiovascular diseases. The research for more DHPs is continuously evolving making them one of the most investigated cardiovascular drugs. In this thesis the Ca^{2+} blocking and antioxidant activities of new synthesized DHPs were evaluated. The main results are described in the following tables:

Table 1. ***DHPs in the binding pocket of LTCC and interaction with selected aminoacid residues***

COMPOUND	BINDING POCKET	AMINO ACID RESIDUES		
		M4i12	Y4i11	Y3i10
NIT	Yes	Yes	Yes	Yes
PDHP	Yes	No	No	Yes
3-OH-DHP	Yes	No	Yes	Yes
3,5-OH-DHP	Yes	Yes	Yes	Yes
4-OH-DH	No	-	-	-
VDHP	No	-	-	-
IDHP	No	-	-	-

Table 2. ***Inotropic and chronotropic effects of new tested DHPs***

COMPOUND	INOTROPY			CHRONOTROPY		
	0.1 µM	1µM	10 µM	0.1 µM	1µM	10 µM
DMSO	-	-	-	-	-	-
NIT	+	+	+	+	-	-
PDHP	-	-	+	-	-	-
3-OH-DHP	+	+	+	+	-	-
3,5-OH-DHP	-	-	-	-	-	-
4-OH-DHP	-	-	-	-	-	-
VDHP	-	-	-	-	-	-
IDHP	-	-	-	-	-	-

Table 3. ***Cell free assays of antioxidant activity of new tested DHPs***

COMPOUND	In vitro reported antioxidant activity In vivo	*In vivo* Basal	Stimulated
NIT	?	Yes?	No
PDHP	Yes[70,90]	No	No
3-OH-DHP	?	No	No
3,5-OH-DHP	Yes[90]	No	No
4-OH-DHP	?	No	No
VDHP	Yes[70]	No	No
IDHP	Yes[70]	No	Yes

LTCC blocking activity

These DHPs had new substituents in the aryl ring in C4, which had never been previously tested for their Ca^{2+} antagonism, it was very appealing and interesting to find out how these new substituents would affect the LTCC blocking activity.

The first step in doing these experiments was to rule out a possible interference of DMSO, as there is controversy in reports that state that it increases LTCCs Ca^{2+} current. Our results proved otherwise, and in Fig. 6 it can be seen that DMSO did not increase or decrease this current, and this result is in agreement with what can be read in the literature.[95-98]

Moving on to our experimental findings about these new DHPs, the results showed that only one of all compounds, namely 3-OH-DHP, demonstrated a blocking activity on the channel at all concentrations used; when looking at all the chemical structures of the DHPs tested, it can be observed that 3-OH resembles the most the structure of the DHP control, NIT. The main difference being the 3-OH substituent in the aryl ring in C4 at m*eta*- position. The nature of the substituent is different in each compound, for instance NIT bears a nitro group, which is an electron withdrawing substituent and 3-OH having a hydroxyl, which is an electron releaser group. Both have the capacity to form hydrogen bonds, but as the literature states, the one with an electron withdrawing substitution would appear to be most effective in blocking LTCCs[41], which is absolutely in agreement with our results, where at all concentrations requires less time to start blocking the calcium channels.

PDHP, which is called the skeleton for not having any substituent in the aryl ring in C4, proved to have an inotropic effect but not a chronotropic effect when assayed at its highest concentration (10 µM); when used at lower concentrations this effect disappeared completely;

raising two ideas, first, the necessity of an ortho- or meta- substituent in the C4 aryl ring to achieve this chronotropic effect, and that in order to maintain either the inotropic or chronotropic effect at concentrations lower than 10 µM, the need for this substituent becomes imperative, and secondly this or these substitutions should bear an electron withdrawing or an electron releasing group.[41]

As expected 3,5-OH-DHP; the disubstituted version of 3-OH-DHP, would exhibit a blocking effect; however, this blocking effect did not come through. If the literature is consulted, felodipine is the only one commercially available DHP, dichloro-substituted in the aryl ring contiguously on same side of the ring at ortho- and meta- positions. It can be inferred that substituents in different sides of the aryl ring may affect the conformation of the molecule, disabling it to achieve its active one, which is boat.[44,85,86] It is possible as well, that hydroxyl groups, being such good hydrogen bond donors, might steer 3,5-OH outside the binding pocket where it gets clogged forming hydrogen bonds with other aminoacid than the ones in the binding pocket.

NIT began its blocking activity faster and perhaps stronger, statiscally at least; than either PDHP, or 3-OH-DHP; in both cases, the explanation for this lies in the structure of the DHPs, assimetry[40] in the dihydropyridine ring as in NIT and the nitro group*, meta*-position, in the aryl ring in C4, may account for a faster insertion into the lipid bilayer,[40] as it was mentioned before, being a lipophilic molecule, the nitro group should confer a more perdurable interaction with the binding site.[44]

There is concern why NIT as well as 3-OH-DHP only reduced the frequency of contraction, at the lowest concentration, 0.1 µM. We postulated three possibilities, first that the compounds precipitated, but if this was the case, then, the minimum concentration would still be there and thus achieving the reduction of frequency would have been the same for all three concentrations used, so this theory was discarded. Second, the idea of an allosteric site to which DHPs could bind and in doing so modifying the canonical binding pocket, rendering it inaccessible to exert the blocking effect. No allosteric binding site has been described so far for DHPs in literature. The only allosteric site described is for the other members of the CCBs, such as diltiazem and verapamil. If two calcium antagonists were to be added together in a solution to cultured cells, a positive cooperative effect would happen, making a stronger block of LTCC.[91] Third, a compensatory mechanism. If there are strong negative inotropic and chronotropic effects the cardiomyocytes would die. In a series of epifluorescence experiments not shown, in which cells were treated first with ryanodyne, 10 µM, and thapsigargin 0.1 µM, for thirty minutes, and then the DHP was added as the experiment was going along, the cells, did not respond well. Although no viability test was performed, when cells were checked, morphologically they did not exhibit their classic rod shape, they were shrinking, vacuolising, going into a death road. When they were treated with ryanodyne alone, they looked better but not as a control. Depletion of SR is therefore a key issue in the integrity of muscle

cells, cardiac muscle cells. It has been suggested by some authors that SR, and its Ca^{2+} release, are responsible for the frequency of contraction, in isolated rat cardiomyocytes.[92,93] Gathering this information gives the notion that a strong blockage of LTCC leading to a powerful inotropic and chronotropic effect, from which the cardiomyocyte might not recover, could cause its death. However if treated with a DHP, at a concentration in which this phenomena is present, grants the cell the need to look for a compensatory mechanism through which it loses either the force of contraction or contraction but not both because it would mean its death. In our case, the ryanodyne receptor releases Ca^{2+}, in absence of CICR, and this Ca^{2+} is able to activate sensitized contractile machinery. Maintaining one of their functions then staying alive and this argument is supported by the literature.[94]

It was proven, as it is also stated in the literature, that any substituent on *para*-position of the aryl ring in C4 has nullifying or abrogating effect on the Ca^{2+} antagonist activity of the molecules.[51-53] Two of our compounds, VDHP and its isomer: IDHP, had two substituents, one adjacent to the other, in meta- and in para-position of the aryl ring in C4 and one had only one substituent in para: 4-OH-DHP. All of them failed to block the LTCC, all the same, compounds similar to VDHP or IDHP, had never been tested, or at least we did not find proof of it bibliographically, being that our main reason to test them. As mentioned previously, in all channel blocking experiments, none of them exerted any effect. This can be associated to a steric effect, predictably one associated to size[87], since the literature states that regardless of the nature of this para-substituent[51], it is then not associated to an attraction or repulsion, the only option left is size, upon acquiring its active boat shape, this substituent would make the DHP too big to enter the binding pocket. This is correlated with our docking studies, which point out that 4-OH-DHP, VDHP and IDHP do not get inside the action site.

Patch clamp

Even though there is not a big population for 3-OH-DHP in this set of experiments and we do have results for NIT yet, this supports the idea of a novel LTCC blocker, because even at concentrations below the minimal tested in confocal microscopy (0.1 µM), there was a remarkable reduction in the Ca^{2+} currents. So summing up, all data obtained in experiments regarding Ca^{2+} antagonizing activity relating to 3-OH-DHP, we have a compound that effectively blocks LTCCs, proven through two different techniques and is confirmed by docking studies, which reveal that the molecule inserts into the binding pocket and even in the absence of one critical interaction maintains the blocking effect on the channel.

Docking studies

Using Zhorov's model of LTCC[45], which was docked with nimodipine, a molecule very similar to our NIT, the only difference is a larger alkyl chain in C3; they found a favorable stern-to-Y3i10 orientation of nimodipine in the LTCC pore and verified key DHP-sensing residues identified in previous mutational studies that confirmed favorable contacts with the nimodipine in their model. To make sure our docking calculations were correct, we validated the nimodipine docking model, compared it to NIT, and NIT docked the same as nimodipine. In all dockings the ligand was in a Ca^{2+} deficient state in which the selectivity filter region was loaded with a single Ca^{2+} ion. This configuration was selected because the Ca^{2+} deficient state was proposed to favor binding of DHP antagonists[88]. Summary of the modeling predictions of favorable DHP contacts with the channel are shown in Figure 15. In 3-OH-DHP, as well as in NIT, the NH group at the stern forms an H-bond with Y3i10. The starboard methyl group fits into a hydrophobic pocket formed by I3i14 and M3i18. The portside group is bound in a hydrophobic pocket formed by M3i19, F3p49, and I4i19. For 3-OH-DHP, the polar OH group at the bowsprit does not interact with the side chain of Y4i11, this would suggest that this hydroxyl it's interacting with another amino acidic residue and that this interaction may be weaker explaining therefore why NIT may be more potent than 3-OH-DHP. The carbonyl oxygen at the starboard accepts an H-bond from Q3o18. The side chain of T3o14 does not interact with the DHP molecule directly, but it is exposed toward the starboard group in such a way that its enlargement would sterically prevent DHP binding. For, PDHP, the interactions were all the same but in the aryl ring in C4 it was different, as there was not substituent, the interaction with M4i12 is absent. The lack of this interaction might explain why PDHP did not block at 1 and 0.1 µM concentrations, as it would render its interaction with the binding site weak, ergo being only present at very high concentrations (10 µM). The most interesting case of all, was the one regarding 3,5-OH-DHP, this molecule inserted into the binding site exactly the same way, as NIT and 3-OH-DHP, this would therefore suggest that a Ca^{2+} channel blocking activity ought to be present, but in practice, this was not true at all. This LTCC model for docking studies, represents the molecule in an ideal state, it does not account possible interactions of the ligand outside its binding site; the odyssey that DHP must endure to get inside the binding pocket, it is discarded; having two hydroxyls, polar substituents, makes 3,5-OH-DHP an easy target to form hydrogen bonds, and get stuck at some point before reaching out its final destination.

Concerning all para-substituted DHPs used in our studies: 4-OH-DHP, VDHP and IDHP, all of them were anywhere in the LTCC but in the binding pocket; confirming our hypothesis about a sterical hindrance, that disables them to bind to the action site.

Antioxidant activity

Our results show that any of these new DHPs have that antioxidant effect. There are various things to consider, let us first recall the results in figure 16, in basal conditions for NIT, the graph suggests that NIT may have an antioxidant effect but not in presence of H_2O_2 (50 µM). The results in Figure 17, point out a very little antioxidant effect for IDHP when incubated with H_2O_2 (100 µM). The results for NIT and IDHP prompted us to say that these molecules have an antioxidant effect but that this effect may be inappreciable in the experimental approach we used. It is certain that these new DHPs exhibited a very promising antioxidant effect in cell free assays; and that they included in their structure as substituents in C4 of the DHP ring, molecules such as vanillin,[70] that have been reported to be very effective antioxidants as well,[71-73] but they lack what is reported as an absolute requirement for having such activity in cells in-vitro assays, and that is a very large or simply large substituent in C2 or C3 of the dihydropyridine ring.[74] This is a characteristic feature of third generation DHPs, such as amlodipine or felodipine.[75,76] This feature of a long substituent in C3 or C5 of the dihydropyridine ring, is what allows compounds such as amlodipine (third generation prototype), not only to easily penetrate the plasma membrane to interact with the LTCC but also have additional biologic or pleiotropic actions that are independent of interaction with the calcium channel,[77] that is antioxidant, in which they seem to insert to a location in the membrane[78-80] near conjugated double bonds, highly lipophilic DHPs are capable of donating protons to lipid peroxide molecules, thereby blocking the peroxidation process.[78-80] So, basically two things can be said about this and our molecules, the first one is that there might not be technique sensitive enough to detect their antioxidant activity or, second, that to improve the antioxidant action of our DHPs, they must be synthesized in a way that the dihydropyridine ring has a long substituent in either C3 or C5.

The matter with these series of experiments for proving a biological antioxidant activity was that none of the techniques was specific for lipid peroxidation. Another important topic in this antioxidant discussion is the concentration of DHPs used, all molecules used in many and different ROS experiments and through various techniques were above or at 100 µM,[81,84] we could not get beyond that concentration due to solubility problems. Besides, all the papers that reported this activity failed to include a toxicity assay at those concentrations in which DHPs were tested. As mentioned above, before finding the antioxidant effect of amlodipine and later on some other third generation DHPs, they were already known for their calcium channel blocking properties. The main aim of this thesis was to test the calcium channel blocking properties of these new DHP agents.

Cell toxicity

Among our aims were also to investigate the effect of these new susbstituents on cell toxicity and their antioxidant properties. When any new drug candidate is synthesized, it must first undergo a series of trials to measure its overall toxicity. This testing allows highly toxic molecules to be identified before proceeding on to more advanced studies to measure therapeutic efficacy.[63,64] Our approach to test a possible toxicity of our new DHPs was to determine cardiomyocyte viability.[65,66] We used three different concentrations (0.1-10 μM); all which are within the range of previously known effective for Ca^{2+} channel blocking. First, we compared all DHPs vs. control, the control compound, NIT, exhibited a slight toxic effect for its highest concentration, the same was observed for all compounds, accordingly in between 1 and 10 μM. A concentration of 0.1 μM seemed to be the most innocuous of all concentrations tested (See Figure 6 for details). It is important to bear in mind that all drugs appear to have a toxic effect,[67] it is sought however that this effect be kept to a minimum,[68,69] which is the case of all our DHPs. It is important to recall that all our assays, be it for LTCC blocking activity or antioxidant activity were short time, being the longest the antioxidants the prolonged ones lasting up to one hour and a half. The conclusion in this aim tell us that the results we would see for the rest of the aims will be the product of a known mechanism for the compounds and not as a by-product of an unlikely toxic effect. One might expect that DHPs would be anything but deleterious; considering that they are reported to be antioxidant, which means a protective outcome, is reasonably expected.

7. FINAL REMARKS

- None of these new synthesized DHPs display toxic effects.
- It is a requisite for any DHP to have a substituent in the aryl in C4 of the dihydropyridine ring in order to exert a LTCC blocking activity.
- This substituent must be either in *ortho* or *meta* position.
- For a faster blocking effect substituents in C3 or C5 of the dihydropyridine ring must be asymmetric.
- There might be also a correlation between the nitro group in the aryl in C4 and a faster course of action in time.
- All *para* substitutions in the aryl in C4 of the dihydropyridine ring abrogate the calcium channel blocking activity as a consequence of steric hindrance.
- Disubstituted DHPs in the aryl ring, in order to block the calcium channel must have these substitutions contiguous and in the same side of ring.
- Electron releasing substituents in aryl ring also exhibit an antagonizing effect on LTCC.
- These new DHPs do have an antioxidant activity, but it is too little to be determined.
- For any DHP to have an assessable antioxidant effect, a long chain substituent in C3 or C5 of the dihydropyridine ring it is compulsory.
- If phenols are inserted in C4 of the dihydropyridine ring and also this ring presents a long chain either in C3 or C5, a powerful antioxidant can be expected, but not a calcium channel blocker.

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