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Fabrice Ivanes

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CARDIOMYOCYTES CONTRE LES LESIONS  
D'ISCHEMIE/REPERFUSION

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## RESUME en français

Les maladies cardiovasculaires constituent un problème de santé publique. Les pré et post-conditionnement ischémiques mais aussi pharmacologiques constituent autant d'avancées qui permettront l'amélioration de la prise en charge des malades en situation d'ischémie/reperfusion myocardique. Néanmoins, la morbi-mortalité des maladies cardiovasculaires reste importante et nécessite le développement de nouvelles techniques.

Les premiers résultats de la thérapie cellulaire myocardique ont été décevants, et s'il est désormais établi que l'on ne peut régénérer le myocarde, les effets bénéfiques observés, notamment avec les cellules souches mésenchymateuses semblent en rapport avec un effet paracrine qui passe par l'activation de la voie de signalisation PI3kinase/Akt sur un mécanisme comparable à celui du post-conditionnement ischémique. Les médiateurs de cet effet sont vraisemblablement des facteurs de croissance comme le VEGF ou l'IGF-1 même si un effet individuel direct de l'une ou l'autre de ces molécules n'a pu être mis en évidence.

La modulation de l'activité de l'ATP synthase mitochondriale est également une cible thérapeutique prometteuse. Cette enzyme inverse son activité et hydrolyse l'ATP durant l'ischémie, conduisant à dépleter le pool d'ATP intracellulaire et accélérer la survenue de la mort cellulaire. De nouvelles molécules ayant un effet similaire à l'IF1 permettent de bloquer cette inversion d'activité de l'ATP synthase, de préserver l'ATP et donc d'améliorer la survie cellulaire par un effet de type préconditionnement ischémique.

Ces 2 techniques, très différentes mais non antinomiques, pourraient faire partie de l'arsenal thérapeutique dans les années à venir.

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## TITRE en anglais

NEW MECHANISMS OF PROTECTION OF CARDIOMYOCYTES FROM ISCHEMIA/REPERFUSION INJURY

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## RESUME en anglais

Cardiovascular diseases are a major problem of public health management. Ischemic and pharmacological pre and postconditioning should significantly improve the prognosis of patients suffering from myocardial ischemia/reperfusion. However, the morbi-mortality of these patients is still high and research must remain active.

The first results of myocardial stem cell therapy show that we cannot regenerate myocardium but a recent meta-analysis reported positive effects that can be explained through a paracrine mechanism. Mesenchymal stem cells protect ischemic cardiomyocytes from reperfusion injury through a paracrine activation of the PI3kinase/Akt pathway in a similar way to ischemic postconditioning. The mediators of this protection could be growth factors such as VEGF or IGF-1 though we couldn't demonstrate a direct effect of one or the other.

Modulating the activity of the ATP synthase during ischemia is another promising therapeutic target. This enzyme reverses its activity and hydrolyses ATP when the supply in oxygen is impaired. This leads to the reduction of the cellular pool of ATP and accelerates cell death. We identified new small molecules with a similar effect to IF1 that can selectively inhibit the reverse activity of the ATP synthase, preserve ATP and thus increase cell survival in a preconditioning-like effect.

These two different techniques could be part of the therapeutic arsenal against ischemia/reperfusion in the next decades.

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## DISCIPLINE : PHYSIOLOGIE CARDIOVASCULAIRE

MOTS-CLES : Ischémie/reperfusion myocardique, Thérapie cellulaire, Cellules souches mésenchymateuses, Mitochondrie, Pore de transition de perméabilité mitochondriale, ATP synthase mitochondriale

# Résumé substantiel en français

Les maladies cardiovasculaires, avec comme chef de file l'infarctus du myocarde, constituent la première cause de mortalité dans le monde. Elles sont la conséquence de l'athérosclérose dont l'évolution va s'accompagner d'épisodes aigus appelé syndrome coronarien en rapport avec l'occlusion partielle ou totale d'une artère coronaire par un thrombus. Dans ces situations d'ischémie aigue, l'essentiel de notre arsenal thérapeutique a pour finalité la désobstruction de l'artère coupable pour obtenir la reperfusion, que ce soit par des traitements anticoagulants et antiagrégants plaquettaires à forte dose ou grâce à l'angioplastie coronaire. Mais, si elle est indispensable, la reperfusion s'accompagne d'un certain nombre de phénomènes pathologiques (surcroît de mort cellulaire, arythmies ventriculaires, phénomène de no reflow) appelés lésions de reperfusion au centre desquelles on trouve la mitochondrie et son pore de transition de perméabilité. De nouvelles techniques permettent de diminuer ces lésions de reperfusion. La première découverte en date a été le préconditionnement ischémique en 1986. Elle consistait en l'application de courtes séquences d'ischémie/reperfusion avant une période d'ischémie prolongée et s'accompagnait d'une diminution significative des tailles d'infarctus chez toutes les espèces étudiées. Le postconditionnement ischémique consiste en l'application de brèves séquences d'ischémie/reperfusion, selon un protocole similaire au préconditionnement, au tout début de la reperfusion, avec un effet comparable en ce qui concerne la réduction de la taille d'infarctus. Cette technique a fait l'objet de quelques essais cliniques très encourageants mais ses bénéfices potentiels sont limités aux seuls patients souffrant d'infarctus du myocarde pris en charge ne angioplastie primaire. Le postconditionnement pharmacologique via la Ciclosporine A (CsA) constitue désormais la technique la plus prometteuse dans le domaine de la pathologie humaine avec des essais cliniques publiés et en cours qui sont jusqu'à présent très positifs. Mais l'importance de la mortalité des maladies cardiovasculaires laisse encore beaucoup de place pour le développement de nouvelles techniques et molécules.

Nous avons choisi de développer dans ce travail 2 approches différentes de protection contre l'ischémie/reperfusion myocardique.

La thérapie cellulaire myocardique a vu le jour il y a maintenant plus d'une dizaine d'années et il est désormais établi qu'on ne peut, en l'état actuel des connaissances, régénérer le myocarde. Mais les essais cliniques dans le domaine de l'infarctus montre qu'il existe bien un effet positif même s'il est modeste, notamment sur la fraction d'éjection ventriculaire gauche et le diamètre télésystolique du ventricule gauche. Plusieurs travaux suggèrent que cet effet pourrait être en rapport avec un mécanisme paracrine, hypothèse que nous avons choisi d'explorer en utilisant un modèle cellulaire d'ischémie/reperfusion. Nous avons décidé d'utiliser les cellules souches mésenchymateuses (MSC) en raison de leurs propriétés immunomodulatrices. Le modèle cellulaire choisi était des cardiomyocytes de rats nouveau-nés qui ont été soumis à des séquences d'ischémie/reperfusion simulée à l'aide d'une chambre à hypoxie. Les MSC étaient issues de la moelle osseuse de fémurs de rats et maintenues en culture au long cours. Après une ischémie simulée de 5 heures, les cardiomyocytes étaient mis en contact en tout début de reperfusion soit à des MSC fraîchement trypsinées, soit à du surnageant de MSC seul. La mort cellulaire était estimée après 16 heures de reperfusion par dosage de l'activité LDH, de la concentration de Troponine I dans le milieu. La viabilité cellulaire était évaluée par test au MTT. Nos résultats montrent que les MSC ont un effet protecteur sur les cardiomyocytes soumis à une telle séquence

d'ischémie/reperfusion, que cette protection passe par un mécanisme paracrine car le surnageant des MSC a une efficacité équivalente à l'ajout de MSC elle-même ou au contrôle positif de cardioprotection qu'est la CsA à la posologie de 200 nM. Cette protection est médiée par l'activation de la voie de signalisation RISK, notamment la PI3kinase/Akt, comme le démontre le fait que l'ajout d'inhibiteur de cette voie (Wortmannine, LY294002) annule l'effet protecteur du surnageant de MSC. Cette activation passe vraisemblablement par la sécrétion de cytokines et facteurs de croissance comme le VEGF et l'IGF-1, retrouvés en quantité significative dans le surnageant de MSC, même si l'utilisation d'anticorps dirigés contre ces molécules n'a pas permis de mettre en évidence un effet individuel direct de l'un ou l'autre de ces facteurs. Cet effet protecteur peut dans tous les cas être assimilé à un effet de type postconditionnement ischémique dans lequel la voie de signalisation de la PI3kinase tient un rôle majeur.

La seconde approche s'est intéressée à la modulation métabolique de phénomènes survenant durant l'ischémie et contribuant à augmenter la mort cellulaire. Durant ces périodes où les cardiomyocytes sont en situation de privation d'oxygène, l'ATP synthase mitochondriale F1Fo, qui d'ordinaire synthétise la majorité de l'ATP dans le cadre de la phosphorylation oxydative, va inverser son activité et se mettre à hydrolyser l'ATP dans le but de transférer des protons de la matrice mitochondriale vers l'espace intermembranaire et essayer ainsi de maintenir le potentiel de membrane mitochondrial, menacé par la défaillance de la chaîne de transport des électrons. Cette hydrolyse d'ATP va rapidement conduire à la consommation complète des stocks d'ATP intracellulaire et à la mort cellulaire via l'ouverture du pore de transition de perméabilité mitochondrial. Il existe des inhibiteurs exogènes de l'ATP synthase comme l'oligomycine B, mais qui ne sont pas spécifiques de son activité reverse. Il existe également un inhibiteur endogène appelé IF1 qui est lui spécifique cette activité reverse. Le laboratoire BMS a publié en 2004 des travaux sur une molécule (BMS199264) ayant un effet de type IF1 et capable de préserver l'ATP et de diminuer la mort cellulaire. En l'absence de nouvelles publications sur cette molécule ou d'autres du même genre, nous avons entrepris de mettre au point et tester (ou acheter et tester) des molécules pouvant potentiellement inhiber sélectivement cette activité reverse sur un modèle cellulaire faisant essentiellement intervenir une lignée de cellules cardiaques : les HL-1. Sur les 8 molécules testées, seules 2 ont démontré des capacités à inhiber sélectivement l'ATP hydrolase sans compromettre de façon notable l'homéostasie cellulaire dans des conditions normoxiques (évaluation sur le potentiel de membrane mitochondriale, l'autofluorescence du NADH, la consommation d'oxygène). Ces molécules ont montré qu'elles préservaient l'ATP (selon un test utilisant le magnésium green, sonde fluorescente) et qu'elle pouvaient retarder/diminuer la mort cellulaire à l'issue d'une séquence d'ischémie/reperfusion prolongée. Pour une de ces molécules (BTB06584), l'action protectrice semble modulée en fonction du niveau d'expression d'IF1. La modulation de la consommation d'ATP par l'ATP hydrolase durant l'ischémie pourrait faire partie des effets du préconditionnement ischémique et jouerait sur les lésions induites par l'ischémie.

Nous avons donc évalué 2 techniques différentes qui sont la thérapie cellulaire myocardique et la modulation d'activité de l'ATP synthase mitochondriale et mis en évidence des pistes futures dans le domaine de la cardioprotection selon un mécanisme en partie comparable aux techniques qui deviennent les gold standards de notre époque, à savoir le pré et le postconditionnement ischémique.

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# LIST OF ABBREVIATIONS

## A

Acetyl-CoA: Acetyl Coenzyme A

ACS: Acute Coronary Syndrome

AMI: Acute Myocardial Infarction

ANOVA: Analysis of Variance

ANT: Adenine Nucleotide Translocase

ATP: Adenosine-5'-Triphosphate

## B

BSA: Bovine Serum Albumine

## C

CD: Cluster of Differentiation

CFP: Cyan Fluorescent Protein

CK: Creatine Kinase

CsA: Cyclosporine A

CVD: Cardiovascular Diseases

## D

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

$\Delta\phi m$ : Mitochondrial Membrane Potential

## E

EDTA: Ethylenediaminetetraacetic acid

EGFP: Enhanced Green Fluorescent Protein

ELISA: Enzyme-Linked Immunosorbent Assay

eNOS: Endothelial Nitric Oxide Synthase

## F

FACS: Fluorescence Activated Cell Sorting

FADH: Flavin Adenine Dinucleotide

FBS: Foetal Bovine Serum

FCCP: Trifluorocarbonylcyanide Phenylhydrazine

FITC: Fluorescein Isothiocyanate

FLIPR: Fluorescence Imaging Plate Reader

FRET: Fluorescence Resonance Energy Transfer

## G

G-CSF: Granulocyte Colony-Stimulating Factor

GLUT 1-4: Glucose Transporter Type 1-4

## H

HGF: Hepatocyte Growth Factor

HIF-1: Hypoxia Inducible Factor 1

HK: Hexokinase

## I

IF1: Inhibitor Protein of the F1 domain of the ATP synthase

IGF-1: Insuline-like Growth Factor 1

## L

LDH: Lactate Deshydrogenase

LDL: Low Density Lipoproteins

LPL: Lipoprotein Lipase

## M

MgG: Magnesium Green

mPTP: mitochondrial Permeability Transition Pore

MSC: Mesenchymal Stem Cells

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue

## N

NADH: Nicotinamide Adenine Dinucleotide

## P

PBR: Benzodiazepine Receptor

PBS: Phosphate Buffered Saline

PCI: Percutaneous Coronary Intervention

PE: Phycoerythrin

PI: Propidium Iodide

PFA: Paraformaldehyde

PI3K: Phosphatidylinositol-3-OH Kinase

PKC: Protein Kinase C

PPAR $\gamma$ 2: Peroxisome Proliferator-Activated Receptor  $\gamma$ 2

Rh 123: Rhodamine 123

RISK: Reperfusion Injury Salvage Kinase

ROS: Reactive Oxygen Species

## S

SD: Standard deviation

SEM: Standard Error of the Mean

## T

TCA: Tricarboxylic Acid

TMRM: Tetramethylrhodamine methyl ester

TTC: Triphenyltetrazolium Chloride

## U

UV: Ultraviolet

## V

VDAC: Voltage Dependent Anion Channel

VEGF: Vascular Endothelial Growth Factor

## Y

YFP: Yellow Fluorescent Prote

# INTRODUCTION AND RATIONALE

## **A. Myocardial ischemia and reperfusion**

Ischemia and reperfusion are two processes accounting for the pathophysiology of cardiovascular diseases (CVD). These pathologies constitute a major problem of public health management worldwide for they are the main cause of morbidity and mortality, before cancer diseases and infections <sup>1</sup>. While their prevalence is stable in “western” high-income countries represented by Europe and North America, it explodes in the “Third-World countries” concomitantly with the increase of life expectancy and the change of lifestyle inherent to the increase of urbanization, industrialization and the “westernization” of feeding behavior, leading to the development of obesity and diabetes in these countries <sup>2-5</sup>. CVD accounts for 40% of the deaths in the high-income countries and 28% in the low and middle-income countries, explaining the need for research in this area.

The best illustration of myocardial ischemia is acute myocardial infarction (AMI). It consists of the abrupt occlusion of a coronary artery by a clot responsible for the disruption of supply of oxygen and nutrients to the myocardium. If total occlusion persists, the cardiomyocytes vascularized by the culprit artery die proportionally to duration of occlusion and will be replaced by a fibrotic scar, devoid of any contractile properties. As a result of AMI, when sudden death doesn't occur due to malignant ventricular arrhythmias, a large proportion of patients suffer from chronic heart failure responsible for significant functional limitations and morbidity in link with recurrent hospitalizations for acute worsening. Thus, restoration of the blood flow by desocclusion of the artery as soon as possible, corresponding to reperfusion, is essential to improve the outcome of patients <sup>6-8</sup>.



## **B. Myocardial ischemia generally occurs in the context of atherosclerosis**

Atherosclerosis is a phenomenon common to all types of cardiovascular diseases and affects every single individual worldwide<sup>9,10</sup>. It concerns the whole of the arterial system, from the aorta to the microcirculation, and localizes preferentially in areas where the blood flow is turbulent, i.e. in the bifurcation of the arteries<sup>11</sup>. This process starts very early in life, from the very first years of the individual<sup>12</sup>. It corresponds to the accumulation in the media of the artery of molecules of oxydated LDL cholesterol that are phagocytated by macrophages, giving rise to spumous cells<sup>13</sup>. They constitute the lipidic core of atherosclerotic plaques, which is concealed into a fibrous cap made of smooth muscle cells and collagen. The pathology evolves silently for decades during which atherosclerotic plaques grow in size and contribute to the reduction of lumen of the concerned arteries<sup>14</sup>. Eventually, patients start to experiment symptoms such as chest pain or breathlessness when the lumen is reduced enough to generate an imbalance between myocardial oxygen supply and oxygen requirement, generally when the need of oxygen is increased, i.e. during exercise.

There are many well-known risk factors associated with atherosclerosis that can explain its increasing incidence worldwide. Smoking, high blood pressure, hypercholesterolemia and diabetes are the strongest precursors of atherosclerosis, as well as obesity, particularly when it predominates in the abdominal area, sedentarity, male sex and increasing age. These risks factors will allow identifying a population at higher risk of CVD that could benefit from the development of preventive actions.

In some cases, a rupture in the fibrous cap occurs that puts the lipidic core, rich in factor VII of the coagulation cascade, in contact with circulating platelets, resulting in the formation of a blood clot<sup>15</sup>. This phenomenon is generally highly symptomatic due to the abrupt myocardial ischemia generated and corresponds to an acute coronary syndrome (ACS). The clot can partially or totally occlude the coronary artery with variable consequences depending on the duration of occlusion, the size of area at risk and the existence of a collateral circulation (constituting a natural bypass between arterial territories and reducing the burden of the ischemia). When the

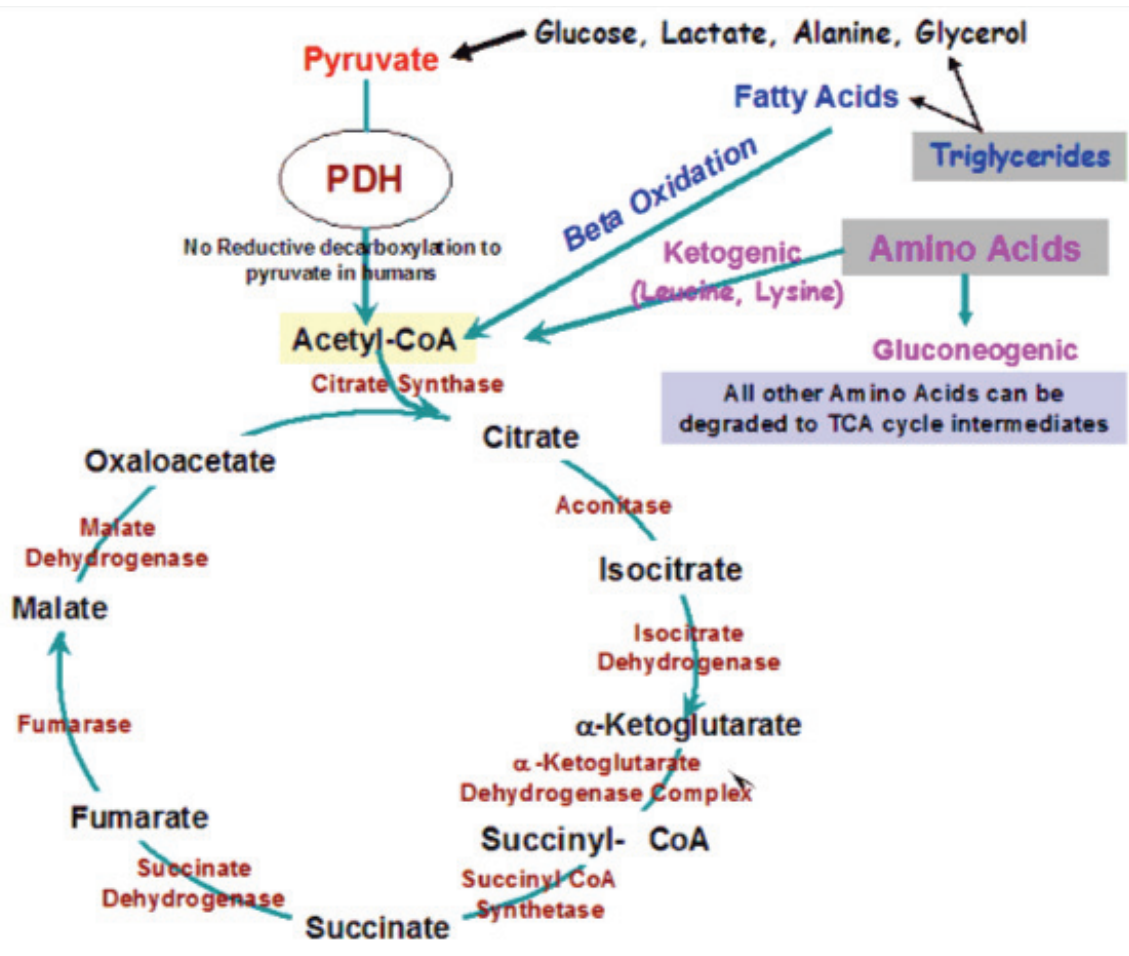
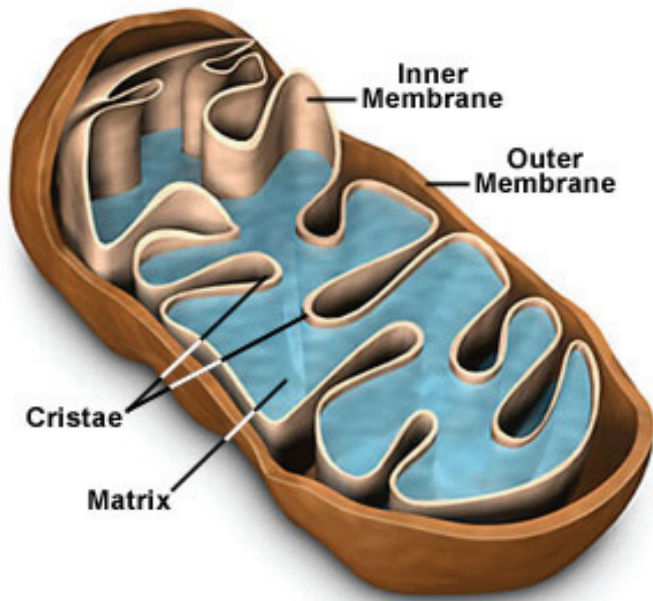
ischemia is long enough, i.e. at least 30 minutes in human, cardiomyocytes in the area at risk start to die, releasing in the blood flow some enzymes (Creatine Kinase CK, Lactate Deshydrogenase...) and also a specific sarcomeric protein called troponin that is easily measured in routine clinical practice and used for the definition of myocardial infarction <sup>16</sup>. Generally the terms AMI, also termed type 1 myocardial infarction, corresponds to the total occlusion of a coronary artery, responsible for a transmural myocardial necrosis <sup>17</sup>.

### **C. Mitochondria at the center of the ischemic process**

Studies of the pathophysiology of AMI highlight mitochondria as a key actor in this process. Mitochondria are at the center of the metabolism of cells.

These organelles, symbiotic to the eukaryotic cells, are composed of 2 membranes (external and internal), which are phospholipid bilayers with embedded proteins. The outer membrane is smooth while the inner has many folds called cristae. These membranes delimitate an intermembrane space and a matrix containing a mitochondrial DNA and numerous enzymes necessary to the oxidation of pyruvate and fatty acids in the Krebs cycle (also called TCA, i.e. tricarboxylic acid, cycle) (Figures 1 and 2).

*Figure 1. Representation of the structure of a mitochondrion. These oblong shaped organelles are present in the cytosol of every single eukaryotic cell and collaborate to their life in a symbiotic manner. The inner and outer membrane separate 3 spaces defined as mitochondrial matrix, intermembrane space and cytosol. The many channels and proteins specific to the mitochondrion contribute to make of these compartments 3 very different spaces ensuring the good functioning of mitochondria and perpetuating the cell homeostasis.*



*Figure 2. Representation of the Krebs cycle and its metabolites. Similar to glycolysis with pyruvate as its main product, the beta-oxidation of fatty acids leads to the production of Acetyl-CoA. It is the first substrate of the TCA cycle. The multiple transformations of the Acetyl CoA molecules ensures a minor production of ATP but mainly the reduction of several molecules of coenzymes NAD<sup>+</sup> and FAD through the many deshydrogenases, which are used as protons donors during the process of mitochondrial respiration.*

The outer membrane is permeable to many different molecules thanks to embedded proteins called porins. On the other hand, the inner membrane is impermeable to most molecules, including ions and metabolites, which transport necessitates specific proteic transporters. The inner mitochondrial membrane also contains the electron transport chain, an association of proteins and enzymes responsible for the oxidative phosphorylation, by far the main source of ATP in the cardiomyocyte <sup>18</sup>. The electron transport chain or mitochondrial respiratory chain is comprised of five complexes (figure 3):

Complex I, also called NADH-ubiquinone oxidoreductase, is the first and biggest complex of the chain, comprised of 45 subunits. It couples the oxidation of NADH with the reduction of ubiquinone, allowing during these reactions the release of electrons. It is inhibited by rotenone.

Complex II, called succinate-ubiquinone reductase or succinate deshydrogenase, is made of 4 subunits and contributes to oxidate succinate to fumarate and FADH<sub>2</sub> to FAD in conjunction with reduction of ubiquinone, allowing again the release of electrons. It is inhibited by malonate.

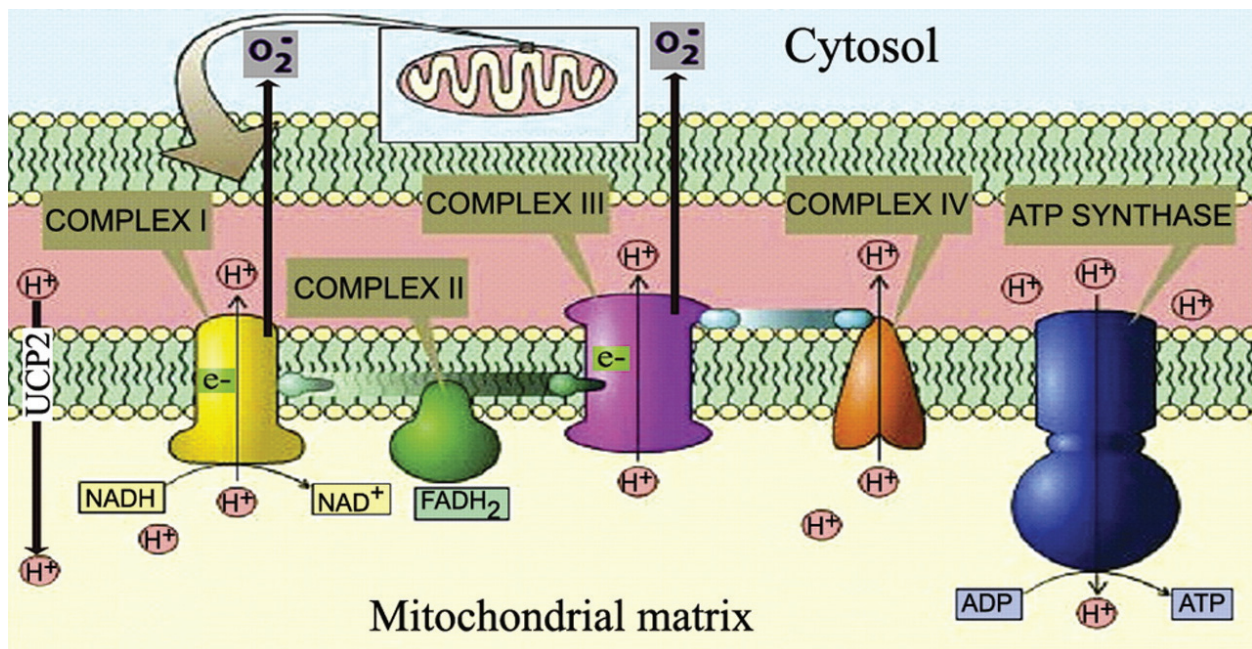
The third complex of the electron transport chain is a composite of 11 subunits, named ubiquinol-cytochrome c reductase, and, as suggested by its name, catalyzes the reduction of cytochrome c with the oxidation of ubiquinone. Its common inhibitor is Antimycin A.

The complex IV or cytochrome c oxidase is a large composite of 14 subunits that oxidizes cytochrome c thanks to the electrons transferred from one complex to the

others, which ends in reducing molecular oxygen to water. Its main inhibitor is cyanide, as well as azide, methanol, and more anecdotally carbon monoxide.

The complex V is the F1Fo mitochondrial ATP synthase (see below).

*Figure 3. Mitochondrial respiratory chain. The transfer of electrons through complexes I, III and IV is coupled to the transfer of protons from the matrix to the intermembrane space, responsible for the protonmotive force. This force is used by the complex V for the synthesis of ATP. Other proteins such as UCP (Uncoupling proteins), substrates of FCCP, can also transfer protons from the intermembrane space back to the mitochondrial matrix.*



The electron transfer from one complex to the other combined with the oxidation of NADH (and FADH<sub>2</sub>) permits transfer of protons from the matrix to the intermembrane space, substrate of the  $\Delta\psi_m$ .

Complex I, III and IV act as proton pumps between the matrix and the intermembrane space, generating a proton gradient also called protonmotive force, and a difference of electric charge between the inside and the outside of the mitochondria (inside is

thus negative) called mitochondrial membrane potential ( $\Delta\psi_m$ ) with a basal value of -140 mV<sup>19</sup>.

In normoxic conditions, the energetic substrates of cardiomyocytes come mainly from the beta-oxidation of fatty acids. It generates ATP through oxidative phosphorylation, which represents up to 60% of the oxygen consumption of the myocardium. Glycolysis is a minor source of ATP for the cardiomyocyte, would it be aerobic, anaerobic or due to the degradation of the stocked glycogen. Oxidative phosphorylation requires a fully functional mitochondrial respiratory chain, a pool of reduced coenzymes such as NADH and FADH<sub>2</sub> from the Krebs cycle that act as donors of electrons and the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase. Thanks to the electrons issued from the oxidation of NADH and FADH<sub>2</sub>, complexes I to IV of the mitochondrial respiratory chain ensure the translocation of H<sup>+</sup> from the mitochondrial matrix to the intermembrane space, generating the protonmotive force. This proton gradient is necessary for the synthesis of ATP. The protons from the intermembrane space are actively translocated back into the matrix through the F<sub>o</sub> subunit of the ATP synthase, also known as complex V of the mitochondrial respiratory chain, generating in the meantime ATP by phosphorylation of ADP by the F<sub>1</sub> subunit.

The stock of ATP in cardiomyocytes being quite low and their needs in oxygen important due to their contractile properties, they are very sensitive to any perturbation in the oxygen and nutrient supply.

But mitochondria don't act solely as energy providers in cardiomyocytes. They play a significant role in the ionic homeostasis of the cell such, ensuring the maintenance of the acidobasic equilibrium, e.g. through the NADP/NADPH buffers systems, and the regulation of calcium fluxes through the calcium uniport or the Na/Ca exchanger<sup>20-22</sup>. They are also a significant source of reactive oxygen species, which often play the role of second messengers in the cell, and, in pathological conditions, contribute to the activation of apoptotic cell death.

## **D. Ischemia**

Ischemia leads to the decrease or the blocking of oxidative phosphorylation due to the lack of oxygen available for the cells<sup>23, 24</sup>. When it is moderate, i.e. when the supply is reduced but not completely stopped, cardiomyocytes respond by inducing adaptative reaction for their survival by activation of the AMP-kinase pathway and their metabolism switches from fatty acid oxidation to a majority of anaerobic glycolysis, with the disadvantage of a reduced output in terms of ATP production and the increase in production of metabolites such as lactates that can be deleterious at high concentration<sup>25, 26</sup>. The decrease in glucose available due to the reduced supply is compensated by the increase in the membrane conductance due to an increase of the number of carrier such as GLUT-1 and GLUT-4. This reduced production of energy implies a reduction in the mechanical activity of cardiomyocytes as their priority is survival. In these conditions, the myocardium is “hibernating” and a restoration of the supply of oxygen and nutrients leads to a total recovery of the contractile activity of the myocardium. Prolonged ischemia also leads to the stimulation of hypoxia-inducible factors (HIF-1 being the best known), which activate the transcription of several genes that allow the cell to adapt to the hypoxic environment.

When the ischemia is severe, the only source of ATP available is anaerobic glycolysis that will be highly stimulated. At the same time, the halt in pyruvate oxidation will lead to proton accumulation (uncoupling between anaerobic glycolysis and pyruvate oxidation contributing to the accumulation of 2 protons for each molecule of pyruvate produced) and thus to acidification of the intracellular compartment. In mitochondria, the lack of oxygen is responsible for a halt in the functioning of the mitochondrial respiratory chain, compromising the maintenance of the proton gradient between the matrix and the intermembrane space and thus of the mitochondrial membrane potential. This leads to a reversal of the mitochondrial F<sub>1</sub>F<sub>0</sub> ATP synthase as a way to maintain a potential: the enzyme actively transfers protons from the matrix to the intermembrane space but at the expense of ATP meaning that it hydrolyses ATP instead of synthesizing it<sup>27-30</sup>. The physiological purpose of such a reversal, despite many theories, is not understood, and it represents a significant

consumption of ATP, termed in the literature as “ATP wastage”<sup>31-33</sup>. The lack of energy available, if persistent, will compromise cell survival leading to necrosis of the cardiomyocyte<sup>34</sup>.

#### **E. Reperfusion, an absolute necessity, but not costless**<sup>35</sup>

In these conditions, it is urgent to restore a correct supply of oxygen and nutrients to the tissues in order to salvage the myocardium and avoid the functional consequences of myocardial necrosis. When ischemia is due to an ACS such as an AMI, the absolute priority is to restore the blood flow in the culprit artery with undiscussed clinical benefit as first illustrated by the GISSI and ISIS trials<sup>36, 37</sup>. All the clinical care strategies are currently oriented toward the desocclusion of the artery through the early use of multiple antiplatelet therapies, powerful parenteral anticoagulants and, where available, mechanical desobstruction of the artery by percutaneous coronary interventions. Reperfusion must be performed as soon as possible in clinical practice according to the dogma “Time is Muscle” and this led to an improvement of the population education in order to rapidly identify the symptoms of AMI, an improvement in the transport logistic and diagnosis in order to allow the earliest start of therapy possible. All these improvements led to a significant reduction of the 30 days mortality of patients with AMI, dropping from more than 30% to 6%.

However, it appears from experimental and clinical studies that reperfusion itself has a significant harmful clinical burden making it a “double-edged sword”<sup>38</sup>. It is responsible for additional cell death meaning that some cells that die at the time of reperfusion would have survived (at least for some additional time) if ischemia had persisted and this is termed lethal reperfusion injury (figure 5). Reperfusion can also generate potentially lethal ventricular arrhythmias, temporary myocardial dysfunction (myocardial stunning persisting after blood flow restoration) and the clinically feared no reflow phenomenon corresponding to the failure of immediate coronary reperfusion despite successful mechanical desobstruction, probably due to an alteration of the arteriolar vasomotricity<sup>39</sup>. In most species including humans, 30 to



40% of the cell death following a reperfused AMI can be attributed to the so-called reperfusion injury that is now identified as a major therapeutic target.

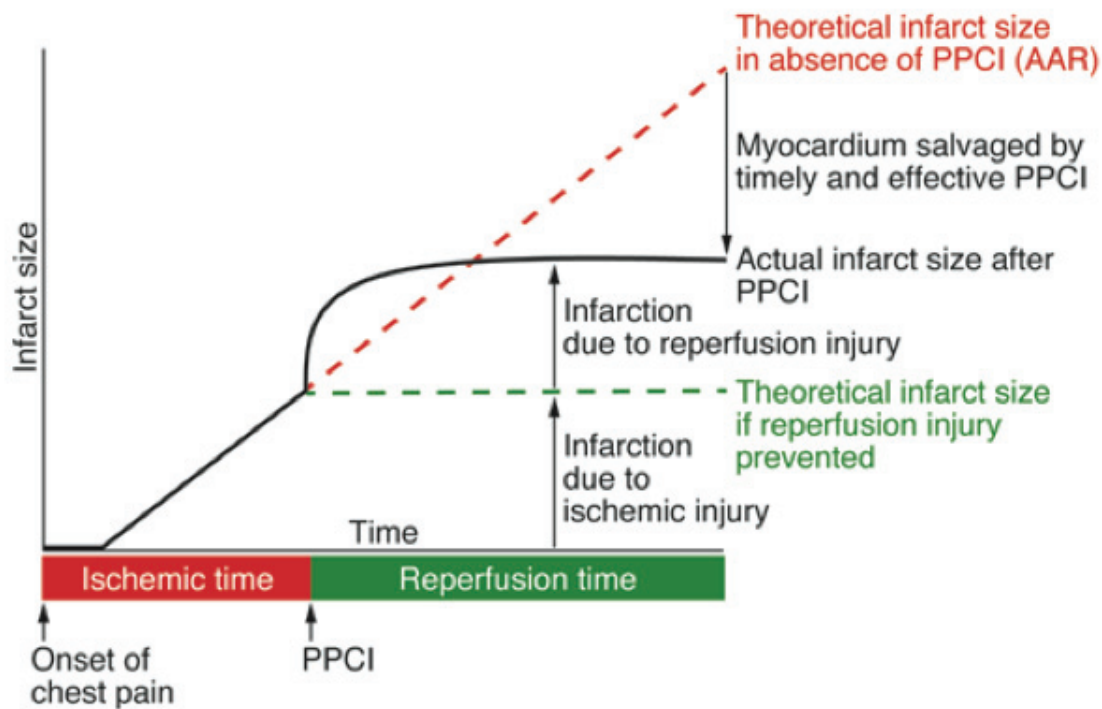


Figure 5. Schematic view of ischemia-induced and reperfusion-induced injuries. AAR: Area at risk, PPCI: Primary percutaneous coronary intervention. Shortly after the onset of ischemia, cardiomyocytes in the area at risk start to die. In the absence of reperfusion, every cardiomyocytes of this area die. In case of reperfusion, the actual infarct size is always more important than expected due to reperfusion injury. The infarct size can be significantly reduced through the prevention of this reperfusion injury.

From a cellular point of view, reperfusion restores the supply of oxygen and nutrients to the cardiomyocytes. This brutal supply of oxygen is responsible for an important peak of production of reactive oxygen species that are deleterious such as the superoxide anion, contributing to paralyze several enzymatic systems in the cells. Reperfusion allows the restoration of a physiologic intracellular pH by the important activation of the membrane antiporter  $\text{Na}^+/\text{H}^+$  exchanger, that excludes protons from the cytosol in exchange of  $\text{Na}^+$ , as well as the activation of the symporter  $\text{Na}^+/\text{HCO}_3^-$ . These two carriers are responsible for a further increase of the cytosolic  $\text{Na}^+$  overload generated during ischemia that the alteration of the functioning of the

$\text{Na}^+/\text{Ca}^{2+}$  exchanger aimed to fight, counteracting the entry of  $\text{Na}^+$  by an influx of  $\text{Ca}^{2+}$ , at the expense of an intracellular  $\text{Ca}^{2+}$  overload. This cytosolic calcium overload is further worsened at the time of reperfusion through dysfunction of the sarcoplasmic reticulum and leads to a mitochondrial calcium overload. This contributes, in conjunction with the excess of ROS production, to the activation of cell death via the release of pro-apoptotic molecules such as cytochrome C and proteins of the Bcl2 family such as Bad and Bax, as well as the opening of the mitochondrial permeability transition pore (mPTP) (figure 6).

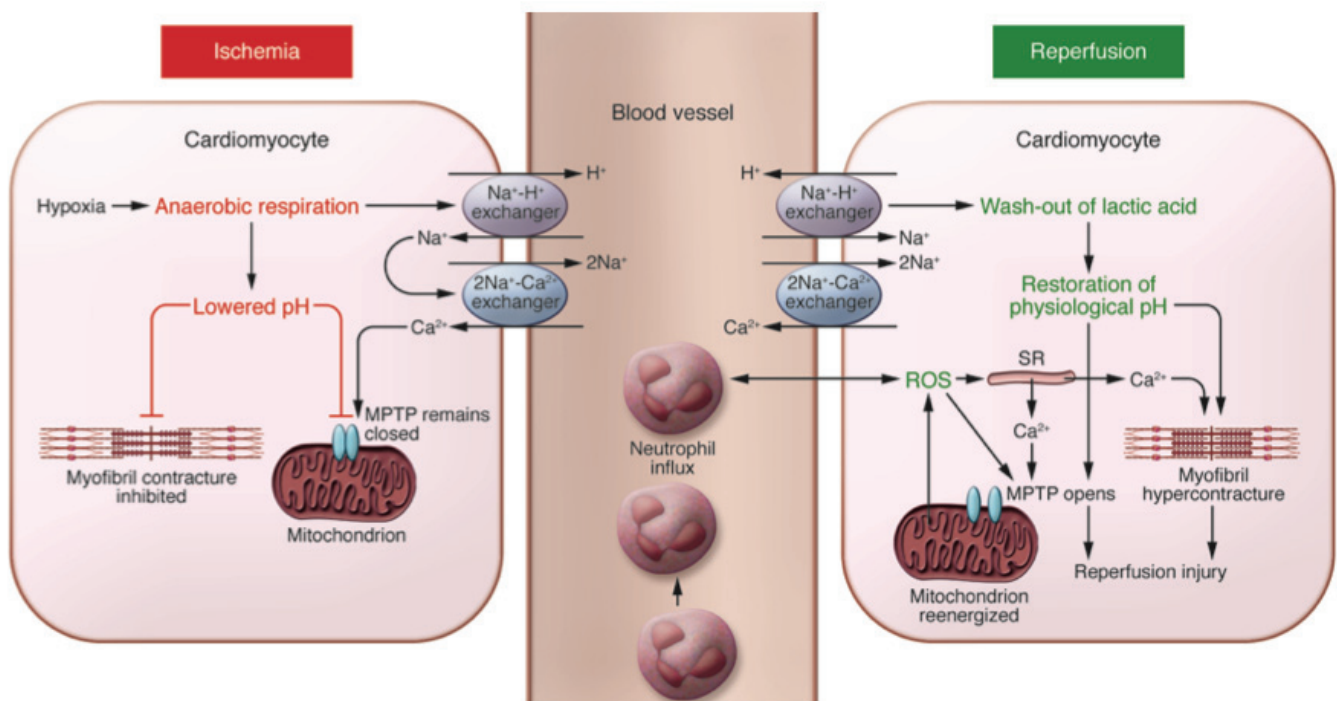


Figure 6. Synthetic view of ischemia and reperfusion consequences on cardiomyocytes. During ischemia, hypoxia triggers the anaerobic glycolysis leading to the acidification of the cytosol, a  $\text{Na}^+$  and a  $\text{Ca}^{2+}$  overload. At this point, the mPTP remains closed due to the acidic pH. At the time of reperfusion, restoration of physiological pH in conjunction with ROS production and sarcoplasmic reticulum dysfunction and thus worsened  $\text{Ca}^{2+}$  overload is responsible for irreversible mPTP opening leading to cell death

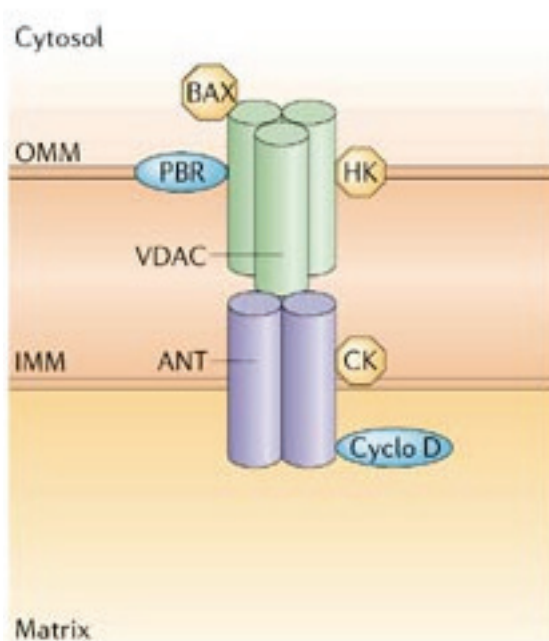
A key actor in reperfusion-induced injury is calcium. Reperfusion is responsible for important influx of  $\text{Ca}^{2+}$  in the cytosol, which are responsible for a great part of the arrhythmias observed during this period and for the phenomenon of myocardial stunning, i.e. the temporary absence of functional recovery despite effective reperfusion. Accumulation of  $\text{Ca}^{2+}$  in the cytosol leads to mitochondrial calcium overload, a substantial signal for cell death.

## **F. The mitochondrial Permeability Transition Pore**

The mPTP is a multi-proteic channel in the inner mitochondrial membrane whose composition is not fully known<sup>40, 41</sup>. It is believed that several proteins are key regulators of the mPTP: cyclophilin D, Adenine Nucleotide Translocase (ANT), Voltage Dependent Anion Channel (VDAC), Creatine Kinase (CK), Hexokinase (HK), Benzodiazepine Receptor (PBR) and several proteins of the Bcl2 family, but the exact composition of the mPTP remains debated (figure 7). This structure allows the transit of small molecules (with a molecular weight below 1.5 kDa) between mitochondrial matrix and the intermembrane space. It can open briefly in physiological conditions but its critical role appears to be in the regulation of cell death when it opens irreversibly, dissipating the mitochondrial membrane potential, releasing large amounts of  $\text{Ca}^{2+}$  and reversing the ATP synthase. When it opens, the matrix swells to the point that the outer membrane ruptures, releasing in the cytosol pro-apoptotic proteins such as Cytochrome C that were confined in the mitochondria.

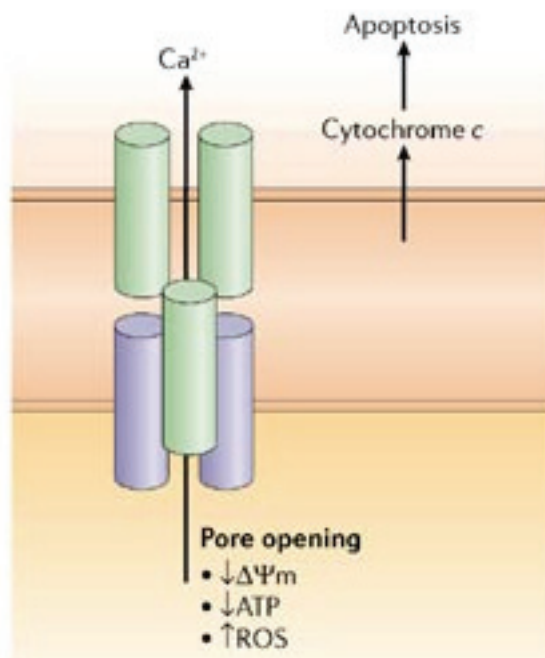
*Figure 7. Supposed composition of the mPTP. Cyclo D : Cyclophilin D ; ANT : Adenine Nucleotide Translocase ; VDAC : Voltage Dependent Anion Channel ; PBR : Benzodiazepine Receptor ; HK : Hexokinase ; CK : Creatine Kinase ; OMM : Outer Mitochondrial Membrane ; IMM : Inner Mitochondrial Membrane. In basal resting conditions, the channel remains closed. During stress, as a consequence of ROS production and  $\text{Ca}^{2+}$  overload, the pore opens, permeable to small molecules and ions such as  $\text{Ca}^{2+}$ , leading to mitochondrial depolarization, increased ATP consumption by the ATP synthase reversed, and further increase of the oxydative stress.*

## Resting



## Stress

- ROS and oxidative stress
- Increased  $\text{Ca}^{2+}$  concentration
- Misfolded mitochondrial proteins



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Its opening is triggered by several phenomena occurring at the time of reperfusion: mitochondrial calcium overload, excess of ROS production, alkalosis, and decrease of the ATP stocks. On the other hand, increasing its opening threshold allows to delay/prevent cell death and is at the basis of the latest cardioprotective techniques. By now, many agents/techniques showed their potential to impair mPTP opening through different direct or indirect mode of actions: Cyclosporine A (a common immunosuppressive molecule used in clinical practice in the prevention of graft rejection), benzodiazepines, many growth factors such as IGF-1 or VEGF, furanes (anaesthetic gas), morphine and ischemic pre and postconditioning<sup>42</sup>.

## **G. mPTP at the crossroads of cardioprotection: pre and postconditioning**

In 1986, Murry et al showed in a dog model of myocardial ischemia reperfusion that when a heart was submitted to 4 sequences of 5 minutes of ischemia followed by 5 minutes of reperfusion, it was significantly protected against a subsequent sequence of ischemia lasting 40 minutes as compared to a heart solely submitted to 40 minutes of ischemia followed by reperfusion<sup>43</sup>. This was defined as ischemic preconditioning. Later, other forms of ischemic preconditioning were defined, differing from the original description according to the delay between the preconditioning cycle(s) and the sustained ischemia, and allowing identification of 3 different windows of preconditioning that imply slightly different mechanisms. In the first classical form, the interval between the preconditioning algorithm and the ischemia doesn't exceed 2 hours and it relies on the activation of already existing signaling molecules. A second window, more sustained but less powerful, appears 24 to 72 hours after the preconditioning cycle and necessitates the synthesis of new signaling molecules<sup>44, 45</sup>. A Third window was described 6 hours following coronary microembolisms<sup>46</sup>. By extension, Zhao et al showed with a similar protocol that applying these short iterative sequences of ischemia reperfusion at the time of reperfusion was also protective against the index ischemia, thus defining the ischemic postconditioning<sup>47</sup>. These concepts have been widely studied and ischemic postconditioning, the most interesting concept in the clinical setting of AMI, was demonstrated to be efficient in most species including humans. In 2005, Staat et al. demonstrated, in a small cohort of patients with AMI reperfused by primary PCI, that 4 iterative 1-minute inflation and deflation of the angioplasty balloon immediately following desocclusion of the culprit artery was cardioprotective<sup>48</sup>. The patients in the postconditioning group displayed significantly smaller infarct size as assessed by CK release. The functional benefit was maintained 6 months after the AMI, as shown by Thibault et al, postconditioned patients displaying a better echographic left ventricular function and a smaller infarct scar as measured by <sup>201</sup>thallium single photon emission computed tomography<sup>49</sup>. But as promising as this technique can be, it has two main downsides. First the therapeutic window to perform this operation is narrow: past the first minute after reperfusion, its efficiency rapidly and significantly decreases. Second, in clinical practice, it is only achievable in patients reperfused by

percutaneous coronary intervention. While this method of reperfusion is the most common in the high-income countries, its use is still minority when we consider the worldwide population. The best compromise would be to develop a molecule that would achieve a pharmacological postconditioning and could be administered prior or concomitantly to any thrombolytic agent or to the percutaneous coronary intervention. While several molecules (adenosine, erythropoietin, atrial natriuretic factor, several isoforms of C-kinase protein and others) were shown to be able to inhibit the opening of the mPTP on the bench, only one by now has been able to achieve efficient pharmacological postconditioning both in all animal models and in the clinical practice: it was the CsA<sup>50-60</sup>. CsA, a far from new molecule, has two main targets in the cell: it inhibits the cyclophilin A, generating an immunosuppressive effect, but it also inhibits cyclophilin D, one of the main components of the mPTP. When cyclophilin D is absent, e.g. in knock out mice, or binded to CsA, i.e. unable to attach to the other components of the mPTP, the latter remains closed, achieving significant protection.

All these techniques aiming to reduce lethal reperfusion injury were shown to reduce infarct size in human by up to 40%. An ongoing international multicenter study tries to assess whether this reduced infarct size translates into an improvement in the clinical outcome of patients with AMI (CIRCUS trial, NCT01502774). A positive result would definitely legitimate the importance of targeting specifically lethal reperfusion injury.

Thus it appears that there's still a lot of room for research in cardioprotection in order to reduce the morbidity and mortality of patients exposed to myocardial ischemia and reperfusion. The fate of the ischemic cardiomyocyte is not irreversible and there are many potential therapeutic targets that could be considered.

## **H. Novel approaches of cardioprotection** <sup>61</sup>

Finding new ways to protect cardiomyocytes from an ischemic insult was the aim of this thesis and I chose to explore the field of cardioprotection with two different approaches.

### **1. First field explored: myocardial stem cell therapy**

This approach considered a technique that is widely used in other fields of medicine such as oncology: the stem cell therapy.

#### **a. Is it possible to regenerate myocardium?**

At all time, scientists considered that the ultimate way to cure AMI was to regenerate the myocardium. Preliminary works were encouraging and basic research quickly translated from bench to bedside with mainly deceiving results but lots of hope still drive research in this field.

The first major paper from Orlic et al., a short letter published in Nature in 2001, aimed to demonstrate that the injection of hematopoietic stem cell in a myocardial infarcted area could result in significant myocardial regeneration <sup>62</sup>. The authors used a C57/BL6 mouse model of myocardial infarction by coronary ligation and isolated hematopoietic cells from bone marrow, identified to stem cells according to their expression of c-kit (CD117, receptor of the stem cell factor and characterizing hematopoietic stem cells) and their absence of expression of lineage markers. The cells were purified, amplified and injected directly into the infarct scar 9 days after the ischemic event. Their results suggested that there was a significant regeneration of new myocardium in the infarcted area and that the newly formed cardiomyocytes derived from the implanted stem cells. Their justification for the origin of the new cardiomyocytes came from the fact that they displayed a Y chromosome and EGFP fluorescence, the stem cells originating from transgenic male mice expressing EGFP

while the experimental infarcted mice were wild type females. Several years and already a couple of clinical trials studying feasibility later, 2 articles studying myocardial stem cell therapy were published in the same issue of Nature<sup>63-66</sup>. Both research groups used a similar mouse model of myocardial infarction where purified and amplified hematopoietic stem cells were again injected directly into the infarct scar. Even if there was a mild improvement in contractile function, both failed to demonstrate transdifferentiation of the injected stem cells into cardiomyocytes. In both works, very few stem cells kept present in the area over time and most if not all adopted traditional hematopoietic fates. The doubt being raised about the transdifferentiation properties of stem cells, several others theories were established in order to explain the mild but still significant protective effect of stem cells administration, one of them being a paracrine effect. It was clearly demonstrated that stem cell injection into a post-ischemic myocardium resulted in a significant preservation of the extracellular matrix, an increase in neoangiogenesis and a decrease in apoptose of cardiomyocytes<sup>67, 68</sup>.

The hypothesis of a paracrine effect derived from the stem cell therapy was even more supported by the work from Togel et al. who demonstrated in a rat model of renal ischemia reperfusion that intra-aortic injection of mesenchymal stem cell at the time of reperfusion was responsible for a clear improvement in renal function and a decrease in renal lesions assessed by histology from as soon as 2 days following the injection<sup>69, 70</sup>. Such an improvement was considered to happen far too early to be the results of any kind of transdifferentiation, especially since the infused cells were shown to spend no more than a couple of hours in the renal parenchyma. Moreover, a similar improvement was observed in several studies after intravenous administration of various growth factors such as IGF1, VEGF, HGF or G-CSF<sup>71-73</sup>.

Over the years, several cell types were considered for myocardial stem cell therapy: foetal cardiomyocytes, skeletal myoblasts, adult cardiomyocytes, smooth muscle cells, hematopoietic stem cells and mesenchymal stem cells (MSC). While most of them gave interesting results regarding the function, MSC display particularly interesting properties that caught our attention. They are easy to isolate from bone marrow, easy to culture and identify. Allogeneic MSC generate little to no immunologic response when they are administered, being able to modulate T cell



proliferation and maturation<sup>74</sup>. This means a reduced risk of graft rejection, making them a perfect alternative to the technically challenging, expensive and time-consuming thus delayed administration of autologous stem cells. Moreover, MSC have a wide range of differentiation properties. In an appropriate extracellular environment, in vitro, they can differentiate into vascular smooth muscle cells or cardiomyocytes, as shown by Fukuda et al, after exposition to 5-azacytidine. Tomita et al. even used autologous MSC pre-treated with 5-azacytidine prior to intra-myocardial injection in a rat model of myocardial infarction. They demonstrated a significant improvement in heart function only in the group that received 5-azacytidine pre-treated cells, with more cells in this group acquiring a cardiac muscle-like phenotype<sup>75</sup>. Another candidate for myocardial stem cell therapy was recently described: cardiac stem cells. These cells, identified in many species, are resident cells in the heart but they express stem cell markers such as c-Kit, Sca-1 or Isl-1. Research mainly focused on their differentiation properties but recent data report paracrine properties involving growth factors<sup>76-81</sup>.

During the last decade, many clinical trials aimed to demonstrate the efficacy and safety of myocardial stem cell therapy, generally through intra-coronary infusion of autologous bone marrow hematopoietic stem cells from 3 to 10 days after a successfully reperfused AMI. A 2007 meta-analysis summarized the main results of these studies, which translated into a mild but still statistically significant increase in the left ventricular ejection fraction of 3.66% and decrease in infarct size of up to 5.49%<sup>82</sup>. While more and more researchers agree that for now this improvement doesn't correspond to clinically significant myocardial regeneration, the mechanism and signaling pathways behind it are still obscure and need to be thoroughly addressed if we want stem cell therapy to be part of our therapeutic arsenal in the next years.

b. How does it work?

In the current work, we emitted the hypothesis that MSC were cardioprotective in a cellular model of simulated ischemia reperfusion, and that this cardioprotection was mediated by a paracrine mechanism. The cellular model used

was neonatal rat ventricular cardiomyocytes. This primary cell culture has the advantage of being closer to physiology than cardiac cell lines such as HL-1 cells or H9C2 cells. They can be kept in culture longer than adult rat cardiomyocytes (6 to 7 days versus up to 24 hours), leaving more time to perform complete range of experiments on a single cell preparation, ensuring improved comparability. We sought for the mechanisms underlying this paracrine protection and searched for agents possibly involved such as VEGF, IGF-1, and HGF according to the literature. Last, we addressed the signaling pathways activated, with our attention focused on the RISK pathway, assuming that this protection is postconditioning-like and allows reduction of lethal reperfusion injury.

The RISK (Reperfusion Injury Salvage Kinase) pathway was well described in recent years as it is strongly involved in the protection generated by ischemic pre and postconditioning and many pharmacological agents such as glucagon-like peptide 1, erythropoietin, atrial natriuretic peptide and even atorvastatin <sup>83</sup>. This pathway is comprised of 2 distinct signaling cascades, both of which are implied in cell survival. The cascade we focused on here is the phosphatidylinositol-3-OH kinase (PI3K)-Akt, as opposed to the p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), as it is strongly linked to mitochondria. The PI3K-Akt cascade can be activated in response to the activation of a wide range of receptors such as those for growth factors like IGF-1 and G-protein coupled receptors <sup>84</sup>. Activation of PI3K-Akt leads to numerous phosphorylation in the cell including glycogen-synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), apoptotic proteins such as Bad, Bax, p53 and others, protein kinase C (PKC), endothelial Nitric Oxide Synthase (eNOS), proteins involved in the glucose metabolism (GLUT 4) and in the transcription processes <sup>85</sup>. One of the most notorious effects of the PI3K-Akt activation is the reduction of the opening probability of the mPTP (figure 8) <sup>86</sup>.

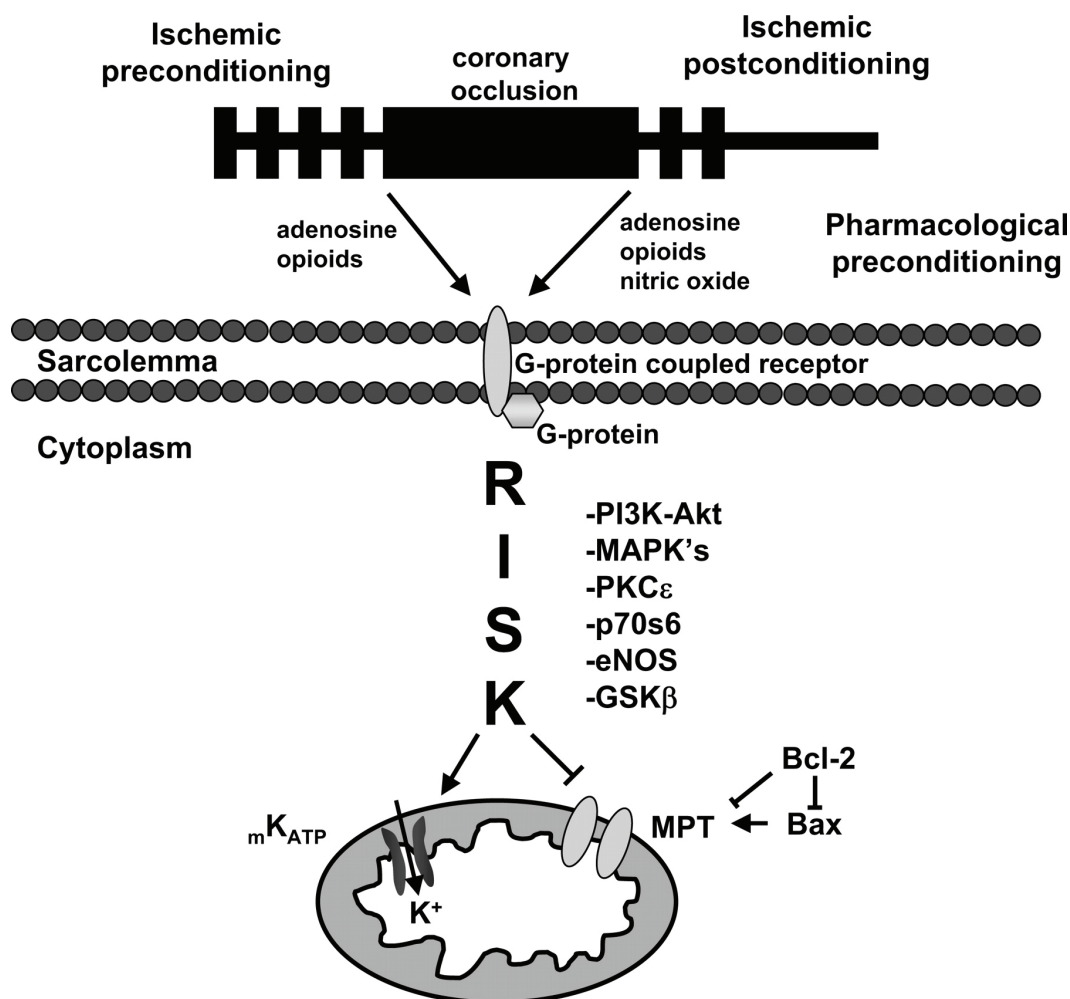


Figure 8. Ischemic pre and postconditioning leads to RISK activation through G-proteins second messengers. Several proteins compose the RISK pathway among which PI3K, Akt, GSK3 $\beta$ . The RISK pathway activation leads to K<sub>ATP</sub> channels activation and inhibition of the mPTP.

2. Second field explored: reduction of cell death through better control of the metabolism

The second approach of cardioprotection that I studied for my thesis aimed to reduce the ischemia-induced lesion. In these conditions, its potential clinical application concerns probably less situations such as AMI but more situations when

the myocardium suffers a scheduled ischemia, e.g. during cardiac surgery implying the use of extracorporeal circulation or in the context of organ transplantation.

As explained above, during ischemia, the failure to supply oxygen to the cell, particularly to the mitochondria, is responsible for the blocking of the respiratory chain. As a result, the mitochondrial membrane potential dissipates and the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase starts to work in reverse mode, hydrolyzing ATP instead of synthesizing it, and generating a proton flux from the matrix towards the intermembrane space in order to try to maintain the membrane potential. This reversal of the enzyme is not only inefficient, but is also responsible for a wastage of ATP, accelerating the consumption of the ATP and, if ischemia is maintained, the cell death.

a. Structure of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase

This key enzyme in the eukaryote cells is composed of two domains:

\_ The F<sub>o</sub> domain contains the subunits a, b<sub>2</sub> and c<sub>12</sub>. It is a transmembrane domain that is normally responsible for the translocation of protons from the intermembrane space toward the mitochondrial matrix.

\_ The F<sub>1</sub> domain is localized on the matrix side of the intermembrane space, connected to the F<sub>o</sub> domain, and contains the subunits  $\alpha$ ,  $\beta$  (3 of each),  $\gamma$ ,  $\delta$  and  $\epsilon$ . It constitutes the catalytic domain.

F<sub>1</sub> and F<sub>o</sub> are attached together through a central stalk of subunits  $\gamma$  and  $\epsilon$ . They are surrounded by the three alternating  $\alpha$  and  $\beta$  subunits forming a cylinder. Between each of these  $\alpha$  and  $\beta$  subunits are the binding sites of the nucleotides, three of which being catalytic. This cylinder, in addition to the  $\delta$ , a and b<sub>2</sub> subunits, constitutes the stator of the enzyme, around which rotates the rotor made of the subcomplex of subunits  $\gamma$ ,  $\epsilon$  and c<sub>12</sub>. The synthesis of ATP from the phosphorylation of ADP implies a unidirectional clockwise rotation of the catalytic rotor, specifically the  $\gamma$  subunit that will contribute to couple the nucleotide-binding catalytic sites to the proton channel (figure 9).

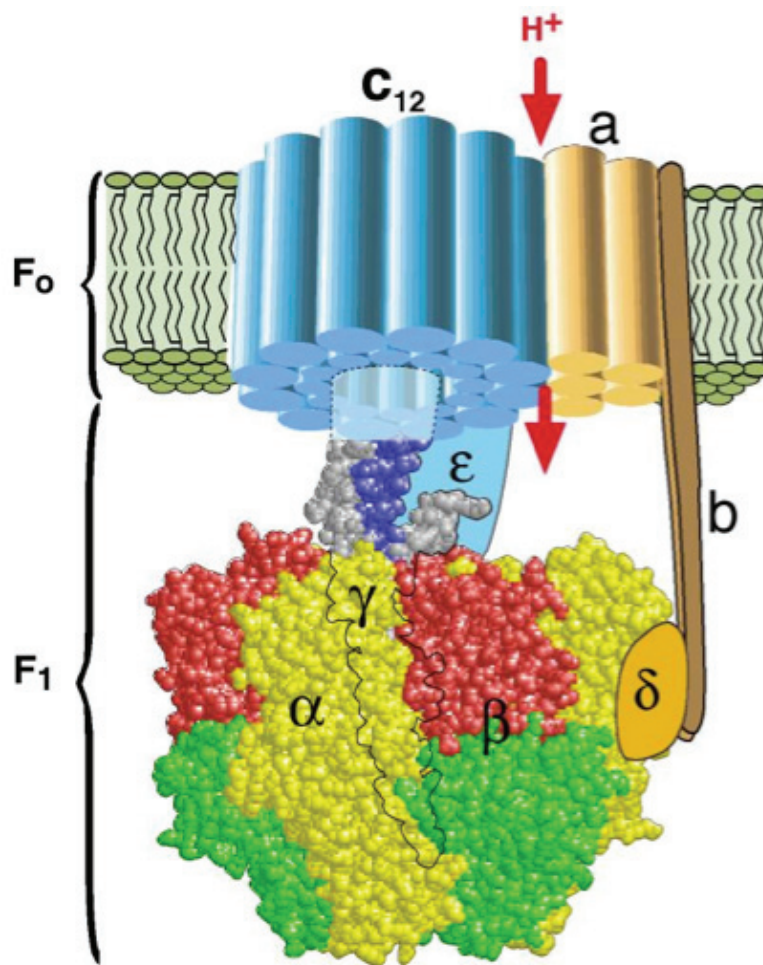


Figure 9. Spatial representation of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase, its F<sub>1</sub> and F<sub>o</sub> domains and their various subunits.

a. IF1, a natural cardioprotective molecule

This F<sub>1</sub>F<sub>o</sub> ATP synthase is subjected to regulation, primarily through the level of ATP and ADP available in the cell. The Inhibitor protein of the F<sub>1</sub> domain (IF1), a small (10 kDa) endogenous nuclear encoded protein, is a major regulator of the ATP hydrolase<sup>87</sup>. This heat-stable proteins binds to the F<sub>1</sub>F<sub>o</sub> ATPase in conditions when it hydrolyses ATP in the absence of a proton gradient and when the pH is acidic, the optimum being 6.7, i.e. all the conditions encountered during ischemia<sup>88</sup>. This highly sequence-preserved protein is found in all living animals but it is expressed in varying quantities according to the species. A difference is clearly made between fast-heart rate species and slow heart rate species<sup>29</sup>. Fast heart rate

species, i.e. mostly rodents, with the exception of guinea pigs, have a low expression level of IF1 and so should be less resistant to ischemia compared to the slow heart rate species whose levels of IF1 are higher. It is found in all tissues but was first discovered in the heart and again, the expression level of IF1 varies according to the tissue considered. All this makes IF1 a potential major actor in the physiopathology of ischemia, but it appears to play a significant role, due to the very same properties, in the physiopathology of tumors<sup>89</sup>. This is explained by the fact that conditions similar to those encountered in ischemia can be found in tumors and peri-tumoral areas, the oxygen levels decreasing from the perivascular regions to the necrotic centers. In these cases, IF1 would contribute to the cancer cell survival and thus the development of the tumor.

During ischemia, the ATP wastage through F1Fo ATP synthase reversal accounts for the majority of the ATP consumption in the cell as most of the other consumers are silenced in an attempt to preserve as much ATP as possible. The binding of IF1 to the F1 subunit is capable of reducing this wastage by up to 90% and it has been shown that overexpression of IF1 was responsible for a significant reduction in the degree of cell injury in a hypoxia reoxygenation cell model<sup>90</sup>. Gene therapy isn't yet a mature therapeutic option in the clinical practice and the possible implication of IF1 in cancer physiopathology would raise concerns about such a technique. But this work demonstrates that being able to punctually block the reversal of the ATP synthase would be a relevant therapeutic option for it would result in significant preservation of ATP and reduction of ischemic cell death. There comes the idea of pharmacologically (and specifically) inhibit the reversal of this enzyme in ischemic conditions.

a. A usable pharmacological alternative to IF1?

There are 2 known classical molecules able to inhibit the reverse activity of the ATP synthase: oligomycin B and aurovertin B<sup>91, 92</sup>. The major problem with these drugs is that they are not specific of the reverse activity of the enzyme and inhibit the synthesis of ATP as well. Thus, the challenge is to synthesize a small molecule able to mimic the effect of IF1 without compromising ATP synthesis. Grover et al. reported in 2004 several benzodiazepine-derived molecules amongst which the compound BMS199264<sup>93, 94</sup>. They showed in an isolated rat heart model that this compound

could reduce, by selective inhibition of the reverse activity of the ATP synthase, ischemia-induced lesions as assessed by the time to the onset of ischemic contracture, the level of LDH release and the measure of ATP concentrations. However, no subsequent work on this compound, or others, was published, raising doubts about the safety of the molecule on others systems, or only traducing the shutdown of the program by the BMS company.

In this work, we sought to identify molecules with an IF1-like effect, based on the structure of compounds published by Grover et al., and tested whether they were really specific of the ATP hydrolase activity, whether they were safe regarding the cellular metabolism in general and could be cardioprotective in a cellular model of simulated ischemia reperfusion.

The various compounds tested were made by Mr Vincent Gray and Dr Jon Wilden from the department of pharmacology of University College London in the United Kingdom based on the published BMS compounds. 2 others compounds, identified as potential interesting drugs using a computational model taking into account the tridimensional geometry of the ATP synthase, were purchased by Dr Michelangelo Campanella from the French company Ambinter in order to be tested in the same model.

All compounds were tested on HL-1 cardiac cell line for they are easy to culture, transfect and still retain a cardiac phenotype, particularly the ability to contract. Several concentrations were tested in basal conditions and in conditions when the F1Fo ATP synthase works in reverse mode (pharmacological reversal). The most promising candidates underwent experiments aiming to demonstrate efficient preservation of the stocks of ATP and finally to delay/reduce ischemic cell death in simulated in vitro ischemia reperfusion assays.

# METHODS

## A. Part one: myocardial stem cell therapy

### 1. Rat bone marrow MSC isolation and culture

All experiments on animals were practiced in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised in 1996 and 2011) <sup>95</sup>. The experimental protocol was approved by our University ethics review board.

We used the technique described by Pittenger et al. to isolate and culture bone marrow MSC <sup>74</sup>. Rapidly, a male adult wistar rat was anesthetized with pentobarbital (50mg/kg) and sacrificed. The femur bones were surgically harvested under a laminar flow hood with cautious asepsis measures, the epiphyses were removed and the bone marrow flushed using cold sterile Phosphate Buffered Saline (PBS) into a culture medium DMEM F12. The cell suspension was then filtered using a 70µm filter and resuspended in a Petri dish containing warm complete culture medium prepared as follow:

Product	Volume necessary to prepare 100mL of complete culture medium
D-MEM/F-12 (available from Invitrogen)	90 mL
Foetal Bovine Serum (FBS, available from Sigma)	9 mL
Penicillin/Streptomycin (available from Invitrogen)	1 mL



The dish was placed into a humidified normoxic incubator with 5% CO<sub>2</sub>. The culture medium was replaced every 3 days to remove the cells that didn't attach to the plate, corresponding to blood cells and hematopoietic stem cells. When the cells reached confluency, they were trypsinized. This sequence consisted in washing twice the cells with warm PBS then incubating them with warm trypsin 0.25% EDTA (1 mL per 10 cm Petri dish) for 5 minutes in the incubator. The dish was rapped to dislodge any cells remaining adherent and the trypsin was inactivated by addition of 10 mL of complete culture medium followed by iterative pipetting up and down. The cells suspension was placed into a 15 mL centrifuge tube and then centrifuged at 1800 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 10 mL of complete medium. The next step consisted in counting the cells using trypan blue and a haemocytometer and plating them at the desired density.

After three passages, the culture was considered purified, the culture medium was replaced on a weekly basis and the cells trypsinized every two weeks. The cells were rapidly tested to ensure that they expressed characteristics of MSC.

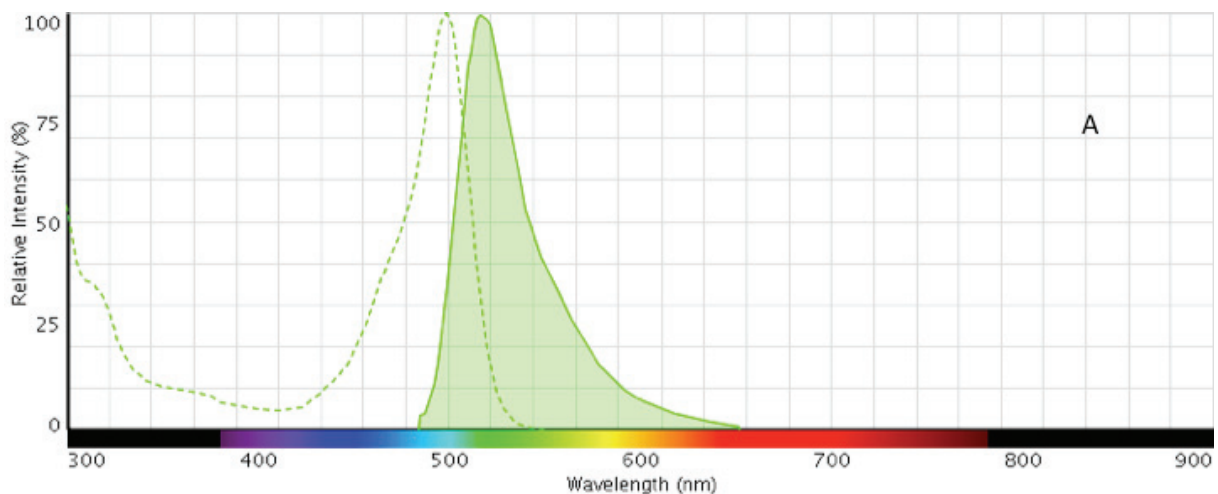
One of the tests conducted consisted in transdifferentiating the MSC into other cell types such as osteocytes and adipocytes. This was achieved by Dr Denis Angoulvant by culturing the cells in a complete culture medium in addition to:

\_ dexamethasone, b-glycerol phosphate and ascorbic acid for differentiation in osteocytes, followed by measure of the expression of alkaline phosphatases

\_ 1-methyl-3-isobutylxanthine, dexamethasone, insuline and indomethacine for differentiation in adipocytes. The effective differentiation in adipocytes was confirmed by the apparition of lipidic vacuoles as observed using a microscope with a x20 objective, and the expression of Peroxisome Proliferator-Activated Receptor  $\gamma$ 2 (PPAR $\gamma$ 2) and of Lipoprotein Lipase (LPL).

The second series of tests aiming to ensure the MSC phenotype was the immunophenotypage of the cells for several differentiation clusters: CD 45, CD 31, CD 90, CD 34 and CD 29. CD 31 (Clone TLD-3A12 from Becton Dickinson) and CD 34 (Clone ICO-115 from Santa Cruz Biotechnology) were conjugated with phycoerythrin (PE, a red fluorescent dye), CD 29 (Clone Ha2/5 from Becton Dickinson), CD 45 (Clone OX-1 from Becton Dickinson) and CD 90 (Clone OX-7 from

Becton Dickinson) were FITC (Fluorescein Isothiocyanate, a green fluorescent dye)-conjugated (figure 10). The protocol consisted in trypsination of the cells according to the usual protocol except that they were resuspended in warm PBS after centrifugation at a concentration of 1 000 000 cells/mL. For each antibody 1 mL of the cell suspension was considered. The cells were washed with cold PBS then centrifugated at 1700 rpm at 4°C for 5 minutes. The supernatant was removed and the pellet resuspended with 1 mL of PBS containing 2% of FBS, then again centrifugated for 5 min at 1700 rpm at 4°C. 2 µL of the antibody solution was added on the pellet after removal of the supernatant and the cells were incubated after resuspension for 30 min in the dark at 4°C. Isotype-identical antibodies groups were prepared as controls. After incubation, the cells were washed with PBS, centrifugated and resuspended in 1 mL of Paraformaldehyde (PFA) 1% and kept away from light at 4°C until analysis by fluorescence-activated cell sorting (FACScan flow cytometer from Becton Dickinson). This part of the work was achieved in collaboration with Dr Serge Nataf.



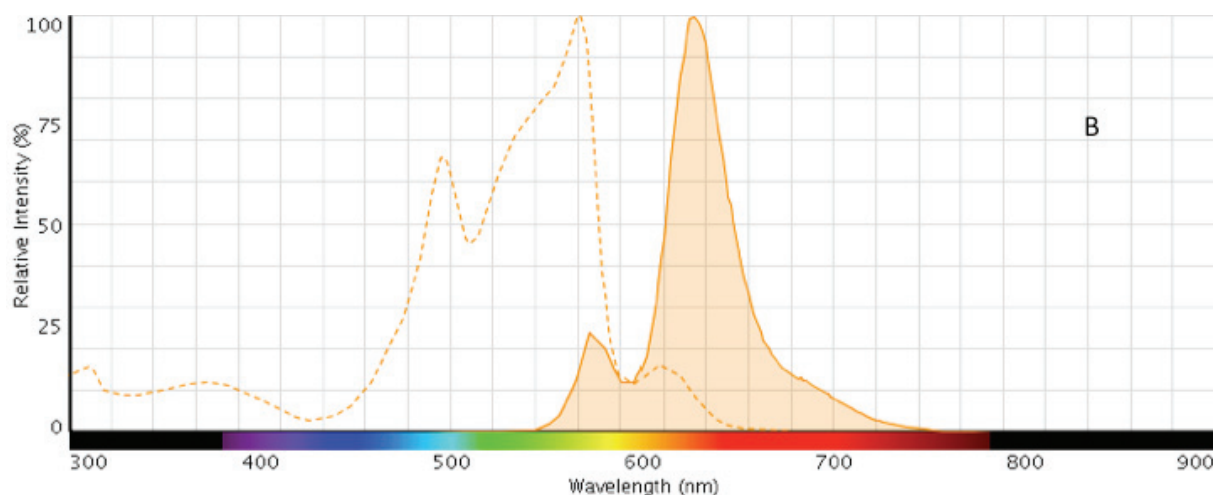


Figure 10. Excitation (dashed lines) and emission (plain lines) spectra of FITC (A: green) and PE (B: orange).

After confirmation of the MSC phenotype, several vials of MSC were frozen and stored in a liquid nitrogen tank for future use. The freezing medium was made of 50% of FBS, 7.5% of Dimethyl Sulfoxide (DMSO) and culture medium.

## 2. Isolation and culture of neonatal rat ventricular cardiomyocytes

2-days old wistar rats were sacrificed by decapitation. Their body was plunged for 20 seconds in alcohol 70°, then, in a laminar flow hood, under strict asepsis, the thorax was transversally sectioned and the ventricles harvested and put in a cold calcium and magnesium-free PBS solution. The hearts were generally still beating when the ventricles were exposed as a criterion for a quick and efficient harvest. The hearts were then finely minced using small scissors during 10 minutes in the cold PBS and then, after 3 washes with cold PBS, submitted to 9 sequential 10-minutes trypsinations at 37°C with a 0.3% trypsin solution. The result from the first trypsination was discarded but the supernatant from each subsequent trypsination was saved in 4 50mL centrifugation tubes containing each 15 mL of warm complete culture medium (the same medium as for MSC) to inactivate the trypsin. After the last trypsination was performed, the content of the trypsinator was transferred into a

homogenizer and the last remaining pieces of hearts were homogenized twice and put in the 4 50 mL tubes. The content of the 4 tubes was then filtered using 70µm cell strainers. The cells were centrifugated during 10 minutes at 2500 rpm and the pellets were resuspended in 20 mL of complete medium and plated in two 100 mm petri dishes. The dishes were left in a normoxic incubator for 150 minutes to allow differential attachment of the fibroblasts that represent the majority of the cells in the dishes. The medium in the dish containing the cardiomyocytes and now very few fibroblasts as they have attached to the plates was gently harvested. The cells were centrifugated during 10 minutes at 2500 rpm, resuspended in 20 mL of new complete medium, counted using a haemocytometer and then plated in 6 or 12-wells plates at the desired concentration, usually 200 000/ cm<sup>2</sup>. In general, 30 rats provided approximatively 90 000 000 cells. The culture medium was subsequently replaced every 2 days (2 mL per well of 6-wells plate, 1.5 mL per well of 12-wells plate).

### 3. Ischemia/reperfusion of neonatal rat ventricular cardiomyocytes

Simulated in vitro ischemia/reperfusion experiments were performed using a non-commercially available chamber designed according to our instructions by a local company named Verretech® (figure 11). It was an airtight glass chamber with 2 gas port allowing continuous flowing with a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub>. The temperature was maintained constant at 37°C by convection using a water bath with circulating water system and the gas flowed was heated through bubbling into warm water, which allowed humidification of the chamber in order to avoid evaporation of the medium in the culture plates. Temperature inside the chamber was monitored with a thermometer as well as oxygen and pH levels using an oxygen electrode coupled to a pH meter (DO-166-NP, Lazar Research Laboratories).

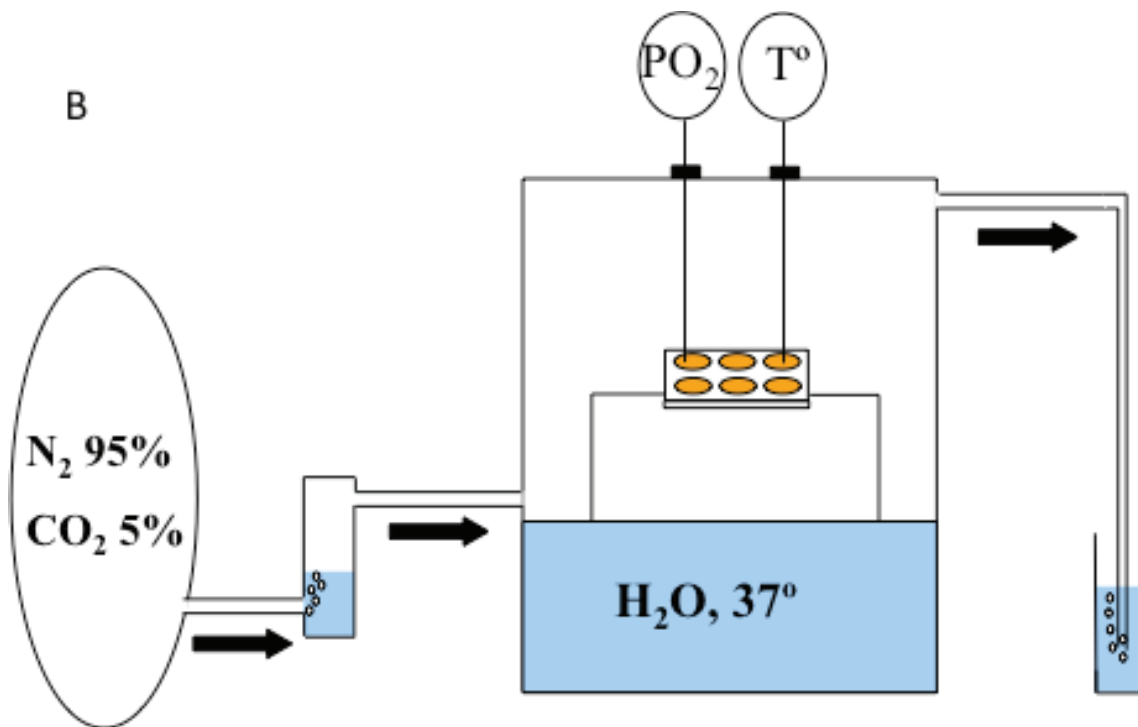


Figure 11. A: Photograph of the hypoxia chamber from Verretech. B: Schematic representation of the system.

To achieve ischemia and not just hypoxia, the culture medium was replaced by warm PBS after 2 washes. Monitoring of the oxygen concentration in the medium showed that oxygen level fell below 10% of the initial concentration (that is 21%) in less than 20 minutes and the pH was reduced to 6.36 after 90 minutes and kept stable during the rest of the ischemia (figure 12).

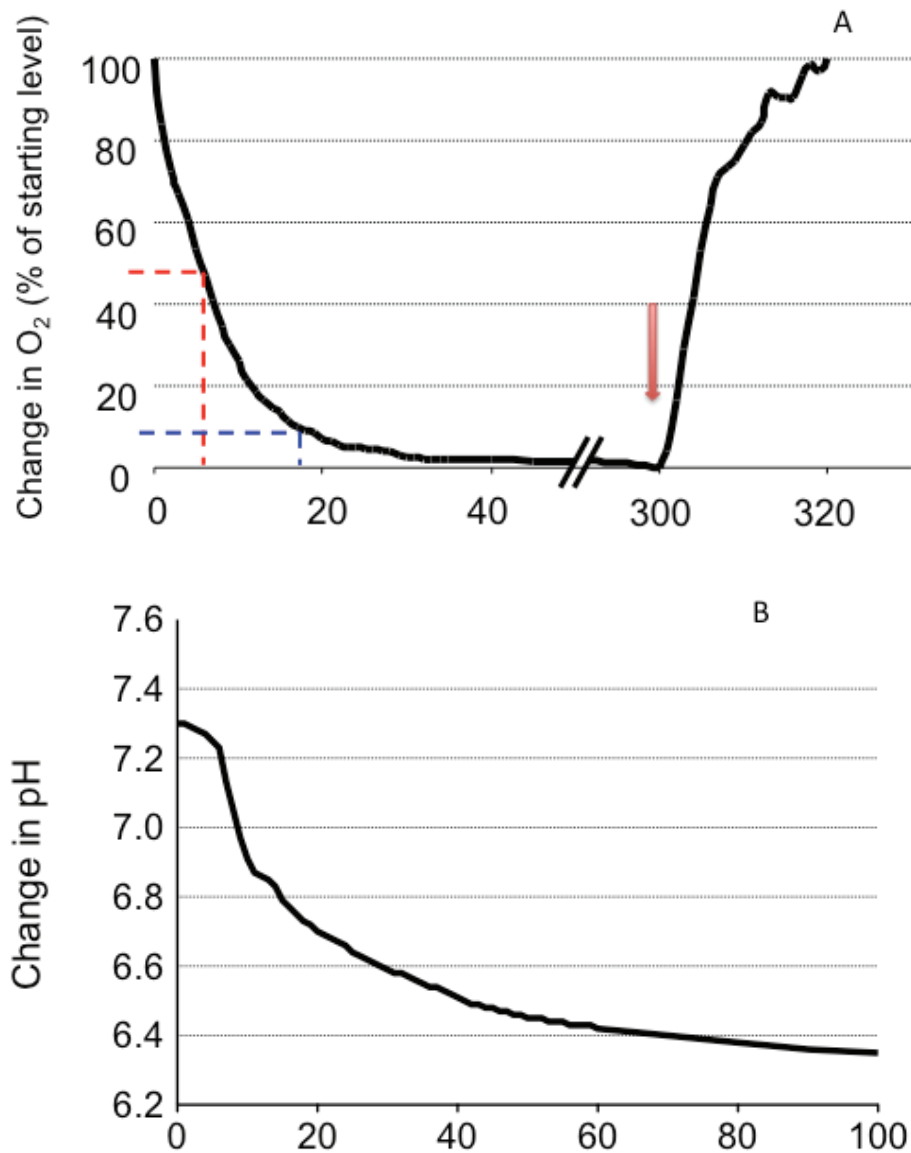


Figure 12. Change in medium during ischemia over time (in minutes). A: change in O<sub>2</sub> dissolved in the medium and expressed as percent of the value in basal normoxic conditions. Red arrow shows the start of reperfusion. B: Change in pH.

Set up experiments identified the optimal ischemia duration with this system as 5 hours (figure 13A).

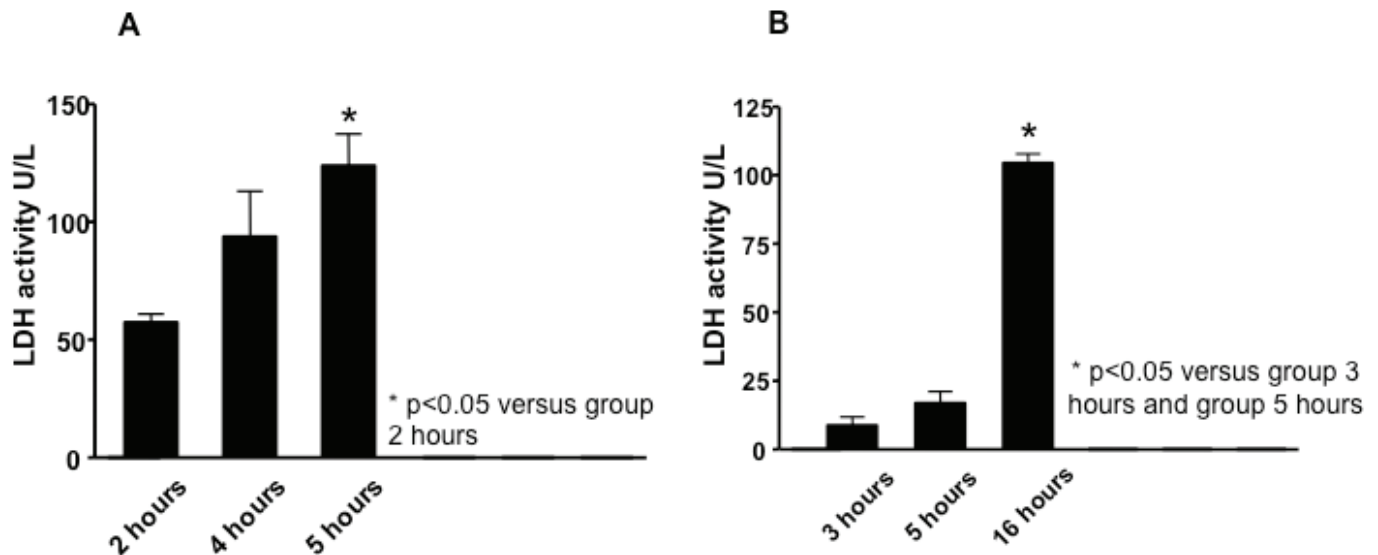


Figure 13. Determination of the optimal duration of ischemia and reperfusion with the Verretech system. A: Variations of LDH activity at the end of reperfusion after different length of ischemia. B: Variations of LDH activity at the end of reperfusion after different length of reperfusion following a 5 hours ischemia.

Reperfusion was achieved by adding to the ischemic medium new D-MEM/F-12 (without FBS as the growth factors contained in it can be cardioprotective) or the MSC conditioned medium in a 1:1 proportion and the plates were replaced in a normoxic incubator. The optimal reperfusion duration that we determined with this system was 16 hours (figure 13B).

The experimental protocol considered 7 different groups (figure14):

\_a sham group that wasn't submitted to ischemia and was kept for 21 hours in the normoxic incubator in D-MEM/F-12,

\_a control group,

\_7 groups with addition of drugs at the time of reperfusion.

One of these groups was a positive cardioprotection control and the drug added was Cyclosporine A at the concentration of 200 nM prepared from Sandimmun (Novartis laboratory) as this preparation contains cremophore, a solvent that ensure penetration and intracellular transportation of the CsA <sup>96</sup>. Two groups received either MSC conditioned medium or freshly trypsinized MSC in D-MEM/F-12. Two groups received MSC conditioned medium with either Wortmannin 100 nM (Calbiochem) or LY294002 15  $\mu$ M (Sigma) <sup>97, 98</sup>. Two last similar groups received MSC with either one or the other of the previous drugs. Wortmannin and LY294002 are 2 inhibitors of PI3K used to determine the implication of the RISK pathway here: wortmannin is a natural competitive inhibitor of the enzyme whereas LY294002 is a synthetic non competitive inhibitor. Naturally the potential toxic effects of these drugs were evaluated on sham.



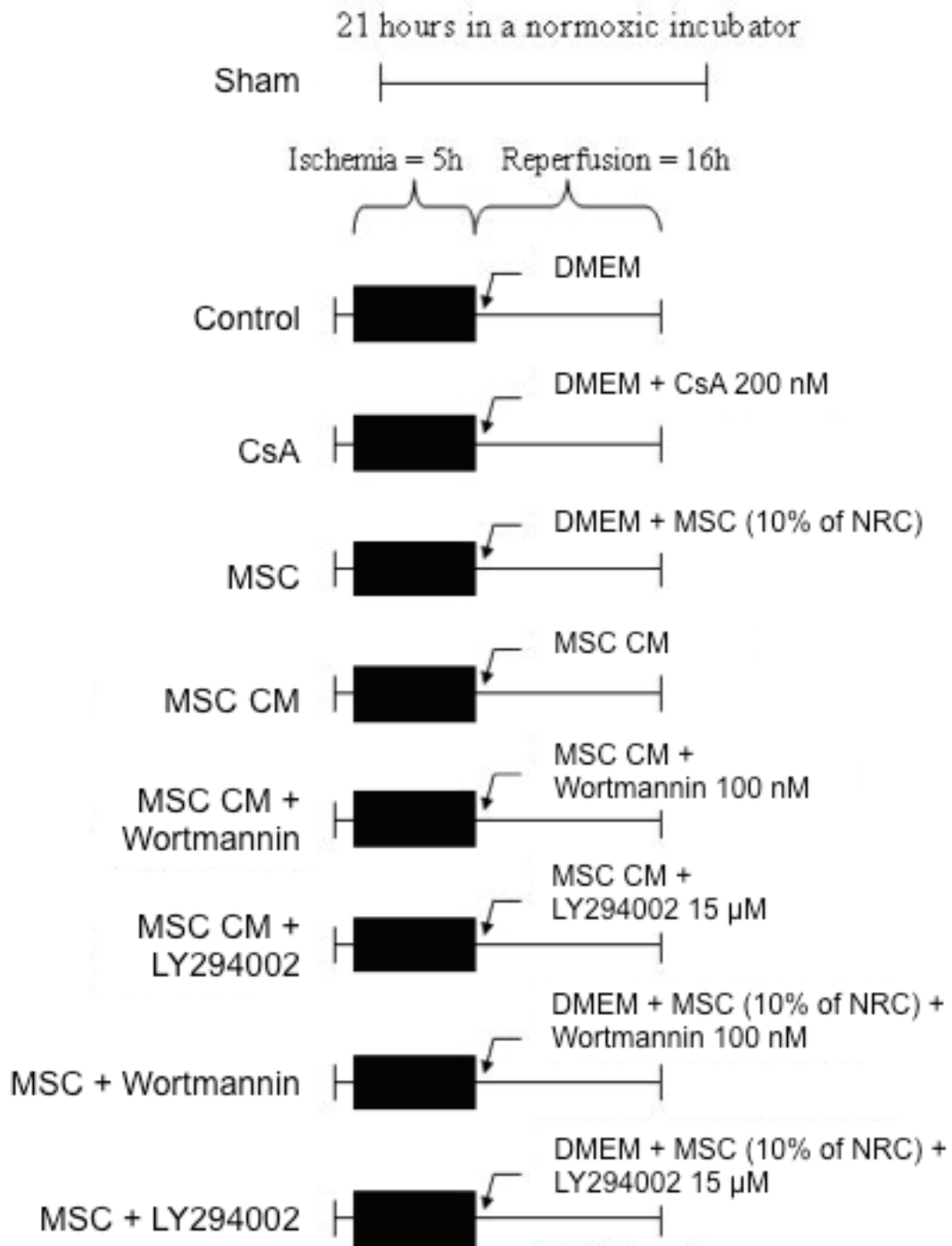


Figure 14. Experimental groups for the *in vitro* simulated ischemia/reperfusion. CsA: Cyclosporine A; MSC: Mesenchymal Stem Cells; MSC CM: MSC Conditioned Medium; DMEM: Dulbecco's Modified Eagle Medium; NRC: Neonatal Rat Cardiomyocyte.

#### 4. Preparation of MSC and MSC conditioned medium for the ischemia/reperfusion experiment

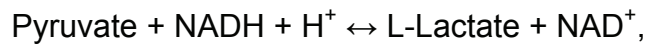
Shortly before the end of ischemia, a 100 mm petri dishes containing near confluent MSC was trypsinized according to the standard protocol. After centrifugation, the cells were resuspended in D-MEM/F-12 without FBS, counted using a haemocytometer and diluted at the desired concentration before addition to the cardiomyocytes at the time of reperfusion. The number of MSC added empirically represented 10% of the total number of cardiomyocytes plated.

The MSC conditioned medium was prepared 8 hours before the beginning of reperfusion: a 100 mm petri dish containing nearly confluent MSC was washed twice with warm PBS and 6 mL of D-MEM/F-12 without FBS was added to the cells. The dish was replaced in the normoxic incubator for 8 hours. At the time of reperfusion, the medium was removed, placed in a 15 mL centrifugation tube and centrifuged at 1800 rpm for 5 minutes before addition to the cardiomyocytes. The objective of this last centrifugation was to avoid as much as possible addition of debris.

#### 5. Assessment of mortality of cardiomyocytes after reperfusion

We considered 2 markers of mortality that could be measured in the medium in the plates at the end of reperfusion: LDH activity and troponin I.

LDH is a ubiquitous enzyme essential in the metabolism of carbohydrates, present in many different tissues amongst which the myocardium that contains mainly the isoform 1. LDH activity is a common non-specific marker of cell death: this intracytoplasmic enzyme can be excluded from the cell as a result of significant cell injury leading to necrosis. It is measured by spectrophotometry using a commercially available kit of measurement (Enzyline ® LDH optimized 10 from Biomerieux Laboratories) and a U-2800 Spectrophotometer (Digilab® Hitachi). This technique was based on the reaction:



using pyruvate as a substrate and the autofluorescence of NADH as a mean of detection. Rapidly, we took samples of the medium of cardiomyocytes at the end of reperfusion in the different experimental groups and stored them immediately in a -80°C freezer to avoid degradation of LDH. At the time of measurement, 100 µL of sample was added to 1 mL of the kit reagent at 37°C and the optical density of the solution at 340 nm was measured during 1 minute (n). The level of LDH activity was determined according to the mean slope of decrease of the optical density (corresponding to the consumption of NADH) using the formula  $U/L = n \times 8095$ , the slope being steeper proportionally to the level of LDH activity. The lower limit of detection of the kit is 18 U/L, the reaction being linear up to 800 U/L.

The second marker of cardiomyocyte necrosis assessed was the release of troponin I in the medium. Troponin I is a protein involved in the contraction of cardiomyocytes and is specific to this cell type. This protein is commonly used in clinical practice for the definition and detection of myocardial infarction as its blood level increases above the detection threshold in case of significant myocardial injury. We used the technique available at Lyon Louis Pradel University Hospital, thanks to the biochemistry laboratory (in particular Dr Regine Cartier) whose members carried on the measurements using an ARCHITECT i2000SR from Abbott Diagnostics on the same conditioned medium than for the LDH measurements, i.e. at the end of reperfusion.

We also used a complementary assay allowing evaluation of the viability of our cardiomyocytes at the end of reperfusion. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue) is a molecule that colors the solutions in yellow. In the presence of living cells, MTT is cleaved into an insoluble purple formazan by their deshydrogenase enzymes. This formazan, once resolubilized by a solvent, e.g. isopropanol, can be measured spectrophotometrically at 550 nm, giving indirect information about the viability of the cells when the measured absorbance is compared to that of similar positive and negative control preparations. We used a MTT kit from Sigma and used the MTT at 5 mg/mL according to the manufacturer's instructions. MTT was added directly on the cardiomyocytes at the end of reperfusion, after removal of the supernatant for the measurements of LDH activity

and troponin I. After an incubation of 24 hours, a 1:1 mix of isopropanol and DMSO was added in the wells for 1 hour before the solution was removed and vortexed during 3 seconds. 100  $\mu$ L of the solution was added in a new 96 wells-plate for spectrophotometric analysis (same U-2800 Spectrophotometer as for the assessment of LDH activity with an appropriate excitation setting). The measurements were done in triplicate for each well and the mean of the 3 measures was considered for statistical analysis, after subtraction of the blank.

#### 6. Attempts to determinate the protective factors involved in the MSC conditioned medium

We considered several growth factors, known for their protective effects against ischemia-reperfusion injury and their ability to activate the RISK pathway, as potential mediators of the protection afforded by MSC conditioned medium. These factors were VEGF, IGF-1 and HGF. We determined whether the MSC conditioned medium contained any of these factors in significant concentration, which could provide us with a relevant hypothesis regarding the phenomena implied here. We used an ELISA (Enzyme-Linked Immunosorbent Assay) technique to measure these factors using a rat VEGF ELISA kit (Quantikine) from R&D Systems, a rat/mouse IGF-1 ELISA kit from IDS and a rat HGF ELISA kit from B-Bridge. The measurements were performed on MSC conditioned medium as well as on freshly resuspended MSC and on fresh D-MEM/F12 as a negative control according to the manufacturer's instructions.

According to the results obtained from these experiments, we considered two additional groups for our experimental protocol, that is a group receiving MSC conditioned medium at the time of reperfusion with an anti-VEGF antibody (anti-rVEGF, R&D Systems, 10 $\mu$ g/mL), and another group receiving MSC conditioned medium and an anti-IGF-1 antibody (anti-IGF-1, clone Sm 1.2, Upstate, 50 $\mu$ g/mL) (figure 15).



Figure 15. Last two groups of the simulated ischemia/reperfusion experiment (same conditions as figure 13). MSC CM: Mesenchymal Stem Cells Conditioned Medium; VEGF: Vascular Endothelial Growth Factor; IGF-1: Insuline-like Growth Factor 1.

#### 7. Ex vivo isolated rat heart experiment

The last part of our research work on MSC was performed by a fellow of our research team, Carine Nemlin, trained in Langendorff experimentation (figure 16). This technique consists in anaesthetizing a male wistar rat weighing 350 to 450g and harvesting their beating heart after heparinization. The hearts were rapidly mounted on a perfusion system through canulation of the aorta and perfused with a cold Celsior solution for 1 minute to achieve cardiac arrest. Global ischemia was then obtained by immersing the hearts during 8 hours into the cold Celsior solution. At the time of reperfusion, the hearts were again mounted on the perfusion system with infusion of a warm Krebs-Henseleit solution with/without additional ingredient according to the experimental group:

- \_No additional drug or solution for the control ischemic group,
- \_Addition of 10 mL of MSC conditioned medium,
- \_Addition of 10 mL of fresh D-MEM/F12.

Myocardial necrosis was assessed by measurement of creatinine kinase release in the coronary effluent at the time of reperfusion, and by determination of the infarct size using the triphenyltetrazolium chloride (TTC) staining.

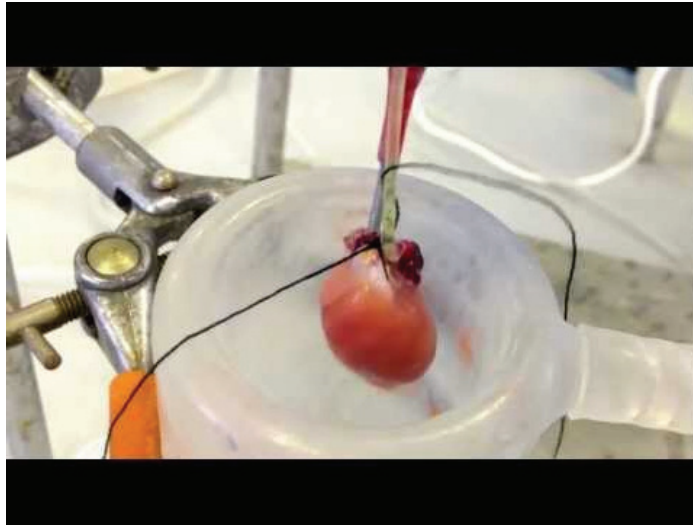


Figure 16. Photograph of an isolated rat heart mounted on a Langendorff perfusion system.

## **B. Part two: Modulation of the mitochondrial F1Fo ATP synthase during ischemia**

### **1. Culture of HL-1 cells**

HL-1 cells are one of the only cardiac muscle cell lines available and were developed by Dr Claycomb<sup>99</sup>. They derive from the AT-1 mouse atrial cardiomyocyte tumor lineage and can be serially passaged. They are able to contract, particularly when the cultures are confluent and display many characteristics similar to wild type cardiomyocytes, such as connexin 43,  $\alpha$ -cardiac actin,  $\alpha$ -cardiac myosin heavy chain.

To retain a cardiac phenotype, these cells must be cultured according to strict protocols and using very specific products. Their typical culture medium is called complete Claycomb medium (from Dr Claycomb), made of:

Product	Volume required to prepare 100 mL of complete Claycomb Medium
Claycomb Medium (commercially available medium from Sigma)	87 mL
Foetal Bovine Serum sourced in USA from Sigma (authorized batch numbers: 058K8426, 8A0177 or 027K8414)	10 mL
Penicilin/Streptomycin	1 mL
Norepinephrine (10 nM stock) in 30 mM ascorbic acid	1 mL
L-Glutamine (200 mM stock)	1 mL

The norepinephrine and ascorbic acid stock solution was made from powder with addition of distilled water; followed by filtration through a 0.2  $\mu\text{m}$  Acrodisc syringe filter.

Once complete, the claycomb medium is usable up to 2 weeks, due to the degradation of L-glutamine, and stored at 4°C in a fridge. The norepinephrine and ascorbic acid solution aliquots are usable up to 4 weeks, stored at -20°C in freezer, and are highly light sensitive. As a consequence, the complete claycomb medium bottle must be protected from light and all culturing procedures on HL-1 cells were performed after switching off the light of the laminar flow hood.

All cell culturing was performed in a laminar flow hood.

To culture HL-1 cells, the culture dishes had to be coated with a gelatin/fibronectin solution. The concentration of gelatin in the solution was 0.02%, prepared by placing 0.1 g of gelatin into 500 mL of distilled water. 1 mL of fibronectin was added to 199 mL of gelatin 0.02%. 1 mL of coating solution was necessary for each well of a 6-wells plate and 3 mL for a T75 flask. The incubation of the coating solution lasted from 2 hours up to 24 hours.

The culture medium was replaced on a daily basis and the cells were trypsinized twice a week, when they were confluent (and beating). T75 flasks were at first briefly rinsed with 2 mL of trypsin 0.05%, warmed at 37°C using a water bath, by pipetting the trypsin onto the bottom of the flask, and not directly on the cells. The trypsin was immediately removed by aspiration and another 3 mL of trypsin was added gently to the flask. The latter was placed back in the incubator during 3 minutes for incubation and then the trypsin was again removed by aspiration. Last, another 3 mL of trypsin was added to the flask and, after 3 minutes of incubation into the incubator, the flask was rapped to dislodge the cells that would still be adherent. At this point, our protocol differed from Dr Claycomb's in that we didn't use any soybean trypsin inhibitor. To inactivate the trypsin, we added 8 mL of complete claycomb medium in the flask and pipetted up and down to completely dislodge the cells and ensure trypsin inactivation. The content of the flask was transferred into a 15 mL centrifuge tube, and centrifuged for 5 minutes at room temperature at 500 g. The supernatant was removed and the pellet resuspended into 10 mL of complete claycomb medium. The resuspended cells were counted using a haemocytometer and seeded in previously coated T75 flasks or 6-wells plates/ 35 mm Petri dishes (immediately after removal of the coating solution). Approximately 5 millions cells were added in the flasks and 150 000 to 500 000 in each well of 6-wells plates or 35 mm petri dishes, according to the subsequent planned experiment.

To avoid loss of the line cell, in case of contamination for example, several vials of cells were frozen and stored in a liquid nitrogen tank for future use. The freezing medium was made of 95% of FBS and 5% of sterile DMSO. Thawing implied rapid warming of the vial in a 37°C water bath with gentle agitation, transfer into a 15 mL centrifuge tube containing 10 mL of warm complete claycomb medium and centrifugation for 5 minutes at 500 g. The supernatant was removed and the pellet resuspended into 10 mL of complete claycomb medium and seeded into a previously coated T75 flask. The culture medium was replaced after 4 hours and then on a daily basis.



## 2. Transfection of HL-1 cells

The cells for transfection were plated on the usual coverslips at the seeding concentration of 150 000 cells per 35 mm dish or well of 6-wells plate the day before transfection so that they were at 10 to 20% confluency at the time of transfection.

We chose FuGENE 6 for transfection as many authors agree that it is one of the less cytotoxic and one of the more easy to use transfection reagents. It is a non-liposomal reagent using the properties of cationic lipidic particles. First, these particles incorporate the DNA of interest, second, when in contact with cells, they merge with the plasmalemmal membrane, ensuring penetration of the DNA in the cytosol.

The transfection preparation was a mix of FuGENE 6 transfection reagent (9  $\mu$ L) with Opti-MEM reduced serum medium (91  $\mu$ L)<sup>100</sup>. A small quantity (1  $\mu$ g) of the DNA of interest, i.e. IF1 construct (a gift from Sir John Walker), IF1 SiRNA (Qiagen), genetically encoded indicators AT1.03 and COX AT1.03, and of a DNA reporter (where necessary), i.e. CFP (Cyan Fluorescent Protein) or YFP (Yellow Fluorescent Protein), was added to this preparation. After appropriate incubation, this solution was gently added to fresh new Claycomb medium, allowing transfection during 24 hours.

Transfected HL-1 cells were ready for experiments 36 to 48 hours after removal of the transfection medium, with an efficiency of transfection of 5 to 10% according to the molecule.

## 3. Cell culture imaging

To perform fluorescence microscope imaging, cells were grown on pre-autoclaved 22 mm glass coverslips placed either in a 6 wells-plate or a 35 mm petri dish. When the desired level of confluency was reached and the cells ready to be imaged, the coverslip was gently harvested from the culture dish with tweezers, in a

laminar flow hood, and mounted with care to avoid breaking it on a custom-made steel imaging ring. All coverslips were washed twice with a normal recording medium (500  $\mu$ L) previously warmed at 37°C in a water bath, then loaded with the appropriate dye for 30 minutes at room temperature (the loading solution is made from the fluorescent dye diluted at the appropriate concentration in normal recording medium). After loading, the cells were once again washed twice with normal recording medium. 500  $\mu$ L of normal recording medium was used to image the cells.

#### Normal recording medium composition

Chemical	Final concentration (mM)
NaCl	156
KCl	3
MgSO <sub>4</sub>	2
KH <sub>2</sub> PO <sub>4</sub>	1.25
CaCl <sub>2</sub>	2
D-Glucose	10
HEPES	10

#### 4. *FLIPR (Fluorescent Imaging Plate Reader)*

This plate reader was designed as an efficient method for optical screening of cell-based fluorescent assays. It can read the fluorescence from 96 wells simultaneously and offers the possibility to add drugs in all wells in different steps. The imaging system is made of a CCD camera and up to 2 drugs can be

added to the wells through a 96-well pipettor, from 2 separate 96-wells plates adjacent to the plate being optically read.

The system available in our facility was equipped with a 488 nm laser allowing measuring fluorescence from only a limited choice of probes. This system measured total mean fluorescence of a 96-wells plate and was used for concentration screening of the different drugs that we had to test.

The 96-wells plates used were specifically designed for the FLIPR, with a glass bottom. As with all the others plates used in our experiments, they were coated with the gelatine/fibronectin solution (50  $\mu$ L per well) at least 2 hours before seeding. 50 000 cells were added to each well, and the cells were imaged the day following the seeding. They were loaded according to the standard protocol with Rhodamine 123 (Rh 123) in quench/dequench mode with Verapamil 10  $\mu$ M during 30 minutes at room temperature (see below). In the meantime, 2 different 96-wells plates were prepared with different drugs at the appropriate concentration. Each condition was tested in triplicates.

The first step was the addition of an inhibitor of the electron transport chain such as rotenone (5  $\mu$ M) followed by the compound to be tested at the chosen concentration. Rotenone is an inhibitor of the electron transfer between complex I and complex II of the mitochondrial respiratory chain, generally assimilated to an inhibitor of complex I. The tested concentrations for all the compounds were 1, 2.5, 5, 10, 25, 50 and 100  $\mu$ M. Several concentrations of oligomycin B were tested: 1, 3 and 10  $\mu$ M. The second step consisted in adding FCCP (Trifluorocarbonylcyanide Phenylhydrazone) at 1  $\mu$ M to ensure maximal mitochondrial membrane depolarization.

##### 5. Assessing the effect of the compounds on mitochondrial membrane potential

To determine the effects of the compounds on mitochondrial membrane potential, we used Rh 123. This green fluorescent cationic dye is cell permeant and accumulates without toxicity in mitochondria in proportion to the proton gradient

between the matrix and the intermembrane space. When excited at 488 nm, its maximum emission wavelength is 534 nm (figure 17). As with TMRM and TMRE, 2 others dyes used to measure  $\Delta\psi_m$ , Rh 123 can be imaged in 2 different modes according to the loading concentration of the dye. When the latter is low, the dye is in distribution/redistribution mode: when the mitochondrion is in a normal state, the dye localizes preferentially in the mitochondrial matrix. But when it depolarizes, the distribution of the dye is modified and there's a leak from the matrix to the cytosol. In this case however, the mean signal of the cell may not change. If the loading concentration of the dye is high, it is said to be in quench/dequench mode. In this case, the dye concentration in the mitochondria is so high that it results in autoquenching. This is due to the formation of aggregates, as the dye is fluorescent only in its monomeric form. When the mitochondrion depolarizes, the redistribution of the dye to the cytosol contributes to relieve the quench and the fluorescence signal significantly increases. The advantage of Rh 123 over TMRM and TMRE is that it is less membrane permeant, meaning that the redistribution across the plasma membrane is slower and the results less affected by the change in plasmalemmal membrane potential.

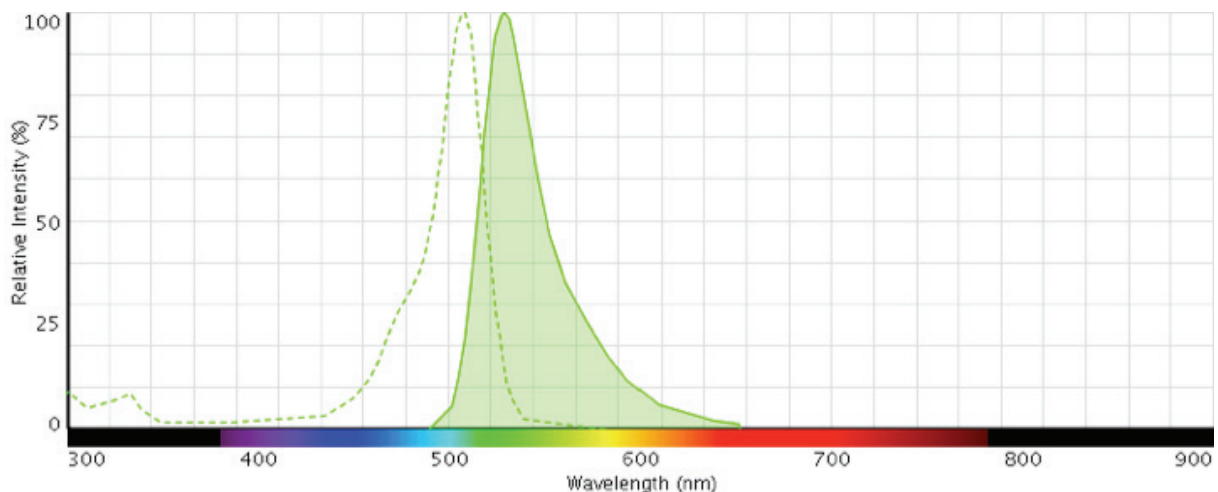
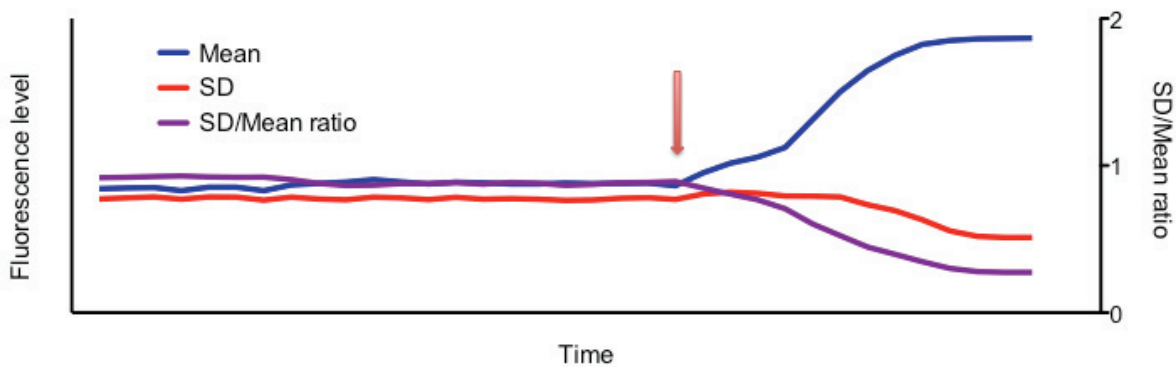


Figure 17. Excitation (dashed line) and emission (plain line) spectra of Rhodamine 123

We found that the best and most sensitive way to explore the changes in  $\Delta\psi_m$  using Rh 123 in quench/dequench mode (10  $\mu\text{g/mL}$ ) was to calculate the ratio SD/mean fluorescence. The standard deviation (SD) of the signal directly reflects the redistribution of the dye: it is high when the mitochondria are normally polarized as the dye is located preferentially in the mitochondria. When the latter depolarizes, the fluorescence of the cytosol increases, contributing to decreasing the difference of signal between cytosol and mitochondria, and thus the SD (figure 18). Monitoring of the SD is potentialized by the monitoring of the mean fluorescence of the cell in quench/dequench mode in that the fluorescence increases when the mitochondria is depolarized. Thus, this ratio provides the best sensitivity possible to detect significant changes in  $\Delta\psi_m$ .



*Figure 18. Changes of mean fluorescence, standard deviation (SD) and SD/mean ratio after loading HL-1 cells with Rh 123 in quench/dequench mode and mitochondrial depolarization. Changes in SD/mean ratio are more sensitive than changes in mean fluorescence and SD individually. Red arrow shows mitochondrial depolarization.*

In this work, the  $\Delta\psi_m$  was assessed in two different situations, aiming to explore the effects of the tested compounds on the mitochondrial F1Fo ATP synthase in normal conditions, to ensure that they aren't toxic, and in conditions when it reverses

a. Testing of the drugs by monitoring the mitochondrial membrane potential in normal basal conditions

The point was to determine whether the compounds could affect ATP synthesis in normal conditions by altering the functioning of the ATP synthase. After mounting the coverslips on the imaging rings, the cells were loaded with Rh 123 in quench/dequench mode according to the protocol. Rh 123 was loaded in conjunction with Verapamil 10  $\mu$ M: the reason is that Rh 123 is one of the numerous substrates of P-glycoprotein. This transmembrane glycoprotein with a ubiquitous distribution is responsible for the efflux of several drugs, Rh 123 being one of them. It is highly involved in the multi-drug resistance mechanism of various cancers. Verapamil, a well-known calcium antagonist, is one of its modulator, acting as an inhibitor of this efflux pump. Application of verapamil in our experimental conditions resulted in a better accumulation of Rh 123 in the cells. The cells were imaged on the Zeiss 700 using the 488 nm laser with the following settings:

Resolution: 1024 x 1024

Laser intensity: 0.2%

Number of series: 75

We used the definite focus system to avoid any unwanted change in focus, especially as we needed to add different drugs during the experiment. At T 15, that is 3 minutes after the start of the experiment, we added the tested drug at the chosen concentration. To ensure a correct mix of the drug in the medium, we prepared an intermediate dilution so that the added volume would represent 10 to 20% of the total volume. The addition was performed with the imaging ring on the stage, between 2 series, with a micropipette, taking great care in not touching the stage and adding the drug in the center of the coverslip, where the image was being recorded. After addition of the drug, the experiment was pursued until T 75, that is during 15 minutes.

What was expected was an absence of any significant change in the signal following the addition of the tested drug. A decrease in the SD/mean ratio could possibly correspond to an inhibition of the synthesis of ATP, which is a highly unwanted effect.

b. Testing of the drugs by monitoring the mitochondrial membrane potential in conditions when the ATP synthase works backwards

This was the main experiment to determine whether the tested drugs could potentially inhibit the reverse activity of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase. In this experiment, performed similarly to the previous, the total number of series was 90. At T 15, we added 2 inhibitors of the electron transport chain: rotenone 5  $\mu$ M and antimycin A 1 mg/mL. Antimycin A is an inhibitor of complex III of the respiratory chain. These 2 drugs act in synergy in order to block the mitochondrial respiration and thus the transfer of protons from the matrix to the intermembrane space. To maintain the proton gradient, the ATP synthase reverses and starts hydrolyzing ATP instead of synthesizing it. At T 30, we added the tested drug according to the standard protocol and monitored the  $\Delta\psi_m$  until T 90, that is during 18 minutes.

What was expected was a significant decrease in the SD/mean ratio following the addition of the tested drug on top of the poisons of the electron transport chain. This would correspond to a significant mitochondrial depolarization, compatible with an inhibition of the reverse activity of the ATP synthase.

For all these experiments, we used several controls to ensure a correct interpretation of the results. To ensure that Rh 123 was a good tool in our experimental conditions for the monitoring of  $\Delta\psi_m$ , we added FCCP 1  $\mu$ M. FCCP (carbonyl cyanide p-trifluoromethoxy-phenylhydrazone) is an uncoupling agent of the inner mitochondrial membrane permeable to protons, resulting in a brutal and complete fall in the  $\Delta\psi_m$ . Oligomycin B was also used, either alone, or after addition of the inhibitors of the electron transport chain. As explained above, oligomycin B is an inhibitor of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase, would it work in reverse mode or not. Addition of oligomycin B on top of the poisons of the respiratory chain results in a fall of the  $\Delta\psi_m$  due to the inhibition of the reverse activity of the ATP synthase.

6. Measurement of NADH autofluorescence

NADH (Nicotinamide Adenine Dinucleotide) is a coenzyme involved in oxidative phosphorylation. It acts as an electron donor to complex I of the electron

transport chain and is autofluorescent <sup>101</sup>. The presence of the adenine results in a strong absorption of the UV light, with peak absorption at 259 nm and 339 nm. In these conditions, NADH emits at 460 nm while its counterpart NAD<sup>+</sup> is not fluorescent (figure 19). Thus, the level of autofluorescence of mitochondria in different conditions could be a direct indicator of the NADH/NAD<sup>+</sup> ratio and so of the functioning of the respiratory chain. In addition, many authors demonstrated that the contribution of cytosolic NADH in the variation of NADH autofluorescence was negligible <sup>102-104</sup>. In our conditions, we tested the effects of the drugs on NADH autofluorescence with the following rationale: NADH is strongly implied in oxidative phosphorylation but also plays a significant role in several other physiological processes. A change in NADH autofluorescence following addition of the drug in cells in basal conditions would imply necessarily an interaction with the cell homeostasis. Inversely, the absence of significant change in NADH autofluorescence following addition of the drug would be a strong signal in favour of an absence of toxicity of the drug, and a specificity of its action toward the reverse activity of the ATP synthase.

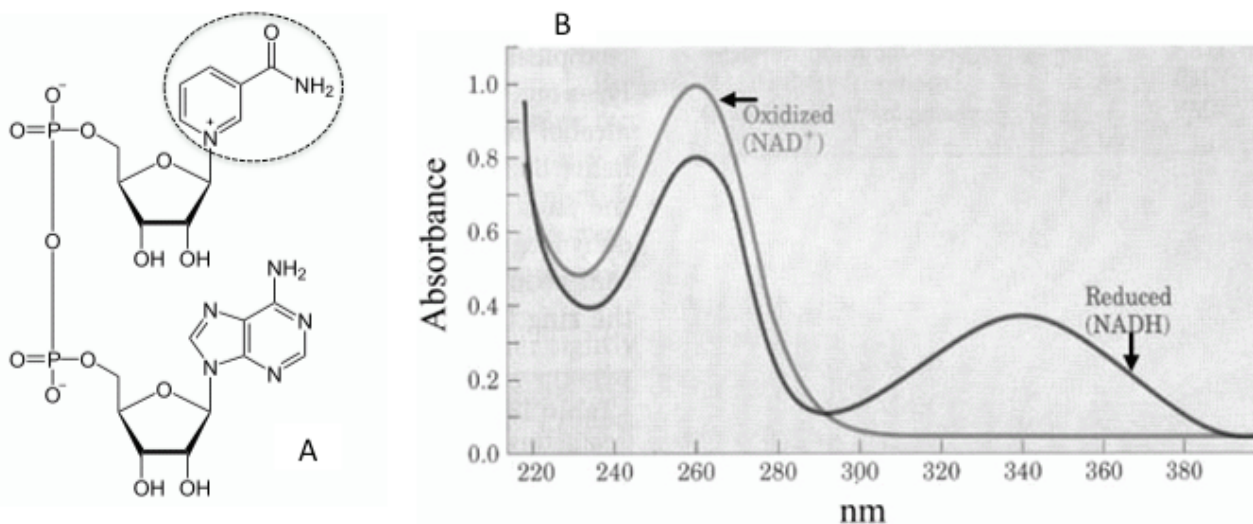


Figure 19. The NADH molecule and its autofluorescence properties. A: Chemical structure of NADH molecule. Inside the dashed circle, the radical bearing the autofluorescent properties. B: Excitation spectra of NADH (black line) and NAD<sup>+</sup> (grey line): only NADH is excited by the UV wavelengths used for microscopy (359 nm).



To assess NADH autofluorescence, HL-1 cells were prepared according to the standard protocol. The coverslips were mounted on the imaging rings and the cells were washed twice with normal recording medium. 500 mL of recording medium was then added and the coverslips were imaged on a Zeiss 510 microscope equipped with a UV laser. The cells were imaged at 359 nm with the following settings:

Laser power: 0.1%

Number of time series: 60

Interval between each series: 10

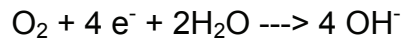
Different controls were again performed, such as addition of an inhibitor of the electron transport such as rotenone (not antimycin A as this molecule is fluorescent by itself when illuminated by ultraviolet light, making such an experiment completely not interpretable) and the uncoupler FCCP. An increase in mean fluorescence is correlated with an accumulation of NADH, similar to what can be seen with the addition of rotenone 5  $\mu\text{M}$ . At the opposite, we expected to see a decrease in fluorescence subsequently to the addition of an uncoupling agent, as it is responsible for the respiratory chain to function at its maximum rate, as a futile attempt to maintain the proton gradient.

Addition of drugs, e.g. rotenone, FCCP or the tested drugs, was performed at T15 using the same technique as in the experiment aiming to monitor the  $\Delta\psi\text{m}$ .

## 7. Oxygen consumption experiments

Another way to assess a possible toxic effect of the compounds was to measure the ability of the cells to consume oxygen in absence and presence of the compounds. The level of oxygen consumption correlates to the functionality of the mitochondrial respiratory chain that shouldn't be affected in basal conditions by the compounds, provided that they act specifically on the reverse activity of the ATP synthase.

Measuring oxygen consumption required the use of a Clark electrode. This system is made of a catalytic platinum electrode placed in a closed chamber and isolated by a thin Teflon membrane that is permeable for molecular oxygen<sup>105</sup>. When the oxygen reaches the electrode, it is electrolytically reduced according to the equation:



The medium in the chamber where the oxygen is measured must be constantly stirred (by a stir bar) so that the current measured is really proportional to the oxygen in solution.

This experiment was performed in very strict conditions to ensure the truthfulness of the results. Temperature of the chamber is an important source of noise, as it affects the amount of oxygen dissolved in solution. This could be overcome by respecting strict timings of recordings. Reproducible timing from one run to the other was also important because the cells could be damaged through mechanical stirring as the run progresses.

Once the system was switched on, we waited for at least 1 hour to ensure that the water bath ensuring the warm up of the chamber was at correct and stable temperature. The solution used in the chamber was normal recording medium warmed at 37°C.

The chamber used to perform the experiment had a capacity of 1 mL. During the warm up of the system, the HL-1 cells were trypsinized according to the standard protocol and resuspended and counted in normal recording medium, ensuring a final concentration of 10 million cells per mL. They were left in a centrifuge tube, put on ice and ready to use. The experiments were conducted as follows (times T are in minutes):

At T0, 700 µL of recording medium was added to the chamber and allowed to equilibrate.

At T5, 100 µL of the cell suspension (that is 1 million cells per run) was added to the recording medium.

At T8, a lid with a hole big enough for a microsyringe was used to seal the chamber and isolate it from outside oxygen, allowing measurement of the basal oxygen

consumption of our cells. It was crucial to ensure that no air bubble remained trapped under the lid as this would distort the measures. When measuring the basal oxygen consumption, the first minute was disregarded.

At T10, a drug was added with the use of a microsyringe: either one of the tested compounds at the appropriate concentration, or oligomycin B at 2.5  $\mu\text{M}$  or FCCP at 1  $\mu\text{M}$ . The variable of interest was the slope of consumption of oxygen, averaged over 1 minute at each step of the run.

For analysis, we set the equilibrated readout at the start of each run to 100% and used the software Chart to measure the slope (proportional to the rate of oxygen consumption). We determined the effect of the drug added using the ratio rate of oxygen consumption after/ rate of oxygen consumption before addition of the drug, i.e. in basal conditions.

Oligomycin B, as an inhibitor of the mitochondrial ATP synthase, is expected to significantly reduce the slope of oxygen consumption. On the contrary, FCCP, as an uncoupler, is responsible for the respiratory chain functioning at full rate, trying vainly to maintain the proton gradient, and this results in an increase in the oxygen consumption (and thus an increase in the measured slope). In order to be considered truly interesting, a compound shouldn't affect at all oxygen consumption. Note that reduction of the slope could mean either inhibition of the ATP synthase or inhibition of the electron transport chain.

A crucial step in this experiment was the wash of the chamber prior and after each run, to ensure that the drugs added during a run wouldn't interfere with the subsequent run. The wash protocol was made of 9 washes: 3 washes with deionized water with careful aspiration in between to avoid damaging the membrane, followed by 3 washes with 100% ethanol (the ethanol was left for 1 minute at the end of the third wash) and again 3 washes with deionized water. Meanwhile, the lid was rinsed with deionized water, then plunged in 100% ethanol for at least 1 minute, then rinsed again with water and shaken to dry.

## 8. Magnesium green experiments

Assessing the level of ATP in a cell is a very challenging goal and few techniques offer the possibility to monitor the changes in ATP either in an indirect or in a direct way. The actual gold standard implies the use of luminescence and the transfection of the cells with luciferase<sup>106</sup>. But the experimental conditions are very difficult to achieve, making the results sometimes difficult to analyze.

An indirect but nonetheless elegant way to monitor the changes in ATP stocks is the monitoring of the changes in free magnesium<sup>107</sup>. Free  $Mg^{2+}$  has a strong affinity with ATP and a low affinity with ADP. Thus when the stocks of ATP are high, the level of free  $Mg^{2+}$  is low, and when the ATP is consumed and the quantity of ADP rises, there's an increase in the level of free  $Mg^{2+}$ . Magnesium green (MgG) is a fluorescent dye highly affine with free  $Mg^{2+}$ . Thus the changes in fluorescence of this dye bear indirect information about the level of ATP in the cell.

In conditions when the oxidative phosphorylation is impaired, glycolysis is a significant provider of ATP in cells, especially in cell lines, and this had to be taken in consideration in such experiments. To limit the impact of glycolysis in this experiment, we used a different recording in which the glucose was replaced by 2-deoxyglucose at the exact same concentration. 2-deoxyglucose cannot undergo glycolysis, making the contribution of this process to the synthesis of ATP in this experiment negligible<sup>108</sup>.

The loading protocol of MgG is similar to that of Rh 123, with an incubation of 30 minutes at room temperature at the concentration of 5  $\mu$ M. Verapamil was not necessary to load the cells with MgG, as opposed to Rh 123, but this dye requires the presence of pluronic acid, at a concentration of 0.02% as it helps disperse the acetoxymethyl (AM) esters of several fluorescent ion indicators such as MgG, and thus optimize the loading quality<sup>109</sup>. The maximum excitation and emission wavelengths were respectively 490 and 530 nm (figure 20).

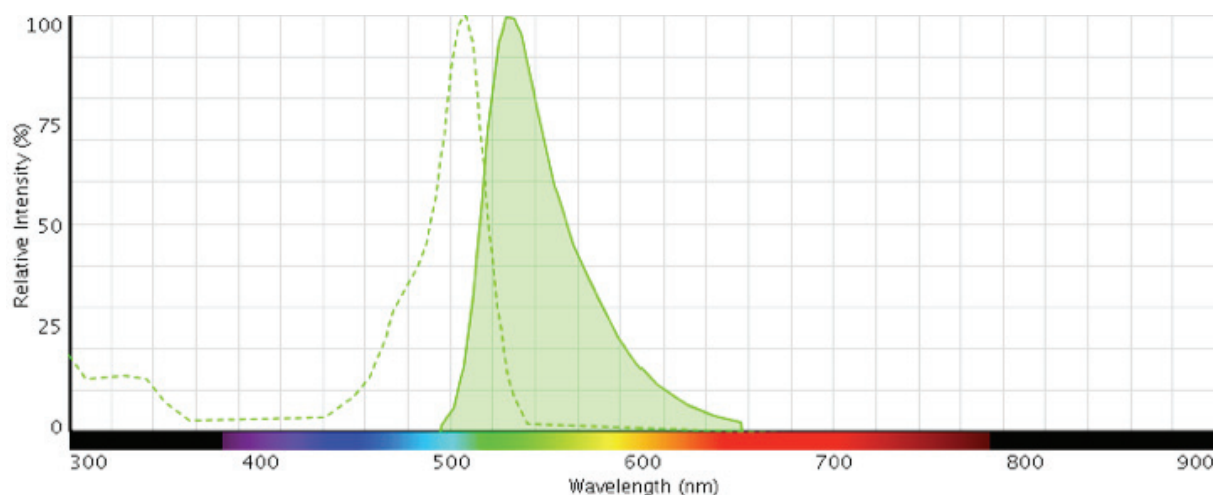


Figure 20. Excitation (dashed line) and emission (plain line) spectra of magnesium green.

The experiments were performed on a Zeiss 510 confocal microscope using a 488 nm laser for excitation. The followed setting applied to this experiment

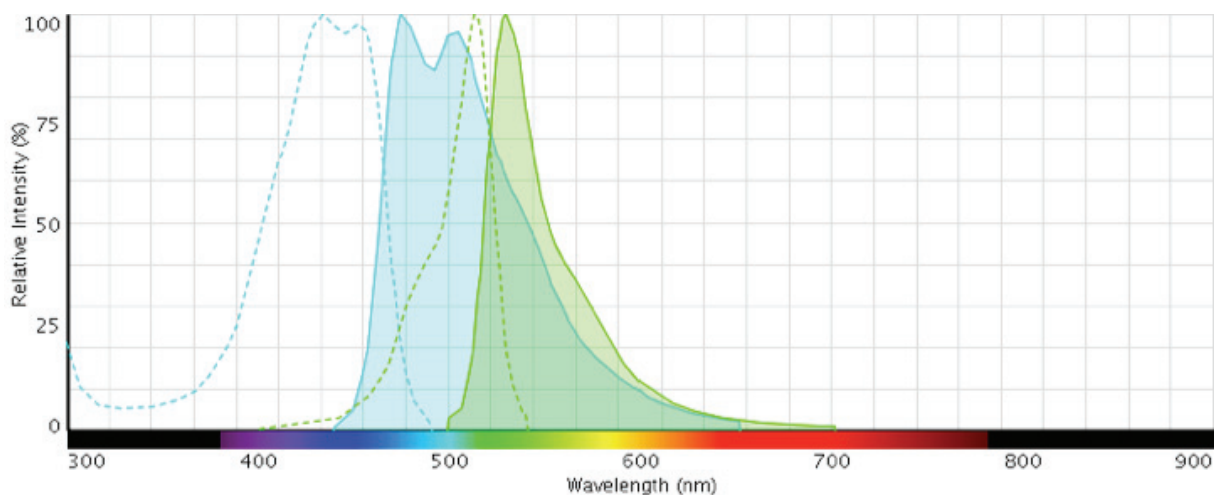
Laser power: 0.2%

Number of time series: 75

Interval between each series: 10

The first few tens of seconds of the time series gave information about the basal level of ATP in the cell. At T3, we added different drugs and monitored the changes in mean fluorescence. All experimental groups received the 2 poisons of the electron transport chain, i.e. rotenone 5  $\mu$ M and Antimycin A 1 mg/mL, either alone or in addition with oligomycin B (as a positive control) or one of the tested drugs. Addition of inhibitors of the respiratory chain was expected to generate a progressive increase in the fluorescence of MgG, as a result of the hydrolysis of ATP by the ATP hydrolase and thus an increase in free  $Mg^{2+}$ . The tested drugs were expected to slow down this increase, suggesting an effective inhibition of the ATP hydrolase activity. The MgG fluorescence was monitored afterwards up to T75, that is 12 minutes and 30 seconds.

In addition to MgG and though the data from this set of experiments cannot be analysed for they were not performed with a high enough  $n$  allowing statistical analysis, we aimed to measure directly the stocks of ATP, cytosolic and mitochondrial, using a genetically encoded fluorescent indicator. These indicators need to be transfected according to the above protocol and use the Fluorescence Resonance Energy Transfer (FRET) principle. They were made (and kindly given by Dr Hiroyuki Noji) from the  $\epsilon$  subunit of the bacterial F1Fo ATP synthase that is supposed to act as a sensor of the intracellular ATP level in order to regulate the activity of the ATP synthase<sup>110</sup>. The FRET reaction implies the presence of 2 fluorescent proteins; in the present case the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) (figure 21). The presence/absence of ATP in the immediate neighbourhood of the probe is responsible for changes in conformation resulting in a variable proximity of CFP and YFP.



*Figure 21. Excitation (dashed lines) and emission (plain lines) spectra of CFP (cyan) and YFP (green). Excitation spectra minimally overlap. There is an important overlap between CFP emission spectrum and YFP excitation spectrum at the basis of the principle of FRET.*

The use of these indicators required transfection using the protocol described above.

During imaging experiments, only CFP is illuminated using a 488nm laser. When YFP is close enough from CFP depending on the conformation of the indicator, CFP emission is responsible for YFP excitation and thus YFP emission can be recorded.

Changes in the YFP/CFP ratio give direct information regarding the changes in ATP stocks in the cell. The indicators were validated by a publication from Imamura et al. We tested 2 indicators: AT1.03 aiming at monitoring the cytosolic ATP level and COX AT1.03 specific of the mitochondrial ATP. We used the same experimental conditions than for the MgG experiments: during a time series, after a few basal pictures, the tested drugs were added at the same time than inhibitors of the electron transport chain and the FRET ratio was serially monitored using a simultaneous 2-channels acquisition with appropriate filters to ensure a minimal overlapping of the fluorescent signals.

#### 9. Time from mitochondrial depolarization to rigor

Another way to “monitor” the hydrolysing activity of the mitochondrial ATPase is the evaluation of the time from mitochondrial depolarization to contracture in adult cardiomyocytes. Rat ventricular adult cardiomyocytes were isolated and kindly given by Dr Bernardo Bollen Pinto (Wolfson Institute) and Richard Tyser (The Hatter Institute). The cells, on coverslips mounted on our imaging rings, were loaded with TMRM in quench/dequench mode (3  $\mu$ M) for 30 minutes at room temperature and imaged on the Zeiss 700 confocal microscope (figure 22). Similar to Rh 123, loading the cardiomyocytes with TMRM in dequench mode means that mitochondrial depolarization generates an increase in fluorescence of the dye. According to the spectra of TMRM, a 555 nm laser was used to illuminate the dye, using a laser power higher than usual to achieve photo-illumination, i.e. mitochondrial damage through excitation of a fluorescent dye localized inside mitochondria. The use of transmitted light allowed monitoring of the changes of shape of the cardiomyocytes. Therefore, the following settings were applied:

Laser power: 2%

Number of time series: 300

Interval between each series: 3s

Reversal of the mitochondrial F1Fo ATPase was triggered by photo-illumination and addition of FCCP (1  $\mu$ M) at T10. This generated mitochondrial depolarization identified as a wave of increase in fluorescence starting from both extremities of the cardiomyocytes, followed by a redistribution of the dye between the mitochondria and the cytosol. Later in the experiment, when the ATP stocks are over, the cardiomyocytes contract and this corresponds to cell death. Preservation of ATP would lead to a significant increase in the time from depolarization to rigor. The cardiomyocytes were pretreated either with oligomycin B as a control of the tested drugs and compared with cells that didn't receive any treatment.

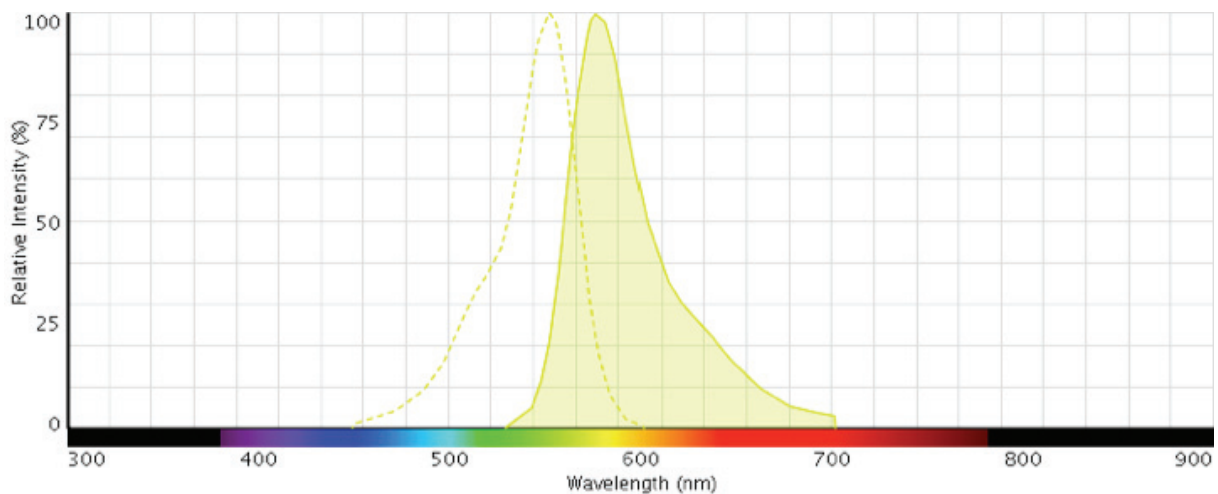


Figure 22. Excitation (dashed line) and emission (plain line) spectra of TMRM.

### 10. Ischemia/reperfusion of HL-1 cells

Simulated ischemia/reperfusion experiments were performed using a commercially available modular incubator chamber (MIC-101) from WolfLabs. It is a high quality polycarbonate airtight chamber with 2 gas port allowing filling the chamber with the desired gas mixture. In order to prevent excessive evaporation of the culture medium, the chamber was humidified by placing a Petri dish filled with 10 mL of sterile water at its bottom. For simulation of ischemia, the cells were washed



twice gently with PBS and then put in a hypoxia buffer under a laminar flow hood. Maximum 26 wells-plates were put at the same time in the hypoxia chamber, each well being filled with 1.5 mL. Then the chamber was closed, ready to be flushed with a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub>. The filling pressure was constantly kept below 2 PSI to avoid rupture of the chamber.

According to the manufacturer's instructions, a rate of 20 liters/min was sufficient to completely purge the chamber in 4 minutes. In our conditions, to ensure that the purge was optimal, the chamber was flowed with the gas mixture during at least 10 to 15 minutes. Once done, the chamber was disconnected from the gas source and sealed by closing the designed plastic clamps. The chamber was then placed in a conventional continuous flow CO<sub>2</sub> incubator with a temperature set at 37°C during the predetermined ischemia time.

At the time of reperfusion, the chamber was taken off the incubator, back under the hood. The chamber was opened and the cells were exposed to a normoxic atmosphere. The hypoxic buffer was then replaced as gently and quickly as possible by a normoxic buffer and the culture plates were placed back in the normoxic conventional continuous flow incubator for the time of reperfusion.

Several experimental groups were considered (figure 23):

\_a sham group, not exposed to ischemia and reperfusion

\_a control group, exposed to ischemia and reperfusion, without any therapeutic intervention

\_a protected group: HL-1 cells were incubated during 1 hour prior to ischemia either with CsA 200 nM or diazoxide 10 μM, a mitochondrial K<sub>ATP</sub> channel opener.

\_groups aiming to test the drugs of interest at the optimal concentration determined during previous experiments: the drugs were incubated during 1 hour prior to ischemia.

The cells were plated according to the standard protocol 24 to 48 hours prior to the experiment. 1 hour before ischemia, the culture medium was replaced by new medium alone (sham, control group) or containing the drug of interest (CsA or

diazoxide for the positive controls, the drugs investigated for the others). The cells were then replaced in the normoxic incubator.

After 1 hour, the culture medium was removed and the cells gently washed twice with PBS warmed at 37°C, then 1.5 mL of ischemic buffer was added per well of 6-well plate (or 35 mm dish). The plates were placed in the hypoxia chamber, which was placed in the normoxic incubator for 15 minutes to allow equilibration.

Afterwards, the chamber was filled with the anoxic atmosphere according to the protocol.

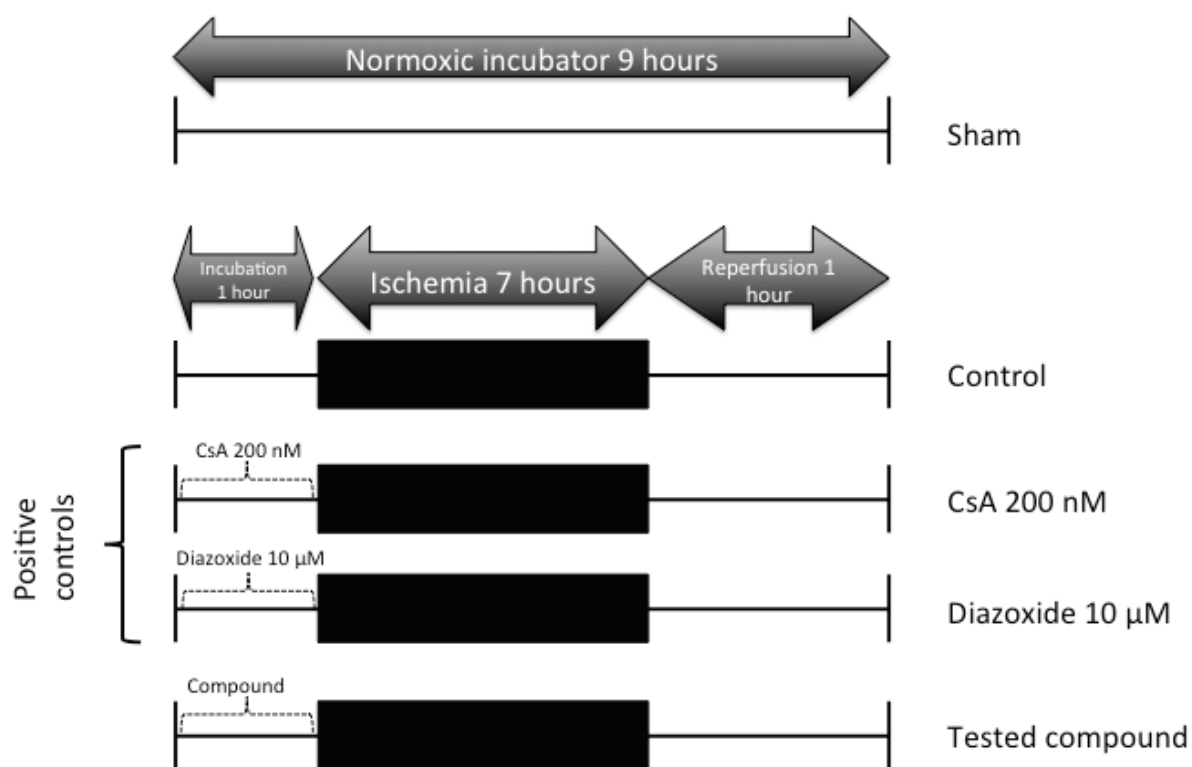


Figure 23. Experimental protocol of the ischemia/reperfusion assay. CsA: Cyclosporine A. The drugs (i.e. positive controls or compounds) were incubated for 1 hour prior to ischemia and washed away.

Several test experiments allowed to identify 7 hours as the best ischemic time for this cell type. Reperfusion was performed according to the protocol and the cells were placed back during 1 hour in the normoxic incubator.

At the end of reperfusion, the normoxic buffer was removed from the plates and the cells were gently washed twice with 2 mL of PBS at 37°C. Protected from light the cells were stained during 10 minutes at room temperature with 2 fluorescent dyes to assess mortality: the Hoechst dye, also known as Hoechst 33342 and propidium iodide.

Hoechst 33342 is a bis-benzimide that binds to the minor groove of the double-stranded DNA, especially the sequences rich in adenine and thymine. As a direct consequence, only the nucleus of cells stained with Hoechst is visible. It stains cells in all state, whether living or apoptotic or even necrotic. It is excited by UV (350 nm) and its maximum emission wavelength is in the blue range of visible light (461 nm). The staining concentration used here was 15 µg/mL.

Propidium iodide is an intercalating agent that binds preferentially to the DNA of necrotic cells. It is used to differentiate necrotic from living cells as it is membrane impermeant and thus excluded from living cells. Used at the concentration of 10 µg/mL, it is excited at 488 nm and its maximum emission wavelength was 617 nm (figure 24).

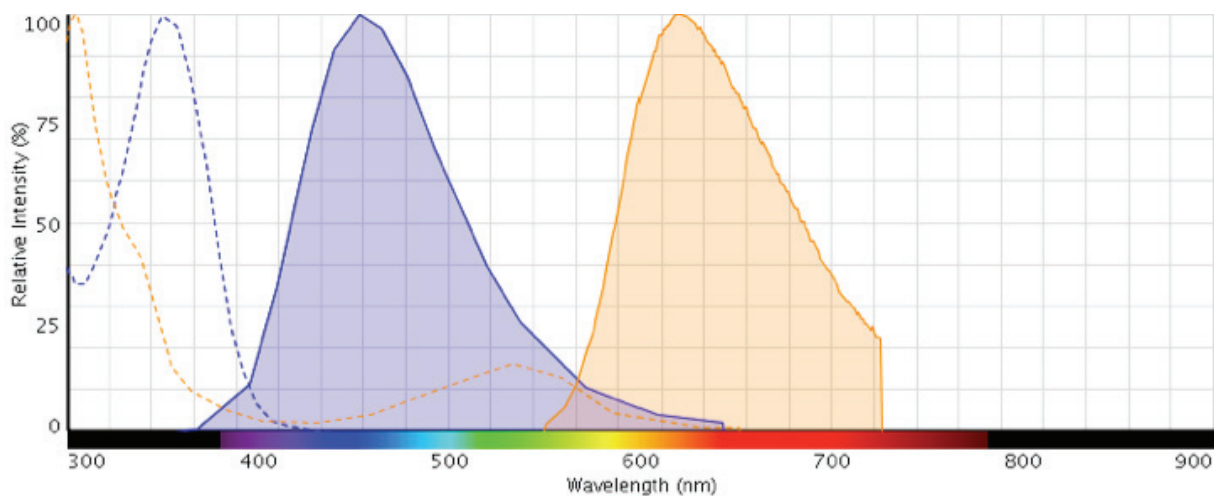


Figure 24. Excitation (dashed lines) and emission (plain lines) spectra of Hoechst (blue) and propidium iodide (orange).

After loading the dyes, the cells were again gently washed twice using warm PBS and then fixed with Paraformaldehyde (PFA) 4% during 10 minutes at room temperature. After 2 new washes with PBS, the cells were ready to be imaged on the fluorescence microscope.

To assess the index of mortality, expressed in percent as the ratio of PI positive cells over Hoechst positive cells, we imaged the cells at the appropriate wavelengths using the Zeiss 510 confocal microscope with a correct choice of filters. For each coverslip, we considered 5 different adjacent fields and counted the number of cells stained with each of the dyes.

#### Buffers preparation

The following quantities are designed for preparation of 500 mL of ischemic or control buffer:

Chemical	Control buffer	Ischemic buffer
NaCl	2.85 g	2.15 g
KCl	0.11 g	0.55 g
D-Glucose	0.9 g	-
Na-Pyruvate	0.11 g	-
Na-Lactate	-	1.4 mL

Each buffer was then bubbled for 30 min: the ischemic buffer was bubbled with Nitrogen and the control buffer with Carbogen. The pH was adjusted to 6.2 for the ischemic buffer and 7.4 for the control buffer and both solutions received 0.6 mL of CaCl<sub>2</sub>.

The next step was sterilization by filtration through Nalgene filter lids into sterile bottles under the laminar flow hood. 5 mL of Penicillin-Streptomycin solution was

added to each bottle of buffer. The buffers were then stored in the fridge and warmed up to 37°C before use using a water bath.

### 11. Immunofluorescence assays

Efficient transfection of HL-1 cells with non-fluorescent indicators like IF1 construct or IF1 SiRNA had to be checked with an immunofluorescence assay. The protocol implied to wash twice the cells with PBS (1 mL per well of 6-wells plate) at room temperature then fix them with PFA 4% (100 µL) during 10 minutes at room temperature. After fixation, the cells were washed thoroughly thrice and permeabilized during 5 minutes by adding 0.2% Triton X-100 in PBS, again at room temperature. 3 new washes with this permeabilizing solution were performed over 10 minutes, allowing addition of the primary antibody (rabbit polyclonal anti-mouse ATPIF1 antibody from ProteinTech, used at 1/100) dissolved in 0.2% Triton X-100 PBS containing 3% Bovine Serum albumin (BSA). After a 60 minutes incubation, the cells were washed four times with the permeabilizing solution and the secondary antibody (donkey anti-rabbit Cy3 antibody from ProteinTech at 1/50) was added and incubated in the dark for 30 minutes in the same 0.2% Triton X-100 PBS containing 3% BSA as the primary antibody. 3 new washes and a loading with Hoechst (15 µg/mL) later, the coverslips were mounted upside down on a microscope slide with a drop of mounting medium, sealed with nail varnish and kept wrapped in a foil at 4°C in the fridge for further imaging analysis with a fluorescence microscope correctly set up.

### 12. Image analysis

The softwares used for imaging with the confocal microscopes were LSM 510 META for the Zeiss 510 and ZEN2009 for the Zeiss 700. The analyses of microscope snapshots and time series were performed using the online free FIJI (FIJI Is Image J) software. A comparable threshold was determined for all images to

subtract the background and noise and for each region of interest, several indicators such as mean fluorescence and standard deviation were considered.

### **C. Statistical analysis**

All statistical analyses were performed using the GraphPad Prism 5 statistic software. Continuous variables were compared using Student's t-test. Multiple-group comparisons were achieved using a one-way analysis of variance (ANOVA) with a post-hoc Dunnett's or Newman-Keuls's multiple comparison test. Data were expressed as mean and SEM (Standard Error of the Mean) and a difference was considered statistically significant if  $p < 0.05$ .

# RESULTS AND PUBLICATIONS

## **A. PART ONE: Mesenchymal stem cell conditioned media attenuates in vitro and ex vivo myocardial reperfusion injury**

### **1. Summary of the publication**

Myocardial stem cell therapy went very quickly from bench to bedside based on the idea that is now believed false that we could regenerate myocardium. The result was an important number of clinical studies with little to no positive results and this was probably due to our misunderstanding of the phenomena implied in stem cell therapy. The work from Togel et al on renal ischemia/reperfusion injury strongly suggested that at least part of the positive effect of stem cell therapy could be paracrine. Thus we chose to explore this hypothesis using a cellular model of simulated ischemia/reperfusion. We assumed that rat mesenchymal stem cells, very interesting according to their immunomodulating properties and easy to harvest and culture, could protect neonatal rat cardiomyocytes from reperfusion injury and that this protection was mediated through a paracrine mechanism involving the PI3kinase/Akt pathway, well described for its implication in the protection induced by ischemic postconditioning.

Mesenchymal stem cells were harvested from an adult rat femur bone and serially cultured. We used FACS to check their phenotype and in particular ensure that they didn't express any CD specific to the hematopoietic lineage. Neonatal rat ventricular cardiomyocytes (NRC) were isolated from newborn rats through a process of serial trypsinations and kept in culture during several days for experimentation. These cells were exposed to a sequence of 5 hours of simulated ischemia followed by 16 hours of reperfusion. Ischemia was achieved using a thermostated hypoxia chamber lowed with a gas mixture containing 95% N<sub>2</sub> and 5% CO<sub>2</sub> and after replacing the culture medium by PBS. At the time of reperfusion, the NRC received additional culture

medium (deprived of FBS) and were replaced in a normoxic incubator until the end of the experiment. Then, the medium was harvested and used to assess cellular mortality by measurement of LDH activity. At the same time, the cardiomyocytes viability was explored using a MTT assay. Different groups were considered: on top of a sham group and a control group exposed to ischemia and reperfusion without any therapeutic intervention, there were a group received our positive control of cardioprotection CsA, groups receiving freshly trypsinized MSC alone or with inhibitors of the PI3kinase (wortmannin or LY294002) and groups receiving MSC conditioned medium either alone or with inhibitors of the PI3kinase as well (see figure 14). All these therapeutic interventions were performed immediately at the beginning of reperfusion.

The results were that both MSC and MSC CM could protect NRC against reperfusion injury and the efficiency of MSC CM confirmed that at least part of the protective effect is paracrine. When inhibitors of the PI3kinase were added, they abolished the protective effect of MSC CM, implying that this signaling pathway is a key actor in this situation involving a paracrine mechanism. However, the inhibitors of PI3kinase didn't affect the protective effect of MSC alone, suggesting that this protection was mediated through a mechanism of action completely different, possibly through a more or less direct contact.

Analysis of MSC CM revealed that it was rich in VEGF and IGF-1, two growth factors with known protective effect and able to activate the PI3kinase pathway. However the direct implication of on or the other of these factors couldn't be demonstrated as the addition of an antibody anti-VEGF or of an anti-IGF-1 individually didn't alter the protection afforded by MSC CM. Thus the protective effect of MSC CM is probably due to a mix action of numerous factors and cannot probably be impaired by the blocking of just one of them.

Last, the protective effects of MSC CM were confirmed on isolated rat heart in a global ischemia/reperfusion experiment. MSC CM significantly reduced CK release and TTC staining compared to an ischemic control.

Thus, our results suggest that addition of MSC CM at the time of reperfusion can be protective against reperfusion injury in two different models. This protective effect appears to involve the PI3kinase pathway but its mediators still need to be identified.



The protection afforded by MSC isn't only paracrine and this needs to be addressed in further studies.



## ORIGINAL PRE-CLINICAL SCIENCE

## Mesenchymal stem cell conditioned media attenuates in vitro and ex vivo myocardial reperfusion injury

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**KEYWORDS:**

mesenchymal stem cells;  
ischemia reperfusion;  
paracrine;  
growth factors;  
PI3K;  
cardioprotection

**BACKGROUND:** Previous studies have suggested that implantation of mesenchymal stem cells (MSC) or their conditioned media (MSC CM) improves heart function after myocardial infarction. We sought to determine whether MSC and MSC CM added at the onset of reperfusion attenuates myocardial reperfusion injury.

**METHODS:** Rat MSC and neonatal rat cardiomyocytes (NRC) were isolated and cultured separately. NRC were subjected to simulated in vitro ischemia/reperfusion (I/R). At the onset of reperfusion, NRC received either fresh medium (control group) or one of the following treatments: MSC in fresh medium; MSC CM alone (without MSC); MSC CM + inhibitors of PI3K (LY294002 or Wortmannin); MSC CM + antibodies neutralizing IGF-1 or VEGF; MSC + inhibitors of PI3K; or cyclosporine. Cell injury was assessed by LDH activity and MTT staining at the end of reperfusion. VEGF, IGF-1 and HGF were measured in each experimental treatment preparation. Ex vivo experimentation on isolated rat hearts subjected to I/R were performed to evaluate the protective effects of MSC CM on myocardial reperfusion injuries measured through CK release and infarct size after TTC staining.

**RESULTS:** In vitro cell injury was significantly reduced by MSC, MSC CM and CsA. PI3K inhibitors significantly attenuated the protection afforded by MSC CM but not growth factor inhibitors. Ex vivo experimentation showed that MSC CM significantly reduced myocardial I/R injury.

**CONCLUSION:** Our data suggest that MSC CM added at the onset of reperfusion can protect myocardium from I/R injury. In vitro data suggest a protection mediated by paracrine activation of the PI3K pathway. J Heart Lung Transplant 2010;xx:xxx

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Mesenchymal stem cells (MSC) are of growing interest in both ischemic heart disease and heart transplantation. Several pre-clinical studies have shown that MSC implantation after myocardial infarction could reduce infarct size and improve heart function.<sup>1,2</sup> Although implanted MSC were shown to express cardiomyocyte and endothelial cell differentiation markers, significant regeneration of cardiac muscle and vessels by implanted MSC transdifferentiation remains controversial.<sup>3</sup> Alternatively, it has been proposed that paracrine mechanisms triggered by cytokines and growth factors secreted by im-

planted MSC in their conditioned media may explain the benefit observed after MSC implantation.<sup>4-6</sup>

In vitro and in vivo data also showed that MSC could reduce proliferation of T lymphocytes and cytotoxic activation involved in the allogeneic immune response.<sup>7-10</sup> In vivo data suggest that addition of MSC could reduce allogeneic graft heart rejection.<sup>11-14</sup> These experiments suggested that some of the immunomodulating effects of MSC resulted from paracrine mechanisms.<sup>15</sup>

Experiments on pre- and post-conditioning have emphasized the emerging role of reperfusion injury in cardiomyocyte loss and infarct size after ischemia/reperfusion.<sup>16-18</sup> Protection afforded by post-conditioning has been shown to involve the RISK (reperfusion injury salvage kinase) pathway that includes the phosphatidylinositol-3-OH kinase (PI3K) pathway.

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Interestingly, the RISK pathway may be triggered by growth factors, including vascular endothelial growth factor (VEGF), human growth factor (HGF) and insulin-like growth factor-1 (IGF-1), secreted by MSC in response to injury.<sup>19–21</sup>

We therefore aimed to determine whether MSC and their conditioned media (CM) added at the onset of reperfusion would attenuate reperfusion injuries. To explore the potential implication of a paracrine activation of the cardiomyocyte PI3K pathway we used an in vitro model of cultured neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion. Ex vivo experiments on isolated rat hearts were performed to better understand the protective effects of MSC CM on myocardial ischemia/reperfusion injuries.

## Methods

### Isolation and expansion of bone marrow MSC

Our investigation was performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Protocol approval was granted by our university ethics review board (Claude Bernard University Lyon 1, Lyon, France).

MSC isolation and expansion were performed following the method described by Pittenger et al.<sup>22</sup> When confluent, MSC were detached with trypsin–ethylene-diamine tetraacetic acid (Invitrogen) and subsequently passaged at  $5 \times 10^3$  cells/cm<sup>2</sup>. The multipotentiality of the resulting cells was verified up to Passage 10 with the use of in vitro assays (R&D Systems, Minneapolis, MN) to differentiate MSC into osteogenic (alkaline phosphatase activity), adipogenic (oil red O staining) and chondrogenic (Type II collagen staining) lineages.<sup>22,23</sup> Passage 10 cells were used for the experiments.

### Flow cytometry

Cultured MSC were analyzed ( $n = 4$ ) by fluorescence-activated cell sorting (FACS; FACScan flow cytometer; Becton Dickinson). Cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythritin (PE)-conjugated mouse monoclonal antibodies against rat. We used CD31–PE (Clone TLD-3A12; Becton Dickinson), CD34–PE (Clone ICO-115; Santa Cruz Biotechnology), CD45–FITC (Clone OX-1; Becton Dickinson) and CD90–FITC (Clone OX-7; Becton Dickinson). Hamster anti-rat CD29–FITC monoclonal antibody (Clone Ha2/5; Becton Dickinson) was also used. Isotype-identical antibodies served as controls.

### Preparation of MSC conditioned media

After evaluating various incubation durations, MSC conditioned media (CM) was obtained by incubating freshly trypsinized MSC in serum-deprived fresh DMEM (Gibco) for 8 hours in a normoxic incubator with 5% CO<sub>2</sub>. Cultured MSC supernatant was then sucked from the dish and centrifuged at 1,800 rpm for 5 minutes to remove detached MSC. Trypan blue control staining performed at the end of the incubation showed 99% cell survival. Experiments were performed using freshly prepared MSC CM. The amount of MSC CM added in the cultured dishes for all in vitro

experiments was 0.2 ml/cm<sup>2</sup>. The amount of MSC CM infused for each sample in the ex vivo experiment was 10 ml. To evaluate whether freezing MSC CM for storage and preservation purposes could affect its protective effect on neonatal rat cardiomyocytes (NRC) in in vitro ischemia/reperfusion-induced death we repeated the in vitro experiments using previously frozen (1-month storage at  $-80^{\circ}\text{C}$ ) MSC CM.

### Isolation of neonatal rat cardiomyocytes

The primary culture of NRC was prepared as previously described.<sup>24</sup> NRC preparation was enriched by incubating the cells for 2.5 hours in culture dishes to allow fibroblasts to attach to the plastic surface. Supernatant was then collected and centrifuged before plating resuspended cells at  $7 \times 10^5$ /ml in culture dishes. Culture medium was changed after 2 days. Cardiomyocyte glucose transporter (Glut-4) immunostaining was used to test for homogeneity of the NRC preparation.<sup>25</sup> The Glut-4 (Abcam) staining showed a 90% homogeneous preparation of NRC. Cultured NRC were used 3 to 5 days after isolation.

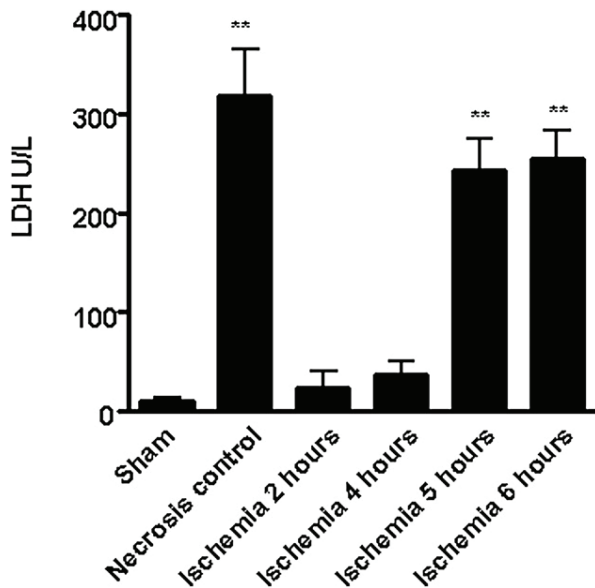
### Simulated ischemia/reperfusion of neonatal cardiomyocytes

The simulated ischemia condition was produced by incubating cultured NRC ( $2 \times 10^5$ /cm<sup>2</sup>) in an air-tight glass humidified chamber (Verretech, Lyon, France) at 37°C ventilated with 5% N<sub>2</sub> 95% + CO<sub>2</sub> after replacing the CM with nutrient-free phosphate-buffered saline (PBS; 0.15 ml/cm<sup>2</sup>). Oxygen concentration, pH and temperature were continuously monitored. To achieve simulated reperfusion immediately after simulated ischemia, nutrient-rich and serum-deprived fresh Dulbecco's modified Eagle medium (DMEM; Gibco) was added in the cultured NRC PBS (v/v) and the culture dish was placed for 16 hours in a normoxic incubator with 5% CO<sub>2</sub>. We first evaluated NRC death induced by various durations of simulated ischemia/reperfusion. We determined an optimal duration of 5 hours of simulated ischemia followed by 16 hours of simulated reperfusion, which was used in all experiments (Figure 1).

### Measurement of neonatal cardiomyocyte death and viability

Cultured NRC conditioned media was collected at the end of simulated ischemia and at the end of simulated reperfusion. Measurement of lactate dehydrogenase (LDH) activity was used as a marker of NRC death.<sup>26–28</sup> LDH was measured in the supernatant using a commercially available kit (Enzyline LDH; Biomérieux) according to the manufacturer's instructions. Optical density was read at 340 nm in a spectrophotometer (Digilab; Hitachi) and translated into LDH activity using a standard curve. Results are expressed as LDH activity (units per liter) in each group.

NRC viability after simulated reperfusion was explored using the thiazolyl blue tetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 550 nm using a spectrophotometer multiplate reader (Thermo). Percent viability was defined as the relative absorbance of all experimental groups vs sham control.



**Figure 1** LDH activity in NRC conditioned medium at the end of simulated ischemia/reperfusion. Various durations of simulated ischemia followed by 16 hours of simulated reperfusion ( $n = 4$  per group) were evaluated and compared with the sham group (no simulated ischemia) and necrosis control group (addition of 12% sodium hypochlorite to induce cell death). In our experimental conditions, 2 and 4 hours of simulated ischemia followed by 16 hours of simulated reperfusion resulted in a non-significant difference for NRC death vs sham. Five or 6 hours of simulated ischemia followed by 16 hours of simulated reperfusion resulted in significant NRC death. One-way ANOVA was significant ( $p < 0.0001$ ); post hoc analysis was a Dunnett's multiple comparison test of all groups vs sham.  $**p < 0.01$  vs sham.

### Experimental design of the in vitro study

Our study included 13 experimental groups (Figure 2). In the sham group ( $n = 14$ ), cultured NRC were incubated at  $2 \times 10^5$  cell/cm<sup>2</sup> in 35-mm dishes with 2 ml of DMEM in a normoxic incubator for 21 hours. All other groups plated at  $2 \times 10^5$  cell/cm<sup>2</sup> in 35-mm dishes with 2 ml of nutrient-free PBS subjected to 5 hours of simulated ischemia followed by 16 hours of simulated reperfusion were run simultaneously. Their culture media (nutrient-free PBS) was collected at the onset of simulated reperfusion ( $n = 6$  to 14/group) and immediately replaced by the same volume (2 ml) of the following experimental preparations. The control group received fresh DMEM. The DMEM + MSC group received freshly trypsinized allogeneic MSC (10% of the number of NRC initially plated in the dish,  $2 \times 10^4$  cell/cm<sup>2</sup>) resuspended in fresh DMEM. Resuspended MSC were plated in the culture dish on top of NRC using a pipette. The MSC CM group received 2 ml of MSC CM prepared as described previously. The DMEM + CsA group received 200 nmol/liter of cyclosporine (CsA; Sandimmune; Novartis) diluted in fresh DMEM.<sup>29</sup> CsA is a potent inhibitor of the opening of the mitochondrial permeability transition pore, which is a key player in cardiomyocyte death during reperfusion after a prolonged ischemic insult<sup>30</sup>; CsA was used here as a positive control for attenuation of lethal reperfusion injury. Because Sandimmune contains Cremophor (BASF Corp.), a solvent facilitating intracellular transportation of cyclosporine, we repeated the experiment with another preparation of CsA (from Sigma) that does not

contain Cremophor to ensure that the effect would not be related to its presence.

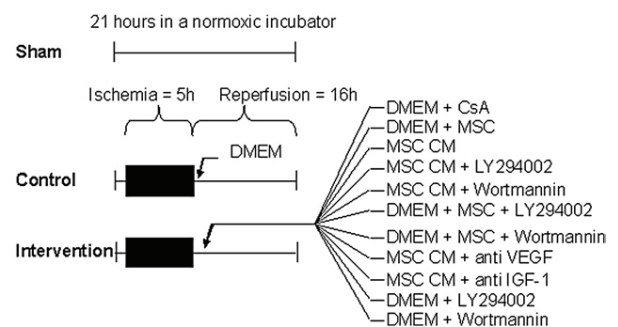
To investigate the role of the PI3K pathway we evaluated experimental groups combining MSC CM or MSC with inhibitors of PI3K: LY294002 (Sigma), a specific non-selective inhibitor of PI3K, and Wortmannin (Calbiochem), a non-specific inhibitor of PI3K. The MSC CM + LY294002 group received 15  $\mu$ mol/liter of LY294002 diluted in MSC CM.<sup>31</sup> The MSC CM + Wortmannin group received 100 nmol/liter of Wortmannin diluted in MSC CM.<sup>32</sup> The DMEM + MSC + LY294002 group received 15  $\mu$ mol/liter of LY294002 mixed with freshly resuspended MSC. The DMEM + MSC + Wortmannin group received 100 nmol/liter of Wortmannin mixed with freshly resuspended MSC. Control groups with addition of LY294002 (DMEM + LY294002) or Wortmannin (DMEM + Wortmannin) alone were also established.

We also investigated the role of growth factors found in MSC CM in the protection by combining MSC CM with blocking antibodies against VEGF (anti-rVEGF; R&D Systems) and blocking antibodies against IGF-1 (anti-IGF-1; Clone Sm 1.2; Upstate Biotechnology). The MSC CM + anti-VEGF group contained 10- $\mu$ g/ml final concentrations of anti-rVEGF antibodies, 25- to 1,000-fold greater than the concentrations for half-maximal inhibition of 10 ng/ml of the recombinant proteins (R&D Systems). The MSC CM + anti-IGF-1 group had 50- $\mu$ g/ml final concentrations of anti-IGF-1 antibodies, 50- to 75-fold greater than the concentrations for half-maximal inhibition of 5 ng/ml of the recombinant proteins (Upstate Biotechnology).

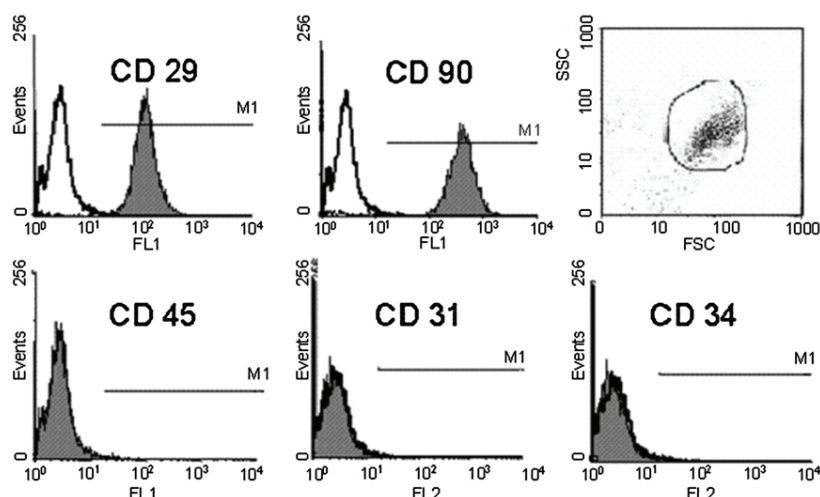
All groups received the same volume of DMEM.

### Measurement of growth factor concentration

VEGF, HGF and IGF-1 concentrations were measured in fresh DMEM, conditioned media of freshly resuspended MSC after 10 minutes of contact, DMEM + CsA and MSC CM ( $n = 6$  per



**Figure 2** Experimental design of the in vitro study. The sham group had no simulated ischemia/reperfusion. All other groups of cultured NRC underwent 5 hours of simulated ischemia followed by 16 hours of simulated reperfusion. Intervention at the onset of simulated reperfusion consisted of adding various conditions of DMEM to NRC conditioned medium: fresh DMEM alone (control); DMEM + CsA (200 nmol/liter); DMEM + MSC (10% of the number of NRC initially plated in the dish resuspended in fresh DMEM); MSC CM + LY294002 (15  $\mu$ mol/liter); MSC CM + Wortmannin (100 nmol/liter); DMEM + MSC + LY294002 (15  $\mu$ mol/liter); DMEM + MSC + Wortmannin (100 nmol/liter); MSC CM + anti-VEGF (10  $\mu$ g/ml); MSC CM + anti-IGF-1 (50  $\mu$ g/ml); DMEM + LY294002 alone (15  $\mu$ mol/liter); and DMEM + Wortmannin alone (100 nmol/liter). NRC, neonatal rat cardiomyocytes. DMEM, Dulbecco's modified Eagle medium; MSC, mesenchymal stem cells; CM, conditioned media.



**Figure 3** FACS analysis of cultured MSC. Results shown were obtained from Passage 10 cells at Day 7 of culture. The cells were 95% to 99% homogeneous and were positive for reactivity to antigens CD29 and CD90. They were negative for reactivity to antigens CD45, CD31 or CD34.

group). VEGF concentration was measured using a rat VEGF enzyme-linked immunoassay (ELISA) kit (Quantikine; R&D Systems). IGF-1 concentration was assessed using a rat/mouse IGF-1 ELISA kit (IDS). HGF concentration was measured using a rat HGF ELISA kit (B-Bridge).

### Ex vivo experiment of myocardial ischemia/reperfusion

Male Wistar rats, weighing 350 to 450 g, were anesthetized with pentobarbital (50 mg/kg). Heparin (200 IU/kg) was injected into the femoral vein. Hearts were quickly removed, aortae were cannulated, and cold Celsior solution was perfused during 1 minute to arrest the hearts. Hearts were then immersed in the cold Celsior solution during 8 hours at 4°C (ischemic period). After hypothermic (4°C) storage, hearts were reperfused on a Langendorff apparatus using Krebs–Henseleit bicarbonate buffer (containing, in millimoles per liter: glucose 11.0, NaCl 118.5, KCl 4.75, MgSO<sub>4</sub> 1.19, KH<sub>2</sub>PO<sub>4</sub> 1.18, NaHCO<sub>3</sub> 25.0, CaCl<sub>2</sub> 1.4) at pH 7.4. The buffer was bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C and perfusion was performed under a hydrostatic pressure of 100 cm H<sub>2</sub>O. The left ventricle was paced at a constant rate of 300 beats per minute (bpm). Animals were randomly assigned to one of the following groups (*n* = 6 per group): a control group (ischemic control)—hearts reperfused without any intervention after the cold ischemic storage period; a control DMEM group—infusion of 10 ml of DMEM using an electric injector prior to reperfusion; and an MSC CM group—infusion of 10 ml of MSC conditioned media performed using an electric injector prior to reperfusion.

Myocardial necrosis was evaluated by measuring creatine kinase (CK) release in the coronary effluent during the reperfusion period (Beckman Coulter kit, Galway, Ireland), as previously validated.<sup>33</sup> Results were expressed as: CK (international units per liter) × volume of coronary effluent (V)/heart dry weight (dW), (CK × V/dW)/1,000. Myocardial necrosis was also assessed by infarct size measurement using triphenyltetrazolium chloride (TTC) staining. Briefly, the hearts were cut into 5 transverse slices, parallel to the atrioventricular groove. After removing the right ventricular tissue, heart slices were weighed and incubated for 30 minutes in a 1% solution of TTC at 37°C to differentiate infarcted (pale) from viable (brick red) myocardial area. The slices were

then photographed. The extent of the area of necrosis was quantified by computerized planimetry. Total area of necrosis (AN) was then calculated and expressed as a percentage of total left ventricular (LV) area.

### Statistical analysis

Continuous variables were compared using Student's *t*-test. For multiple-group comparisons we used a 1-way analysis of variance (ANOVA) with a post hoc Dunnett's multiple comparison test comparing all groups with the control group if *p* < 0.05. All data are expressed as mean ± SD.

## Results

### Identification of MSC

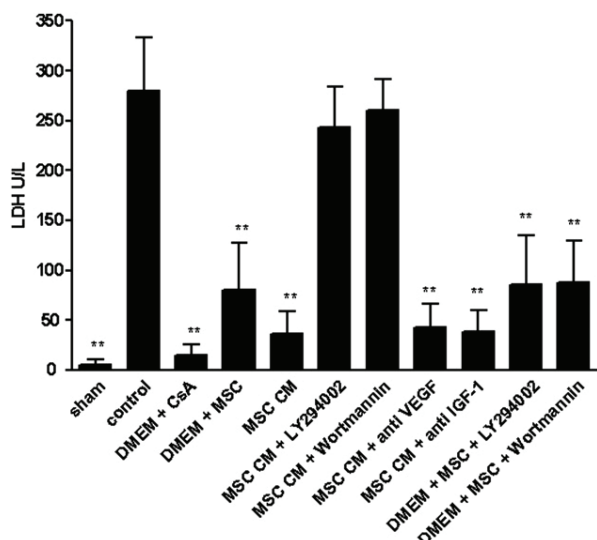
FACS analysis of Passage 10 MSC showed 99% expression on the cell surface of CD29 and CD90. The cells did not express CD31, a marker of endothelial progenitor cells, nor CD34 or CD45, markers of hematopoietic progenitor cells (Figure 3).

### LDH activity and MTT assay after simulated ischemia and simulated reperfusion

Oxygen concentration monitoring in the conditioned medium showed 90% reduction after 20 minutes of simulated ischemia compared with baseline. Low oxygen concentration (2%) remained stable during the 5 hours of simulated ischemia. Oxygen concentration returned to the baseline value (21%) after 20 minutes of reperfusion.

### CsA significantly reduced cell death markers and preserved viability after simulated ischemia/reperfusion

LDH activity at the end of simulated ischemia was not statistically different from sham in any of the groups. LDH



**Figure 4** LDH activity in NRC conditioned medium at the end of simulated ischemia/reperfusion. Both CsA, freshly resuspended allogeneic MSC and MSC CM added at the onset of simulated reperfusion significantly reduced LDH activity. Addition of PI3K inhibitors to MSC CM significantly blocked the reduction of LDH activity. Addition of antibodies neutralizing VEGF or IGF-1 did not modify the effect of MSC CM on LDH activity. Addition of PI3K inhibitors to freshly resuspended allogeneic MSC did not modify the effect of MSC on LDH activity. Values are expressed as LDH activity in units per liter. Bar graph shows mean  $\pm$  SD. Numbers in each group were as follows: sham,  $n = 14$ ; control,  $n = 14$ ; DMEM + CsA,  $n = 12$ ; DMEM + MSC,  $n = 14$ ; MSC CM,  $n = 14$ ; MSC CM + LY294002,  $n = 8$ ; MSC CM + Wortmannin,  $n = 8$ ; MSC CM + anti-VEGF,  $n = 6$ ; MSC CM + anti-IGF-1,  $n = 6$ ; DMEM + MSC + LY294002,  $n = 6$ ; and DMEM + MSC + Wortmannin,  $n = 6$ . Additional controls with DMEM + Wortmannin and DMEM + LY294002 alone were not different from control DMEM (data not shown). One-way ANOVA was significant ( $p < 0.0001$ ); post hoc analysis was a Dunnett's multiple comparison test of all groups vs control.  $**p < 0.01$  vs control.

activity at the end of simulated reperfusion was significantly elevated in the control group, averaging  $276 \pm 72$  UI/liter vs  $10.6 \pm 9$  UI/liter in the sham group. MTT assay showed a  $68 \pm 11\%$  reduction of viability in the control group compared with sham. Addition of CsA (Sandimmun; Novartis) at the onset of simulated reperfusion significantly reduced LDH activity, averaging 4% of control ( $p < 0.01$ ). CsA significantly preserved NRC viability assessed by MTT assay, averaging 182% of control ( $p < 0.01$ ) (Figures 4 and 5). The same results were observed using the CsA (Sigma) preparation (data not shown).

#### MSC significantly reduced cell death markers and improved viability after simulated ischemia/reperfusion

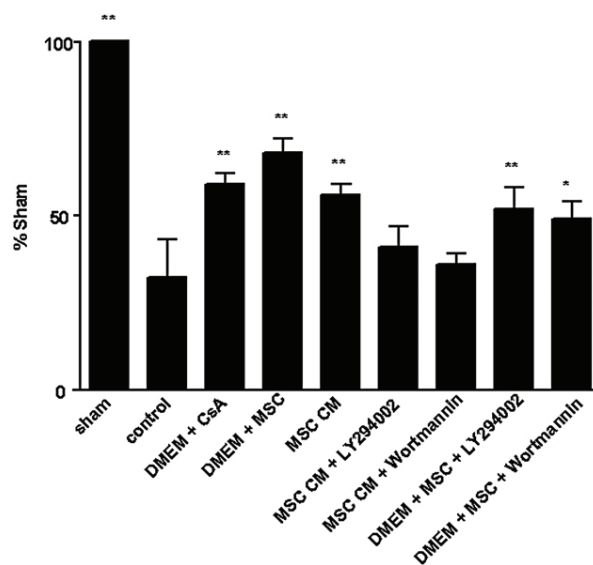
Addition of freshly resuspended allogeneic MSC at the onset of simulated reperfusion significantly reduced LDH activity, averaging 29% of control ( $p < 0.01$ ). MTT assay showed an improved viability, averaging 211% of control (Figures 4 and 5).

#### MSC CM significantly reduced cell death markers and improved viability after simulated ischemia/reperfusion

Addition of MSC CM at the onset of simulated reperfusion resulted in a significant reduction of LDH activity, averaging 12% of control ( $p < 0.01$ ). MTT assay showed an improved viability, averaging 175% of control (Figures 4 and 5). An additional experiment using previously frozen (1-month storage at  $-80^{\circ}\text{C}$ ) MSC CM ( $n = 6$ ) resulted in a significant reduction of LDH activity that averaged 15% of control ( $p < 0.01$ ).

#### Addition of PI3K inhibitors abolished the protection afforded by MSC CM, but not by freshly resuspended MSC

Addition of LY294002 or Wortmannin to MSC CM at the onset of simulated reperfusion significantly abolished the reduction of LDH activity as well as the improvement in NRC viability observed with MSC CM alone. Addition of LY294002 or Wortmannin to freshly resuspended allogeneic MSC had no significant effect on LDH activity and MTT assay compared with freshly resuspended MSC alone (Figures 4 and 5). Additional control groups with addition



**Figure 5** MTT assay measuring NRC viability after simulated ischemia/reperfusion. CsA, freshly resuspended allogeneic MSC and MSC CM added at the onset of simulated reperfusion significantly improved NRC viability as compared with control. Addition of PI3K inhibitors to MSC CM significantly abolished the preservation of NRC viability. Addition of antibodies neutralizing VEGF or IGF-1 did not modify the effect of MSC CM on NRC viability. Values are expressed as percent of viability compared with sham. Bar graph shows mean  $\pm$  SD. Numbers in each group were as follows: sham,  $n = 6$ ; control,  $n = 6$ ; DMEM + CsA,  $n = 6$ ; DMEM + MSC,  $n = 6$ ; MSC CM,  $n = 6$ ; MSC CM + LY294002,  $n = 6$ ; MSC CM + Wortmannin,  $n = 6$ ; MSC CM + anti-rVEGF,  $n = 6$ ; DMEM + MSC + LY294002,  $n = 6$ ; and DMEM + MSC + Wortmannin,  $n = 6$ . One-way ANOVA was significant ( $p < 0.0001$ ); post hoc analysis was a Dunnett's multiple comparison test of all groups vs control.  $*p < 0.05$  vs control;  $**p < 0.01$  vs control.

of LY294002 or Wortmannin alone did not modify LDH activity compared with control (data not shown).

#### MSC CM contains growth factors known to activate the PI3K pathway

We sought to determine whether the observed protection by MSC CM is related to the presence of growth factors known to be potential activators of the PI3K pathway. We measured VEGF, IGF-1 and HGF concentrations in the MSC CM, MSC, CsA and DMEM groups. VEGF concentration in the MSC CM group averaged  $217 \pm 91$  pg/ml, whereas it was below the detection level in all other groups. IGF-1 concentration averaged  $115 \pm 25$  ng/ml in MSC CM,  $50 \pm 22$  ng/ml in MSC,  $53 \pm 18$  ng/ml in CsA and  $52 \pm 28$  ng/ml in DMEM ( $p < 0.001$  for MSC CM vs all other groups). These data show that the concentration of IGF-1 found in MSC and CsA is entirely related to the presence of IGF-1 in fresh DMEM. HGF concentration was below the detection level in all groups.

#### Addition of antibodies blocking anti-IGF-1 and VEGF did not modify the protection afforded by MSC CM

Addition of antibodies blocking VEGF or IGF-1 to MSC CM had no effect on LDH activity when compared with MSC CM alone (Figure 4).

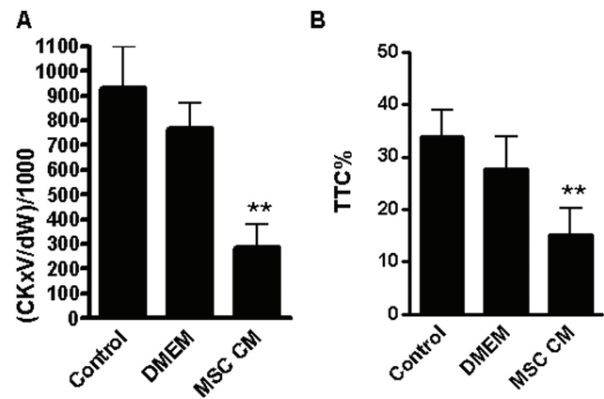
#### Ex vivo experiments on isolated heart suggest that addition of MSC CM at the time of reperfusion after prolonged ischemia could reduce infarct size

Infusion of MSC CM at the onset of reperfusion resulted in a significant reduction in CK release in the coronary effluent during the reperfusion period. Measurement of infarct size after TTC staining also showed a significant reduction in the group receiving MSC CM infusion at the onset of reperfusion (Figure 6).

## Discussion

In the present study, we report that freshly resuspended rat MSC, added to NRC at the time of simulated reperfusion after simulated ischemia, can significantly reduce reperfusion-induced cell injury in an in vitro model. This protective effect was reproduced by the administration of MSC CM alone. Addition of PI3K pathway inhibitors abolished the protection afforded by MSC CM but had no effect on the protection afforded by resuspended MSC. Data from our ex vivo model suggest that infusion of MSC CM at the onset of myocardial reperfusion after ischemia may also reduce infarct size.

The novelty of our work relates to the use of MSC and MSC CM at the onset of simulated reperfusion to investigate a potential “post-conditioning-like” protective effect. Reports have demonstrated that implantation of MSC in cardiac tissue results in a significant functional improvement.<sup>4</sup> In most instances, however, MSC had been injected either in non-reperfused tissue or minutes to hours after reperfusion.<sup>34,35</sup> In contrast, in our experimental prepara-



**Figure 6** Ex vivo evaluation of MSC conditioned media on isolated rat heart ischemia reperfusion injuries. (A) Measurement of creatine kinase (CK) release in the coronary effluent during the reperfusion period after ischemia–reperfusion. MSC CM significantly reduced CK release compared with control; DMEM did not significantly reduce CK release compared with control. Results are expressed as: CK (international units per liter) × volume of coronary effluent (V)/heart dry weight (dW), (CK × V/dW)/1,000. Bar graph shows mean ± SD. Number in each group was 6. One-way ANOVA was significant ( $p < 0.0001$ ); post hoc analysis was a Dunnett’s multiple comparison test of all groups vs control. \*\* $p < 0.01$  vs control. (B) Measurement of infarct size using triphenyl-tetrazolium chloride (TTC) staining. MSC CM significantly reduced infarct size compared with control; DMEM did not significantly reduce infarct size compared with control. Total area of necrosis (TTC%) is expressed as a percentage of total left ventricular (LV) area. Bar graph shows mean ± SD. Number in each group was 6. One-way ANOVA was significant ( $p < 0.0001$ ); post hoc analysis was a Dunnett’s multiple comparison test of all groups vs control. \*\* $p < 0.01$  vs control.

tions, we added MSC or MSC CM at the onset of reperfusion. Recent studies of ischemic post-conditioning have clearly demonstrated in vivo that most of the lethal reperfusion injury is activated within the first minutes of reflow.<sup>36</sup> Therefore, our data suggest that the observed reduction of myocardial injury observed in the present in vitro and ex vivo studies is likely related to attenuation of reperfusion injury. The effect of MSC and their conditioned media is comparable to that observed by Sun et al, who assessed the effect of hypoxic post-conditioning on cultured NRC.<sup>28</sup>

A second important observation of our study was that MSC CM alone was as efficient as MSC in the in vitro model, suggesting a paracrine mechanism. Our results are consistent with a study by Hung et al, who reported that human MSC CM protected human aortic endothelial cells from hypoxia-induced apoptosis and cell death, and that the protection was partly related to the presence of anti-apoptotic and pro-angiogenic factors in MSC CM.<sup>21</sup> However, in that study, the observed protection applied to hypoxic but non-reperfused cells.

In our model, MSC CM was collected after incubation in serum-deprived culture medium to avoid the potential interference with MSC-secreted soluble factors.<sup>37</sup> This condition is known to stress cultured MSC and favor secretion of growth factors involved in cell protection against apo-

ptosis.<sup>38</sup> In a different model, Wang et al showed that MSC produced VEGF, HGF and IGF-1 in response to tumor necrosis factor (TNF).<sup>20</sup> In our experimental conditions, rat MSC secreted significant concentrations of VEGF and IGF-1 in the supernatant. However, addition of neutralizing antibodies to VEGF and IGF-1 did not block the protective effect of MSC CM. This observation suggests that the protection afforded by MSC-conditioned media may involve combined effects of several components secreted by cultured MSC.<sup>21</sup>

Regarding the protection afforded by freshly resuspended allogeneic MSC, no significant secretion of VEGF, HGF or IGF-1 was found in the preparations, and their protective effect was not blocked by addition of PI3K inhibitors. These results suggest that freshly resuspended allogeneic MSC may protect cardiomyocytes through other mechanisms, such as cell-to-cell connection and/or secretion of growth factors once in contact with reperfused NRC, as suggested by Cselenyák et al.<sup>39</sup> Further work is required to address this specific issue.

Our data suggest that the protection afforded by MSC CM may result from the activation of the PI3K pathway in NRC. This is consistent with previous findings by Hung et al, who showed that LY294002 was able to prevent the beneficial effect of MSC CM on hypoxia-induced apoptosis of endothelial cells.<sup>21</sup> Other groups have reported the protective effect of MSC and MSC CM on cultured cardiomyocytes stressed by hypoxia and reoxygenation.<sup>40,41</sup> In these models, MSC or MSC CM were added before stressing cultured cardiomyocytes. Our results are also consistent with Timmers et al, who reported the beneficial effect of intravenous injection of concentrated conditioned media from embryonic stem cell-derived MSC-like cultures 5 minutes before reperfusion in a pig model of myocardial ischemia/reperfusion.<sup>42</sup>

Some limitations our study must be acknowledged. First, in our *in vitro* model, we used neonatal rat cardiomyocytes because they can be easily cultured for several days while keeping their contractile properties. Adult human cardiomyocytes may behave differently. Second, our observations were limited to the effects of MSC and MSC CM *in vitro* on NRC, and MSC CM *ex vivo* in isolated rat's hearts. We can only speculate on the effect of MSC CM on *in vivo* myocardial ischemia/reperfusion injuries. Importantly, the CsA used in our experiment as a positive control has been shown to protect both animal and human myocardium against ischemia/reperfusion injury.<sup>30,43</sup> Future *in vivo* experiments comparing the effect of MSC and MSC CM with ischemic and pharmacologic post-conditioning are warranted. Third, we did not entirely explore the mechanisms involved in the protection afforded by freshly trypsinized MSC in which growth factor secretion was low and reduction of cell death was not affected by PI3K inhibitors.

A major limitation of MSC therapy at the time of acute myocardial infarction or heart transplantation is the availability of autologous MSC. It takes 2 to 3 weeks to grow sufficient numbers of MSC, and MSC from older patients or patients with severe heart conditions often show impaired

biologic properties.<sup>44</sup> Use of allogeneic MSC injected at the time of myocardial reperfusion would provide "off-the-shelf" cell therapy.<sup>45</sup> These cells are currently being investigated in clinical trials but more data are needed regarding the safety of allogeneic MSC therapy. Use of MSC conditioned media would provide an "off-the-shelf" alternative to allogeneic stem cell therapy at the time of acute myocardial reperfusion.<sup>42</sup>

In conclusion, our results support the proposition that addition of MSC conditioned media at the time of reperfusion significantly reduces reperfusion injury. This protection may be mediated *in vitro* by a paracrine activation of the PI3K pathway in NRC. Further studies are required to verify the intracellular pathways activated in this protection and to explore which mediators are involved in the protective effect afforded by MSC and MSC CM.

## Disclosure statement

We thank Dr Odile Gateau-Roesch for her help with the biochemical experiments.

The authors have no conflicts of interest to disclose.

## References

1. Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 2003;9:1195-201.
2. Tomita S, Mickle DA, Weisel RD, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg* 2002;123:1132-40.
3. Psaltis PJ, Zannettino AC, Worthley SG, et al. Concise review: mesenchymal stromal cells: potential for cardiovascular repair. *Stem Cells* 2008;26:2201-10.
4. Gnechi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* 2006;20:661-9.
5. Zhang M, Mal N, Kiedrowski M, et al. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB J* 2007;21:3197-207.
6. Zimmet JM, Hare JM. Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. *Basic Res Cardiol* 2005;100:471-81.
7. Angoulvant D, Clerc A, Benchalal S, et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. *Bio-rheology* 2004;41:469-76.
8. Tse WT, Pendleton JD, Beyer WM, et al. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003;75:389-97.
9. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;99:3838-43.
10. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;30:42-8.
11. Zhou HP, Yi DH, Yu SQ, et al. Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft. *Transplant Proc* 2006;38:3046-51.
12. Wu GD, Nolte JA, Jin YS, et al. Migration of mesenchymal stem cells to heart allografts during chronic rejection. *Transplantation* 2003;75:679-85.



13. Popp FC, Eggenhofer E, Renner P, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. *Transplant Immunol* 2008;20:55-60.
14. Inoue S, Popp FC, Koehl GE, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. *Transplantation* 2006;81:1589-95.
15. Yagi H, Soto-Gutierrez A, Parekkadan B, et al. Mesenchymal stem cells: mechanisms of immunomodulation and homing. *Cell Transplant* 2010;June 3. [Epub ahead of print].
16. Argaud L, Gateau-Roesch O, Raisky O, et al. Postconditioning inhibits mitochondrial permeability transition. *Circulation* 2005;111:194-7.
17. Zhao ZQ, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 2003;285:H579-88.
18. Ferrera R, Bopassa JC, Angoulvant D, et al. Post-conditioning protects from cardioplegia and cold ischemia via inhibition of mitochondrial permeability transition pore. *J Heart Lung Transplant* 2007;26:604-9.
19. Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev* 2007;12:217-34.
20. Wang M, Crisostomo PR, Herring C, et al. Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol* 2006;291:R880-4.
21. Hung S-C, Pochampally R, Chen S-C, et al. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 2007;25:2363-70.
22. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-7.
23. Sun S, Guo Z, Xiao X, et al. Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. *Stem Cells* 2003;21:527-35.
24. Ferrera R, Cuchet D, Zaupa C, et al. Efficient and non-toxic gene transfer to cardiomyocytes using novel generation amplicon vectors derived from HSV-1. *J Mol Cell Cardiol* 2005;38:219-23.
25. Montessuit C, Rosenblatt-Velin N, Papageorgiou I, et al. Regulation of glucose transporter expression in cardiac myocytes: p38 MAPK is a strong inducer of GLUT4. *Cardiovasc Res* 2004;64:94-104.
26. Bes S, Vandroux D, Tissier C, et al. Direct, pleiotropic protective effect of cyclosporin A against simulated ischemia-induced injury in isolated cardiomyocytes. *Eur J Pharmacol* 2005;511:109-20.
27. Li L, Hessel M, van der Valk L, Bax M, et al. Partial and delayed release of troponin-I compared with the release of lactate dehydrogenase from necrotic cardiomyocytes. *Pflugers Arch* 2004;448:146-52.
28. Sun HY, Wang NP, Kerendi F, et al. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca<sup>2+</sup> overload. *Am J Physiol Heart Circ Physiol* 2005;288:H1900-8.
29. Sharov VG, Todor A, Khanal S, et al. Cyclosporine A attenuates mitochondrial permeability transition and improves mitochondrial respiratory function in cardiomyocytes isolated from dogs with heart failure. *J Mol Cell Cardiol* 2007;42:150-8.
30. Argaud L, Gateau-Roesch O, Muntean D, et al. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. *J Mol Cell Cardiol* 2005;38:367-74.
31. Bhamra GS, Hausenloy DJ, Davidson SM, et al. Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic Res Cardiol* 2008;103:274-84.
32. Germack R, Dickenson JM. Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* 2005;39:429-42.
33. Ferrera R, Michel P, Ovize M. Paradoxical toxicity of cardioplegic compounds on ischemic cardiomyocyte using optimal design strategy. *J Heart Lung Transplant* 2005;24:904-11.
34. Halkos ME, Zhao ZQ, Kerendi F, et al. Intravenous infusion of mesenchymal stem cells enhances regional perfusion and improves ventricular function in a porcine model of myocardial infarction. *Basic Res Cardiol* 2008;103:525-36.
35. Tang J, Xie Q, Pan G, et al. Mesenchymal stem cells participate in angiogenesis and improve heart function in rat model of myocardial ischemia with reperfusion. *Eur J Cardiothorac Surg* 2006;30:353-61.
36. Kin H, Zhao ZQ, Sun HY, et al. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc Res* 2004;62:74-85.
37. Dai W, Hale SL, Kloner RA. Role of a paracrine action of mesenchymal stem cells in the improvement of left ventricular function after coronary artery occlusion in rats. *Regen Med* 2007;2:63-8.
38. Bhakta S, Hong P, Koc O. The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation. *Cardiovasc Res* 2006;7:19-24.
39. Cselenyák A, Pankotai E, Horvath EM, et al. Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections. *BMC Cell Biol* 2010;11:29.
40. Xu M, Uemura R, Dai Y, et al. In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. *J Mol Cell Cardiol* 2007;42:441-8.
41. Dai Y, Xu M, Wang Y, et al. HIF-1 $\alpha$  induced-VEGF overexpression in bone marrow stem cells protects cardiomyocytes against ischemia. *J Mol Cell Cardiol* 2007;42:1036-44.
42. Timmers L, Lim SK, Arslan F, et al. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;1:129-37.
43. Piot C, Croisille P, Staat P, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med* 2008;359:473-81.
44. Xin Y, Wang YM, Zhang H, et al. Aging adversely impacts biological properties of human bone marrow-derived mesenchymal stem cells: implications for tissue engineering heart valve construction. *Artif Organs* 2010;34:215-22.
45. Hare JM. Translational development of mesenchymal stem cell therapy for cardiovascular diseases. *Tex Heart Inst J* 2009;36:145-7.

## 2. Unpublished data

In complement to LDH activity and cell viability assessed by the MTT experiment, measurement of troponin I confirmed the protective effect of MSC and MSC CM, as well as the implication of the PI3kinase pathway and the inefficacy of the single blocking of VEGF or IGF-1 using specific antibodies (figure 25).

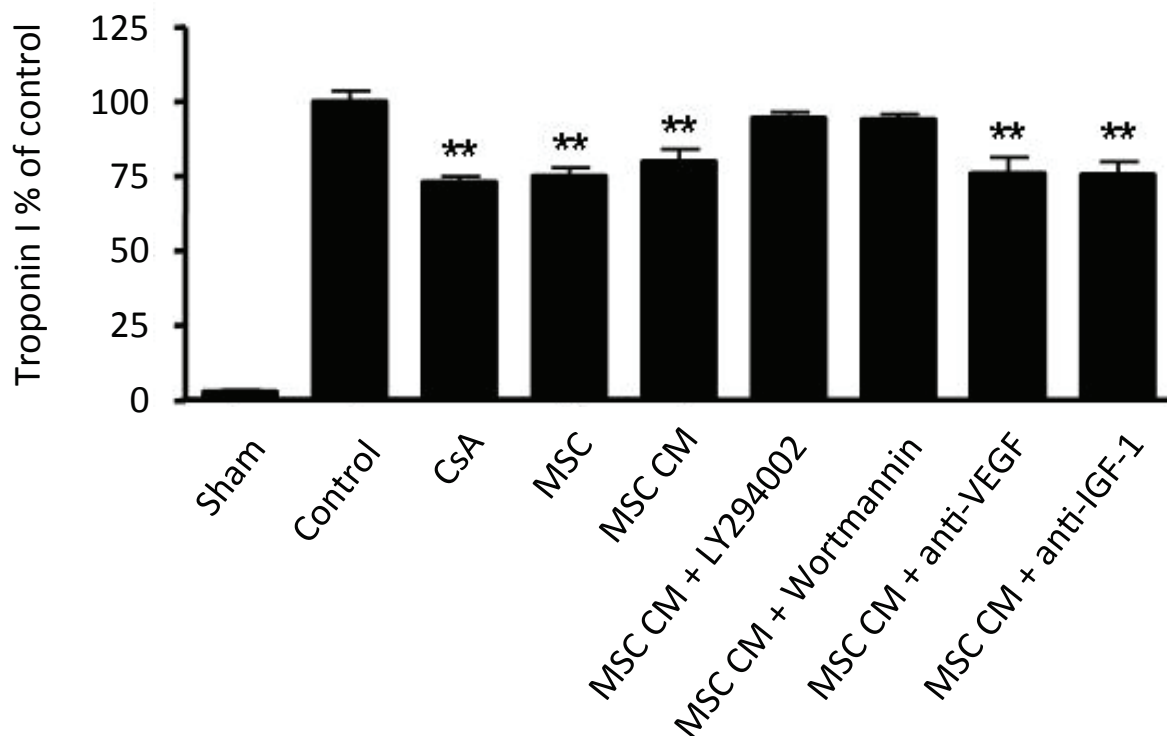


Figure 25. Troponin I at the end of reperfusion, expressed in % of the ischemic control value. \*\*  $p < 0.01$  versus control.

## 3. Additional elements for discussion

An important point is missing from the discussion above: the role of cardiofibroblasts. These cells, whose role is often neglected, constitute the major cell type in the heart and have mainly a role of support for the cardiomyocytes, producing

extracellular matrix and secreting growth factors involved in the maintenance of cardiac function <sup>111</sup>. In our model, the protocol of isolation of NRC contains a stage of differential attachment of fibroblasts with the objective of a significant reduction of the proportion of fibroblasts in the culture. Therefore, the modified ratio of cardiomyocytes/fibroblasts could impact our results in one way or the other. Moreover, unpublished data from our research group suggest that fibroblasts do have a significant role during ischemia/reperfusion, altering the response of cardiomyocytes to ischemia/reperfusion. These data suggest that the presence of fibroblasts at an adequate concentration protects cardiomyocytes up to a certain point against ischemia/reperfusion injury and this protection could again be mediated by the various growth factors secreted by these cells, such as Fibroblast Growth Factor-2 <sup>112-114</sup>.

In this study, we leak as well data regarding the role of the cardiac extracellular matrix and the impact of inflammation on cell survival, particularly as MSC were shown to reduce oxidative stress and systemic inflammation <sup>115, 116</sup>.

Since this publication, there has been in the literature several article supporting this hypothesis of a protective paracrine effect of stem cells against ischemia/reperfusion in different tissues, suggesting that the future of myocardial stem cell therapy could be the identification and production of the appropriate mix of protecting factors produced by the stem cells <sup>117-120</sup>. Reprogrammation of the resident cells through genetic exchange via microvesicles is another suggested mechanism of action <sup>121</sup>.

#### 4. Conclusion

Thus, despite poor initial clinical results, myocardial stem cell therapy still appears to be a viable therapeutic tool, thanks to all the fundamental studies aiming at understanding its various potential mechanisms of action. Our data suggest an activation of the RISK pathway as mediator of protection, i.e. a postconditioning-like effect. Hopefully, this doesn't necessarily imply that this technique will be in direct competition with ischemic postconditioning. In fact, combination of several new

techniques might be the key to significant improvement of the prognosis of patients, as suggested by Chen et al <sup>122, 123</sup>.

## **B. Part two: Inhibition of the reverse activity of the mitochondrial F1Fo ATP synthase protects against ischemia-induced injury**

### **1. Targeting mitochondrial F1Fo ATP synthase reduces the impact of myocardial ischemia**

This article is ready for submission in the British Journal of Pharmacology.

#### a. Summary

Mitochondria, through their F1Fo ATP synthase, are the powerhouse of cells. When the supply of oxygen and nutrients is impaired, e.g. during ischemia, these organelles are subjected to many changes, generally to promote survival. However one of these changes appears to be clearly deleterious: during ischemia, the lack of oxygen contributes to the cessation of activity of the mitochondrial respiratory chain. Protons stop being transferred from the mitochondrial matrix to the intermembrane space, which leads to the dissipation of  $\Delta\psi_m$ . At this point, the mitochondrial F1Fo ATP synthase reverses its activity and starts hydrolysing ATP and transferring protons from the matrix to the intermembrane space in a vain attempt to maintain the  $\Delta\psi_m$ . This “ATP wastage” results in an accelerated depletion of the ATP stocks and reduced cell survival. IF1, the endogenous inhibitor of this reverse activity, has protective effects against ischemia and can improve cell

survival. A compound from the BMS Company, BMS199264, was shown to be cardioprotective in a Langendorff model of ischemia/reperfusion. However, no subsequent experimental publication followed the initial report. Therefore, in collaboration with the Department of Chemistry of UCL, we developed several benzodiazepine-derived compounds based on the BMS compounds and determined whether they could selectively inhibit the reverse activity of the ATP synthase, preserve ATP and reduce ischemic cell death.

We chose the cardiac cell line HL-1 cells for this work. We exposed the HL-1 cells to inhibitors of the electron transport chain to cause the reversal of the ATP synthase and monitored their  $\Delta\psi_m$  using fluorescence microscopy and the dye Rh 123. We expected addition of one of the tested compounds to produce mitochondrial depolarization as the result of an effective inhibitory effect on the reverse activity of the ATP synthase. We also determined the effect of the compounds on several mitochondrial parameters, i.e. NADH/NAD<sup>+</sup> ratio, oxygen consumption and  $\Delta\psi_m$ , in basal conditions to ensure that they wouldn't compromise the cellular homeostasis. If a compound was considered able to selectively inhibit the ATP hydrolase, we determined whether it could effectively preserve ATP in indirect ATP monitoring using the dye MgG, and whether it could prevent cell death in a simulated ischemia/reperfusion assay. In this last experiment, we calculated an index of cell death using fluorescence microscopy based on the ratio of PI positive cells/Hoechst positive cells.

Seven compounds were produced and tested at 10 and 100  $\mu\text{M}$ . Addition of only four compounds, respectively VG007 at 100  $\mu\text{M}$ , VG013 at 10 and 100  $\mu\text{M}$ , VG019 at 100  $\mu\text{M}$  and VG025 at 10 and 100  $\mu\text{M}$ , resulted in mitochondrial depolarization in conditions when the ATP synthase was reversed by rotenone and antimycin A. Out

of these compounds only VG013 at 10  $\mu\text{M}$  appeared to be selective of this reverse activity, as we didn't observe in basal conditions any significant effect on  $\Delta\psi\text{m}$ , NADH/NAD<sup>+</sup> ratio of oxygen consumption. The MgG experiment suggested that this compound could reduce ATP consumption in conditions when the ATP synthase was reversed, supporting the hypothesis that it was a selective inhibitor of the ATP hydrolase activity. Last, pre-treatment of HL-1 cells with this compound prior to an ischemia/reperfusion sequence was followed by a significant reduction of the index of cell death, emphasizing the potential cardioprotective effect of VG013.

Thus, we identified a novel molecule that selectively inhibited the reversal of the ATP synthase encountered in ischemic conditions. This compound displayed in vitro a cardioprotective effect that needs to be confirmed in other models.

# TARGETING MITOCHONDRIAL F1Fo ATP SYNTHASE REDUCES THE IMPACT OF MYOCARDIAL ISCHEMIA

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Running head: Inhibiting the F1Fo mitochondrial ATP synthase

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## Abstract

The mitochondrial F1Fo-ATP synthase is responsible for the synthesis of ATP. During ischemia, mitochondrial respiration is compromised and the enzyme reverses, acting as an ATPase, hydrolysing ATP and translocating protons and so maintaining mitochondrial membrane potential. ATP consumption by the F1Fo-ATPase can rapidly deplete ATP and accelerate cell death. The compound BMS199264 inhibits the ATPase activity without compromising ATP synthesis by the enzyme and is cardioprotective. We have

therefore sought similar small molecule inhibitors that will selectively inhibit the ATP hydrolase activity that might preserve ATP and delay hypoxic cell death.

We developed benzodiazepine analogues based on the published BMS compounds and tested their ability to selectively inhibit the ATPase activity. All experiments were performed on HL-1 cells and, using fluorescence microscopy, we studied the effects of the compounds (at 10 and 100  $\mu\text{M}$ ) on the mitochondrial membrane potential (in presence or absence of inhibitors of the electron transport chain), the NADH autofluorescence and the ATP stocks. We also determined their effect on basal oxygen consumption using a Clark electrode and performed an ischemia-reperfusion assay to establish their cardioprotective effect.

Four compounds significantly inhibited the reverse activity of the ATPase. However all of them, except VG013 at 10  $\mu\text{M}$ , revealed adverse effects including inhibition of ATP synthesis or uncoupling effects. VG013 also showed significant preservation of the ATP and significant cardioprotection reducing ischemic cell death by 70%.

Thus, we have found a novel compound that selectively inhibits the reverse activity of the mitochondrial F<sub>1</sub>F<sub>o</sub>-ATPase and is protective against ischemia-induced injury.

Keywords: Mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase, myocardial ischemia-reperfusion, IF1.



## Introduction

During the past decade most of the research in the field of ischemia-reperfusion aimed at reducing lethal reperfusion injury with significant success, as attested by the exciting results of the clinical trials evaluating ischemic postconditioning or pharmacological postconditioning with Cyclosporine A (CsA) (Piot *et al.*, 2008; Staat *et al.*, 2005; Thibault *et al.*, 2008). However we should not completely forget the importance of ischemia-induced injury, which still accounts for at least 60% of the total lesion in pathologies such as acute myocardial infarction, as not everything has been discovered and tempted yet to reduce the ischemic burden (Yellon *et al.*, 2007). Moreover, with the progresses of surgery and organ transplantation, there are more and more situations of predictable ischemia that could benefit of any significant reduction of the ischemia-induced injuries.

Considering the metabolic changes occurring during ischemia, the reversal of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase appears to be a valuable therapeutic target in order to improve the survival of tissues suffering ischemia. This enzyme, fifth complex of the mitochondrial respiratory chain, is responsible for the majority of ATP production in the mammalian cell in normoxic conditions. It phosphorylates ADP into ATP using the protonmotive force, i.e. the proton gradient between the mitochondrial matrix and the mitochondrial intermembrane space resultant from the normal functioning of the respiratory chain (Senior, 1988). The disruption of oxygen supply due to ischemia contributes to the blocking of the electron transport chain, affecting the transfer of protons from the matrix into the intermembrane space and thus leading to a fall in the mitochondrial membrane potential ( $\Delta\psi_m$ ). The

mitochondria “adapt” to this situation by reversing the activity of their ATP synthase, which starts to hydrolyze ATP in order to transfer protons to the intermembrane space in an attempt to maintain the  $\Delta\psi_m$  (Jennings *et al.*, 1991; Rouslin *et al.*, 1990). This phenomenon is responsible for the commonly called “ATP wastage” that accounts for the consumption of up to 80% of the ATP stocks during ischemia, precipitating the cell to death when the stocks are over (Dimroth *et al.*, 2000).

Blocking the reversal of the ATP synthase by overexpression of IF1, i.e. the natural inhibitor of the ATP hydrolase activity, allows preservation of the ATP stocks and improves cell survival, as demonstrated by Campanella *et al.* (Campanella *et al.*, 2008). Oligomycin B and Aurovertin B are pharmacological inhibitors of the ATP hydrolase but none of them is specific for this activity and they do also inhibit ATP synthesis in normal conditions. Grover *et al.* reported that a small molecule called BMS199264 was able to selectively inhibit the reversal of the ATP synthase, preserve ATP and reduce infarct size in an isolated rat heart model of ischemia-reperfusion (Grover *et al.*, 2004). However no subsequent publications can be found regarding this drug and no similar work aiming to impact the reversal activity of the ATP synthase was published since then.

So we ought to develop several compounds based on BMS published compounds that could selectively inhibit the reverse activity of the ATP synthase and test whether they could reduce the ischemia-induced cell death in a cellular model of simulated myocardial ischemia-reperfusion.

## Methods

All chemical and reagents were purchased from Sigma, the fluorescent dyes from Molecular Probes. The IF1 construct used for transfection was a kind gift from Sir John Walker.

*Compounds production.* Several compounds were manufactured in the department of pharmacology based on the published BMS compounds according to the paper from Hamann et al.(Hamann *et al.*, 2004). These compounds were then dissolved in DMSO and the stock solutions were kept at 4°C.

*Cell cultures.* All experiments were performed on Claycomb's cardiac cell line: HL-1 cells(Claycomb *et al.*, 1998). They were cultured according to Claycomb's instructions. Transfections with IF1 construct or SiRNA in conjunction with either CFP or YFP were done using the FuGENE 6 transfection reagents kit from Roche.

*Imaging experiments.* All imaging experiments were performed on a Zeiss 510 or a Zeiss 700 confocal microscope with appropriate settings regarding excitation and emission filters.  $\Delta\psi_m$  was determined using the green fluorescent probe Rhodamine 123 loaded in dequench mode at the concentration of 10 mg/mL. Indirect measurement of ATP stocks was achieved using the probe Magnesium green, another green fluorescent probe loaded at 5 mM(Leyssens *et al.*, 1996). NADH/NAD<sup>+</sup> ratio were studied using the autofluorescence properties of NADH (blue fluorescence) under UV light

excitation. Last, cell death following the ischemia-reperfusion experiments was assessed by calculating the dead cells/total cells ratio after loading the cells with Hoechst (1  $\mu\text{g}/\text{mL}$ ) and propidium iodide (15  $\mu\text{g}/\text{mL}$ ). All dyes were loaded at room temperature for 30 minutes with the exception of Hoechst and propidium iodide that were loaded for 10 minutes.

*Assesment of the  $\Delta\psi_m$ .* Monitoring the  $\Delta\psi_m$  in the presence or absence of inhibitors of the electron transport chain gives direct information regarding the action of the tested compounds on the F1Fo ATP synthase. The addition of inhibitors of the mitochondrial respiratory chain, i.e. rotenone 5  $\mu\text{M}$  (inhibits complex I) and Antimycin A 1  $\mu\text{g}/\text{mL}$  (inhibits complex III) leads, similarly to oxygen deprivation in ischemia, to the reversal of the ATP synthase: the blocking of the electron transport chain compromises the maintenance of the  $\Delta\psi_m$ , a direct result from the translocation of protons from the matrix toward the intermembrane space, and the ATP synthase starts to work backward and consume ATP in order to maintain this potential. In these conditions, the monitoring of  $\Delta\psi_m$  shows little to no change at all. But addition of oligomycin B (3  $\mu\text{M}$ ), a known inhibitor of ATP synthase activities, forward as well as reverse, leads to mitochondrial depolarization, accurately characterized by a decrease in the Standard Deviation (SD)/mean fluorescence ratio. Addition of the different tested compounds at 10 and 100  $\mu\text{M}$  in the same conditions aimed to prove their ability to inhibit the reverse activity of the ATP synthase. In the absence of inhibitors of the electron transport chain, if selective of the reverse activity of the enzyme, the tested compounds shouldn't affect the  $\Delta\psi_m$ .

*Determination of NADH autofluorescence.* NADH and NAD<sup>+</sup> are essential substrates of complex I of the electron transport chain. A modification of the NADH/NAD<sup>+</sup> ratio in basal conditions could be the sign of a significant perturbation of the metabolic homeostasis of the cell and the result of a potential toxic effect such as, in this case, inhibition of the ATP synthase activity, inhibition of the electron transport chain or uncoupling effect. NADH is autofluorescent under UV light excitation, and its oxidation in NAD<sup>+</sup>, devoid of autofluorescence properties, results in a reduction of the mean fluorescence of the mitochondria. The effect of the addition of the tested compounds on NADH fluorescence in basal conditions was determined and compared to that of rotenone 5  $\mu$ M, which leads to the accumulation of NADH by blocking of Complex I activity as attested by an increase in fluorescence.

*Oxygen consumption experiments.* Complementary to the monitoring of NADH autofluorescence, assessment of the potential toxicity of the compounds can be achieved by studying oxygen consumption of freshly trypsinized HL-1 cells in suspension in absence or presence of the compounds in comparison to oligomycin B (3  $\mu$ M) and FCCP. Using a Clark electrode, oxygen consumption can be derived from the slope of oxygen decrease in the extracellular medium. Oligomycin B in basal conditions inhibits the ATP synthase and thus reduces oxygen consumption. FCCP, by its uncoupling effect, maximises oxygen consumption by the cell. In conditions when the ATP synthase is not reversed, one expects from a selective compound an absence of effect regarding oxygen consumption.

*Insight of the ATP stocks of the cells.* Only few techniques provide direct information regarding the stocks of ATP in the cells and they are difficult to set and interpret. Reliable indirect appreciation of the stocks of ATP can be made through measurement of the concentration of free  $Mg^{2+}$ :  $Mg^{2+}$  is strongly affine with ATP as compared to ADP meaning that the higher the ATP/ADP ratio is, the lower the concentration of free  $Mg^{2+}$ . This ion concentration can be determined using the fluorescent probe Magnesium green, which fluorescence is inversely proportional to the ATP/ADP ratio. Thus, in conditions when the ATP synthase reverses, the mean fluorescence of Mg green increases over time as ATP is consumed by the ATP hydrolase. Addition of an inhibitor of ATP hydrolase such as oligomycin B is responsible for a slowing down in the increase in fluorescence, i.e. a reduced rate of consumption of ATP. The compounds that successfully demonstrated selective inhibition of the reverse activity of the ATP synthase were tested in this experiment. These experiments were performed in a recording medium where glucose was replaced by 2-deoxyglucose to ensure that the impact of ATP synthesis from anaerobic glycolysis would here be minimal.

*Ischemia-reperfusion experiments.* Once the selective inhibition of the reversal of ATP synthase demonstrated, the ability of the compounds to prevent ischemic cell death, through preservation of the ATP stocks, was determined in simulated ischemia-reperfusion assays. These experiments were performed on HL-1 cells using a commercially available ischemia chamber (Wolflabs) flowed with a mixture of 95%  $N_2$ / 5%  $CO_2$ . Prior to

ischemia, the cells were incubated with the compounds at the determined concentration for 1 hour, then gently washed twice and the culture medium was replaced with an ischemic medium free from nutrients. The plates were placed in the chamber, flowed for 20 minutes with hypoxic gas according to the manufacturer's instructions, and the chamber was placed in an incubator at 37°C. 7 hours of ischemia were necessary to generate a suitable ischemia resulting in approximately 60% cell death. Ischemia was followed by 1 hour of reperfusion consisting in replacing the ischemic medium by a normoxic medium containing nutrients and placing the plates back in a normoxic incubator. At the end of reperfusion, the cells were loaded with Hoechst and propidium iodide, fixed with paraformaldehyde 4% and stored in the dark at 4°C for further analysis on the fluorescence microscope.

*Data analysis.* Microscope images analyses were performed using the free online available software FIJI. Statistical analyses were achieved with the commercially available GraphPad Prism 5 software. One-way ANOVA followed by post tests (Dunnett's multiple comparison tests) were used with a difference considered as statistically significant when  $p < 0.05$ .

## Results

Seven compounds (VG002, VG006, VG007, VG013, VG015, VG019 and VG025) were manufactured and considered as suitable candidates for the following experiments. All compounds were initially tested at 100  $\mu\text{M}$  and, when considered appropriate (i.e. when the 100  $\mu\text{M}$  concentration produced results), at 10  $\mu\text{M}$ . Figure 1 shows the chemical structure of the compounds.

*Inhibition of the reverse activity of the ATP synthase.* Among the seven tested compounds, only VG007, VG013, VG019 and VG025 induced a significant decrease in Rh123 SD/mean fluorescence ratio in the presence of inhibitors of the electron transport chain (-42.61%, -40.79%, -29.74% and -64.12% respectively versus baseline,  $p < 0.05$ ), suggesting an inhibition of the reverse activity of the ATP synthase (Figure 2). This SD/mean ratio decrease was similar to that of oligomycin B, our positive control, and that of FCCP (-49.37% and -52.52% respectively versus baseline,  $p < 0.05$ ) for VG007 and VG013. Out of these 4 compounds, only VG013 and VG025 displayed a significantly comparable effect at 10  $\mu\text{M}$  (-30.15% and -46.03% respectively versus baseline,  $p < 0.05$ ), though the decrease of the ratio was significantly smaller with VG013 compared to oligomycin B.

*Potential toxicity of the compounds.* VG025 at both concentrations was the only compound out of the 4 responsible for a significant decrease in SD/mean ratio in the absence of inhibitors of the mitochondrial respiratory chain (-47.48% for 100 $\mu\text{M}$  and -7.30% for 10 $\mu\text{M}$  versus baseline,  $p < 0.05$ ), and thus in  $\Delta\psi\text{m}$  (Figure 3A).



Regarding NADH autofluorescence, addition of VG007, VG013 and VG019 at 100  $\mu\text{M}$  were followed by a significant increase in NADH fluorescence (+116.50%, +30.54%, +20.51% respectively versus baseline,  $p < 0.05$ ), though, for VG007, this effect seems due to a possible autofluorescence of the compound (Figure 3B).

Last of the toxicity experiments, monitoring of the oxygen consumption in basal conditions showed that only VG013 at 10 $\mu\text{M}$ , VG019 at 100  $\mu\text{M}$  and VG025 at 10  $\mu\text{M}$  didn't affect the slope of oxygen decrease in the medium (+3.32%, -0.34% and -1.19% respectively versus baseline,  $p = \text{ns}$ ), i.e. oxygen consumption in the cells (Figure 3C).

All these results put together suggested that only VG013 at 10  $\mu\text{M}$  doesn't significantly affect the homeostasis of HL-1 cells while being able to significantly and thus selectively inhibit the reverse activity of the ATP synthase.

*Indirect assessment of the effect of VG013 on ATP stocks.* Addition of VG013 at 10  $\mu\text{M}$  to HL-1 cells after inhibition of the electron transport chain was responsible for a significant slowing down of the increase in Magnesium green fluorescence (+6.34% versus +25.93% when the ATP synthase runs backward with no further intervention,  $p < 0.05$ ), traducing a slowing down in the hydrolysis of ATP and thus preservation of ATP by VG013. This effect was similar to that of oligomycin B (+4.27%) (Figure 4).

*Effect of VG013 on cell survival after ischemia.* VG013 at 10  $\mu\text{M}$  significantly reduced the ratio of propidium iodide positive cells (-54.83% versus ischemic

control,  $p < 0.05$ ) suggesting that, it could prevent or at least delay ischemic cell death at this concentration (Figure 5). This effect was comparable to the effect of well described protective molecules such as Cyclosporine A (200 nM) and Diazoxide (10  $\mu$ M) (-62.6% and -56.3% respectively versus ischemic control,  $p < 0.05$ ).

## Discussion

Thus, among seven potential candidates, we identified a drug that can selectively inhibit the reverse activity of the ATP synthase without any sign of immediate toxicity regarding the cell metabolism in basal conditions. This inhibition of the ATP hydrolase was associated with a significant preservation of the stocks of ATP and a significant reduction of ischemia-induced cell death. Our work highlights VG013 as a potential new therapeutic drug in the setting of ischemia-reperfusion.

These data echo the results from Grover et al. regarding the compound BMS199264. However, we managed to perform a more complete study of the effect of VG013 than Grover et al. did for the BMS199264. The literature doesn't reveal any information regarding the effect of BMS199264 at the cellular level other than its effect on the ATP synthase (and only on submitochondrial particles) and little is known regarding a potential perturbation of the cell homeostasis. Our experiments with VG013 focused on the monitoring of several sensible cellular indicators of cell homeostasis and didn't reveal any significant detrimental effect of VG013 on the normal cellular metabolism, suggesting that this compound is devoid of significant toxicity at the concentration of 10 $\mu$ M (our work focused on immediate toxicity though and our experiments weren't design to address late toxicity). We confirmed in our cardiac cell line that inhibition of the reverse activity of the ATP synthase was correlated with an effective preservation of the stocks of ATP leading to a reduction of the ischemic necrotic cell death. However, the action mode behind this cardioprotective effect remains to be elucidated. We questioned whether the protection afforded by VG013 could be linked to IF1 itself,

potentializing its own protective effect. Unfortunately, we failed to demonstrate such a link as overexpression or silencing of IF1 in our HL-1 cells didn't impact the effect of VG013 (data not shown). The fact that HL-1 cells are derived from mice, which are among the species with the lowest expression level of IF1, like most rodents, could however be an issue here in our attempt to show a link with IF1 and similar experiments would be worth performing in larger mammals expressing high level of IF1(Rouslin, 1991). A valuable alternative hypothesis is that VG013 interacts directly with the F1Fo ATP synthase and its selectivity could be the consequence of a change in conformation of the enzyme when it reverses its activity, change that still needs to be demonstrated.

Inversely, the cardioprotective effect of VG013 might not be due to a direct inhibitory effect on the ATP hydrolase. VG013 is a member of the benzodiazepine family and research on the mitochondrial permeability transition pore and the RISK pathway already demonstrated that activation of the benzodiazepine receptors could account for a significant cardioprotective effect through the inhibition of the opening of the mPTP and the activation of the PI3K pathway, i.e. an ischemic preconditioning-like effect(Hausenloy *et al.*, 2007). Inhibition of the reversal of the ATP synthase was linked by some authors to ischemic preconditioning as it was suggested that this could be responsible for part of the effect of preconditioning(Murry *et al.*, 1990). In the work from Vuorinen *et al.*, no direct link between IF1 and preconditioning was observed, and a direct role of adenosine was excluded but it was clear that preconditioning was accompanied by significant energy preservation and ATPase inhibition such as that seen in our study(Vuorinen *et al.*, 1995).

Activation of the benzodiazepine receptor, through the AMPc second messenger pathway, is responsible for a pharmacological preconditioning effect and such a mechanism could explain the protective effect of our VG013 compound. Whether VG013 effectively binds to the benzodiazepine receptor needs to be addressed, though this could be a plausible explanation of the similar effect of this drug and the mPTP inhibitor CsA in the ischemia-reperfusion experiments.

There are still debates in the literature regarding the real consequences of the reversal of the ATP hydrolase. Chinopoulos et al. support the idea that, despite an effective reversal of the ATP synthase into an ATP hydrolase, this enzyme doesn't significantly accounts for the reduction in the "stocks of ATP" as it doesn't necessarily have access to ATP (Chinopoulos, 2011; Chinopoulos *et al.*, 2010). The Adenine Nucleotide Translocase (ANT) is responsible for the translocation of ATP from the mitochondria to the cytosol and it was shown to reverse its activity during ischemia, translocating ATP from the cytosol to the matrix where it can be used as a substrate for the ATP hydrolase. According to these authors, ANT and the ATP synthase don't share the same reversal potential, that of ANT being the lowest, meaning that we could under experimental or physiopathological conditions (possibly during ischemia) encounter a situation where the ATP synthase works in reverse mode while the ANT is still in forward mode. In this case, there would be very little fuel for the ATP hydrolase, which wouldn't account for a significant loss in the stocks of ATP. Our work however supports the hypothesis of a significant consumption of ATP by the ATP hydrolase in ischemia-like situations, the supposed selective inhibition of this enzyme being able to preserve the stocks

of ATP, and this is in favor of reversal of both ATP synthase and ANT. This doesn't necessarily negate Chinopoulos's theory. Swietach et al. showed that under some pathological conditions (and ischemia could certainly be part of those), we could encounter pH microdomains in the rat ventricular myocytes (Swietach *et al.*, 2005). These microdomains, even transient, could result in the ANT (and possibly the F<sub>1</sub>F<sub>o</sub> ATPase) to alternate between forward and reverse mode, still sufficient to contribute to fuelling the ATP hydrolase with ATP and thus to the ATP wastage. This however remains to be demonstrated.

Thus VG013 appears to be a valuable drug, potentially deserving to be added in our inventory to reduce ischemia-induced lesions according to its ability to safely and selectively inhibit the reverse activity of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase. However, more work needs to be done to explore its action mode and confirm that the protective effect observed here at the cellular level persists at the tissular level and in vivo.

#### Acknowledgements

To Drs Nadeene Parker and Rachel Choon Tan for their technical assistance and for providing the HL-1 cells.

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#### Disclosures

None

## References

Campanella M, Casswell E, Chong S, Farah Z, Wieckowski MR, Abramov AY, *et al.* (2008). Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1. *Cell Metab* **8**(1): 13-25.

Chinopoulos C (2011). Mitochondrial consumption of cytosolic ATP: not so fast. *FEBS Lett* **585**(9): 1255-1259.

Chinopoulos C, Gerencser AA, Mandi M, Mathe K, Torocsik B, Doczi J, *et al.* (2010). Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation. *Faseb J* **24**(7): 2405-2416.

Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, *et al.* (1998). HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A* **95**(6): 2979-2984.

Dimroth P, Kaim G, Matthey U (2000). Crucial role of the membrane potential for ATP synthesis by F(1)F(o) ATP synthases. *J Exp Biol* **203**(Pt 1): 51-59.

Grover GJ, Atwal KS, Sleph PG, Wang FL, Monshizadegan H, Monticello T, *et al.* (2004). Excessive ATP hydrolysis in ischemic myocardium by mitochondrial F1F0-ATPase: effect of selective pharmacological inhibition of mitochondrial ATPase hydrolase activity. *Am J Physiol Heart Circ Physiol* **287**(4): H1747-1755.

Hamann LG, Ding CZ, Miller AV, Madsen CS, Wang P, Stein PD, *et al.* (2004). Benzodiazepine-based selective inhibitors of mitochondrial F1F0 ATP hydrolase. *Bioorg Med Chem Lett* **14**(4): 1031-1034.

Hausenloy DJ, Yellon DM (2007). Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev* **12**(3-4): 217-234.

Jennings RB, Reimer KA, Steenbergen C (1991). Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. *J Mol Cell Cardiol* **23**(12): 1383-1395.

Leyssens A, Nowicky AV, Patterson L, Crompton M, Duchon MR (1996). The relationship between mitochondrial state, ATP hydrolysis, [Mg<sup>2+</sup>]<sub>i</sub> and [Ca<sup>2+</sup>]<sub>i</sub> studied in isolated rat cardiomyocytes. *J Physiol* **496** ( Pt 1): 111-128.

Murry CE, Richard VJ, Reimer KA, Jennings RB (1990). Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* **66**(4): 913-931.

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, *et al.* (2008). Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med* **359**(5): 473-481.

Rouslin W (1991). Regulation of the mitochondrial ATPase in situ in cardiac muscle: role of the inhibitor subunit. *J Bioenerg Biomembr* **23**(6): 873-888.

Rouslin W, Broge CW, Grupp IL (1990). ATP depletion and mitochondrial functional loss during ischemia in slow and fast heart-rate hearts. *Am J Physiol* **259**(6 Pt 2): H1759-1766.

Senior AE (1988). ATP synthesis by oxidative phosphorylation. *Physiol Rev* **68**(1): 177-231.

Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, *et al.* (2005). Postconditioning the human heart. *Circulation* **112**(14): 2143-2148.

Swietach P, Vaughan-Jones RD (2005). Spatial regulation of intracellular pH in the ventricular myocyte. *Ann N Y Acad Sci* **1047**: 271-282.

Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, *et al.* (2008). Long-term benefit of postconditioning. *Circulation* **117**(8): 1037-1044.

Vuorinen K, Ylitalo K, Peuhkurinen K, Raatikainen P, Ala-Rami A, Hassinen IE (1995). Mechanisms of ischemic preconditioning in rat myocardium. Roles of adenosine, cellular energy state, and mitochondrial F<sub>1</sub>F<sub>0</sub>-ATPase. *Circulation* **91**(11): 2810-2818.

Yellon DM, Hausenloy DJ (2007). Myocardial reperfusion injury. *N Engl J Med* **357**(11): 1121-1135.



Figures

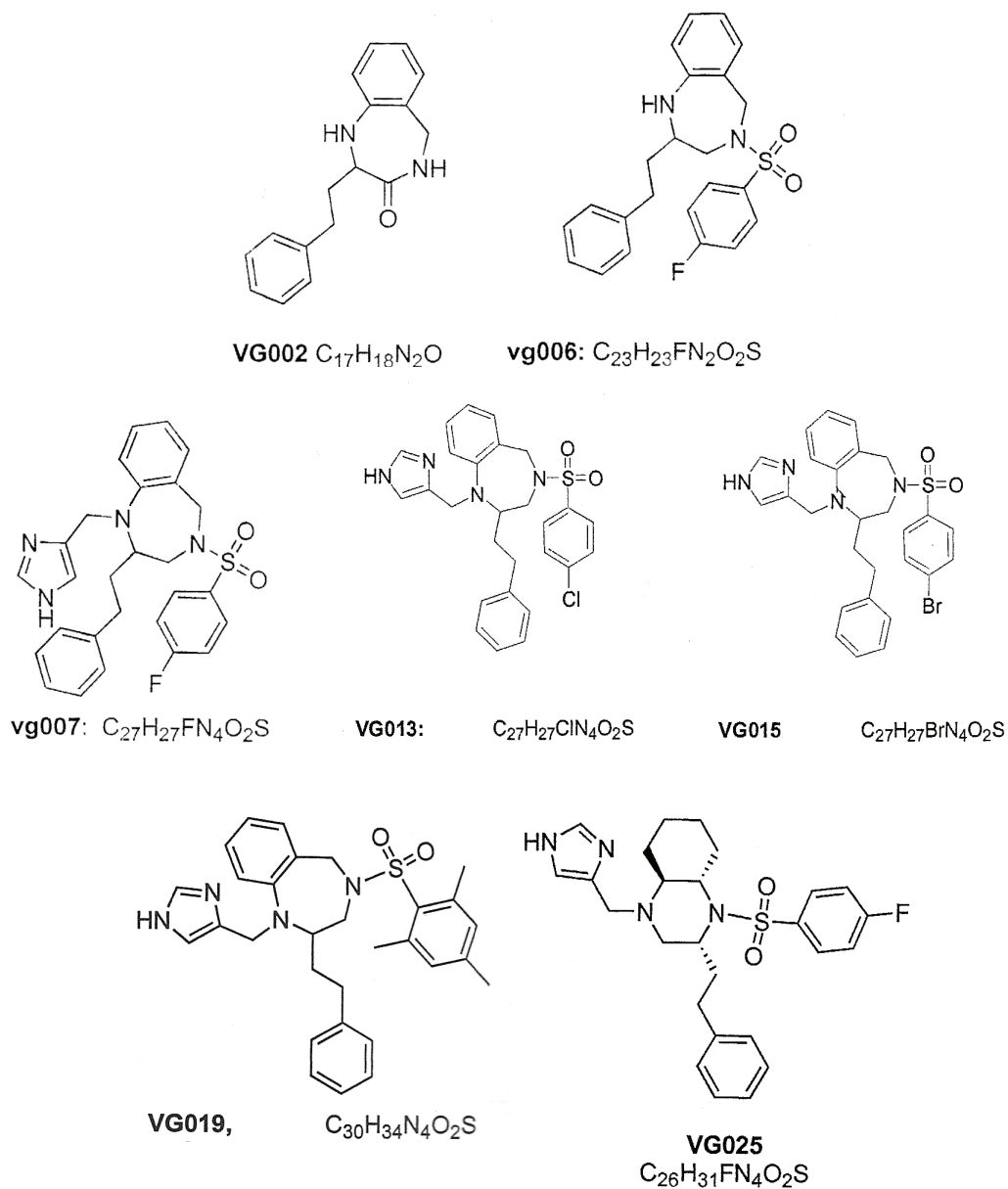


Figure1. Chemical structure and formula of the tested compounds.

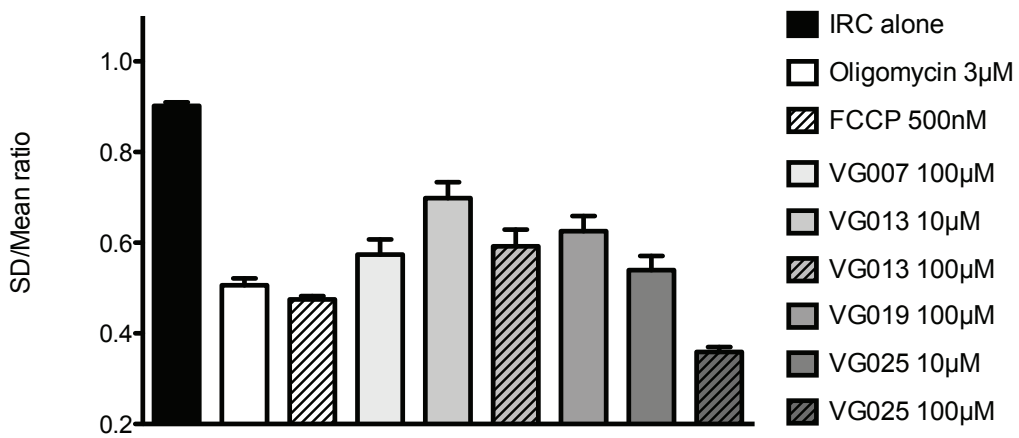


Figure 2. Compounds with IRC. SD/mean ratio, i.e. estimated change in  $\Delta\psi_m$ , 15 minutes after addition of inhibitors of the respiratory chain (IRC) followed by vehicle, the controls (oligomycin, FCCP) or the test compounds. Controls and all compounds addition resulted in mitochondrial depolarization when F1Fo ATP synthase is reversed by IRC. \*  $P < 0.05$  versus IRC alone. Data are shown as Means  $\pm$  SE.

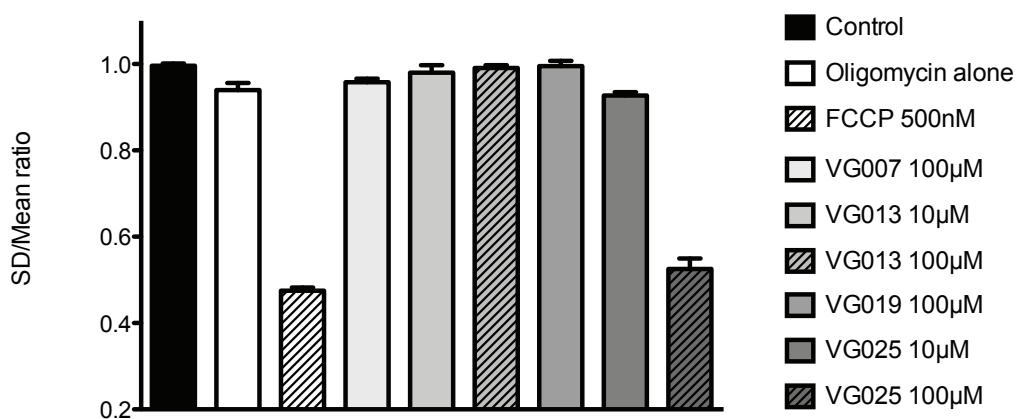


Figure 3A. Compounds without IRC. SD/mean ratio, i.e. estimated change in  $\Delta\psi_m$ , 15 minutes after addition vehicle (control), the controls (oligomycin,

FCCP) or the test compounds, i.e. in basal conditions. Only addition of the positive control FCCP, VG025 10 $\mu$ M and VG025 100 $\mu$ M led to mitochondrial depolarization, i.e. alteration of basal cellular metabolism. \* P<0.05 versus control. Data are shown as Means  $\pm$  SE.

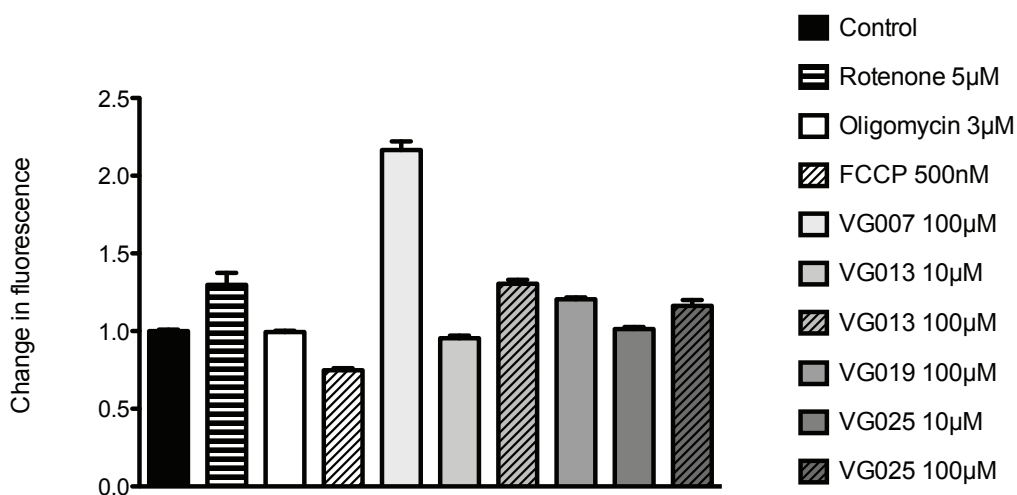


Figure 3B. NADH autofluorescence 5 minutes after addition of the vehicle (control), the controls (rotenone, oligomycin, FCCP) or the test compounds, i.e. in basal conditions. Only addition of the positive control rotenone, FCCP, VG007 100 $\mu$ M, VG013 100 $\mu$ M and VG019 100 $\mu$ M led to significant change in the cellular NADH/NAD<sup>+</sup> ratio. \* P<0.05 versus control. Data are shown as Means  $\pm$  SE.

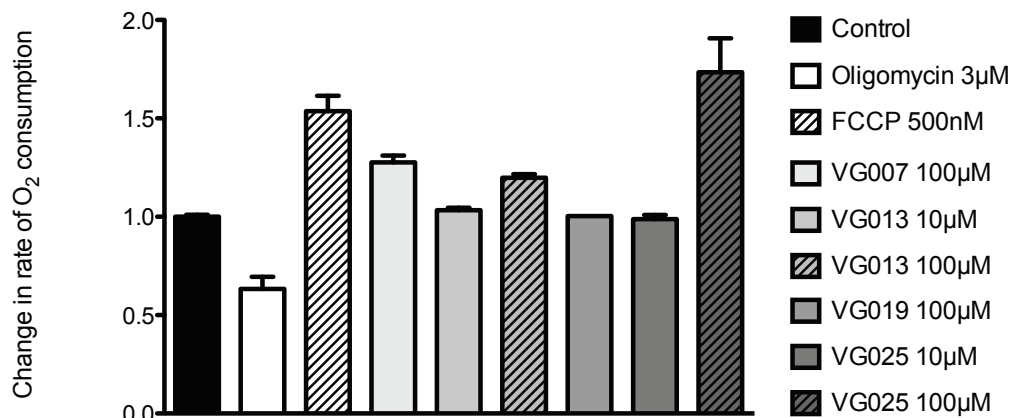


Figure 3C. Oxygen consumption measured using a Clark electrode after addition of the vehicle (control), the controls (oligomycin, FCCP) or the test compounds, in cells in suspension. Only addition of the controls, VG007 100µM, VG013 100µM and VG025 100µM led to significant change in O<sub>2</sub> consumption, i.e. aerobic metabolism in basal conditions. \* P<0.05 versus control. Data are shown as Means ± SE.

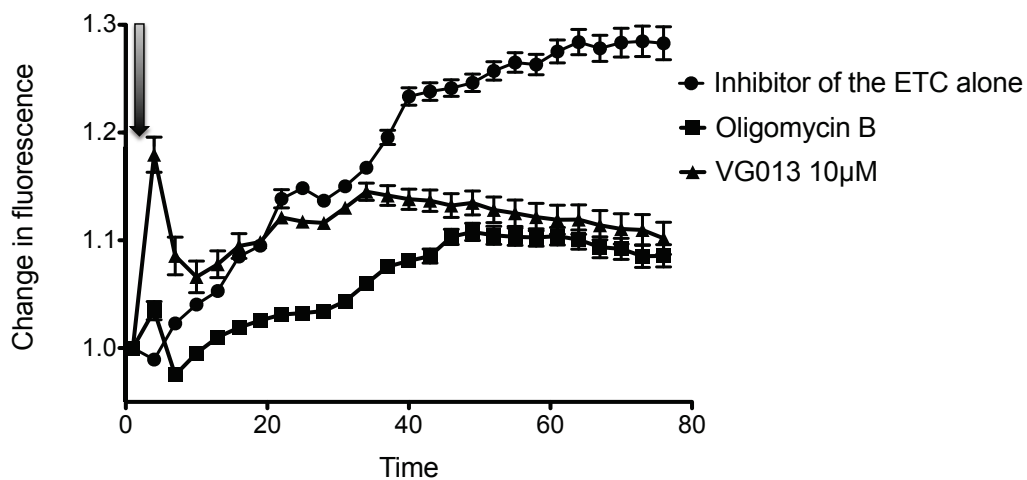


Figure 4. Indirect ATP consumption through Magnesium green fluorescence.

Magnesium green fluorescence changes over time following addition of inhibitors of the electron transport chain (ETC) and the vehicle, the positive control (oligomycin) or the VG013 10 $\mu$ M (black arrow). Addition of the positive control oligomycin and VG013 significantly slowed the increase in magnesium green fluorescence, i.e. the rate of ATP consumption in an ischemic-like cell.

Data are shown as Means  $\pm$  SE.

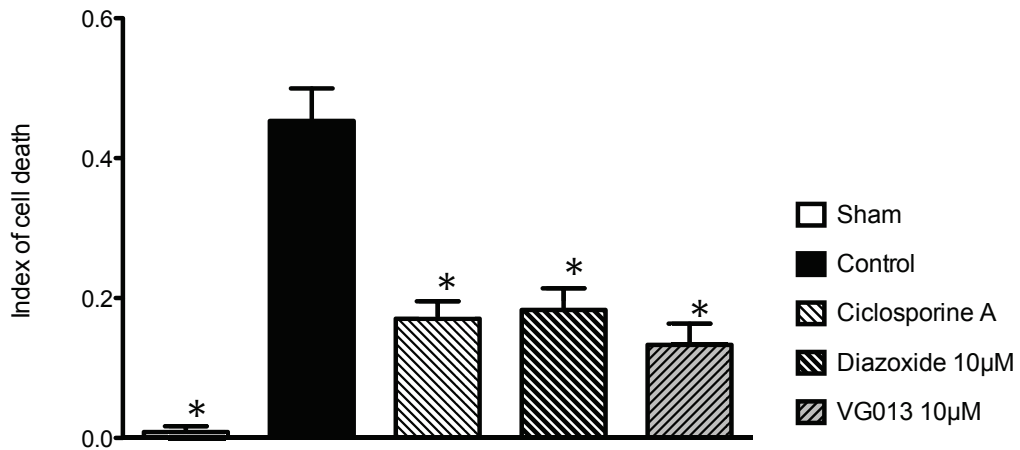


Figure 5. Cell death after ischemia/reperfusion. Protective effect of the positive controls (oligomycin, diazoxide 10 $\mu$ M) and the VG013 10 $\mu$ M in a simulated ischemia-reperfusion assay. Addition of the positive controls and VG013 10 $\mu$ M significantly decreased the ratio of propidium iodide positive cells after ischemia-reperfusion, i.e. cell death. \*  $P < 0.05$  versus control. Data are shown as Means  $\pm$  SE.

b. Data not included in the draft: direct assessment of the stocks of ATP

AT1.03 and COX AT1.03 are genetically encoded fluorescent proteins that are direct indicators of the cytosolic and mitochondrial stocks of ATP, based on the FRET principle. Experiments using these indicators couldn't be included in the draft as no statistical analysis could be performed according to the low n number of experiments. However, if we considered only the tendencies, these direct indicators of the stocks of ATP appear to be really promising.

In basal conditions, addition of VG013 at 10  $\mu\text{M}$  didn't seem to induce a notable change in the FRET ratio normalized to control (vehicle). On the contrary, addition of Oligomycin B was responsible for a decrease in the normalized FRET ratio, suggesting a decrease in the ATP stocks of the cells, in line with its inhibitory effect on the ATP synthase. The results appeared similar with AT1.03 and COX AT1.03 (figure 26).

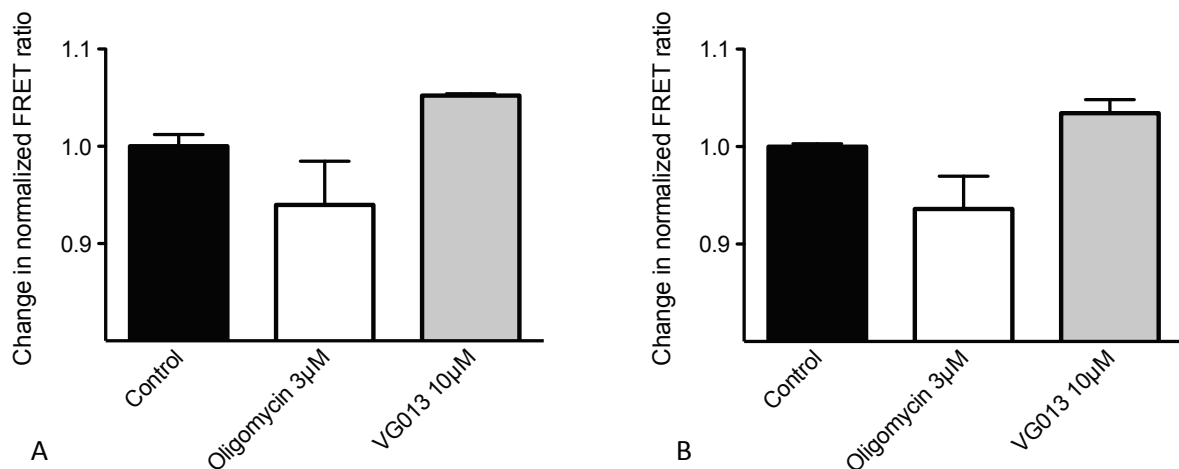


Figure 26. Preliminary results with the FRET-based genetically encoded ATP indicators COX AT1.03 (A) and AT1.03 (B).  $n=2$

2. Selective inhibition of the reversal of the mitochondrial F1Fo ATP synthase: a potential therapeutic target to reduce ischemia-induced lesions

This article is ready for submission to JMCC as a rapid communication.

a. Summary

During ischemia, the reversal of the mitochondrial F1Fo ATP synthase into an ATP hydrolase is one of the main mechanisms of accelerated ischemic cell death. This switch constitutes a vain attempt in maintaining the proton gradient between the mitochondrial matrix and the intermembrane space and thus the  $\Delta\psi_m$ . It is however responsible for an accelerated consumption of ATP called “ATP wastage” that significantly contributes to empty the cellular stocks of ATP.

IF1 is a small endogenous inhibitor of this hydrolase activity and displayed significant protective effect when overexpressed in a neuronal model of ischemic cell death. BMS199264, a compound from the BMS Company, was shown to be cardioprotective in a model of ischemia/reperfusion in isolated rat heart, through an IF1-like inhibitory effect of the reverse activity of the ATP synthase. However, we lack additional data regarding this compound that seemed promising in reducing the ischemia-induced injury. Thus, using a computational model studying 3-dimensional structure, we searched for existing compounds that could display a similar IF1-like effect able to selectively inhibit the reverse activity of the ATP synthase and reduce myocardial ischemic cell death without compromising basal cellular homeostasis.

We identified a candidate from the Ambinter Company called BTB06584. Using the cardiac cell line HL-1, we determined the optimal working concentration using a FLIPR assay by monitoring the  $\Delta\psi_m$  with the fluorescent dye Rh 123 in situation of reversal of the ATP synthase by blocking the electron transport chain with rotenone. The concentration tested ranged from 1 to 100  $\mu\text{M}$ . In these conditions, mitochondrial depolarization following addition of the compound suggested an inhibition of the ATP hydrolase activity and this was achieved only with the 100  $\mu\text{M}$  concentration and confirmed using confocal fluorescence microscopy. These data were confirmed in HeLa cells in a similar assay involving the dye TMRM after blocking of the ATP synthase by cyanide. We searched for a toxic effect of BTB06584 that would result in

an alteration of basal parameters such as  $\Delta\psi_m$  still with fluorescence microscopy and oxygen consumption with a Clark electrode. In our experimental conditions, BTB06584 didn't significantly impair these parameters. Preservation of the ATP stocks being the theoretical result of inhibition of the ATP hydrolase activity, we monitored indirectly the changes in cellular ATP concentration using the free  $Mg^{2+}$ -affine fluorescent probe MgG. In conditions of reversal of the ATP synthase using inhibitors of the electron transport chain, addition of BTB06584 resulted in a significant reduction of ATP consumption. A simulated in vitro ischemia reperfusion assay showed that BTB06584 could reduce ischemic cell death after incubation prior to ischemia. Last we sought whether this protective effect was dependent of the level of IF1 expression of the cell and performed genetic modification of HL-1 through transfection in order to overexpress or silence IF1. When IF1 was overexpressed, the efficacy of BTB06584 in inhibiting the reversal of ATP synthase was significantly increased while it was reduced (but not abolished) when IF1 was silenced.

In conclusion, our data strongly suggest that BTB06584 is a selective inhibitor of the reversal of the ATP synthase, able in ischemic conditions to preserve the stocks of ATP and reduce ischemic cell death, probably through an interaction with IF1.



## SELECTIVE INHIBITION OF THE REVERSAL OF THE MITOCHONDRIAL F1FO ATP SYNTHASE: A POTENTIAL THERAPEUTIC TARGET TO REDUCE ISCHEMIA-INDUCED LESIONS.

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### Abstract

The mitochondrial F1Fo-ATP synthase is responsible for the synthesis of ATP. During ischemia, mitochondrial respiration is compromised and the enzyme reverses, acting as an ATPase, hydrolysing ATP and translocating protons and so maintaining mitochondrial membrane potential ( $\Delta\psi_m$ ). ATP consumption by the F1Fo-ATPase can rapidly deplete ATP and accelerate cell death. The compound BMS199264 inhibits the ATPase activity without compromising ATP synthesis by the enzyme and is cardioprotective. We have therefore sought similar small molecule inhibitors that will selectively inhibit the ATP hydrolase activity that might preserve ATP and delay hypoxic cell death.

Using computational modelling, we identified a molecule named BTB06584 similar to the published BMS compounds and tested its ability to selectively inhibit the ATPase activity. All experiments were performed on HL-1 cells and/or HeLa cells and, using fluorescence microscopy, we studied the effects of the compounds on the  $\Delta\psi_m$  (in presence or absence of inhibitors of the electron transport chain) and the ATP stocks. We also determined their effect on basal oxygen consumption using a Clark electrode and performed an ischemia-reperfusion assay to establish their protective effect. We last questioned a possible interaction between this drug and IF1, the endogenous inhibitor of the ATP hydrolase, by overexpression/silencing of IF1 and repeat of the cell death experiments

BTB06584 significantly and selectively inhibited the reverse activity of the ATPase, i.e. without any significant effect on  $\Delta\psi_m$  or oxygen consumption in basal conditions. BTB06584

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also showed significant preservation of the ATP and significant protection reducing ischemic cell death in a similar way to diazoxide or cyclosporine A. Last, BTB06584 efficiency was potentialized when IF1 was overexpressed and reduced when it was silenced, suggesting a strong interaction between these molecules.

Thus, we have found a novel compound that selectively inhibits the reverse activity of the mitochondrial F<sub>1</sub>F<sub>o</sub>-ATPase and is protective against ischemia-induced injury.

### Highlights

ATP wastage plays a significant role in ischemia-induced cell death. BTB06584, a drug with similar structure to the BMS compound, selectively inhibits the mitochondrial ATP hydrolase activity. The preservation of ATP by BTB06584 prevents ischemic cell death. The action of BTB06584 is strongly linked to the level of expression of IF1.

### Keywords

Mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase; ischemia-induced lesion; IF1; ATP wastage

### Abbreviations

ANT, Adenine Nucleotide Translocase; CFP, Cyan Fluorescent Protein; CsA, Cyclosporine A; ETC, Electron Transport Chain; FCCP, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; Fluorescent Imaging Plate Recorder; IF1, Inhibitor protein of F<sub>1</sub> subunit;  $\Delta\psi_m$ , Mitochondrial Membrane Potential; Rh123, Rhodamine 123; FLIPR, TMRM, TetraMethylRhodamine Methyl Ester; YFP, Yellow Fluorescent Protein;

### Acknowledgements

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## 1. Introduction

In eukaryotic cells, ATP is mainly produced through oxidative phosphorylation by the mitochondrial F<sub>1</sub>F<sub>0</sub> ATP synthase. When the oxygen supply is compromised, e.g. during ischemia, the ATP synthase activity reverses into an ATP hydrolase activity in order to maintain the proton motive force and thus the mitochondrial membrane potential ( $\Delta\psi_m$ ) at the expenses of the cellular stock of ATP fueled by the glycolytic activity. This major ATP consumption, referred to as ATP wastage, contributes to significant cell death [1].

IF1 is a nuclear-encoded endogenous inhibitor of the hydrolase activity of the F<sub>1</sub>F<sub>0</sub> mitochondrial ATP synthase, expressed in various quantities according to the specie and the tissue considered [2]. Though it can inhibit ATP synthase, it binds essentially to the enzyme when the cellular pH is acidic, conditions encountered when the cell suffers ischemia leading to the ATP synthase working backwards [3]. Overexpression of IF1 was shown to render the cells resistant to conditions mimicking ischemia, identifying the inhibition of the ATP hydrolase as a new potential therapeutic target [4]. Hence, producing a molecule able to mimic the effect of IF1 in ischemic conditions could translate into a significant improvement in the treatment of pathologies where ischemia plays a significant detrimental role. Grover et al. developed such a compound named BMS199264 able to reduce the infarct size in a model of isolated rat heart but no study was performed at the cellular level and no other original publication confirmed its positive effects [5]. So the question remains open: could a drug with similar effects to IF1 be clinically useful and how could we explain its specificity toward the hydrolase activity of the ATP synthase?

Using a computational model, we screened a database of pharmacological molecules in order to identify a drug potentially sharing the same biological action as BMS199264 thanks to its conformation and selected a molecule from Ambinter: BTB06584. We first performed several experiments on different cellular models in order to confirm its ability to inhibit selectively the reverse activity of the ATP synthase and, by this action, to protect the cell from ischemic cell death. Second, we sought its action mode through the study of a possible interaction with IF1.

## 2. Methods

### 2.1. Chemicals and reagents

All materials were purchased from Sigma unless stated otherwise, and the fluorescent dyes were purchased from Molecular Probes. All probes were loaded at room temperature for 30 minutes.

### 2.2. Cell cultures

HL-1 cells, a cardiac cell line derived from a mouse atrial cardiomyocyte tumor lineage, were cultured according to the instructions from Dr Claycomb [6]. HeLa cells, derived from a human cervix cancer lesion, were grown in standard DMEM supplemented with foetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (4 nM). FuGENE® 6 transfection reagents (Roche) were used for the transfection experiments in both HL-1 cells and HeLa. Cells were co-transfected with either CFP or YFP in addition to IF1 construct (a gift from Sir John Walker) or IF1 siRNA (Qiagen) to overexpress or silence IF1.

### 2.3. Fluorescent Imaging Plate Reader (FLIPR) experiments

To assess the appropriate concentration of BTB06584 that could result in an efficient inhibition of the F<sub>1</sub>F<sub>o</sub> ATP hydrolase, several concentrations from 1 to 100 µM were tested on HL-1 cells after exposure to rotenone (5 µM), an inhibitor of complex I of the electron transport chain (ETC) responsible for the reversal of the activity of the ATP synthase. The cells were loaded with Rhodamine 123 (Rh123) in dequench mode (20 mM), a fluorescent dye commonly used to assess the  $\Delta\psi_m$ . In dequench mode, when the  $\Delta\psi_m$  falls, the dye, initially located in the mitochondria, redistributes to the cytosol and its mean fluorescence increases [7]. In our experimental conditions, such a response corresponds to an effective inhibition of the reverse activity of the mitochondrial ATP synthase.

### 2.4. Microscope experiments

All imaging experiments were performed with either a Zeiss 510 or Zeiss 700 confocal microscope using the appropriate lasers and emission filters.  $\Delta\psi_m$  was assessed using Rh123 or TMRM (Tetramethylrhodamine methyl ester, another fluorescent dye used to monitor

$\Delta\psi_m$ ). Cells were imaged intermittently during 18 minutes using a 488nm laser and the SD/mean ratio was monitored after addition of inhibitors of the ETC (Rotenone 5  $\mu$ M and Antimycin A 1mg/mL for HL-1, cyanide 1 mM for HeLa) followed by addition of either BTB06584 or oligomycin B (3  $\mu$ M) as a positive control. Indirect assessment of cellular ATP content was achieved with the fluorescent dye magnesium green (5  $\mu$ M) [8]. All cells were imaged in a normal recording medium. In ischemia/reperfusion experiments, cell death ratio was measured after loading the cells with Hoechst (1  $\mu$ g/mL) and propidium iodide (15  $\mu$ g/mL).

## 2.5. Ischemia/reperfusion

Ischemia/reperfusion experiments in HL-1 cells were performed using an airtight gas chamber (Wolflabs) filled with 95% N<sub>2</sub> / 5% CO<sub>2</sub> after replacing the claycomb medium with an ischemic medium. The chamber was stored for 7 hours in an incubator at 37°C. Reperfusion consisted in replacing the ischemic medium by an oxygenated reperfusion medium and storing the culture plates for 1 hour in a normoxic incubator. Seven experimental groups were considered:

- \_ sham
- \_ control (ischemia/reperfusion without any intervention)
- \_ Cyclosporine A (CsA) 200nM (a potent inhibitor of the mitochondrial permeability transition pore)
- \_ Diazoxide 10 $\mu$ M (a mitochondrial K<sub>ATP</sub> channel opener)
- \_ BTB06584 incubated with HL-1 cells, HL-1 cells overexpressing IF1 (IF1+) and HL-1 cells silenced for HL-1 (IF1-), prior to ischemia

## 2.6. Immunofluorescence assay

Successful overexpression or silencing of IF1 was demonstrated by immunofluorescence experiments. We used a rabbit polyclonal anti-mouse ATP1F1 antibody (ProteinTech, 1/100) in conjunction with an anti-rabbit Cy3 antibody (1/50). Cells were fixed with paraformaldehyde 4% and stored at 4°C before imaging.

## 2.7. Oxygen consumption experiments

HL-1 cells were trypsinized and resuspended in normal recording medium at a concentration of 10 millions/mL. 1 million non permeabilized cells were placed in a chamber in contact with a Clark electrode and oxygen tension was continuously monitored in basal condition and after addition of either BTB06584, oligomycin B or FCCP to determine a possible inhibiting effect on the ATP synthesis or an uncoupling effect.

## 2.8. Data analysis

Confocal images were processed using the FIJI software. Statistical analysis was performed using the GraphPad Prism 5 software. We used one-way ANOVA, with either Dunnett's or Newman-Keuls's multiple comparison test as a post-test. Data are expressed as mean and SEM. A  $p < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Determination of the optimal working concentrations and effect on the ATP hydrolase activity

Seven concentrations of BTB06584 were tested using the FLIPR: 1, 2.5, 5, 10, 25, 50 and 100  $\mu\text{M}$ . Only the cells receiving 100  $\mu\text{M}$  of BTB06584 displayed a significant increase in total Rh123 fluorescence, suggesting an effective inhibition of the F<sub>1</sub>F<sub>o</sub> ATP hydrolase activity. The effect of 100  $\mu\text{M}$  of BTB06584 was similar to that of oligomycin B at 3  $\mu\text{M}$  (figure 1A).

This was directly visualized and confirmed in confocal microscopy. Compared to basal conditions, addition of rotenone and antimycin A in HL-1 cells resulted in a slight but non significant decrease in the  $\Delta\psi\text{m}$  as assessed by a decrease in the SD/mean ratio. Subsequent addition of 100  $\mu\text{M}$  of BTB06584, similarly to 3  $\mu\text{M}$  of oligomycin B, resulted in a significant decrease in this ratio, reflecting the inhibition of the ATP hydrolase activity (figure 1B). Similar observations statistically significant were made with HeLa cells through the monitoring of TMRM fluorescence after addition of cyanide (data not shown).

#### 3.2. Assessment of a potential toxic effect of BTB06584 at 100 $\mu\text{M}$

To ensure that, at this concentration, BTB06584 didn't induce any toxicity, the same experiment as above was reproduced without addition of an inhibitor of the ETC. The aim was to determine the effect of BTB06584 on the ATP synthase in basal conditions, i.e. when it produces ATP. Addition of BTB06584 didn't induce any significant decrease in the SD/mean ratio, supporting its selectivity toward the ATP hydrolase activity (data not shown).

This was further emphasized by the results of the oxygen consumption experiments. Addition of oligomycin B inhibited the ATP synthase, resulting in a reduction of the oxygen consumption compared to basal. On the contrary, addition of the uncoupler FCCP increased the rate of mitochondrial respiration to its maximum, as shown by the increase in oxygen consumption. BTB06584 didn't induce any significant change in oxygen consumption compared to basal conditions, supporting again its selectivity on the ATP hydrolase (figure 1C).

### 3.3. BTB06584 preserves the intracellular stock of ATP

Monitoring of free intracellular magnesium through the mean fluorescence of magnesium green in microscopy revealed that, in conditions when the ATP synthase runs backwards, i.e. in this experiment when the ETC is inhibited (by rotenone and antimycin A), BTB06584 partly prevented the increase in fluorescence (figure 1D). The level of free magnesium in a cell being inversely correlated with the stock of ATP as its affinity to ATP is high whereas its affinity to ADP is low, this further emphasizes a selective inhibition of the ATP hydrolase by this molecule, resulting in ATP salvation in ischemic-like conditions.

### 3.4. Preservation of ATP by BTB06584 induces protection against ischemic cell death

Pre-treatment with BTB06584 during 1 hour prior to ischemia resulted in a significant reduction in the percentage of PI positive cells, i.e. ischemic cell death, compared to control (figure 2A). This reduction was similar to that observed with either CsA or diazoxide.

### 3.5. Going deeper into the action mode of BTB06584

To further understand the protection induced by BTB06584, we questioned whether its action could involve a participation of IF1. Thus, we transfected HL-1 cells with either the IF1 construct to induce overexpression of IF1 or the IF1 siRNA to silence this protein, in conjunction with CFP or YFP. Immunofluorescence experiments revealed a significant overexpression/silencing of IF1 as shown in figure 2C. When we considered the effect of BTB06584 in IF1+ and IF1- HL-1 cells and monitored the changes in  $\Delta\psi_m$  in conditions when the ATP synthase is reversed (i.e. after inhibition of the ETC by rotenone and antimycin A), we observed a significant additional decrease in the SD/mean ratio in the IF1+ cells compared to the non transfected cells and a significantly lower decrease in this ratio in IF1- cells (figure 2B). These variations depending on the level of expression of IF1 suggested that the efficacy of BTB06584 was dependent on the interaction between the ATP hydrolase and IF1. We tested whether this increase in protection was linked to a decrease in the pH of mitochondria by BTB06584, which would lead to an increased effect of IF1 itself but, in basal conditions, BTB06584 didn't affect the pH in the mitochondrial compartment (data not shown).



#### 4. Discussion

We identified a drug able to selectively inhibit the reverse activity of the ATP synthase, responsible for ATP wastage in ischemic conditions. This molecule allowed preservation of the stocks of ATP in ischemic-like conditions and reduced the amount of cell death compared to control. Moreover, its specificity toward the hydrolase activity seemed to be the results of an interaction, could it be direct or not, with IF1.

These results support the work of Grover et al. on the BMS199264 compound with significantly more details regarding the pharmacodynamics of the BTB06584 drug. Experiments performed at the cellular levels on two different cell models provide substantial information regarding the selectivity of the drug towards the enzyme of interest and its hydrolase activity in particular. Hence, Grover et al. successfully demonstrated on a submitochondrial particles model that their compound was selective of the hydrolase activity of the ATP synthase as opposed to the synthase activity but their model didn't allow showing the absence of effect on other cell structures/systems [9]. In addition we showed that modification of the expression of IF1 was responsible for a significant modulation of the effect of the BTB06584 compound. This suggests that the BTB06584 compound doesn't act directly as an inhibitor of the hydrolase activity but might instead potentialize the effect of IF1 once bound to the enzyme, but not through a drop in mitochondrial pH.

In any case, the direct role of the reversal of ATP synthase in the ischemia-induced cell death still remains controversial in the literature. Chinopoulos et al. support in their work the fact that the ATP synthase and the Adenine Nucleotide Translocase (ANT) don't share the same "reversal potential" [10, 11]. The reversal potential of the ANT would be less negative than that of the mitochondrial ATP synthase, meaning that the mitochondrial ATP synthase could work in reverse mode with the ANT working in forward mode. This configuration, which could possibly exist in conditions such as ischemia, would result in the ATP hydrolase hydrolyzing the ATP stocks in the mitochondria but without any supply of cytosolic ATP through the ANT and thus without any significant effect on the cytosolic ATP stocks maintained by the glycolytic activity. There is no doubt though that there is a decrease in the cellular ATP stocks during ischemia and that BTB06584 seems to preserve them. If Chinopoulos's theory is right, then the protective effects of BTB06584 regarding ischemic cell

death would imply a beneficial action of the molecule on others actors implied in these pathological processes, which we didn't question here. In any case, such effects wouldn't necessarily be exclusive.

BTB06584's protective effect in ischemic-like conditions seems to be potentialized by the overexpression of IF1, meaning that depending on the expression level of IF1 in the tissue exposed to ischemia, the effect of BTB06584 could vary. However, if we consider myocardial ischemia, the heart is one of the richest tissues in IF1, making BTB06584 an interesting candidate to prevent myocardial ischemia-induced lesions. Still remains the question of potential adverse effects in case the patient would suffer any neoplastic pathology as IF1-mediated survival is a described way participating in cancer growth [12]. This remains to be assessed in further experiments. In addition, a cellular effect of a drug does not necessarily imply a tissular effect and the efficiency of BTB06584 in decreasing the ischemia-induced lesions at the tissular level remains to be determined, particularly in *in vivo* models.

Thus, we have identified an interesting potential candidate to reduce ischemia-induced lesions directed toward a mitochondrial phenomenon not targeted before and which could increment our therapeutic arsenal in situations of ischemia-reperfusion.

## References

- [1] Jennings RB, Reimer KA, Steenbergen C. Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. *J Mol Cell Cardiol.* 1991;23:1383-95.
- [2] Green DW, Grover GJ. The IF(1) inhibitor protein of the mitochondrial F(1)F(0)-ATPase. *Biochim Biophys Acta.* 2000;1458:343-55.
- [3] Power J, Cross RL, Harris DA. Interaction of F1-ATPase, from ox heart mitochondria with its naturally occurring inhibitor protein. Studies using radio-iodinated inhibitor protein. *Biochim Biophys Acta.* 1983;724:128-41.
- [4] Campanella M, Casswell E, Chong S, Farah Z, Wieckowski MR, Abramov AY, et al. Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1. *Cell Metab.* 2008;8:13-25.
- [5] Grover GJ, Atwal KS, Sleph PG, Wang FL, Monshizadegan H, Monticello T, et al. Excessive ATP hydrolysis in ischemic myocardium by mitochondrial F1F0-ATPase: effect of selective pharmacological inhibition of mitochondrial ATPase hydrolase activity. *Am J Physiol Heart Circ Physiol.* 2004;287:H1747-55.
- [6] Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A.* 1998;95:2979-84.
- [7] Duchen MR, Surin A, Jacobson J. Imaging mitochondrial function in intact cells. *Methods Enzymol.* 2003;361:353-89.
- [8] Leyssens A, Nowicky AV, Patterson L, Crompton M, Duchen MR. The relationship between mitochondrial state, ATP hydrolysis,  $[Mg^{2+}]_i$  and  $[Ca^{2+}]_i$  studied in isolated rat cardiomyocytes. *J Physiol.* 1996;496 ( Pt 1):111-28.
- [9] Atwal KS, Wang P, Rogers WL, Sleph P, Monshizadegan H, Ferrara FN, et al. Small molecule mitochondrial F1F0 ATPase hydrolase inhibitors as cardioprotective agents. Identification of 4-(N-arylimidazole)-substituted benzopyran derivatives as selective hydrolase inhibitors. *J Med Chem.* 2004;47:1081-4.
- [10] Chinopoulos C. Mitochondrial consumption of cytosolic ATP: not so fast. *FEBS Lett.* 2011;585:1255-9.

[11] Chinopoulos C, Gerencser AA, Mandi M, Mathe K, Torocsik B, Doczi J, et al. Forward operation of adenine nucleotide translocase during FOF1-ATPase reversal: critical role of matrix substrate-level phosphorylation. *Faseb J.* 2010;24:2405-16.

[12] Capuano F, Guerrieri F, Papa S. Oxidative phosphorylation enzymes in normal and neoplastic cell growth. *J Bioenerg Biomembr.* 1997;29:379-84.

Figures

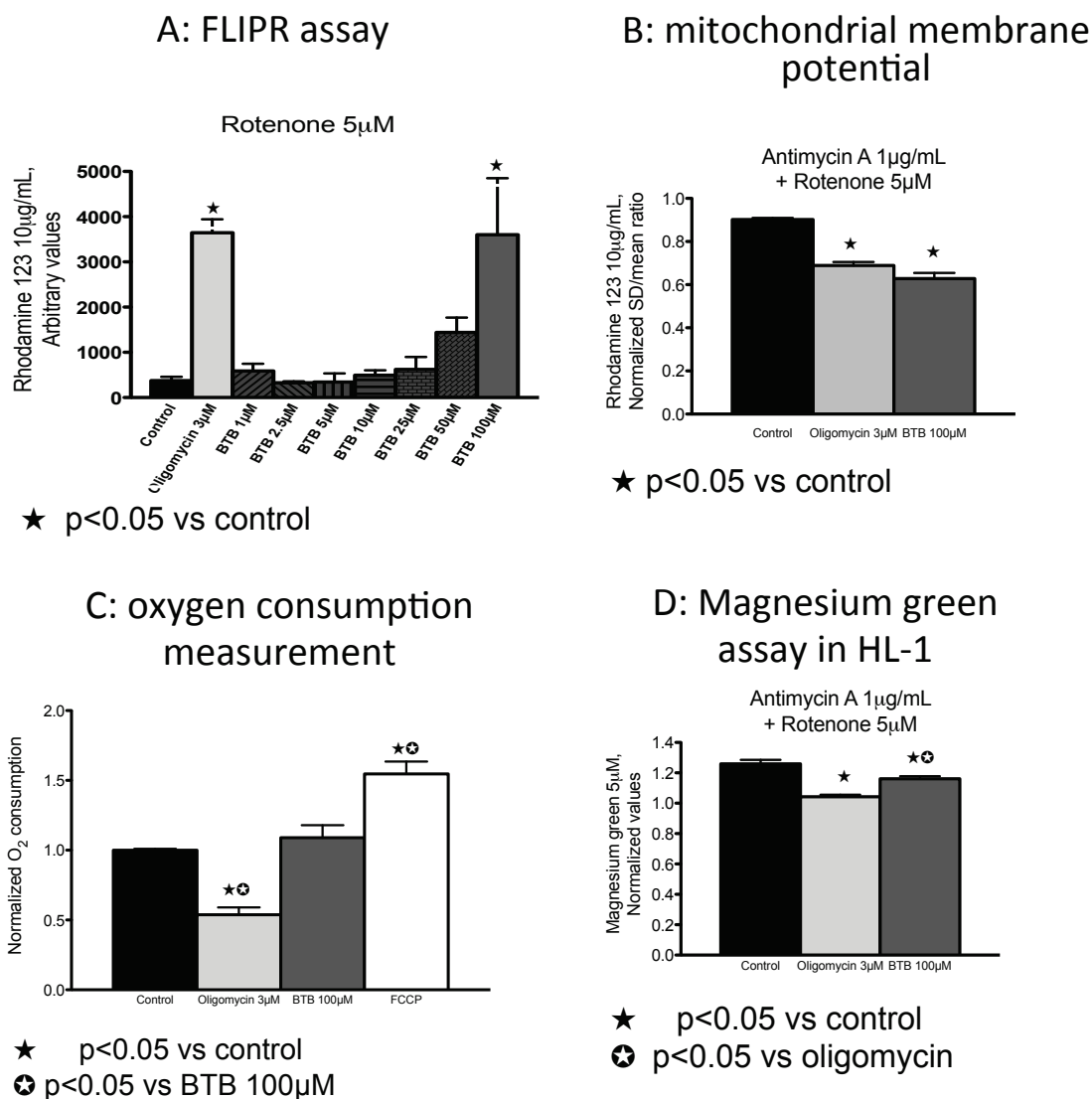
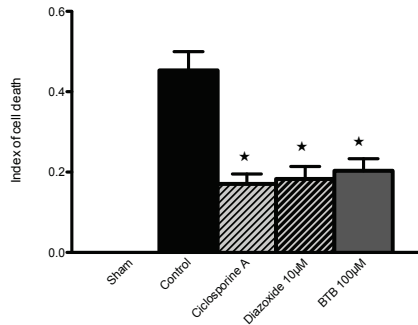


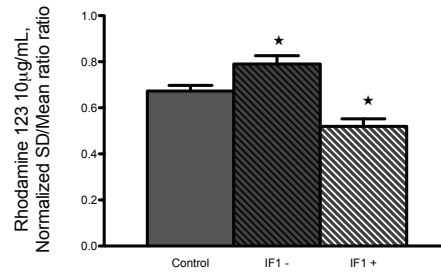
Figure1. A: Optimal concentration of BTB06584 through a FLIPR assay in HL-1 cells. B: Effect on mitochondrial membrane potential assessed by SD/mean fluorescence ratio of Rh123 in conditions of reversal of the ATP synthase as a consequence of inhibition of the electron transport chain by antimycin A and rotenone. C: Effect on oxygen consumption as a way to evaluate a possible disturbance in cell homeostasis. D: Indirect assessment on ATP stocks through monitoring of Magnesium green fluorescence.

A: Effect of BTB in simulated ischemia/reperfusion in HL-1



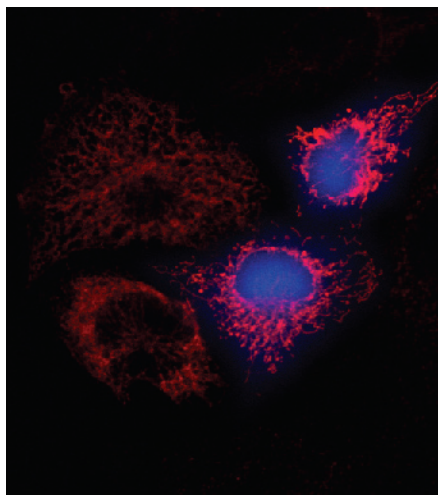
★ p<0.05 vs control

B: Effects of IF1 expression on BTB efficiency through monitoring of  $\Delta\psi_m$

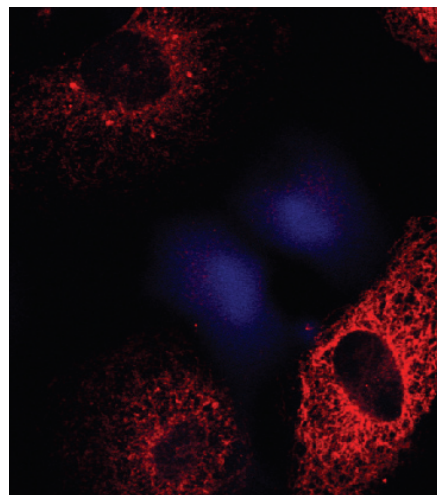


★ p<0.05 versus control  
Newman-Keuls multiple comparison test

C: Overexpression/Silencing of IF1 in HL-1



Overexpression of IF1 in HL-1 cells



Silencing of IF1 in HL-1 cells

Figure 2. A: Protective effect of BTB06584 against ischemia/reperfusion in HL-1 cells. B. Effect of the modulation of IF1 expression on the efficiency of BTB06584 in a similar experiment as in figure 1B. C: Immunofluorescence assay confirming an efficient IF1 overexpression/silencing.

b. Data not included in the draft

We developed another assay to indirectly but in a significantly more elegant manner to demonstrate preservation of the ATP stocks. This technique consisted in monitoring the time from mitochondrial depolarization to rigor in freshly isolated adult rat ventricular cardiomyocytes after loading with TMRM in quench/dequench mode and photo-illumination of the probe in addition to FCCP to trigger reversal of the ATP synthase. While addition of the positive control oligomycin B significantly increased the time from mitochondrial depolarization to contracture, addition in BTB06584 in this assay didn't have any effect compared to control, despite various protocols of addition (pre-incubation for 1 hour, addition on the microscope stage while imaging (figure 27).

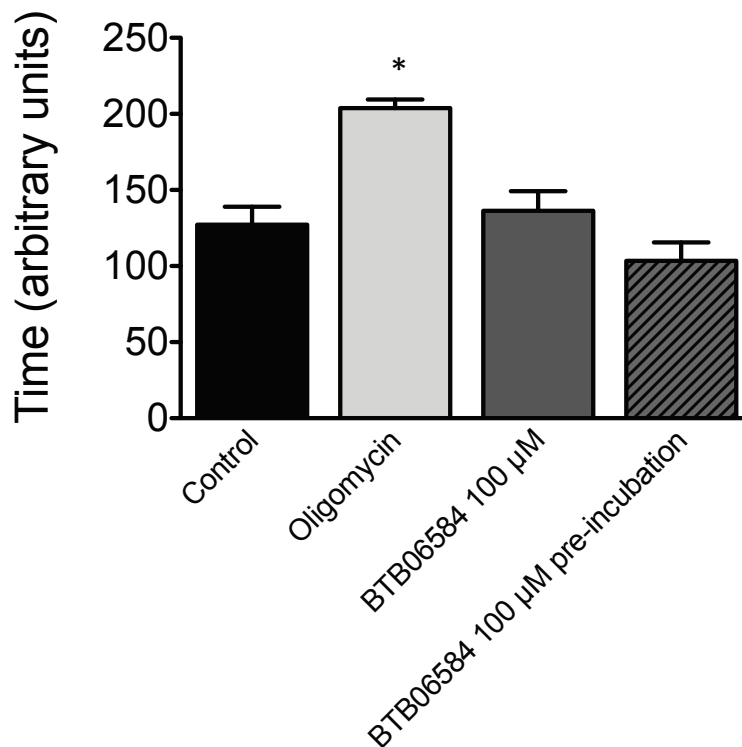


Figure 27. Time from mitochondrial depolarization to cell shortening in adult ventricular cardiomyocytes. \*  $p < 0.05$  versus control.

Considering the results of the modulation of IF1 expression on BTB06584 efficiency, a plausible explanation for the absence of efficiency of the molecule in this assay in

the low level of IF1 expression in rat cells. As published by Rouslin et al., rodents with the exception of guinea pigs have a low expression level of IF1. In the case of rat ventricular cardiomyocytes, this expression level is probably too low to allow visualization of an effect of the drug using these experimental conditions. This isn't the case for HL-1 cells as cell lines and cancer cells in general have a higher expression level of IF1. An elegant way to confirm this hypothesis would be to repeat this experiment after transfection of these adult ventricular cardiomyocytes with the IF1 construct through adenoviral transfection or electroporation.



# CONCLUSIONS

In this work we demonstrated that, despite the significant improvements in the management of ischemia/reperfusion injury, there remains interesting therapeutic trails to explore.

Myocardial stem cell therapy is more and more focused on cardioprotection and less on cardioregeneration and our work support further the hypothesis of a paracrine effect sharing the same signaling pathways than ischemic postconditioning, in the present case the PI3kinase/Akt pathway. If the vectors of this paracrine protection remain to be identified, many growth factors and cytokines demonstrated similar protective effect in the literature and are just so many candidates as mediators of this protection. Reality is probably that paracrine protection resulting from stem cell therapy is the sum of the effect of many effectors, which will make even harder precise identification of these effectors. In any case, bearing in sight the objective of a large use of stem cell therapy to improve CVD, confirmation of the positive paracrine effect makes easier the translation into patient care as it frees from the eventual problems of graft rejection when cells are administered. A lot of fundamental work remains to be done before we can consider myocardial stem cell therapy (or its derivatives) as part of our daily therapeutic arsenal against ischemia/reperfusion injury but the recent progresses in the understanding of the underlying mechanisms encourage to keep on exploring this therapeutic option.

In a completely different register, modulation of the metabolic changes during ischemia offers new possibilities to reduce the ischemic burden. Though less transposable to modern management of myocardial ischemia/reperfusion injury, particularly as a result of AMI, inhibition of the reversal of the mitochondrial F<sub>1</sub>F<sub>o</sub> ATP synthase still has many potential applications, one of which is organ transplantation. It appears to be an efficient way to reduce ischemia-induced lesions, probably comparable to ischemic preconditioning (inhibition of the reverse activity of the ATP synthase was described as one of its mechanism of protection). The theoretical advantage of this technique over preconditioning is that it relies on a

pharmacological agent. Amongst many potential candidates, and even more that weren't considered here, we identified 2 molecules with promising in vitro effects that deserve to push further the investigations, in particular using in vivo models. This could be the first step toward the development of new drugs that would further enlarge our therapeutic arsenal.

Thus, we demonstrated through these 2 different approaches of cardioprotection that research ought to remain active in the domain of CVD as there are many potential therapeutic targets that still need to be addressed in order to reduce the burden of these pathologies, first cause of mortality worldwide.

# BIBLIOGRAPHY

1. McGovern PG, Pankow JS, Shahar E, Doliszny KM, Folsom AR, Blackburn H, et al. Recent trends in acute coronary heart disease--mortality, morbidity, medical care, and risk factors. The Minnesota Heart Survey Investigators. *N Engl J Med.* 1996; **334**(14): 884-90.
2. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation.* 2011; **123**(4): e18-e209.
3. Mathers CD, Salomon JA, Ezzati M, Begg S, Hoorn SV, Lopez AD. Sensitivity and Uncertainty Analyses for Burden of Disease and Risk Factor Estimates. In: Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL, editors. *Global Burden of Disease and Risk Factors.* Washington (DC); 2006.
4. Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet.* 1997; **349**(9061): 1269-76.
5. Celermajer DS, Chow CK, Marijon E, Anstey NM, Woo KS. Cardiovascular disease in the developing world: prevalences, patterns, and the potential of early disease detection. *J Am Coll Cardiol.* 2012; **60**(14): 1207-16.
6. Mandelzweig L, Battler A, Boyko V, Bueno H, Danchin N, Filippatos G, et al. The second Euro Heart Survey on acute coronary syndromes: Characteristics, treatment, and outcome of patients with ACS in Europe and the Mediterranean Basin in 2004. *Eur Heart J.* 2006; **27**(19): 2285-93.
7. Fox KA, Dabbous OH, Goldberg RJ, Pieper KS, Eagle KA, Van de Werf F, et al. Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). *Bmj.* 2006; **333**(7578): 1091.
8. Fox KA, Carruthers KF, Dunbar DR, Graham C, Manning JR, De Raedt H, et al. Underestimated and under-recognized: the late consequences of acute coronary syndrome (GRACE UK-Belgian Study). *Eur Heart J.* 2010; **31**(22): 2755-64.

9. Velican C. The coronary atherosclerotic plaque: natural history, pathogenesis, relation to risk factors. *Med Interne*. 1979; **17**(4): 323-39.
10. Velican C, Velican D. Study on the onset of atherosclerotic lesions in human coronary arteries. *Med Interne*. 1979; **17**(2): 131-49.
11. Suo J, Oshinski JN, Giddens DP. Blood flow patterns in the proximal human coronary arteries: relationship to atherosclerotic plaque occurrence. *Mol Cell Biomech*. 2008; **5**(1): 9-18.
12. Haust MD. The genesis of atherosclerosis in pediatric age-group. *Pediatr Pathol*. 1990; **10**(1-2): 253-71.
13. Plenz G, Robenek H. Monocytes/macrophages in atherosclerosis. *Eur Cytokine Netw*. 1998; **9**(4): 701-3.
14. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation*. 1995; **91**(9): 2488-96.
15. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005; **111**(25): 3481-8.
16. Kehl DW, Iqbal N, Fard A, Kipper BA, De La Parra Landa A, Maisel AS. Biomarkers in acute myocardial injury. *Transl Res*. 2012; **159**(4): 252-64.
17. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, et al. Third universal definition of myocardial infarction. *Circulation*. 2012; **126**(16): 2020-35.
18. Senior AE. ATP synthesis by oxidative phosphorylation. *Physiol Rev*. 1988; **68**(1): 177-231.
19. Mitchell P. Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: power transmission by proticity. *Biochem Soc Trans*. 1976; **4**(3): 399-430.
20. Szabadkai G, Duchen MR. Mitochondria: the hub of cellular Ca<sup>2+</sup> signaling. *Physiology (Bethesda)*. 2008; **23**: 84-94.
21. Duchen MR. Mitochondria and Ca<sup>2+</sup> in cell physiology and pathophysiology. *Cell Calcium*. 2000; **28**(5-6): 339-48.
22. Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. *Immunol Today*. 1997; **18**(1): 44-51.
23. Jennings RB, Murry CE, Steenbergen C, Jr., Reimer KA. Development of cell injury in sustained acute ischemia. *Circulation*. 1990; **82**(3 Suppl): II2-12.

24. Solaini G, Baracca A, Lenaz G, Sgarbi G. Hypoxia and mitochondrial oxidative metabolism. *Biochim Biophys Acta*. 2010; **1797**(6-7): 1171-7.
25. Szczepanek K, Chen Q, Larner AC, Lesnefsky EJ. Cytoprotection by the modulation of mitochondrial electron transport chain: the emerging role of mitochondrial STAT3. *Mitochondrion*. 2012; **12**(2): 180-9.
26. Krebs HA. The Pasteur effect and the relations between respiration and fermentation. *Essays Biochem*. 1972; **8**: 1-34.
27. Nicholls DG, Lindberg O. Inhibited respiration and ATPase activity of rat liver mitochondria under conditions of matrix condensation. *FEBS Lett*. 1972; **25**(1): 61-4.
28. Jennings RB, Reimer KA, Steenbergen C. Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. *J Mol Cell Cardiol*. 1991; **23**(12): 1383-95.
29. Rouslin W, Broge CW, Grupp IL. ATP depletion and mitochondrial functional loss during ischemia in slow and fast heart-rate hearts. *Am J Physiol*. 1990; **259**(6 Pt 2): H1759-66.
30. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem*. 1985; **54**: 1015-69.
31. Rouslin W, Erickson JL, Solaro RJ. Effects of oligomycin and acidosis on rates of ATP depletion in ischemic heart muscle. *Am J Physiol*. 1986; **250**(3 Pt 2): H503-8.
32. Dimroth P, Kaim G, Matthey U. Crucial role of the membrane potential for ATP synthesis by F(1)F(o) ATP synthases. *J Exp Biol*. 2000; **203**(Pt 1): 51-9.
33. Gaballo A, Zanotti F, Papa S. Structures and interactions of proteins involved in the coupling function of the protonmotive F(o)F(1)-ATP synthase. *Curr Protein Pept Sci*. 2002; **3**(4): 451-60.
34. Vinogradov AD. Steady-state and pre-steady-state kinetics of the mitochondrial F(1)F(o) ATPase: is ATP synthase a reversible molecular machine? *J Exp Biol*. 2000; **203**(Pt 1): 41-9.
35. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med*. 2007; **357**(11): 1121-35.
36. Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI). *Lancet*. 1986; **1**(8478): 397-402.

37. Randomised trial of intravenous atenolol among 16 027 cases of suspected acute myocardial infarction: ISIS-1. First International Study of Infarct Survival Collaborative Group. *Lancet*. 1986; **2**(8498): 57-66.
38. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *J Clin Invest*. 1985; **76**(5): 1713-9.
39. Kloner RA, Ganote CE, Jennings RB. The "no-reflow" phenomenon after temporary coronary occlusion in the dog. *J Clin Invest*. 1974; **54**(6): 1496-508.
40. Ricchelli F, Sileikyte J, Bernardi P. Shedding light on the mitochondrial permeability transition. *Biochim Biophys Acta*. 2011; **1807**(5): 482-90.
41. Halestrap AP. What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol*. 2009; **46**(6): 821-31.
42. Hausenloy DJ, Yellon DM. Preconditioning and postconditioning: underlying mechanisms and clinical application. *Atherosclerosis*. 2009; **204**(2): 334-41.
43. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986; **74**(5): 1124-36.
44. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation*. 1993; **88**(3): 1264-72.
45. Bolli R. The late phase of preconditioning. *Circ Res*. 2000; **87**(11): 972-83.
46. Skyschally A, Gres P, Hoffmann S, Haude M, Erbel R, Schulz R, et al. Bidirectional role of tumor necrosis factor-alpha in coronary microembolization: progressive contractile dysfunction versus delayed protection against infarction. *Circ Res*. 2007; **100**(1): 140-6.
47. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol*. 2003; **285**(2): H579-88.
48. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, et al. Postconditioning the human heart. *Circulation*. 2005; **112**(14): 2143-8.
49. Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, et al. Long-term benefit of postconditioning. *Circulation*. 2008; **117**(8): 1037-44.
50. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med*. 2008; **359**(5): 473-81.

51. Mahaffey KW, Puma JA, Barbagelata NA, DiCarli MF, Leesar MA, Browne KF, et al. Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction Study of Adenosine (AMISTAD) trial. *J Am Coll Cardiol*. 1999; **34**(6): 1711-20.
52. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J Am Coll Cardiol*. 2005; **45**(11): 1775-80.
53. Fokkema ML, Vlaar PJ, Vogelzang M, Gu YL, Kampinga MA, de Smet BJ, et al. Effect of high-dose intracoronary adenosine administration during primary percutaneous coronary intervention in acute myocardial infarction: a randomized controlled trial. *Circ Cardiovasc Interv*. 2009; **2**(4): 323-9.
54. Desmet W, Bogaert J, Dubois C, Sinnaeve P, Adriaenssens T, Pappas C, et al. High-dose intracoronary adenosine for myocardial salvage in patients with acute ST-segment elevation myocardial infarction. *Eur Heart J*. 2011; **32**(7): 867-77.
55. Theroux P, Chaitman BR, Danchin N, Erhardt L, Meinertz T, Schroeder JS, et al. Inhibition of the sodium-hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations. Main results of the GUARDIAN trial. Guard during ischemia against necrosis (GUARDIAN) Investigators. *Circulation*. 2000; **102**(25): 3032-8.
56. Mentzer RM, Jr., Bartels C, Bolli R, Boyce S, Buckberg GD, Chaitman B, et al. Sodium-hydrogen exchange inhibition by cariporide to reduce the risk of ischemic cardiac events in patients undergoing coronary artery bypass grafting: results of the EXPEDITION study. *Ann Thorac Surg*. 2008; **85**(4): 1261-70.
57. Kitakaze M, Asakura M, Kim J, Shintani Y, Asanuma H, Hamasaki T, et al. Human atrial natriuretic peptide and nicorandil as adjuncts to reperfusion treatment for acute myocardial infarction (J-WIND): two randomised trials. *Lancet*. 2007; **370**(9597): 1483-93.
58. Ott I, Schulz S, Mehilli J, Fichtner S, Hadamitzky M, Hoppe K, et al. Erythropoietin in patients with acute ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention: a randomized, double-blind trial. *Circ Cardiovasc Interv*. 2010; **3**(5): 408-13.

59. Najjar SS, Rao SV, Melloni C, Raman SV, Povsic TJ, Melton L, et al. Intravenous erythropoietin in patients with ST-segment elevation myocardial infarction: REVEAL: a randomized controlled trial. *Jama*. 2011; **305**(18): 1863-72.
60. Mochly-Rosen D, Grimes KV. Myocardial salvage in acute myocardial infarction--challenges in clinical translation. *J Mol Cell Cardiol*. 2011; **51**(4): 451-3.
61. Gerczuk PZ, Kloner RA. An update on cardioprotection: a review of the latest adjunctive therapies to limit myocardial infarction size in clinical trials. *J Am Coll Cardiol*. 2012; **59**(11): 969-78.
62. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001; **410**(6829): 701-5.
63. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002; **106**(15): 1913-8.
64. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation*. 2002; **106**(24): 3009-17.
65. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature*. 2004; **428**(6983): 668-73.
66. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature*. 2004; **428**(6983): 664-8.
67. Angoulvant D, Fazel S, Li RK. Neovascularization derived from cell transplantation in ischemic myocardium. *Mol Cell Biochem*. 2004; **264**(1-2): 133-42.
68. Sam J, Angoulvant D, Fazel S, Weisel RD, Li RK. Heart cell implantation after myocardial infarction. *Coron Artery Dis*. 2005; **16**(2): 85-91.
69. Togel F, Isaac J, Westenfelder C. Hematopoietic stem cell mobilization-associated granulocytosis severely worsens acute renal failure. *J Am Soc Nephrol*. 2004; **15**(5): 1261-7.
70. Lange C, Togel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int*. 2005; **68**(4): 1613-7.



71. Davani EY, Brumme Z, Singhera GK, Cote HC, Harrigan PR, Dorscheid DR. Insulin-like growth factor-1 protects ischemic murine myocardium from ischemia/reperfusion associated injury. *Crit Care*. 2003; **7**(6): R176-83.
72. Dawn B, Guo Y, Rezazadeh A, Huang Y, Stein AB, Hunt G, et al. Postinfarct cytokine therapy regenerates cardiac tissue and improves left ventricular function. *Circ Res*. 2006; **98**(8): 1098-105.
73. Chao W, Matsui T, Novikov MS, Tao J, Li L, Liu H, et al. Strategic advantages of insulin-like growth factor-I expression for cardioprotection. *J Gene Med*. 2003; **5**(4): 277-86.
74. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res*. 2004; **95**(1): 9-20.
75. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation*. 1999; **100**(19 Suppl): II247-56.76. Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature*. 2009; **460**(7251): 113-7.
77. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A*. 2003; **100**(21): 12313-8.
78. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell*. 2003; **114**(6): 763-76.
79. Matsuura K, Honda A, Nagai T, Fukushima N, Iwanaga K, Tokunaga M, et al. Transplantation of cardiac progenitor cells ameliorates cardiac dysfunction after myocardial infarction in mice. *J Clin Invest*. 2009; **119**(8): 2204-17.
80. Huang C, Gu H, Yu Q, Manukyan MC, Poynter JA, Wang M. Sca-1+ cardiac stem cells mediate acute cardioprotection via paracrine factor SDF-1 following myocardial ischemia/reperfusion. *PLoS One*. 2011; **6**(12): e29246.
81. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature*. 2005; **433**(7026): 647-53.

82. Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, et al. Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med.* 2007; **167**(10): 989-97.
83. Hausenloy DJ, Yellon DM. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev.* 2007; **12**(3-4): 217-34.
84. Vivar R, Humeres C, Varela M, Ayala P, Guzman N, Olmedo I, et al. Cardiac fibroblast death by ischemia/reperfusion is partially inhibited by IGF-1 through both PI3K/Akt and MEK-ERK pathways. *Exp Mol Pathol.* 2012; **93**(1): 1-7.
85. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. *Exp Cell Res.* 2000; **256**(1): 34-41.
86. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation.* 2008; **118**(19): 1915-9.
87. Pullman ME, Monroy GC. A Naturally Occurring Inhibitor of Mitochondrial Adenosine Triphosphatase. *J Biol Chem.* 1963; **238**: 3762-9.
88. Cabezon E, Butler PJ, Runswick MJ, Walker JE. Modulation of the oligomerization state of the bovine F1-ATPase inhibitor protein, IF1, by pH. *J Biol Chem.* 2000; **275**(33): 25460-4.
89. Sanchez-Cenizo L, Formentini L, Aldea M, Ortega AD, Garcia-Huerta P, Sanchez-Arago M, et al. Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H<sup>+</sup>-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype. *J Biol Chem.* 2010; **285**(33): 25308-13.
90. Campanella M, Casswell E, Chong S, Farah Z, Wieckowski MR, Abramov AY, et al. Regulation of mitochondrial structure and function by the F1Fo-ATPase inhibitor protein, IF1. *Cell Metab.* 2008; **8**(1): 13-25.
91. Lardy H, Reed P, Lin CH. Antibiotic inhibitors of mitochondrial ATP synthesis. *Fed Proc.* 1975; **34**(8): 1707-10.
92. van Raaij MJ, Abrahams JP, Leslie AG, Walker JE. The structure of bovine F1-ATPase complexed with the antibiotic inhibitor aurovertin B. *Proc Natl Acad Sci U S A.* 1996; **93**(14): 6913-7.
93. Grover GJ, Atwal KS, Sleph PG, Wang FL, Monshizadegan H, Monticello T, et al. Excessive ATP hydrolysis in ischemic myocardium by mitochondrial F1F0-ATPase: effect of selective pharmacological inhibition of mitochondrial ATPase hydrolase activity. *Am J Physiol Heart Circ Physiol.* 2004; **287**(4): H1747-55.

94. Atwal KS, Wang P, Rogers WL, Sleph P, Monshizadegan H, Ferrara FN, et al. Small molecule mitochondrial F1F0 ATPase hydrolase inhibitors as cardioprotective agents. Identification of 4-(N-arylimidazole)-substituted benzopyran derivatives as selective hydrolase inhibitors. *J Med Chem.* 2004; **47**(5): 1081-4.
95. *Guide for the Care and Use of Laboratory Animals.* 8th ed. Washington (DC); 2011.
96. Sharov VG, Todor A, Khanal S, Imai M, Sabbah HN. Cyclosporine A attenuates mitochondrial permeability transition and improves mitochondrial respiratory function in cardiomyocytes isolated from dogs with heart failure. *J Mol Cell Cardiol.* 2007; **42**(1): 150-8.
97. Germack R, Dickenson JM. Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. *J Mol Cell Cardiol.* 2005; **39**(3): 429-42.
98. Bhamra GS, Hausenloy DJ, Davidson SM, Carr RD, Paiva M, Wynne AM, et al. Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic Res Cardiol.* 2008; **103**(3): 274-84.
99. Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci U S A.* 1998; **95**(6): 2979-84.
100. Jacobsen LB, Calvin SA, Colvin KE, Wright M. FuGENE 6 Transfection Reagent: the gentle power. *Methods.* 2004; **33**(2): 104-12.
101. Mayevsky A, Rogatsky GG. Mitochondrial function in vivo evaluated by NADH fluorescence: from animal models to human studies. *Am J Physiol Cell Physiol.* 2007; **292**(2): C615-40.
102. Chapman JB. Fluorometric studies of oxidative metabolism in isolated papillary muscle of the rabbit. *J Gen Physiol.* 1972; **59**(2): 135-54.
103. Jobsis FF, Stainsby WN. Oxidation of NADH during contractions of circulated mammalian skeletal muscle. *Respir Physiol.* 1968; **4**(3): 292-300.
104. Nuutinen EM. Subcellular origin of the surface fluorescence of reduced nicotinamide nucleotides in the isolated perfused rat heart. *Basic Res Cardiol.* 1984; **79**(1): 49-58.

105. Clark LC, Jr., Wolf R, Granger D, Taylor Z. Continuous recording of blood oxygen tensions by polarography. *J Appl Physiol.* 1953; **6**(3): 189-93.
106. Nakamura M, Mie M, Funabashi H, Yamamoto K, Ando J, Kobatake E. Cell-surface-localized ATP detection with immobilized firefly luciferase. *Anal Biochem.* 2006; **352**(1): 61-7.
107. Leyssens A, Nowicky AV, Patterson L, Crompton M, Duchen MR. The relationship between mitochondrial state, ATP hydrolysis,  $[Mg^{2+}]_i$  and  $[Ca^{2+}]_i$  studied in isolated rat cardiomyocytes. *J Physiol.* 1996; **496 ( Pt 1)**: 111-28.
108. Brown J. Effects of 2-deoxyglucose on carbohydrate metabolism: review of the literature and studies in the rat. *Metabolism.* 1962; **11**: 1098-112.
109. Manzini I, Schweer TS, Schild D. Improved fluorescent (calcium indicator) dye uptake in brain slices by blocking multidrug resistance transporters. *J Neurosci Methods.* 2008; **167**(2): 140-7.
110. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, et al. Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci U S A.* 2009; **106**(37): 15651-6.
111. Weber KT. Fibrosis in hypertensive heart disease: focus on cardiac fibroblasts. *J Hypertens.* 2004; **22**(1): 47-50.
112. Virag JA, Rolle ML, Reece J, Hardouin S, Feigl EO, Murry CE. Fibroblast growth factor-2 regulates myocardial infarct repair: effects on cell proliferation, scar contraction, and ventricular function. *Am J Pathol.* 2007; **171**(5): 1431-40.
113. Rosenblatt-Velin N, Lepore MG, Cartoni C, Beermann F, Pedrazzini T. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. *J Clin Invest.* 2005; **115**(7): 1724-33.
114. Iwakura A, Fujita M, Kataoka K, Tambara K, Sakakibara Y, Komeda M, et al. Intramyocardial sustained delivery of basic fibroblast growth factor improves angiogenesis and ventricular function in a rat infarct model. *Heart Vessels.* 2003; **18**(2): 93-9.
115. Borg TK, Rubin K, Carver W, Samarel A, Terracio L. The cell biology of the cardiac interstitium. *Trends Cardiovasc Med.* 1996; **6**(2): 65-70.
116. Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate

- PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2013; **10**(3): 301-12.
117. Ravi S, Caves JM, Martinez AW, Xiao J, Wen J, Haller CA, et al. Effect of bone marrow-derived extracellular matrix on cardiac function after ischemic injury. *Biomaterials.* 2012; **33**(31): 7736-45.
118. Alfarano C, Roubexis C, Chaaya R, Ceccaldi C, Calise D, Mias C, et al. Intraparenchymal injection of bone marrow mesenchymal stem cells reduces kidney fibrosis after ischemia-reperfusion in cyclosporine-immunosuppressed rats. *Cell Transplant.* 2012; **21**(9): 2009-19.
119. Pan GZ, Yang Y, Zhang J, Liu W, Wang GY, Zhang YC, et al. Bone marrow mesenchymal stem cells ameliorate hepatic ischemia/reperfusion injuries via inactivation of the MEK/ERK signaling pathway in rats. *J Surg Res.* 2012; **178**(2): 935-48.
120. Li D, Fang Y, Wang P, Shan W, Zuo Z, Xie L. Autologous transplantation of adipose-derived mesenchymal stem cells attenuates cerebral ischemia and reperfusion injury through suppressing apoptosis and inducible nitric oxide synthase. *Int J Mol Med.* 2012; **29**(5): 848-54.
121. Bruno S, Bussolati B. Therapeutic effects of mesenchymal stem cells on renal ischemia-reperfusion injury: a matter of genetic transfer? *Stem Cell Res Ther.* 2013; **4**(5): 55.
122. Chen S, Chen L, Wu X, Lin J, Fang J, Chen X, et al. Ischemia postconditioning and mesenchymal stem cells engraftment synergistically attenuate ischemia reperfusion-induced lung injury in rats. *J Surg Res.* 2012; **178**(1): 81-91.
123. Fang J, Chen L, Fan L, Wu L, Chen X, Li W, et al. Enhanced therapeutic effects of mesenchymal stem cells on myocardial infarction by ischemic postconditioning through paracrine mechanisms in rats. *J Mol Cell Cardiol.* 2011; **51**(5): 839-47.

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#### RESUME en français

Les maladies cardiovasculaires constituent un problème de santé publique. Les pré et post-conditionnement ischémiques mais aussi pharmacologiques constituent autant d'avancées qui permettront l'amélioration de la prise en charge des malades en situation d'ischémie/reperfusion myocardique. Néanmoins, la morbi-mortalité des maladies cardiovasculaires reste importante et nécessite le développement de nouvelles techniques.

Les premiers résultats de la thérapie cellulaire myocardique ont été décevants, et s'il est désormais établi que l'on ne peut régénérer le myocarde, les effets bénéfiques observés, notamment avec les cellules souches mésenchymateuses semblent en rapport avec un effet paracrine qui passe par l'activation de la voie de signalisation PI3kinase/Akt sur un mécanisme comparable à celui du post-conditionnement ischémique. Les médiateurs de cet effet sont vraisemblablement des facteurs de croissance comme le VEGF ou l'IGF-1 même si un effet individuel direct de l'une ou l'autre de ces molécules n'a pu être mis en évidence.

La modulation de l'activité de l'ATP synthase mitochondriale est également une cible thérapeutique prometteuse. Cette enzyme inverse son activité et hydrolyse l'ATP durant l'ischémie, conduisant à dépleter le pool d'ATP intracellulaire et accélérer la survenue de la mort cellulaire. De nouvelles molécules ayant un effet similaire à l'IF1 permettent de bloquer cette inversion d'activité de l'ATP synthase, de préserver l'ATP et donc d'améliorer la survie cellulaire par un effet de type préconditionnement ischémique.

Ces 2 techniques, très différentes mais non antinomiques, pourraient faire partie de l'arsenal thérapeutique dans les années à venir.

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#### TITRE en anglais

NEW MECHANISMS OF PROTECTION OF CARDIOMYOCYTES FROM ISCHEMIA/REPERFUSION INJURY

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#### RESUME en anglais

Cardiovascular diseases are a major problem of public health management. Ischemic and pharmacological pre and postconditioning should significantly improve the prognosis of patients suffering from myocardial ischemia/reperfusion. However, the morbi-mortality of these patients is still high and research must remain active.

The first results of myocardial stem cell therapy show that we cannot regenerate myocardium but a recent meta-analysis reported positive effects that can be explained through a paracrine mechanism. Mesenchymal stem cells protect ischemic cardiomyocytes from reperfusion injury through a paracrine activation of the PI3kinase/Akt pathway in a similar way to ischemic postconditioning. The mediators of this protection could be growth factors such as VEGF or IGF-1 though we couldn't demonstrate a direct effect of one or the other.

Modulating the activity of the ATP synthase during ischemia is another promising therapeutic target. This enzyme reverses its activity and hydrolyses ATP when the supply in oxygen is impaired. This leads to the reduction of the cellular pool of ATP and accelerates cell death. We identified new small molecules with a similar effect to IF1 that can selectively inhibit the reverse activity of the ATP synthase, preserve ATP and thus increase cell survival in a preconditioning-like effect.

These two different techniques could be part of the therapeutic arsenal against ischemia/reperfusion in the next decades.

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#### DISCIPLINE : PHYSIOLOGIE CARDIOVASCULAIRE

MOTS-CLES : Ischémie/reperfusion myocardique, Thérapie cellulaire, Cellules souches mésenchymateuses, Mitochondrie, Pore de transition de perméabilité mitochondriale, ATP synthase mitochondriale

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