Regulating Mitochondrial Permeability Transition: A Chemical Biology Approach to Preserve Mitochondrial Integrity

A THESIS
SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

By

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To

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June 2015

DECLARATION

I **Govardhan. K. S**, hereby declare that the work presented in this thesis entitled **"Regulating Mitochondrial Permeability Transition: A Chemical Biology Approach to Preserve Mitochondrial Integrity"** submitted by me under guidance of Dr. Prasenjit Mitra, Dr. Reddy's Institute of Life Sciences, Hyderabad, is an original research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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DECLARATION

I **Govardhan. K. S**, hereby declare that this thesis entitled **"Regulating Mitochondrial Permeability Transition: A Chemical Biology Approach to Preserve Mitochondrial Integrity"** submitted by me under guidance and supervision of Dr. Prasenjit Mitra, Dr. Reddy's Institute of Life Sciences, Hyderabad, is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Shodganga/INFLIBNET.

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Regulating Mitochondrial Permeability Transition: A Chemical Biology Approach to Preserve Mitochondrial Integrity"** has been carried out by **Govardhan. K. S**, under the guidance of **Dr. Prasenjit Mitra** at Dr. Reddy's Institute of Life Sciences, Hyderabad and is being submitted to Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, for a full period prescribed under the PhD ordinance of this University.

We recommend this thesis for submission for the award of the degree of Doctor of Philosophy of this University.

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Dedicated to my father

Late Shri TARANATH. K. S

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enriched my understanding immensely. I convey my special thanks to Mithila and Natesh for helping me to acquire experimental data even during odd hours. I thank Dr. Manika Pal Bhadra IICT, Hyderabad for allowing me to work in her lab and for the excellent support of all the lab members for her team. I thank ICMR for providing me fellowship and DST and DBT for funding the research work. Special thanks to Vamshi Krishna, for listening and giving me friendly support all the time. Special thanks to Dr. Vasundhra Kain for her excellent support and insightful suggestions. Special thanks to Mrs. Santilata mitra for her encouragement and support. I thank my seniors Dr. Krishnaveni, Dr. Ashraf for their enduring support. Thanks to all my friends, lab mates and many people who were instrumental during my research work.

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Synopsis

The thesis entitled, **"Regulating Mitochondrial Permeability Transition: A Chemical Biology Approach to Preserve Mitochondrial Integrity"** contains three chapters:

Introduction

In this chapter I introduce the concept of mitochondrial permeability transition pore (MPTP) and its role in preserving mitochondrial function and structural integrity. I discussed the molecular composition in terms of the canonical paradigm as well as the presently evolved dynamic concept of the pore to describe its physiological and pathophysiological role in type 2 diabetes, cardiovascular disorder. Further, the chapter describes the chemical modulators of the mitochondrial permeability transition pore and their mechanism of action in context of cardiac reperfusion injury and progressive loss of pancreatic beta cell mass. Finally a model has been proposed to describe how inhibition of cis-trans peptidyl prolyl isomerase activity of cyclophilin D prevents mitochondrial pore opening and prevents cell death.

Chapter 1: Screening and Identification of a small molecule that inhibits mitochondrial permeability transition and prevents pancreatic β cell death. (*Eur. J. Org. Chem.* **2014**, 1151–1156)

Thapsigargin is known to inhibit SERCA (sarco-endoplasmic reticulum calcium ATPase) pump and prevents the entry of calcium to endoplasmic reticulum. However Korge and Weiss (ref) have shown that in isolated heart and liver, thapsigargin directly induces mitochondrial permeability transition at micromolar concentrations. We employed this strategy to develop a cell based model using cultured pancreatic beta cells to screen for inhibitors that prevents the depolarization of mitochondrial membrane potential **(Fig 1a)** which is one of the read out for mitochondrial permeability transition. Enantioenriched, benzofuran derived compounds with 12 member macrocyclic rings was screened in our cell based assay system among which compound 2.4c and its analogues was found to inhibit the depolarization of mitochondrial membrane potential in a dose dependent manner **(Fig 1b)**. The assay also revealed that the macrocyclic architecture of the compound is required for the activity as the acyclic precursor (2.3c) did not show any effect **(Fig 1c).**The final readout of the prolonged opening of the mitochondrial permeability transition pore is the release of cytochrome c from mitochondria. The data

presented in this chapter shows that treatment of 2.4c prevents the thapsigargin induced cytochrome c release from pancreatic beta cell mitochondria **(1d).**

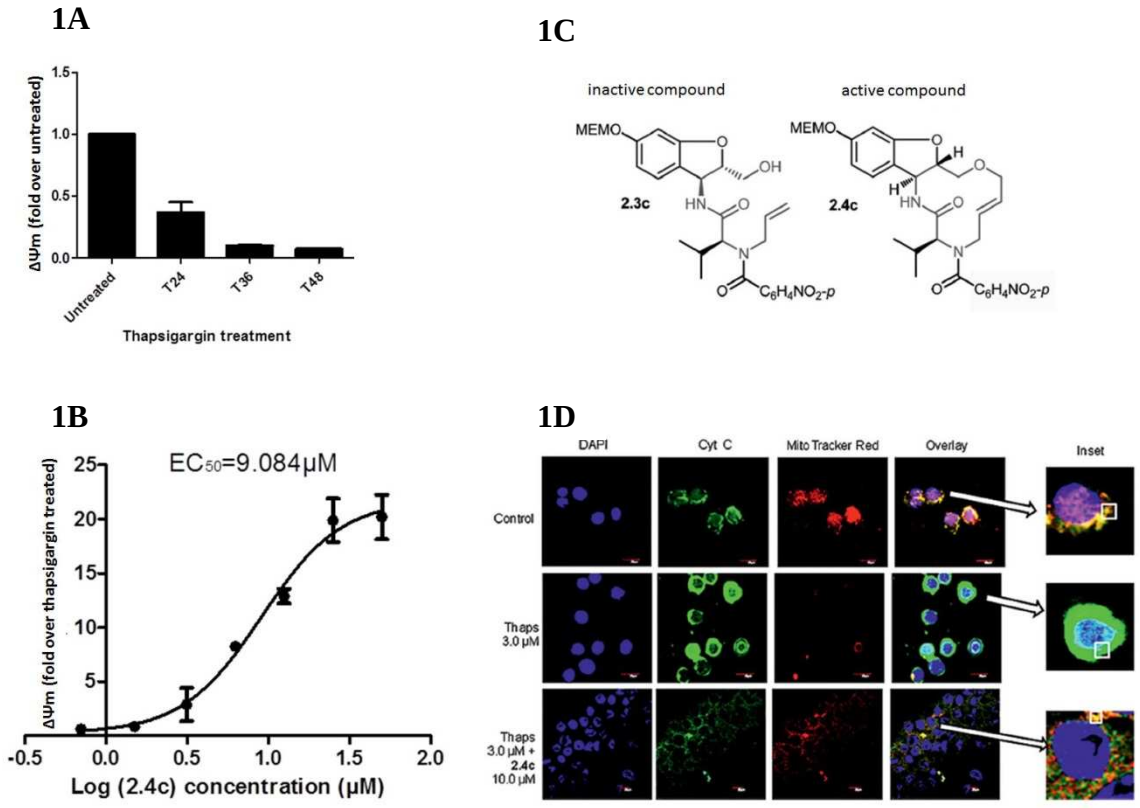

Figure 1: Compound 2.4c inhibits the Mitochondiral Permability Transition and release Cytochrome C. **1A**, Temporal depolarization of the mitochondrial membrane potential (ΔΨm) in pancreatic β-cells; **1B**, Dose response curve for the rescue of thapsigargin-induced depolarization of ΔΨm by 2.4c; **1C**, Structure of Compound 2.3c and Compound 2.4c; **1D**,Prevention of Cytochrome C release from thapsigargin-treated cells upon treatment with 2.4c. Confocal microscopy images of Brin-BD11 cells immune labeled with primary monoclonal mouse anti-cytochrome C antibody (green) depicting release of cytochrome C from the mitochondria. Mitochondria are labeled by Mito-tracker red (red) and the nucleus is visualized by DAPI (blue) staining. Yellow indicates co-localization of cytochrome C with Mito-tracker Red in control and 2.4c treated cells in mitochondria. Diffused staining in thapsigargin-treated cells shows the release of cytochrome c from the mitochondria.

Selected references:

1. C. J. Rhodes, *Science. (*2005), *307*, 380–384.
2. Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. Biochem J *341 (Pt 2)*, 233-249.
3. Johnson, J.D., Bround, M.J., White, S.A., and Luciani, D.S. (2012). Nanospaces between endoplasmic reticulum and mitochondria as control centres of pancreatic beta-cell metabolism and survival. Protoplasma *249 Suppl 1*, S49-58.
4. Ramadan, J.W., Steiner, S.R., O'Neill, C.M., and Nunemaker, C.S. (2011). The central role of calcium in the effects of cytokines on beta-cell function: implications for type 1 and type 2 diabetes. Cell calcium *50*, 481-490.
5. Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiol Rev *87*, 99-163.
6. D Zhang., JS Armstrong. (2007). Bax and the mitochondrial permeability transition cooperate in the release of cytochrome c during endoplasmic reticulum-stress-induced apoptosis. Cell death and Differentiation *14*, 703-715.

Chapter 2: Interaction of compound 2.4c with Cyclophilin D and in silico analysis (manuscript submitted).

In the next step I explored whether compound 2.4c interacts with any member of the protein complex that constitutes the MPTP. Cyclophilin D is the only undisputed regulatory protein which modulates the opening and closure of MPTP; its role in MPTP has been proved by genetic studies. The in silico analysis by Swiss online Molecular docking platform of compound 2.4c with protein PPIF (PDB ID 3R49) results in 32 clusters being identified in several different pockets of the protein. The top scoring cluster was used for further analysis; it had significantly lower Fullfitness value -674.75 than others. The binding site is formed within two hydrophobic pockets; wherein Glycine 114 is predicted to form Hydrogen bond with NH_2 group of ring in ligand with a bond length of 2.62Å (Figure 2A). The docking of other open ring structure 2.3c gives full fitness score of -690.07 which is higher than that of 2.4c. Due to its rigid structure 2.4c gives less of possible rotation of bonds which is reflected in the full fitness score. The site of

binding of cyclosporine A and 2.4c to PPIF might be different based on predicted docking model.

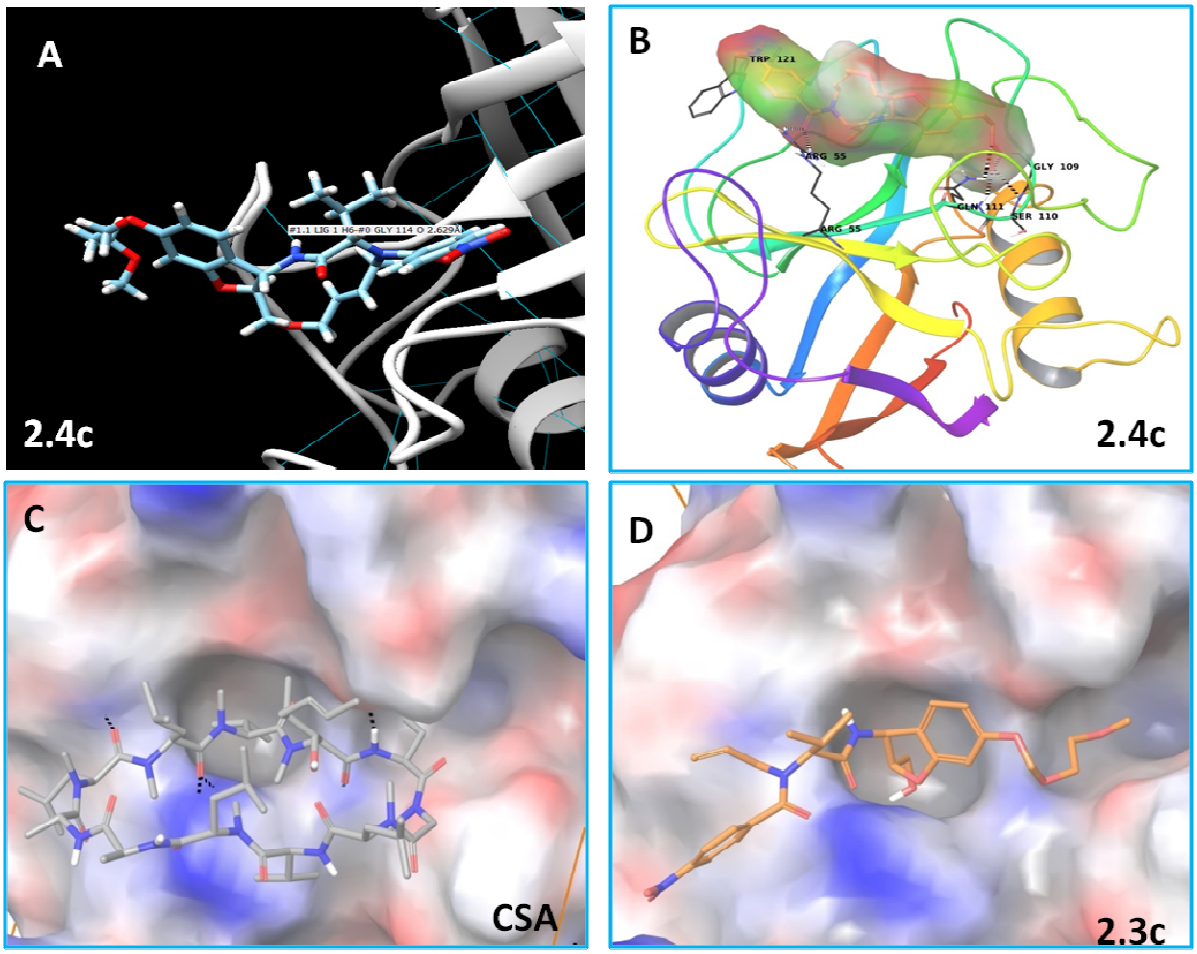

Figure 2: Molecular docking of compound 2.4c with protein Cyclophilin D/PPIF. **A**, Molecular modeled binding site of compound 2.4c, partial view of protein Human PPIF showing location of the proposed binding site of compound 2.4c (top score position from Swiss dock with full fitness), the residue Gly 114 forms a Hydrogen bond with NH2 group of ring of compound 2.4c, which is shown as a blue line. **B**, Compound 2.4c and protein PPIF molecular docking shows interaction with both the pockets, various active site amino acids Arginine (Arg) 55, Glycine (Gly) 109, Serine (Ser) 110, Glutamine (Glu) 111 and Tryptophan (Trp) 121 of PPIF protein interacting with 2.4c (shown with shaded molecular surface) and there H-bonds formation is highlighted with black dash lanes. **C,** Cyclosporine A (CSA) molecular docking with protein PPIF shows a strong binding to one pocket. **D,** Compound 2.3c docking with protein PPIF is shown.

We analyzed further in collaboration with Dr. Ben Ross lab, Queensland University, Australia and used Maestro (version 9.8) for molecular modeling simulations of molecules using Schrödinger molecular modeling suite-2014 (Schrödinger Release 2014-2). The compound 2.4c, 2.3c and CSA were drawn in 3D format using Build panel tools of Maestro; the structures were energy minimized using OPLS 2005 force field. The protein Peptidyl prolyl Cis-Trans isomerase F model was acquired from Protein data bank (PDB) id 2Z6W (Kajitani et al., 2008).The structure was further refined by addition of Hydrogen and missing residues and minimized by using OPLS 2005 force field. The grid for docking simulations was generated with bound co-crystallized cyclosporine A molecule. The docking analysis of compound 2.4c reveals it to be a better Cyclophilin D binder compared to compound 2.3c. Memo and isopropyl moieties of the molecule are well occupied by both deep binding pockets of PPIF protein. The hydrogen bonding of =O group with Arg-55 facilitates the entry of the isopropyl group in the hydrophobic pocket of protein .The highly flexible structure of 2.3c does not support its compatible binding orientation in deep hydrophobic pocket of Cyclophilin D (Fig 2B).

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1. Aurelian Grosdidier, *et al*. PROTEINS: Structure, Function, and Bioinformatics 67, 1010-1025(2007).
2. Ana Jorge-Finnigan. *et al*. Human Molecular Genetics, 22, 3680-3689 (2013).
3. Kajitani, K.; Fujihashi, M.; Kobayashi, Y.; Shimizu, S.; Tsujimoto, Y.; Miki, K. Crystal Structure of Human Cyclophilin D in Complex with Its Inhibitor, Cyclosporin A at 0.96-A Resolution. *Proteins* 2008, *70*, 1635–1639.
4. Schrödinger Release 2014-2: Maestro, Version 9.8, Schrödinger, LLC, New York, NY, 2014.

Chapter 3: Validation of in silico data in enzymatic as well as cell based assays (Manuscript submitted).

In Chapter 4, we explored further the biochemical assessment of compound **2** (compound 2.4c renamed henceforth) in inhibiting the peptidyl prolyl *cis-trans* isomerase activity of cyclophilin D. As shown in Figure 4, compound **2** decreases the peptidyl prolyl *cis-trans* isomerase activity of cyclophilin D similar to cyclosporin A as measured by the chymotrypsin activity on Suc-Ala-Ala –Pro-Phe Para nitroanilide synthetic substrate. We studied further in cell based system using H9c2 cardiomyocytes we compared the activity of CSA and compound **2** in the regulation of cytochrome c release in. As the data reveals, both CSA (**1**) and compound **2** decrease the cytochrome c release by 47.41% and 47.23% respectively validating our *in vitro* biochemical studies. We collaborated with Dr. Sandhya Sitasawad laboratory at National Centre of Cell Science, Pune, for evaluation of cytochrome c release by flowcytometer.

We also evaluated the effect of compound **2** and CsA (**1**) on the mitochondrial structural integrity in H9c2 cells. Thapsigargin has been reported to induce biphasic fragmentation of mitochondria (ref) As shown in Figure 4, both CsA (**1**) and compound **2** treatments prevent thapsigargin-induced mitochondrial fragmentation in H9c2 cells indicating at their role in preserving mitochondrial integrity

Selected references:

1. James L Kofron. *et al*. Biochemistry, 30, 6127-6134(1991).
2. Jennifer R Hom. *et al*. J. Cell. Physiol.212, 498-508(2007).

Concluding remarks:

Protection of mitochondria structurally and functionally has implications in clinical scenarios associated with Cardiac reperfusion injury and Progressive loss of pancreatic beta cells in Type II Diabetes. Cyclosporine A is used clinical for the treatment of Cardiac reperfusion injury; it inhibits PPIF enzyme activity and blocks MPT. Our study was to search for small molecule with similar function like Cyclosporine A. We devised a cell based method to screen for such compounds and found compound 2.4c with MPT inhibitory activity. Next, we hypothesized that whether the molecule interacts with PPIF, a well known MPT regulator. The compound was found in in silico studies to have strong interaction with PPIF similar to Cyclosporine A. Further to validate the study, we performed PPIF enzyme assay and found that compound 2.4c inhibits PPIF similar to Cyclosporine A. Additionally it also protected mitochondria structurally and also form release of cytochrome c. The study reports discovery of a novel small molecule which blocks MPTP change by inhibiting PPIF enzyme activity, and subsequent structural protection of mitochondria. To my knowledge this is first report of a small molecule protecting Mitochondria structurally by enzymatic inhibition of PPIF. To this end compound 2.4c (or 2) has wide spread application for treatments of features of mentioned disorders albeit further study in this direction are required on animal models of cardiac reperfusion injury.

Govardhan K Shroff Peer reviewed publications:

1. Govardhan KS, Ramyasri K, Kethora D, Ravishekar Y, Prasenjit M. **Harnessing impaired energy metabolism in cancer cell: small molecule- mediated ways to regulate tumorigenesis**. Anticancer Agents Med Chem. 2011 Mar; 11(3):272-9. PMID: 21434854.

2. Govardhan K. Shroff, Ravikumar Jimmidi, M. Satyanarayana, B. Ramesh Reddy, Jahnavi Kapireddy, Mithila A. Sawant, Sandhya L.Sitaswad, Prabhat Arya and Prasenjit Mitra. **Prevention of Mitochondrial Membrane Permeabilization and Pancreatic β- Cell Death by an Enantioenriched Macrocyclic Small Molecule.** European Journal of Organic Chemistry. Volume2014, Issue 6, Pages: 1151–1156.

3. Govardhan K. Shroff, Ravikumar Jimmidi, Mithila A. Sawant, Sandhya L.Sitaswad,Kiranam Chatti, Prabhat Arya and Prasenjit Mitra. **A Macrocyclic Small Molecule Having Cyclosporin A Like Acitivity Prevents the Induction of Mitochondrial Permeability Transition and Cytochrome C Release** (Manuscript submitted)

Seminars/Conferences/Workshops Attended:

1. Raman bhai 5th International symposium on Translational medicine, Feb 1-4, 2011, Ahmedabad, Gujarat.
2. Indo-US workshop on mitochondrial medicine, 2008 Manipal University, Manipal.
3. Historical aspects of drug discovery, 2008 MCOPS, Manipal.

Table of contents

List of Tables

List of Figures

List of Abbreviations

Abu- L-α-aminobutyric acid

ADP- Adenine di phosphate

AKT- protein kinase B

Ala- Alanine

AMBER- Assisted Model Building and Energy Refinement

ANT- Adenine nucleotide translocase

Arg- Arginine

Asp- Aspartic acid

Asn- Asparagine

ATP- Adenosine Tri Phosphate

ATCC- American type collection center

A23187- Calcium ionophore

BAX- Proapoptotic BCL2 family related protein

BCL2 – B cell lymphoma protein 2 antiapoptotic

Brin-BD11- electrofused rat pancreatic cell line

BSA- Bovine Serum albumin

Ca^{2+}- Calcium ions

CCCP -Carbonyl cyanide *m*-chlorophenyl hydrazone

CHARMM- Chemistry at HARvard Molecular Mechanics

CICR- Calcium induced calcium release

CO_2- Carbon di oxide

CPB- Cardiopulmonary bypass surgery

CsA- Cyclosporine a

cTnI- Cardiac troponin I

Cyt C- Cytochrome C

CypA- cyclophilin A

Cys- Cysteine

Cyp- cyclophilin

Da- Daltons

DAPI- 4', 6-diamidino-2-phenylindole stains nucleus

DMEM- Dubelco's Minimal Essential medium

DMSO- Dimethyl sulfoxide

DNA-Deoxyribo Nucleic Acid

ECACC-European collection of cell cultures

E.Coli- *Escherichia coli*

EC_{50}- half maximal effective concentration

EGTA- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid glycol ether diamine tetra acetic acid

FBS- Fetal bovine serum

GM-CSF- Granulocyte Monocyte Colony stimulating factor

Gln-Glutamine

Glu- Glutamic acid

Gly- Glycine

GSK-3β- glycogen synthase kinase 3- beta

H+- Hydrogen ions

[^{3}H]- Tritium ion

HCV- Hepatitis C virus

HEPES-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HIV-1- Human Immuno deficiency virus 1

His- Histidine

HKII- Hexokinase II

Hrs-hours

HSP- Heat shock protein

IFN-γ- Interferon gamma

IMM-inner mitochondrial membrane

IMS-inter membrane space

IL-2- Interleukin 2

Ile- Isoleucine

IP3- Inositol triphosphate

IPTG-Isopropyl β-D-1-thiogalactopyranoside

K+- Potassium ions

KCl- Potassium chloride

kDA- Kilo Daltons

K113I- Lysine 133 Isoleucine mutant of Cyp D

LB broth- Luria bertani broth

Leu- Leucine

Lys- Lysine

MAPK- Mitogen activate protein kinase

Mbps- Mega bytes per second

MeBmt- (4R)-4-[(E)-2-butenyl]-4

MEFs- Mouse embryonic fibroblasts

MEMO- methoxy ethoxy methyl group

MeLeu- *N*-methylleucine

MeVal- *N*-methylvaline

Met- Methionine

Mg^{2+}-Magnesium ions

MI- mycocardial infarction

mM- milli Molar

mmp- mitochondrial membrane potential

mPTP- Mitochondrial Permeability Transition Pore

mtCK- mitochondrial creatine kinase

MTT- (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)

Na+-Sodium ions

NAD+- Nicotinamide adenine dinucleotide

NADH- reduced Nicotinamide adenine dinucleotide

NFAT- Nuclear factor of activated T-cells

Ni-NTA- Nickel- Nitrilotriacetic acid

NMR- Nuclear magnetic resonance

nS- nano Siemens

OD- optical density

OMM-outer mitochondrial membrane

OPLS- Optimized Potentials for Liquid Simulations

OSCP-Oligomycin sensitivity conferring protein

PAGE- Pole acrylamide gel

PBR- peripheral benzodiazepine receptor also known TSPO- Translocator protein

PBS- phosphate buffered saline

PDB- protein data bank

Pdx1- Pancreatic duodenal homeobox gene 1

PEG- Poly ethylene glycol

pH- Per Hydrogen ion concentration

Phe- Phenylalanine

PiC- inorganic phosphate carrier protein

PKCε-protein kinase C epsilon

PMI-posterior myocardial infarction

pNA- para nitro anilide

PPIase- Peptidyl-Prolyl *cis-trans* isomerase

PPIF- Peptidyl-Prolyl *cis-trans* isomerase F

Pro- Proline

pS- pico siemens

PT- permeability transition

PTP- Permeability Transition Pore

RAM- random access memory

RINm5F- Rat insulinoma

RMSD- Root means square deviation

RNA- Ribonucleic acid

RNAi- RNA interference

S-seconds

Sar- Sarcosine

SASA- solvent accessible surface area

SDS- Sodium deoxy lauryl sulphate

SERCA- smooth endoplasmic reticulum calcium ATPase

Ser- Serine

SfA- Sangliferin A

SMV- Sub mitochondrial vesicles

SP- Standard precision

Sr^{2+}- Strontium ions

STZ- Sterptozotocin induced diabetic rats

Suc- A-A-P-F-pNA- succinyl Ala- Ala- Pro-Phe- para nitro anilide

T4- T even umbered bacteriophage

TFE- Tri fluoro ethanol

Thr- Threonine

TMRE- Tetra methyl rhodamine ethyl ester

TNF- α- Tumour necrosis factor alpha

Trp- Tryptophan

Tyr- Tyrosine

UNT- untreated

Val- Valine

VDAC- Voltage dependent anionic channel

Ver- Version

XP- extra precision

ZO- Zucker Obese rats

Δψm- mitochondrial membrane potential

µm- Micro meter

β-ME- beta mercaptoethanol

µL- Micro liter

Aim of the Thesis

Mitochondrial Permeability Transition Pore (mPTP) regulates the solute exchange between the mitochondrial matrix and cytoplasm and plays an essential role in regulating ROS signaling and calcium efflux. However, the prolonged opening of mPTP results in the depolarization of mitochondrial membrane potential and the release of cytochrome c which causes ischemic cellular injury and necrotic cell death. Understanding the mechanism of mPTP and *more importantly*, the identification of specific pharmacological inhibitors that regulate mPTP opening is of profound disease relevance. The aim of my present study is to develop a screening paradigm to identify novel chemical entities that could prevent the depolarization of mitochondrial membrane potential and the release of cytochrome c from mitochondria under patho-physiological conditions. I further studied the molecular target to explore the mechanism of action of the lead compound and related analogs which reveals that the *enantiopure*, 12-membered macrocyclic compound we identified in our screen inhibits the peptidyl prolyl *cis-trans* isomerase activity of cyclophilin D, a component of mitochondrial permeability transition pore, much similar to cyclosporin A. Like cyclosporin A, the molecule also reverses the thapsigargin-induced rupture of mitochondria and the subsequent release of cytochrome c in cultured cardiomyocytes.

Chapter 1: Literature review

Mitochondria: Determinator of Life or Death of a Cell.

Among the cell organelles mitochondrion has a special importance due to its complexity and involvement in an array of cellular physiological process. Being endosymbiotic in origin, arising from engulfment of α proteo-bacterium by eukaryotic cells around 2 billion years ago (Lane and Martin 2010), mitochondria have their own circular DNA and have been conjectured to be domesticated for generation of ATP through oxidative phosphorylation of nutrients. However recent evidences suggest a wider role of the endosymbiont in regulating the cell nucleus: the organelle at one end supports the physiological reactions pertaining to the liveliness of the cell; on the other hand it is involved in the release of caspase activators and the regulation of pro and anti-apoptotic family of proteins that delineate cell death. These dramatically opposite functions reflects on the intricacy of the relationship of mitochondria with its host: in return of serving as a platform for cellular metabolism, the organelle regulates the fate of the cell; in fact, the architecture, location and activity of mitochondria serves as a key marker of cellular pathophysiology which defines various metabolic and genetic disorders extant in human population.

1.1 Mitochondrial architecture:

Mitochondria are about 0.5–1 μm in diameter and up to 7μm in length (Krauss 2001). Their shape and number per cell varies from one tissue to the another; in humans, for example, erythrocytes do not contain any mitochondria while heart liver and muscle cells have them in thousands (Alberts et al. 2002; Voet and Voet 2004). Like ancestral bacterial cells, mitochondria also have a double membrane organization (Bui, Bradley, and Johnson 1996) with two membranes having distinct physico-chemical properties and different biochemical function.

Phospholipid compositions and protein-to-lipid ratios are also different in outer and inner membrane of mitochondria. In the inner membrane of mitochondria the protein to-lipid ratio is 80:20 where as for the outer membrane this ratio is about 50:50 (Krauss 2001). Moreover, the inner membrane of mitochondria is unique in possessing a unique phospholipid cardiolipin which constitutes about 20% of the total lipid composition and is essential for the optimal function of enzymes involved in mitochondrial energy metabolism (M Schlame, Brody, and Hostetler 1993; Michael Schlame et al. 2003; M Schlame and Haldar 1993).The outer membrane is permeable to ions and larger molecules, but the inner mitochondrial membrane, unlike the outer membrane, is much less permeable and sequestered the mitochondrial matrix from the cytosolic environment. The inner mitochondrial membrane, in fact serves as an electrical insulator and chemical barrier which allows only specific carriers, ion transporters and shuttles to cross the barrier thus generating a difference in potential which is prerequisite for ATP synthesis (Krauss 2001).

1.2 Mitochondrial Permeability Transition Pore:

In addition to transporters and shuttles, different electrophysiological and biochemical studies reveal the existence of an inducible nonspecific voltage gated pore across the mitochondrial inner membrane that allows molecules less than 1500 daltons to traverse the membrane **(Figure 1.1).** The pore, termed as Mitochondrial Permeability Transition Pore (mPTP) allows the solute exchange between matrix and cytosol on transient opening (low conductance opening)**(Figure 1.1)**, however, on prolonged opening (long lasting PTP) the pore releases pro-apoptotic factors in cytoplasm causing cell death **(Figure 1.1B).**

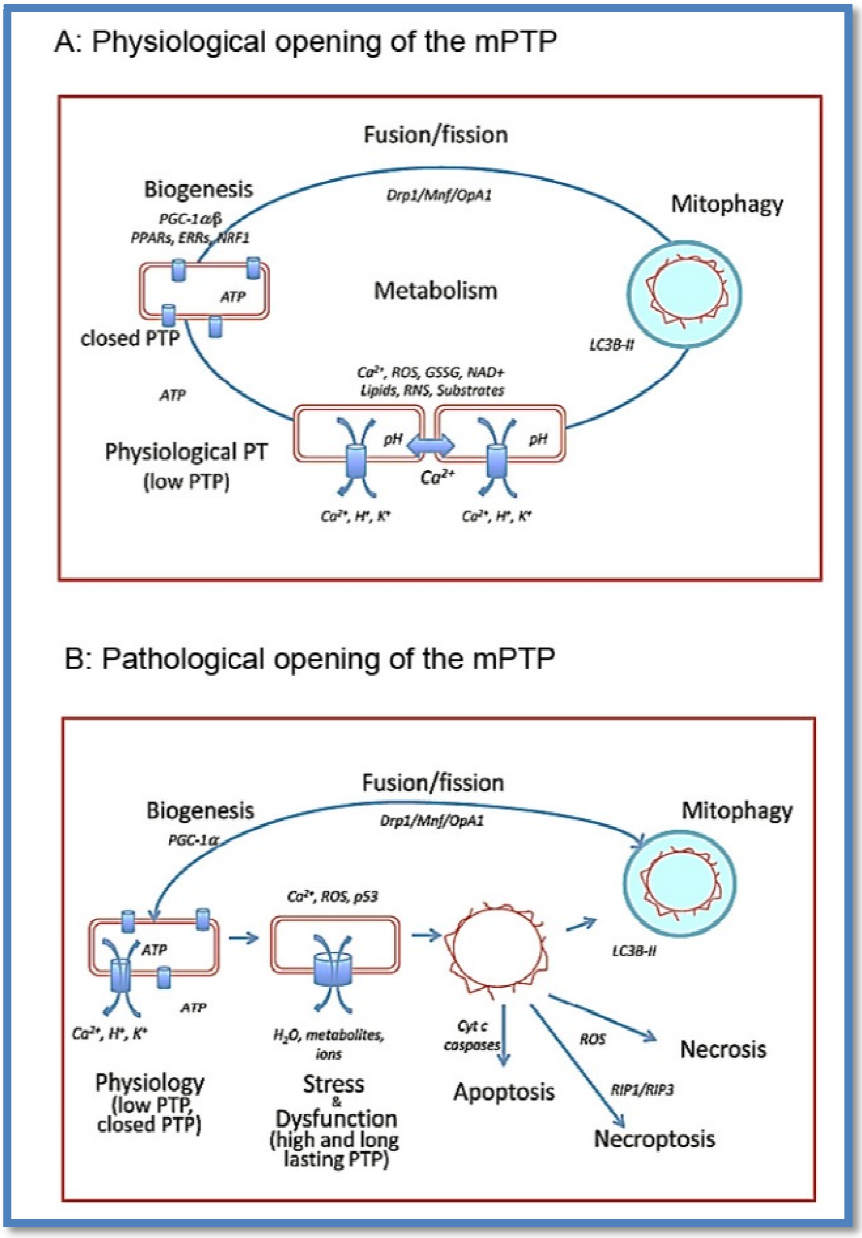

Figure 1.1: Schematic diagram explaining the differences between physiological and pathological conditions of mPTP opening (Brenner and Moulin 2012).

The mPTP thus has an essential role in regulating cellular physiology and pathophysiology and has been implicated in various diseases including ischemia–reperfusion damage (D. J.

Hausenloy, Boston-Griffiths, and Yellon 2012), liver damage (Christopher P Baines et al. 2005), and chronic disorders of the nervous system (Shevtsova et al. 2014).

1.3 mPTP: A Historical Perspective:

The permeability transition(PT) is defined as an increase of mitochondrial inner membrane permeability to ions and solutes with molecular masses up to about 1500 Daltons which leads to the matrix swelling (Elrod and Molkentin 2013). The phenomenon has been reported since early 1950s (Raaflaub 1953; F. E. Hunter and Ford 1955; Tapley 1956; A. L. Lehninger and Remmert 1959; Wojtczak and Lehninger 1961; Zborowski and Wojtczak 1963; Azzone, Azzi, and A 1965; Azzone and Azzi 1965; A Azzi and Azzone 1966; Chappell and Crofts 1965); but the observation being prior to the emergence of chemiosmotic concept (P Mitchell and Moyle 1967; Peter Mitchell 2011) could not evoke substantial attention. Later, the pioneering work of Hunter, Howarth and Southard (D R Hunter, Haworth, and Southard 1976) on heart mitochondria provided the basis of the mechanism that regulates the phenomenon. They coined the term permeability transition (PT) and showed the PT was due to the reversible opening of a proteinaceous pore in the inner mitochondrial membrane whose physiological role remained undefined.

1.4 Mitochondria swelling phenomenon: First step of mPTP Discovery:

Mitochondria, isolated from animal tissues were observed to undergo “swelling” when placed in a sucrose deficient isotonic medium (Gotterer, Thompson, and Lehninger 1961) which accounted for the variations in their optical density (A Azzi and Azzone 1965). Several agents, namely, inorganic phosphate, calcium, reduced glutathione (A. L. Lehninger 1959) was reported to induce mitochondrial swelling which was blocked by ATP, azide and arsenate. Interestingly,

mitochondrial swelling induced by some agents was reversible through ATP-driven mechanism, while swelling by glutathione, carbon tetrachloride and digitonin were not found to be readily reversible by ATP + Mg^{2+} (a L. Lehninger 1959; a L. Lehninger and Schneider 1959) adding complexity to the observation. Among various inducers, calcium and phosphate mediated mitochondrial swelling received major attention because of its stoichiometric synchrony with oxidative phosphorylation (Rossi and Lehninger 1964). However, the electron microscopic studies of swelling found out that phosphate induced swelling completely disrupted the size and shape of the particles of the inner membrane while leaving the outer membrane intact and the phenotype was not restored by the addition of ATP. This observation ruled out the possibility of any coupled system in mitochondrial membrane which regulates the swelling and contraction as well as the production of ATP (Bartley and Enser 1964). The calcium induced mitochondrial swelling stimulated respiration, and was not inhibited even in presence of cyanide and dinitrophenol but this additional treatment caused extensive swelling and eventual release of calcium into the medium. The calcium induced swelling occurred even in anaerobic conditions and in the presence of oligomycin (Crofts et al. 1965) which clarified the concept that calcium induced mitochondrial swelling did not require any energy in the process.

1.5 Calcium induced Permeability Transition:

Mitochondria swell when its membrane becomes permeable to ions of medium while in the presence of an oxidizable substrate or ATP, the ions were pumped out causing shrinkage. The efflux of ions from mitochondria did not occur down the concentration gradient and was found to be coupled to metabolism. These observations led to the proposal of two basic mechanisms: a) an operating contractile system in mitochondrial membrane or b) the active extrusion of ions (Angelo Azzi and Azzone 1967). Haworth and Hunter, in their study on calcium induced

mitochondrial swelling, used a specialized medium (D R Hunter, Haworth, and Southard 1976) that effectively block both the electron transport chain and the energy generation by substrate catalyzed reaction. In these conditions, the Ca^{2+}induced swelling required neither electron transport chain nor energy, but appeared to have an internal site blocked by some unknown endogenous protective agents (Robert A. Haworth and Hunter 1979; R A Haworth and Hunter 1979; Douglas R Hunter and Haworth 1979). Mitochondria in presence of low levels of Ca^{2+} accumulated it rapidly with no change in shape; however, after a period of time, they underwent a sudden increase in permeability of the inner membrane allowing the molecule to enter the matrix and maintain ionic equilibrium and tonicity. This phenomenon they coined as **calcium induced transition**; in fact, identical transitions were also produced by agents like phosphate, arsenate or fatty acids, but they were found to be mere potentiators of the activity of calcium which was the common physiological agent in all the cases (Robert A. Haworth and Hunter 1979).

The calcium induced transition was inhibited by ruthenium red and was found to occur only in case Ca^{2+} with Sr^{2+}having no effect. The data suggested that the site at which Ca^{2+} acts might be a ruthenium red-sensitive carrier protein or space. The study was further supported by observation that calcium induced transition could be reversed by Ca^{2+}ionophore A23187 which led to the speculation of a Ca^{2+}binding 'trigger site" that allows permeability transition (R A Haworth and Hunter 1979). Haworth and Hunter employed a permeability assay to determine the size limit by using osmotically active solute like PEG (poly ethylene glycol) and reported that molecules less than 1500 daltons are able to cross the barrier defining the size limit of permeability transition (Douglas R Hunter and Haworth 1979). The most probable molecules in less than 1500 daltons molecular weight range are adenine nucleotides, NAD+ and other Krebs

cycle cofactors implying at the physiological relevance of calcium induced permeability transition. Moreover, permeability transition was found to be non-specific as this could be modulated by adjusting the concentration of available Ca^{2+} in the medium and could be reversed by addition of EGTA. Thus the pioneering work of Haworth and Hunter finally established the fact that Ca^{2+} induced transition of mitochondrial permeability does not require a source of energy and is insensitive to inhibitors and uncouplers of mitochondrial respiration and energy synthesis pathways.

1.6 Voltage dependency of mPT: presence of low conductance and high conductance states:

Mitochondria function in a cytosolic environment containing Na^{+}, K^{+}, Ca^{+2} as well as other cations and anions. The inner mitochondrial membrane, however, is impermeable to these ions and their flux to mitochondrial matrix is regulated by specific channels and transporters. Patch clamp experiments of inner mitochondrial membrane provided reveling information about the presence of different kind of channels which were voltage sensitive. In patch clamp experiments with 150mM KCl, the conductance of these channels varied from 10-20, 45, 80, 120-150, 200, 350 and 1000 pS (pico Siemens of conductance) with different selectivity (Kathleen W. Kinnally, Campo, and Tedeschi 1989; K W Kinnally et al. 1989) **(Table 1.1)**.

Conductance(in pico Siemens) in 150mM KCl	**Negative Voltage effect**	**Positive Voltage effect**	**Selectivity**
10-20	not measured	not measured	not measured
45	None	None	Anion
80-150	Open	Close	Cation

350	Open	Close	Cation
1000	Close	Open	Non selective

Table 1.1: Various conductance channels present in inner mitochondrial membrane. (Redrawn from K.W. Kinnally (Kathleen W. Kinnally, Campo, and Tedeschi 1989))

Zoratti, DeMarchi and Szabo, in a series of seminal papers reported full mPTP conductance in the range 0.9–1.5 nS, which had a preferred value of 1.3nS (Zoratti and Szabo 1995; De Marchi et al. 2006; Szabo and Zoratti 2014). More importantly, the mPTP conductance was found to have many lower substates, including a prominent half-size one which is often termed as a "way station" during the closures or openings of the full conductance (Szabo and Zoratti 2014). The data gave rise to the present concept that the pore complex operates at least in two modes: a low conductance mode that provides transient opening (flickering of the pore) for solutes with MW <343 Da which allows exchange of small ions such as H^+, Ca^{2+}, or K^+ and serves the purposes of generating Ca^{2+}waves in the cell and a high conductance mode which is permeable to solutes with MW <1,500 Da and results in the release of apoptogenic materials thus contributing to cell death.

Patch clamp studies also provided information about the structural feature of the pore. Reconstitution of adenine nucleotide translocase (ANT) in liposomes and treatment with Bongkrekic acid and carboxyataractlyate (Known inhibitors of ANT) showed that ANT may not be an integral part of inner membrane channel. On the other hand, electrophysiological evidences for the presence of half-mPTP gave rise to the concept that the pore may be dimeric in nature (reviewed in (Szabo and Zoratti 2014).

1.7 Cyclosporin A: inhibitor of mPTP:

Fournier et.al in their pioneering work observed that when calcium was added in high concentrations, mitochondria could not accumulate it completely and the membrane potential gets dissipated. However, the addition of Cyclosporin A (CsA) restored the ability to store calcium and prevented the dissipation of mitochondrial membrane potential (Fournier, Ducet, and Crevat 1987). Prior to this observation, Mihatsch *et.al* reported the presence of giant mitochondria (Mihatsch et al. 1981) due to CsA treatment in their electron micrograph observations of renal biopsies of patients which was suggestive of matrix Ca^{2+} overload being induced by CsA. These observations set the stage for Crompton et.al who first identified CsA as an inhibitor of the Ca^{2+}-dependent pore of the inner membrane responsible for mitochondrial permeability transition (M Crompton, Ellinger, and Costi 1988; Broekemeier, Dempsey, and Pfeiffer 1989; Broekemeier and Pfeiffer 1989). Later, the patch clamp experiments on mitoplasts in the presence of CsA showed that CsA inhibited the conductance activity of 1.3 nano Siemens (~ 1000pico Siemens) channel of the inner membrane and the inhibitor acted when present on the matrix side of membrane (Szabo and Zoratti 1991). The experiment established CsA as a pharmacological inhibitor of MPTP; Szabó and Zoratti also showed that the 1.3nS conductance channel responded to the activation by Ca^{2+}, inhibition by Mg^{2+}, CsA and ADP and confirmed that the minimum pore size derived from conductance corresponds to independent estimates of minimum size of permeabilization pore (Szabó and Zoratti 1992).

1.8 Cyclosporin A inhibits mitochondrial peptidyl prolyl *cis-trans* isomerase:

CsA, a cyclic undecapeptide of fungal origin was known for its ability to prevent the immune response against xenografts (Borel et al. 1976; Borel et al. 1977). Mechanism of action of Cyclosporin A was through its interaction with cytosolic cyclophilinA (CypA) resulting in the

formation of a CsA-Cyp A complex which inhibited calcineurin, a Ca^{2+}/calmodulin-dependent cytosolic phosphatase (Jun Liu et al. 1991). Due to this inhibition, NFAT remained phosphorylated and could not translocate to the nucleus to trigger IL-2 dependent activation of the immune response (Jun Liu et al. 1991). There are 17 cyclophilins in the human genome sharing a common domain of about 109 amino acids, the Cyp-like domain (Wang and Heitman 2005) which possess Peptidyl-Prolyl *cis-trans* isomerase (PPIase) activity (G. Fischer et al. 1989; Takahashi, Hayano, and Suzuki 1989) and inhibited after the binding to CsA (Borel et al. 1977). Site-directed mutagenesis of CyP-A allowed the PPIase activity to be separated from CsA binding and calcineurin inhibition (Zydowsky et al. 1992), which suggested that cyclophilins function through interactions with a specific set of partner proteins rather than serving as general mediators of protein folding (Wang and Heitman 2005). To decipher the mechanism of CsA mediated inhibition of MPTP, a photoactive, radio-labeled CsA derivative was used to tag the putative CsA 'receptor' in mitochondria (Andreeva, Tanveer, and Crompton 1995; Tanveer et al. 1996). In photo-labeling experiments, a number of mitochondrial components became covalently labeled by the CsA derivative, but only photo-labeling of CypD was promoted by ADP and abolished by Ca^{2+} thereby identifying CypD as the pore-associated CsA-binding component (Andreeva, Tanveer, and Crompton 1995; Tanveer et al. 1996).

The full-length CypD protein comprises of 207 AA (22 kDa) having 109 AA cyclophilin domain that imparts prolyl-isomerization activity. Baines *et al.,* showed that cyclophilin D (Arg 96 Gly), an isomerase deficient mutant, unlike its wild type counterpart, was unable to rescue mitochondrial swelling or ROS-induced cell death in PPIF –/– mouse embryonic fibroblasts (MEFs), highlighting the importance the isomerase domain of CypD in modulation of the mPTP (Christopher P Baines et al. 2005). The X-ray crystal structure of the complex between

recombinant cyclophilin B with Cyclosporin A refined at 1.85-A resolution provided the insight on the mechanism of CsA inhibition (Mikol, Kallen, and Walkinshaw 1994). CsA has unmodified alkyl side chains, which occupies the hydrophobic pocket in cyclophilin corresponding to the active site. Residues 1, 2, 3, 9, 10 and 11 provide the Cyp-binding domain and the residues 4 to 8 are reported to be exposed to the solvent (reviewed by (M Crompton 1999). Modification of the CsA residue 4 from methyl-leucine to methyl-valine did not alter the affinity of the pore or Cyp D (for details read refer chapter 3) towards the inhibitor; however, the modification of residue 8 from alanine to dansyl lysine decreased cyclosporin potency as pore inhibitor which is in concert with equivalent decrease in its binding affinity of free CypD (Nicolli, Redetti, and Bernardi 1991). Thus CsA mediated inhibition of mPTP was conjectured to be due to the occupancy of the active site of CypD, unlike Cyp-A-CsA-calcineurin complex, no data has yet suggested that CypD-CsA complex binds to other mitochondrial proteins to regulate the pore opening (M Crompton 1999).

1.9 Physiology of mPT Pore:

Ichas et.al first reported that in living cells, mitochondrial calcium uptake during inositol trisphosphate (IP3)-induced calcium mobilization triggers a mitochondrial efflux of Ca^{2+} (mCICR) that is generated in concert with depolarization spikes (mDPS) initiated due to transient opening of mPTP (Ichas, Jouaville, and Mazat 1997). The mPTP thus served as an amplifier and propagator of depolarization and calcium waves that was emitted from endoplasmic reticulum and contributed to the intracellular calcium induced calcium release (CICR) which regulates the different physiological function in different cell types ranging from insulin secretion (Santulli et al. 2015) to nerve regeneration (Duregotti et al. 2015). The mechanism of mDPS generation and mCICR is explained in the following figure **(Figure 1.2)**

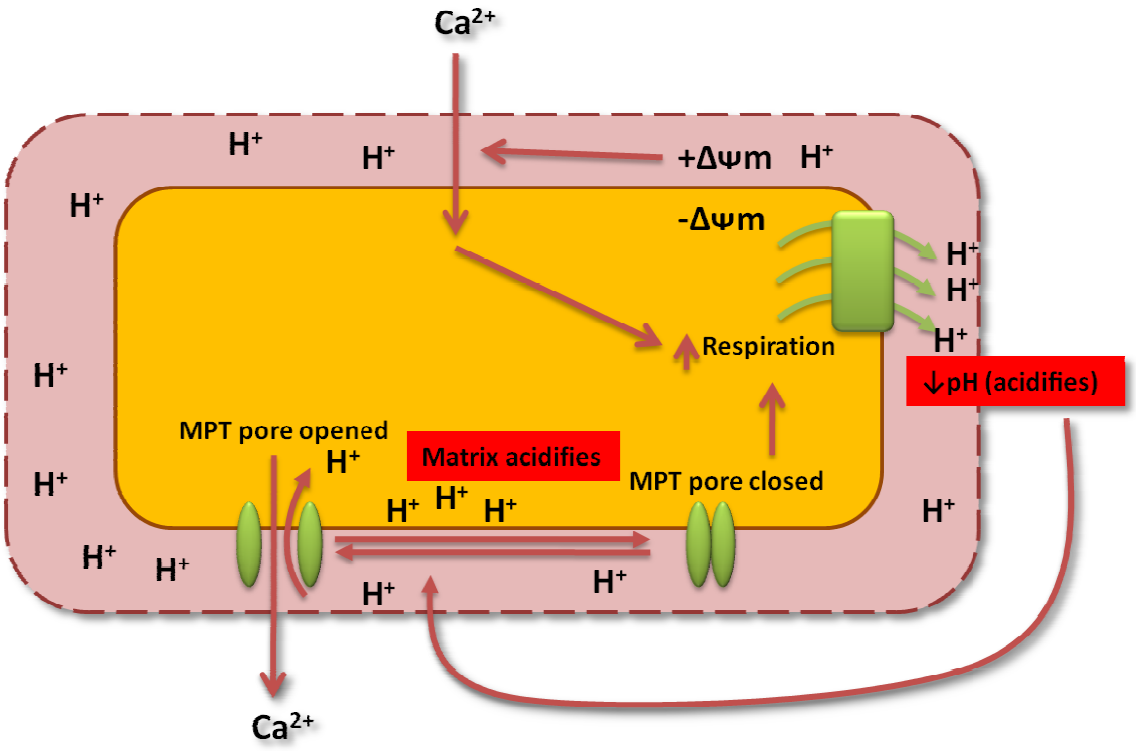

Figure 1.2: Schematic representation showing cross talk between MPT pore and respiration, matrix, pH, Δψm (mitochondrial membrane potential) and Ca^{2+}(Ichas, Jouaville, and Mazat 1997).

As the Figure describes, mDPS and mCICR is pH dependent and is initiated by the entry of calcium in mitochondria due to ΔΨm which is negative inside the organelle. This enhances the the activity of electron transport chain which increases pH. High pH triggers the PTP opening that collapsed the proton gradient and dissipated the ΔΨm causing outward efflux of calcium and concomitant matrix acidification which closes the PTP. The Electron transport chain then generates the proton gradient restoring ΔΨm thus facilitating calcium reuptake (Ichas, Jouaville, and Mazat 1997). Thus the flickering of the pore at low conductance mode represents the excitability of the organelle which is associated with diverse cellular functions.

1.10 Early models of mPT pore complex:

The CsA binding to the mitochondrial cyclophilin (CypD) contributed immensely to the understanding of the molecular composition of the pore complex. The CypD-GST affinity matrix identified voltage dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) as its interacting partners. Moreover, artificial liposomes having GST-CypD, VDAC and ANT could be permeabilized by Ca^{2+} and phosphate which is blocked by CsA. These biochemical properties shown by these proteo-liposomes were similar to the putative pore in mitochondria (Martin Crompton, Virji, and Ward 1998). Detergent based extraction and further characterization by chromatography showed peripheral benzodiazepine receptor (PBR) to be co-purified with ANT and VDAC (McEnery et al. 1992).Currently known as Translocator Protein (TSPO), the protein had been shown to be present at mitochondrial outer membrane and cooperate with steroidogenic acute regulatory protein (StAR) to facilitate the cholesterol transport to mitochondria (Selvaraj and Stocco 2015). Further characterization of the outer and inner membranes by electron microscopy using gold labeled antibodies and selective disruption of the outer membrane by digitonin identified several possible protein-protein interacting partners like hexokinase and creatine kinase (Kottke et al. 1988). It was presumed that there is contact between both outer and inner membrane of mitochondria which facilitated mPT phenomenon; therefore the putative contact site was further characterized by density gradient centrifugation from osmotically disrupted mitochondria, and in agreement with the previous reports, the fraction was found to be rich in hexokinase and was also found to have creatine kinase (Adams et al. 1989).

Studies on freeze-fractured liver mitochondria also revealed the existence of the contact sites between outer and inner membrane and its occurrence was observed in the presence of ADP and carboxy-atractyloside, a ligand that binds with ANT despite inhibition by antimycin A. ATP was ineffective in inducing the contact sites irrespective of the membrane potential; further, the binding analysis of [3H] atractyloside showed that ANT is localized in the peripheral part of the inner membrane (Bücheler, Adams, and Brdiczka 1991).

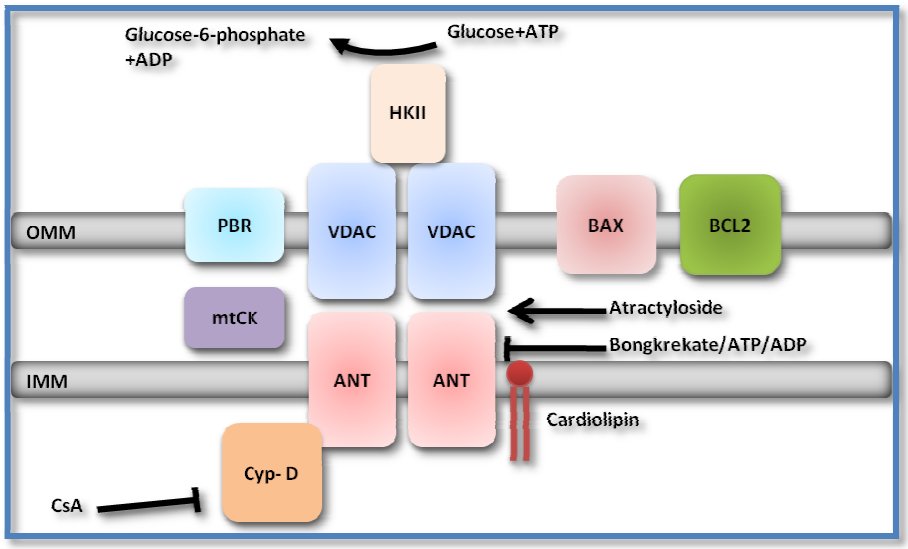

Figure 1.3: Illustrative diagram of mitochondrial permeability transition pore complex (Zamzami and Kroemer 2001). ANT-adenine nucleotide translocator, VDAC-voltage-dependent anion channel, PBR- peripheral benzodiazepine receptor, mtCK- mitochondrial creatine kinase, HKII- hexokinase II, Cyp D- cyclophilin D, CsA- Cyclosporin A.

ANT was found to have interactions with the Bcl2 family of proteins as well (Marzo et al. 1998). Bax and ANT have been shown to interact in co-immunoprecipitation experiments as well as in yeast two hybrid systems. In addition, ectopic expression of Bax could not induce death in ANT deficient yeast (Marzo et al. 1998). These set of observations led to a model of mPT pore as a

multi-protein complex spanning both the membranes and composed of ANT, VDAC, PBR(~TSPO), hexokinase II, Bcl2, Bax and Cyclophilin D as shown in **Figure 1.3** (Zamzami and Kroemer 2001). However, the model was seriously questioned by genetic ablation and knock down studies as CsA sensitive mPT could still be detected in the absence of ANT isoform1/2 (Kokoszka et al. 2004), VDAC isoform 1/2/3 (Krauskopf et al. 2006; Christopher P Baines et al. 2007) and TSPO (Šileikytė et al. 2014). Another model of formation of mPT by phosphate carrier (PiC) was proposed due to its interaction with CypD and ANT (Leung, Varanyuwatana, and Halestrap 2008). However, the electrophysiological experiments with reconstituted PiC did not show similar conductance and properties like mPT. Also the genetic ablation experiments showed that PiC is not required for MPT (Herick, Krämer, and Lühring 1997; Kwong et al. 2014). CypD, on the other hand, was found to be a regulator of mPT but not a structural component of the pore since mitochondria isolated from heart, brain and liver of Peptidyl prolyl isomerase F(PPIF) knockout mice showed mPTP opening in a CsA insensitive manner at a higher calcium concentration and after prolonged exposure (Christopher P Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005).

1.11 Present model of MPT pore complex:

The blue native gel electrophoresis along with the immunoprecipitation and extraction with digitonin showed that CypD associated with ATP synthase complex which was again confirmed by cross-linking experiments (Giorgio et al. 2009).The data further showed that interaction of CypD happened with the lateral stalk of ATP synthase, where the main interacting subunits were OSCP (oligomycin sensitivity conferring protein) and b and d subunits which bind with Cyp D at a ratio of 1:1:1:1 (Giorgio et al. 2009). Further, reconstituted ATP synthase dimerizes in liposomes and shows conductance of 1.0-1.3nS in 150mM KCl whose activity was inhibited by

$Mg^{2+}$with ADP and not inhibited by CsA owing to absence of CypD in preparation , which is similar to earlier reports of mega channel (reviewed in (Bernardi 2013).

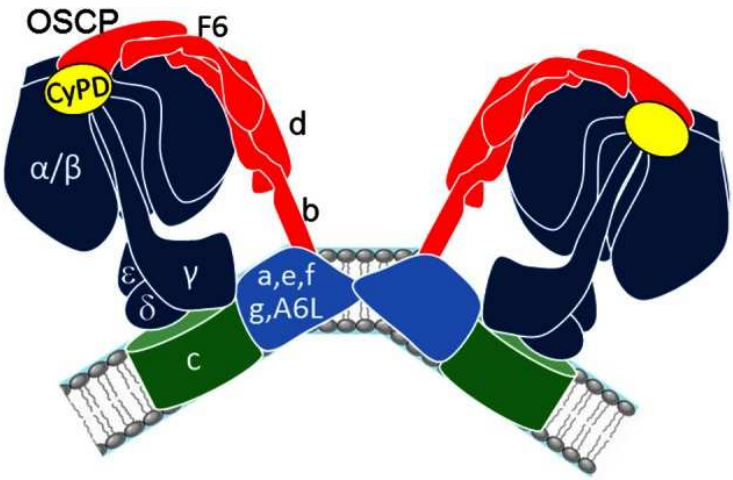

Figure 1.4: Schematic model of F0F1 ATP synthase subunit and its association with cyclophilin D(Cyp D)(Bernardi 2013).

The model was taken further by Alavian et.al who proposed that the c-subunit of the F0-F1ATPase synthase creates the regulated pore (Alavian et al. 2014). Proteoliposomes having purified c subunit demonstrated the presence of multi-conductance channel having 100pS sub-conductance states with peak conductance upto ~1.5–2 nS similar to the reported conductance reported for mPTP (Alavian et al. 2014). In a series of elegant electrophysiological experiments to delineate the structure of mPTP they have shown that the conductance in proteoliposomes having c subunit was independent of modulation by calcium or CsA. Proteoliposomes having monomeric ATP synthase showed infrequent channel activity which is significantly enhanced by the addition of recombinant CypD and inhibited by CsA. Whole mitochondria and sub mitochondrial vesicles (SMV) exhibited robust channel activity and calcium and CsA responsiveness which is lost in urea denatured SMVs where F0-F1ATPase, OSCP and CypD

were removed. The data confirmed that Ca^{2+}, CypD, and CsA regulate the inner mitochondrial membrane conductance by binding to an extra membrane protein site, possibly within the OSCP-subunit of ATP synthase. The work supported the previous observation of Bonora and Pinton (Bonora et al. 2013) who have used calcein fluorescence in presence of cobalt quencher to demonstrate that genetic ablation of c subunit of F0-F1ATPase prevents the mPTP opening.

Contrasting views exist regarding the outer membrane component of mPTP. Karch et.al reported proapoptotic Bcl-2 family members Bax and Bak to be a member of the outer membrane component of mPTP (Karch et al. 2013). Mitopatching experiments revealed that Bax and Bak double deleted fibroblasts were resistant to Ca^{2+}-induced swelling and lacked permeability/conductivity (Karch et al. 2013) however the current model predicted that outer membrane and inner membrane mPTP may function independently; outer membrane mPTP in presence of Bax and Bak is largely permissive while that of inner membrane permeation is highly regulatory (Karch et al. 2013). Morciano summarized the current model of molecular identity of mPTP in the following **Figure 1.5** (Morciano et al. 2015).

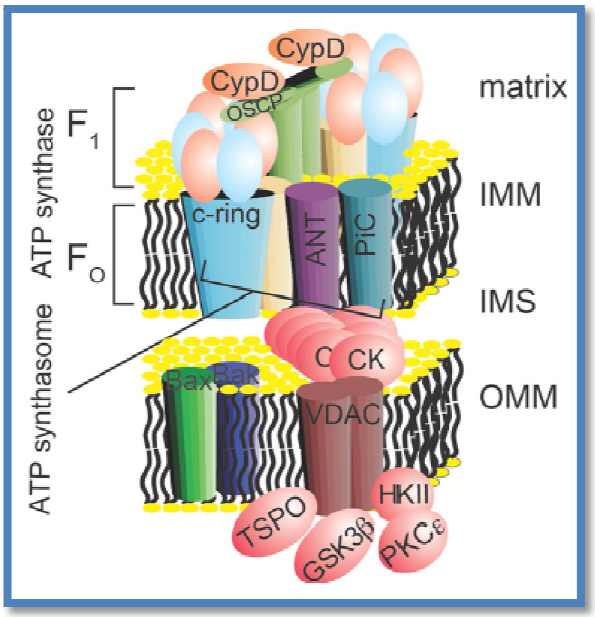

Figure 1.5: Present model of MPT pore and the mitochondrial contact site between outer and inner membrane. (Morciano et al. 2015) IMM-inner mitochondrial membrane, IMS-inter membrane space, OMM-outer mitochondrial membrane, PKCε-protein kinase C epsilon, GSK-

3β- glycogen synthase kinase 3- beta, TSPO- Mitochondrial translocator protein (also known as PBR).

Emerging evidences and observations from genetic ablation studies and patch clamp experiments of reconstituted proteins of inner membrane of mitochondria put in favor of the present model of mPT pore complex (Bernardi and Di Lisa 2015; Morciano et al. 2015; Murphy 2015). A critical assessment of old and new candidates of mPT pore complex by Halestrap's group suggested that perhaps a fraction of 'ATP synthasome complex' composed of F0F1ATPase, PiC, ANT, Cyp D and VDAC might form the site of mPT induction (Andrew P Halestrap and Richardson 2015). The endogenous pathway modulators of mPT phenomenon were also explored and it was found that GSK3-β inhibitors impair the ANT function and thus may serve hitherto unknown regulatory role (Miura and Miki 2009). Protein Kinase C family of kinases was found to have interactions with VDAC+ANT+ Hexokinase II complex which increases VDAC phosphorylation and may regulate mPT pore opening (Christopher P. Baines et al. 2003). Thus, the debate regarding the composition of mPT exists till date, but the application of this mPT phenomenon in context of cardiac reperfusion injury and ischemia in neuronal cells makes it an attractive drug candidate.

1.12 mPT and relevance to Cardiac Reperfusion injury:

Patients undergoing cardiopulmonary bypass (CPB) surgery suffers from perioperative myocardial infarction resulting in increased morbidity and mortality (Roques et al. 1999). The resultant aortic cross clamping-unclamping induced a global myocardial ischemia-reperfusion sequence which results in myocardial injury reflected through increased cardiac troponin I (cTnI) levels (diagnostic marker for acute coronary syndromes) (Chiari et al. 2014). During the initial

periods of reperfusion, rapid correction of acidosis (rise in H^+ ion concentration in body fluids) via the Na+/H+ exchangers causes activation of the Na+ /Ca^{+2} exchanger in the reverse mode resulting in cytosolic Ca^{2+} accumulation (Argaud et al. 2005). In addition, abrupt re-exposure of the ischemia (lack of normal oxygen levels) generated ΔΨm which stimulated ATP synthesis but also caused rapid matrix Ca^{2+} overload and massive generation of oxygen free radicals resulting in mPTP opening (S. Javadov et al. 2008; S. A. Javadov et al. 2000). Griffith et.al used 2[^{3}H]-deoxyglucose (radio labeled glucose analog) entrapment kinetics in mitochondria as a marker of mPTP opening (Griffiths and Halestrap 1995). The technique has been used further to demonstrate that mPTP did not open during ischemia; on the other hand, opening happened within the first five minutes of reflow following a 30 min ischemia in the isolated rat heart (Griffiths and Halestrap 1995). In fact, the time course of mPTP opening was found to match the rapid correction of pH that happened during reperfusion (Ovize 2006). CsA, a potent inhibitor of mPT, when administered intravenously just before reperfusion, has been reported to reduce myocardial injury in patients with acute ST-elevation myocardial infarction (where ECG shows the line of ST segment in unusually higher than base line) (Piot 2007). This protective effect was further evaluated in randomized controlled clinical trials on patients undergoing perioperative myocardial surgery (D. Hausenloy et al. 2014) which revealed that in higher-risk patients with longer cardiopulmonary bypass times, there was a significant reduction in PMI (posterior myocardial infarction) with CsA therapy that reduced postoperative cTnI levels rise when compared with placebo group (D. Hausenloy et al. 2014). However CsA, in addition to cyclophilin D, interferes with several molecular targets including HSP70, HSP90, ERK, p38MAPK, and Akt thereby evoking several off target effects (C. W. Yang et al. 2003; Rezzani

et al. 2003). This necessitates the development of novel inhibitors to modulate MPT during cardiac reperfusion.

1.13 mPT and pancreatic beta cell:

Targeting mPT promotes pancreatic beta cell survival and prevents diabetes during Pancreatic Duodenal homeobox gene-1 (Pdx-1) deficiency (Fujimoto et al. 2010). Mutations in Pdx-1gene have been reported to cause heritable diabetes in humans and mice (Stoffers, Thomas, and Habener 1997; Kulkarni et al. 2004). RNAi mediated ablation of Pdx-1 in Min-6 cells has been shown to dissipate mitochondrial membrane potential and promote cell death which is inhibited by cyclosporin A (Fujimoto et al. 2010). Mitochondrial abnormalities in Pdx-1 haplo-insufficient pancreatic beta cells were found to be ameliorated by genetic ablation of PPIF(Fujimoto et al. 2010). Most importantly, Pdx1+/− mice maintained on a high-fat diet develop increased fasting blood glucose and impaired glucose clearance due to β-cell insufficiency and defective insulin secretion which is not found in Pdx1+/− PPIF −/− mice which defines the link between mPT and Pdx-1. However the mechanism of the regulation of mPT through PDX-1 is hitherto undiscovered.

1.14 Modulation of mPT pore complex by inhibition of Cyp D:

There are both endogenous factors and exogenous chemical agents that can modulate mPT pore opening and closing, but in a clinical scenario it becomes apparent that the later attains more importance.

Table 1.2: Modulators of Cyclophilin D and MPT pore

Name of compound	**Description of activity**	**Preclinical/Clinical status**	**References**
Cyclosporin A (CsA)	Binds to family of proteins called cyclophilins, clinical use for graft rejection prevention, Reperfusion injury in acute Myocardial Infarction(MI)	Phase III for MI www.ClinicalTrials.gov ID : NCT01650662	(Andrew P Halestrap and Richardson 2015) (Nathan Mewton et al. 2010; Gallay 2009)
Quinoxaline	Inhibits CypD enzyme activity and MPT	Preclinical	(Guo et al. 2005)
NVP018 (Sangamide based)	Amide derivatives of Sanglifehrin A, (Allosteric modulator of Cyp D)	Preclinical	(Gallay 2009)
NIM811	A non-immunosuppressive	Phase II for Chronic Hepatitis C	(Ma et al. 2006; Gallay 2009)

	analog of CsA, inhibits Hepatitis C Virus replication	www.ClinicalTrials.gov ID: NCT00983060	
Alisporivir (DEB025)	A non-immunosuppressive analog of CsA, inhibits Hepatitis C Virus replication	Several Phase III studies for chronic hepatitis, www.ClinicalTrials.gov ID: NCT01446250, NCT01500772	(Landrieu et al. 2010; Gallay 2009)
Sanglifehrin A	Novel immune-suppressive cyclophilin inhibitor.	In pipeline (Rat heart reperfusion injury model)	(Samantha J. Clarke, McStay, and Halestrap 2002; Gallay 2009)
Antamanide	Cyclic decapeptide inhibitor of cyclophilin activity	Cellular model and Biochemical tests	(Azzolin et al. 2011)
GNX-5086 or mPTPi	Reduces cardiac reperfusion injury induced cell death in rabbit	Phase I	From company website (http://www.congenia.it/rd-preclinical-programs.html)

CsA was approved by US FDA in 1983 for its immunosuppressive properties in the treatment of graft rejection (Borel and Kis 1991), and is in use till date. It inhibits the activity of a family of proteins called cyclophilins which has Prolyl *cis-trans* isomerase activity and is required for

protein folding. CsA binds to Cyclophilin A and forms a complex which further binds to calcium dependent calmodulin phosphatase-calcineurin and inhibits its activity (Jun Liu et al. 1991). The calcineurin inhibition by CsA-CyPA complex, stops translocation of NFAT (Nuclear factor of activated T-cells) which leads to the blockage of expression of cytokine genes like IL2, TNF-α, GM-CSF and IFN-γ (Walsh, Zydowsky, and McKeon 1992). However, the specific inhibitor of the calcineurin, tacrolimus did not show any effect on infract size in reperfusion injury models of rat hearts, attributing the fact that cardioprotective effect of CsA was solely due to mPT inhibition (D. J. Hausenloy et al. 2002). Hence the non-immunosuppressive variants of CsA which do not show inhibition of calcineurin pathway were in great demand which paved the way for discovery of NIM811 and DEB025. Both of them have shown promising cardio-protective effect, but were having some undesirable property similar to CsA when it comes to mass, bioavailability and lack of specificity (D. J. Hausenloy, Boston-Griffiths, and Yellon 2012). Sanglifehrin A (SfA) which is a cyclic peptide with high molecular mass and showed cyclophilin D inhibition also shows protection of cardiac cells from reperfusion induced death. The SfA inhibits mitochondrial swelling by Ca^{2+} in a sigmoidal pattern with not much activity at low concentration, while CsA was giving a progressive curve of activity. GST tagged Cyp D affinity chromatography and subsequent immunoblot analysis for detection of ANT, revealed pattern of interaction between Cyp D and ANT in the presence of CsA and SfA; binding of SfA to Cyp D enhanced ANT-Cyp D interaction while CsA inhibited the interaction, suggesting that CsA binds to the same site where ANT binds and SfA bind to a distinct site (S J Clarke, McStay, and Halestrap 2002). SfA like CsA also showed mPT inhibition and cell protection in reperfusion injury of rat hearts induced for ischemia (when pretreated before perfusion). The table 2.5.1 lists some known Cyclophilin inhibitors of which many are derivatives of CsA. Among them

NIM811 and DEB025 have been studied for their activity in blocking viral replication. The immune-suppressive effect of CsA is much sought for treatment of graft rejection during organ transplantation. CsA, among various compounds tested also showed suppressive effect on Hepatitis C Virus (HCV) replication both at RNA and protein levels in cultured hepatocytes infected with the virus (Watashi et al. 2003) which indicates at its pleiotropic role in disease pathophysiology.

1.15 Limitations of Cyclosporin A:

The protective effect of CsA has been shown to be due to its binding to Cyp D, but it has been shown to interact with other cyclophilins as well. Firstly CsA-CypA complex inhibits calcineurin and brings about its immunosuppressive activity, which has adverse outcomes for patients undergoing cardiac surgery. The complex structure of CsA does not have much room for structural modifications, yet, analogs NIM811 and DEB025 were found to have same activity of CsA without having interaction with calcineurin. SfA, an mPT inhibitor and a promising candidate for treatment of ischemia induced cell death shows immunosuppressive activity as well inspite of having different macrocyclic architecture. In a nut shell, the high molecular mass and low bioavailability as well as non-specificity are the major concerns for the use of CsA and analogs in the treatment of ischemia reperfusion injury. The other drawbacks of CsA is the reduction of efficacy in presence of co-morbid conditions like type II diabetes, and hypertension with left ventricular hypertrophy (Whittington et al. 2012; Dongworth et al. 2014). Rat hearts isolated from Zucker Obese (ZO) rats, which are normoglycemic but pre-diabetic, were resistant to CsA- mediated cardio-protective effect in case of reperfusion during myocardial infarction (Huhn et al. 2010). CsA has been tested both at preconditioning stage and post conditioning stage in streptozotocin induced diabetic (STZ) rats and one of studies have identified that CsA

could reduce ischemia induced death in hearts of STZ treated diabetic rats when given at post conditioning stage (Najafi et al. 2014). The underlying mechanism for variation in mPT in each diabetic model is yet unclear, however CsA based cardio-protective treatment still stands as a viable option till specific inhibitors of mPT are discovered.

1.16 References:

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Chapter 2: Prevention of Depolarization of Mitochondrial Membrane Potential & Cytochrome C Release by Novel Small Molecules

2.1 Abstract:

The inhibition of mitochondrial permeability transition (mPT) by CsA has been known to rescue cells from reperfusion injury during ischemia reperfusion sequence. The underlying mechanism behind this rescue has been attributed to enzymatic inhibition of cyclophilin D (CypD), which regulates the opening and closure of mPT pore complex. The existing CypD inhibitors have nonspecific effect and low bioavailability. In the first part of my work, I developed a screening platform to analyze small molecules that can rescue cultured pancreatic beta cells from mPT-mediated cell death. We selected a chemical library comprising of Benzofuran based macrocycles; the selection being based on the previous observation of the inhibitory activity of mPT by Benzofuran based small molecule Amiodarone (2-butyl-3-benzofuranyl-4-[2-(diethyl amino)-ethoxy]-3, 5-diiodophenyl-ketone hydrochloride). The macrocycle architecture of the compounds in the chemical library with diverse functionality and stereochemical complexity in a conformational pre-organized ring structure were conjectured to modulate protein- protein interactions required for the opening and closure of the mPTP. The compounds were screened against thapsigargin induced loss of mitochondrial membrane potential and cytochrome c release which are the hall marks of mPT. The TMRE based flowcytometry assay identified the compound **2.4c** to be having protective effect against thapsigargin induced loss of mitochondrial membrane potential. Further, the small molecule 2.4c was found to inhibit cytochrome c release and prevent death of cultured pancreatic beta cells.

2.2 Introduction:

Mitochondrion is the site of cell fate determination; its well-being is an indicator of cellular homeostasis (Jang et al. 2015; Tower 2015). They have two lipid bilayer membranes of which outer membrane is permeable to the solutes of 5kDa size while the inner membrane is impermeable. However, in response to elevated intracellular calcium levels, the inner membrane undergoes a transition which opens up a channel to allow molecules of less than 1500 Dalton to cross the barrier without any charge specificity. The above phenomenon, termed as mitochondrial permeability transition (mPT), dissipates the proton gradient, depolarizes mitochondrial membrane potential (mmp) and on prolonged opening of the pore, releases the apoptogenic materials which causes cell death.

Ca^{2+} levels have been reported to be increased during cellular apoptosis/ necrosis (Pinton et al. 2008) which opens up mPT pore and aids the release of cytochrome c (cyt c) from mitochondria. The agents that like CsA that blocks mPT were known to arrest cell death in several cellular models by protecting the mitochondrial membrane potential and inhibiting cyt c release (Waring and Beaver 1996). Apoptosis is a programmed cell death pathway which requires energy, while necrosis is spontaneous without energy requirement. However, both apoptosis and necrosis have a common intermediate cyt c whose release from mitochondria serves as a marker of cell death (Li et al. 1999; Jemmerson, LaPlante, and Treeful 2002). Thus, the measurement of mmp and cyt c release are the important parameters to study the pathological opening of mPTP (Zhang and Armstrong 2007; Zhang et al. 2008) For the measurement of mmp, the cationic rhodamine based dyes were introduced in 1980's. The dyes, on binding to intact mitochondria showed increased fluorescence based on the mitochondrial membrane potential which could be estimated by microfluorimeter (Ehrenberg et al. 1988). The tetra methyl

rhodamine esters (TMRM and TMRE) displayed a readily reversible membrane potential-dependent staining of mitochondria and thus were used for studies like fluorescence activated cell sorting and digital fluorescence microscopy. The dyes followed nernstian distribution (Farkas et al. 1989) and showed distinct pattern of staining cytoplasmic and mitochondrial fluorescence allowing for the quantification of mmp-based studies (Scaduto and Grotyohann 1999; Perry et al. 2011; Lemasters and Ramshesh 2007).

For our study we have induced mPT by thapsigargin, a plant alkaloid from plant *Thapsia gargnica*. Thapsigargin inhibits smooth endoplasmic reticulum calcium ATPase (SERCAs) (Hakii et al. 1986) which transiently elevates Ca^{2+} levels of cytoplasm, however, at micromolar concentrations it has been found to induce mPT which is CsA dependent (Korge and Weiss 1999).

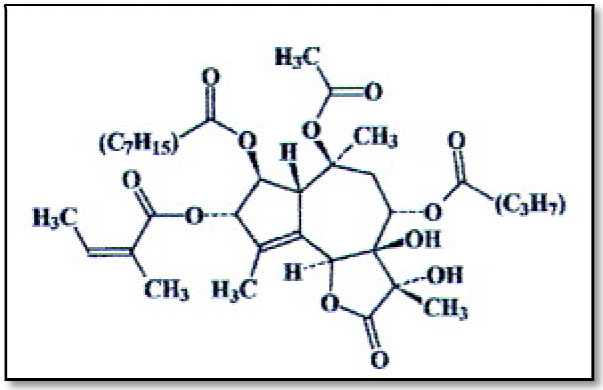


Figure 2.1: Structure of Thapsigargin (Hakii et al. 1986; Treiman, Caspersen, and Christensen 1998).

Thapsigargin has been speculated to act directly on mitochondria to induce mPT (Korge and Weiss 1999) and has been used by several authors to induce the opening of mPTP in different cell systems to study necrotic cell death (Zhang and Armstrong 2007; Quintanilla et al. 2013). In

our study, we used Brin-BD11 pancreatic beta cells which were first developed in Prof. Peter Flatt's laboratory by electro-fusing RINm5F and primary rat pancreatic beta cells (McClenaghan et al. 1996). The chemical tool box that I used comprised of molecules based on Benzofuran core scaffold which was synthesized in Prof. Prabhat Arya's lab at Dr.Reddy's Institute of Life Sciences. Benzofuran has a benzene ring fused to furan ring and is also called as coumarone, whose derivatives are found in various natural products like Liphagal, a specific inhibitor of PI3kinase α, which is isolated from marine sponge *Akacoralliphaga. sp.* (Marion et al. 2006), Morphine, an opioid analgesic drug isolated from *Papaver somniferum* (Yoshimatsu et al. 2005) and Moracins isolated from *Morus alba* having anti-inflammatory, antimicrobial, anticancer and antioxidant activity (Naik et al. 2015) .

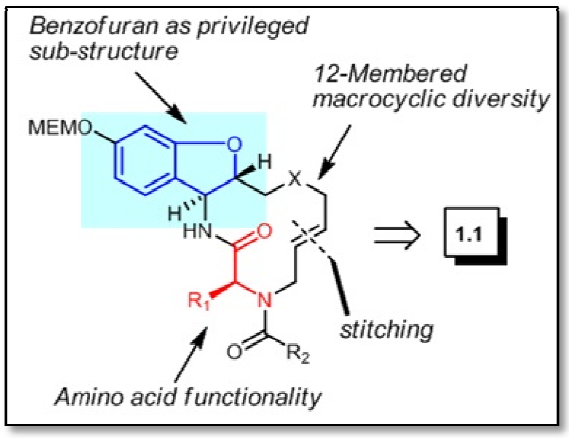


Figure 2.2: Proposed modular approach for synthesis of different benzofuran derived macrocyclic compounds. (Jimmidi, **Govardhan KS,** Arya P and Prasenjit Mitra. 2014)

Benzofuran derivatives, in fact, have a plethora of diverse pharmacological properties.2-Butyl, 3 – (4-hydroxybenzoyl)-benzofuran has anti-histamine property, and Benziodarone is a highly potent coronary dilator. Amiodarone has antianginal property and causes coronary dilation in addition to its activity of mPT inhibition at lower micromolar concentrations. The chemical tool

box I used are based on benzofuran scaffold, however they have additional large ring macrocyclic architecture which enabled them to function in a 3 dimensional space where dimers and multimers of proteins interact to regulate a complex phenomenon like permeability transition. The presence of amino acid moiety within the macrocycle architecture of the chemical library provided attractive features to introduce a diverse array of chiral side chains having a variety of polarities. Our data identified three molecules from the chemical tool box that could prevent the depolarization of mitochondrial membrane potential and inhibit cytochrome c release from thapsigargin treated cultured pancreatic beta cells.

2.3 Materials and Methods:

2.3.1 Cell culture

Brin-BD 11 pancreatic beta cells were purchased from ECACC and maintained at 37%, 5% CO_2 in RPMI 1680 medium, supplemented with 10% Fetal Bovine Serum (FBS), and standard antibiotics. All media and components were purchased from Invitrogen, Cell passages were maintained every 48hrs following ECACC guidelines.

2.3.2 Treatment of Brin-BD11 cells

Brin-BD11 pancreatic beta cells were seeded in 6 well plates a day before the treatment which was carried out at 60-70% confluence. Before treatment, cells were washed once with 1X PBS and the treatment was carried out in fresh complete media. Final DMSO concentration in treatment media is 0.1%. The cells were treated with thapsigargin (3µM) in presence and absence of small molecules at different concentrations and at different time points as per the requirement of the experiment.

2.3.3 TMRE based flowcytometry assay

Brin BD11 cells were plated in 6 well plates (80000 cells/well) and allowed to grow for 24h. After respective treatments, 50nM TMRE (prepared in 1X PBS 0.2% BSA) solution was added to the cells for 10mins. The stained cells were gently pipetted and the cell suspension was analyzed by BD canto II flowcytometer using weasel 2.0 software (Battye 2013).

CCCP (Carbonyl cyanide *m*-chlorophenyl hydrazone) at the concentration of 100 µM was used as a negative control to represent maximum mitochondrial membrane depolarization wherever necessary.

2.3.4 Immunofluorescence studies

Cells were grown on coverslips till they reach 70% area on coverslips and were treated with thapsigargin (3µM) with or without compound 2.4c (10µM). The cells were stained with MitoTracker red stain (100nM) and the images were acquired using confocal microscopy. Images were processed by Bio image XD software (Kankaanpää et al. 2012).

The sub cellular localization of cytochrome C was performed by dual-labeling confocal fluorescence imaging. The cells were treated with 100nM MitoTracker Red for 10min and fixed with 3% paraformaldehyde, washed with 1X PBS twice, permeabilized with 0.02% Triton X 100 and incubated in blocking buffer (5%BSA in PBS) for 10 min. After blocking, the cells were treated with primary rabbit polyclonal cytochrome C antibody (1:50) from Cell Signalling Technology, MA, USA for 2 h at room temperature, washed thrice with 1X PBS and incubated with Cy2 conjugated goat anti-rabbit antibody (1:500) for 1h. After washing with 1X PBS thrice, cells were treated with DAPI for 2 minutes and mounted with Anti-fade (Jackson Immuno-research) mounting medium on glass slides. Cellular images were acquired in Leica confocal

microscope and were acquired using Leica Application Suite (2.6.3) Advanced Fluorescent Lite software (Leica microsystems).

2.3.5 Cell viability assay by MTT dye

Cell viability was measured quantitatively by assessing mitochondrial dehydrogenase activity on MTT dye reduction. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by the action of dehydrogenase enzymes NADH and NADPH of metabolically active cells to produce purple formazan crystals that can be solubilized and quantified spectrophotometrically at 570nm. Cells were seeded at 10,000 cells/well in 96 well plates and were allowed to grow for 24hours before being treated with thapsigargin with or without compound 2.4c. After respective treatment, MTT dye (10mg/ml) was added at 10uL per 100uL media and incubated for 2hours which leads to the formation of formazan crystals based on the levels of cellular dehydrogenase activity. The formazan crystals were solubilized by 100ul Solution of 20% SDS prepared in 50% DMF solution and the absorbance was measured at 570nm using microplate reader (Perkin Elmer Victor 1420 multi label counter). The untreated cell absorbance was considered 100% viability. The Graphpad prism 5.0 was used for statistical calculations and plotting graphs.

2.4 Results:

2.4.1 Thapsigargin induced loss of mitochondrial membrane potential in Brin-BD11 cells

Depolarization of mitochondrial membrane potential is the primary indication of mPT (Brenner and Moulin 2012). In our present study we followed the method of Zhang and Armstrong (Zhang and Armstrong 2007) to assess the depolarization of mitochondrial membrane potential (mmp) by Thapsigargin in Brin-BD11 cells. As the **Figure 2.3** reveals, thapsigargin initially increased

TMRE signal at 6hrs time point, fold Ψ_m activation being 1.5 fold compared to untreated control cells. By 24 hrs time point the fold Ψ_m activation as measured by TMRE signal was down by 3 fold compared to untreated control. Eventually the TMRE signal becomes negligible by 36hrs, 42 and 48hrs indicating the loss of mmp on thapsigargin treatment. The data provided us a temporal assay platform to screen molecules to restore mmp which is one of the salient features of mitochondrial permeability transition (mPT).

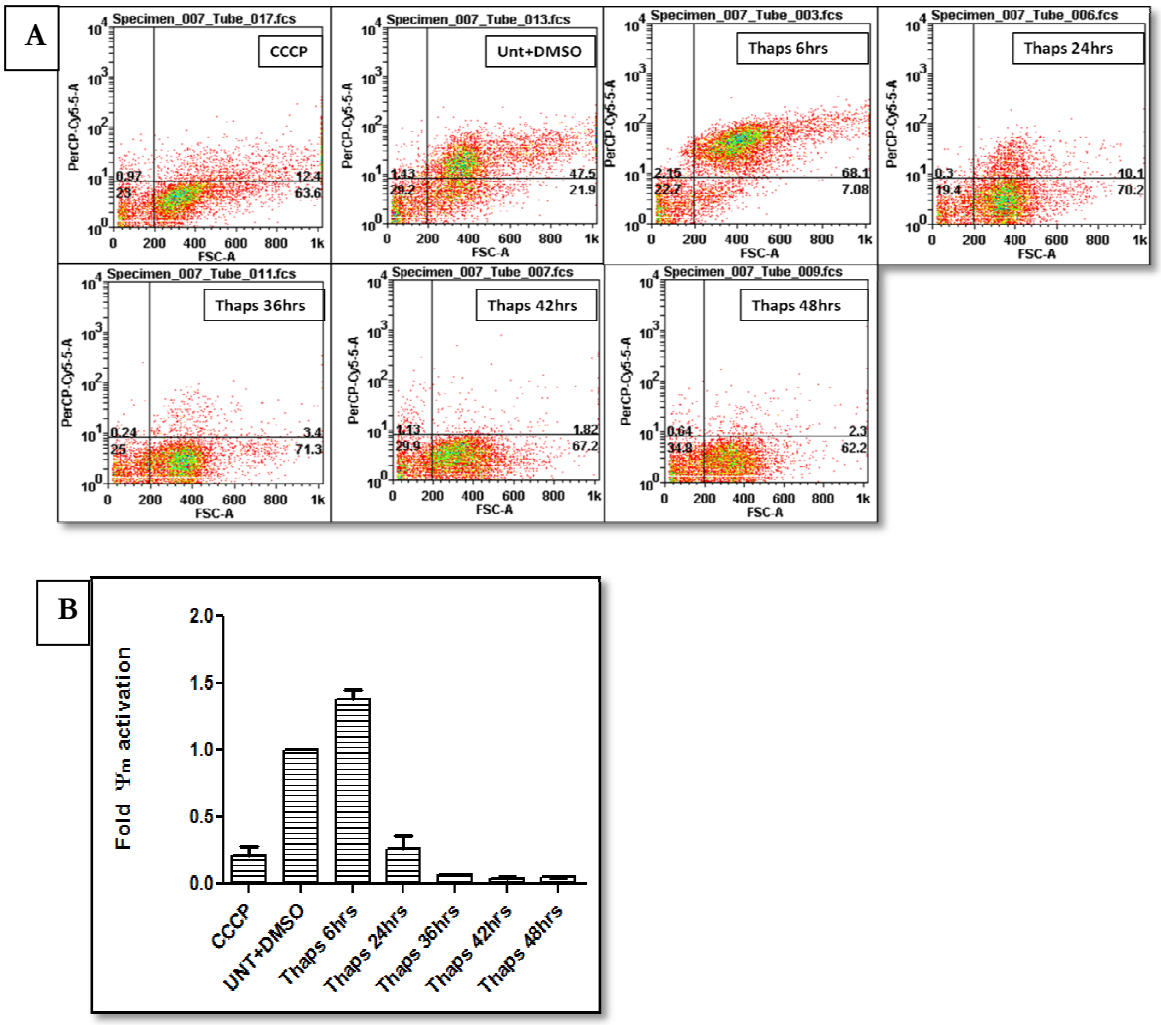
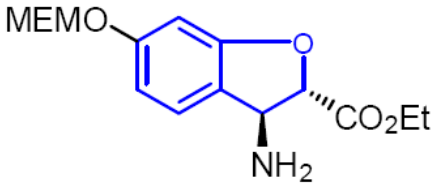
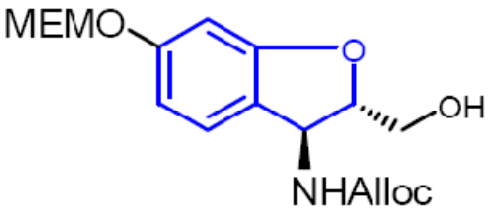


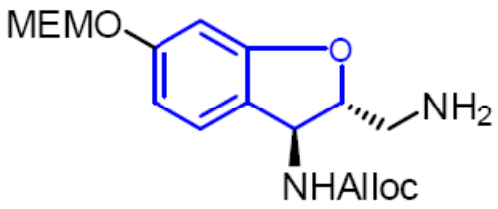
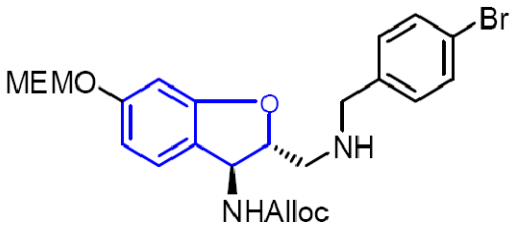
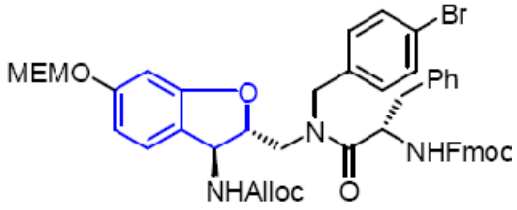
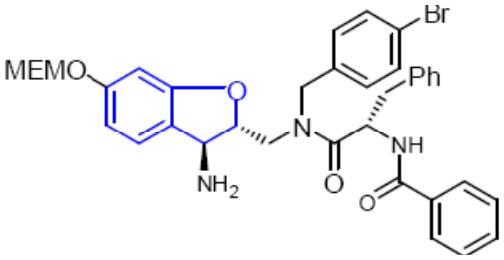
Figure 2.3: Thapsigargin induced MMP loss, **A**, Representative Flow-cytometric data plotted as 2D graph, X axis- FSC (Forward Scatter events) and Y axis- PerCP-CY5-5A region positive events (excitation 482nm and emission 690nm corresponds for red fluorescence region) data

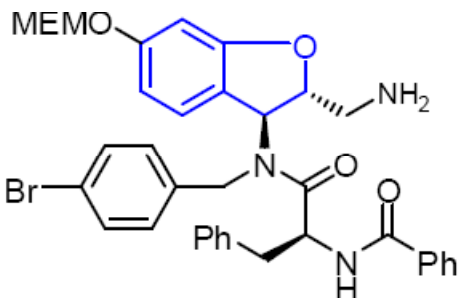
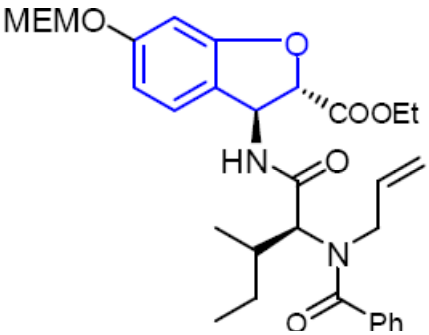
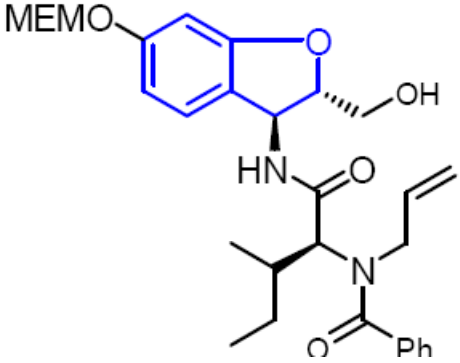
conveniently represented as quadrants by Weasel software. **B**, CCCP- Carbonyl cyanide m-chlorophenyl hydrazone (uncouples and depolarizes MMP), UNT- untreated, DMSO- Dimethyl sulfoxide, Thaps- Thapsigargin, hrs- hours. Fold Ψ_m as represented in bar graph, TMRE positive events in untreated plus DMSO is considered as 1fold. The data represents experiments done in triplicate.

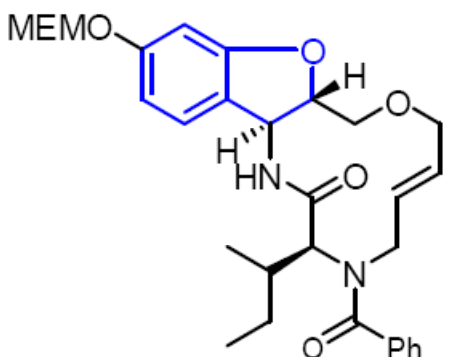
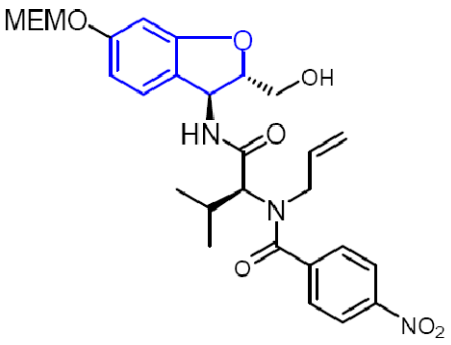
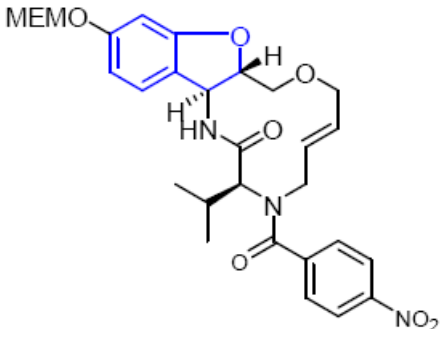
2.4.2 Screening of Benzofuran based small molecules

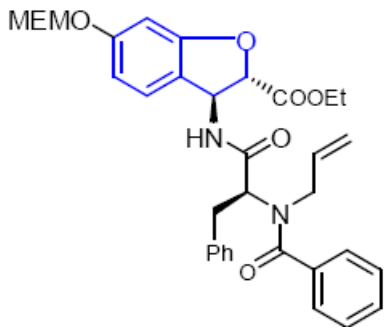
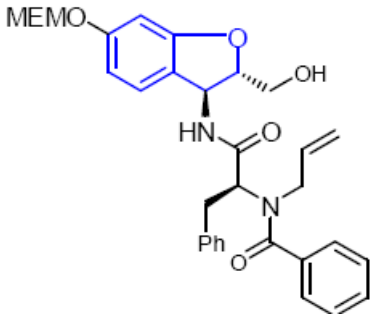
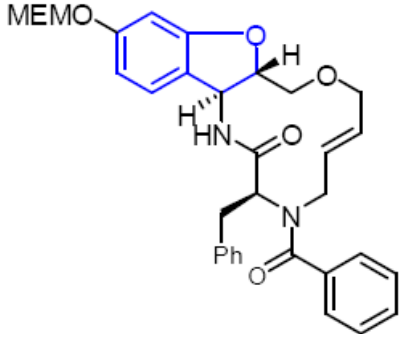
The chemical library that was screened comprised of 24 enantioenriched benzofuran based small molecules synthesized at Dr. Reddy's Institute of Life Sciences (contributor: Prof. Prabhat Arya). These molecules were screened for the prevention of Thapsigargin (3µM) induced depolarization of mitochondrial membrane potential. The compounds which are precipitated at 10µM concentration are excluded from the study. The following **Table 2.1** describes the results of the screening.

No.	Name	Structure	Mol. formula	Mol.wt	Fold [b]
1.	**A1**		**$C_{15}H_{21}NO_6$**	**311.33**	**0.23±0.04**
2.	**A3**		**$C_{17}H_{23}NO_7$**	**353.37**	**0.25±0.03**

3.	**A5**		**$C_{17}H_{24}N_2O_6$**	**352.38**	**0.05±0.03**
4.	**A6**		**$C_{24}H_{29}BrN_2O_6$**	**521.40**	**0.11±0.07**
5.	**A7**		**$C_{48}H_{48}BrN_3O_9$**	**890.81**	**0.28±0.02**
6.	**A8**		**$C_{36}H_{38}BrN_3O_6$**	**688.61**	**0.60±0.08**

7.	**A12**		**$C_{36}H_{38}BrN_3O_6$**	**688.61**	**0.54±0.07**
8.	**B1**		**$C_{31}H_{40}N_2O_8$**	**568.66**	**0.55±0.09**
9	**B2**		**$C_{29}H_{38}N_2O_7$**	**526.62**	**0.51±0.04**

10.	**B3**		**$C_{30}H_{38}N_2O_7$**	**538.63**	**0.62±0.02**
11.	**B4**		**$C_{28}H_{35}N_3O_9$**	**557.59**	**0.06±0.01**
12.	**B5**		**$C_{29}H_{35}N_3O_9$**	**569.60**	**1.60±0.05**

13.	**B6**		**$C_{34}H_{38}N_2O_8$**	**602.67**	**0.28±0.04**
14.	**B7**		**$C_{32}H_{36}N_2O_7$**	**560.64**	**0.09±0.03**
15.	**B8**		**$C_{33}H_{36}N_2O_7$**	**572.65**	**0.19±0.08**

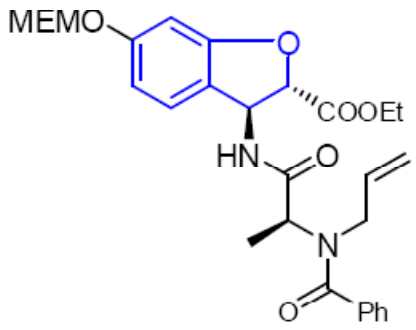
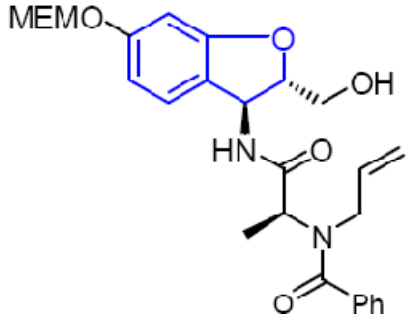
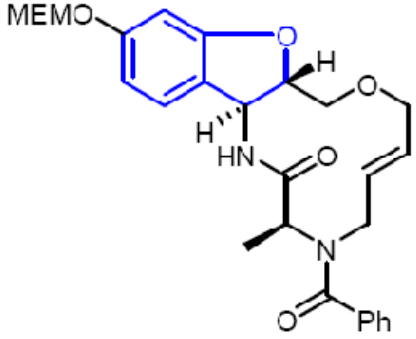
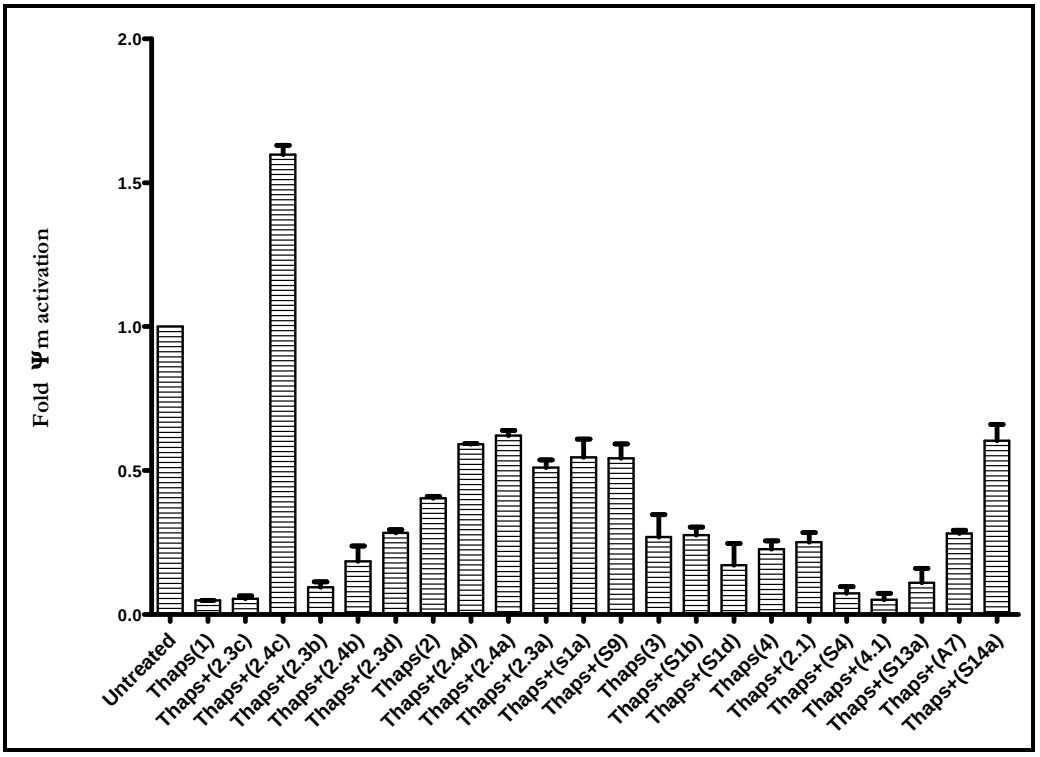
16.	**B9**		**$C_{28}H_{34}N_2O_8$**	**526.58**	**0.17±0.11**
17.	**B10**		**$C_{26}H_{32}N_2O_7$**	**484.54**	**0.28±0.02**
18.	**B11**		**$C_{27}H_{32}N_2O_7$**	**496.55**	**0.59±0.003**

Table 2.1: Screening of compounds for the rescue of thapsigargin induced depolarization of ΔΨm. Fold [b]: fold increase of Ψm over untreated mitochondria.

Figure 2.4A describes the graphical presentation of the screening results. As the **Table 2.1** and **Figure 2.4A** reveals, compound 2.4c (B5 in **Table 2.1**) which has a 12 member macrocyclic ring

with an N-(4-Nitrobenzoyl) Valine amino acid moiety fused to the benzofuran scaffold showed the highest activity for prevention of thapsigargin induced depolarization of mitochondrial membrane potential. To further validate the data we evaluated C104 and C108 which are the analogues of 2.4c. C104 has N – benzoyl valine unit without NO_2 attached to the benzene ring whereas in C108, the amino acid moiety is replaced by leucine **(Figure 2.5)**. Both C104 and C108 have comparable efficacy as that of 2.4C **(Figure 2.4A)**. However when valine amino acid was replaced with Phenylalanine (2.4b, B8 in **Table 2.1**) group there is a dramatic decrease in the activity emphasizing the importance of amino acid side chains in conferring the prevention from thapsigargin- induced depolarization of mitochondrial membrane potential.



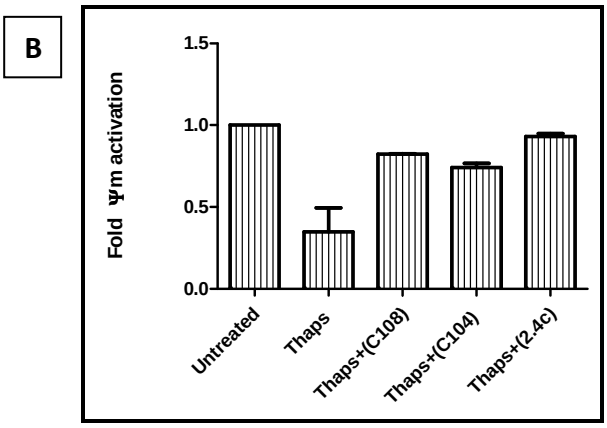


Figure 2.4: Graphical representation of Fold Ψ_m activation of a series of benzofuran based small molecules; Compound 2.4c rescues cells from thapsigargin induced MMP loss in the compound screening assay. Thaps- Thapsigargin, Fold Ψ_m activation, the TMRE positive events in case untreated was considered as 1 fold and the rest were calculated. **A**. Entire screening data of small molecules tested, **B**. The fold Ψ_m activation of compound C104 and C108 and 2.4c is shown as graphical chart (Jimmidi et al. 2014).

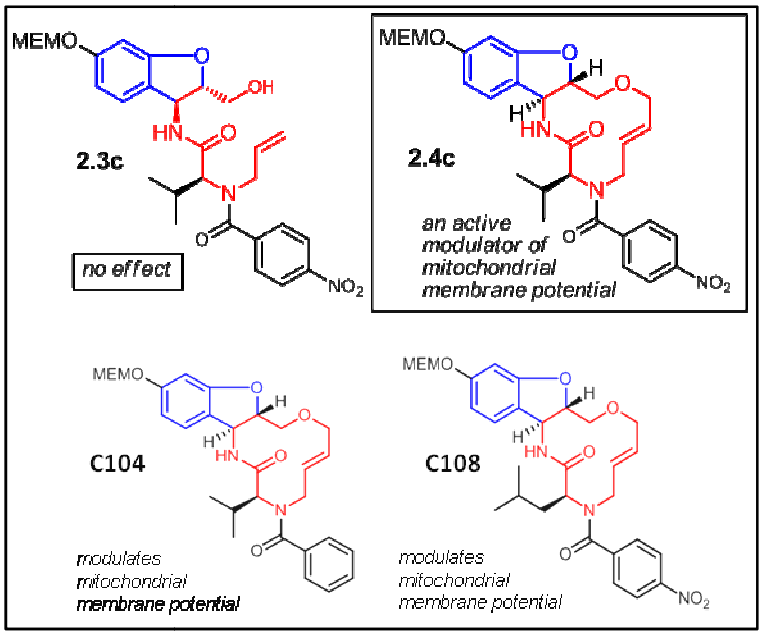
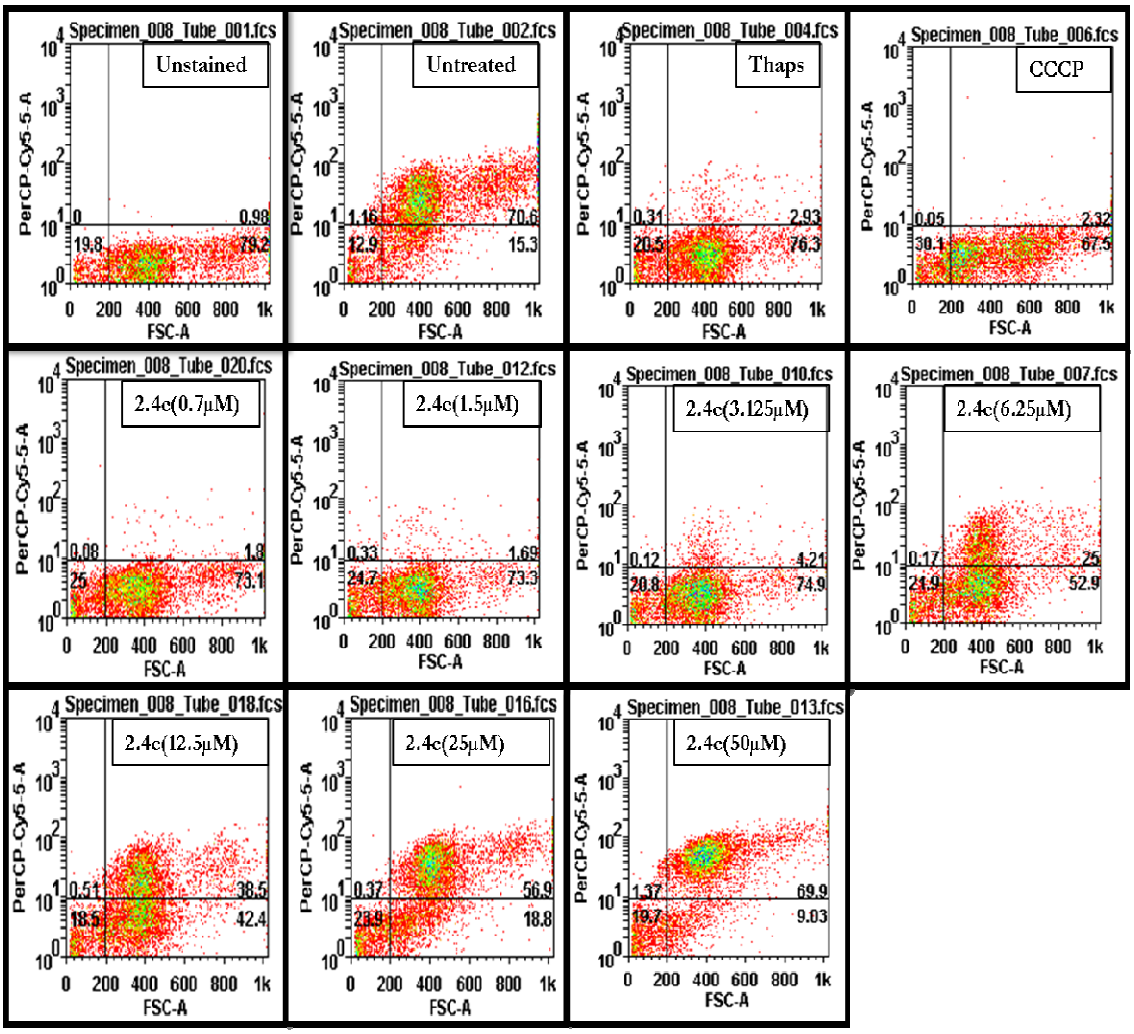


Figure 2.5: Structure of compounds 2.3c, 2.4c, C104 and C108 are shown as graphical images. – OMEM: methoxy ethoxy methyl group, red color region- central ring structure of which two variation are present closed ring and open ring., blue color region- benzofuran core, other groups on the right corner is a benzene ring with or without NO_2 (C104)., the amino acid moiety attached to red region.

2.4.3 Dose response curve of compound 2.4c

In the next step we determined the dose dependent response of 2.4c in preventing thapsigargin induced depolarization of mitochondrial membrane potential (fold Ψ_m activation). The fold Ψ_m in only thapsigargin treated cells is considered as 1 fold activity. The assay response values were taken as Y-axis and log Dose of compound used was taken as X-axis, which generated a typical sigmoidal curve as plotted in Graph Pad Prism software **(Figure 2.4B)**. The EC_{50} which is the half maximal effective concentration was determined to be 9.04μM.

A


Specimen_008_Tube_001.fcs
Unstained
Specimen_008_Tube_002.fcs
Untreated
Specimen_008_Tube_004.fcs
Thaps
Specimen_008_Tube_006.fcs
CCCP
Specimen_008_Tube_020.fcs
2.4c(0.7µM)
Specimen_008_Tube_012.fcs
2.4c(1.5µM)
Specimen_008_Tube_010.fcs
2.4c(3.125µM)
Specimen_008_Tube_007.fcs
2.4c(6.25µM)
Specimen_008_Tube_018.fcs
2.4c(12.5µM)
Specimen_008_Tube_016.fcs
2.4c(25µM)
Specimen_008_Tube_013.fcs
2.4c(50µM)
PerCP-Cy5-5-A
FSC-A

B

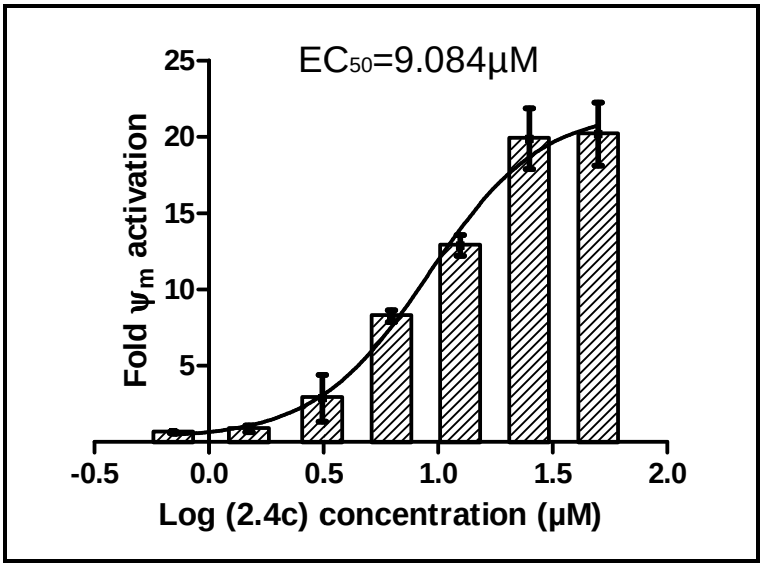

EC50=9.084µM
Fold ψm activation
Log (2.4c) concentration (µM)

Figure 2.6: Dose response curve of compound 2.4c, determined by the linear regression analysis in Graph pad prism version 5.0.**A**., Flow cytometer data depicted as graphical chart prepared in Weasel software, **B**., Dose response curve showed that the EC_{50} to be 9.084µM. (Govardhan KS and Prasenjit Mitra 2014).

2.4.4 Prevention of cytochrome c release

The release of cytochrome c from mitochondria is a salient feature of mitochondrial permeability transition. Thapsigargin treatment is known to mediate cytochrome c release and promotes cell death. In my present study I assessed whether compound 2.4c could prevent the cytochrome c release from pancreatic beta cell mitochondria. As the **Figure 2.7** reveals, in control cells there is a complete overlap of cytochrome c staining with Mito-Tracker Red indicating its presence in mitochondria. Treatment with Thapsigargin for 18h causes a marked loss of Mito-Tracker Red staining and a concomitant release of cytochrome c to the cytoplasm **(Figure 2.7, Second row)** which is totally prevented on treatment with compound 2.4c **(Figure 2.7, Third row)**.

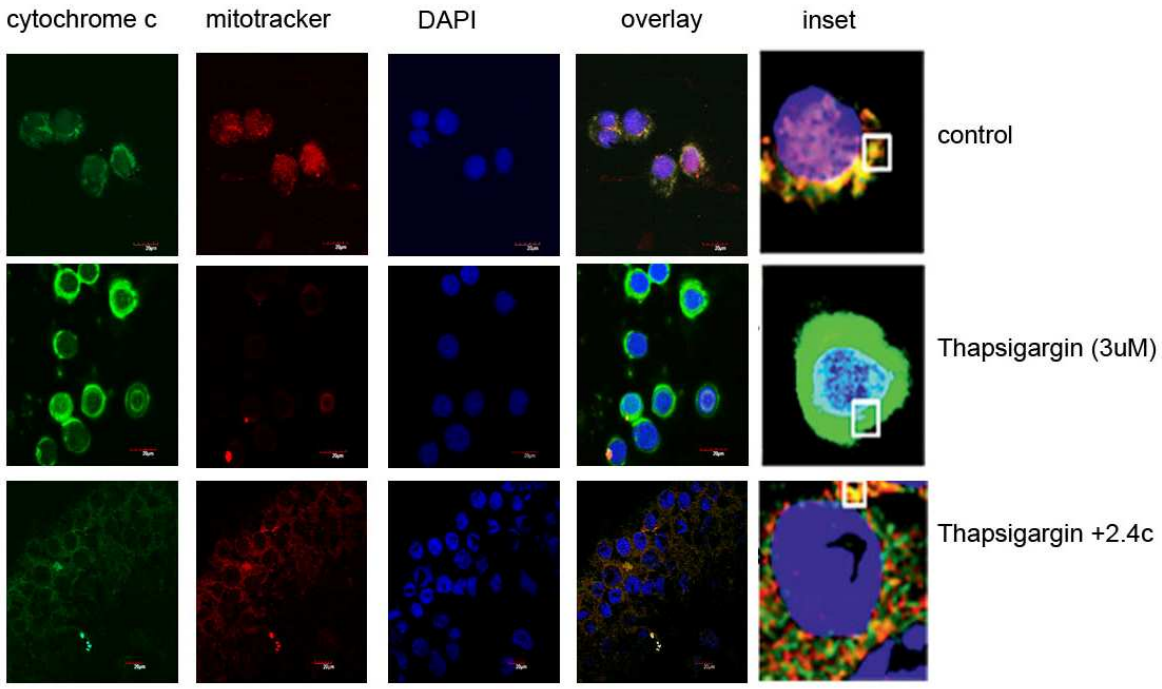


Figure 2.7: Compound 2.4c prevents Thapsigargin induced release of cytochrome c from pancreatic beta cell mitochondria. Mitotracker Red (red) stains the mitochondria and cytochrome C is immune stained with Cy2 anti -rabbit antibody (green) Nucleus is visualized with DAPI staining (blue) Yellow dots in overlay shows the presence of cytochrome c in mitochondria. (Govardhan KS and Prasenjit Mitra 2014)

2.4.5 Cell viability assay

The cells were treated with Thapsigargin (3µM), CCCP (10µM), Tunicamycin (10µM) and Staurosporine (10 µM) in presence and absence of compound 2.4c (10 µM). **Figure 2.8** explains the efficacy of the compound 2.4c in preventing the cell death as measured by MTT assay. As the data reveals, 2.4c prevents thapsigargin induced cell death but has no effect on antimycin (inhibitor of electron transport chain), staurosporine (ATP-competitive kinase inhibitor) or tunicamycin (N-linked glycosylation blocker) induced reduction of cell viability. The dose

dependency of amelioration of Thapsigargin induced cell death is shown in **Figure 2.8**. The experiment was carried out in serum free medium for 18h. As shown in the figure, Thapsigargin treatment at the concentration of 5µM causes 58.5±1.87% cell death which is reduced to 46.81±4.31% on treatment with 5µM 2.4c, 34.05 ±1.57 % on treatment with 10µM 2.4c and 30.92 ± 2.42 % on treatment with 25 µM 2.4c

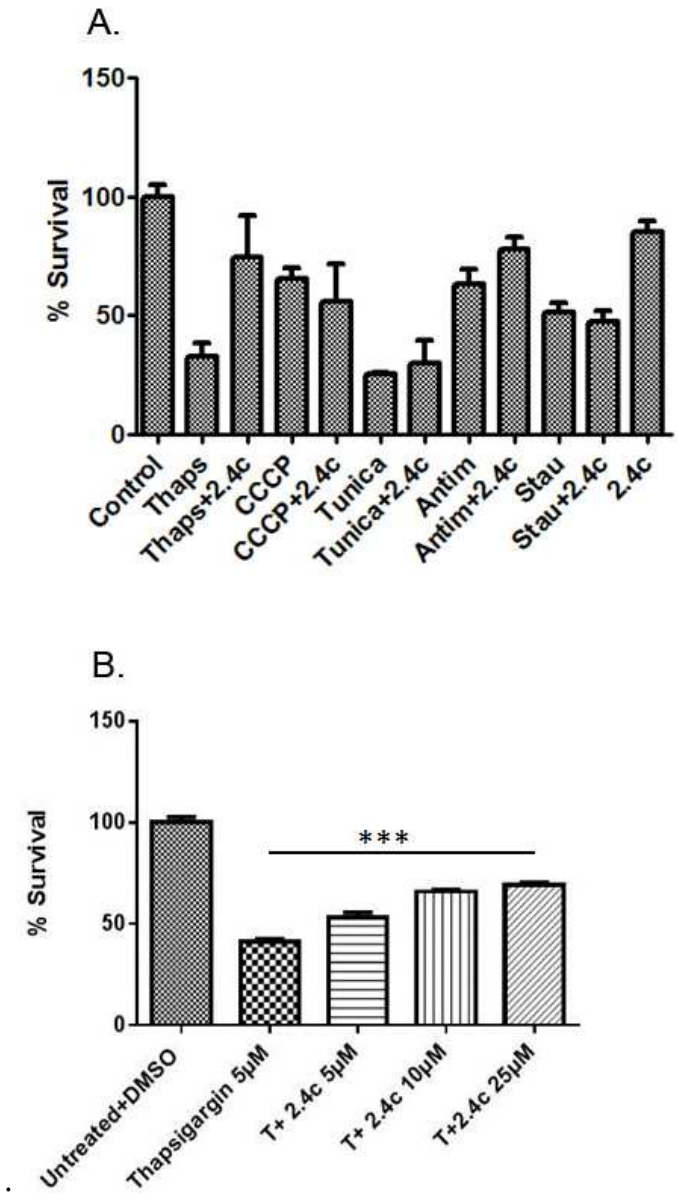


Figure 2.8: Cell viability assay as determined by MTT. A. % cell viability on treatment with different inhibitors in presence and absence of 2.4c Thaps: Thapsigargin; Anti: Antimycin; Tunica: Tunicamycin; Stau: Staurosporine B. Dose dependent effect of 2.4c in rescuing Thapsigargin induced cell death in Brin-BD11 cells (*** p value<0.0001).

2.5 Discussion:

There is a growing interest in evaluating small molecules having 3D-dimensional architectures to study complex protein- protein interactions. They could either have multiple rings or could also have the macrocyclic architectures. The exploration of the latter is gaining momentum since macrocycles can potentially make more extensive contact with the protein target (Villar et al. 2014). More importantly, macrocycles are conformationally constrained, although not rigid, which allows them to have sufficient flexibility to efficiently interact with the binding site of the proteins keeping the internal entropy penalty to a minimum which is associated with a change from unbound to a bound state of the ligand (Mallinson and Collins 2012). The data I presented in this chapter shows that while the small molecule (2.4c) with its macrocyclic architecture possess the efficacy in inhibiting the thapsigargin induced depolarization of Ψm and cytochrome c release , its acyclic precursor 2.3c do not show any activity emphasizing the role of macrocyclic architecture in regulating mPT. The small molecule 2.4c does not have any effect on Thapsigargin induced modulation of cytosolic calcium indicating that the compound does not have any direct effect on SERCA pump (data not shown).

The small molecules evaluated in this study have macrocyclic rings incorporated to benzofuran scaffold. Benzofuran based small molecule amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethyl

amino)-ethoxy]-3, 5-diiodophenyl-ketone hydrochloride), a class III anti angina drug, has been previously reported to prevent mPT at lower concentrations in cardiomyocytes as well as during ischemia and reperfusion in Langendorff-perfused rat hearts (Varbiro et al. 2003). However, the drug is metabolized in liver and its metabolite desethyl-amiodarone (the absence of the ethyl side chain from the amino group), unlike amiodarone could not prevent mPT (Hellebrand and Varbiro 2010). In addition, the use of amiodarone is severely limited due to its side effects including pulmonary fibrosis (Martin 2nd and Rosenow 3rd 1988), thyroid abnormalities liver, and pancreas fibrosis (Amico et al. 1984; Martin and Howard 1985). In MTT assay, amiodarone shows significant cytotoxicity in PANC-1 cells and cardiomyocytes (Varbiro et al. 2003); on the contrary, we did not notice any toxicity of 2.4c in cardiomyocytes and cultured pancreatic beta cells. Moreover, compound 2.4c prevents thapsigargin induced cell death; it has no effect on staurosporine, antimycin or tunicamycin induced attenuation of cell viability which indicates at its precise mechanism of action.

In summary, the work describes the first report of the involvement of macrocycle based small molecule 2.4c in regulating the thapsigargin induced depolarization of mitochondrial membrane potential and the release of cytochrome c which are the salient features of mPT. The interaction of compound 2.4c with components of mitochondrial permeability transition pore has been evaluated in the Chapter III & Chapter IV.

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Chapter 3: Molecular modeling of interaction of compound 2.4c with Cyclophilin D/PPIF

3.1 Abstract:

The computational modeling of protein with small molecules and their study based on known x-ray crystallographic data defines the characteristics of interaction, the binding pockets, and the active site and is also a widely used technique for in silico drug discovery analysis. The protein human PPIF (Cyclophilin D) is a mitochondrial cyclophilin of 207 amino acids in length, it has 3 helices, 12 parallel beta sheets and 5 turns. It binds to cyclosporin A (CsA) and the bound complex hinders mPTP opening. However CsA has some adverse physiological effects and as a consequence there is an unmet medical need for the discovery of small molecules which could hinder the mitochondrial permeability transition. The screening platform I developed (detailed in Chapter 2) identified the compound 2.4c as a “hit molecule” that inhibits thapsigargin-mediated depolarization of mitochondrial membrane potential and prevents cytochrome c release, both the criteria being the functional hall mark of mPTP opening. Genetic scrutiny as well as randomized clinical trials identified Cyclophilin D as the regulator of mPTP which encouraged us to carry out in silico analysis of the interaction between Cyp D and compound 2.4c. Based on known X-ray crystallographic structures of Cyp D/PPIF bound to some ligands, we explored how compound 2.4c might interact with the protein. The Swiss dock based preliminary docking analysis showed that compound 2.4c binds to the hydrophobic region near the active site, while the non-active analog compound 2.3c showing low full fitness values did not show binding to the active site of the protein. To further validate our study, we did molecular docking in Schrödinger Maestro platform which revealed that Cyclophilin D/PPIF unlike the compound 2.3c interacts very well with compound 2.4c and forms several hydrogen bonds as well as hydrophobic interactions with the compound. The compound 2.4c is conformationally constrained and hence have minimized internal entropy while being converted from unbound to bound state. On the

other hand, the compound 2.3c is flexible with un-fused ring and hence prone to excessive entropic penalty during its interaction with Cyclophilin D/PPIF**.** Our in silico analysis described in this chapter provides an insight into probable molecular interaction between compound 2.4c and Cyclophilin D/ PPIF**.**

3.2 Introduction:

Cyclophilin family of proteins (PPIases) were discovered and named after their property of binding to Cyclosporin A (CsA). The human genome codes for 17 cyclophilins and they share a common domain called cyclophilin domain which catalyzes the *cis-trans* isomerization of proline (Davis et al. 2010)). Some of the members of cyclophilins have clinical relevance. Cyclophilin A is known to form a ternary complex with CsA and inhibit calcineurin, a calcium-calmodulin-activated serine/threonine-specific protein phosphatase. As a consequence, NFAT mediated IL-2 dependent activation of immune response against the implant is hindered (Belshaw 1996). Cyclophilin A is also involved in Hepatitis C (Jyothi KR 2015) and HIV-1 infectivity (Delaco A 2014). Cyclophilin D on the other hand is present in mitochondria and is known to regulate mitochondrial permeability transition and consequent cell death which has implications during myocardial reperfusion injury (Kwong and Molkentin 2015) as well as in neurodegeneration (Thomas et al. 2012). Among diverse member of PPIases family, cyclophilin A (abundant, cytoplasmic) has been studied extensively to determine its active site. The PPIases catalytic site comprised of the following amino acids: Arg 55, Phe 60, Met 61, Gln 63, Ala 101, Phe 113, Trp 121, Leu 122 and His 126 numbered in accordance to their presence in CyPA (Howard et al. 2003; Davis et al. 2010) and they are conserved among CyP A, CyP B and CyP D. In depth analysis of CyPA active site reveals the presence of two deep hydrophobic pockets named as S1 and S2 pockets; S1 is the site where proline binds; S2 site, according to molecular

modeling studies, confers specificity by binding to other amino acids of the peptide.S2 pocket is deep and relatively nonspecific and thus can accommodate long short, polar and hydrophobic side chains. However, the site is guided by a set of gatekeeper residues which restrict access to the pocket. In Cyp A, the gatekeeper residues comprised of Thr73, Glu81, Lys 82, Ala107, Thr 107, Ser 110 and Glu111 which also represent an attractive site to design isoform specificity.(Davis et al. 2010).

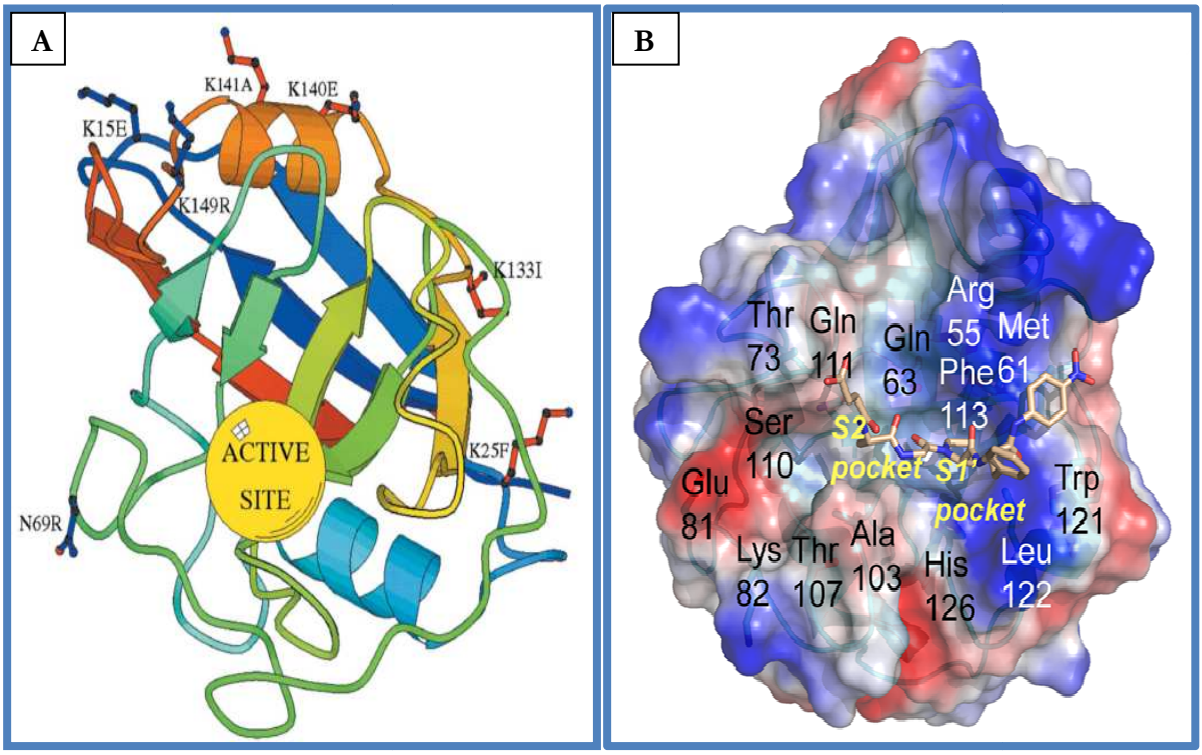


Figure 3.1: Protein PPIF and PPIases modeled molecular structure. **A**, Illustrative diagram of CyP D/ PPIF with depiction of active site and other mutations screened for aiding crystallization (Schlatter et al. 2005). **B**, Computational model of cyclophilin a bound to synthetic peptide showing Pocket S1 and S2 which define the active site of Cyclophilin group of proteins(Davis et al. 2010). Abbreviations: standard amino acid single letter and triple letter code were used.

The several mPTP pore complex model and genetic knockdown studies put Cyp D as undisputed protein part of the complex capable of regulating mPT phenomenon. The data I presented in

Chapter 2 clearly reveals that the compound 2.4c prevents the depolarization of mitochondrial membrane potential and the release of cytochrome c which are the salient features of mPTP pore opening. The observation encouraged us to speculate that the regulation of mPTP by 2.4c might happen due to its interactions with protein Cyp D/PPIF. To study the interaction in silico we performed a preliminary docking study using Swiss dock online docking server and conducted a detailed docking and simulation study by Maestro (done in collaboration with Mr. Girdhar Deora and Dr. Ben Ross, University of Queensland, Australia). Currently, there are 28 structures coordinates of human PPIF which are available in Protein Data Bank(PDB) of varying resolution from 0.85 to 2.01 Å. Dr. Hennig and coworkers authored Cyp D/PPIF structures at resolution of 1.7 Å which are unbound to ligand and are therefore unfit for molecular docking studies (Schlatter et al. 2005). However the most important outcome of the study is the identification of K133I mutant (Lysine at 133 positions is replaced by Isoleucine) which have similar biochemical or enzymatic property (Schlatter et al. 2005) to that of wild type Cyp D/PPIF but could be crystallized with relative ease. The Kajitani K group in 2008 authored the PPIF bound to CsA protein structure where they used K133I mutant form of PPIF. The reported structure has a resolution of 0.96 Å, revealing additional details that contributed immensely in the molecular understanding of Cyp D-CsA interaction. The structure revealed that half of the CsA residues were buried in hydrophobic pocket with nitrogen and oxygen atoms of CsA being engaged in hydrogen bond formation(Kajitani et al. 2008). Dr. Guichou and his coworkers deposited PPIF structures with different ligands bound to the protein; which were basically built upon scaffolds of quniolines, sulfamoyl benzoic acids, carbaldehydes, indazolamines, aminophenyls, oxalamines and diaryl ureas. In our in silico analysis, we selected PDB ID: 3R49, protein PPIF bound to a ligand quinolin-8-amine for the preliminary study using Swiss molecular docking

server; for detailed molecular docking study we have chosen the PDB ID: 2Z6W(Kajitani et.al, 2008), where PPIF is bound to CsA.

3.2.1 Cyclosporin A and its interaction with cyclophilins

Cyclosporin A (CyPA) is a cyclic undecapeptide isolated first from fungi *Tolypocladium inflatum*, by Dr. Hans Peter Frey in 1969 which is of non-ribosomal in origin (Svarstad, Bugge, and Dhillion 2000). It is widely used to prevent or treat graft versus host rejection during organ transplantation and also used as an immunosuppressant for treatment of rheumatoid arthritis and psoriasis. The CsA structural studies by NMR and X ray crystallography revealed that it might have 4 intramolecular hydrogen bond (three shown in figure), and one *cis* peptide bond between methyl Leucine 9 and 10 (Thériault et al. 1993).

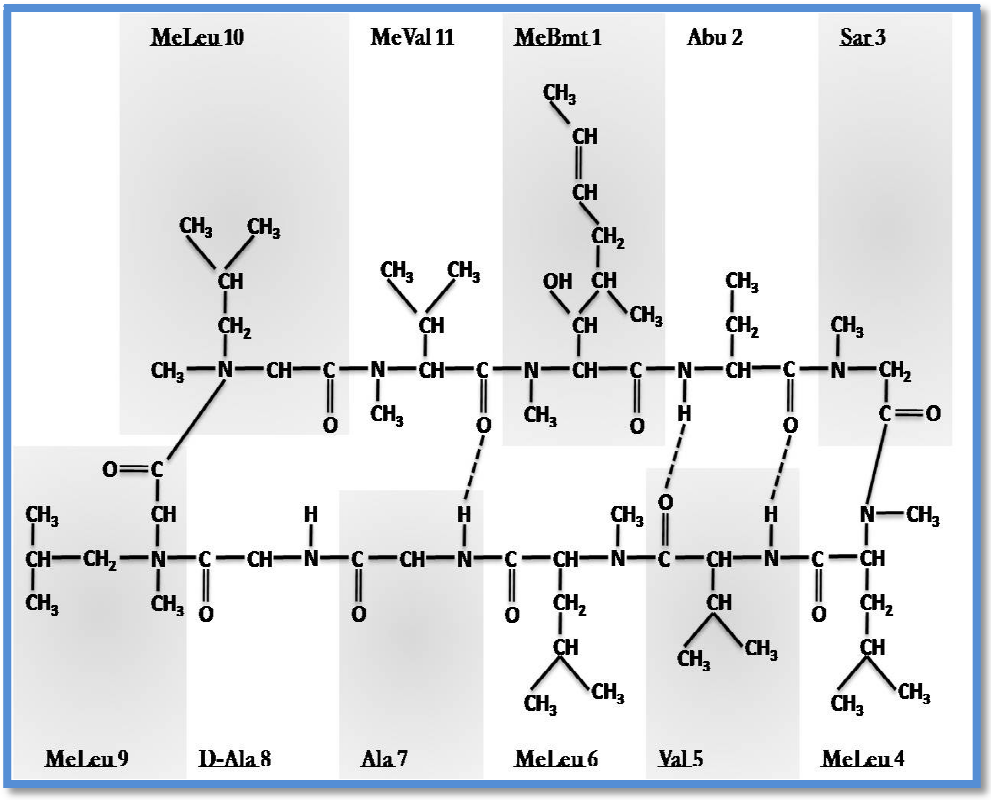


Figure 3.2: The chemical structure of cyclosporin a as described by Dreyfuss(Dreyfuss et al. 1976), MeBmt- (4R)-4-[(E)-2-butenyl]-4, *N*-dimethyl-L-threonine, Abu- L-α-aminobutyric acid, Sar- Sarcosine, MeLeu- *N*-methylleucine, MeVal- *N*-methylvaline.

CsA can be divided into two domains based on its interaction with cyclophilins, 'effector domain' composed of residues 1, 2, 3, 9, 10 and 11 are the actual cyclophilin binding region of CsA. The other residues 4, 5, 6, 7, and 8 are part which are exposed to solvent phase and are deemed to interact with calcineurin (shown in **Figure 3.2**). Supporting this notion we found a report from Liu et.al(Liu et.al 1991) that Tryptophan 121 mutation to Phenyl alanine led to low affinity binding of CsA to CyPA, and Tryptophan 121 is known to interact with MeLeu9 of CsA (J Liu, Chen, and Walsh 1991). Another notable modification in CsA, where MeVal 11 was replaced with MeAla 11, showed a decrease in cyclophilin binding but enhancement in immunosuppressive activity. Moreover, binding studies report that MeVal 11 interacts and binds to the hydrophobic pocket and any change of this moiety to lower hydrophobic amino acids yields to a lower order of interaction which is reflected in its ability to bind to the cyclophilins (R M Wenger and Payne 1989; Roland M. Wenger, Payne, and Schreier 1986).

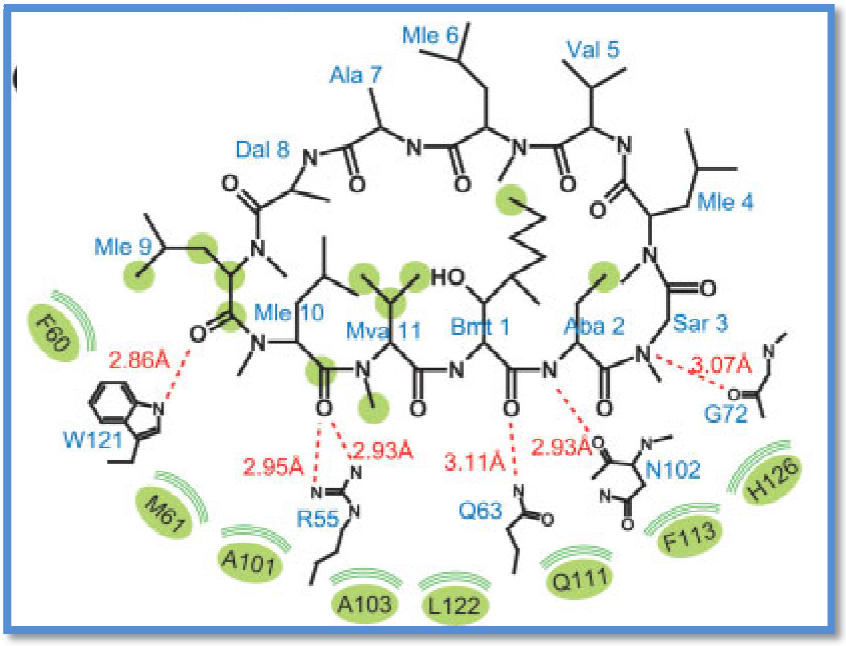


Figure 3.3: Illustrative diagram of CsA interaction with Cyp D from Kajitani group, the hydrophobic interaction between CsA atoms and Cyp D are marked as green circles (without any letters) and green ellipsoids (with letters) respectively, hydrogen bond interactions are represented as red dash lanes (Kajitani et al. 2008).

CsA residue number	**Cyclophilin A amino acid**	**Type of interaction**
MeBmt (1)	Arg 55, Gln 63, Asn 102	Hydrogen bond (Gln 63)
Abu (2)	Gly 72, Thr 73, Ala 101, Asn 102, Ala 103, Thr 107 and Gln 111	Hydrogen bond (Asn 102, Gln111)
Sar(3)	Gly72, Thr 73	Hydrogen bond (Gly 72, Thr 73)
MeLeu(9)	Phe 60, Trp 121	vander Waals interaction (Phe 60), Hydrogen bond (Trp 121)
MeLeu(10)	Arg 55, Phe 60, Lys 125	Hydrogen bond (Arg 55), van der Waals interaction (Phe 60)
MeLeu(11)	Arg 55, Phe 60, Met 61, Gln 63, Ala	vander Waals interaction

	101, Asn 102, Phe 113, Leu 122, His 126	(Met 61, Phe 113, His 126)

Table 3.1: Interacting partners of residues from CsA and amino acids from CyPA (Kajitani et al. 2008).

3.2.2 Structural aspects of lead compound 2.4c and 2.3c

Compound 2.4c was built on benzofuran scaffold; it incorporates some interesting structural features, a fused 12 member ring and a critically placed amino acid moiety. The 2.4c related compounds with changes in amino acid region and closed and open ring, showed marked variations in their biological activity.

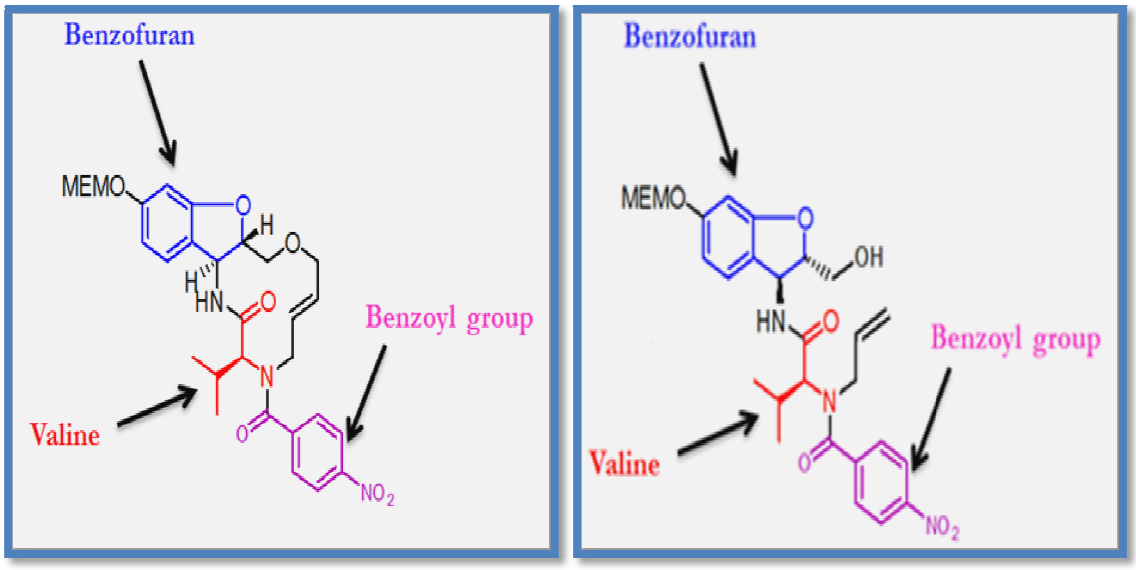


Figure 3.4: Chemical structure of Compound 2.4c and 2.3c, with specific moieties highlighted.

Compared to cyclopsorin a structure, 2.4c is a small macrocycle and has valine in common which is present as N-(4-nitrobenzoyl) valine fused to benzofuran scaffold. Replacing the valine group with N-benzoylvaline unit (without NO_2 group, compound C104) or leucine (compound C108) did not alter its biological activity while the acyclic form, compound 2.3c, does not show

any biological activity. The replacement of valine group with phenylalanine also reduced the activity emphasizing the importance of isopropyl moiety in conferring the inhibitory activity.

3.2.3 Molecular docking

The structural model of enzyme binding to a substrate had been referred to as 'Lock and Key model' proposed by Emil Fischer (E. Fischer 1894). Later, Daniel Koshland modified the model by proposing 'Induced fit model'(Koshland 1958) where the enzymes are flexible structures and are reshaped in the presence of substrate. Both the models carry similar references how a polymer like protein recognize other molecules and bind specifically and carry out catalysis which is termed as 'conformational proofreading'. The studies based on these models became the basis for understanding of the interaction pattern between protein and small molecules and is coupled with rapid expansion of available protein structures and its atomic co-ordinates determined by both X-ray crystallography or NMR based methods which established the field of molecular modeling (Warren et al. 2006; DesJarlais et al. 1988). It involved computational determination of how a molecule binds to a protein with details involving amino acids interaction and also the type of bonds or interactions formed. Such studies actually contribute towards the understanding of how better we could design a molecule that binds to the protein. In reality, protein is not rigid and is a polymer of amino acids, so the number of probable configuration for protein is out of reach for computational determination. However, to ease out the study, protein can be kept rigid or flexible form in computational programs and their interaction with the ligand can be determined. This kind of study is termed as 'Molecular docking'. There are several traditional algorithms used for molecular docking, classified according to their protein conformation prediction strategies and genetic algorithms which work basically by calculating low energy conformation of atomic structures of protein, while molecular dynamics based

algorithm simulates the movement of atoms and rotations of bonds which takes into account all possible confirmation of protein and is computationally expensive. For our study, we have used Swiss dock online docking server for preliminary docking and Maestro for detailed study.

Swiss dock is based on EADock evolutionary algorithm, which starts with the initial input of protein structural co-ordinates as well as low energy structure of ligand and prepares a population of diverse structures and pulls out a management system for them based on RMSD (root mean square deviation) variation between protein structure before docking and after docking. EADock utilizes two fitness functions for the discrimination; they are simple fitness and full fitness.

$$\boldsymbol{SimpleFitness = E^{ligand\ intra} + E^{receptor\ intra} + E^{inter}}$$

Where the E stand for internal energy calculated according to CHARMM program (based on number of internal bonds, angles and electrostatic and van der Waals interactions), Ligand intra- internal energy of ligand, receptor intra-internal energy of receptor, inter- interaction energy between ligand and receptor (Grosdidier, Zoete, and Michielin 2007). Next the fullfitness values are determined based on RMSD values of all complexes in the population; the threshold of RMSD values to be fixed is deduced by taking the mean of most favorable effective energy (G_{eff}).

$$\boldsymbol{G_{eff} = E^{ligand\ intra} + E^{receptor\ intra} + E^{inter} + \Delta G_{elec.solv} + \sigma \times SASA}$$

Where $\Delta G_{elec.solv}$ is determined from electrostatic and non-polar interactions of molecules, SASA- solvent accessible surface area, σ-empirical atomic solvation parameter which is equal to 7.2 cal/(mol $Å^2$)(Still et al. 1990). In effect, EADock fitness function like SimpleFitness are related to electrostatic and van der Waals interactions which get grouped according to their

scores, which is further refined by FullFitness values which are related to the solvent effect and search region. This fitness value is utilized for ranking and reanalyzing the interaction until a number of user defined cycles are reached.

Maestro docking platform from Schrödinger is a collection of program pertaining to molecular docking and modeling. It uses Glide program for ligand docking, wherein the ligand is kept flexible and the protein rigid, additionally, if the protein has already bound to a known ligand we can define the region of interest by grid based docking. The initial step is generation of low energy structure of ligand. For this purpose, OPLS (Optimized Potentials for Liquid Simulations) force field molecular mechanics programs are used, which remove the predicted structures containing abnormal bonds and torsion angles as well as high energy conformations. OPLS (developed by William L Jorgensen) is built on a study based on reported 25 peptide residues plus neutral and charged terminal group's intermolecular potential functions; these parameters were tested in combination with Monte Carlo mechanical simulations of 36 organic compounds and aqueous solvents of organic ions. It is similar to AMBER (Assisted Model Building and Energy Refinement) and CHARMM (Chemistry at HARvard Molecular Mechanics)force field chemistry programs and a little superior in terms of RMSD from crystal structure of atomic positions (Jorgensen and Tirado-Rives 1988; Jorgensen, Maxwell, and Tirado-Rives 1996). The several optimized structures are docked onto refined protein structure with or without defined grid region and best scoring interactions are ranked and presented. Scoring is the important function in docking programs; Maestro uses GlideScore for examining the binding affinity and ordering ligand interaction models, additional ligand receptor mechanics interaction energy and ligand strain energy are also used to further refine the ranking pattern. For example, a predicted hydrogen bond crossing 2.0 Å will result in penalty score and for a

predicted angle crossing the limit of 30º to 180º is also penalized. Glide Score are of two types: (i) SP- standard precision, (ii) XP- extra precision, SP – is designed to identify the ligand that have nominal probability of binding and does not take into account of imperfections in ligand poses, while XP- is designed to put penalty functions for those poses which does not fit into established biophysical and chemistry principles Thus Maestro serves as one of the best platform for extensive molecular modeling and docking studies.

3.3 Materials and methods:

3.3.1 Computer and software programs

Lenovo desktop with following configurations, Intel Pentinum D 2GB RAM, Windows 7 32 bit operating system with Microsoft office 2007 and 100Mbps internet connection, Chem Office and Marvin sketch for drawing ligand structure, Raswin and UCSF Chimera for viewing and analyzing PDB structures as well as docking results, Swiss dock online docking platform (http://www.swissdock.ch/docking) for academic users, Maestro docking program (version 9.8) implemented from Schrödinger molecular modeling suite-2014 has been used.

3.3.2 Dataset preparation

The compound 2.4c and 2.3c structures were drawn in ChemDraw professional and also in Marwin sketch as 2D diagrams and rendered as 3D structures with refining by addition of hydrogen atoms and checking for abnormal bonds and angles, energy minimized and saved in Mol2 or SDF format for further analysis.

3.3.3 PDB structural details of Cyp D/PPIF

All protein structure and its atomic co-ordinates were accessed in PDB bank (http://www.rcsb.org/pdb), we choose human PPIF/Cyp D protein PDB ID: 3R49 for Swiss dock and 2Z6W for Maestro.

3.3.4 Swissdock server based molecular docking

Swiss dock server allows for uploading PDB file of our choice or enter and search by PDB code, the PDB ID 3R49 was entered and searched , the web displayed the hit after which the exact peptide chain A with 166 amino acids was selected for docking, successful set up and inspection is confirmed by green tick mark. The compound structures were drawn in ChemDraw 3D software and Marwin sketch and energy minimized; these files were uploaded in mol2 format. The job name was given and docking was started, after 2-3hrs the docking results link was active and available. The docking results were downloaded and the zip file was extracted and analyzed by UCSF Chimera software (Ver 1.9). On the File drop menu the option open was clicked and respective docking results folder was selected to open a file named target.pdb. The chimeras opens into protein structure, select all to color protein white and then select negatively charged amino acid to color them red and positively charged amino acid to color them blue. For analyzing and opening ligand binding pose, view dock option was selected and then clusters.doc4.pdb was opened, then a new window shows where dock 4, 5 or 6 option was selected. The docking ligand in the protein surface is displayed; docking is further analyzed by checking position of ligand. Along with the structure displayed another window remained open, showing the details of cluster rank number, energy, SimpleFitness and FullFitness values. Based on the fitness values and known interaction studies further conclusion about docking could be drawn.

3.3.5 Maestro docking platform

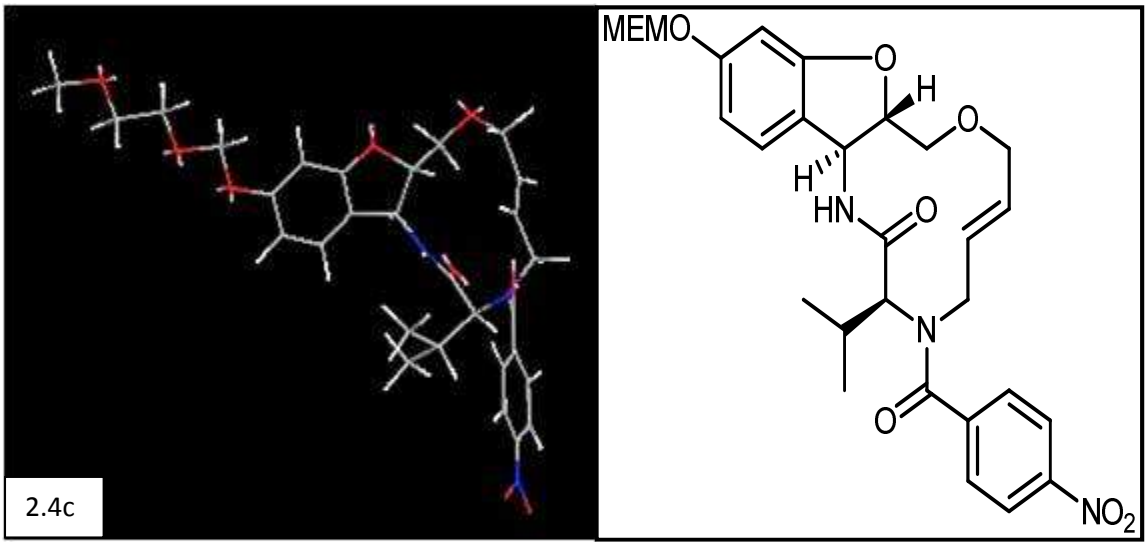
We adapted method from Woody Sherman's report (Sherman et al. 2006). The Molecular modeling simulations of molecules were performed using Maestro (version 9.8), implemented from Schrödinger molecular modeling suite-2014("Schrödinger Release 2014-2: Maestro, Version 9.8, Schrödinger, LLC, New York, NY, 2014."). All molecules were sketched in 3D format using build panel of maestro and energy minimized using OPLS-2005 force field to produce low-energy conformers. The structural coordinates of peptidyl prolyl cis-trans isomerase F were taken from protein data bank (PBD) with PDB id 2Z6W(Kajitani et al. 2008). Raw PBD protein structure was prepared by giving preliminary treatment like adding hydrogen, adding missing residues, refining the loop with prime and finally minimized by using OPLS-2005 force field. The grid for docking simulations was generated with bound co-crystallized CsA molecule. Molecules were docked using Glide module in Extra-precision (XP) mode, with up to three poses saved per molecule. The ligand was kept flexible, whereas, the protein PPIF was kept rigid throughout the docking studies. The lowest energy conformations were selected and, the ligand interactions (H-Bond and Hydrophobic interactions) with target protein were determined.

3.4 Results:

3.4.1 Ligand preparation

Compound 2.4c and control compound 2.3c (without the macrocycle ring) were drawn in ChemDraw 3D, and energy minimized. We utilized MM2 force filed for energy minimization (Allinger 1977), it showed the 3D structure of 2.4c as a rigid molecule with less possible conformations, while 2.3c was flexible with more possible conformations. As shown in the figure the central core fused 12 member ring restricts the possible rotations of bonds and angles;

while compound 2.3c presents a more flexible structure where the distance between nitrobenozyl group and benzofuran is less compared to 2.4c. We also have shown in the OPLS force field the chemistry determined structures of compound 2.4c, 2.3c and CsA.



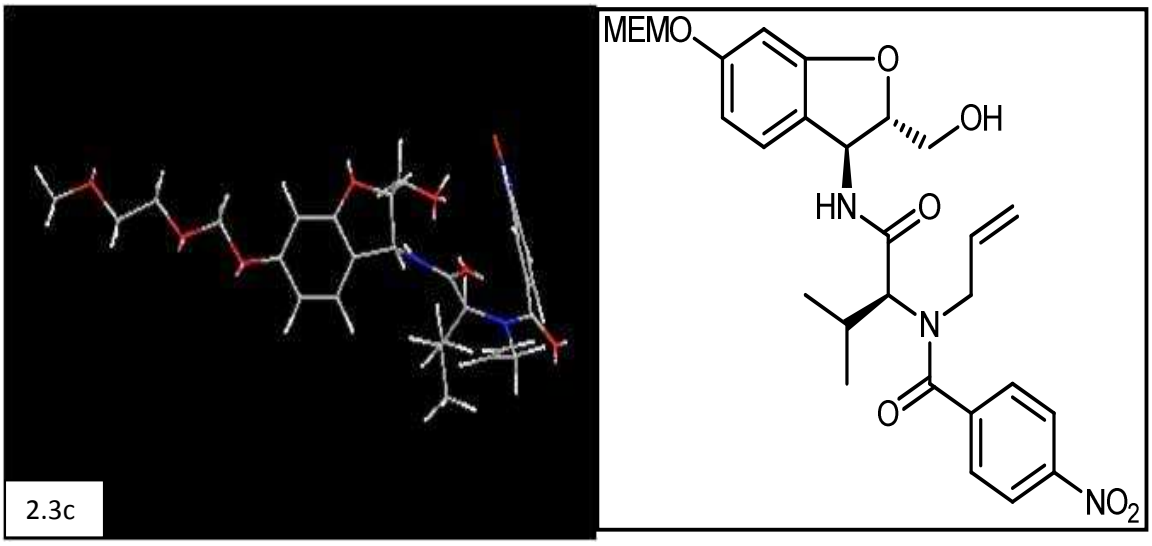


Figure 3.5: Energy minimized structures of compound 2.4c and compound 2.3c generated by MM2 program in ChemDraw 3D.

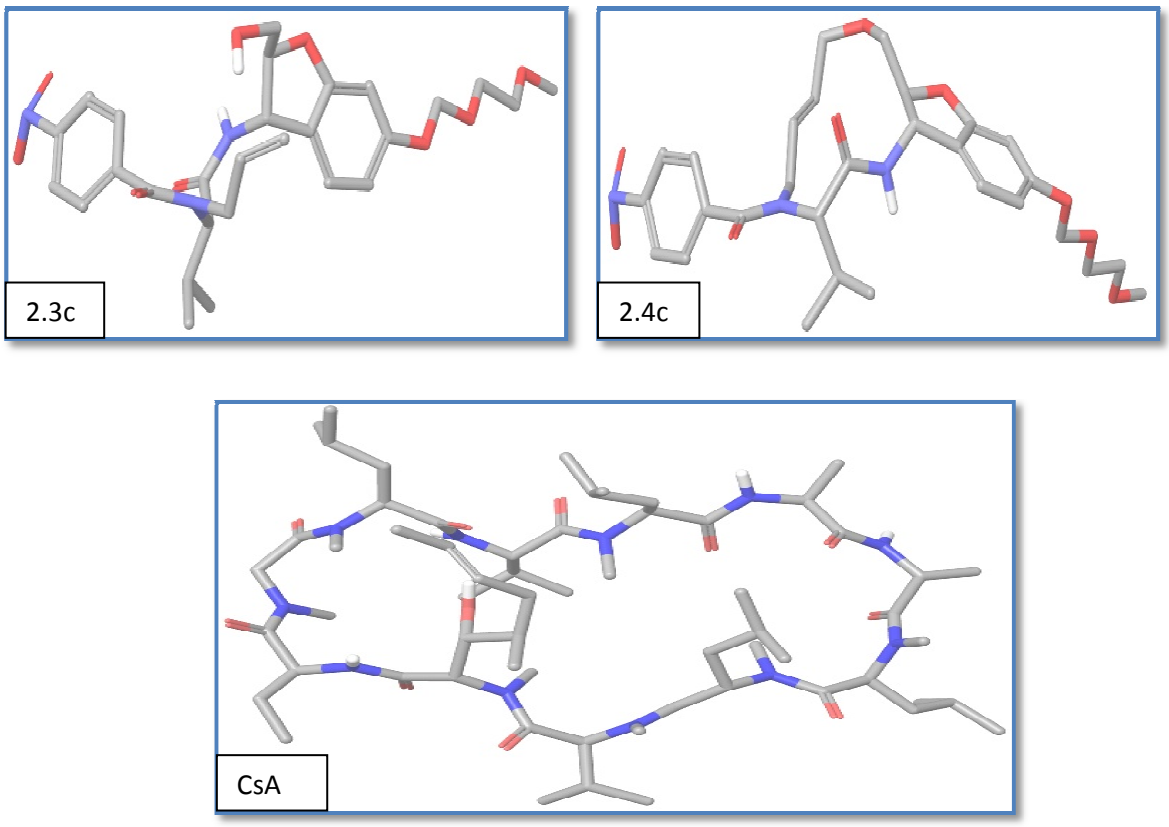
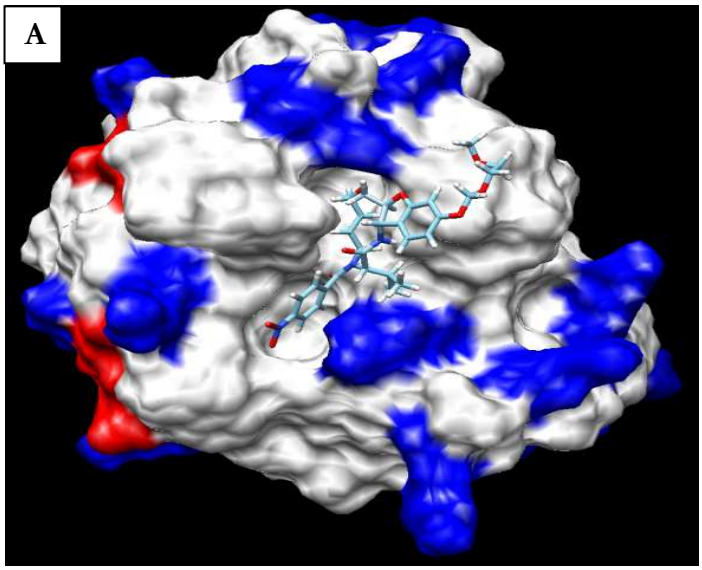


Figure 3.6: Energy minimized structures of Compound 2.3c, 2.4c and CsA generated by OPLS-2005 force field.

3.4.2 Swiss dock results analyzed by UCSF chimera (Ver. 1.9)

Chimera analysis of Swiss molecular docking results showed that, compound 2.4c binds near the active site of human Cyp D/PPIF enzyme. The active site comprised of catalytic Arginine and possesses two deep hydrophobic pockets S1 and S2 as described by Davis et.al (2010). On the other hand, the compound 2.3c binds at the other region far from the active site. Protein PPIF is a rigid protein showing very few structural changes upon binding with solvent molecules like DMSO and PEG400 (Davis et al. 2010). Hence, we believe that PPIF and small molecule (2.4c) interaction and binding are more or less like Lock and Key model of Daniel Koshman. Since

2.4c shows to be binding to active site and 2.3c does not, we speculate a correlation might exist between their biological activities which can be explained by structural interaction study.



Energy: 55.3545

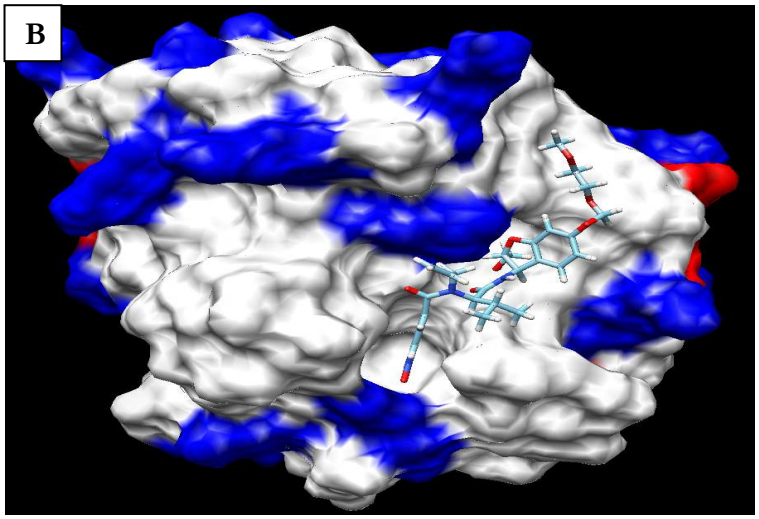
SimpleFitness: 55.3545

FullFitness: -674.75934

deltaG: -7.1456785

Cluster: 0

ClusterRank: 0



Energy: 32.6383

SimpleFitness: 32.6383

FullFitness: -690.07056

deltaG: -8.587084

Cluster: 0

ClusterRank: 0

Figure 3.7: Snapshot images of results of molecular docking by Swiss dock as analyzed by Chimera 1.9 software.

3.4.3 Maestro docking platform

The maestro platform allowed us to achieve a more detailed analysis of protein–ligand interaction. Compound 2.4c has several oxygen and nitrogen atoms at key positions which

increase the chance of having a hydrogen bond interaction. The carbonyl group of the compound between macrocycle and the phenyl ring establishes Hydrogen bonding with catalytic Arginine of PPIF, the valine of compound 2.4c sets up hydrophobic interactions with Phe113, Ala101, Leu122 at the S1 hydrophobic pocket of the active site while –OMEM remained well occupied in a unique pocket in the active site of the protein with its terminal oxygen making 4 hydrogen bonds with Gly109, Ser110 and Gln111 are shown in 2D interactive map **(Figure 3.9D)**.

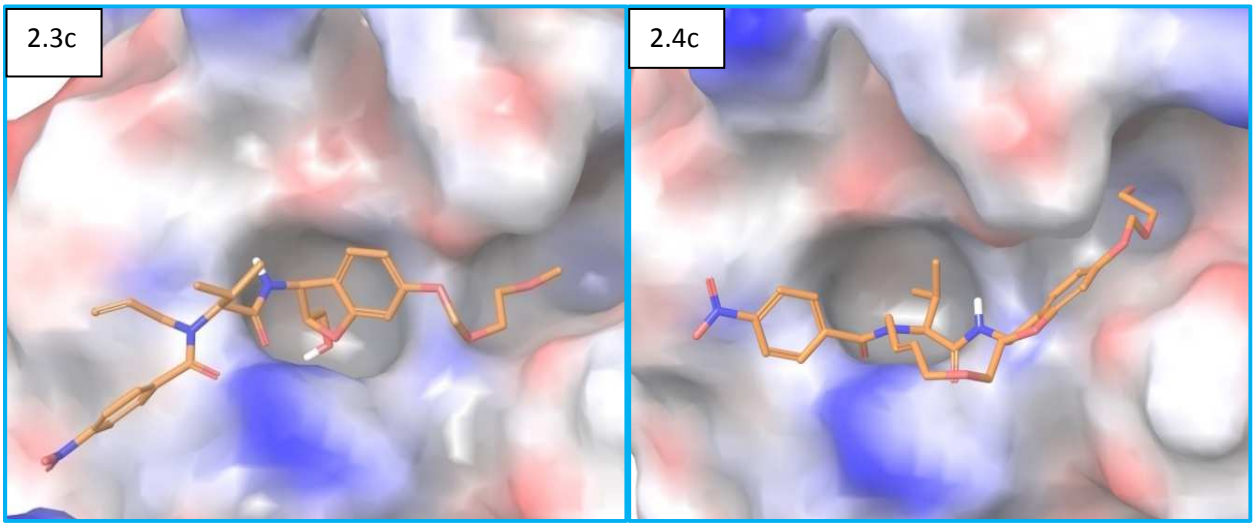


Figure 3.8: Snapshot images of docking pattern observed in Maestro docking analysis of compound 2.3c/2.4c with PPIF protein.

In our study we compared the CsA and 2.4c interaction as well. 2.4C like CsA reveals hydrogen bonding with catalytic Arg55 and hydrophobic interactions with Phe 60, His126 and Leu 122. However, the interaction of -OMEM group with Gln111, Ser110 and Gly 109 could not be extrapolated to CsA which highlight the novel hot spots of interaction between Cyp D and compound 2.4c.

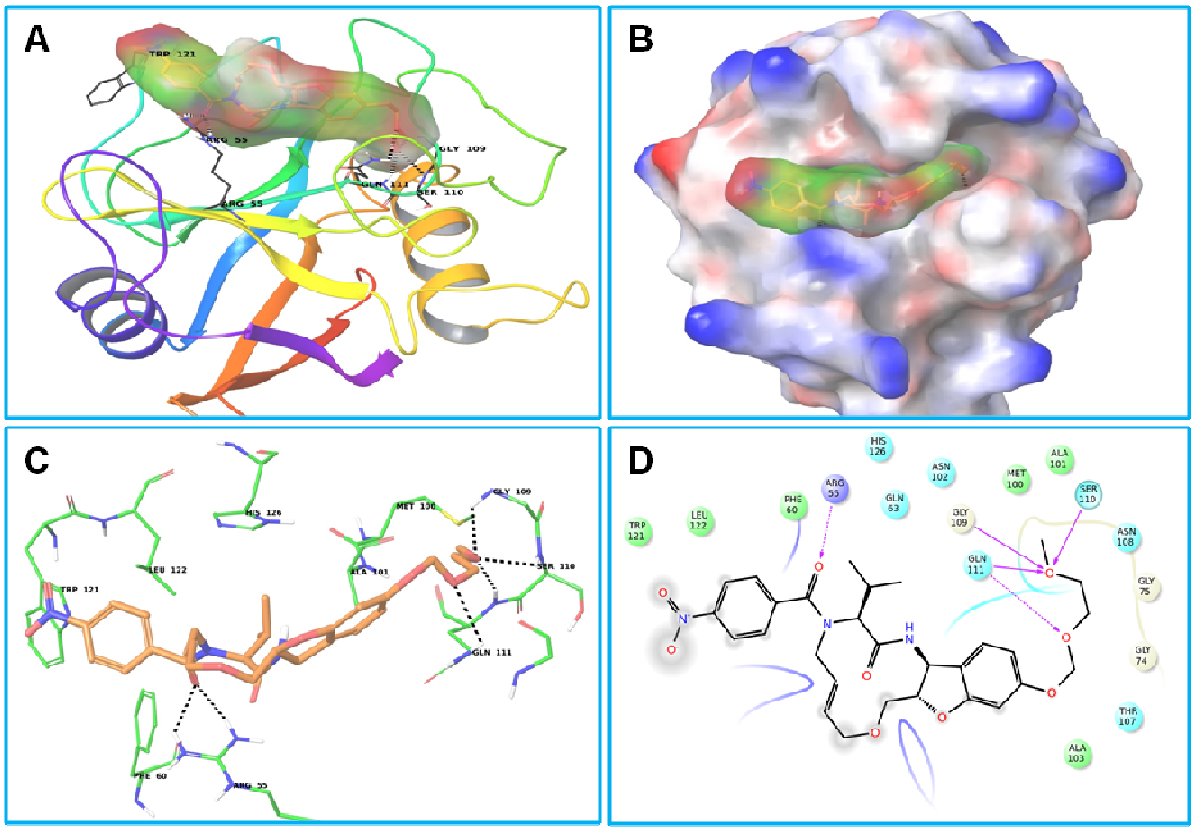


Figure 3.9: Snapshot of docking analysis as performed by Maestro, A, docking picture highlighting the hydrogen bond involved on the secondary structure of protein PPIF, 2.4c is shown with full molecular surface graphics, B, Molecular surface depiction of both protein and ligand of the docking model, C, 3D interactive map showing amino acids their structural positions while highlighting the bonds involved, D, 2D interactive map showing different type of interactions of 2.4c with the amino acids of the protein under study.

3.5 Discussion:

The molecular docking technique we employed in our study enabled us to understand the interactions between compound 2.4c and cyclophilin D/PPIF. The PPIF is structurally rigid protein which sans the mitochondrial signal peptide is of 160 amino acids in length. The rigidity of the protein PPIF was determined by reports of X ray crystal structures of PPIF complexed with tetra peptide substrate and PPIF complexed with CsA; these features put molecular docking

and modeling studies to a great advantage. The high resolution structure of PPIF bound to ligand was available in PDB bank, for preliminary study we selected human PPIF PDB ID 3R49 which was bound to ligand quinoline-8-amine. As shown in **Figure 3.7**, the compound 2.4c docks near the active site and also shows low Full Fitness score (-674.75) than the 2.3c docking on PPIF. The 2.3c docks to an altogether different region and also does not show consistency in several clustered docking poses and gives high Full Fitness score (-690.07) compared to 2.4c. Nearly, 28 different clusters of docking pose were formed in case of 2.4c, majority of them were showing high tendency to dock around S1 and S2 pockets of the active site. Swiss dock relays on global docking, hence we find best possible outcome poses both in case of 2.4c and 2.3c. We noted a drawback of our docking posed of 2.4c with PPIF where hydrophobic valine was exposed to solvent environment which could not be the case in real space due to the proximity of regional S1 and S2 hydrophobic pockets. Nevertheless, 2.4c and 2.3c docking by Swiss dock could help us to understand and explain their respective biological activity, which is exactly reflected again in terms of its molecular docking with protein PPIF. To understand in depth the nature of interaction we carried out detailed in silico analysis using Maestro docking platform.

Compound 2.4c has a fused ring structure which constrains the possible rotations of important groups and in turn makes it more rigid compared to 2.3c. The other compound is CsA which is a cyclic peptide and has a similar locked configuration like 2.4c due to the closure of the peptide chain. Another structural similarity we found was the presence of valine amino acid in both the compounds. So we speculated that 2.4c and CsA might bind to similar site in a predictable manner. Docking studies by Maestro's glide program validated our speculation. The compound 2.4c was found to have good interactions compared to 2.3c, the valine was found buried in the deep pocket and –OMEM moiety revealed Hydrogen bond interactions with some novel

hotspots. As revealed in the 2D interactive map, Arg55 forms a hydrogen bond with oxygen atom near the benzoyl nitro group, similarly we found three other amino acids having propensity to from hydrogen bonds. The 3D interactive map further states the positioning of atoms and shows bond formation. This molecular docking study further strengthens our understanding of interactions between compound 2.4c and PPIF. The high flexibility of 2.3c does not support this kind of interaction, though we observe it's binding near to the deep pocket of active site. The molecular docking of 2.4c with Cyp D /PPIF shows promising in silico interaction which encouraged us to carry out further explorations in biochemical and cell based assays (described in Chapter 4).

3.6 References:

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Chapter 4: Compound 2.4c mediated inhibition of mitochondrial permeability transition (mPT) in cardiomyocytes: a mechanism based evaluation

4.1 Abstract:

The pathological opening of mitochondrial permeability transition pore (mPTP) causes mitochondrial dysfunction and necrotic cell death. This has special implication in case of cardiomyocytes as increasing body of evidence indicates at the role of mPTP in mediating cardiac dysfunction and cell death. My present work describes the discovery of a novel benzofuran-based macrocycle compound 2.4c that prevents the depolarization of mitochondrial membrane potential and the cytochrome c release (described in Chapter 2) which are the salient features of mPTP opening. To understand the mechanism, we carried out an in silico analysis (described in Chapter 3) which predicted the interaction of compound 2.4c with the active site pocket of cyclophilin D (Cyp D), the molecule which has been reported to be the regulator of the pore. In the present chapter, we evaluated the potential of compound 2.4c in inhibiting the peptidyl prolyl cis-trans isomerase activity (*Ppif* activity) of Cyp D. The data reveals that like cyclosporin A the compound 2.4c also inhibits the *Ppif* activity of Cyp D. More importantly, in cultured cardiomyocytes, the compound 2.4c, like cyclosporin A, preserves mitochondrial integrity and inhibits the cytochrome C release from thapsigargin treated mitochondria at a comparable efficacy indicating at its role in inhibiting the phenomenon of mPT.

4.2 Introduction:

CsA binds to cyclophilin family of proteins whose binding inhibits the enzymatic activity of the protein. Earlier CsA was known as immunosuppressive agent used in treating graft rejection cases. The mechanism of action of CsA was found to be involved with calcineurin; the CsA-cyclophilin complex binds to calcineurin and blocks its activity that eventually leads to inhibition of expression key immuno-modulating factors. CsA was also found to inhibit that calcium induced mPT phenomenon, and also that it had potential cell protective effect during reperfusion injury treatment segment of myocardial infarction cases, but immune-modulator function of CsA was not necessary for this action. There have been several reports where CsA has been used to treat myocardial infarction both in animal models as well humans (D. J. Hausenloy, Boston-Griffiths, and Yellon 2012; S. Javadov and Karmazyn 2007; A P Halestrap, Clarke, and Javadov 2004; Dongworth et al. 2014; Najafi et al. 2014; C P Baines 2007; Duchen et al. 1993; A P Halestrap 2002; N Mewton et al. 2010; Argaud et al. 2005). Hence, CsA analogs which were non-immunosuppressive gained lot of importance, there are two such molecular as mentioned in **Table 1.2** chapter 1, they are NIM811 and DEB025. These molecules do not have strong interaction with calcineurin protein due to modifications of in the region of CsA peptide which is exposed after binding to cyclophilins (refer chapter 3). These studies of molecular and structural interaction between CsA and cyclophilins has been well established by X ray crystallographic and solution NMR biophysical experiments (Zurini et al. 1990; Thériault et al. 1993; Mikol, Kallen, and Walkinshaw 1994; Kallen et al. 2005; Ke et al. 1994; Schlatter et al. 2005; Kajitani et al. 2008), based on this study many different molecules could be designed which bind specifically to Cyp D and have no interaction with calcineurin (Guichou et al. 2006). The current studies also involve fragment based designing of molecule that bind to cyclophilins specifically

(Fu et al. 2014; Valasani et al. 2014; Sweeney, Fu, and Wiedmann 2014) and inhibit its enzyme activity.

In our study, we found out that compound 2.4c dock to the active site of CypD protein much similar to how CsA binds to cyclophilins, we speculated that this might lead to the inhibition of enzyme activity of Cyp D. Hence we carried out a study from recombinant His tagged rat cyclophilin D/PPIF to ascertain whether compound 2.4c inhibits its enzyme activity. The assay was initially developed by Fischer *et.al* in 1984 who synthesized tetra peptides with 4-nitroanilide group as chromogenic substrate for estimation of chymotrypsin enzyme activity. When proline was present at second position, chymotrypsin cleaves only trans-proline isomer containing peptide which serves as the basis PPIF enzyme activity analysis (G. Fischer et al. 1984).

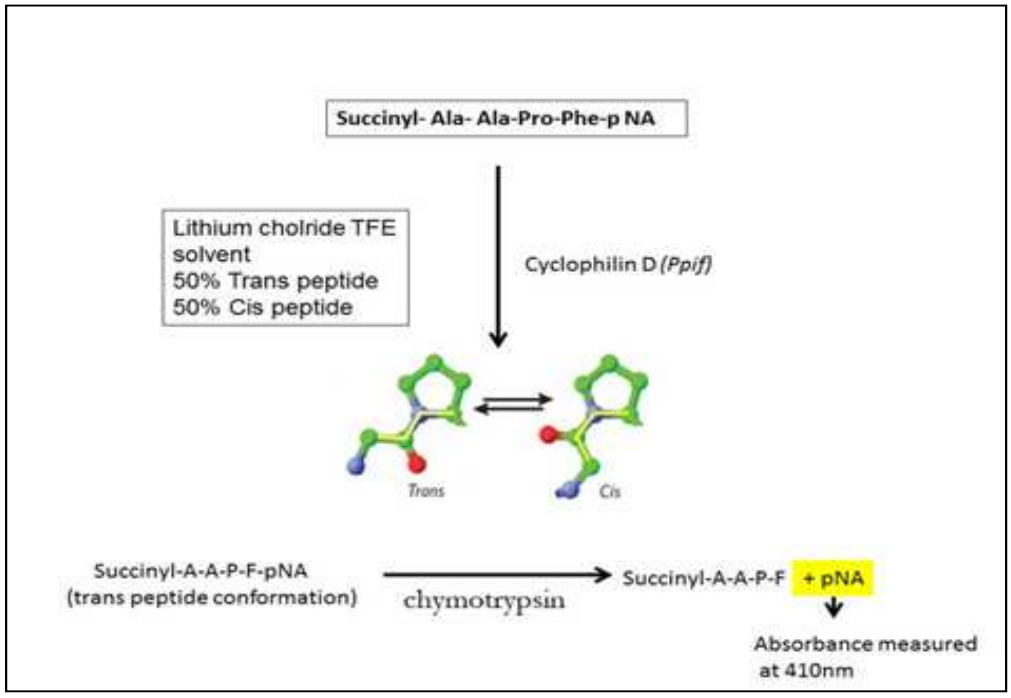

Figure 4.1: Illustrative diagram of enzymatic estimation of Cyp D/ppif activity. TFE-Trifluoroethanol, Ala- Alanine, Pro-Proline, Phe-Phenyl alanine, pNA- para nitroanilide, nm-nanometer.

The synthetic substrate where proline is present at third position has high conformational selectivity for chymotrypsin against trypsin; the hydrolysis of terminal p-nitroaniline (pNA) bond occurs preferably in trans X-pro conformation (G. Fischer et al. 1984). Kofron *et.al* coupled the proline isomer specific cleavage of substrate chymotrypsin for estimation of activity of peptidyl prolyl *Cis-Trans* isomerases (PPIases). The enzyme activity estimation was limited due to kinetic complexity of assay and physical properties of substrates and products (pNA). In water based solutions, the proline exists as 10% *cis* and 90% *trans* isomer, which lead to high signal to noise ratio for estimation of PPIase activity. The substrate concentration could also be not increased due to limited solubility of pNA. The other limitations which was encountered was that in order to determine velocities reaching Michaelis constant, the enzyme must be saturated with excess substrate, which was not possible due to pNA solubility. To circumvent these issues, Kofron *e.t al.,* formulated a unique method, wherein substrate is prepared in an organic solvent with high salt concentration, here the cis isomer concentration was found to be increased to more than 50%. The chymotrypsin concentration was kept at highest levels so that noise ratio due to trans- peptide cleavage could be lowered down, *PPIF* concentration was kept as low as possible to estimate its velocity and the entire assay carried at 0º degree celcius so that *PPIF* activity could estimated favorably in time. Using this principle Kofron estimated Km (of cis substrate) of tetra peptide for human Cyp D/*PPIF* to be ~ 870µM, which was found to be confirmed by NMR based assays of enzyme activity (Kofron et al. 1991; Janowski et al. 1997). Cyclophilin D

catalyzed *cis-trans* isomerisation is reversible; however, till date, there are no method devised to estimate *trans* to *cis* isomerase enzyme activity of PPIases.

Our first screening study was performed in pancreatic beta cells, but we found out that Cyp D inhibition has more direct correlation with cardiac muscle cells death due sudden change of ions and oxygen concentrations levels which happens during treatment of Myocardial infarction while reperfusing the clogged blood vessels (D. J. Hausenloy, Boston-Griffiths, and Yellon 2012). Therefore we wanted to check whether the compound 2.4c has similar mitochondrial protective effect in cardiac muscle cells. Cellular death both by necrosis and apoptosis has been estimated by cytochrome c levels detected by flowcytometer, immunoflouresence, western blot and ELISA (Naniche, Sau, and Pasinelli 2011). Cytochrome c is a protein which is present in mitochondrial membrane space, and released upon increase in porosity or damage to membranes, which happen due to cell death or injury (Tait and Green 2010). Anti apoptotic protein Bcl2 and pro apoptotic protein Bax have also been associated with mPT induced cell death and cytochrome c has served as a valuable marker in these studies (Zhang and Armstrong 2007; Chen et al. 2015). Nevertheless, mitochondrial permeability transition pore (mPTP) acts as a critical determinate of cellular viability and regulates cytochrome c release (Zhang et al. 2008). The opening of the pore causes swelling of mitochondria, uncouples the mitochondrial oxidative phosphorylation and release the apoptogenic material which results in cell death. During myocardial infarction and reperfusion sequence, mPTP has been found to open only during reperfusion when mitochondrial Ca^{+2} and inorganic phosphate ions concentration is high and the organelle is under oxidative stress and in ATP depleted state (Ovize 2006). Suppression of the opening of mPTP at the onset of reperfusion thus offers an attractive clinical perspective for cardio protection.

Mitochondria are structurally dynamic in nature, they tend to undergo swelling and rupture and have characteristic interconnected structure. They are stained by several dyes and visualized under microscope; we have utilized MitoTracker red dye for staining mitochondria. Thapsigargin is known to induce swelling and rupture of mitochondria when cells are treated for longer durations (Zhang and Armstrong 2007) and it is also known induce mPT at micro molar concentrations (Korge and Weiss 1999). We sought to examine whether compound 2.4c protects mitochondrial structurally and prevents cytochrome c release as compared to CsA which has been reported to preserve mitochondrial integrity against mPT inducing agents (Zhang and Armstrong 2007; Cho et al. 2010; Quintanilla et al. 2013).

4.3 Materials & Methods:

4.3.1 Materials

DMEM (Dulbecco's Modified Eagle's medium), FBS (Fetal Bovine Serum), Penicillin-Streptomycin, Trypsin , MitoTracker Red ,Alexa Fluor 488 goat anti-mouse IgG and DAPI were purchased from Life technologies; Thapsigargin, Tunicamycin, α-Chymotrypsin, Succinyl Ala-Ala-Pro-Phe-pNA, Urea, HEPES, Trifluoroethanol, Paraformaldehyde, Lithium Chloride, Cyclosporin A were purchased from Sigma-Aldrich; coverslips and glass slides, Culture dishes from Corning Life sciences, H9c2 cardiomyocytes was obtained from ATCC, Mouse monoclonal anti-cytochrome c (SC-13156) was purchased from Santa Cruz Biotechnology.

4.3.2 Cell culture

H9c2 cells were grown on cover slips or in 60mm culture dishes in complete media (DMEM with 10% FBS and standard antibiotics) at 37ºC in 5% CO_2. After the cells reached 60% confluency, they were treated with thapsigargin with or without compound 2.4c or CsA. The

cells treated with 0.1% DMSO served as vehicle control. Final DMSO concentration is maintained at 0.1% in assay media. After respective treatment, cells were either stained and mounted on glass slides or harvested for flow cytometric analysis.

4.3.3 Rat PPIF cloning and Protein expression

Total RNA was isolated from rat L6 myocytes by using Trizol reagent following manufacturer's protocol. The First strand cDNA was synthesized from rat total RNA by Superscript II cDNA synthesis kit, by utilizing random hexamers as primers and following kit manual. The first strand cDNA was used as template for amplification of cyclophilin D (*Ppif*) by using the following primers Forward (5' to 3') - CGCGGATCCATGCTAGCTCTGCGCTGCGG with BamHI restriction site, Reverse (5' to 3') – CCCAAGCTTAGCTCAACTGGCCACAGTC with HindIII restriction site. Around 500ng of pET 28a vector was digested with BamHI and HindIII for 8h and heat inactivated at 85ºC. The gel purified PCR amplicon was added at a molar ratio of 3:1 (insert: vector) and the ligation was performed by adding T4 DNA quick ligase for 15min at 16ºC. The resulting recombinant plasmid was used to transform DH5 alpha E.coli strain to generate high quantity of recombinant plasmid DNA.

E. Coli BL21 cells were transformed by pET 28a recombinant plasmid containing rat *Ppif* insert and bacterial culture (in LB broth with ampicillin 50µG/mL) was grown till O.D. at 600nm reaches 0.4 and then induced with 1mM IPTG for 4h. After induction, 2ml culture was spun down and analysed in SDS PAGE to confirm protein expression.

4.3.4 Rat PPIF protein purification

PPIF protein was expressed in *E. coli* BL21 cells, in 1L LB broth medium with 0.1mg/ml Ampicillin; the medium was incubated at 37ºC till OD reaches 0.4 after which 1.5mM

isopropylβ-D-1 -thiogalactopyranoside (IPTG) was added and another 8hrs of incubation was carried out. After incubation, 50ml culture containing the cells was pelleted by spinning at 3000 rpm for 10 min and supernatant was discarded. The pellet was resuspended in 1X Phosphate Buffered Saline (137 mM Sodium chloride; 2.7 mM Potassium chloride; 8.1 mM Disodium hydrogen phosphate pH 7.4), centrifuged twice to have cell pellets free of debris. We adapted method of protein purification with little modification of two step denaturing and refolding method described in (Z. Yang et al. 2011) .Cell pellets were then resuspended in10 ml of 8M Urea denaturing buffer containing 50mM Tris pH 7.5 and 150mM NaCl; the solution was sonicated 5 times at 45 kHz for 30 seconds with a 60second pause in ice between each sonication. After sonication, the mixture was centrifuged for 30min at 10000 rpm, supernatant was discarded and the resulting pellet was again resuspended in fresh 8M Urea denaturing buffer. The resultant crude protein extract was applied in Ni-NTA column equilibrated with 8M Urea denaturing buffer and the flow through was collected by gravity and stored in ice. The column was washed twice with 8M Urea denaturing buffer and then washed 4 times with 10ml 8M Urea denaturing washing buffer containing 10mM Immidazole and the flow through was collected and stored.

The column was subjected to step gradient renaturation using 4 buffer systems each being applied twice consecutively. All the buffers contain 50mM Tris-Cl pH 7.5, 150mM NaCl, 10% Glycerol, 10mM β-mercaptoethanol, with a gradual decrease of Urea concentration from 5M (Buffer A) , 3M (Buffer B), 1M (Buffer C) and lastly with no Urea (Buffer D). Finally, the His tagged protein CypD was eluted in Buffer D containing 500mM Imidazole and the eluted protein was dialyzed against 1X PBS at 4ºC overnight with four buffer changes of 1000ml volume. The protein was then concentrated in Centricon with a cut-off of 10kD. The

concentrated protein was estimated by Bradford reagent and stored in -80ºC freezer for further use.

4.3.5 Cyclophilin D(PPIF) enzyme assay

The enzyme assay was carried out following the method of (Kofron et al., 1991) with modifications using synthetic substrate Succinyl Ala-Ala-Pro-Phe pNA (s7388) and the purified Cyclophilin D (*Ppif*) protein prepared in our laboratory. The α-chymotrypsin mediated para-nitro aniline (pNA) release was the read out in this experiment. The principle of the assay is based on the fact that chymotrypsin specifically cleaves peptide with trans-proline but not with cis-proline. The ratio of cis-proline to trans-proline in Succinyl A-A-P-F pNA (s7388) varies in different solvents: while it is 90% *trans* proline: 10% *cis* proline in distilled water, the ratio shifts to 50% *trans* proline: 50% cis proline in Trifluoroethanol (TFE) containing Lithium chloride. The 50% *trans* proline peptide is readily cleaved by α-chymotrypsin while remaining 50% cis proline peptide remains un-cleaved. When the synthetic substrate cleavage was carried out in TFE in the presence of CypD, it catalyzed the conversion of *Cis*-proline to *Trans*-proline, thus isomerized peptide is further cleaved by α-chymotrypsin and the increase in paranitroaniline release is considered as a read out to estimate CypD (PPIF) enzyme activity. The principle of the assay is explained in **Figure 4.1**.

The α-chymotrypsin was dissolved in double distilled water at a concentration of 30mg/ml, and 100mM succinyl Ala-Ala-Pro-Phe pNA substrate was prepared and stored in TFE with 470mM Lithium chloride. For enzymatic inhibition assays, CsA and Compound 2.4c was incubated with PPIF at 10µM CsA concentration for 1hour in ice prior to the addition of substrate. The enzyme assay was carried in 1ml of 50mM HEPES buffer pH 8.0 with 100mM NaCl; 10µL of α-chymotrypsin and 100nM of Cyp D (PPIF) were pre-incubated in ice for 10mins. The reaction

was initiated by addition of substrate at 100µM concentration, and the increase of absorbance at 410nm was monitored for 240 s.

4.3.6 Cytochrome c release analysis

A quantitative Cytochrome c release assay was carried out through flowcytometry following the method of Waterhouse and Trapani (cell death and differentiation 2003). A stock solution of digitonin was prepared just before each experiment by dissolving digitonin in phosphate buffered saline (PBS), by heating in a boiling water bath, to form a 20 mg/ml solution. The stock solution was diluted with 100 mM KCl in PBS (the NaCl concentration of the PBS was decreased so that the PBS/KCl solution was isotonic) to form 30µg/ml working solutions, which were placed on ice. Washed cells (100µl aliquots) were mixed with digitonin working solution (300µl) and the suspension was kept on ice for exactly 5min. The cells were then washed and fixed with 200µl of 3.7% formaldehyde for 20 min at room temperature, washed twice with PBS, resuspended in 1 ml blocking buffer (3% bovine serum albumin, 0.05% saponin in PBS) for 1 hrs at room temperature and centrifuged. Mouse monoclonal anti-cytochrome c was added to the cell pellet and the cells were left at 4°C overnight. Cells were washed once with blocking buffer and the cell pellet was incubated with secondary Alexa Fluor 488 goat anti-mouse IgG for 1 hrs at room temperature. Cells were then washed, resuspended in PBS and then acquired on BD FACS calibur, followed by analysis using Cell Quest Pro software.

4.3.7 Assessment of mitochondrial integrity

H9c2 cells were grown cover slip in DMEM media supplemented with 10% FBS and standard antibiotics at 37ºC in 5% CO_2. At 60% confluency, the cells were treated with thapsigargin with or without compound 2.4c or CsA while treatment with 0.1% DMSO served as vehicle control.

In the next step, cells were stained with 100nM MitoTracker red for 10min and fixed with 3% paraformaldehyde for 5min following which they were washed with PBS twice and the cover slips were mounted with antifade containing DAPI. Cellular images were acquired in Zeiss confocal microscope.

4.4 Results:

4.4.1 Cloning of rat Cyp D in pET 28 a vector

Rat cyclophilin D was amplified by PCR with the designed primers having specific restriction sites. The amplified product was digested with Hind III and BamHI and the pET 28a vector mediprep sample was digested with the same enzyme. The admixture was subject to ligation by T4 DNA ligase and resulting mixture was used for bacterial transformation. The **Figure 4.2** shows the restriction enzyme digested plasmids isolated from transformed colonies, two colonies came up positive for the insert DNA fragment which were later confirmed by DNA sequencing.

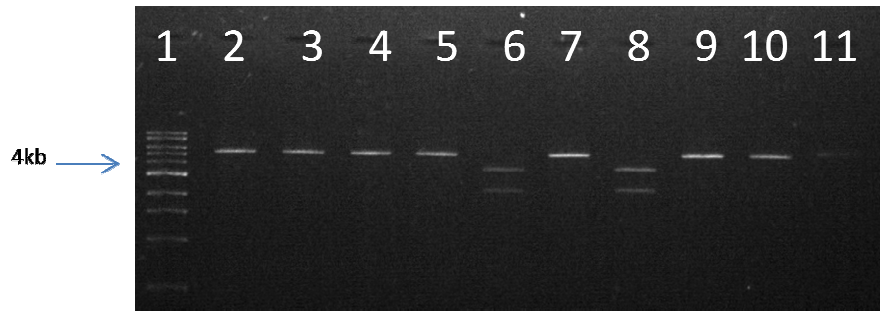

Figure 4.2: Agarose gel picture showing the two colonies which came to be positive for presence of insert Cyclophilin D in pET28a vector. Positive colonies were inoculated for miniprep and resulting DNA is digested with same restriction enzymes and checked for insert pop out. The positive colonies C5 (lane6) and C7 (lane8) were sent and confirmed by DNA sequencing.

4.4.2 Rat Cyp D protein expression and purification

Rat Cyp D/*PPIF* was expressed in *E coli* BL21 cells and induced for expression with 1mM IPTG for 8hrs at 37ºC, the expression was found to be max at these conditions. The crude extract obtained from sonication was used for testing quantity of expression by SDS page (data not shown). After initial confirmation the crude was centrifuged and the inclusion bodies stuck to tube was redissolved in 8M urea denaturing solution.

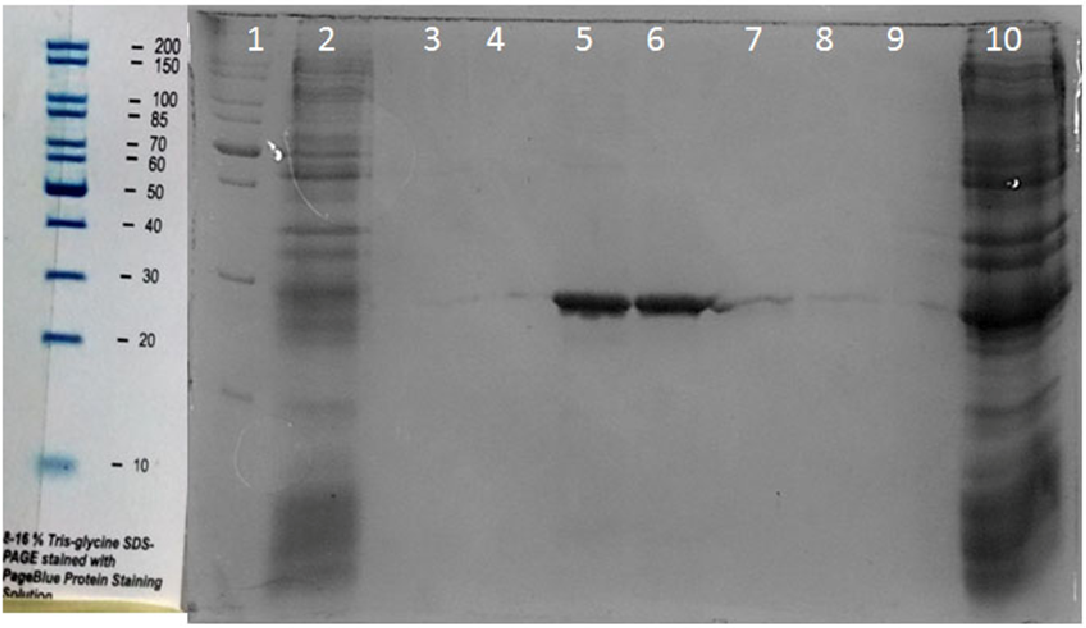

Figure 4.3: Purification of His-Tagged Cyclophilin D. Lane 1: Protein ladder; Lane 2: Flow through; Lane 4, 5, 6: Imidazole elution; Lane 10: Induced Cell Lysate.

4.4.3 Assessment of enzyme activity

Peptidyl prolyl cis-trans isomerase (*Ppif*) activity of purified cyclophilin D was measured in a coupled enzyme assay using synthetic substrate succinyl Ala-Ala-Pro-Phe pNA. *Ppif* activity is measured as increase of chymotrypsin mediated peptidase activity on addition of Cyp D over the

basal value **(Figure 4.4).** As the Figure reveals, compound 2.4c, at 10µM concentration, showed a significant reduction of *Ppif* activity in enzyme assay, however unlike CsA compound 2.4 c does not show significant reduction of *Ppif* activity in 1µM and 0.1 µM inhibitor concentration.

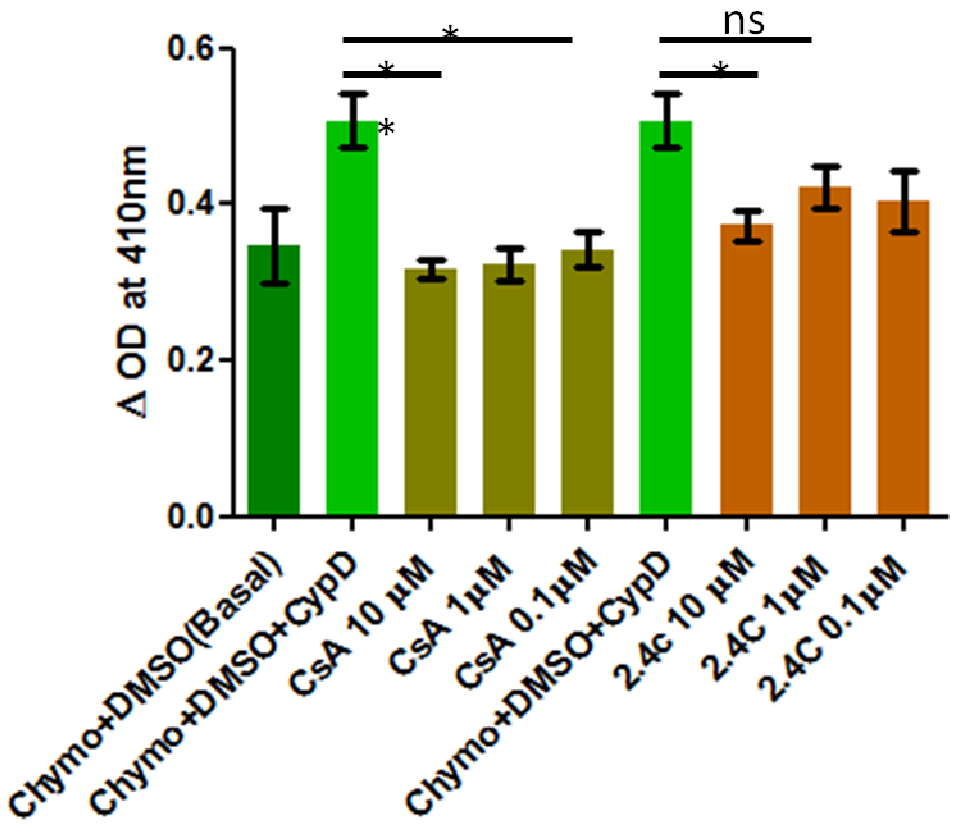

Figure 4.4: Coupled enzyme assay to determine inhibition of Cyp D (*Ppif*) enzyme activity by CsA and compound 2.4c; (*p<0.05; **p<0.01; ns: non-significant)

4.4.4 Prevention of cytochrome c release in cardiomyocytes

In the next step, we compared the activity of CsA and compound 2.4c in the regulation of cytochrome c release in H9c2 cardiomyocytes. The cells were treated with 3.0 µM thapsigargin for 36 h in the presence and absence of CsA or compound 2.4c and the cytochrome c release was analysed through flowcytometry **(Figure 4.5).** As the data reveals, both CsA (1) and compound 2

decrease the cytochrome c release by 47.41% and 47.23% respectively in H9c2 cardiomyocytes which is in concert to our in vitro enzyme inhibition studies.

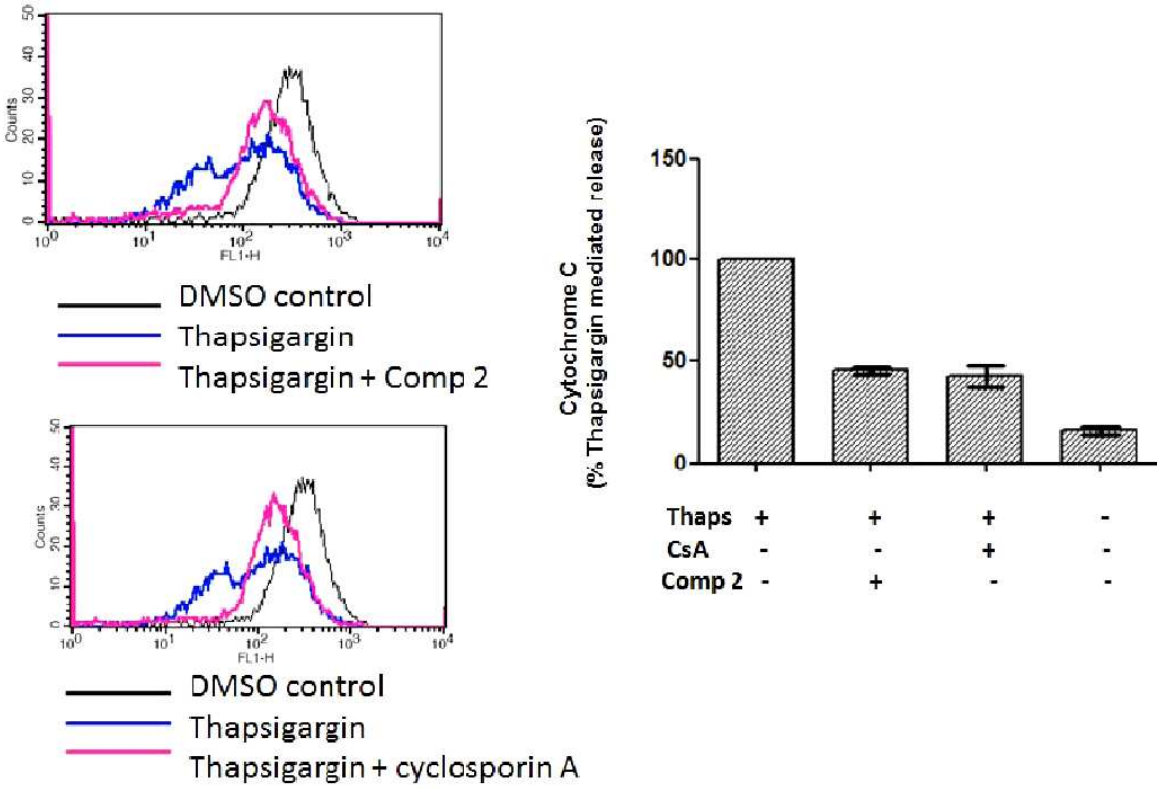

Figure 4.5: The effect of CsA and compound 2 on thapsigargin-mediated cytochrome c release from H9c2 cardiomyocytes. Thapsigargin (3.0 µM) mediated cytochrome c release is considered as 100%.

4.4.5 Preservation of mitochondrial integrity

Bonora et.al, reported the involvement of c subunit of F0 ATP synthase in mPT as well as in mitochondrial fragmentation and cell death (Bonora et al. 2013). In the next step of our study we evaluated whether effect of compound 2.4c and CsA could preserve mitochondrial integrity in H9c2 cardiomyocytes which is compromised on thapsigargin treatment (**Figure 4.6**). As the data reveals, thapsigargin at 3µM concentration induces mitochondrial morphological alteration

including swelling and fragmentation (**Figure 4.6** panel B) which is prevented by both CsA (**Figure 4.6** panel C) and compound 2.4c (**Figure 4.6** panel D) further confirming the role of the compound 2.4c in preservation of mitochondrial architecture.

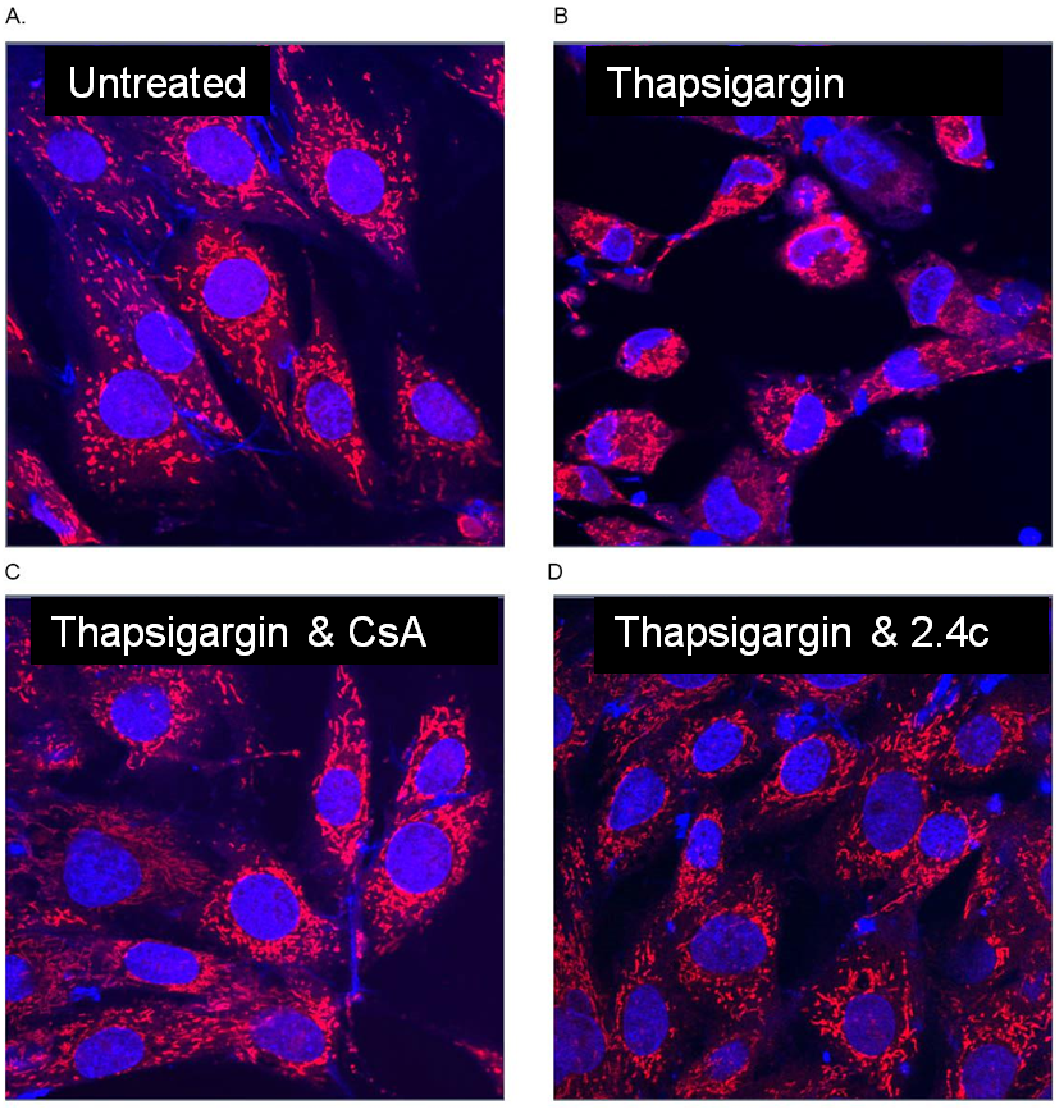

Figure 4.6: The effect of CsA and compound 2.4c on the preservation of mitochondrial integrity in thapsigargin treated H9c2 cardiomyocytes. A: untreated; B: treated with 3.0 µM thapsigargin for 18 h; C: treated with 3.0 µM thapsigargin for 18h in the presence of 10.0 µM CsA; D: treated with 3.0 µM thapsigargin for 18 h in the presence of 10.0 µM Compound 2.4C

4.5 Discussion:

Mitochondrial permeability transition (mPT) is a pathologically relevant process which precipitates to cell death in response to various stimuli. The phenomenon which is activated by ischemia/reoxygenation is a leading cause of acute loss of cardiomyocytes and thus considered as an attractive target for cardioprotection. Molecular nature of the pore is yet unclear; however the regulatory role of cyclophilin D in the opening and closure of mitochondrial permeability transition pore (mPTP) is established in animal models as well as in clinical settings (Elrod and Molkentin 2013). The CsA which inhibits the *Ppif* activity was found to be protective to patients immediately after myocardial infarction (MI) when applied during the revascularization phase (Piot 2007) but the compound itself has limitations due to its several off targets effects (Wilkins and Molkentin 2004). The development of new inhibitor of mPTP is therefore an unmet medical need in the field of cardioprotection. The benzofuran derivative amiodarone and its derivatives are known to inhibit mPT but the compound showed adverse side effects and the mechanism of action is unclear (Hellebrand and Varbiro 2010).

The data presented in this chapter clearly reveals that the benzofuran based macrocycle identified in my screening inhibits the *PPIF* activity of Cyp D and prevents the mitochondrial swelling and cytochrome c release. The compound unlike another benzofuran based small molecule amiodarone does not show any effect on the survival of cultured pancreatic beta cells and cardiomyocytes which indicates at its different mechanism of action. The small molecule 2.4c may find its application in reducing the perioperative myocardial injury during cardiopulmonary bypass (CPB) surgery which remains a challenging problem in developed as well as developing countries.

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Concluding Remarks

Macrocyclic drugs represent an emerging class of therapeutics which targets biological spaces that is inadequately addressed by small chemical entities. Cyclosporin A (CsA) is the classic example of such a drug where a cyclically constrained peptide with modifications at its backbone (N-methylation) as well as on selected side chains ("non-natural" amino acids) offers unique attributes of intracellular targeting that has role in immunosuppression as well as in the prevention of myocardial ischemia reperfusion injury (IRI); CsA's role in regulating mitochondrial permeability transition pore (mPTP) is well documented in literature. Treatment of rodent model with CsA prior to coronary artery ligation had been reported to reduce reperfusion induced arrhythmias as well as myocardial necrosis. The observation was supported by Griffith and Halestrap (1993) who reported the ability of CsA to protect against sustained myocardial ischemia reperfusion injury in isolated rat heart. The data has clinical implication as CsA when administered intravenously just before reperfusion was found to reduce the extent of myocardial injury in patients with acute ST-elevation myocardial infarction.

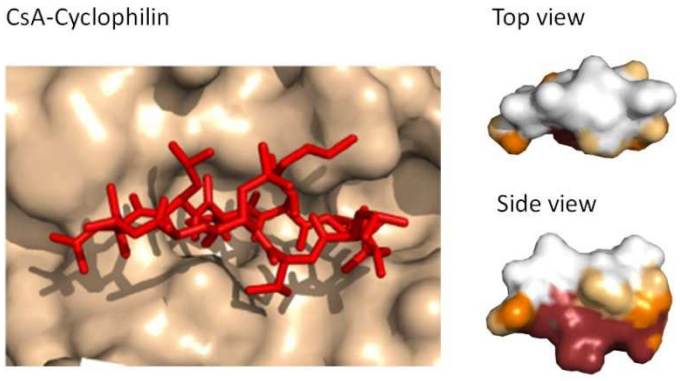

Figure 4.7 : Model of Macrocycle(MC) CsA binding interaction with cyclophilin active site, molecular surface of CsA is color coded based on their amount of contact with protein, white: <25% buried, gold: 25-50% buried; orange: 50-90%; red >90%burried. (Villar et al. 2014) The reference bibliography is found in chapter 4.

The shortcoming of CsA in cardioprotection is its interaction with Cyclophilin A which forms a ternary complex with catalytic subunit of calcineurin and inhibits its phosphatase activity. The macrocycle (MC) of CsA adopts an "edge on ˝ binding geometry in which the MC ring lies face on to the protein surface (**Figure 4.7**) making contact across a large contact area with substituent interacting with pockets and clefts in the protein while the outer edge of the ring being exposed to the solvent. In case of CsA around 50% of the MC remains solvent exposed and thus have a chance to interact with calcineurin through Met-Leu6 which contacts with Tyr-341, Pro-344, and Trp-352 of Calcineurin A and Leu-123 of Calcineurin B.

In case of compound 2.4c the MC is smaller and hence adopts a compact, roughly globular conformation and bind in a cleft or pronounced depression on the protein. Nearly 70% of the MC of compound 2.4c is buried while 30% is solvent exposed which is in stark contrast to that of CsA. Compound 2.4c like CsA establishes hydrogen bonding with catalytic Arg 55 and hydrophobic interaction with Phe60, Phe113, His126 Leu-122, and its –OMEM moiety establishes novel interaction with Cyp D through hydrogen bonding with Gly109, Ser110 and Gln111 which is unexploited by CsA. The data indicates at the unique interaction pattern of 2.4c with Cyp D, however whether compound 2.4c –Cyp D complex could inhibit the phosphatase activity of calcineurin has to be addressed in separate studies.

The CsA Cyclophilin binding affinity has been studied in details by several authors. The equilibrium dissociation constant of CsA (K_{dCsA}) and CypA was estimated to be 23± 6nM (Wear et al. 2005). In my present study EC_{50} for restoration of mitochondrial membrane potential was found to be in micromolar range. However the work is presently in progress in our lab to determine the potency at which the compound 2.4c could inhibit cytochrome c release, the experiments are also being carried out to find out equilibrium dissociation constant of compound

2.4c ($K_{d2.4c}$) in surface plasmon resonance studies which would reveal more details on Cyp D-compound 2.4c interaction.

In summary, the present research work reports the discovery of a novel small molecule 2.4c which could inhibit Cyp D inhibition and prevent cardiomyocytes from mPT induced cell death. The following Figure (Fig 4.8) describes the proposed mechanism of action of 2.4c in regulating mitochondrial permeability transition.

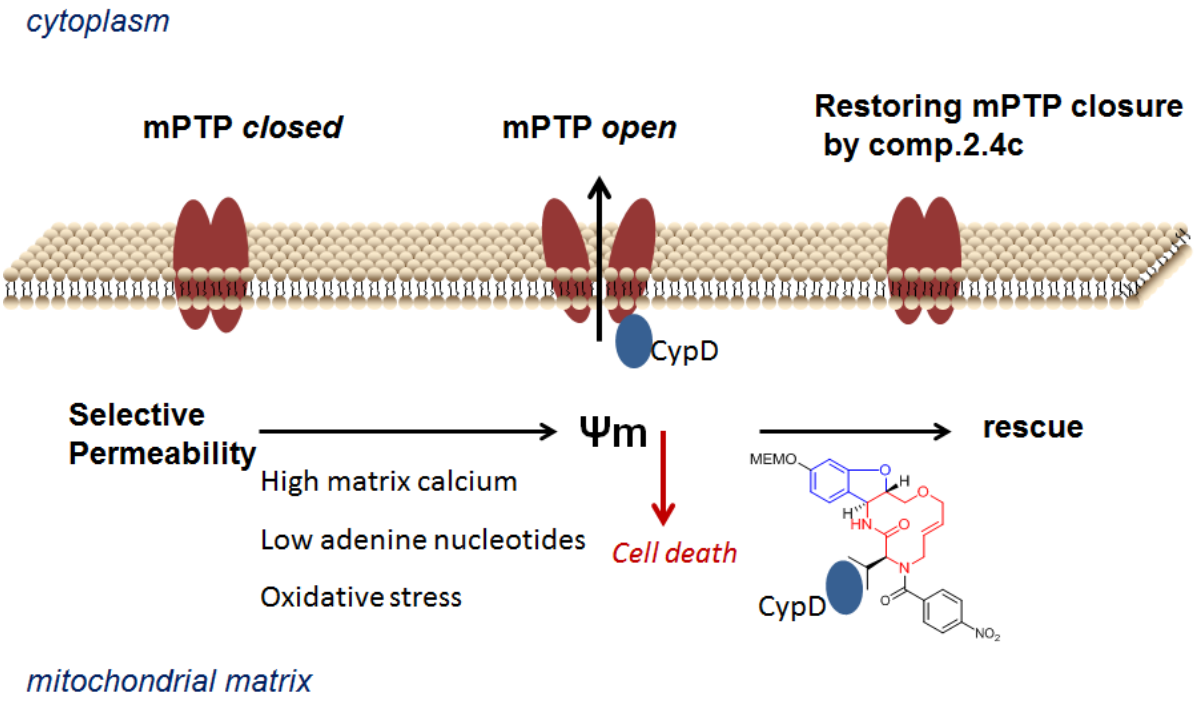

Fig 4.8: Proposed model of mechanism of action of compound 2.4c in regulating mitochondrial permeability transition.

Appendix

Harnessing Impaired Energy Metabolism in Cancer Cell: Small Molecule- Mediated Ways to Regulate Tumorigenesis

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Abstract: Altered cellular metabolism is a hallmark of tumorigenesis. Described first in 1924 by Otto Warburg, a cancer cell undergoes complete metabolic reprogramming to attain nutrient self-sufficiency for proliferation and survival. Interplay between diverse signalling cascades confers this metabolic advantage. In this review we focus on signalling molecules that regulate this altered metabolic paradigm in a cancer cell with emphasis on small molecule mediated intervention for attenuation of growth and progression of tumor.

Keywords: Warburg effect, glutaminolysis, aerobic glycolysis, Myc, Akt, mTOR, Hexokinase II

INTRODUCTION

A living cell must produce energy to remain viable and perform its genetically programmed function. This energy is stored as ATP and is released during the hydrolysis of the terminal phosphate bond. The standard energy of ATP hydrolysis under physiological conditions is known as ΔG'ATP and is tightly regulated between -53 to -60 kJ/mol in all cells [1]. In a normal cell with functional mitochondria, ΔG'ATP is derived mostly from oxidative phosphorylation; only a fraction of the amount is produced through glycolysis in the cytoplasm.

A constant ΔG'ATP of approximately -56 kJ/ mol is maintained in quiescent liver or kidney cell as well as in proliferating cancer cell [2]. However, the major difference in cellular energy metabolism between a normal cell and a cancer cell is in the origin rather than in the amount of energy generation. Most cancer cells, in contrast to their normal counterpart, generate energy through an inefficient way of aerobic glycolysis even in the presence of abundant oxygen. First observed by Otto Warburg in 1924, the phenotype is considered as an essential hall mark of cancer and possibly a causal event of tumor formation [2, 3].

A cancer cell's preference for glycolysis over energy- efficient oxidative phosphorylation has been an enigma right from the day of Warburg's observation. Several hypotheses have been put forward to address the issue, however the phenomenon still remains incompletely understood. In this review we explore the signalling networks that drive a tumor cell to shift to aerobic glycolysis thereby ensuring its progression and survival. We also discuss the differences in signalling events occurring under normal homeostatic conditions and those in a tumorigenic milieu with an aim to identify candidates amenable for therapeutic intervention.

A CANCER CELL SURVIVES IN AN ENVIRONMENT OF GLUCOSE ABUNDANCE

Cancer cells require a high ATP/ADP ratio and a high ATP/AMP ratio to ensure its proliferation and survival. This is achieved by overexpression of facultative glucose transporters (GLUT1-9) which ensures adequate glucose abundance in majority of cancer cells. Ciampy *et al*., [4] carried out expression analysis of GLUT isoforms in thyroid tumor cell lines derived from anaplastic, papillary, follicular and medullary human thyroid carcinoma as well as in malignant and benign thyroid tissues. They reported overexpression of GLUT1 but not GLUT3 in malignant human thyroid tumors. A high level of GLUT1 expression has also been documented in human colorectal adenocarcinoma [5], in primary squamous cell carcinoma of head and neck [6] and also in renal cell carcinoma [7] making it a potential target for tumor cell therapy. Wood *et al*., [8] reported that N-[4-chloro-3-(trifluoromethyl) phenyl]-3-oxobutanamide (Fasentin), a small molecule chemical sensitizer to the death receptor stimuli FAS, interact with a unique site in the intracellular channel of GLUT1 and sensitize cells to FAS induced apoptosis.

Similar to GLUT1, Sodium dependent glucose co-transporters (SGLT) induction has also been exploited by cancer cells to enhance glycolysis. Ishikawa *et al*., [9] studied the expression of SGLT1 and SGLT2 in autopsies from normal lung and lung primary tumors along with their metastatic lesions and reported SGLT2 expression to be significantly higher in metastatic lesions. These observations provide evidences that tumor cells evolve different adaptive mechanisms to ensure adequate glucose supply so that in spite of having an inefficient method of energy generation through aerobic glycolysis, the ATP/ADP ratio remains sufficiently high to ensure proliferation and survival.

GLUCOSE ENTRAPMENT IN CANCER CELL IS ACCOMPLISHED BY HEXOKINASE II

Once inside the cell glucose needs to be irreversibly entrapped through phosphorylation to glucose-6-phosphate; the reaction being carried out by one of the hexokinase present in the cell. For entrapment of glucose, hexokinase needs to have low K_M for glucose, the ability to escape product inhibition and an adequate and continuous supply of ATP. Mammalian cells harbour four hexokinases isoform HK1-4 [10] of which HK-1, HK-3 and HK-4 are present in cytoplasm. HK-4, also known as glucokinase, possesses higher K_M for glucose and function as glucose sensor in liver and pancreas. The predominant hexokinase isoform present in cancer cell is HK-2 which is associated with the outer mitochondrial membrane protein Voltage dependent anion channel (VDAC). The strategic location gives the enzyme preferential access to mitochondrial generated ATP *via* mitochondrial adenine nucleotide translocator (ANT). Moreover, location of HK-2 in mitochondrial membrane protects the enzyme from inhibition by its product Glucose-6 phosphate [11]. Thus by overproducing HK-2 and stationing it with VDAC on the mitochondrial outer membrane, tumor cell executes a clever manipulation to maintain glycolysis at an enhanced rate.

The pivotal role of HK-2 in regulating cancer cell metabolism makes it an attractive target for anti-cancer therapy. Ko *et al*., [12] and others have independently demonstrated that the use of a small-molecule analog of pyruvate, 3-bromopyruvate, eliminates large

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tumors in animals by targeting both HK-2 and mitochondrial ATP synthasome. Pre-clinical studies using 3-bromopyruvate against a multitude of tumors are currently being conducted in many laboratories [13, 14] indicating at the interest on the target for anti-cancer therapy.

EXCLUSIVE EXPRESSION OF PKM2 IN CANCER CELL ENSURES ACCUMULATION OF GLYCOLYTIC INTERMEDIATES TO BE USED AS SYNTHETIC PRECURSORS FOR NUCLEIC ACID, LIPID AND AMINO ACIDS REQUIRED FOR UNCONTROLLED PROLIFERATION

The last and rate limiting step in glycolysis is the transfer of a phosphate group from phosphoenol pyruvate to ADP to generate one molecule of pyruvate and one molecule of ATP. The reaction is catalysed by Pyruvate kinase (PK) which is largely conserved through evolution. Four mammalian PK isozymes exist (M1, M2, L and R) which are generated through alternative splicing and are expressed in different cell types. Normal adult cells constitutively express M1 while M2 is expressed in foetal cells and is re-expressed when a cell acquires tumorigenic potential. The M1 and M2 isoforms are encoded by the M gene and result from alternative splicing of exon 9 and 10. These exons encode a stretch of 56 amino acids that have amino acid differences between M1 and M2 isoforms in 22 positions [15]. These amino acid differences are concentrated near the site required for binding and allosteric activation of Pyruvate kinase M2 (PKM2) by fructose1, 6, biphosphate (FBP). Binding of FBP is known to tetramerize PKM2 to a higher active form while its release causes dissociation to a lower active form.

Switching of PKM2 to a lower active form confers a distinct advantage to cancer cell since it can shunt the key glycolytic intermediates towards pathways where they can be used to synthesize precursors for lipids, nucleic acid and amino acids. In cancer cells this is accomplished by binding of the PKM2 isoform to tyrosine-phosphorylated proteins [16] which impair binding of PKM2 to FBP. Similarly, Fibroblast growth factor receptor (FGFR)-dependent phosphorylation of PKM2 at tyrosine 105 residue causes its dimerization by the release of FBP favouring the stability of dimeric form over tetrameric form. Tumor cells thus have multiple pathways to regulate PKM2 to ensure energy metabolism through aerobic glycolytic pathway.

Studies on nude mice with cancer cells engineered to express only PKM1 shows delayed tumor formation. Moreover tumor formed in this model re-expresses PKM2 making the enzyme an attractive target for anti-cancer therapy. Boxer *et al*., recently identified substituted N, N'- diarylsulfonamides as activators of PKM2 [16] which can be an attractive option to restore oxidative phosphorylation and attenuation of lactate production thereby regulating tumorigenic potential (Fig. **1**).

THE PYRUVATE DEHYDROGENASE COMPLEX SERVES AS A GATE POINT FOR CELL'S ENTRY TO OXIDATIVE PHOSPHORYLATION

The next step of glucose metabolism is the irreversible decarboxylation of pyruvate to acetyl –CoA, NADPH and carbon dioxide [17] which is catalysed by pyruvate dehydrogenase complex (PDC). PDC determines whether carbons derived from carbohydrates will be used to generate energy or biosynthetic intermediates necessary for cell proliferation (Fig. **1**). Three catalytic components comprise PDC; pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3) and these are organized into large multimeric complexes together with structural subunit E3 binding protein (E3BP). The E1 pyruvate dehydrogenase complex is a hetero tetramer of two alpha and two beta subunits which catalyzes the first step of pyruvate decarboxylation. The activity of PDC is regulated by reversible phosphorylation of three serine residues of E1 α. Pyruvate dehydrogenase kinase (PDK1-4) inactivates PDC while pyruvate dehydrogenase phosphatase (PDP1-2) activates the complex [17].

Regulation of PDC by PDK and PDP provides an important tool for regulation of cancer cell metabolism. Dichloroacetate (DCA) has been identified as PDC activator through its ability to inhibit PDK activity [17]. The crystal structure of PDK2 in complex with DCA shows that DCA occupies the pyruvate binding site in the N-terminal regulatory domain. Of the four isozymes, PDK2 is most sensitive to DCA inhibition, PDK1 and PDK4 are moderately sensitive and PDK3 is least sensitive.

The importance of DCA in inhibiting tumor growth emerges from the work of Bonnet *et al*., who demonstrated that DCA suppresses the growth of A549 lung tumor xenografts in rats [18]. Papandreou *et al*., [17] also reported that daily DCA treatment of mice with pancreatic SU86.86 xenografts causes significant tumor growth delay. These results are in agreement with initial clinical trial data [19] where DCA has been used in combination with surgery, temozolomide and radiation for treatment of five patients with glioblastoma multiforme. The authors reported promising clinical results in four out of five patients with significant change in mitochondrial membrane potential, increased amounts of mitochondrially generated oxygen radicals and increased tumor cell apoptosis. Mechanistic studies on DCA mediated regulation of growth and progression of cancer cells reveal altered level of HIF1 signaling, p53 activation and decreased angiogenesis indicating involvement of multitudes of signaling events downstream of PDKs in regulating the process.

AEROBIC GLYCOLYSIS IS ASSOCIATED WITH CREATION OF A MICROENVIRONMENT SUITABLE FOR TUMOR CELL METASTASIS

Attenuation of oxidative phosphorylation in cancer cells ensures generation of lactic acid through glycolysis which conditions the environment for tumor invasion [20]. Muller recently reported that primary lesions with metastasis contain significantly higher amount of lactate than non-metastatic tumors and survival of patients with high-lactate tumours is worse than low lactate-tumours [21]. One of the major advantages that lactic acid generation confers is to help the tumor evade the host's immune response by suppressing the proliferation and cytokine production of human cytotoxic T lymphocytes (CTLs) [22]. However the most important aspect of lactate generation is the establishment of an exquisite symbiosis in which glycolytic and oxidative tumor cells mutually regulate their access to energy metabolites [23]. A tumor in fact offers a heterogeneous microenvironment where a subpopulation remains in aerobic environment and another subpopulation remains in a hypoxic milieu. Sonveaux *et al*., [24] showed that the lactate produced by hypoxic cells is taken up by aerobic cells which in turn use it as a prominent source of energy defining a concept of metabolic symbiosis.

Shuttling of lactate between hypoxic and aerobic tumor cells is carried out by monocarboxylate transporters (MCT). At least 9 MCT (MCT1-9)-related genes have so far been identified in mammals, each having a different tissue distribution. Among all MCTs, MCT1 is expressed in a variety of cell lines and primary tumors including breast, head and neck and lung cancer [24] as well as in neuroblastoma [26], brain [27] and colon cancer [28]. It has been shown that while lactate is released from cells through the low affinity transporter MCT4, lactate uptake is carried out by the high affinity transporter MCT1. Immunostaining reveals presence of MCT1 in tumor cell populations at well vascularized area and MCT4 in hypoxic regions justifying their respective function in the shuttling phenomenon [24].

Broad MCT1 expression among human cancers and its role in fuelling tumor cells opens up opportunity for the development and clinical evaluation of MCT1 inhibitors as potential anticancer

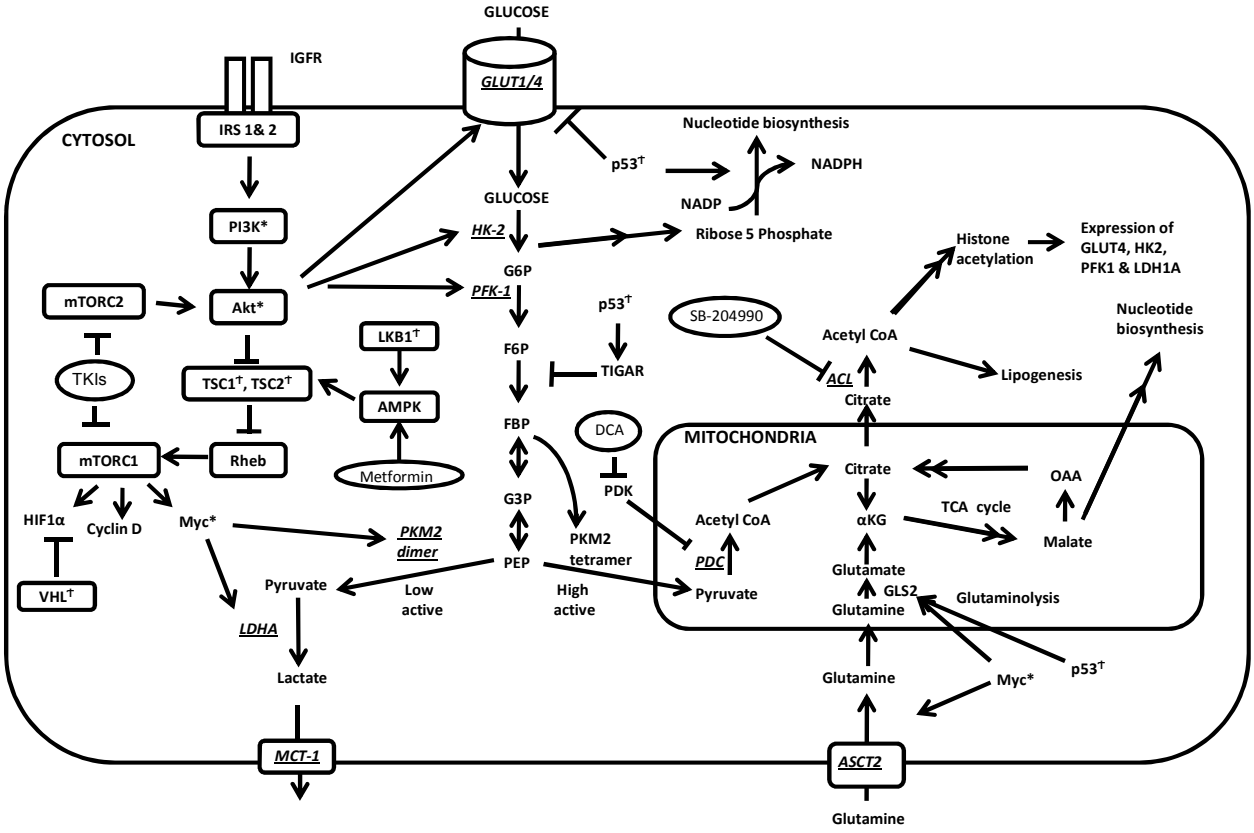


Fig. (1). Signaling networks that regulate cancer cell metabolism. The figure summarizes salient feature of regulation of glucose and glutamine metabolism in a cancer cell. Growth factor stimulation activates PI3K/Akt pathway which promotes glucose uptake and stimulates glycolysis. Phosphorylated Akt activates mTORC1which stimulates Myc resulting in increased PKM2 gene expression and enhanced lactate generation. Tyrosine kinase signaling impairs binding of FBP to PKM2 keeping the latter in low active state thus ensuring availability of intermediates of glycolytic pathway for generation of biosynthetic precursors necessary for cell proliferation. Myc regulates LDH A and also promotes glutamine uptake and glutaminolysis. Citrate exported to cytoplasm is converted to acetyl CoA by ATP-citrate lyase which regulates lipogenesis and epigenetically controls aerobic glycolysis through regulation of expression of Glut4, HK2, PFK1 and LDHA. The enzymes which are targets for cancer are in italics and underlined, ***** indicates oncogenes and indicates tumor suppressors; small molecules that intervene in the pathways are depicted in oval, double arrow in one side indicates multiple steps in a pathway and curved arrow signifies allosteric activation. HK-2:Hexokinase 2; PFK-1:Phospho fructo kinase1; LDH A: Lactate dehydrogenase A; PKM2: Pyruvate kinase M2; TSC1& 2: Tuberous sclerosis protein 1& 2; mTORC1& 2: mammalian target of rapamycin complex1&2; VHL: von Hippel-Lindau Protein; TKIs: ATP competitive mTOR kinase inhibitors; TIGAR: TP53 Induced Glycolysis and Apoptosis Regulator; DCA: Dichloroacetate; PDK: Pyruvate dehydrogenase kinase; SB-204990:ATP citrate lyase inhibitor; ACL: ATP citrate lyase; PDC: Pyruvate dehydrogenase complex; KG: Ketoglutarate; GLS2: Glutaminase 2; OAA: Oxalo acetic acid, ASCT2- Sodium-dependent neutral amino acid transporter type 2.

agents. Hypoxic tumor cells are mostly resistant to chemotherapy and radiation, thereby leading to treatment failure or disease relapse [25]. Sonveaux *et al*., [24] has made a pioneering observation that treatment of tumor- bearing mice with MCT1 specific siRNA or with α-cyano-4-hydroxycinnamate, a small molecule inhibitor of MCT1, provides a new mechanism to kill cancer cells. The authors conjectured that MCT1 inhibition disrupts the lactate shuttle between aerobic and hypoxic tumor cells. Aerobic cells increase their uptake of glucose thereby depriving hypoxic cells from adequate nutrition. In their seminal paper, Sonveaux *et al*., also showed that growth delay of mouse tumor xenografts induced by radiotherapy is increased when combined with MCT1 inhibition [24].

ATP CITRATE LYASE LINKS AEROBIC GLYCOLYSIS WITH DE-NOVO FATTY ACID SYNTHESIS IN TUMOR CELLS

In addition to aerobic glycolysis, *de novo* lipogenesis is also a salient feature of cancer cell metabolism. Fatty acids play an essential role in variety of cellular process [29]. They are obtained from the diet or can be synthesized from carbohydrate precursors in liver and adipose tissue. *De novo* fatty acid synthesis is very active during embryogenesis; however, in adults, it is limited only to lactating breast and cycling endometrial tissue.

Cancer cells, on the contrary, display high level of fatty acid synthesis [30]. ^{14}C glucose studies have shown that in tumor cells almost all fatty acids are derived from *de novo* synthesis despite having abundance of extracellular lipids [31,32]. This glucose dependent increased lipogenesis results as a consequence of truncated TCA cycle in tumor cells [33, 34,] which instead of ATP generation exports citrate to cytoplasm to be converted by ATP citrate lyase(ACL) to lipogenic precursor acetyl CoA (Fig. **1**).

In addition to its role in linking glucose metabolism to tumor-associated enhanced lipogenesis, ACL has very recently been implicated in global transcriptional control through regulation of histone acetylation [35]. Wellen *et al*., [35] showed that suppressing ATP-citrate lyase expression by siRNA decreases global histone acetylation in several different mammalian cell types. Their studies also describe an interesting connection between ATP-citrate lyase mediated acetyl CoA production and expression of Glut4,

hexokinase2 (HK-2), phosphofructokinase-1(PFK-1) and lactate dehydrogenase A (LDH-A) in adipocytes which are the key regulators of glycolysis. This effect on glucose metabolism seems to be selective, since markers of adipocyte differentiation are not modulated by ACL ablation [35].

Marked elevation of ACL expression and activity has been reported in different cancer cells [36,37]. The expression of phosphorylated ACL in human lung adenocarcinoma correlates with stage, differentiation grade and poor prognosis of the tumor [38]. Moreover ACL inhibition by siRNA or selective inhibitor SB-204990 supresses the growth of tumor cells *in vitro* and *in vivo* [36, 37, 38] making it an attractive target for cancer therapy. The ability of ACL inhibition to suppress tumor growth is in correlation with glycolytic phenotype of the tumor [37]; cancer cells with high rates of glucose metabolism are severely affected while those with low rate of aerobic glycolysis are largely unaffected. This differential sensitivity of tumor cells to ACL inhibition indicates at the crucial role of the enzyme in regulating the growth and proliferation of glycolytic tumors. ACL expression increases on growth factor stimulation [35] and identification of ACL as Akt substrate [39] highlights its potential to contribute to tumorigenesis.

ACTIVATION OF ONCOGENES AND LOSS OF TUMOR SUPPRESSOR GENES CONTRIBUTE TO METABOLIC REPROGRAMMING REQUIRED FOR CANCER CELL'S PROLIFERATION AND SURVIVAL

Mounting evidence indicates that ATP production and generation of biosynthetic precursors are under direct regulation of oncogenes and tumor suppressors. Activation of oncogenes such as Myc, Ras, Akt and phosphatidylinositol-3-kinase and loss of tumor suppressor genes such as p53, LKB1and VHL (Von Hippel Lindau) triggers transcriptional and post-transcriptional alterations that rewires the signalling network of a tumor cell and helps it to attain metabolic autonomy [40].

PI3K/Akt pathway is considered as a major signalling cascade which regulates both growth control and glucose metabolism. Akt activation regulates glucose transporter expression, enhances glucose capture by hexokinase, increases phosphofructokinase activity [41] and stimulates glucose to lactate metabolic pathway thereby promoting tumorigenesis [42]. In addition, IGF1/PI3K/Akt pathway is known to regulate mTORC1which acts as a scaffold to recruit downstream substrates such as eukaryotic translation initiation factor 4E binding protein 1(4EBP1) and ribosomal S6 kinase (S6K1). mTORC1 controls the translation of many cell growth regulators including cyclin D1, hypoxia inducible factor 1a (HIF1a) and Myc which promotes cell cycle progression, cell growth and angiogenesis [43]. mTORC1 is nutrient sensitive and is regulated by tumor suppressors like LKB1, TSC1and TSC2 (Fig. **1**). Loss of function mutation of these tumor suppressors plays a critical role in tumor initiation and progression.

The mTOR signalling is deregulated in many different human tumors making it an attractive target for cancer therapy. A well-known mTOR inhibitor is rapamycin which is a macrocyclic natural product that inhibits mTOR kinase activity by forming a complex with the immunophilin FK506-binding protein of 12kDa (FKBP12). FKBP12 binds to the C terminus of mTOR that is adjacent to catalytic site. This protein–protein interaction interferes exclusively with the kinase activity of the mTORC1 complex, but does not inhibit all the functions of mTOR; it does not block mTORC2 kinase activity either [44].

Currently, several of mTOR inhibitors are in clinical trials (Table **1**) and among them rapamycin analogs temsirolimus and everolimus have been approved by US Food and Drug Administration (USFDA) for treatment of advanced kidney cancer. However rapamycin has limited efficacy in majority of tumors due to a negative feedback loop that activates Akt in preclinical and clinical settings [45]. Recent research advocates the use of ATP-competitive mTOR inhibitors (TKIs) that bind to the active site of mTORC1 and mTORC2 thereby effecting a global inhibition on mTOR activity [46]. Global inhibition of mTOR has a selective advantage for inhibition of tumor progression since attenuation of mTORC2 activity by TKIs impair phosphorylation of Akt at serine 473 residue inhibiting aerobic glycolysis, lipogenesis and lactate production in a tumor cell.

A pyrazolopyrimidine derivative PP242 is one of the first selective ATP-competitive mTOR inhibitor described in literature [47], (Table **1**). Several studies have shown that PP242 blocks tumor cell proliferation and phosphorylation of mTORC1 substrate 4EBP1 more effectively than rapamycin (reviewed in 44). Most importantly, the compound inhibits mTORC2 complex and attenuates downstream Akt activation underlying its immense importance for the development of anticancer therapeutic agents.

One of the potential pitfalls of ATP-competitive mTOR inhibitors is their toxicity [48]. However TKIs are reported to be well tolerated in short term xenografts studies [49]. A second caveat is that mTORC1 inhibition may activate autophagy which in turn may promote cancer cell survival [50]. In that context specific mTORC2 inhibitors might be of future therapeutic relevance.

Another mechanism of regulating mTOR pathway is by using low doses of metformin, a biguanide which is also a standard antidiabetic agent [51]. Metformin exerts its effects by activating LKB1 and its downstream target AMPK which in turn suppresses mTORC1 through activation of tumor suppressor TSC2 (Fig. **1**), [51]. More recently metformin has also been reported to regulate mTORC1 in AMPK independent manner through Rag family of trans membrane GTPases [51].

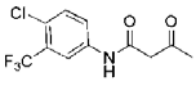
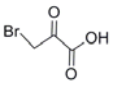
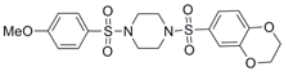
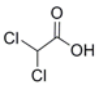
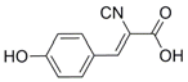
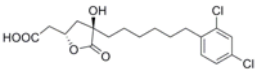
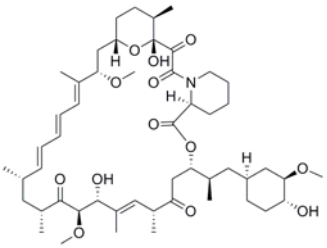
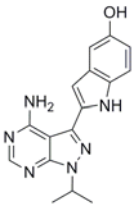
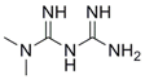
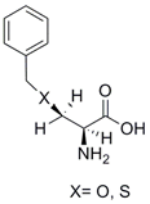
Inhibition of mTOR pathway by metformin makes the latter an attractive molecule for potential anticancer therapy. Several preclinical models have demonstrated the efficacy of metformin in reducing the tumor burden. Intraperitoneal administration of metformin reduces NNK (4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone) induced lung tumorigenesis in A/J mice [52]. Treatment with metformin selectively suppresses the tumor growth of HCT116 $p53^{-/-}$xenografts in nude mice [53]. Also nude mice bearing tumor xenografts of the triple negative breast cancer cell line MDAMB231 shows significant reduction of tumor growth when treated with metformin [54].

Several clinical trials are currently underway to test the efficacy of metformin as an adjuvant to conventional chemotherapy as well as in combination with new targeted agents in various settings such as breast cancer and other solid malignancies [51]. The outcome of these clinical trials will be of great importance in understanding the role of metabolic perturbations in regulating tumor growth and survival.

Amino acids also can activate mTORC1 signaling cascade [55]. Leucine and glutamine has been reported to cause maximum activation of mTORC1 [56, 57]. Glutamine, in addition to its role in protein translation, is involved in production of α ketoglutarate, an oxidative substrate of TCA cycle and also in production of lactate by glutaminolysis. Real time ^{13}C NMR studies have shown that a significant amount of glutamine carbon is converted to lactate and secreted out of the cell. This is analogous to the effect described by Warburg on glucose metabolism in cancer cell [58]. Conversion of glutamine to lactate requires the activity of malic enzymes which oxidatively decarboxylates malic acid producing carbon dioxide, pyruvate and NADPH, the latter being used for lipid and nucleotide biosynthesis [59]. Thus glutamine serves as important source for cellular energy and anabolic carbon and nitrogen required for growth and progression of tumor.

Recent studies using quantitative RT-PCR and chromatin immunoprecipitation in multiple cell systems reveal the participation of Myc oncogene in regulating glutamine metabolism. Myc activa-

Table 1. Small Molecule Mediators of Cancer Cell Metabolism

Target	Inhibitors/Activators	Chemical Structures	Action	Stages of Therapeutic Development	Reference
GLUT1	N-[4-Chloro-3-(trifluoromethyl)phenyl]-3-oxobutanamide (Fasentin)		blocks Glut1 mediated glucose uptake	Preclinical	[8]
Hexokinase II	3-bromopyruvate		Inhibits Hexokinase II	Preclinical	[11]
PKM2	*N, N'* diarylsulfonamides		Stabilize and activate the tetramer form of PKM2	Preclinical	[16]
	TLN-232	Cyclic heptapeptide	Inactivates dimeric form of PKM2	Phase II	[65]
Pyruvate dehydrogenase complex	Dichloroacetate (DCA) (salts or esters)		Inhibit Pyruvate dehydrogenase kinase.	Phase II	[17]
MCT1	Cyano-4-hydroxycinnamate		Reversible inhibition of MCT1	Preclinical	[26]
ATP citrate lyase	SB-204990		Inhibits lipogenesis	Preclinical	[64]
mTORC1 (Allosteric)	Rapamycin		mTORC1 allosteric inhibitor	Rapamycin analogues Temsirolimus and Everolimus are US FDA approved for treatment of advanced renal carcinoma	[44]
mTOR C1 &mTOR C2 (ATP-competitive)	Pyrazolopyrimidine		mTORC1 and mTORC2 dual inhibitors	Preclinical	[44]
AMPK	Metformin		AMPK activator	Phase III study initiated for breast cancer	[51]
ASCT2	Benzyl serine, Benzyl cysteine	 X= O, S	Compete with glutamine for ASCT2 binding.	Preclinical	[63]

tion and amplification is one of the common events in human tumors and Myc transformed cells are particularly sensitive to glutamine withdrawal resulting in rapid loss of TCA cycle intermediates and cell death [60]. Myc up regulation has recently been linked to increased glutaminolysis (reviewed in 59) and transcriptional activation of two high affinity glutamine transporters: SLC38A5 (SN2) and SLC1A5 (ASCT2) which are required for glutamine-dependent mTORC1 activation. In addition, Myc promotes conversion of glutamine to glutamate and ultimately to lactate facilitating tumor growth under hypoxic condition.

Glutamine dependency of cancer cell lines has stimulated research on glutamine analogues as anticancer agents; however major caveat against glutamine analogues is their dose limiting neurotoxicity, gastrointestinal toxicity and myelosuppression which limit their use under clinical settings. Present research focuses on attenuation of cancer cell glutamine uptake by using L-γ-glutamyl-ρ-

nitroanilide, one of a panel of SLC1A5 inhibitors, or by suppression of glutamine-dependent anaplerosis by transaminase inhibitor amino-oxy acetic acid (AOA) through blocking the entry of glutamine carbon in TCA cycle. The extensive *in vivo* utility of these approaches are currently under investigation [63, 64].

CONCLUDING REMARKS

Metabolism essentially directs the functionality of a cell. The evolving evidence indicates that cell metabolism is reprogrammable appropriately adjusted to specific functional necessities of a cell. The necessity of cancer cells to attain uncontrolled proliferation and nutrient self-sufficiency requires metabolic rewiring and this is mostly accomplished by activation of oncogenes and loss of tumor suppressor genes. Tumorigenesis is an ever evolving process where a cell adapts to constantly changing microenvironment by modifying its metabolism in synchrony with the altered milieu it has to deal with. A cell's journey to malignancy may be interpreted as a gradual shift of metabolic balance between oxidative phosphorylation and aerobic glycolysis accomplished in part by upregulation of IGFR/PI3K/Akt signaling cascade as well as by deregulated expression of Myc oncogene. The extent of activation/expression and regulation by tumor suppressors determines the metabolic advantage bestowed on a tumor cell at a particular stage of malignancy and whether small molecule mediated intervention can convert that advantage to therapeutic vulnerability will be unfurled by outcome of future research.

ABBREVIATIONS

G6P	=	Glucose 6 phosphate
F6P	=	Fructose 6 phosphate
FBP	=	Fructose1, 6 bisphosphate
G3P	=	Glyceraldehyde 3 phosphate
PEP	=	Phosphoenolpyruvate
SGLT	=	Sodium glucose transport proteins
MCT-1	=	Monocarboxylate transporter1
HIF1A	=	Hypoxia inducible factor 1A
NADPH	=	Nicotinamide adenine dinucleotide phosphate bonded with Hydrogen
Acetyl CoA	=	Acetyl coenzyme A
TCA cycle	=	Tricarboxylic acid cycle

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SHORT COMMUNICATION

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Prevention of Mitochondrial Membrane Permeabilization and Pancreatic β-Cell Death by an Enantioenriched, Macrocyclic Small Molecule

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Mitochondria produce the majority of cellular energy through the process of oxidative phosphorylation and play a central role in regulating the functionality and survival of eukaryotic cells. Under physiological stress, mitochondrial membrane permeabilization results in the release of apoptogenic material such as cytochrome c in the cytoplasm, which thereby initiates caspase activation and the consequent cell death. In our present study, we screened a series of compounds for their ability to inhibit mitochondrial membrane permeabilization and to prevent cytochrome c release during the endoplasmic reticulum stress in cultured pancreatic β-cells. Three benzofuran-based macrocyclic small molecules, that is, **2.4c**, **c104**, and **c108**, were found to restore the depolarization of mitochondrial membrane potential and to prevent the release of cytochrome c from mitochondria. Interestingly, the acyclic precursor of **2.4c** (i.e., **2.3c**) did not show any effect, whereas the macrocyclic derivative obtained by utilizing ring-closing metathesis as the "stitching technology" led to this function. The macrocyclic architecture seems to play a crucial role in presenting various functional moieties in the right orientation to observe this effect.

Introduction

Mitochondria play an essential role in pancreatic β-cell homeostasis through their involvement in the modulation of stimulus-coupled insulin secretion[1] and in the regulation of cell survival.[2,3] The permeabilization of mitochondrial membranes under the influence of various cytokines results in the release of cytochrome c, which is known to activate the caspase cascade.[4] Most importantly, during chronic endoplasmic reticulum (ER) stress, the accumulation of unfolded proteins in the ER results in leakage of calcium from the ER, which leads to calcium overload in the mitochondria[5,6] and the consequent opening of the mitochondrial permeability transition pore. The latter process plays a decisive role in the depolarization of the mitochondrial membrane potential and programmed cell death.

In our present study, we induced chronic ER stress in cultured BRIN-BD11 pancreatic β-cells by treating them with the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pump inhibitor thapsigargin (note: structure not shown). Thapsigargin treatment causes the depolarization of the mitochondrial inner membrane potential and compromises cell survival.[7] To prevent this depolarization, we utilized a small-molecule toolbox having 18 enantioenriched, benzofuran-derived compounds with 12-membered macrocyclic rings that are rich in 3D architectures and that can be considered members of the broad family of natural-product-inspired compounds. Our data reveals that enantioenriched benzofuran-based macrocycles having a 12-membered ring, which we synthesized, prevent the depolarization of the mitochondrial membrane potential and inhibit apoptogenic cytochrome c release from mitochondria in cultured pancreatic β-cells.

Results and Discussion

There is growing interest in accessing small molecules that are inspired by bioactive natural products having 3D architectures to explore their biological functions.[8] They could have either multiple rings or macrocyclic architectures. The latter is quite interesting,[9] because there are numerous examples of complex macrocyclic natural products exhibiting a wide range of biological properties.[10] Owing to several advantages that are associated with macrocyclic rings, there is also growing interest[9,11] in developing modular synthesis methods that allow a diverse chemical toolbox having different types of macrocyclic architectures to be ob-

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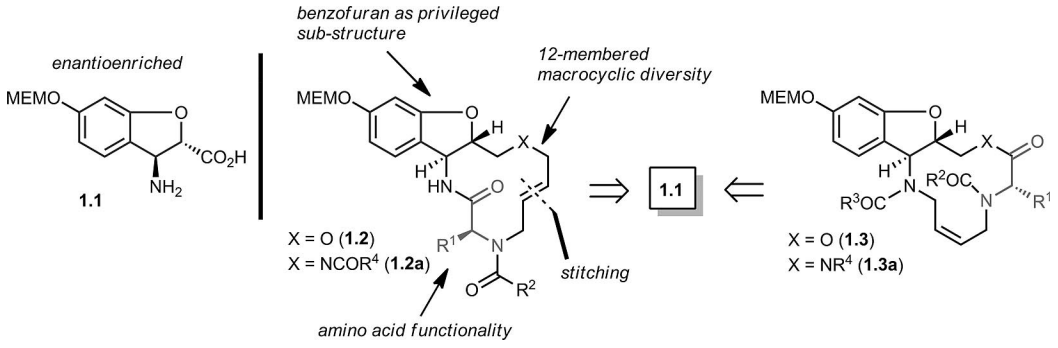
tained. Some of these advantages[10] include (1) preorganization, (2) enhanced cell permeation properties, and (3) the possibility of having numerous binding interactions, a property that could be highly relevant to search for small molecule modulators of protein–protein[12] and other types of biomacromolecular (e.g., DNA/RNA–protein)[13] interactions.

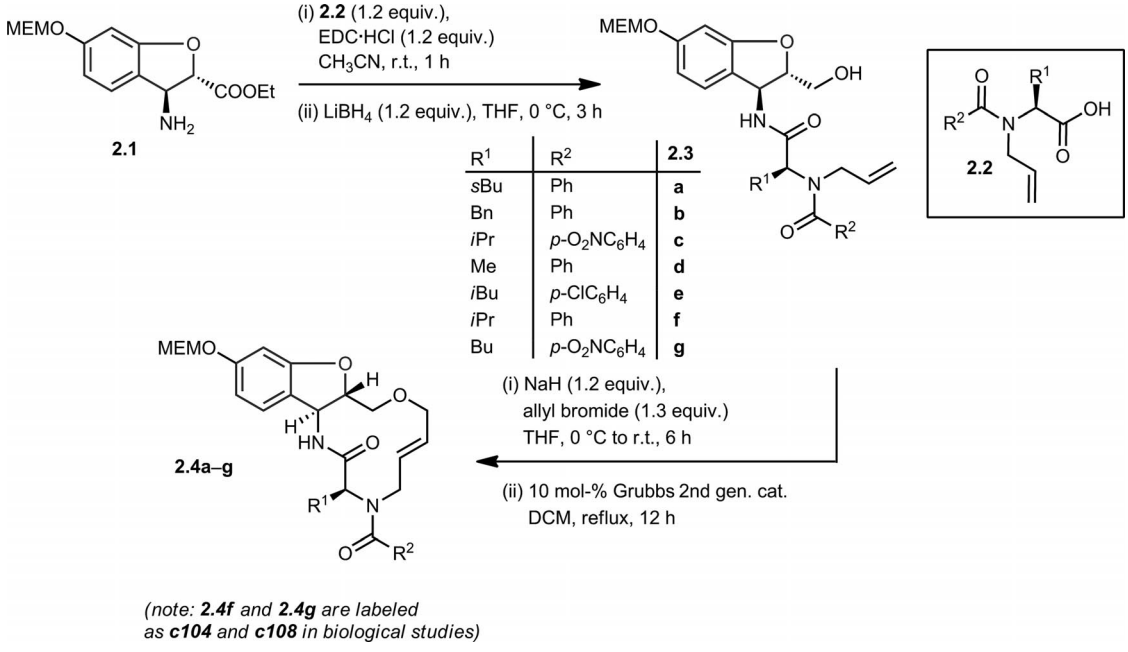
Earlier, we reported an enantioenriched synthesis of benzofuran-based, 1,2-*trans*-β-amino acid **1.1** (Scheme 1).[14] In this article, with the objective to explore further the additional large-ring chemical space, we report here a modular approach that allowed us to incorporate two different types of 12-membered macrocyclic rings onto the benzofuran scaffold (Scheme 1; see **1.2/1.2a** and **1.3/1.3a**). The presence of an amino acid moiety within the macrocyclic architecture is an attractive feature to introduce a diverse array of chiral side chains having a variety of polarities.

Shown in Scheme 2 is our plan to obtain a 12-membered macrocyclic ring that utilizes benzofuran-based *trans*-β-amino ester **2.1**. Compound **2.2**, a derivative of an *N*-protected amino acid, was prepared (see Supporting Information). In each case, following the derivatization of –OH as –OAllyl, the sample was submitted to ring-closing metathesis[15] (RCM, Grubbs 2nd generation catalyst) as the "stitching technology". All macrocyclic compounds that were synthesized are stable at room temperature.

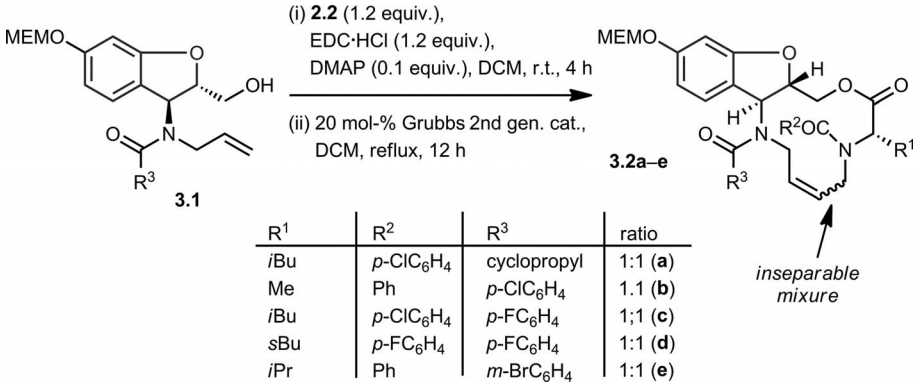
In our next approach, we were interested in accessing a different type of macrocyclic architecture, that is, **3.2** (Scheme 3), that would arise from coupling the amino acid moiety to the primary hydroxy side of the benzofuran scaffold. To achieve this, we synthesized **3.1** as the *N*-protected chiral amine-based benzofuran derivative. This was further coupled with the *N*-allyl derivative of the *N*-protected amino acid that produced the starting material required for the Grubbs RCM-based stitching technology. Our last ap-



Scheme 1. Proposed synthesis of four different types of benzofuran-derived macrocyclic compounds, that is, **1.2/1.2a** and **1.3/1.3a**, from enantioenriched **1.1**. MEM = (2-methoxyethoxy)methyl.



Scheme 2. Synthesis of 12-membered macrocyclic derivative **2.4**, which is derived from *N*-functionalized 1,2-*trans*-amino alcohol **2.3**. EDC = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide,.

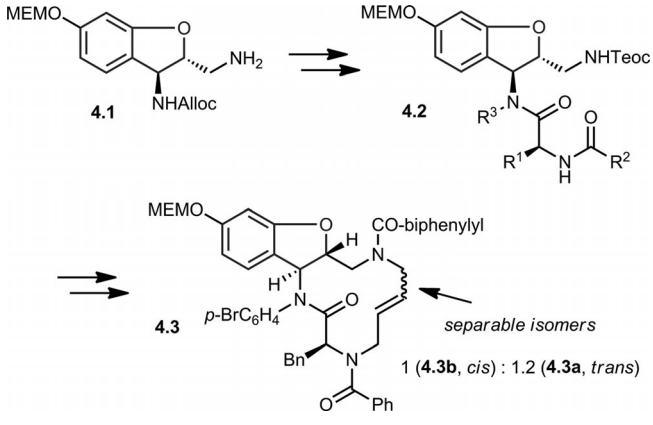


R^1	R^2	R^3	ratio
*i*Bu	*p*-ClC_6H_4	cyclopropyl	1:1 (**a**)
Me	Ph	*p*-ClC_6H_4	1.1 (**b**)
*i*Bu	*p*-ClC_6H_4	*p*-FC_6H_4	1;1 (**c**)
*s*Bu	*p*-FC_6H_4	*p*-FC_6H_4	1:1 (**d**)
*i*Pr	Ph	*m*-BrC_6H_4	1:1 (**e**)

Scheme 3. Macrocycle **3.2** from *N*-functionalized, enantioenriched, 1,2-*trans*-amino alcohol **3.1**.

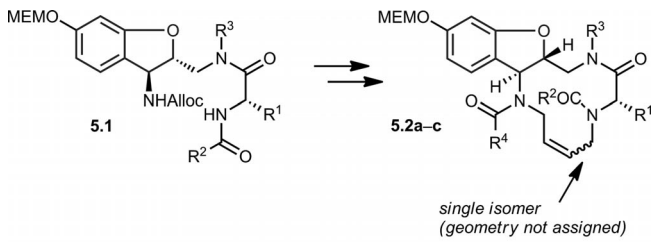
proach involved the synthesis of **3.2** (Scheme 3; see also Supporting Information) that would arise from coupling the amino acid moiety to the primary hydroxy side of the benzofuran scaffold. The detailed synthesis plan is shown in the Supporting Information.

In another plan (Scheme 4; see also Supporting Information), the goal was to replace the primary –OH group by an $–NH_2$ group to allow the incorporation of three diversity sites into the 12-membered ring. To achieve this synthesis, **4.1** as the starting material was utilized. This produced **4.2** in several easy steps that included (1) primary $–NH_2$ protection as an –NTeoc group {Teoc = [2-(trimethylsilyl)ethoxy]carbonyl}, (2) –NAlloc removal (Alloc = allyloxycarbonyl), (3) reductive alkylation (R^3 as the diversity point), (4) coupling with the protected amino acid (to introduce two diversity sites as R^1 and R^2). Finally, **4.2** was subjected to –NTeoc removal, amidation, which further upon bis(allylation) produced the key precursor for the RCM-based stitching technology. Once again, the RCM approach worked well, and **4.3** as the macrocyclic derivative was obtained as a mixture of two olefinic compounds. The details of the synthesis plan are provided in the Supporting Information.



Scheme 4. 12-Membered macrocycle **4.3** from 1,2-*trans* chiral diamine **4.1** (see Supporting Information).

As described earlier, a similar approach that utilized **5.1** (Scheme 5) as a starting material led to the synthesis of macrocyclic derivative **5.2**. Using the orthogonally protected chiral amine derivative of benzofuran, **5.1** was easily prepared. This compound has the coupled protected amino acid moiety on the primary amine and contains three diversity sites (R^1, R^2, and R^3). Once subjected to –NAlloc removal, it was then derivatized through amide coupling, followed by bis(allylation) needed for the RCM technology. This approach was attempted with three cases, and macrocyclic product **5.2** was easily obtained as a single isomer, although the olefin geometry remains to be assigned. The detailed synthesis methods are provided in the Supporting Information.



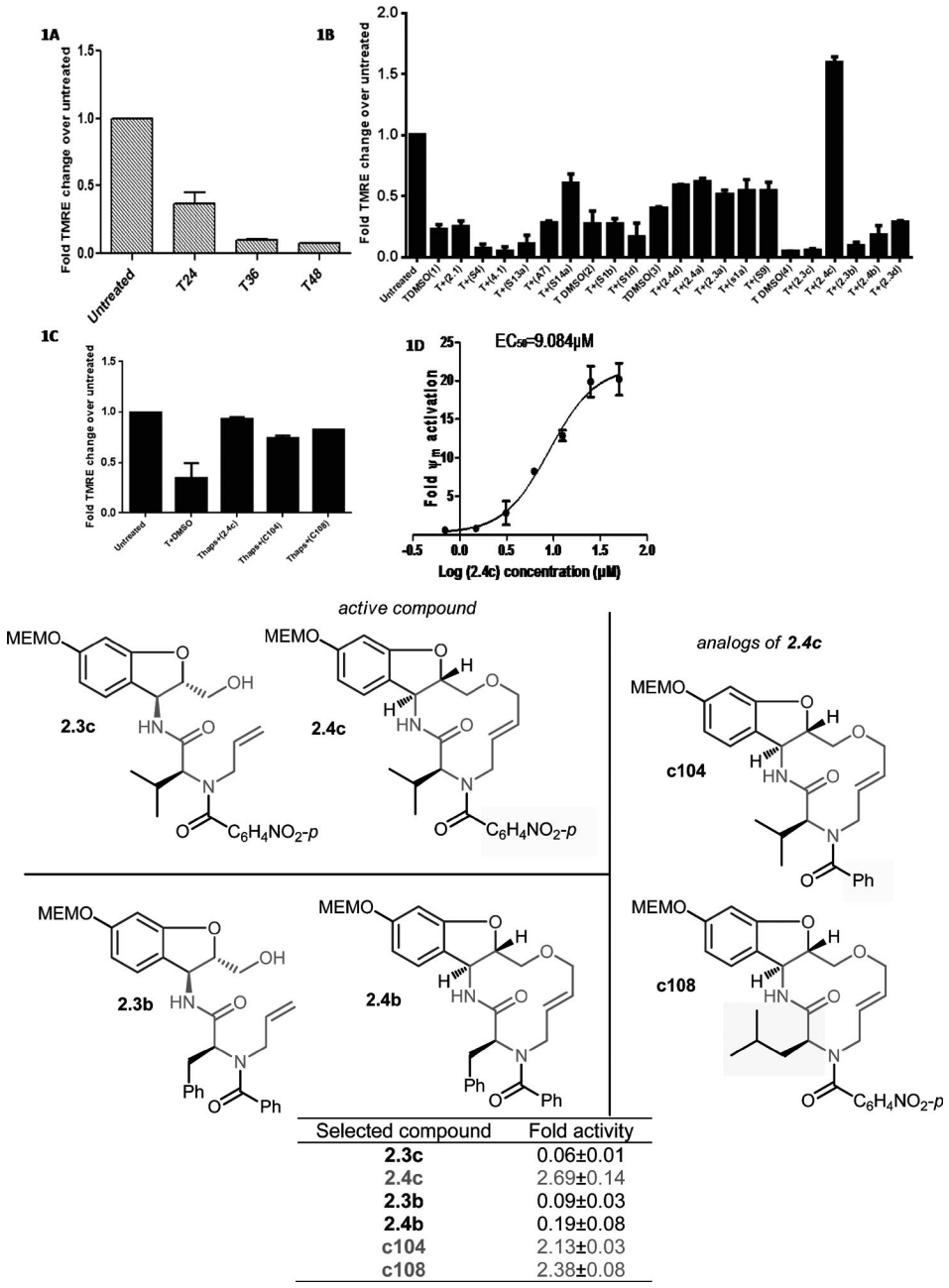
R^1	R^2	R^3	R^4	**5.2**
Bn	Ph	*p*-$BrC_6H_4C(=O)$	biphenylyl	**a**
*i*Pr	naphthyl	*p*-$BrC_6H_4C(=O)$	Ph	**b**
*i*Bu	*p*-ClC_6H_4	Bn	*m*-BrC_6H_4	**c**

Scheme 5. Synthesis of macrocycle **5.2** from *N*-functionalized, chiral 1,2-*trans* diamine **5.1** (see Supporting Information).

Prevention of Thapsigargin-Induced Mitochondrial Depolarization

Benzofuran-based macrocycles reported in this study showed remarkable activity to prevent the depolarization of the mitochondrial membrane potential (MMP) under thapsigargin-induced ER stress. Figure 1a shows the temporal effect of thapsigargin on the depolarization of the MMP. As the data reveals, a 36 h treatment of thapsigargin at a concentration of 5.0 μM caused a 10-fold reduction in the mitochondrial membrane potential. To prevent this depolarization of the MMP, we screened a library of benzofuran-based compounds to study their efficacy to rescue the phenotype (Figure 1b,c). Compound **2.4c** was found to prevent the depolarization of the MMP induced on thapsigar-

gin treatment in cultured pancreatic β-cells. Compound **2.4c** possessing a 12-membered macrocyclic ring and having an *N*-(4-nitrobenzoyl)valine amino acid moiety fused to the benzofuran scaffold showed the highest activity for the prevention of thapsigargin-induced depolarization of the mitochondrial membrane potential. To validate the structural features of this compound, we further synthesized two more related macrocyclic compounds, that is, **c104** and **c108**. In **c104**, the *N*-(4-nitrobenzoyl)valine is replaced by an *N*-benzoylvaline unit (i.e., no NO_2 group), whereas the amino acid moiety in **2.4c** is replaced by leucine to obtain **c108**. A comparative account of the efficacy of all these macrocycles to prevent thapsigargin-induced depolarization of the MMP is shown in Figure 1. Compounds **2.4c**, **c104**, and **c108** showed comparable efficacy in the prevention of thapsigargin-induced depolarization of the MMP at a concentration of 10.0 μM. Interestingly, the acyclic precursor of **2.4c** (i.e. **2.3c**) did not show any effect. In addition, replacement of the *N*-benzoylvaline amino acid moiety with an *N*-benzoyl-(phenylalanine) (i.e., **2.4b**) group dramatically reduced the



Selected compound	Fold activity
2.3c	0.06±0.01
2.4c	2.69±0.14
2.3b	0.09±0.03
2.4b	0.19±0.08
c104	2.13±0.03
c108	2.38±0.08

Figure 1. Attenuation of thapsigargin-induced mitochondrial depolarization: (a) Temporal depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) in pancreatic β-cells. (b and c) Screening potential of compounds that prevent the depolarization of the MMP. (d) Dose–response curve for the rescue of thapsigargin-induced depolarization of $\Delta\Psi_m$ by **2.4c**. (Note: for detailed structural information of all compounds tested, see Supporting Information).

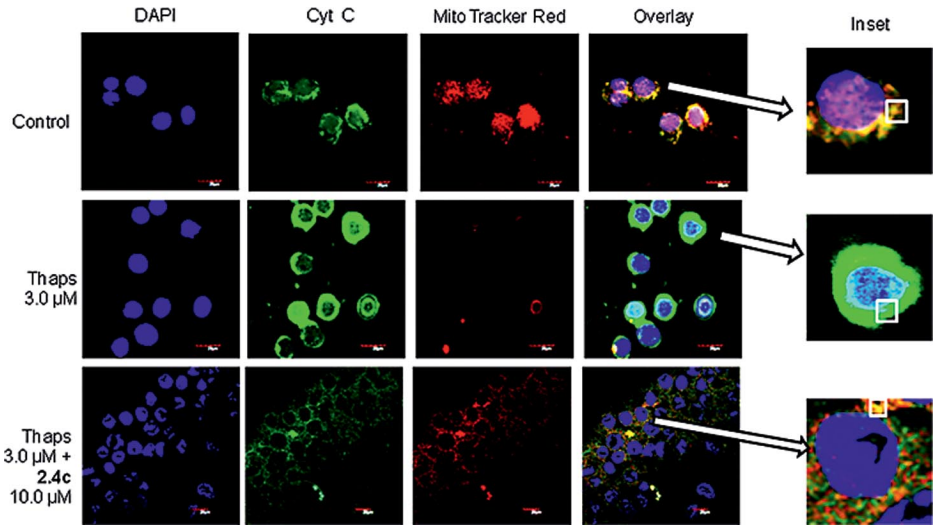


Figure 2. Prevention of cytochrome c release from thapsigargin-treated cells upon treatment with **2.4c**. Confocal microscopy images of BRIN-BD11 cells immune-labeled with primary monoclonal mouse anti-cytochrome c antibody (green) depicting release of cytochrome c from the mitochondria. Mitochondria are labeled by Mito-tracker Red (red), and the nucleus is visualized by DAPI (blue) staining. Yellow indicates co-localization of cytochrome c with Mito-tracker Red in control and **2.4c**-treated cells in mitochondria. Diffused staining in thapsigargin-treated cells shows the release of cytochrome c from the mitochondria.

activity. A dose–response curve for the prevention of the depolarization of the MMP by **2.4c** is shown in Figure 1d, and the EC_{50} of the response was found to be 9.04 μM (Figure 1c).

Prevention of Cytochrome C Release

In the next step, we evaluated the distribution of cytochrome c in untreated, thapsigargin-treated, and **2.4c**-treated cells. As Figure 2a reveals, in normal cells, there was a complete overlap of cytochrome c staining with Mito-Tracker Red; this indicated its presence in the mitochondria. Treatment with thapsigargin for 18 h caused a marked loss of Mito-Tracker Red and a concomitant release of cytochrome c into the cytoplasm (Figure 2b), which was totally prevented with the use of **2.4c** (Figure 2c). The data suggest the role of **2.4c** in preventing the release of cytochrome c from the mitochondria, which is known to activate cell death in pancreatic β-cells.

Conclusions

To the best of our knowledge, this is the first report of a macrocyclic small molecule that modulates the mitochondrial membrane potential ($\Delta\Psi_m$) and further prevents the release of cytochrome c from mitochondria from thapsigargin-induced ER stress in pancreatic β-cells. Given the role of small molecule **2.4c** in preventing mitochondria from high cytosolic calcium insult, this compound may also have interesting applications related to neurological disorders, such as, cortical spreading depression (CSD). Whether **2.4c** regulates protein misfolding and/or clustering in mitochondria or participates in chaperone-mediated regulation of mitochondrial membrane permeabilization is yet to be determined.

Supporting Information (see footnote on the first page of this article): Experimental details, characterization data, and copies of the ^1H and ^{13}C NMR spectra of all key intermediates and final products.

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