ELUCIDATING MOLECULAR MECHANISM OF ANTIGLYCATION COMPOUNDS BY PROTEOMIC APPROACHES

THESIS SUBMITTED

TO

SAVITRIBAI PHULE PUNE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

MR. SANDEEP BALWANTRAO GOLEGAONKAR

UNDER THE GUIDANCE OF

DR. MAHESH J. KULKARNI

BIOCHEMICAL SCIENCES DIVISION

CSIR-NATIONAL CHEMICAL LABORATORY

PUNE - 411 008, INDIA.

OCTOBER 2014

Dr. Mahesh J. Kulkarni
Scientist,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Pune 411 008.

+91 20 2590 2541
mj.kulkarni@ncl.res.in

CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Elucidating molecular mechanism for antiglycating compounds by proteomic approaches"** submitted by **Mr. Sandeep B. Golegaonkar**, was carried out by the candidate at CSIR-National Chemical Laboratory, Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

Date:

Place:

Dr. Mahesh J. Kulkarni
(Research Supervisor)

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled **"Elucidating molecular mechanism of antiglycation compounds by proteomic approaches"** submitted for the award of degree of ***Doctor of Philosophy*** in Biotechnology to the 'Savitribai Phule Pune University' has not been submitted by me to any other university or institution. This work was carried out by me at CSIR-National Chemical Laboratory, Pune, India. Such materials as obtained from other sources have been duly acknowledged in the thesis.

Mr. Sandeep B. Golegaonkar

(Research Student)

Biochemical Sciences Division,

CSIR-National Chemical Laboratory,

Pune- 411 008.

Date:

Place:

Dedicated to My Family

Acknowledgement

During my PhD carrier there are many individuals that have influenced my life both personal and professional levels. This gives me immense pleasure to acknowledge their help in achieving this important milestone.

First and foremost, I would like to express my deep and sincere gratitude to my research supervisor, Dr. Mahesh J Kulkarni for this continuous support and guidance. I must appreciate his intellectual thinking, organizational skill, painstaking and caring nature for his students. His hunger for developing novels ideas and approach to do better has always inspired me. He always encouraged me to develop own ideas and provided necessary freedom to execute the ideas. He has always given freedom and opportunity to learn proteomics, guided me when needed and always stood behind me.

I am highly indebted to Dr. Arnab Mukhopadhyay, Scientist at National Institute of Immunology, New Delhi. The project taken shape with his active collaboration. I express my deep sense of gratitude towards him, for his mentoring skills, constant support, valuable suggestions, inspiration, encouragement and faith in me. He is perfect blend of scientific and professional skills. He guided me to understand cell biology, molecular biology using C. elegans model system. I always owe my high regards to him.

I am also grateful to current Director NCL, Dr. Sourav Pal, and previous Director Dr. S. Shivram for infrastructure and lab support to accomplish research work at NCL. I extend my sincere thanks to Dr. Vidya Gupta, Head, Biochemical Sciences division and Dr. C.G Suresh, Chairman Student Accademic Office (SAO) for their support and help. I sincerely thank to Prof. J. K Pal, Dr. Rajesh Gokhale, Dr. Ramanmurthy Bopanna, Dr. Kausik Chakraborty, Dr. Venkat panchagnula, Dr. Mugdha Gadgil and Dr. Absar Ahmed, for evaluating my progress reports and presentations. I thank Dr. Jomon Joseph, Dr. H.V. Thulasiram, Dr. A. P. Giri, Dr. Moneesha Fernandes, Dr. S. N. Nene, Dr. R. V. Gadre, Dr. Narendra Kadoo, Dr. H. V. Adikane and Mr. V. V. Jogdand for their help and suggestions during this research work. I wish to thank staff member's library and SAO at NCL for their timely help.

I am grateful to Mrs B. Santhakumari, Mr. S. S. Deo, Mr. R. S. Gholap, Mr. Y. Ramanjaneyulu, Mr. Suraprasad R., Mrs Shashikala Ranjane, Dr. D. M. Thakar, Mr. Jogdand, Dr. Rakesh KS, and Mr M. L. Shaikh, for their support and encuragement.

I am extremely grateful to my friends who are continuously with me in good and bad time and added smile on my face, Prabhakar Lal Srivastava, Harekrishna Punjal, Dr. Bhalchandra V, Dr. Laxman S, Pankaj D, Suresh K K, Dr. Pradeep S, Atul anand, Dr. Prasnna kumar Chikade, Mangesh M, Atul More, Sarang R, Sunil D, Unmesh B, Dr. Santosh D, Dr. Mukesh M, Dr Shailesh S, Dr Sanjay M, Dr. Dadasaheb P, Dr. Bhagyashree K, Shailendra S, Dr. Pratap, Dr. Rahul and Abhijeet Karale. I am grateful to Dr. Bhushan Dholakia for helping in correcting my thesis versions.

I would like thanks to my labmates Dr. Arvind K, Dr. Ashok C, Dr. Hemangi B, Shweta B, Santosh B, Sachin K, Swapnil B, Rubina K, Arti D, Sagar B, Reema B, Kedar B, Jagdeeshaprasad M.G, Vannur swamy, Rashmi G, Dr. Yogesh K, Gouri P, Yougendra P, Sakuntalabai, Sneha B, Ramesha H.J, Saikat, Devdat, Dipesh, Harshal, Rincy, Nilofer, Krithika, Avinash, Shiva, Uttara, and Shrikant. I would like to take opportunity to thank NII friends who made my stay pleasant Dr. Awdesh Pandit, Dr. Neeraj, Manish Chamoli, Anupama Singh, Latika Matai, Syed Shamsh Tabrez, Sonu Gupta, Sonia, Urmila Jagtap, Yasir Malik, Anita, Shalini Setu, Kanika, Mukund Sudersan, Balaji, Vaibhav Jain, Avinash, Bharat, Ajay, Pravin, Amrish, Suhas and Amit.

I would like to extend my thanks to NCL friends Bhausaheb T, Dr. Namdev, Dr. Sudhir, Dr. Abasaheb, Dr. Ankush, Dr. Sane, Dr. Nishant, Dr. Pitambar, Dr. Puspesh, Dr. Vijayanand, Dr. Kiran, Dr. Dhanaraj, Dr. Pradeep, Dr. Digambar, Shekhar, Chinmay, Majid, Dr. Venu, Dr. Khaja Mohaideen, Dr. Bharmana, Dr. Namrata, Harshit, Manoj, Anjan, Ramsundar R M, Dr. Satish B, Chakadola Panda, Ajeet Singh, Nivedita B, Deepika D, Dr. Nagesh K, Nagesh Kolhe, Kailas P, Eldho M, Venugopal, Alson M, Rajesh T, Parin Shah, Yashwant kumar, Dr. Bhusan, Dr. Vishal D, Shon Mary, Anurag, Rahul, Anand, Yashpal, Vijay and Montu.

I am highly obliged to my teachers Dr. S. S. Jadhav, Dr. Suresh K, Dr. S. S. Pisal and Dr.Varsha Pokharkar for inculcating scientific interest and encouraging me to explore various dimensions.

Unconditional love, support, faith and care from family is the greatest gift of life. I would like offer regards and respect to my family. My parents Shri. Balwant Golegaonkar and Smt. Vijaya Golegaonkar for their blessings. My elder brother Dr. Pankaj and my sister-in-law Dr. Smita always stood on my side. Words are less to express my gratitude towards my life partner Mrs. Rajani for providing me strength to achieve this memorable event. I extend my regards to my father-in-law Shri Rajeshwarrao Kulkarni and mother-in-law Smt.Vasundhara Kulkarni for their support along with all member of their family. I would like thank all member of Golegaonkar family for their support.

I duly acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi, for financial support, which facilitated me to carry out my work.

Finally I am grateful to almighty God for providing me patience, determination, and strength for achieving goals in research as well as in family issues.

Sandeep B. Golegaonkar

October 2014

Contents

Abbreviations

AGEs	-	Advanced glycation end products
AUC	-	Area under curve
ACN	-	Acetonitrile
AMG	-	Aminoguanidine
Amp	-	Ampicillin
BSA	-	Bovine serum albumin
CID	-	Collision induced dissociation
Da, kDa	-	Dalton, Kilodalton
3-DG	-	3-deoxyglucosone
EDTA	-	Ethylenediamminetetraacetic acid
ESI MS	-	Electro spray ionisation mass spectrometry
ELISA	-	Enzyme-linked immunosorbent assay
Xg	-	Relative centrifugal force
g, mg, µg, ng	-	Gram, milligram, microgram, nanogram
µM	-	Micromolar
Hr	-	Hours
HbA_{1C}	-	Hemoglobin glycation
HA	-	Hydroxylamine
HDMS	-	High definition mass spectrometry
INH	-	Isoniazid
ITC	-	Isothermal calorimetric titration
LB	-	Luria broth
LC	-	Liquid chromatography
MALDI MS	-	Matrix associated laser desorption ionization MS
MS^E	-	MS at elevated energy
min	-	Minutes
mL	-	Millilitre
MG	-	Methylglyoxal
MET	-	Metformin
PARA	-	Paracetamol

PLGS	-	Protein lynx global server
PVDF	-	Polyvinylidene fluride
qRT	-	Quantitative real time
RPM	-	Rotation per minute
ROS	-	Reactive oxygen species
RNS	-	Reactive nitrogen species
RT	-	Retention time
RAGE	-	Receptor for advanced glycation end products
RIF	-	Rifampicin
RSV	-	Rifamysin SV
RMN	-	Rifaximin
TBS	-	Tris buffered saline
UPLC	-	Ultra high performance liquid chromatography
w.r.t	-	With respect to
V, kV	-	Volt, kilovolt

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Thesis abstract

Glycation is a non-enzymatic reaction between glucose and protein, one of the major causes for developing diabetic complications. Inhibiting glycation is considered as an intervention strategy in the treatment of diabetic complications. In view of this, an *in vitro* insulin glycation assay was developed by using MALDI-TOF-MS. This assay was further extended to screening glycation inhibitors, using this assay rifampicin (RIF) were identified as a potent glycation inhibitor. We evaluated the *in vivo* efficacy of RIF using *Caenorhabditis elegans (C. elegans),* as this is an excellent model system for study aging, a glycation associated process. RIF increased the lifespan of *C. elegans* in euglycemic, hyperglycemic and hyper-methylglyoxal conditions. Mass spectrometric and western blotting analysis of *C. elegans*, suggested that the drug inhibited *in vivo* glycation. In addition, RIF inhibits amyloid beta and polyQ proteotoxicity in *C elegans*. Genetic analysis suggested that the drug activated the DAF-16 (a mammalian homolog of FOXO transcription factor), thus regulated a subset of its target genes and it requires DAF-16, DAF-18 and JNK-1 for the extension of lifespan in *C. elegans*.

Chapter 1: Introduction

Hyperglycemic condition in diabetes leads to protein glycation due to prolonged exposure of proteins and tissues to high glucose[1]. Glycation is a non-enzymatic reaction where glucose reacts with proteins preferentially at epsilon amino groups of lysine or arginine[2]. Further, the dicarbonyl intermediates of glycation reaction react with the proteins leading to formation of Advance glycation end products (AGEs), which is implicated in development of diabetes, atherosclerosis, cardiovascular, Alzheimer's and Parkinson's diseases as well as in aging[3]. AGEs formation is accelerated in diabetic condition and during the aging process[4]. Pharmaceutical intervention for reducing AGEs is required in order to manage glycation-associated diseases. Molecules like OPB-9195[5], ALT-711[6], LR series[7], carnosine[8] are in safety clinical trials. Aminoguanidine, a known antiglycating molecule[9], had shown nephrotoxicity in clinical trials[10]. Therefore, there is an urgent need to identify novel and safe glycation inhibitors. Drug repositioning is the process of finding new uses for existing drugs, outside the scope of the original use. This process reduces the cost of drug discovery and has a lower risk of failure[11]. Hence, in

present thesis we used a drug repositioning approach to discover novel glycation inhibitors.

Chapter 2: Establishing an assay for screening novel glycation inhibitors and their possible mechanism

An *in vitro* MALDI-TOF-MS based insulin glycation assay or screening glycation inhibitors. The assay involves monitoring of glycated insulin in presence or absence of drug. Using this assay, RIF was discovered as potent antiglycating compound. This was further validated by a BSA fluorescence assay. Further determination of IC_{50} of RIF and its analogs including rifamycin SV, rifaximin and aminoguanidine, suggested that RIF was a more potent glycation inhibitor. Rifamycin SV showed relatively lesser IC_{50} than RIF while, rifaximin didn't show any antiglycation activity. Molecular mechanism of antiglycation activity of RIF was explained using high resolution mass spectrometry and Isothermal calorimetric titration assay. RIF appears to inhibit glycation by binding to the proteins.

Chapter 3. Effect of rifampicin in regulation of *in vivo* glycation

C. elegans is a well-established model for studying aging and age related disorders like Alzheimer's disease[12,13]; Parkinson's disease (α-synuclein)[14]; Huntington's disease (polyQ)[15]; and diabetes[16,17]. AGEs are also implicated in aging and pathogenesis of age-related diseases[18]. Thus, pharmaceutical interventions that can reduce AGEs may delay age-onset diseases and extend the lifespan. Using *C. elegans* as a model system, we studied the effects of RIF, rifamycin SV, rifaximin and aminoguanidine on life span. RIF and rifamycin SV imparted similar enhancement of longevity while aminoguanidine and rifaximin had limited effects. RIF and rifamycin SV were also able to completely overcome high glucose toxicity, reduce AGEs and increase life span under hyperglycemic conditions. RIF ameliorated AGEs in *glod-4(gk189)* mutant, as determined by western blotting and mass spectrometric analysis, as well as increased longevity. In two different models of proteotoxicity, RIF and rifamycin SV offered significant protection against proteotoxicity.

Chapter 4. Molecular mechanism of rifampicin mediated longevity

In order to understand the longevity effects of RIF, genetic analysis was performed using *C. elegans* mutants. The increased lifespan on RIF treatment was found to be dependent on DAF-16 (a homolog of mammalian FOXO). RIF was able to activate DAF-16 transcription factor and influence its nuclear-cytoplasmic distribution. Genetic analysis suggested that RIF regulate insulin signaling differently. In addition, microarray based analysis was performed to study differential gene expression in response to RIF treatment. Around 102 genes were up regulated while 101 down regulated by 2 fold upon RIF treatment with a $p<0.05$. It was found that vitellogenins, lipid transporters, heat shock proteins, lysozymes and few DAF-16 target genes were down-regulated while xenobiotic genes and oxidative stress tolerance genes were up regulated with RIF treatment. Interestingly, RIF activates different subset of genes in regulating DAF-16/FOXO transcription factor. Finally, the genetic analysis has suggested that RIF mediated lifespan extension required DAF-18 (mammalian PTEN homolog), DAF-16 and in *C. elegans*.

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Chapter 1

Introduction

1.1 Diabetes

Diabetes is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion or action or both. The estimated number of people suffering from diabetes globally is increasing and predicted to rise alarmingly. It is expected that the number of people suffering from diabetes will rise to 380 million by 2025 globally[1]. The symptoms of diabetes include hyperglycemia along with polyuria (frequent urine), polydipsia (frequent thirst), weight loss, sometimes with polyphagia (increased apatite), and blurred vision. Acute cause of hyperglycemia in some individuals leads to ketoacedosis. Prolonged hyperglycemia leads to chronic and progressive development of the diabetic complications with long-term damage, dysfunction, and failure of various organs[2]. These complications include retinopathy, nephropathy, neuropathy, arthrerosclerosis, cardiovascular and sexual dysfunction. In developing countries diabetic complication is the major cause of morbidity and mortality. One of the major inevitable consequences of hyperglycemia is protein glycation.

1.2 Glycation

Glycation, a non-enzymatic reaction between reducing sugars and protein, was first observed by Louis Maillard in 1912, when these reactants were turned into brown color upon heating at physiological condition[3]. Hence, it is also referred as Maillard's reaction or Browning reaction. This process is different from enzymatically catalyzed glycosylation of proteins by glycosyl transferases[4]. Enzymatic glycosylation is based on the attachment of oligosaccharides to specific protein side chains such as asparagine (N-linked), serine (O-linked) and threonine (O-linked). While in glycation, reducing sugars such as glucose, fructose, and ribose etc. react with epsilon amino groups of lysine or guanidino groups in arginine residues leading to formation Schiff's base[5], which is the first of step glycation reaction and is reversible in nature[6]. In the next step, Schiff's base rearranges to form a stable reversible Amadori product, which undergoes series of cyclization, oxidation, dehydration and fragmentation reactions and result into a variety of dicarbonyl compounds including methylglyoxal, glyoxal, glucosones, 3-deoxyglucosone (3-DG) etc[7]. These carbonyl compounds are more reactive than the original sugar and act as propagators of reaction leading to the formation of Advanced

Glycation End Products (AGEs). AGEs are heterogeneous in nature caused by irreversible protein cross-linking and the formation of AGEs is illustrated in Figure 1.1 and various structures of AGEs are depicted in Figure 1.2.

These AGEs have been divided into following catagories; 1. Fluorescent crosslinked AGEs such as pentosidine and crossline; 2. Non-fluorescent crosslinked AGEs such as MOLD (Methylglyoxal lysine dimer), GOLD (Glyoxal lysine dimer) and AFGP (Alkyl formyl glycosyl pyrroles); and 3. Non-crosslinked AGEs such as CML (Carboxymethyl lysine), CEL (Carboxyethyl lysine) and pyralline represented in Figure 1.3[8-10].

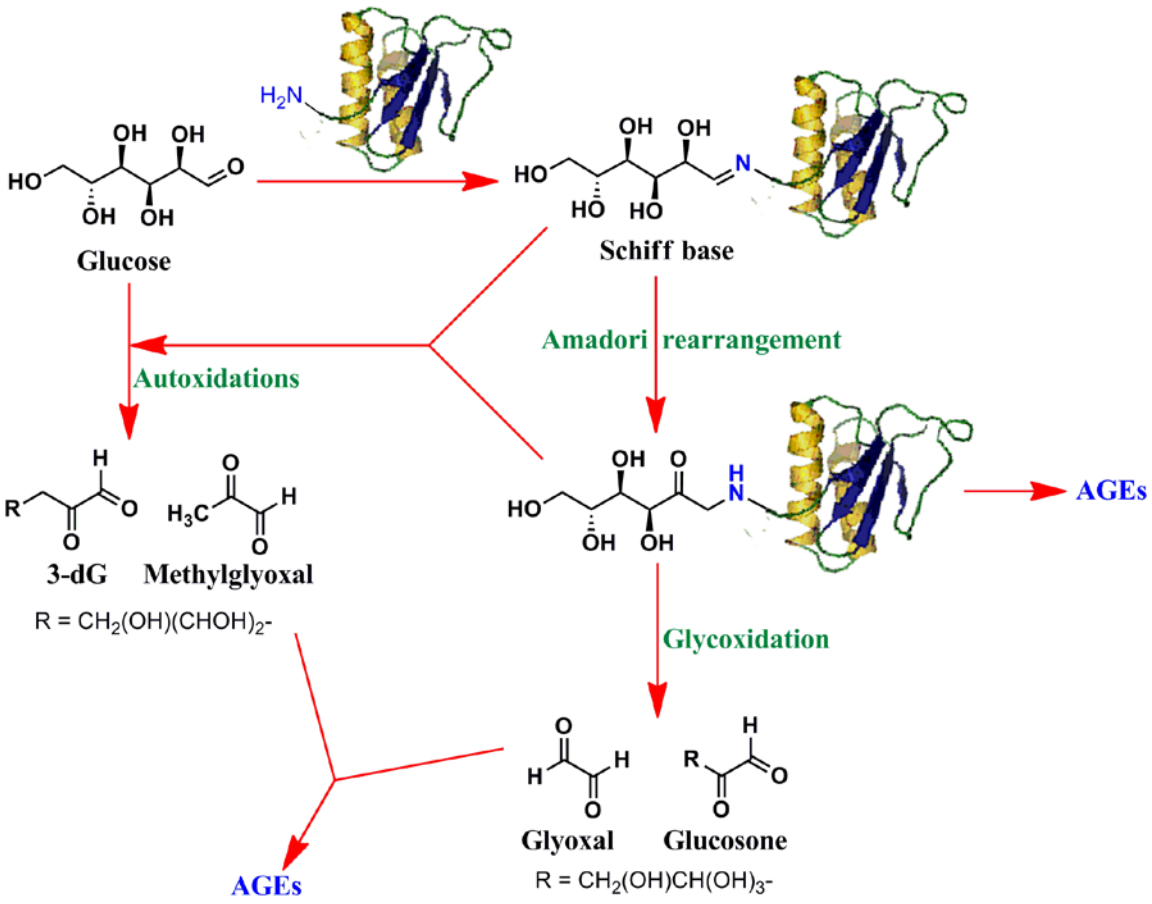
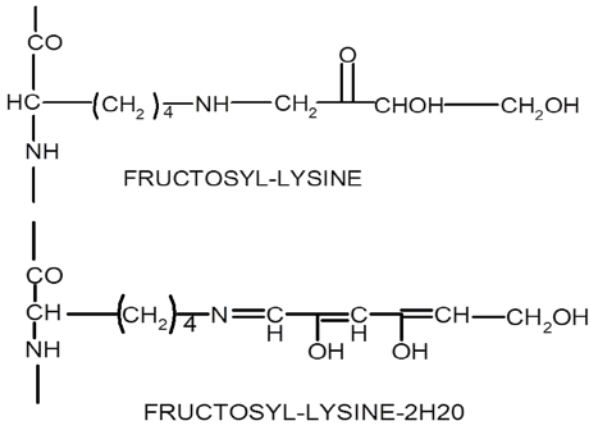


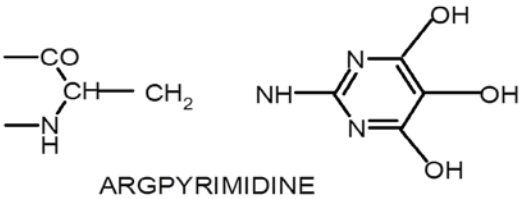
Figure 1.1: Mechanism of Advanced Glycation End products (AGEs) formation. The figure is adapted from Reddy et al.,(2006).

Pentosidine is a crosslink between lysine and arginine residues and its levels have been shown to increase in diabetes[11,12]. Crossline was first identified in the kidneys of diabetic rats and can be formed both *in vitro* and *in vivo*[13]. Imidazolium dilysine crosslinks are also known as GOLD or MOLD are also detected *in vivo*[14]. AFGP is formed by a single lysine residue with two sugar molecules, but *in vivo* has limited significance[15]. Pyrraline is detected in human skin, plasma and in brain plaques which is a non-cross-linked AGE[16]. Glycation leads to formation of reactive oxygen species (ROS). Increased levels of ROS are responsible for oxidation of proteins, lipids and nucleic acids which further accelerates formation of AGEs. The pathway of formation of carbonyl compounds by autoxidation of reducing sugars and glyceraldehydes, is referred as Wolff pathway[17,18]. Lipid peroxidation leads to formation of dicarbonyl compounds, and is called as Namiki pathway[19,20]. AGEs play an important role in the pathogenesis of diabetic complications. Increased protein glycation can alter enzymatic activity, decrease ligand binding, modify protein half-life and alter immunogenicity[21]. Autoantibodies against serum AGEs are capable of forming AGE-immune complexes in diabetic patients that may play a role in atherogenesis[22]. Formation of AGEs in non-diabetic subjects is at extremely low level. However, this process accelerates in poor glycemic condition[23], but the levels of different types of AGEs varies. For example, serum levels of CML increase in diabetic patients with retinopathy but not with nephropathy, whereas the levels of pentosidine increase in both groups[24].

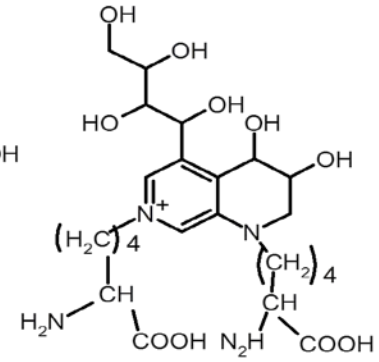


FRUCTOSYL-LYSINE

FRUCTOSYL-LYSINE-2H20



ARGPYRIMIDINE



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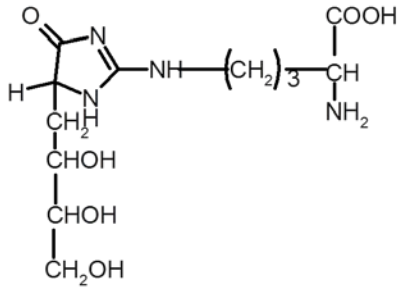
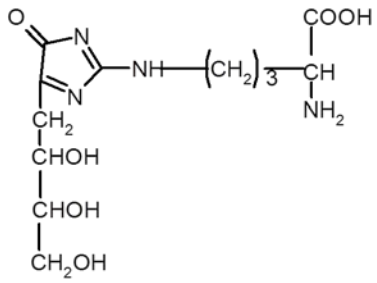


IMIDAZOLONE-A



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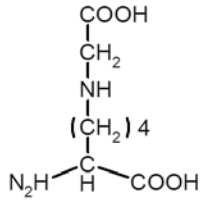
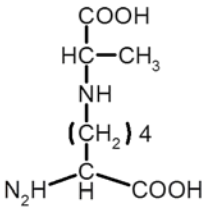


N-(CARBOXYMETHYL)-LYSINE
(CML)



N-(CARBOXYETHYL)-LYSINE
(CEL)

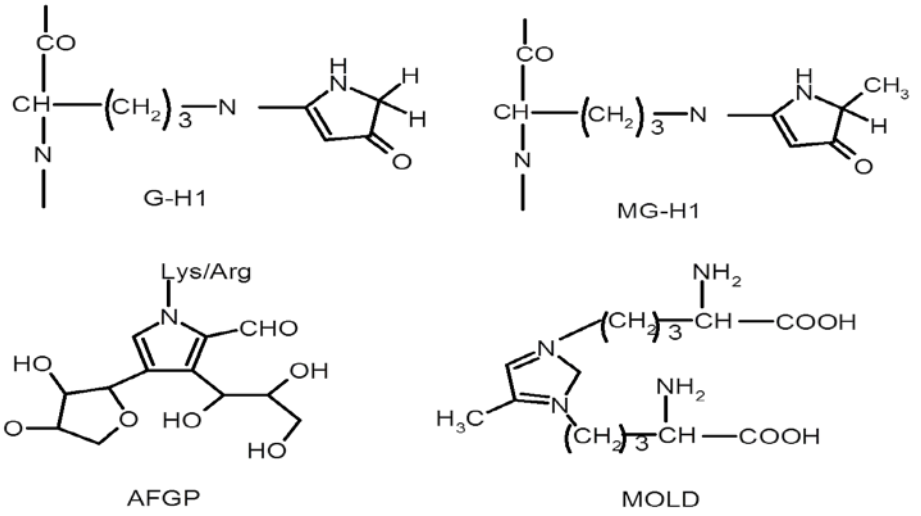


Figure 1.2: Advanced Glycation End products (AGEs) with heterogeneous structural forms.

1.3 Characterization of AGE modification

Characterization of AGE modifications is a challenging task, as varieties of AGEs are formed in the body. One of the characteristic features of few AGEs; they emit fluorescence, which can be monitored by fluorescence spectroscopy for their quantification and characterization. Fluorescence based quantification was used to analyze the AGE levels in diabetes associated atherosclerotic condition. Enzyme linked immunosorbant assay (ELISA) is also being used for quantification of AGEs. Qualitative and quantitative measurement of AGEs have been reported in human lens proteins with age[25], in human serum[26] and red cell[27] proteins in diabetes, and renal[28] collagen in diabetic rats. Volatile and semi-volatile products of Maillard's reaction are analyzed by Capillary electrophoresis (CE)[29]. In addition to these methods, enrichment of proteins and peptides improves detection of AGEs. Boronic acid chromatography (BAC) is used for enriching glycated proteins; it involves esterification between boronic acid and 1, 2- cis-diol compounds like glucose under alkaline conditions. Based on this principle, an electrophoretic method called Methylacrylamido phenylboronate acrylamide (mPAGE) was developed that can effectively used for identification of early and late AGEs[30]. Mass spectrometric analysis provides the tool for comprehensive characterization of AGE

modifications. However, the identification and characterization of AGE modification in proteins is technically challenging due to (1) relatively low concentration of glycated proteins; and (2) low ionization efficiency of glycated peptides in mass spectrometry analysis[31]. Lapolla et al.1995, for first time observed characteristic mass shift (Δm 162 Da) of Amadori product on protein by MALDI-TOF-MS[32,33]. Mass spectrometry-based approaches involving neutral loss, electron transfer dissociation (ETD), electron capture dissociation (ECD) are useful for characterization of glycation modifications. Isotope labelling with reducing sugars has been used for the analysis of glycated proteins from human plasma. Recently, glycating activities of seven important AGE-precursors, including glucose and their reactive carbonyl compounds, glucosone, 3-DG, 3-deoxygalactosone, 3,4-dideoxyglucosone-3-ene, methylglyoxal, and glyoxal were determined by multiple reaction monitoring using ultra-HPLC/MS/MS. MS^E (MS at elevated energy) is another strategy for identification of glycated protein in a complex mixture. The peptides that are eluted from LC (Liquid chromatography) are analyzed at lower energy CID (Collision induced dissociation) and high energy CID. Thus, MS and MS/MS occur at the same time for each peptide eluting. The fragments generated by high energy CID are time aligned with its precursor ion by Protein Lynx Global Server software (Waters, USA). Using this approach plasma AGE modified proteins were identified and characterized[34-38] List of AGEs modifications are listed in Table 1.1.

1.4 Diabetes and glycation

Hyperglycemic condition in diabetes results in increased oxidative stress and glyco-oxidation, which promote AGE formation[39]. AGEs causes macro and micro vascular complications. They accumulate in the various organs in hyperglycemic condition. The intermolecular collagen cross-linking caused by AGEs leads to diminished arterial and myocardial compliance and increased vascular stiffness. AGEs affect various organs such as eye, nervous system, heart, kidneys and small blood vessels[5]. AGEs have been detected in retinal blood vessel walls, they contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage[40]. AGEs are toxic to pericytes, which possess AGE receptors and damage to pericytes, which is observed in diabetic retinopathy[41]. In lens accumulation of AGEs cause conformational

changes and aggregation of lens protein, which is responsible for opacification. Increased glycation of Na–K ATPase *in vitro* reduces its activity, altering intracellular ion concentration and subsequent water movement via osmosis, this factors contribute in formation of diabetic cataract[42-44]. Deposition of arthrosclerotic plaques inside the arterial wall causes resistance in blood flow, which may lead to myocardial infarction. Atherosclerosis is one of the serious consequences of long standing diabetes due to AGEs[45]. Glycation of high-density lipoprotein (HDL) increases in diabetes and reduces its efficiency during reverse cholesterol transport[46].

Table 1.1: Glycation modifications with mass increase (Δm) for different AGEs.

S. No	Abbreviations	Modification	Δm (Da)
1	Amadori	Amadori-lysine	162.0528
2	CEL	N_{ε}-carboxyethyl-lysine	72.0211
3	CML	N_{ε}-carboxymethyl-lysine	58.0055
4	PYRRALINE	Pyrraline	108.0211
5	FL-$2H_2O$	Fructosyl-lysine- $2H_2O$	126.0317
6	IMIDAZOLONE-A	Imidazolone-A	144.0300
7	IMIDAZOLONE-B	Imidazolone-B	142.0266
8	ARGPYR	Argpyrimidine	80.0262
9	MG-H1	Nε-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine	54.0106
10	G-H1	Nε-(5-hydro-4-imidazolon-2-yl)ornithine	39.9949
11	AFGP	1-alkyl-2-formyl-3,4-glycosyl-pyrrole	270.0740
12	MOLD	2-ammonio-6-[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]hexanoate	49.0078
13	CROSSLINE	Crossline	252.1100

Diet containing elevated levels of AGEs promotes atherosclerotic lesions in diabetic apoE null mice, in comparison to diet with low levels of AGEs[47]. Kidney is the major site for clearance of AGEs from the body. In diabetic nephropathy kidney function is

affected as basement membrane thickening, expansion of the mesangium, reduced filtration, albuminuria and ultimately renal failure. The thickening of basement membrane occurs because of two reasons, first, increased glycation activates the release of transforming growth factor-β (TGF-β) which in turn stimulates synthesis of collagen matrix components and second, accumulation of AGEs on the collagen matrix[48]. Diabetic neuropathy disorder is associated with neurons affected due to diabetes leading to pain and numbness of limbs. AGEs on myelin can trap plasma proteins such as IgG, IgM and C3 to elicit immunological reactions that contribute towards neuronal demyelination[49]. AGEs also shown to reduce sensory motor conduction velocities, nerve action potentials and blood flow in peripheral[50]. Diabetic embryopathy is characterized by congenital anomalies or foetal/neonatal complications in an infant that are linked to diabetes in the mother. Hyperglycemia may give rise to oxidative stress which has been implicated in the pathogenesis of embryopathy. In embryopathy, glycation of DNA and histones by reactive intracellular sugars is observed and increased AGEs have been detected on histones isolated from diabetic rats[51]. All these studies establish the role of AGEs in diabetes and its complications.

1.5 Glycation and aging

Aging is an irreversible physiological process associated with reduced cell metabolism and function, decline in tissues and organ functions, eventually causing death. One of the consequences of aging is accumulation of damaged proteins due to decline in protein degradation machinery that removed damaged proteins[52-54]. Accumulation of damaged proteins is mainly due to free radicals[55] including reactive oxygen species (ROS), RNS (Reactive nitrogen species) etc. On the other hand glycation of proteins leads to formation of AGEs, which causes impaired functioning of proteins due to the chemical modifications, as well as affect the proteolytic degradation leading to protein accumulation or aggregation during aging. Glycation process is relatively constant throughout ageing in a healthy population. In diabetes, it is observed that glycation process is accelerated and this acceleration is proportional to the increase in blood glucose concentration[56]. Accumulation of fluorescent and cross-linked AGEs decreases transparency of the lens with age. AGEs affect the elasticity of the extracellular matrix[57]

and thus the turnover of (extracellular matrix) ECM proteins[58]. Glycated collagen plays a central role in the vascular complications of aging. Glycation of histones implicating in proteome glycation in the pathophysiology of aging and age-related diseases[59]. Aging leads to increased accumulation of AGEs in brain, affecting the central nervous system[60].

In addition AGEs binds to its receptor known as receptor for advanced glycation end products (RAGE). RAGE is a member of immunoglobulin superfamily, found on endothelial cells, mononuclear phagocytes and vascular smooth muscles, neurons, microglia and leukocytes including T-cells, granulocytes and dendritic cells[61,62]. RAGE up regulates different signaling pathways upon binding with AGEs via activation of nuclear factor kappa B (NFκB)[63], inducing secretion of pro-inflammatory and prothrombotic molecules, growth factors and the expression of RAGE itself [64-66]. Binding of AGEs to RAGE induces the NADPH oxidase system[67]. This causes an enhanced generation of ROS, which in turn promote formation of AGEs. The AGE modified proteins are accumulated during aging due to decline in protein degradation machinery[68]. AGEs play an important role in aging as well as age-related pathologies[69]. AGEs are involved in the genesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[70,71].

1.6 Intervention strategies

AGEs are heterogeneous in nature and thus involved in various disease conditions. Efforts are being made to reduce AGE levels, but limited success has been achieved till date. AGEs can be reduced *in vivo* by metabolites, amino acids, and enzymes, which are referred as cellular approaches. AGE inhibition can also be accomplished using chemical inhibitors of glycation reaction, which is referred as chemical strategies. Both these strategies are discussed below.

1.6.1 Cellular strategies

Cells have evolved mechanisms to counteract AGEs and such mechanisms can be exploited to find novel intervention strategies against AGE-related disorders. The reversible products of glycation reaction, Schiff's base/Amadori product, are deglycated by a class of enzymes called "Amadoriase" including fructosylamine oxidase, fructose

lysine 3-phosphokinase, and fructoselysine-6-phosphate deglycase, which are known to occur in mammalian, fungal, and other prokaryotes[72,73]. Auto-oxidation of glucose during glycation reaction leads to the formation of reactive dicarbonyls such as glyoxal, methylglyoxal, and 3-DG, which are detoxified by glyoxalase system or aldo–keto reductases[74]. Glucose-dependent insulinotropic polypeptide (GIP), an incretin secreted in response to nutrition plays a crucial role in developing vascular damage in diabetes. This polypeptide blocked the deleterious effects of AGEs by reducing RAGE mRNA and protein levels in endothelial cells. In addition, an antioxidant *N*-acetylcysteine mimics the effects of GIP on RAGE signaling, suggesting a role for GIP in blocking AGE–RAGE signaling[75]. Administration of recombinant sRAGE (soluble form of RAGE) in animal models has shown to suppress atherosclerosis, neuronal dysfunction, and diabetes[76,77]. In mice db/db model, neutralizing RAGE by anti-RAGE antibodies increases kidney weight, glomerular volume, mesengial volume, and decrease urinary albumin excretion and creatinine level[78].

1.6.2 Chemical strategies

Many steps are involved in the formation of AGEs. Prevention of glycation reaction is one of the approaches that can reduce AGEs. This can be achieved by keeping the low levels of glucose in blood. Inhibition of Schiff's base or Amadori product formation is the one of the strategies of intervention. Compounds that can interact with the reducing sugars and thereby inhibit the reaction can possibly inhibit the Schiffs base/ Amadori reaction. Examples of such compound include amines, polyamines, and small peptides[79]. Some of these molecules have the ability to knock of glucose moiety bound to protein due to their nucleophillicity a process known as transglycation. Examples of such molecules include carnosine and hydralazine[25, 80]. Certain molecules can modify or block amino acid side chain of proteins and prevent glycation. For example, aspirin protect protein glycation by transferring acetyl group to lysine, and the acetylated lysine cannot react with glucose[81]. Scavenging of the dicarbonyl compounds such as glyoxal, methyglyoxal and 3-DG is important to prevent AGE formation. Aminoguanidine[82,83] is one of the well studied AGE inhibitor acts by scavenging dicarbonyls. OPB-9195[(±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yl acetanilide] is also known to trap

carbonyl intermediates more efficiently than aminoguanidine[84] and inhibits the formation of AGEs *in vitro* and *in vivo*. "Amadorin" (the post-Amadori inhibitors), inhibits the conversion of Amadori intermediates to AGE[85]. The first Amadori inhibitor identified was pyridoxamine that showed a great potential for treatment of diabetic nephropathy. It inhibits AGE formation at different levels by scavenging carbonyl products of glucose and lipid degradation, sequestering catalytic metal ions, blocking oxidative degradation of Amadori intermediate, and trapping of ROS. Aminoguanidine is well known antiglycating compound, however it has shown side effects, and lesser efficacy in clinical trial[86]. Metformin, one of the oldest antihyperglycemic agents used for type 2 diabetes also shows interaction with dicarbonyl compounds[87]. Most of the AGE inhibitors show chelating activity that can inhibit metal catalyzed oxidation reaction and inflammatory responses[88]. Lalezari-Rahbar (LR) compounds are potent Cu^{2+} chelators, which inhibit post-Amadori AGE formation *in vitro* more efficiently than Pyridorin[89]. AGE crosslink breakers such as *N*-phenacyl thiazolinium bromide (PTB) and *N*-phenacyl-4,5-dimethylthiazolium chloride (ALT-711/alagebrium) have shown to cleave the AGE–protein cross-link in vitro. ALT-711 is currently under phase II clinical trials for its ability to reduce blood pressure and complications of aging and diabetes[90]. Eight, "RAGE blockers" comprise of agents that trap AGE and block AGE-RAGE interaction, thus inhibiting signal transduction mediated by RAGE[76,78].

1.7 Drug repositioning

Drug repositioning is also called as drug repurposing, therapeutic switching, drug re-profiling or drug re-tasking. It involves either of two processes, first, finding new indications for existing drugs, and second, the novel indication for a drug that has failed during clinical development owing to lack of efficacy[91,92]. The increasing interest in drug repositioning is encouraged by the high failure rates as well as the costs associated with attempts to bring new drugs to market[93,94]. Repositioning drug molecules reduced the risk, as the molecules were already pass through several stages of clinical development and therefore have well-known safety and pharmacokinetic profiles. It is well known that *de novo* drug discovery and development is about 10–17 year process from idea to marketed drug. Drug repositioning makes path shorter around 3–12 years, as *in vitro* and

in vivo screening, chemical optimization, toxicity, clinical development for many of those have been completed. This process consumes many years and substantial cost and risk involved in normal *de novo* drug discovery process[95]. Repositioning can offer a better risk versus reward trade-off compared with other drug development strategies due to low risk involved[94]. Benefiting effects of the repositioning creates opportunity to create the pipeline, thus it has high value. On the other hand drug repositioning faces some challenges related to intellectual property issues surrounding the original drug. Nevertheless, it is a useful approach and many successful therapies have been developed by drug repositioning (Table 1.2)

Table 1.2: Repositioned molecules with their new indications

Drug	**Old Indication**	**New Indication**
Celcoxib (Cycloxygenase-2 Inhibitor)	Osteoarthritis and adult rheumatoid arthritis	Familial adenomatous polyposis, colon and breast cancer
Minoxidil (β-adrenoceptor Blocked)	Hyper tension	Hair loss
Paclitaxel (Disturb normal microtubule dynamics)	Cancer	Restenosis
Phentolamine (α-adrenoceptor antagonism)	Hypertension	Impaired night vision
Raloxifene	Breast and prostate cancer	Osteoporosis
Thalidomide (TNF-α inhibition)	Sedation, nausea and insomnia	Leprosy
Zidovudine (reverse transcriptase inhibitor)	Cancer	HIV/AIDS
Topiramate	Epilepsy	Obesity

Ropinirole (dopamine-2 agonism)	Hypertension	Parkinson's disease
Chlorpromazine (dopamine receptor blocked)	Anti-emitic/anti-histamine	Non-sedating tranquillizer
Bupropion (SSRI*)	Depression	Smoking cessation
Duloxetine (SSRI*)	Depression	Stress urinary incontinence

* SSRI-Selective Serotonin Reuptake Inhibitor

1.8 Rationale of work

AGEs are implicated in various diseases including aging, diabetic complications and neurodegenerative disease. Reducing AGE levels is considered as an intervention strategy in the treatment and management of AGE associated diseases. Well known glycation inhibitor aminoguanidine had proven to be the best therapeutic molecule but showed nephrotoxicity in the clinical trials, and so it was discarded from the trials. Drugs like ALT-711 (algebium), thiazolium analogs, LR series of molecules, are still in drug discovery pipeline. In such scenario drug repositioning is better strategy than conventional drug discovery process. Using this approach, we screened several molecules for the glycation inhibition.

We developed *in vitro* insulin glycation assay by using MALDI-TOF for screening of the glycation inhibitors. Using this assay we identified rifampicin as potent glycation inhibitor. We evaluated *in vivo* efficacy of rifampicin in *C. elegans* model system. Rifampicin increases life span in euglycemic as well as hyperglycemic condition in *C. elegans*. In glyoxylase I (*glod-4*) mutant AGE accumulation will be more, leading to shorter lifespan. Rifampicin effectively increased lifespan of *glod-4* mutants. Further with mass spectrometric and western blotting analysis has shown that rifampicin decreases *in vivo* protein glycation. Using *C. elegans* model we also have shown the molecular mechanism for longevity at both genomic as well as proteomic level. In general, rifampicin increases the lifespan by inhibiting glycation, as well as it activates DAF-16, and requires DAF-18 and JNK-1.

1.9 Thesis Outline

- **Chapter 1:** Introduction
- **Chapter 2:** Establishing glycation assay for screening novel glycation inhibitors and its possible mechanism
- **Chapter 3:** *In vivo* effects of rifampicin in regulation of glycation
- **Chapter 4:** Molecular mechanism of rifampicin mediated longevity
- **Chapter 5:** Summary and Future Perspective
- Appendix I
- Appendix II
- Appendix III

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Chapter 2

Establishing glycation assay for screening novel glycation inhibitors and its possible mechanism

2.1 Introduction

AGEs have been implicated in diabetic complications such as nephropathy, neuropathy, retinopathy and arthrosclerosis[1]. AGE modification results in change in conformation and function of various proteins such as hemoglobin, albumin, IgG, collagen, crystalline, and metabolic enzymes. In diabetic patients, the rate of AGE modification and extent of protein cross linking is accelerated due to rise in blood glucose levels[2]. Inhibiting glycation and AGE accumulation has been considered to be one of the intervention strategy to prevent glycation mediated diabetic complications. Glycation inhibitors such as aminoguanidine (AMG)[3], carnosine[4,5], alagebrium chloride (ALT-711)[6], N-phenacyl thiazolium bromide[6] etc are not yet FDA approved and AMG has shown severe side effect. FDA approved drugs such as metformin, aspirin, diclofenac show antiglycating activity[7], however they are not very efficient inhibitors of glycation. Therefore, it is important to find array of molecules with an ability to inhibit glycation.

Currently the screening for glycation inhibitors is performed with assays such as AGE fluorescence[8], hemoglobin-δ gluconolactone assay[9], ELISA[10] etc. AGE fluorescence is monitored in presence or absence of inhibitors. Hemoglobin-δ gluconolactone assay determines early stage of hemoglobin glycation (HbA_{1C}). In the ELISA based technique, inhibition of the cross linking of glycated-BSA (AGE-BSA) to a rat tail-tendon-collagen is monitored. However, each method has its own advantages and disadvantages with respect to simplicity, rapidity, throughput and sensitivity. Here we report a MALDI-TOF-MS based insulin glycation assay for screening molecules that inhibit the glycation reaction. In this assay, insulin and glucose were allowed to react *in vitro* leading to formation of glycated insulin. Glycation inhibition of insulin is monitored in presence or absence of glycation inhibitor using MALDI-TOF-MS. For the first time we show that anti-tuberculosis drug rifampicin (RIF) show strong anti-glycation activity in comparison with aminoguanidine. These results suggest the possibility for repositioning anti-tuberculosis drugs for inhibiting glycation mediated diseases such as diabetic complication, Alzheimer, and aging.

2.2 Materials and methods

2.2.1 Chemicals

Insulin (Biocon, Ltd), bovine serum albumin fraction IV, glucose, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, aminoguanidine hydrochloride (AMG), rifampicin (RIF), rifamycinSV (RSV), rifaximin (RMN) and sinapic acid were procured from Sigma USA. Metformin (MET), isoniazid (INH), paracetamol (PARA), hydroxylamine (HA) were generous gift from Emcure Pharmaceuticals, Pune, India.

2.2.2 Insulin glycation assay

For insulin glycation assay, 25µL of insulin (1.82 mg/mL) was incubated at 37°C with 25µL of 0.5 M of glucose prepared in 0.1 M sodium phosphate buffer (pH 7.4) for 7 days. This reaction serves as glycation control. Different concentrations of aminoguanidine, metformin, hydroxyl amine, isoniazid (0.5 mM, 5 mM and 10 mM for each drug) and RIF (0.5 mM and 1 mM) were prepared in milli Q water. Each of these molecules was incubated along with insulin and glucose at 37°C for 7 days in ratio of 2:1:1, respectively.

2.2.3 BSA-AGE fluorescence assay

Glycation inhibition leads to decreased AGE modification and thus decreased AGE fluorescences. This assay was performed to corroborate the findings of MALDI based insulin glycation assay. In this assay fluorescence emission of glycated BSA was monitored in presence or absence of drugs. Triplicates of BSA 50 mg/mL and 0.5 M glucose in 0.1 M phosphate buffer pH 7.4 were incubated under aseptic conditions at 37°C for 7 days. The reaction volume was 3 mL in the presence or absence of various concentrations of the inhibitors. Glycation of BSA was examined after 7 days for AGE specific fluorescence (Excitation 370 nm; Emission 440 nm) using Varian spectrofluorometer. The inhibition of glycation reaction was calculated by (C-T)/C X 100 for each inhibitor compound. Where C is fluorescence of glycated BSA in absence of

inhibitor, T is fluorescence of glycated BSA in presence of inhibitor. AMG was used as a known glycation inhibitor.

2.2.4 Sample preparation for MALDI-TOF-MS

Five µL of the each assay mixture kept at 37°C was mixed with 45 µL of freshly prepared sinapinic acid (30% Acetonitrile, 0.1% Trifluoroacetic acid) and loaded onto the stainless steel MALDI plate separately by dried-droplet method[11] and incubated at 37°C till it get dried.

2.2.5 MALDI-TOF-MS analysis

The mass spectral analysis was done on Voyager-De-STR (Applied Biosystems, USA) MALDI-TOF. A 337 nm pulsed nitrogen laser was used for desorption and ionization. An Aquiris 2GHz dizitiger board was used for all experiments. MALDI analysis of insulin in presence or absence of glycation inhibitors was done using same instrumental settings. Spectra were acquired in the range of 2500 Da to 7500 Da, on a positive reflector mode with the following settings; an accelerating voltage 25kV, grid voltage 68.5% of accelerating voltage, delayed ion extraction time of 350 ns, low mass ion gate was set to 500 Da. For all spectral acquisition, the laser power was set just above the ion generation threshold to obtain peaks with highest possible signal to noise ratio. Experiment was repeated for four times and all spectra were acquired with 50 shots of laser in eight replications. The spectra were processed for advanced base line correction, noise removal and mass calibration. The percent inhibition of glycated insulin peak was calculated using following formula

$$(C - T)/C \times 100$$

Where

C – Intensity of glycated insulin in absence of inhibitor

T- Intensity of glycated insulin in presence of inhibitor

2.2.6 Determination of IC_{50}

Percent glycation inhibition was determined at different concentrations of AMG (0.5 mM to 30 mM) and RIF, RSV and RMN (0.0625 mM to 1 mM) in MALDI based insulin glycation assay as described above. IC_{50} was determined by plotting the per cent glycation inhibition versus drug concentration. All the experiments were performed in triplicate.

2.2.7 LC-MS analysis of RIF

LC-MS analysis of RIF was performed using UPLC system was connected with Q-Exactive (hybrid quadrupole orbitrap mass analyzer) (Thermo Scientific, USA) consisting of a reverse phase Hypersil GOLD C18, 8 µM, 4.6 X 150 mm column. The binary solvent system included 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). RIF and RIF + glucose was incubated at 37° C for 7 days and samples were eluted with a flow rate of 0.5 mL/min using sequential gradient of mobile phase B for 0 – 90% in 15 min. The data was acquired by Xcalibur and processed by Quant software (Thermo Scientific, USA). All the experiments were performed in triplicate.

2.2.8 Isothermal calorimetric titration

Isothermal titration calorimetry (ITC) experiments were performed by injecting RIF in the cell containing either glucose or bovine serum albumin (BSA) in MicroCal ITC_{200} (GE Healthcare, USA). Glucose and BSA was in 10 mM phosphate buffer (pH 7). Experiment was performed at 20^{o}C. Reference titration was subtracted from experimental titration. Affinity constants (Kd), the molar reaction enthalpy (ΔH) and the stoichiometry of binding (N), by fitting the integrated titration peaks.

2.2.9 Statistics

Statistical analyses for all the experiments were performed using Sigma Plot version 10.

2.3 Results

2.3.1 Insulin glycation assay

We have developed high throughput *in vitro* insulin glycation assay for screening glycation inhibitors assay using MALDI-ToF-MS. The glycation reaction was monitored by measuring the intensity of the glycated insulin peak using MALDI-ToF-MS. The mass of unglycated insulin was 5808 Da (Figure 2.1A), while the mass of glycated insulin is 5970 Da (Figure 2.1B). The glycated insulin peak has shown an increase in mass of (162 Da) with a relative intensity ranging from 30-50% w.r.t unglycated insulin peak. The intensity of glycated peak decreases in presence of aminoguanidine, a well established glycation inhibitor (Figure 2.1C). This assay was used to screen molecules for antiglycation activity. The antituberculosis drug RIF showed higher glycation inhibition than aminoguanidne (Figure 2.1D).

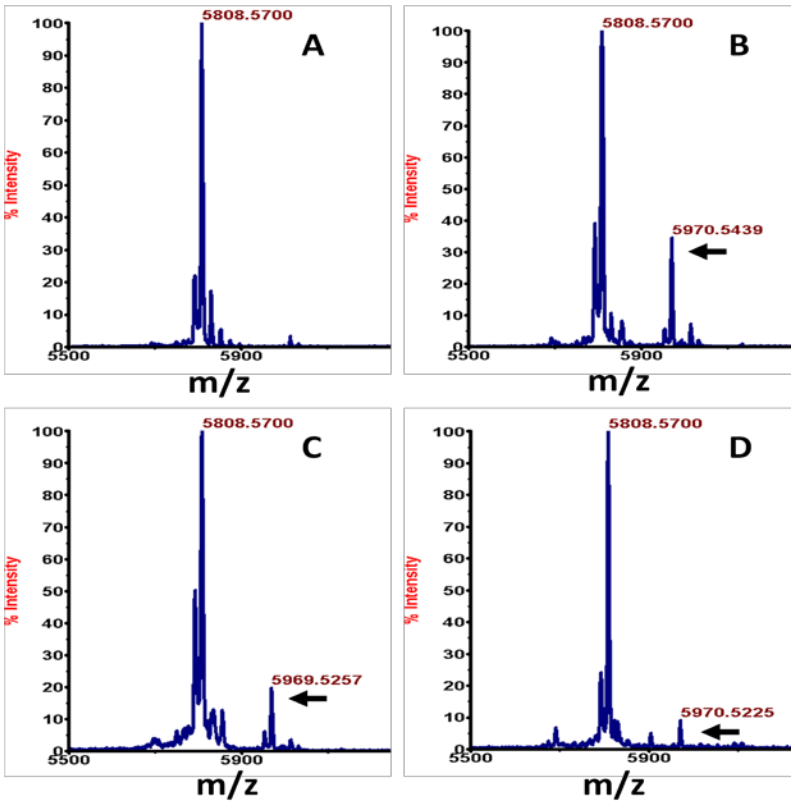

Figure 2.1. Insulin glycation assay using MALDI-TOF-MS. A. control insulin; **B.** glycated insulin without inhibitor; **C.** glycated insulin in presence of 10 mM aminoguanidine; **D.** glycated insulin in presence of 1 mM RIF.

2.3.2 Screening of glycation inhibitors

In this experiment, the intensity of glycated peak (5970 Da were determined in presence and absence of drug) using MALDI-ToF-MS. AMG, INH, HA and RIF showed concentration dependent inhibition of glycation (Figure 2.2). While drugs like MET and PARA did not show concentration dependent inhibition of glycation (Figure 2.2). The antituberculosis drugs, INH and RIF showed potent antiglycation activity in comparison with some of the known antiglycating compounds such as AMG, MET, PARA, and HA. Further, it was observed that rifampicin showed highest (40%) antiglycation activity even at lowest concentration of 0.5 mM of the drug. RIF has shown highest glycation inhibition up to 75% at 1 mM concentration (Figure 2.2). RIF was not completely soluble above 1 mM concentration. Therefore, 5 mM and 10 mM concentration of RIF was not considered for comparison.

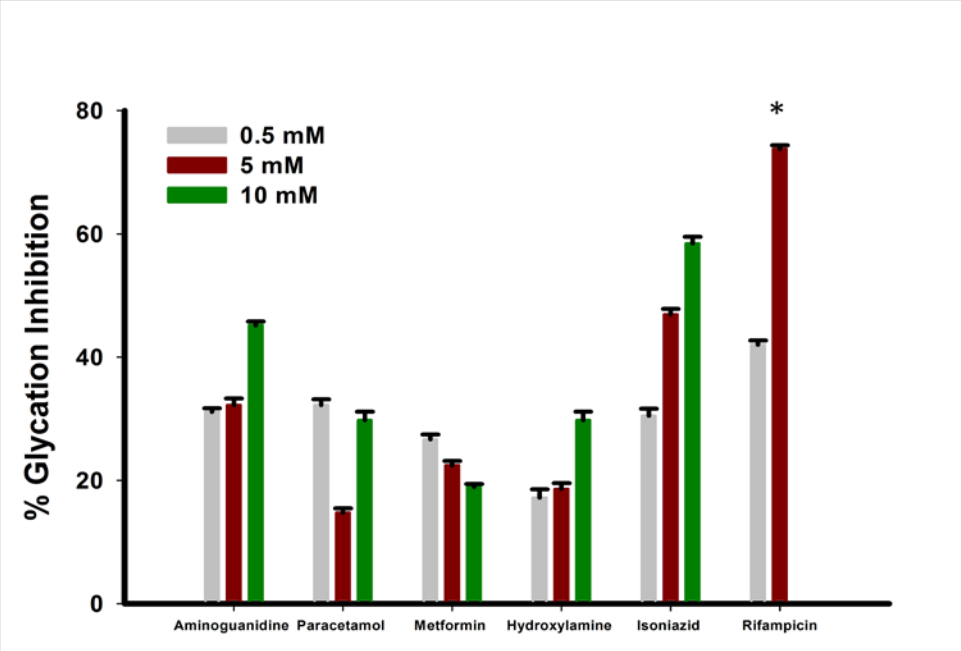

Figure 2.2: MALDI-TOF-MS based insulin glycation assay for screening glycation inhibitors. The graph represents % glycation inhibition on y axis w.r.t contol Vs molecules screened. The values represent mean ± standard error (n =8), * Indicates RIF concentration was 1 mM.

2.3.3 *In vitro* fluorescence assay

One of the characteristic features of AGEs, they emit fluorescence at 440 nm upon excitation at 370 nm. The emitted fluorescence was recorded by detector in fluorescence spectroscopy (Cary Eclipse, Varian), percent glycation inhibition of molecules were calculated from fluorescence emitted at 440 nm the values w.r.t glycated control fluorescence has shown (Table 2.1). AMG, HA, MET, PARA and INH were having antiglycation activity in the range of 30 to 60% at all concentrations studied. RIF was having highest antiglycation potentional, it inhibit glycation up to 90 to 95% at 1 mM concentration.

Table 2.1: Percent glycation inhibition using BSA-Fluorescence assay; the values represented mean ± standard error (n=3)

Concentration of Drug / Inhibitors	0.5 mM	5 mM	10 mM
Aminoguanidine	34.634 ± 2.982	44.652 ± 7.414	45.051 ± 7.902
Paracetamol	40.183 ± 5.714	34.830 ± 4.190	33.354 ± 3.647
Metformin	36.102 ± 2.327	36.611 ± 4.882	36.747 ± 4.118
Hydroxylamine	33.391 ± 4.549	40.920 ± 3.901	42.00 ± 5.016
Isoniazid	39.019 ± 6.585	48.575 ± 5.564	51.401 ± 4.700
Rifampicin	90.920 ± 6.681	94.175* ± 2.913	------

* **Indicates RIF concentration was 1 mM**

2.3.4 IC_{50} determination

IC_{50} is defined as the concentration at which 50% inhibition of glycation reaction was achieved in presence of inhibitor. It was determined by plotting a graph of concentration of inhibitor Vs percent glycation inhibition in a MALDI based insulin glycation assay. The IC_{50} for RIF, RSV and AMG were determined to be 0.12 mM, 0.22 mM and 14.4 mM, respectively (Figure 2.3). Whereas, RMN unable to show antiglycation activity. This suggests that RIF and RSV are more potent glycation inhibitors than AMG. Although RIF, RSV and RMN are analogs, they have different antiglycation properties as RMN < RSV < RIF.

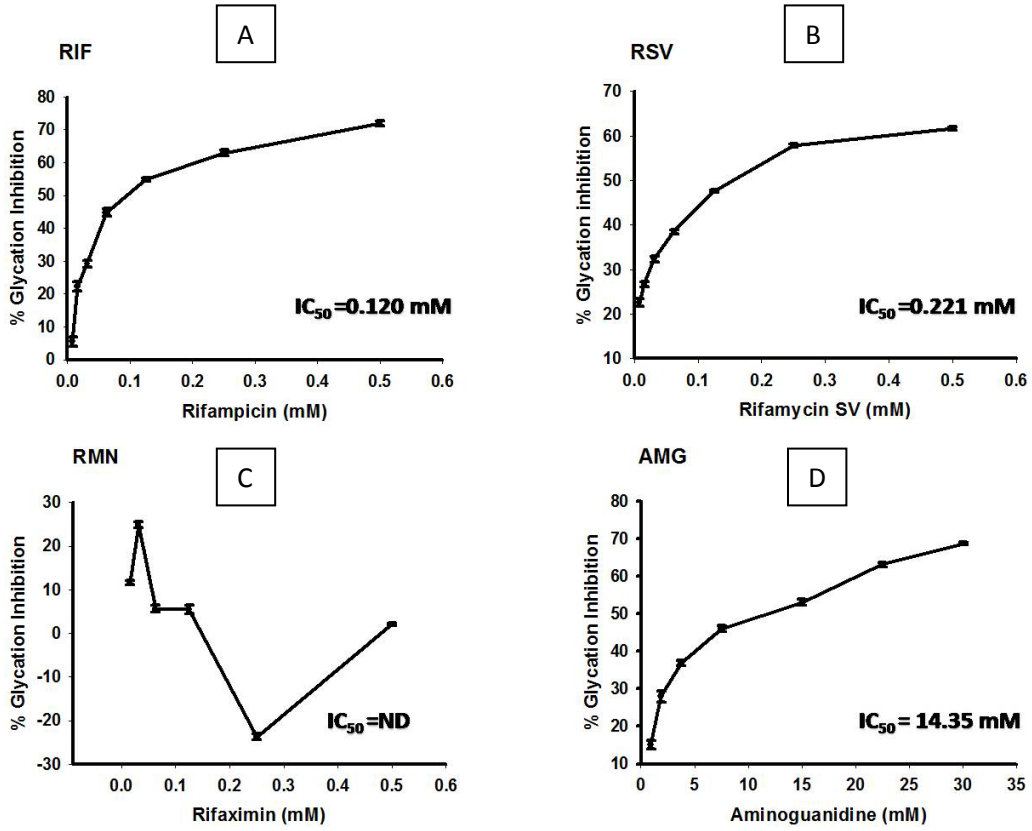

Figure 2.3. **IC_{50} values determined using insulin glycation assay. A.** RIF; **B.** RSV; **C.** RMN and **D.** AMG. The values represent the mean ± standard error.

2.3.5 *In vitro* mechanism for RIF

We have reported for the first time, RIF has a strong antiglycation activity even at a very low concentration. It is important to explore the possible mechanism of antiglycation action of RIF. The possible ways by which RIF can inhibit is 1) can react with glucose and prevent glycation, and 2) can bind to protein and block binding of glucose. The possibility of RIF reacting with glucose was studied by monitoring RIF-Glucose adducts in LC-MS analysis. RIF and glucose were incubated at 37°C for 7 days. The reaction was monitored at different time points by analyzing the area under curve (AUC) for RIF, glucose and the RIF-glucose adducts. LC-MS analysis revealed that there was no formation of RIF glucose adduct, which was also reflected in no change in the AUC of RIF or glucose. This suggests the fact that RIF is not reacting to glucose in preventing glycation *in vitro* (Figure 2.4).

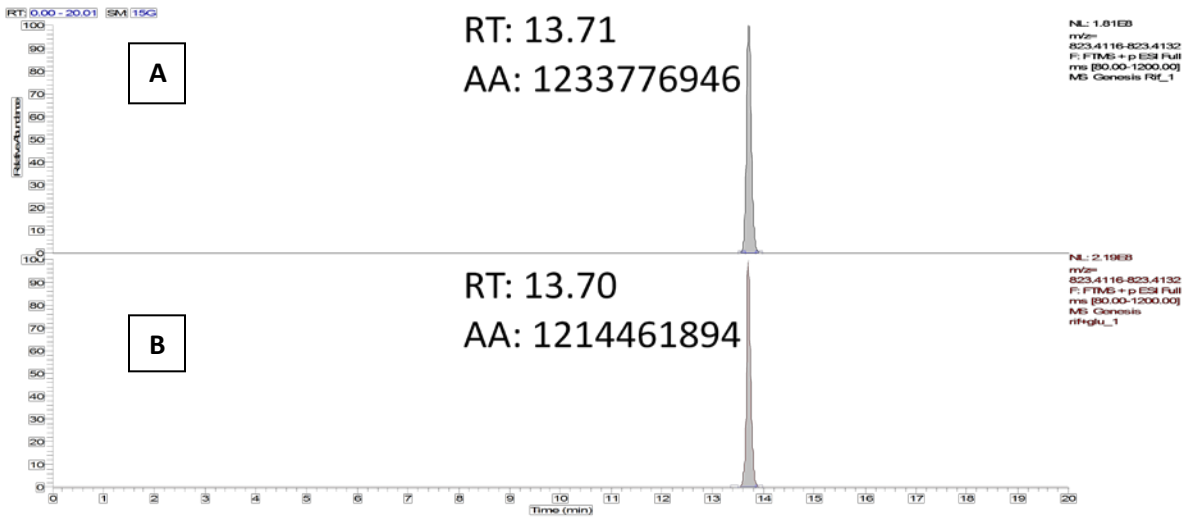

Figure 2.4: LC-MS analysis RIF and RIF + Glucose incubated samples. A. RIF and **B.** RIF + glucose. There is no change in area between RIF and RIF + glucose.

Further we evaluated whether RIF binds to protein and blocks binding of glucose using isothermal calorimetric titration (ITC). ITC is a biophysical technique used to determine the thermodynamic parameters of interactions in solution. The technique is most often used to study the binding of small molecules to proteins. ITC is a quantitative technique

that can determine the binding affinity (K_a), enthalpy changes (ΔH), and binding stoichiometry (n) interaction between two molecules in solution. We used RIF as a ligand and titrated against glucose and BSA separately at 20°C. We observed that RIF was unable to bind to glucose when it was titrated against glucose. But interestingly RIF binds to BSA, as it was observed that binding of RIF leads to change in reaction enthalpy and it was found that two molecules of RIF bind to one molecule of BSA (Figure 2.5).

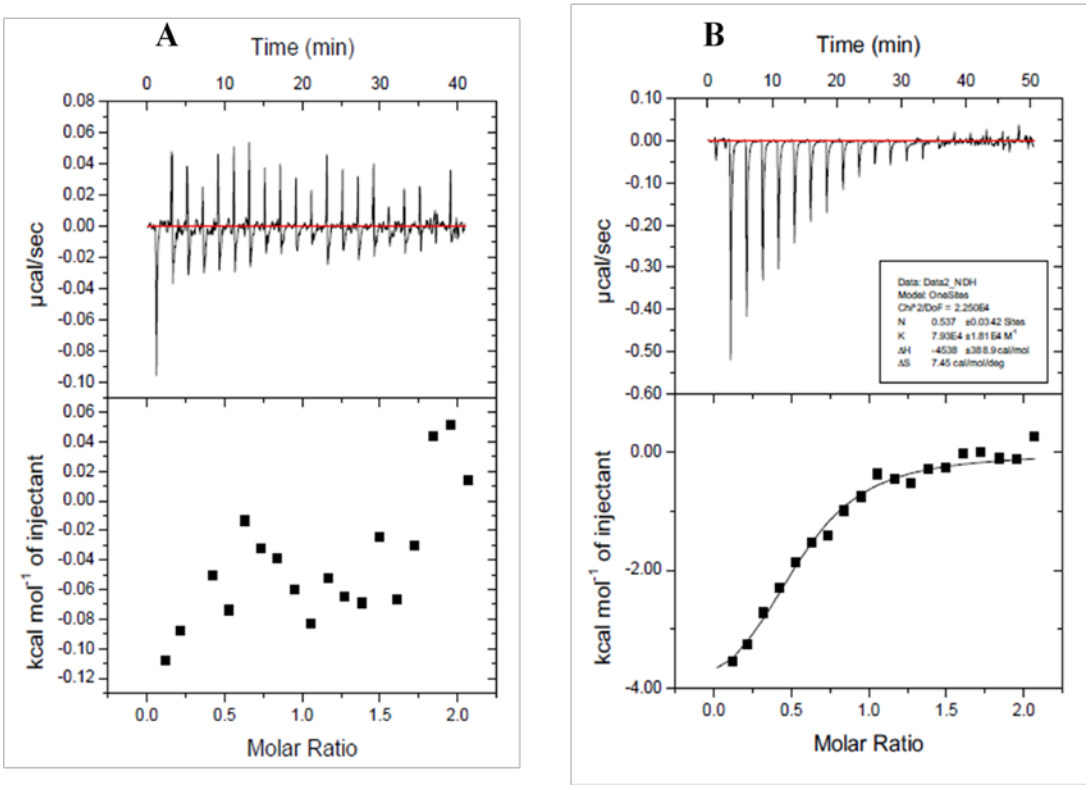

Figure 2.5: Isothermal calorimetric titration assay of RIF. A. RIF + Glucose; **B.** RIF + BSA.

2.4 Discussion

MALDI-TOF-MS is rapid, sensitive and amenable for high throughput studies. In addition several studies have suggested that MALDI-TOF-MS can also be used for quantitative purpose. For example, it has been used for quantification of substrates and products of an immobilized lipase-catalyzed reaction in non-buffered organic solvent[12]. Similarly the conversion of D-fructose into glucosamine-6P catalyzed by glucosamine-6P synthase was quantified by MALDI-MS[13]. The levels of free and bound cystatin were

quantified by MALDI-TOF-MS[14]. This approach was also used screen the enzyme inhibitors[15,16, 19]. In the context of glycation, mass spectrometry is powerful tool for characterization of protein modification by AGEs. Mass spectrometry has been extensively used to study glycation *in vitro* as well as *in vivo*[17]. In particular, MALDI-TOF-MS has been widely used for understanding the glycation process. However, this technique was not explored to screen glycation inhibitors. In present study, MALDI based insulin glycation assay was developed to screen the inhibitors. RIF was identified to a potent inhibitor of glycation. In the earlier studies RIF was known to inhibit protein aggregation[18]. As glycation leads to formation of fluorescent AGEs, antiglycation activity of RIF was also reflected in decreased AGE- BSA fluorescence. Amongst the drugs that were screened in this study, AMG, MET[7,19], PARA[7], INH[20], HA[20] have already been reported to have antiglycation activity. For the first time, we report that RIF has a strong antiglycation activity at a very low concentration. The IC_{50} of RIF for glycation inhibition was found to be minimal (0.12 mM) as compared to other molecules studied. The possible mechanism of antiglycation activity of RIF was studied by LC-MS and ITC experiments, which suggested that RIF bouds to BSA and prevented it from getting glycated.

The finding of an additional therapeutic property of RIF as a glycation inhibitor is very significant considering the fact that RIF has been FDA approved drug for the treatment of tuberculosis. And therefore, this drug can be repositioned as a potent antiglycating molecule for the treatment of diabetic complications, aging and Alzheimer's diseases. Repositioning of drugs has become an emerging approach in drug discovery. One of the classical examples of repositioning of drugs is thalidomide, which was used as a sleeping aid, is now being used for the treatment of leprosy[21,22].

2.5 Conclusions

In this study we have developed a MALDI-TOF-MS based insulin glycation assay for screening glycation inhibitors. Considering the advantages of MALDI-TOF-MS such as amenability for high throughput screening, sensitivity, resolution, and the ability to monitor the specificity of reaction, this assay can used for identifying specific inhibitors of glycation. Furthermore, we show that anti-tuberculosis drug RIF has a potent anti-

glycation activity in comparison with known antiglycation drugs. We studied IC_{50} for RIF structural analogues such as RSV and RMN; and among these RSV showed potent antiglycation activity similar to RIF, while RMN unable to show antiglycation activity. Further we have attempted to understand the mechanism of RIF mediated glycation inhibition. LC-MS and ITC analysis suggested that RF might inhibit glycation by interacting with protein.

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Chapter 3

In vivo effects of rifampicin in regulation of glycation

3.1 Introduction

Glycation is complex and heterogeneous reaction between protein and glucose, or glucose derived products. AGE formation is accelerated during diabetics as compared to non-diabetics[1]. In diabetic condition, AGEs alters structure and function of the proteins and promotes protein aggregation. AGEs binds to its receptor known as "receptor for advanced glycation end products" (RAGE) and activates the NADPH oxidase system[2], resulting in reactive oxygen species (ROS) formation. During glycation, dicarbonyls such as 3-deoxyglucosone and methylglyoxal (MG) are formed. However, *in vivo* enzymes such as aldehyde reductase and glyoxalase detoxify these reactive dicarbonyls. Glyoxalase I is an enzyme that detoxify MG to D-lactate[3]. This activity is markedly reduced with age leading to increased accumulation of methylglyoxal in the system. Interestingly, over expression of glyoxalase gene can decrease methylglyoxal-induced mitochondrial protein modifications and increase lifespan in *C elegans*[4]. Accumulation of methyglyoxal promotes protein modification and aggregation[5]. These modified proteins are removed by proteasomal system. With advancing age, the efficiency of proteasomal function decreases leading to accumulation of AGE modified proteins, which in turn promotes aging and neurodegenerative disease such as Alzheimer's, Parkinson's and Huntigton's disease[6]. Thus, pharmaceutical interventions that can suppress AGE formation may be an effective way to increase lifespan and health span[7].

Caenorhabditis elegans (*C. elegans*) is a nematode used as model organism for studying aging. This model organism was first introduced by Sydney Brenner in 1963. The adult hermaphrodite worm is 1 mm in length and 80 µM in diameter. The worms have short life cycle, and grow on *Escherichia coli* (*E. coli*), on agar plate or in liquid medium. Worms have transparent body thus allow easy visualization and monitoring of cellular processes. Gravid hermaphrodite comprises of 959 cells including muscle cells, hypodermis, a nervous system, intestine, gonads, glands, and an excretory system. *C. elegans* genome size is 100 Mb and has been fully sequenced and has 60-80% proteins similarity with human proteins[8-10]. The molecular and cellular pathways involved in majority of the human disease genes are highly conserved in the worm. In addition, mutants for majority of the genes are available. Thus, *C. elegans* is an ideal model system

for studying aging and other age related human diseases. In addition, growing of worms is cost-effective and can be used in drug discovery process including target identification, screening of molecules, lead identification, optimization and validation of drug targets.

Therefore, in this study we have used *C. elegans* as the model system to evaluate the antiglycation activity of rifampicin (RIF). Our results suggest that RIF extends lifespan of wild type (WT) *C. elegans* by 50%. It completely rescues glucose toxicity, increasing lifespan under hyperglycemic conditions by more than 100%. It even offers protection against protein aggregation diseases in the nematode model.

3.2 Materials and methods

3.2.1 Strain maintenance

All strains of *C. elegans* were maintained at 20°C with a standard maintenance procedure [11]. Strains used were N2 Bristol as wild-type, *glod-4 (gk189), CL200 dvIs2[pCL12(unc-54/human Abeta peptide 1-42 minigene) + pRF4]. Q35::YFP rmIs132 [unc-54p::Q35::YFP]*

3.2.2 Lifespan assays

All the lifespan assays were performed at 20°C. Eggs were collected from well fed gravid worms and placed on new *E. coli OP50* NGM agar plate. The plates were incubated at 20°C till young adult stage. Worms were washed and collected with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, H_2O to 1 litre, sterilize by autoclaving); centrifuged at 2000 rpm for a minute, pellet was washed with M9 buffer for a minimum of 4 times to remove all live bacteria. Finally the worms were suspendened in S-complete buffer (1 litre S-basal buffer, 10 mL of 1M potassium citrate pH-6, 10 mL of trace metals, 3 mL of 1M calcium chloride, 3 mL of 1M magnesium sulphate and 0.5 mL of 10 mg/mL cholesterol was filter sterilized using 0.2µ filter). Liquid lifespan assay was set up in 96 well sterile flat bottom plates. Each well contained 8 to 12 live worms suspended in S-complete buffer with 100 mg/mL heat killed *E. coli OP50* and 0.6 mM Fudr (5'-fluoro-2'-deoxyuridine), 50 mg/mL carbenicillin and 250 µg/µL amphotericin B. All steps were performed in laminar air flow; plates were wrapped with parafilm and

incubated at 20°C till the assay was completed. Next day, 10 µL of S-complete buffer in each well followed by 10 µL of Di-methyl sulfoxide (DMSO, vehicle control) or RIF, RSV, RMN and AMG was added in respective wells. This day was considered as 1st day of lifespan. In glucose experiments 10 µL of 2% glucose was added. On 5th day of lifespan 9µL of 100 mg/mL heat killed feed was added in each well. The plates were scored after seven days on every alternate day till all worms were alive. Each experiment was repeated for a minimum of 3 times. Data plotted in lifespan graph was cumulative for each experiment. Lifespan statistics analysis was carried with the help of 'OASIS' an online software [12]

3.2.3 Paralysis assay

CL2006 worms were bleached and eggs were placed on NGM agar plates seeded with *E. coli OP50*. The plate was incubated at 15°C till all worms grown to young adult stage. Worms were collected in M9 buffer; centrifuged at 2000 rpm for minute and the supernatant was removed. This procedure was repeated for 4 times with M9 buffer to remove all live bacteria. Finally the worms were suspendened in S-complete buffer (filter sterilized) with 100 mg/mL heat killed and 0.6 mM Fudr (5'-fluoro-2'-deoxyuridine), 50 mg/mL carbenicillin and 250 µg/µL amphotericin B in sterile 96 well plate, all the above steps were performed in laminar air flow. The plates were covered with the help of parafilm and incubated at 20°C. Next day, DMSO or RIF, RSV, RMN or AMG were added to the plates. On 5th day again 9µL of 100 mg/mL heat killed feed was added to each well. On day 3rd, 6th and 13th day, 3 to 4 wells were harvested and worms were transferred on to the 35 mm NGM agar plate with *E. coli OP50*. The plates were allowed to dry and the worms were scored by prodding. All the experiments were performed minimum 3 times.

3.2.4 PolyQ35 aggregation assay

PolyQ35::YFP worms were bleached and eggs were placed on NGM agar plates seeded with *E. coli OP50.* The assay was set as per procedure described above for paralysis assay. Three wells were harvested on 4th day, worms were mounted on 2% agarose slide with a drop of 10 mM sodium azide in S-basal buffer. YFP (yellow fluorescent protein)

expression for each worm was observed under 10 X magnifications with Zeiss Axio imager M2 digital microscope (Carl Zeiss, USA), and images were acquired using Axio Cam software (Carl Zeiss, USA). Analysis was performed by counting fluorescent protein aggregates in rifampicin treated worms with respect to control treated worms.

3.2.5 Protein extraction

In a 250 mL flask, 17.78 mL S-complete buffer, 1.10 mL of young-adult worm suspension (5000 worms), 1.9 mL heat-killed *E. coli OP50* (100 mg/mL sotck), 33 µL of Carbenicillin (Sigma, USA), 13.2 µL of Fungizone (Sigma, USA) and 6.6 mL of 0.6 mM FuDr was added. The next day, 2.2 mL of 200 µM drugs or 0.14% DMSO with or without 2.0% glucose was added. The culture was grown for 15 days (fed once again on day 5 with 1.98 mL *E. Coli OP50*) at 20^{o}C and harvested. The pellet was washed 4 times with M9 buffer and flash frozen and stored at -80 ^{o}C till further use. The pellet was freeze-thawed 4 times in protein extraction buffer (20 mM Hepes buffer pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$ hexahydrate , 0.2 mM EDTA dihydrate, 0.5 mM DTT) with protease inhibitor, the pellet was sonicated using Bioruptor (Diagenode, Belgium) and centrifuged at 10,000 rpm for 10 minute. The protein concentration in the supernatant was estimated using Bradford reagents (BioRad, USA).

3.2.6 Western blotting

About 30 µg of total protein was separated on 12% SDS-PAGE and transferred to PVDF membrane. The membrane was probed with 1:1000 dilution of CML monoclonal antibodies (Cosmo Bioscience, Japan) by incubating overnight at 4^{o}C. Later, the membrane was washed thrice with TBST (Tris-Buffered Saline with 0.1% Tween 20) and incubated in 1:10000-diluted secondary antibody conjugated to HRP (Horseradish peroxidase) for 1 hr. The blot was developed using chemiluminiscent substrate (Millipore, USA).

3.2.7 Proteomics analysis

Prior to LC-MS^{E} analysis, 100 µg of total protein was washed with 0.1 % RapiGest (Waters Corporation, USA) and digested with trypsin after reduction and alkylation with 100 mM DTT and 200 mM iodoacetamide (IAA), respectively. Two µL of digested

peptides (final concentration of 100 ng/μL) were analyzed using online nano ACQUITY UPLC coupled to a SYNAPT HDMS (Waters Corporation, USA) by MS^{E}[13,14]. After MS^{E} analysis, data was analyzed with Protein Lynx Global Server software (PLGS version 2.5 Waters Corporation, USA). A preliminary search of processed samples was performed for protein identification against the UniProt *C. elegans* database. Glycation modification sites were identified using PLGS search against subset of protein databases that were identified in the preliminary search as described earlier[15]. The false positive rate was set to a default of 1% in the PLGS workflow. The PLGS-identified glycated peptides were manually validated using the following criteria: (a) all the modified peptides should be present in at least duplicates; (b) have correct parent ion mass shift values; (c) presence of precursor ion in the MS/MS spectra ensured the precursor and fragment ions are time-aligned with retention time of the peptide; (d) glycated peptides should have both b and y ions in the MS/MS spectra and (e) glycated peptides should have a minimum of seven fragment ions matching with the sequence.

3.2.8 Statistics

Statistical analyses for all the experiments were performed by Student's *t*-test. Lifespan statistics analysis was carried out using Mantel-Cox log rank test with 'OASIS' an online statistical software (http://sbi.postech.ac.kr/oasis).

3.3 Results

3.3.1 RIF increases lifespan in *C. elegans*

RIF increases lifespan of *C. elegans* both in live and heat killed *E. coli OP50* feed. The drug increased lifespan in a concentration dependent manner, fed with live *E. coli OP50* (Figure 3.1A). Since *C. elegans* were fed live *E. coli OP50* that can act as an opportunistic pathogen in older worms and decrease lifespan, it is possible that RIF simply increases life span by killing the bacteria. Additionally, it may also be possible that the increased lifespan may be due to the bacteria metabolizing the drug to produce an unrelated product that indirectly affect lifespan. To rule of out these possibilities, worms were grown on heat killed *E. coli OP50*. RIF was able to increase the lifespan of WT

worms up to 50% at all concentrations studied (Figure 3.1B). Thus, further experiments were performed using heat-killed *E. coli OP50*.

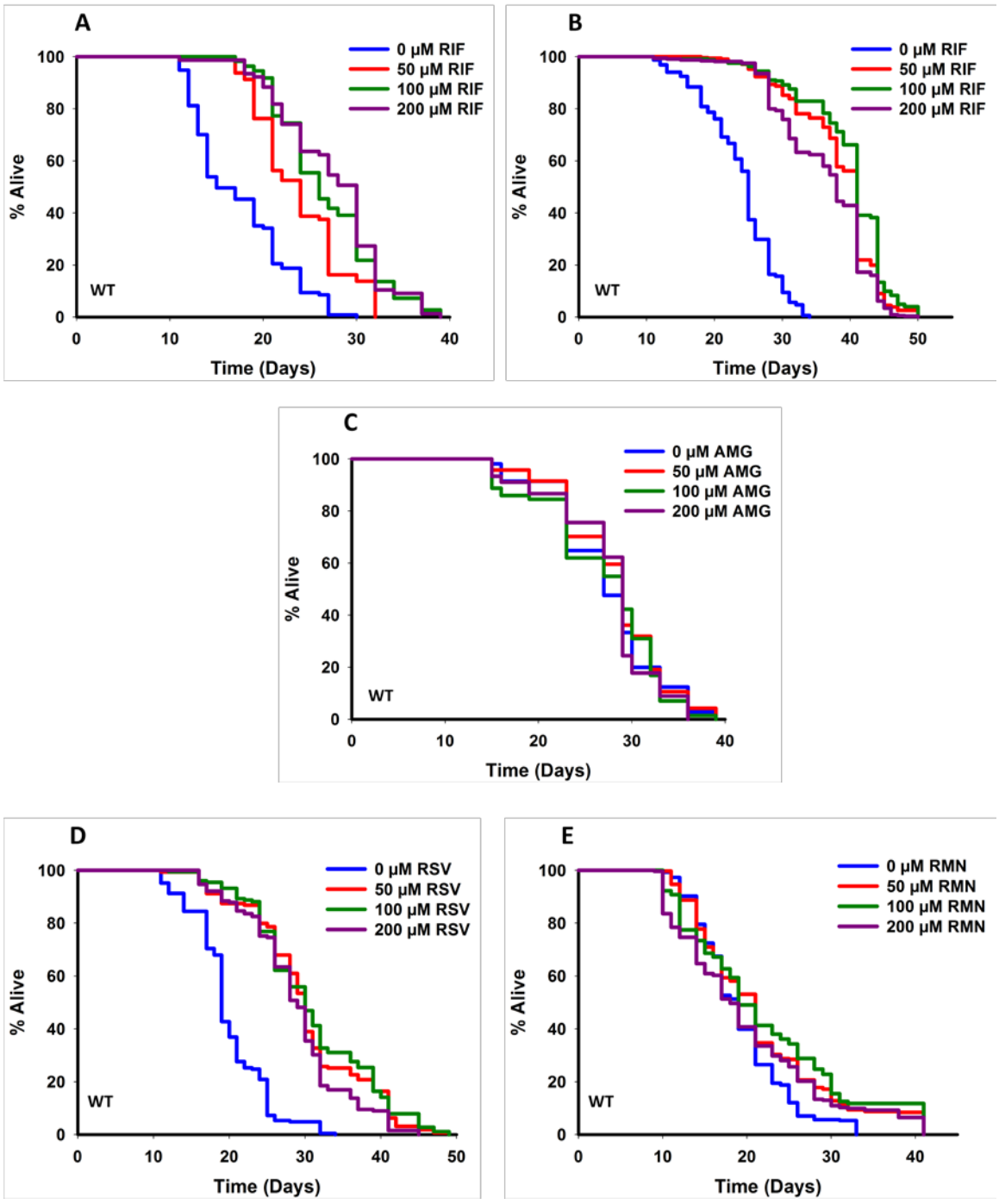

Figure 3.1: *In vivo*, RIF treatment increases lifespan of *C. elegans* (WT) worms. A. RIF supplementation increases the lifespan of worms fed with live *E. coli OP50*; **B.** RIF extends lifespan of worms fed on heat-killed *E. coli OP50*. Equivalent concentrations of DMSO treatment served as control (0 µM RIF); **C.** AMG failed to increase lifespan; **D.**

RSV, an analog of RIF increases lifespan; **E.** RMN, another analog of RIF, was unable to increase lifespan.

Additionally we evaluated *in vivo* efficacy of AMG, RSV, and RMN as shown in Figure 3.1C, D and E respectively (Table 3.1). We observed RSV was able to increase the lifespan up to 20% and had antiglycation action *in vitro* (described in Chapter 2). On the other hand, AMG and RMN were unable to extend the lifespan corresponding to the fact that they lacked *in vitro* anti-glycation activity (described in Chapter 2). It is well established that calorie restriction increases lifespan[16,17]. However, it is possible that under diabetic condition, especially glucose can react with proteins and form AGEs. AGE-modified proteins have altered function and could contribute to decreased lifespan. Therefore, we evaluated the effect of glycation inhibitors RIF and its analogs on *C. elegans* lifespan in high glucose condition. Glucose, as expected reduced the lifespan of worms by 48% (Figure 3.2A). While worms treated with RIF from 1st day of lifespan, showed increase in lifespan by 100% w.r.t control (Figure 3.2B). Similarly, RSV was able to increase the lifespan by 80% (Figure 3.2C). Both RIF and RSV were found to be potent inhibitor of glycation *in vitro*, which suggested that RIF and RSV might extend *C. elegans* lifespan by inhibiting glycation. However, it was observed that RMN also increased lifespan by more than 35%, despite of the absence of *in vitro* glycation inhibition activity (Figure 3.2D). However, RMN was unable to increase the lifespan in WT *C. elegans* (Figure 3.1E). Therefore it could be plausible that RMN might increase lifespan by a different mechanism other than glycation.

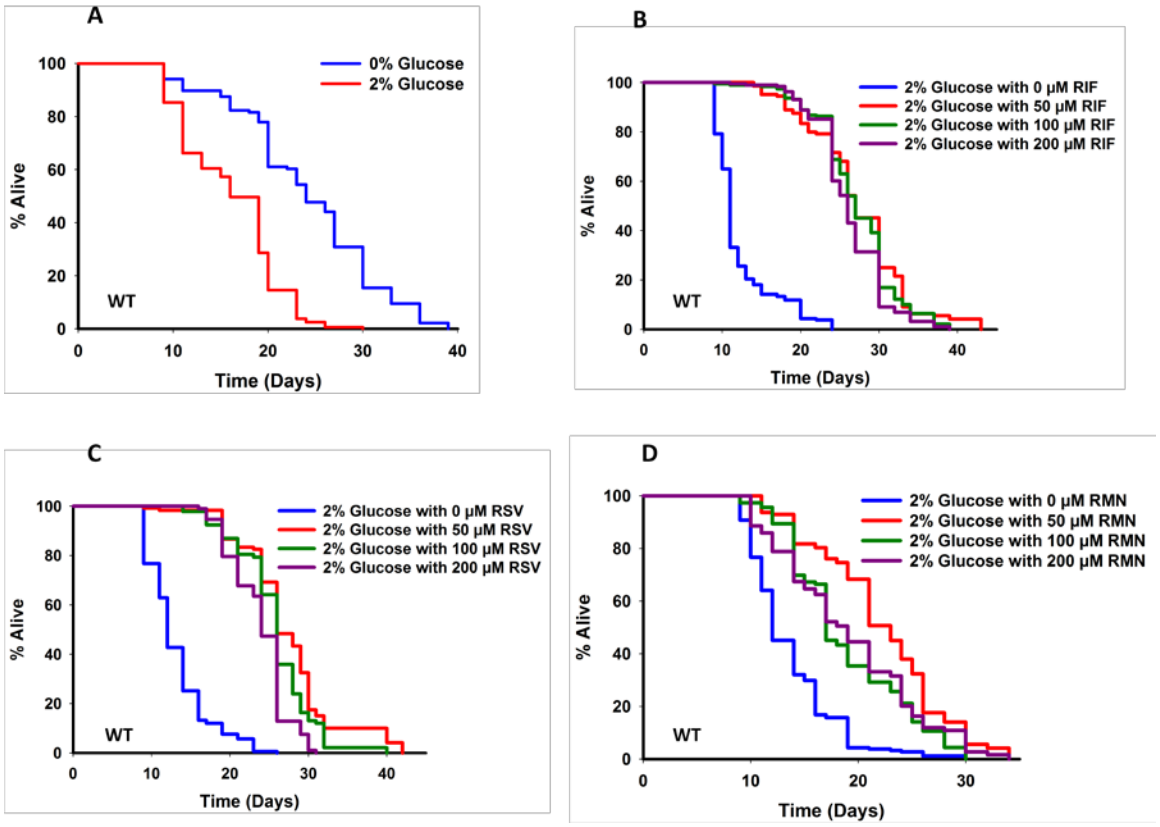

Figure 3.2: RIF ameliorate glucose toxicity and increases lifespan. A. 2% glucose decreases lifespan in *C. elegans* by 48%; **B.** RIF extends lifespan significantly in WT worms by 100% after supplementation with 2% glucose; **C.** RSV extends lifespan significantly in WT worms by 80%; **D.** RMN extends lifespan in presence of glucose more than 30%.

3.3.2 Effect on lifespan with delayed treatment of RIF

In present study, it was observed that RIF was able to increase the lifespan consistently when the worms were treated in the initial stages of lifespan, thus we asked whether delayed treatments of RIF have any beneficial effects on lifespan. We observed that treatment on 7th and 9th day increased lifespan, although this increase was relatively less as compared to 1st and 3rd day RIF treatment (Figure 3.3). Further, delayed treatment of RIF was evaluated in presence of glucose. Worms were treated with glucose on 1st day followed by treatment of RIF on i.e. 7th and 9th day. RIF treatment initiated on 7th and 9th day partially rescued glucose toxicity and increased the lifespan minimum of 30% and 20% respectively (Figure 3.4). Although early treatment was certainly more beneficial,

delayed treatment of RIF was also useful to certain extent under conditions of glucose induced toxicity.

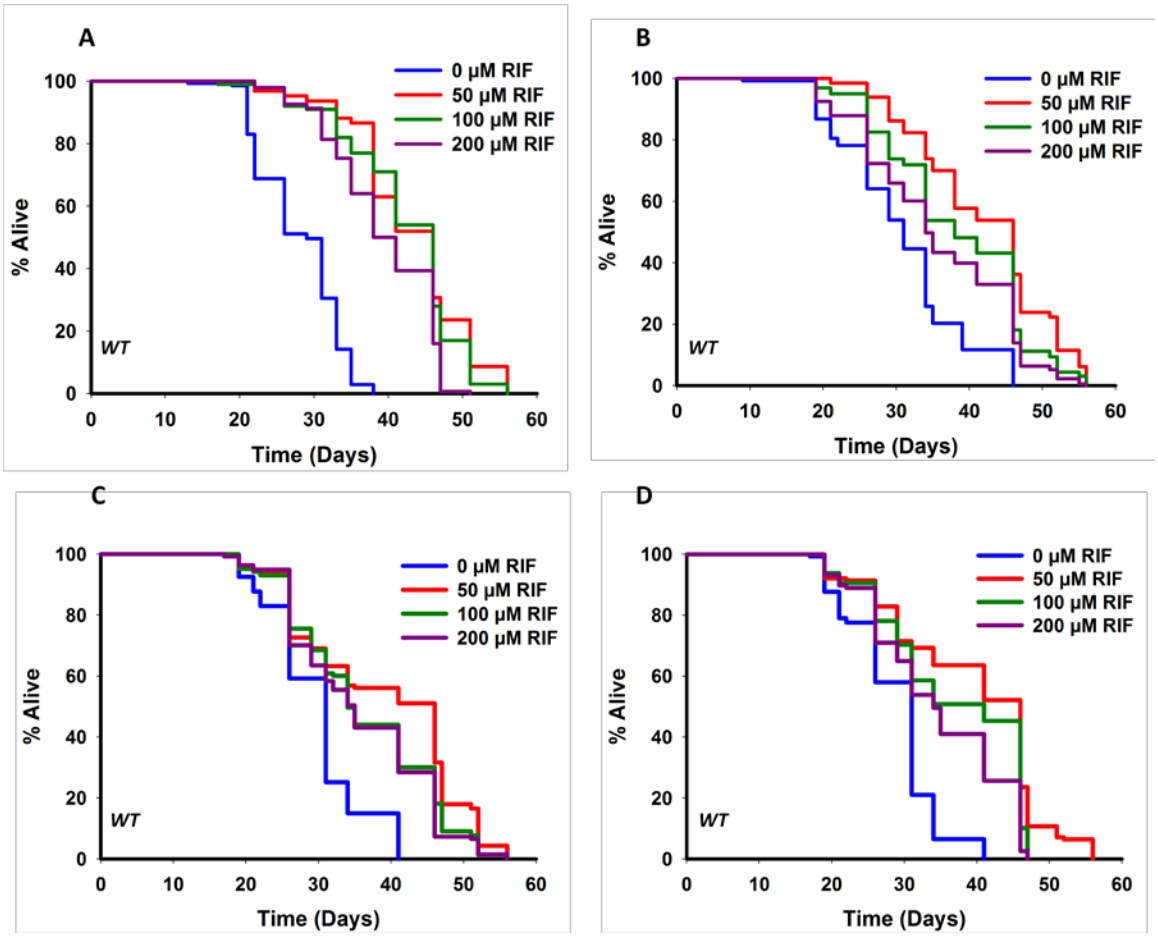

Figure 3.3: *In vivo* effect of delayed RIF treatment under euglycemic condition; RIF treatment was initiated on 1st, 3rd, 7th or 9th day. A. RIF treatment initiated on 1st day dramatically increases lifespan; **B.** RIF treatment initiated on 3rd day increases lifespan but less effective than 1st day; **C and D.** RIF treatment on 7th day or 9th has less efficacy, respectively.

Table 3.1: Lifespan data for *C. elegans*

Background	Treatment	Mean ± SEM	No. of Subjects	Percent increase (+) or decrease (-) w.r.t. control	*p*-value
Wild Type	0 µM RIF with live *E. coli OP50*	17.47 ± 0.47	117		
	50 µM RIF with live *E. coli OP50*	23.90 ± 0.52	80	(+) 36.806	≤0.0001
	100 µM RIF with live *E. coli OP50*	26.82 ± 0.51	110	(+) 53.520	≤0.0001
	200 µM RIF with live *E. coli OP50*	27.45 ± 0.64	77	(+) 57.127	≤0.0001
Wild Type	0 µM RIF	24.89 ± 0.28	644		
	50 µM RIF	39.57 ± 0.29	625	(+) 58.980	≤0.0001
	100 µM RIF	39.61 ± 0.31	618	(+) 59.140	≤0.0001
	200 µM RIF	37.28 ± 0.27	698	(+) 49.779	≤0.0001
	2 % Glucose with 0 µM RIF	12.23 ± 0.26	211		
	2 % Glucose with 50 µM RIF	27.38 ± 0.52	144	(+) 123.876	≤0.0001
	2 % Glucose with 100 µM RIF	27.18 ± 0.37	189	(+) 122.240	≤0.0001
	2 % Glucose with 200 µM RIF	26.22 ± 0.33	188	(+) 114.391	≤0.0001
Wild Type	0 µM RMN	24.89 ± 0.28	644		
	50 µM RMN	21.48 ± 0.46	320	(-) 13.700	≤0.0001
	100 µM RMN	21.98 ± 0.57	271	(-) 11.691	0.0472
	200 µM RMN	19.95 ± 0.52	292	(-) 19.847	≤0.0001
	2 % Glucose with 0 µM RMN	13.61 ± 0.30	184		
	2 % Glucose with 50 µM RMN	22.11 ± 0.50	142	(+) 62.454	≤0.0001
	2 % Glucose with 100 µM RMN	18.81 ± 0.51	113	(+) 38.207	≤0.0001
	2 % Glucose with 200 µM RMN	19.07 ± 0.48	184	(+) 40.118	≤0.0001
Wild Type	0 µM RSV	24.89 ± 0.28	644		
	50 µM RSV	30.09 ± 0.61	159	(+) 20.892	≤0.0001
	100 µM RSV	30.90 ± 0.59	177	(+) 21.254	≤0.0001
	200 µM RSV	28.74 ± 0.48	189	(+) 15.468	≤0.0001
	2 % Glucose with 0 µM RSV	13.18 ± 0.30	159		
	2 % Glucose with 50 µM RSV	27.39 ± 0.56	120	(+) 107.815	≤0.0001
	2 % Glucose with 100 µM RSV	25.75 ± 0.49	108	(+) 95.372	≤0.0001
	2 % Glucose with 200 µM RSV	23.88 ± 0.38	113	(+) 81.184	≤0.0001
Wild Type	0 µM AMG	27.31 ± 0.55	105		
	50 µM AMG	28.13 ± 0.80	47	(+) 3.003	0.5925
	100 µM AMG	26.89 ± 0.74	71	(-) 1.538	0.8423
	200 µM AMG	27.44 ± 0.81	45	(+) 0.476	0.8439
Wild Type	0 µM RIF 1 Day	28.01 ± 0.46	141		
	50 µM RIF 1 Day	43.01 ± 0.70	127	(+) 53.552	≤0.0001
	100 µM RIF 1 Day	42.16 ± 0.79	100	(+) 50.518	≤0.0001
	200 µM RIF 1 Day	39.23 ± 0.57	150	(+) 40.057	≤0.0001
Wild Type	0 µM RIF 3 Day	30.81 ± 0.73	128		
	50 µM RIF 3 Day	42.14 ± 0.81	130	(+) 36.774	≤0.0001
	100 µM RIF 3 Day	38.43 ± 0.77	160	(+) 25.996	≤0.0001
	200 µM RIF 3 Day	35.69 ± 0.77	173	(+) 15.352	0.0057

Background	Treatment	Mean ± SEM	No. of Subjects	Percent increase (+) or decrease (-) w.r.t. control	*p*-value
Wild Type	0 µM RIF 7 Day	29.81 ± 0.52	147		
	50 µM RIF 7 Day	38.92 ± 0.94	139	(+) 30.560	≤0.0001
	100 µM RIF 7 Day	36.34 ± 0.81	143	(+) 21.905	≤0.0001
	200 µM RIF 7 Day	35.72 ± 0.81	137	(+) 19.826	≤0.0001
Wild Type	0 µM RIF 9 Day	28.62 ± 0.50	138		
	50 µM RIF 9 Day	39.46 ± 0.88	140	(+) 37.876	≤0.0001
	100 µM RIF 9 Day	36.84 ± 0.85	128	(+) 28.721	≤0.0001
	200 µM RIF 9 Day	34.62 ± 0.83	117	(+) 20.964	≤0.0001
Wild Type	0 Glucose	24.10 ± 0.65	136		
	2 % Glucose	16.15 ± 0.41	746	(-) 48.880	≤0.0001
Wild Type	2 % Glucose with 0 µM RIF 1 Day	12.30 ± 0.28	158		
	2 % Glucose with 50 µM RIF 1 Day	25.33 ± 0.59	108	(+) 105.935	≤0.0001
	2 % Glucose with 100 µM RIF 1 Day	25.27 ± 0.41	100	(+) 105.447	≤0.0001
	2 % Glucose with 200 µM RIF 1 Day	24.44 ± 0.36	105	(+) 98.699	≤0.0001
Wild Type	2 % Glucose with 0 µM RIF 3 Day	15.62 ± 0.38	181		
	2 % Glucose with 50 µM RIF 3 Day	26.37 ± 0.39	150	(+) 68.822	≤0.0001
	2 % Glucose with 100 µM RIF 3 Day	28.17 ± 0.45	129	(+) 80.346	≤0.0001
	2 % Glucose with 200 µM RIF 3 Day	26.36 ± 0.36	149	(+) 68.758	≤0.0001
Wild Type	2 % Glucose with 0 µM RIF 7 Day	14.92 ± 0.27	181		
	2 % Glucose with 50 µM RIF 7 Day	19.92 ± 0.57	133	(+) 33.512	≤0.0001
	2 % Glucose with 100 µM RIF 7 Day	21.95 ± 0.56	153	(+) 47.118	≤0.0001
	2 % Glucose with 200 µM RIF 7 Day	21.32 ± 0.50	158	(+) 42.895	≤0.0001
Wild Type	2 % Glucose with 0 µM RIF 9 Day	13.85 ± 0.24	214		
	2 % Glucose with 50 µM RIF 9 Day	16.65 ± 0.52	127	(+) 20.217	0.0017
	2 % Glucose with 100 µM RIF 9 Day	17.67 ± 0.59	148	(+) 27.581	0.0031
	2 % Glucose with 200 µM RIF 9 Day	17.21 ± 0.42	173	(+) 24.260	≤0.0001
***glod-4* (*gk189*)**	0 µM RIF	22.78 ± 0.23	379		
	50 µM RIF	36.88 ± 0.31	278	(+) 61.896	≤0.0001
	100 µM RIF	35.86 ± 0.34	254	(+) 57.419	≤0.0001
	200 µM RIF	32.42 ± 0.31	305	(+) 42.318	≤0.0001

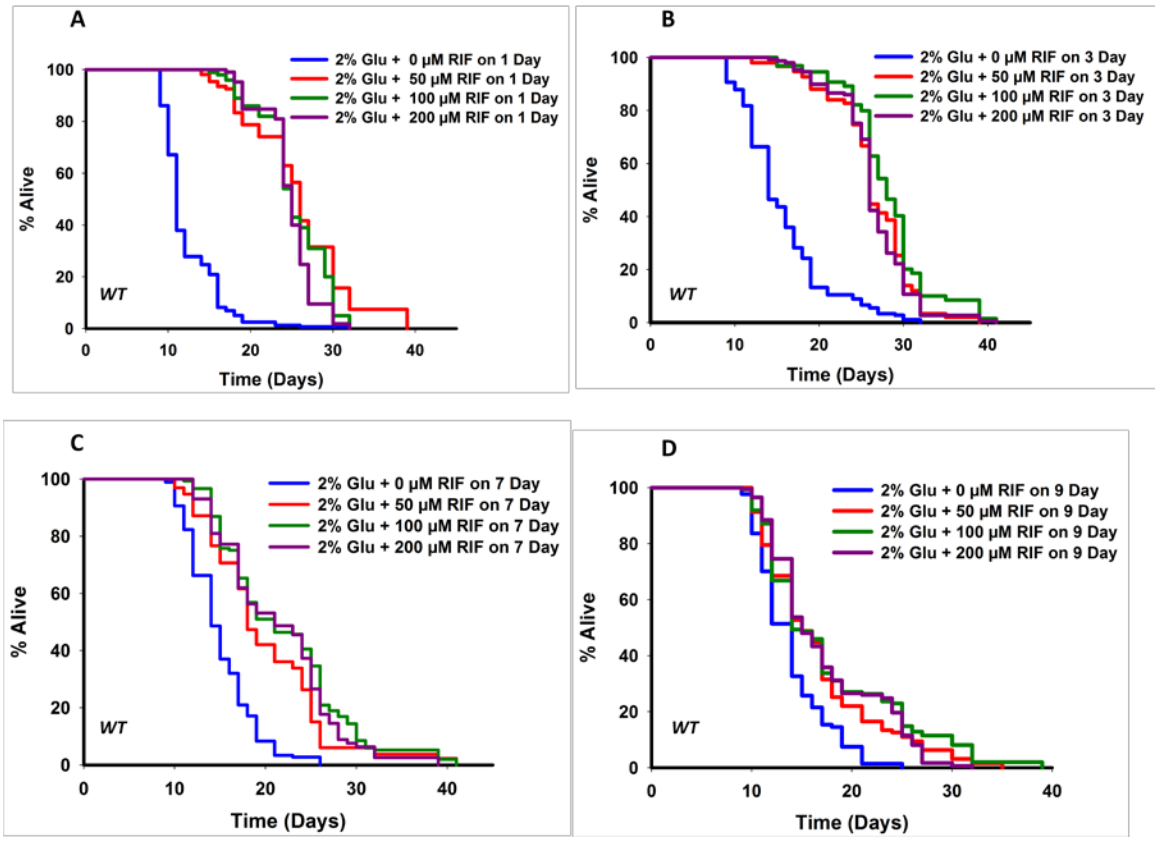

Figure 3.4: RIF ameliorates glucose toxicity and increases *C. elegans* lifespan on early drug treatment. **A.** RIF treatment on 1st day extends lifespan by 100% w.r.t control; **B** RIF treatment on 3rd day extends lifespan by 70 % w.r.t control **C and D.** RIF treatment on 7th and 9th day protects worms from glucose toxicity but with a decreased efficiency minimum of 30% and 20% respectively.

3.3.3 RIF rescues methyglyoxal toxicity

Methylglyoxal (MG) is a highly reactive dicarbonyl that intereacts with epsilon amino group of lysine and sulfhydryl groups of proteins leading to the formation of AGEs. Glyoxylase system normally detoxifies MG, but with advancing age glyoxalase activity declines, leading to the increased accumulation of AGEs. Increased AGE levels were observed when glyoxalase was knocked down[4]. In this study, we used the glyoxalase mutant, *glod-4(gk189)* which lacked MG detoxifying enzyme glyoxylase I, thus had shorter lifespan. We evaluated RIF antiglycation activity using *glod-4(gk189)* mutant model. RIF was able to significantly increase the lifespan of *glod-4(gk189)* mutant minimum of up to 40%. Western blot was performed to analyze the AGE modified

proteins in *glod-4 (gk189)* mutant in presence of RIF. Interestingly, RIF was able to decrease AGE modification of proteins in the *glod-4(gk189)*. Thus, RIF could act as a glycation inhibitor *in vitro* and *in vivo* and affected lifespan positively (Figure 3.4). Next we used LC-MS^E, a Data-Independent Acquisition (DIA) approach to identify AGEs in *glod-4(gk189)*. LC-MS^E is a unique approach wherein all eluted peptides are fragmented and the fragment ions are time-aligned with the retention time of the peptides[13,18]. This method allows analysis of low-intensity AGE modified peptides as well as heterogenous AGEs[19]. A total of 24 proteins carrying 59 AGE modifications were detected in control treatment, while in RIF treatment the modifications decreased to 25 (i.e up to 57%) (Table 3.1). We observed that Vitellogenin (VIT-6) had more number of modifications. Nakamura et al. (1996) also reported that VIT-6 was heavily carbonylated in worms[20]. The other proteins that were AGE modified include ALDO-2, EEF-2, CRT-1, HEX-1, ACO-2, UNC-15, TBA-2, MDH-1, AHCY-1 CST-1, UBQ-2 and FAR-1&2. RIF treatment decreased AGE modification of these proteins. Marked decrease in glycation was also reported in mitochondrial proteins when glyoxalase I was overexpressed in *C. elegans*[4]. We also found lesser number of modifications on a Calreticulin and a beta hexoaminidase protein after RIF treatment. While the aldolase, aconitase, malate dehydrogenase and citrate synthase are important rate-limiting enzymes of glycolysis and Kreb's cycle, Calreticulins prevent misfolded proteins from exiting the endoplasmic reticulum. On the other hand, beta-hexosaminidase A deficiency causes Tay-Sachs and Sandhoff diseases due to accumulation of its substrate, GM2 ganglioside, in neuronal lysosomes[21]. Modifications on these important proteins may disrupt normal cellular functions leading to aging. RIF has beneficial effects in retaining functional proteins, therefore it affected lifespan positively.

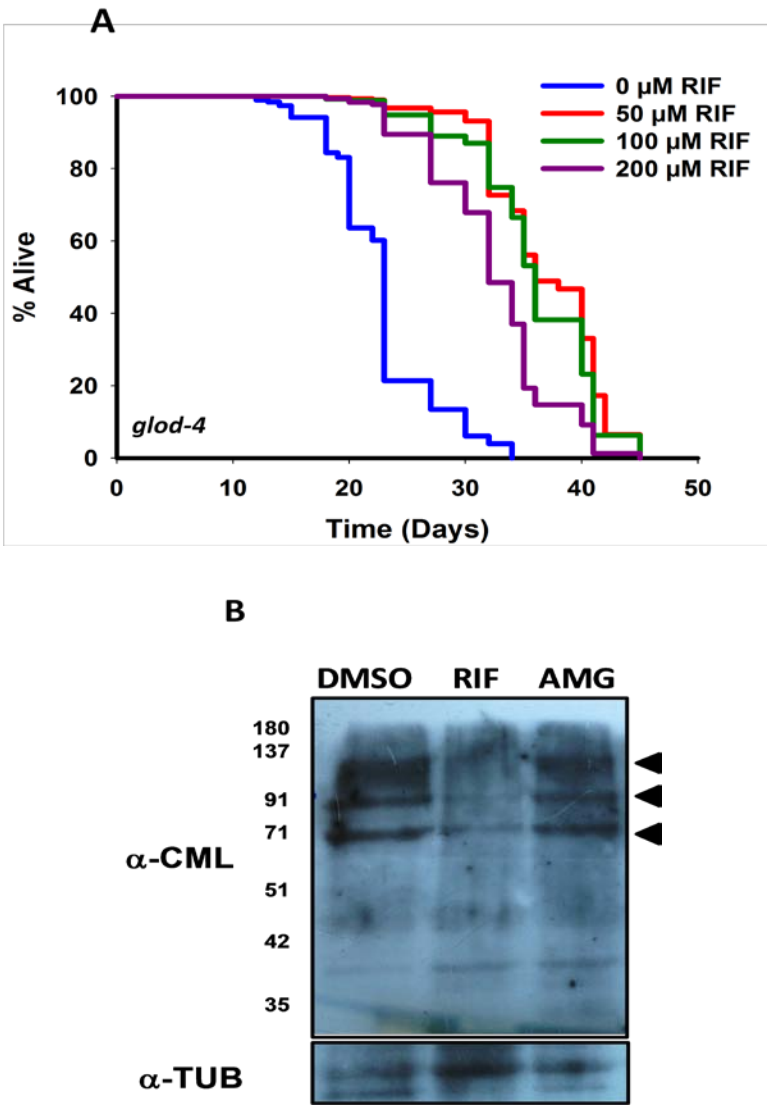

Figure 3.5: RIF ameliorate glycation in *glod-4* (*gk189*) mutant. A. RIF extends lifespan of *glod-4* (*gk189*) significantly; **B.** Western blot performed with *glod*-4 protein lysate using 30 µg total protein per lane. α-CML antibody was used for detection of AGE modified proteins and α-tubulin (TUB) used as control. RIF has effectively decreases CML modification in *glod-4* mutant (lane 2) while AMG has less effective (lane 3).

Table 3.2: List of proteins glycation status after treatment with RIF

Gene Name	Protein Name	No of peptides modified with DMSO Treatment	Modification Type (number of modifications of this type)	No of peptides modified with RIF Treatment	Modification Type (number of modifications of this type)
vit-6	**Vitellogenin 6**	5	AM(3), PEN(1), IMD-A(1), IMD-B(1), ARGPYR(1), MG-H1(1), PYR(2), AFGP(1)	1	CEL(1), IMD-A(1),
unc 54	**Myosin 4**	3	AM(1), ARGPYR(1), PYR(1), CROSS(1), MG-H1(1)	4	CEL(1), IMD-A(1), MOLD(4), CROSS(1)
aldo-2	**Fructose bisphosphate aldolase 2**	3	AM(1), PEN(1), CEL(1), MG-H1(1), AFGP(1), CROSS(1), GH1(1)	0	N/A
eef-2	**Elongation factor 2**	2	AM(1), MOLD(1)	1	AM(1), MG-H1(1)
hex-1	**Beta hexosaminidase A**	2	AM(1), CML(1)	0	N/A
F37C4.5	**Protein F37C4 5**	2	IMD-A(1), IMD-B(1), ARGPYR(1)	0	N/A
cts-1	**Probable citrate synthase mitochondrial**	2	MOLD(1), CROSS(2)	0	N/A
crt-1	**Calreticulin**	2	AM(1), CROSS(2)	0	N/A
vit-2	**Vitellogenin 2**	1	IMD-A(1), IMD-B(1)	1	AM(1), GH1(1)
unc-15	**Paramyosin**	1	MOLD(1), MG-H1(1)	0	N/A
aco-2	**Probable aconitate hydratase mitochondrial**	1	PEN(1), CROSS(1)	0	N/A
eft-3	**Elongation factor 1 alpha**	1	CML(1)	2	CEL(2), CROSS(1)
tba-2	**Tubulin alpha 2 chain**	1	CEL(1)	0	N/A
ahcy-1	**Adenosylhomocysteinase**	1	CML(1)	0	N/A
act-4	**Actin 4**	1	CEL(1)	1	MOLD(1)
asp-6	**Aspartic protease 6**	1	AM(1)	1	AM(2)

mdh-1	**Probable malate dehydrogenase mitochondrial**	1	MOLD(2)	0	N/A
far-1	**Fatty acid and retinol binding protein 1**	1	PEN(1), MOLD(1), CROSS(1)	0	N/A
far-2	**Fatty acid and retinol binding protein 2**	1	PEN(2), MOLD(1)	0	N/A
sod-1	**Isoform b of Superoxide dismutase Cu Zn**	1	CEL(1)	1	MOLD(1)
ttr-2	**Transthyretin like protein 2**	1	CML(1), AFGP(1)	1	CML(1)
ubq-2	**Ubiquitin 60S ribosomal protein L40**	1	PYR(1)	0	N/A
atp-2	**ATP synthase subunit beta mitochondrial**	0	N/A	1	CEL(2)
H28O16.1	**Isoform d of ATP synthase subunit alpha mitochondrial**	0	N/A	1	GH(1)
F08B12 4	**Uncharacterized protein F08B12 4**	0	N/A	1	ARGPYR(1)
	Total	**35**	**59**	**15**	**25**

Decreased glycation w.r.t. control

No decrease or increase w.r.t control

Increased glycation w.r.t. control

3.3.4 Effect of RIF on proteotoxicity

With progression of age the removal of damaged proteins declines, probably due to the reduced activity of proteolytic system leading to toxic protein aggregation[22]. Apart from aging and diabetic complication, AGEs also play important role in the genesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease[23,24](PD). AD is one of the most common type of age-related dementia[25,26].

Accumulation of toxic Aβ1-42 peptide in the human brain is the hallmark of AD. AGEs can be detected in amyloid plaques and neurofibrillary tangles and the extent of glycation of these amyloid peptides correlates well with pathology of the disease[24,27]. In *C. elegans*, constitutively expressed human Aβ1-42 peptide in the body wall muscles of *CL2006* strain results in progressive paralysis with age[28]. Since RIF treatment led to increased lifespan, we checked whether this treatment with it will have beneficial effects on age-onset proteotoxicity. We found that RIF was able to significantly reduce age-dependent paralysis in *CL2006* (Figure 3.5A). Similar inhibition of Aβ aggregation by RIF treatment has also been reported in rat pheochromocytoma-derived PC12 cells [29]. This protective nature of RIF appears to be linked to the glycation inhibition efficacy of the drug as RSV had a similar role (Figure 3.5B). RMN has shown beneficial effects at higher concentration against proteotoxicity that may be due to potential of DAF-16 translocation in nucleus. AMG effect decreases with age progress (Figure 3.5C), perhaps higher concentration might be required to have protection against amyloid beta toxicity. Additionally, in the Huntington disease[30] model of *C elegans*, RIF was able to significantly delay the age-dependent accumulation of poly Q35 aggregates in the *rmIs132* strain (Figure 3.6).

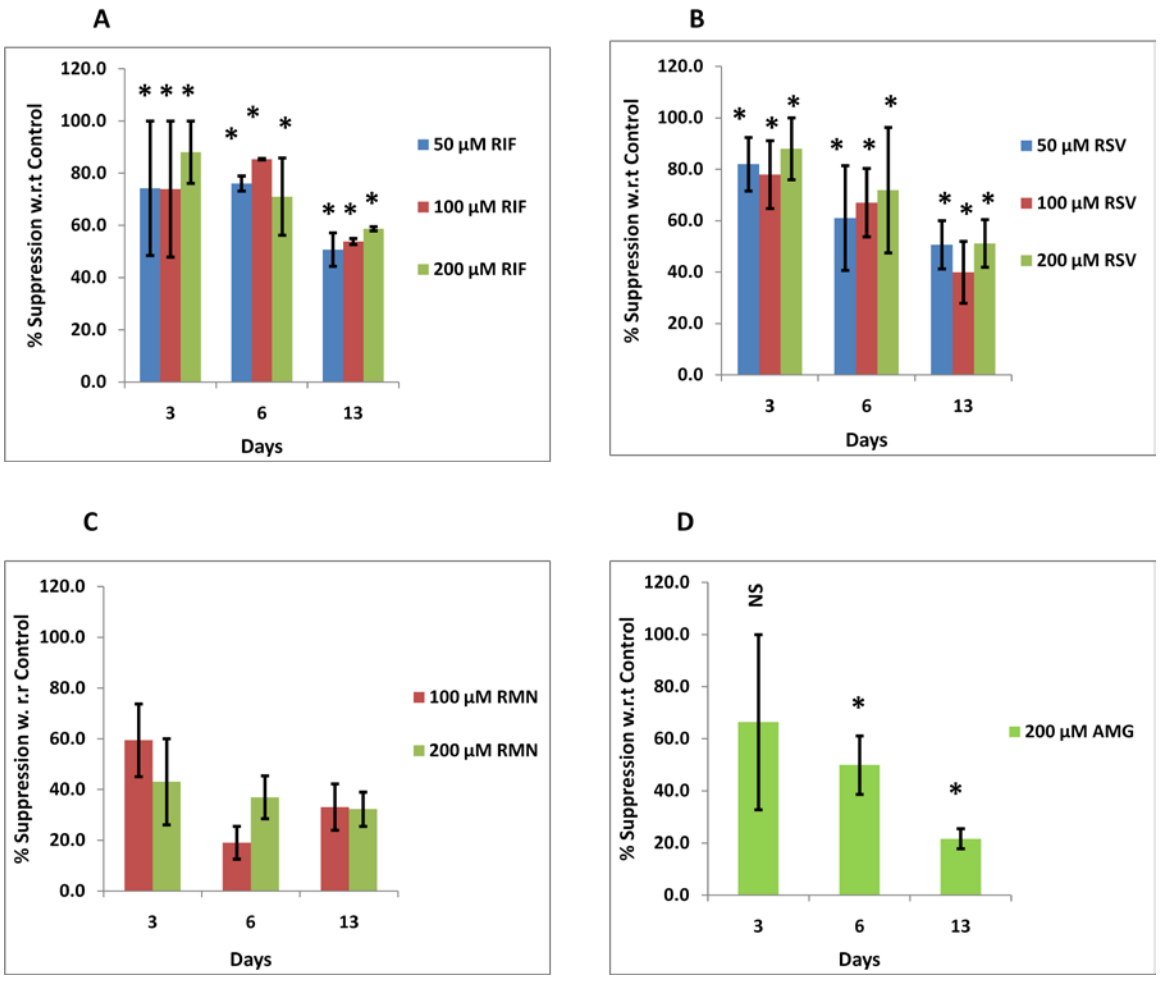

Figure 3.6: RIF and RSV protects against proteotoxicity. A and B. RIF and RSV treatment reduces Aβ$_{1\text{-}42}$-induced paralysis by 40% on 13 day in *CL2006* transgenic strain significantly; **C.** RMN Treatment shows slight protection effect at higher concentration; **D.** AMG also has slight protective effect in preventing paralysis * $p \leq 0.01$, NS-non significant.

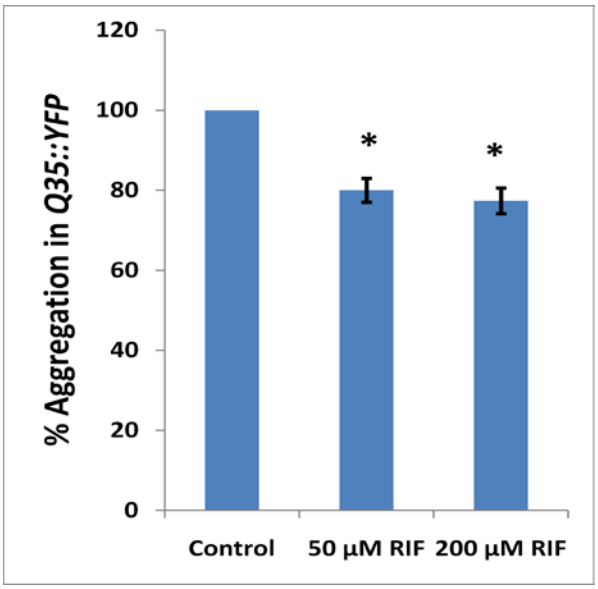

Figure 3.7: RIF significantly reduce polyglutamine (Q35) aggregation in *rmIs132* strain. * $p \leq 0.01$

3.4 Discussion

In this study, we have shown that RIF and its analog RSV were effective in extending wild type worm's lifespan, reduction in glucose toxicity and proteotoxicity. Though RIF extended lifespan but its efficiency of lifespan extension was decreased when the worms were treated at the late stage, which was perhaps due to the onset of aging process. AMG is also a known glycation inhibitor that has been shown in many studies to reduce *in vivo* AGE formation and offer protection against diabetic neuropathy, nephropathy and retinopathy[31]. However, we observed that AMG was unable to reduce *in vivo* glycation as effectively as RIF and increase lifespan. In *Drosophila*, AMG was not able to increase lifespan, but AMG had marginal decrease in AGE formation in old flies[32]. MG is a highly reactive dicarbonyl that accelerates AGEs formation and is normally detoxified by the glyoxalase system. In the glyoxalase mutant *glod-4 (gk189)*, higher AGE levels were observed because of continuous formation of MG[4]. With progressive age, glyoxalase activity was reduced leading to increased accumulation of AGEs. RIF was able to rescue lifespan of *glod-4 (gk189)* mutant and protected proteins from glycation. In addition we have shown by proteomic approach, RIF treatment reduces AGE modification on major cellular proteins that control metabolism at post-translational level. RIF targets the

vitellogenins that are required for growth and development of progeny. However, the vitellogenin proteins appear to be toxic at old age in worms, as knockdown of *vit-2* and *vit-5* by RNAi increases lifespan (Murphy *et al.* 2003). Vitellogenins are required during the self-fertile reproductive phase of hermaphrodites and later for reproduction by cross-fertilization. In the absence of cross-fertilization in older worms as well as in aging *Drosophila*, vitellogenins accumulate in the body cavity and are subjected to oxidative damage. In later life, they become one of the most prevalent proteins in aggregates[20,33]. AGE modifications on these proteins may accelerate aggregation and toxicity[34]. Additionally with the help of *C. elegans* Aβ1-42 peptide model *CL2006* and polyglutamine Q35::YFP, we have shown that RIF effectively reduce age related proteotoxic disorders. Thus, the multi-dimensional effects of RIF results in one of the most dramatic effect of a drug on longevity in *C. elegans*. This study using a *C. elegans* model shows how a FDA-approved drug might be repositioned to potentially treat age-associated pathologies like amyloid diseases and hyperglycaemia as well as increase lifespan.

3.5 Conclusions

C. elegans model system was used to evaluate *in vivo* efficiency of RIF, RSV, RMN and AMG. RIF has been able to increase lifespan in euglycemic as well as hyperglycaemic conditions. RSV is an analog of RIF that has shown similar trend in *C. elegans* while the same time, RMN and AMG unable to do so. Delayed treatment of RIF on worms in euglycemic condition does not show any beneficial effects. However, in hyperglycemic condition, delayed treatment of RIF offered a slight protection against glucose toxicity. Further, RIF protect against MG toxicity in glyoxalase lacking mutant *glod-4(gk189)* in *C. elegans* model. Using LC-MS^E, we show that RIF reduces glycation of important cellular proteins *in vivo* and increases lifespan in *glod-4(gk189)* mutant by minimum of 40%. Apart from increasing lifespan, RIF offers considerable protection against proteotoxicity in worm models of Alzheimer's and Huntington disease. Thus, pharmaceutical interventions with RIF can reduce AGEs; and may delay age-onset diseases and extend lifespan.

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Chapter 4

Molecular mechanism of rifampicin mediated longevity

4.1 Introduction

Aging is an unavoidable and irreversible process involving complex multiple cellular processes. Insight into the molecular mechanisms of aging was gained after the discovery of Target of rapamycin (TOR), AMP-activated protein kinase (AMPK), Transforming growth factor-β (TGF-β) and insulin/IGF signaling (IIS) pathways that regulate aging across the animal kingdom. The TOR kinase is a major amino acid and nutrient sensor that stimulates growth and metabolism. In response to nutrients, TOR up regulates translation by activating ribosomal subunit S6 kinase and inhibiting 4E BP (translation initiation factor 4E-binding protein 1). This effect mediates longevity in yeast, worms, *Drosophila*, and mice[1-4]. AMP kinase is a sensor for nutrient and energy; it activates catabolic pathways and represses anabolic pathways if the AMP/ATP ratio increases[5]. TGF-β signaling plays a crucial role in cell proliferation, differentiation and death. This pathway operates through IIS pathway[6]. Genetic and physiological studies in various models suggest that the IIS is evolutionary conserved[7]. Cellular levels of IIS regulates multiple processes such as carbohydrate, lipid metabolism, cell growth, DNA synthesis and protein synthesis [8,9]. The DAF-2 gene encodes for IIS receptor in *C elegans*. For the first time, Kenyon et al. 1993, demonstrated that reduced IIS leads to extension of lifespan in *C elegans*[10]. AGE-1 is a phosphoinositide 3 Kinase (PI3K) that acts downstream to DAF-2 and signals to the serine/threonine kinase PDK-1[11,12]. DAF-18, (a mammalian homolog of PTEN) acts as negative regulator of the IIS pathway by converting PIP3 to PIP2. As a result, *daf-18* mutants have shorter lifespan[13,14] . PDK-1, in turn, activates downstream serine-threonine kinases AKT-1, AKT-2 and SGK-1[15]. The major output of the IIS pathway is DAF-16 (the mammalian homolog of FOXO3a and a fork-head containing transcription factor). Under the normal insulin signaling condition, the upstream kinases phosphorylate DAF-16 and retain it in the cytoplasm. However, under low insulin signaling conditions or in case of mutations in components of the DAF-2 pathway, DAF-16 is translocated into the nucleus, thus leading to expression of its target genes. C-Jun N terminal kinase (JNK) belongs to a subgroup of the mitogen-activated protein kinase (MAPK) super family. In *C. elegans* and Drosophila, JNK phosphorylates DAF-16/FOXO and overexpression of JNK-1 results in lifespan

extension[16,17]. Since DAF-16 is a key protein in lifespan regulation in *C. elegans*, therefore, we studied the effect of rifampicin (RIF) on DAF-16 to elucidate RIF induced lifespan extension.

4.2 Materials and methods

4.2.1 Strain maintenance

All strains were maintained at 20°C with a standard maintenance procedure[18]. Strains used were *daf-16(mgDf50), daf-18(e1375), pdk-1(mg142), akt-1(mg144), jnk-1(gk7),* and *jkk-1(km2).*

4.2.2 Microarray

Microarray analysis was carried out commercially at iLife Technology (Gurgaon, India) using an Affymatrix platform. This whole genome Gene Chip array represents 22,500 transcripts from *C. elegans*. Data was analysed using CLC Genomics Workbench 4 (CLC Bio, USA). 2 fold up and down expression changes with p <0.05 were reported. This microarray data is available at GEO repository with number GSE45292. Up and down regulated genes were mentioned in Appendix I and II.

4.2.3 RNA sample preparation

Eggs were collected from well fed gravid worms and placed on new *E. coli OP50* NGM agar plate. The plates were incubated at 20°C till young adult stage, at this stage worms were washed and collected with M9 buffer. Worms were centrifuged at 2000 rpm for a minute; pellet was washed for 4 times with M9 buffer for complete removal of live bacteria. Finally the worms were suspendened in S-complete buffer (1 litre S-basal buffer, 10 mL of 1M potassium citrate pH-6, 10 mL of trace metals, 3 mL of 1M calcium chloride, 3 mL of 1M magnesium sulphate and 0.5 ml of 10 mg/mL cholesterol was filter sterilized using 0.2µ filter); with 100 mg/mL heat killed *E. coli OP50*; 0.6 mM Fudr (5'-fluoro-2'-deoxyuridine); 50 mg/mL carbenicillin and 250 µg/µL amphotericin B in a sterile flask. All the steps were performed in laminar air flow. The flasks were kept in shaker incubated with 120 rpm at 20°C. Next day, Di-methyl sulfoxide (DMSO) or RIF, RSV, RMN or AMG were added each flask. Additional experiment was done with or

without glucose. On 5th day again 1.8 mL of 100 mg/mL heat killed *E. coli OP50* were added in each flask. On 8th day, worms of different condition were collected in separate 15 mL falcon tubes and centrifuged at 2000 rpm for a minute. The worm pellet was washed with M9 buffer at least 4 times. The worm pellet was transferred to a 1.5 mL eppendrof tube, centrifuged to remove M9 buffer. Around 150 µL of trizol (Life Technologies, USA) was added and vortex the tube. Further the tube were flash frozen in liquid nitrogen followed by quick thawing twice, the tubes were stored at -80°C till use.

4.2.4 RNA extraction

Worms grown under different treatment were thawed at room temperature (RT) and kept on ice. Tubes were vortexed after adding 150 µL of trizol (Life Technologies, USA) in each tube. Chloroform was added to each tube; tubes were vortexed for 3 minute and allowed to stand at RT for 10 minute. The tubes were centrifuge at 13000 rpm for 15 minute at 4°C. Supernatant was collected in fresh RNAase free tubes separately. 2-propanol was added such a way that supernatant and 2-propanol volume ratios were 1:1. The tubes were gently inverted and allowed to stand for 3 minute. The tubes were centrifuged at 13000 rpm for 10 minute at 4°C. The pellet was washed with 500 µL of 70% ethanol (freshly prepared). The tubes were centrifuged at 14000 rpm for 5 minute at 4°C; pellet was kept for drying at RT. Pellet was dissolved in RNAase free water. RNA was quantified using Nanodrop (Thermo, USA), and RNA purity was determined using Bioanalyzer (Agilent, USA).

4.2.5 cDNA and quantitative real time (qRT-PCR)

cDNA was prepared using SuperScript III cDNA synthesis kit (Life Technologies, USA), reaction was initiated with 2.5 µg of RNA concentration. Gene expression was determined by quantitative real time PCR (qRT-PCR) using the Mesagreen master mix (Eurogentec, Belgium) using Realplex PCR system (Eppendorf, USA) platform. Relative gene expression was determined after normalizing actin between worms DMSO treated control and RIF treated. Statistical analysis was performed by Student's *t*-test. Primers are listed in Appendix III.

4.2.6 DAF-16::gfp Translocation assay

The translocation assay was performed according to Oh et al. (2005), with slight modification in protocol as DMSO induce stress so all experiment carried out at 15°C[16]. *E. coli OP50* seeded plates were overlaid with 500 µM DMSO, RIF, rifamysin SV (RSV), and rifaximin (RMN) and aminoguanidine (AMG). *daf-16::gfp* (*TJ356*) worms were bleached and the eggs were placed on respective plates, and incubated at 15°C till L3 Stage. L3 stage worms were picked and mounted on slide containing 2% agarose pad with a drop of 10 mM sodium azide in S-basal buffer. GFP (Green fluorescent protein) expression was analyzed at 10X and 40X magnifications under Zeiss Axio imager M2 digital microscope (Carl Zeiss, USA), and images were acquired using Axio Cam software. Worms were scored and divided in to three categories depending upon their GFP expression pattern such as cytosolic, intermediate and nuclear. The experiment was performed in 3 biological and 3 technical repeats of each. DMSO and water were used as control for RIF, RSV, RMN and AMG, respectively depending on their solubility.

4.2.7 Lifespan assays

All lifespan assays were performed at 20°C. A well fed gravid worms on *E. coli OP50,* was bleached and eggs were placed on new *E. coli OP50* seeded NGM agar plate. The plates were incubated at 20°C till young adult stage. Worms were washed and collected with M9 buffer; centrifuged at 2000 rpm for a minute, pellet was washed for minimum of 4 times to remove all live bacteria. Finally the worms were suspendened in S-complete buffer (1 liter S-basal buffer, 10 mL of 1M potassium citrate pH-6, 10 mL of trace metals, 3 mL of 1M calcium chloride, 3 mL of 1M magnesium sulphate and 0.5 mL of 10 mg/mL cholesterol was filter sterilized using 0.2µ filter). Liquid life span assays were set up in 96 well sterile flat bottom plates. Each well contained 8 to 12 live worms suspended in S-complete buffer with 100 mg/mL heat killed *E. coli OP50* and 0.6 mM Fudr (5'-fluoro-2'-deoxyuridine), 50 mg/mL carbenicillin and 250 µg/µL amphotericin B. All the steps were performed in the laminar air flow; plates were wrapped with para film and incubated at 20°C till the completion of assay. Next day, 10 µL of DMSO or S-complete buffer (vehicle control) or RIF, RSV, RMN and AMG was added in respective wells, this

day was considered as 1st day of lifespan. In case of glucose, all the steps were same except addition of glucose. On 5th day again 9 µL of 100 mg/mL heat killed *E. coli OP50* feed was added to each well. The plates were scored after seven days on every alternate day till all worms were alive. All the experiments were repeated minimum of 3 times. Data plotted in lifespan graph was cumulative for each experiment. Lifespan statistics analysis was carried out using Mantel-Cox log rank test with 'OASIS' an online statistical software[19].

4.2.8 Statistics

Statistical analyses for all the experiments were performed using Student's *t*-test. Lifespan statistics analysis was carried out using Mantel-Cox log rank test with 'OASIS' an online statistical software (http://sbi.postech.ac.kr/oasis).

4.3 Results

4.3.1 RIF activates FOXO homolog, DAF-16

Insulin signaling is an important pathway in *C. elegans* aging. Mutation in the IIS component like DAF-2 reduces insulin signaling and increases *C. elegans* lifespan[20]. Downstream to DAF-2, lipid phosphatase *daf-18* (mammalian homolog PTEN) is present which negatively regulates DAF-16 and this has shorter lifespan[13] . When *daf-18(e1375)* worms were treated with RIF, we observed that RIF was unable extend lifespan (Figure 4.1A). This result was suggested that *daf-18* is required for RIF mediated lifespan extension. *daf-2* mutants showed increased lifespan, which was completely dependent on DAF-16[21,22]. Further *daf-18* also interacts with DAF-16, so it was important to investigate the effect of RIF in *daf-16* mutation. We observed that RIF was unable to extend lifespan in *daf-16 (mgdf50)*, a null mutant (Figure 4.1B) suggesting RIF requires DAF-16 for extension of lifespan.

We have described in Chapter 3 that wild type worms supplemented with 2% glucose suppresses the life span up to about 48 % but with RIF treatment the lifespan increases up to 120%, thus RIF protects the wild-type worms from toxicity caused by glucose. Glucose normally activates IIS pathway, eventually phosphorylates DAF-16, thus impart its toxic effects of high glucose. Therefore, we have studied the influence of high glucose

in *daf-16* mutation. We observed that in *daf-16(mgdf50)*, glucose causes slight decrease in lifespan (about 10%) and similar results were observed by Lee et al., 2009[23] (Figure 4.1C). RIF at 50 µM and 100 µM concentration protects glucose toxicity with significant increases lifespan up to 7.8% and 7.2%, respectively (Figure 4.1D).

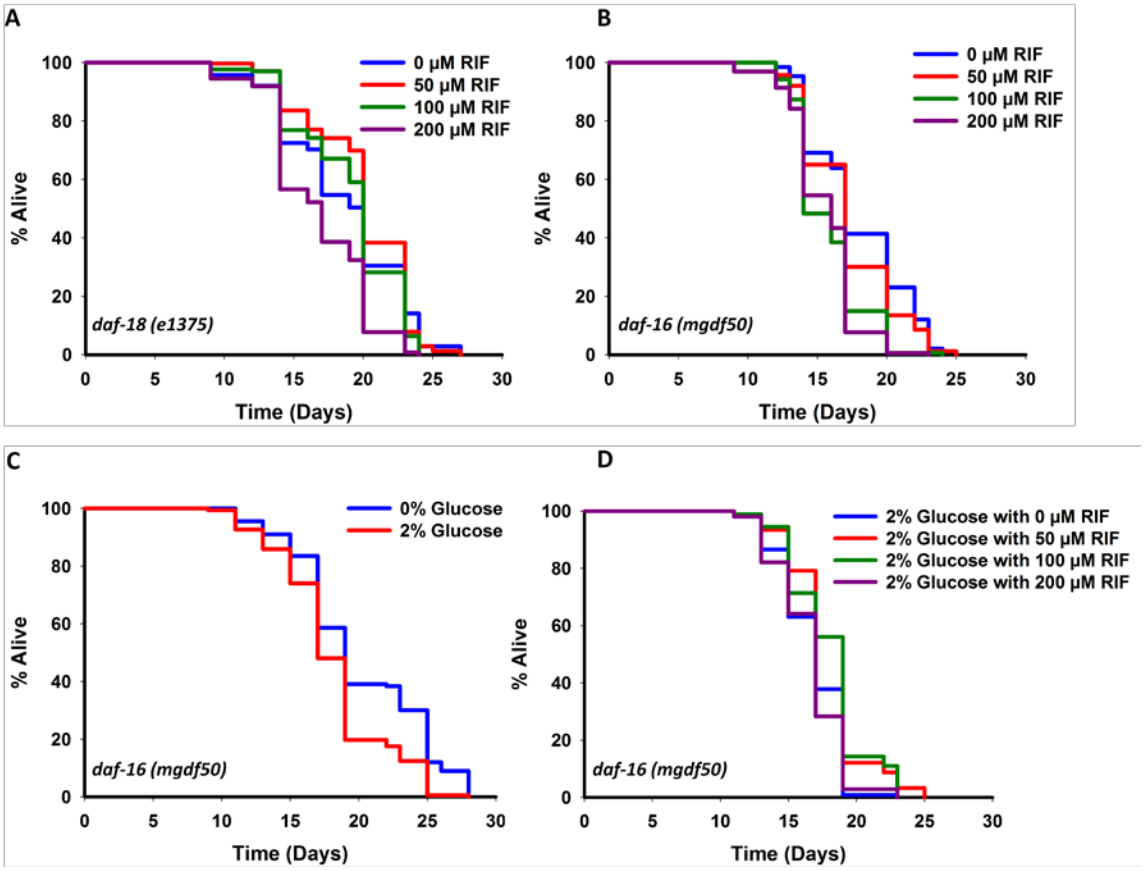
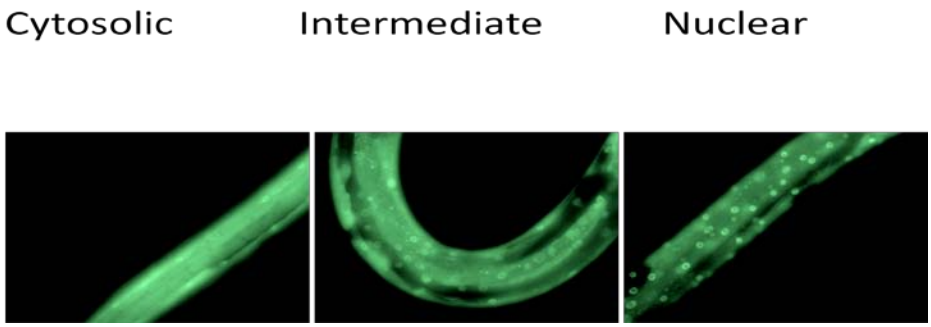


Figure 4.1: RIF requires components of the IIS pathway to increase life span. A. RIF failed to extend life span of a mutant *daf-18* (*e1375*); **B.** similarly RIF unable to extend *daf-16* (*mgdf50*) a component of IIS pathway; **C.** in presence of glucose *daf-16* (*mgdf50*) mutant unable to reduce the lifespan which was observed in *wild type* worms; **D.** RIF marginally extends lifespan with 50 µM and 100 µM significantly but not observed in 200 µM.

Environmental stresses, such as starvation, heat and oxidative stress cause rapid nuclear localization of DAF-16[24]. DAF-16 remains unphosphorylated under the reduced IIS condition, allowing its nuclear translocation and consequent transcriptional activation. Translocation can be studied using transgenic DAF-16::GFP (*TJ365*) strain to evaluate whether RIF, RSV, RMN or AMG treatment can lead to movement of DAF-16 into the nucleus. RIF, RSV and RMN were able to translocate DAF-16::GFP to the nucleus significantly such that nuclear localization was observed in 55.93%, 48.86% and 59.031% of the animals, respectively. However, in case of DMSO treatment nuclear translocation of DAF-16 was in 37.84% of the worms and for AMG it was observed in 38.01%. Thus, AMG treatment was unable to translocate the DAF-16::GFP more than control, as shown in Figure 4.2B.

A



B

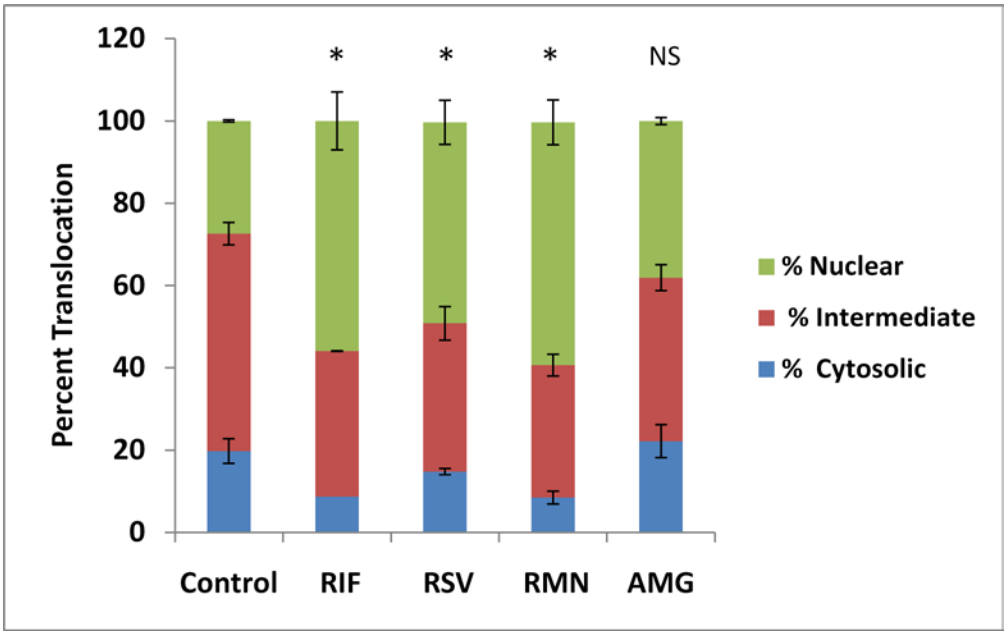


Figure 4.2: DAF-16::GFP translocation assay with RIF, RSV, RMN and AMG. A. DAF-16::GFP pattern i.e. cytosolic, intermediate and nuclear; **B.** RIF, RSV and RMN translocates DAF-16::GFP, while AMG was unable to translocates at the same time * $p \leq 0.05$, **NS** not significant.

4.3.2 Gene expression analysis on RIF treatment

In order to understand how RIF affects cellular physiology, we performed gene expression profiling using Affymetrix array of *C. elegans*. The data was normalized by RMA (Robust Multichip Average) algorithm. Principle component analysis (PCA) of the

normalized data suggested that control and test samples were distinctly separated apart from each other with an altered and contrasting gene expression as shown in Figure 4.3.

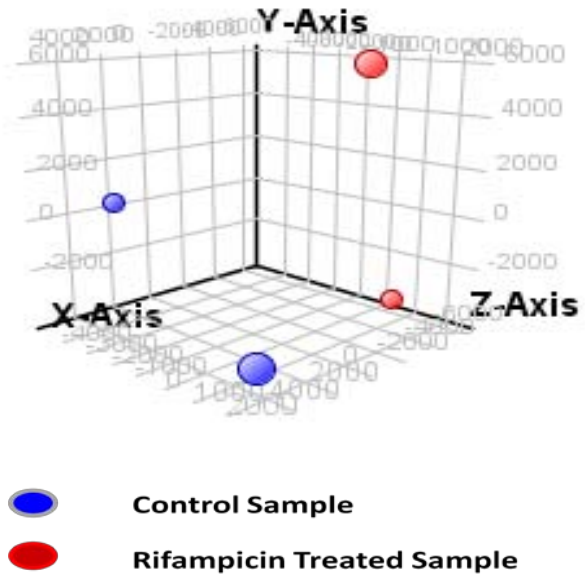


Figure 4.3: PCA analysis of gene expression Control vs. Test. From the 3D plot above, the control (Blue solid circle) and test (red solid circle) samples suggests that control and test samples are significantly different.

Normalized data was visualized for differential expression of genes by volcano plot analysis (Figure 4.4), where X axis represent log2 fold change and Y axis represented log10 P Value change. The figure represents 2 fold up and down regulated genes with $p \leq 0.05$ represented by red spots. We observed that 102 genes were significantly up-regulated (Appendix I), while 104 genes were down-regulated (Appendix II) in response to RIF treatment. Most of genes that were found to be up regulated are uncharacterized. Among the annotated genes, cytochrome 450 family genes, UDP-glucuronosyl transferase, P-glycoprotein, ubiquitin ligase and ubiquitin conjugating enzyme, superoxide dismutase, and unfolded protein response transcription factor *Xbp-1* were up regulated. Cytochrome P450 is involved in phase I detoxification while UDP-Glucuronosyl transferase works in phase II detoxification and P-glycoprotein related proteins in phase III detoxification. It has been recently proven that upregulated xenobiotic detoxification increases lifespan[25]. Ubiquitin ligase and ubiquitin conjugating enzyme facilitate removal of nonfunctional proteins[26] while *Xbp-1* is involved in proper

folding of proteins along with degradation of misfolded protein [27]. Most of the highly down regulated genes were lysozymes, peptidases and remaining were uncharacterized. The decreased expression of lysozymes and peptidases could be perhaps due to low levels of AGEs in RIF treated samples. Vitellogenin genes (*vit-3*, *vit-4* and *vit-5*) were found to be down-regulated significantly. These proteins accumulate in the intestine following reproductive development and are subjected to oxidative damage leading to toxicity. Thus, lower levels of these proteins may promote longevity as shown earlier [28,29]. Interestingly, SOD is up regulated suggesting RIF is effective in protecting cellular system from oxidative stress. These results suggest that RIF can activates longevity associated genes.

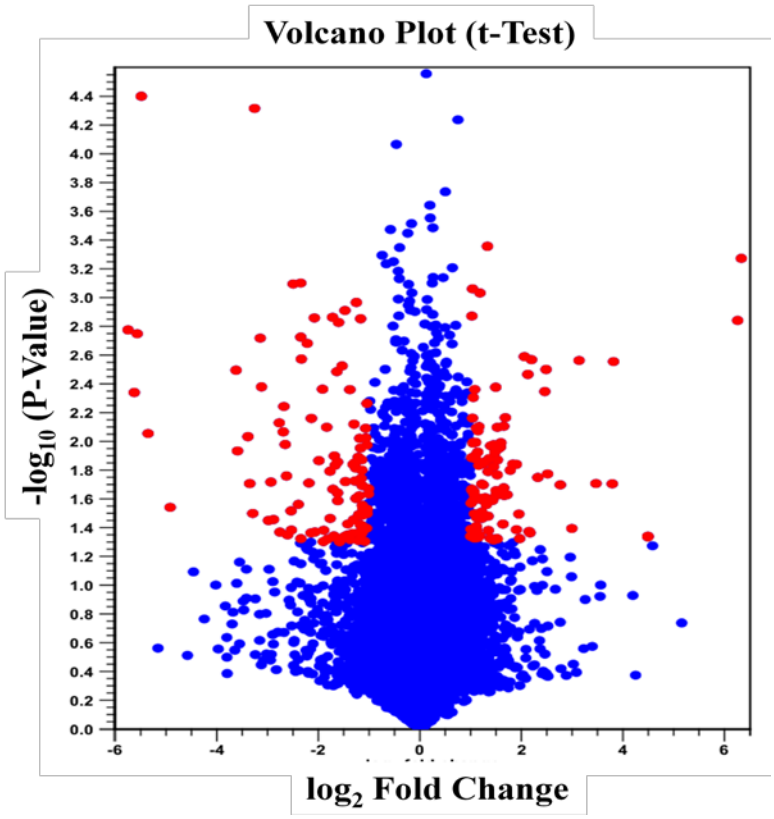


Figure 4.4: Volcano plot of differential gene expression analysis of RIF Vs DMSO. X-axis represent *log2 fold change* and Y-axis represents *log10 P Value*. Red spots show differentially expressed genes, while blue spots show unaltered genes or differentially expressed genes that are not statistically significant.

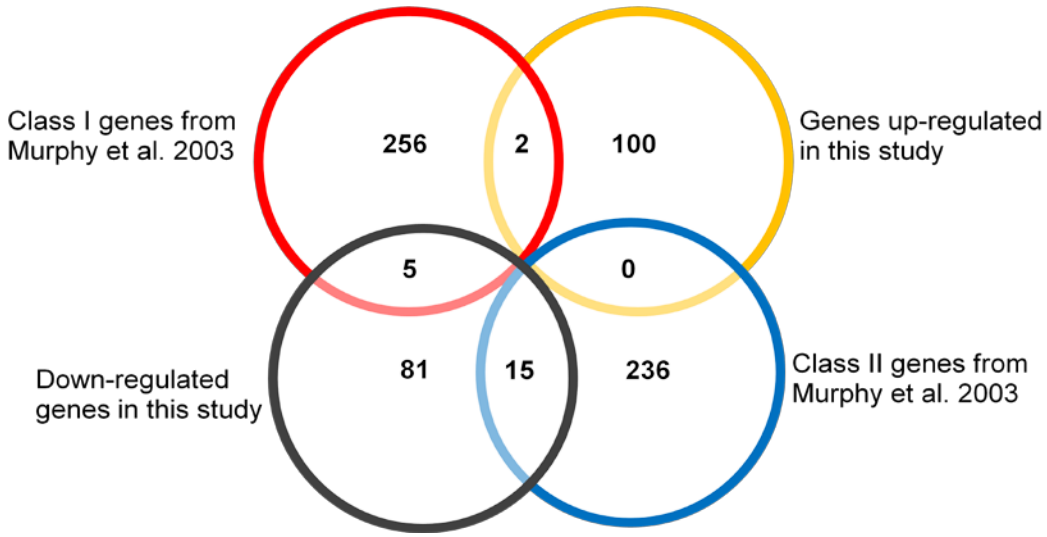


Figure 4.5: Overlap of genes identified between RIF treatment and genes reported by Murphy et al. (2003) as targets of DAF-16. Class I genes are upregulated and class II genes are down regulated in *daf-2(e1370)* mutant, in a *daf-16*-dependent manner.

Earlier microarray analysis of *daf-2* and *daf-16; daf-2* double mutants by Murphy et al. 2003, resulted in identification of two classes of genes. The class I genes were up regulated while class II genes were down regulated in the *daf-2* mutant in a DAF-16-dependent manner. Comparison of our microarray data with that of showed only 2% similarity in upregulated genes and 14 % similarity in the down regulated genes (Figure 4.5) This along with DAF-16::GFP has suggested that RIF treatment activates DAF-16 in a different manner. Kenyon et al. 2006, had identified DAF-16 binding site by Mobydick' algorithm and found two sequences, GTAAAC/TA as the canonical DAF-16 binding element (DBE) and CTTATCA known as the DAF-16 associated element (DAE) that are present in the promoter region of genes upregulated in the *daf-2* mutant in a *daf-16*-dependent manner. Though genes that were upregulated upon RIF treatment in the present study quite different from the Murphy et al.2003, 92-95% of differentially expressed genes on RIF have both canonical DBE and DAE in the promoter region (5 KB upstream)[28,30] as shown in Table 4.1.

Quantitative real-time PCR (qPCR) was performed for evaluating effect of RIF on direct targets of DAF-16. The results suggested that *sod-3, sod-4* was significantly up regulated

in presence of RIF while *scl-1*, *lys-7*, *hsp-12.6* were significantly down regulated on RIF treatment w.r.t control[31] shown in Figure 4.6. This along with comparison analysis with Murphy microarray data highlights the fact that RIF activates DAF-16 differently. Thus we have evaluated the expression of top 10 up regulated genes the gene expression of wild type on RIF treatment, along with DAF-16 (*mgdf50*) on RIF were compared. The results were shown that 7 genes were differentially expressed in a *daf-16*-dependent manner (Table 4.2) upon RIF treatment. Thus, we conclude that RIF may activate DAF-16 differently and modulate a separate subset of target genes compared to the *daf-2* mutant.

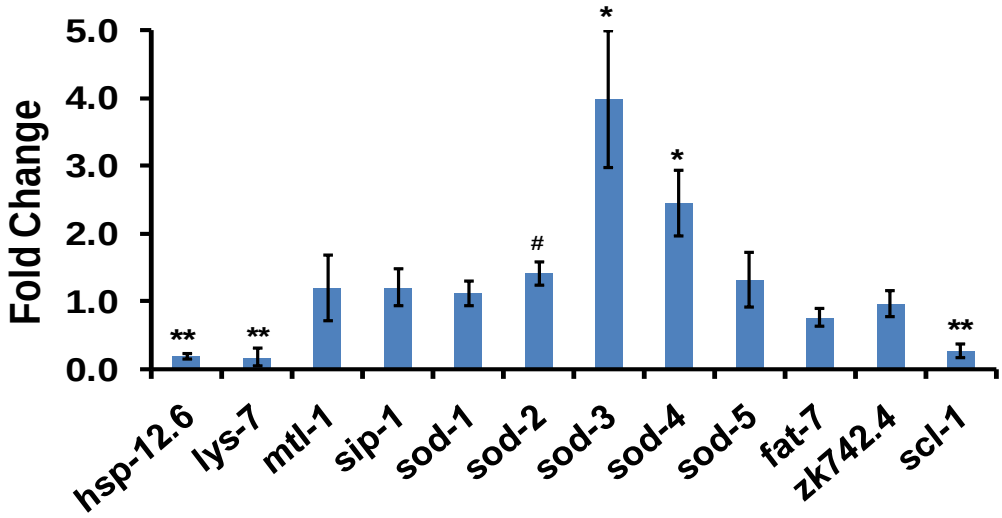


Figure 4.6: RIF mediated differential regulation of DAF-16 targets w.r.t DMSO treatment, using quantitative real-time PCR (qPCR). The data was normalized with actin and fold change was calculated by ΔΔCT method. The experiment was repeated 5 times, ** $p \leq 0.001$, * $p \leq 0.01$, # $p \leq 0.05$

Table 4.1: Promoter analysis (5 kb upstream region) of differentially expressed genes on RIF treatment. This suggested that significant enrichment of DAF-16 binding element (DBE) and DAF-16 associated element (DAE).

		No. of DAF-16 binding site motifs		
	Genes	**DBE / (%) (GTAAAC/TA)**	**DAE / (%) (CTTATCA)**	**Both / (%) DBE+DAE**
Up regulated on RIF treatment	102	88 / 86%	63 / 61%	94 / 92%
Down regulated on RIF treatment	101	85 / 84%	78 / 76%	96 / 95%

Table 4.2: RIF mediated identification of new DAF-16 targets. Quantitative real-time PCR assay comparison of wild type Vs DAF-16 revealed that 7 out of 10 up regulated genes that showed change in expression in a *daf-16*-dependent manner upon treatment with RIF.

S. No	Sequence Name	Gene Name	Function	Fold change in wild type on RIF treatment Vs DMSO treatment	Fold change in daf-16 (mgdf50) on RIF treatment Vs DMSO treatment	Gene expression in *daf-16 (mgdf50)* on RIF treatment
1	T16G1.6	-	Hypothetical Protein	27.615	14.355	Down
2	R05D8.8	*dhs-14*	Dehydrogenases	3.915	9.8	UP
3	F09G2.6	*ugt-36*	UDP-Glucuronosyl Transferase	6.37	13.28	UP
4	C47A10.1	*pgp-9*	P-glycoprotein related	1.71	9.55	UP
5	C45H4.2	*cyp-33*	Cytochrome P450 Family	8.325	8.7	UP
6	F13D11.4	-	Dihydroflavonol-4-reductase	1.64	2.18	UP
7	B0331.2	-	Phosphate permease like	5.41	1.625	Down
8	T24H10.7	*jun-1*	JUN trancription factor	0.995	1.04	No Change
9	W10G6.2	*sgk-1*	serum glucocorticoid kinase-1	1.215	0.855	No Change
10	K09A9.2	*rab-14*	RAB Family	1.44	1.38	No Change

4.3.3 RIF acts through JNK signaling

PDK-1 and AKT-1 act downstream to DAF-18 and upstream to DAF-16. Therefore, we inspected the effect of RIF in the mutants of these genes. We observed that RIF increases lifespan of activated *pdk-1(mg 142)* significantly with 23.34%, 24.88% and 8.53% for 50, 100 and 200 µM treatment, respectively (Figure 4.7A). RIF also increased lifespan in

activated *akt-1(mg144)* significantly with 50, 100, 200 µM by 99.24%, 94.60% and 62.69%, respectively (Figure 4.7B). These results pointed towards a parallel pathway that controls DAF-16/FOXO when worms are treated with RIF. Oh et al. (2005) had shown that JNK signaling acts parallel to the IIS and activate DAF-16, resulting in increased lifespan and stress tolerance. We found that RIF was unable to extend the lifespan of *jnk-1*(*gk7)* (Figure 4.7C). In MAP kinase pathway, JKK-1 is the upstream kinase that phosphorylates and signals to JNK-1. Further we studied effect of RIF in *jkk-1(km2)* mutant of *C. elegans*. We observed that RIF was unable to extend lifespan at all concentrations studied. The details of lifespan are shown in Table 4.3.

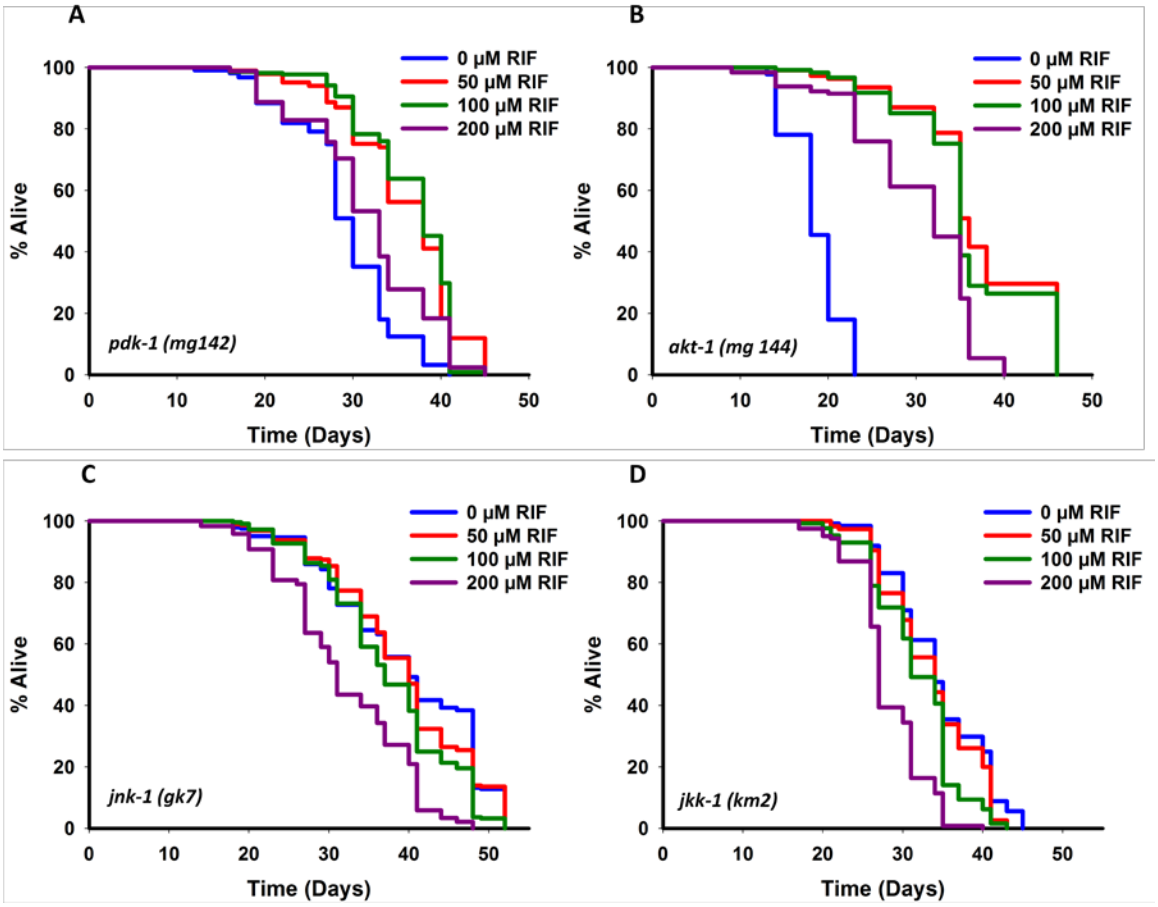


Figure 4.7: RIF activates IIS differently and requires *jnk-1* & *jkk-1*. A. RIF enhances the life span significantly in *pdk-1*, mutant; **B.** RIF extends life span significantly in *akt-1* mutant; **C.** RIF was unable to extend lifespan significantly in *jnk-1* mutants; **D.** RIF was not able to extend lifespan significantly in *jkk-1* mutant.

Table 4.3: Lifespan data for *C. elegans* mutant

Background	Treatment	Mean ± SEM	No.of Subjects	Percent increase (+) or decrease (-) w.r.t. control	*p*-value
***daf-16* (*mgdf50*)**	0 µM RIF	17.80 ± 0.25	191		
	50 µM RIF	17.11 ± 0.25	163	(-) 3.876	0.122
	100 µM RIF	15.64 ± 0.18	174	(-) 12.135	≤0.0001
	200 µM RIF	15.43 ± 0.17	196	(-) 13.315	≤0.0001
	0 Glucose	20.03 ± 0.41	133		
	2 % Glucose	18.03 ± 0.29	177	(-) 9.985	≤0.0001
	2 % Glucose with 0 µM RIF	16.75 ± 0.21	119		
	2 % Glucose with 50 µM RIF	17.97 ± 0.26	102	(+) 7.881	≤0.0001
	2 % Glucose with 100 µM RIF	17.71 ± 0.27	100	(+) 7.224	≤0.0001
	2 % Glucose with 200 µM RIF	16.57 ± 0.23	106	(-) 1.075	≤0.0001
***daf-18*(*e1375*)**	0 µM RIF	18.60 ± 0.26	276		
	50 µM RIF	19.84 ± 0.21	305	(+) 6.667	0.0739
	100 µM RIF	18.99 ± 0.21	298	(+) 2.097	0.05
	200 µM RIF	16.66 ± 0.22	272	(-) 10.430	≤0.0001
***pdk-1*(*mg142*)**	0 µM RIF	29.18 ± 0.40	216		
	50 µM RIF	35.99 ± 0.47	185	(+) 23.338	≤0.0001
	100 µM RIF	36.44 ± 0.36	221	(+) 24.880	≤0.0001
	200 µM RIF	31.67 ± 0.54	169	(+) 8.533	≤0.0001
***akt-1*(*mg144*)**	0 µM RIF	18.55 ± 0.22	178		
	50 µM RIF	36.96 ± 0.70	108	(+) 99.245	≤0.0001
	100 µM RIF	36.10 ± 0.66	121	(+) 94.609	≤0.0001
	200 µM RIF	30.18 ± 0.61	129	(+) 62.695	≤0.0001
***jnk-1*(*gk7*)**	0 µM RIF	39.52 ± 0.61	242		
	50 µM RIF	39.14 ± 0.51	287	(+) 0.698	0.6307
	100 µM RIF	37.40 ± 0.54	220	(-) 5.364	0.0001
	200 µM RIF	31.95 ± 0.51	239	(-) 19.155	≤0.0001
***jkk-1*(*km2*)**	0 µM RIF	34.51 ± 0.52	124		
	50 µM RIF	33.58 ± 0.58	115	(-) 2.695	0.1146
	100 µM RIF	31.73 ± 0.47	128	(-) 8.056	0.0001
	200 µM RIF	28.12 ± 0.39	122	(-) 18.516	≤0.0001

4.4 Discussion

The first major breakthrough in aging research has come from the study carried out by Kenyon et al., (1993). They showed for the first time that mutation in IIS receptor *daf-2* leads to extension of lifespan in wild type worms. Apart from hormonal signaling many other pathways are responsible for increase lifespan. Among them IIS is key pathway for increasing lifespan across the species. We have observed that RIF mediated lifespan increase required DAF-18/PTEN a component of IIS. DAF-16 is an important transcription factor, which controls many target genes affecting lifespan. The RIF

treatment modulates DAF-16 transcription by activating different subset of target genes, those not controlled by the conserved Insulin-IGF-1-like signaling cascade.

JNK (MAP Kinase pathway) is a parallel signaling pathway to IIS, which increases lifespan by translocating DAF-16 into nucleus. We have observed that RIF requires a wild-type *jnk-1* allele for its longevity effects. It could be possible that the strong mutant allele of *daf-18* leads to a highly activated IIS pathway and thus leads to DAF-16 sequestration in the cytoplasm. Under this condition, RIF activates JNK was not sufficient to translocate DAF-16 into the nucleus. Interestingly, PDK-1 and AKT-1 are components of IIS, hyper-activated mutations in *pdk-1* or *akt-1* may not be sufficiently strong to sequester DAF-16 in cytoplasm; thus JNK activation can over-ride this inhibition with RIF treatment. It is also possible that DAF-18 and JNK-1 interact independent of PDK-1 or its downstream kinases, by some other mechanism. DAF-16::*GFP* was remarkably translocated by RIF, RSV and RMN but AMG was unable to do so. Dillin et al. 2002, have shown that translocation of DAF-16 is not only sufficient but requires to be activated in nucleus for extension of lifespan[32]. This may be true in case of RMN, where DAF-16 was translocated to nucleus but failed to increase the lifespan in wild type worms. In addition, differentially expressed genes have DAF-16 binding sites in their promoter region. Further genetic analysis was performed to identify mechanism by which RIF mediates longevity.

4.5 Conclusions

Microarray analysis revealed that RIF treatment causes upregulation of detoxifying genes, genes involved in proteasome, stress responsive genes. The differentially expressed genes have shown enrichment in DAF-16 binding (DBE and DAE) sites up to 92-96% in the promoter region. RIF and RSV successfully translocate DAF-16::GFP into nucleus and increases lifespan, on the other hand AMG was unable to do the same. Further genetic analysis upon RIF treatment regulate IIS pathway differently and it requires DAF-18, DAF-16 and JNK signaling pathway for its longevity effect.

4.6 References

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Chapter 5

Summary and Future Perspective

5.1 Summary

The thesis dealt with identification of glycation inhibitor, further we envisaged it's *in vivo* efficacy and molecular mechanism. We have MALDI-TOF-MS based assay for screening glycation inhibitor was developed using insulin as a model protein. Using this assay rifampicin (RIF) identified as a potent glycation inhibitor. The *in vivo* efficacy of RIF was evaluated in *C. elegans,* which is an excellent model system for studying aging, a glycation associated process. RIF increased the lifespan of *C. elegans* system in euglycemic, hyperglycemic and hyper-methylglyoxal conditions. In addition to this, RIF structural analogs like rifamycin SV (RSV) and rifaximin (RMN) were also evaluated. RSV increased *C. elegans* lifespan similar to RIF, but RMN could not increase lifespan to the same extent as that of RIF. Mass spectrometric and western blotting analysis of *C. elegans*, suggested that the drug inhibited *in vivo* glycation. In addition, RIF inhibited amyloid beta and polyQ proteotoxicity in *C. elegans*. Genetic analysis suggested that RIF requires the DAF-16 (a mammalian FOXO transcription factor), to regulate a subset of its target genes for the extension of lifespan. In addition RIF also depended on DAF-16, DAF-18 and JNK-1 for lifespan extension.

5.2 Future perspectives

- The results were promising from an invertebrate's model like *C. elegans,* thus it will be added advantage to study the RIF, RSV and RMN efficacy in vertebrate model such as mice, rat or guinea pig and pre-clinical studies for diabetic complications, Alzheimer's, Parkinson's and other age related disorders.

- As RIF is well known antibiotics and a drug of choice for treatment of tuberculosis. It selectively inhibits bacterial DNA dependent RNA polymerase and therefore it cannot be easily acceptable as a glycation inhibitor for the treatment of diabetes. In order to overcome the antibiotic effect, structural analogs need to be synthesized with high antiglycation activity and least antibiotic activity. The intellectual property from the work can be patented for antiglycation action.

Appendix

Table 7.1: List of genes up regulated on RIF treatment determined by microarray. The analysis along with the number of DAF-16 binding sites in their 5 kb upstream region

Sequence Name	Gene Name	Functional annotation	Fold Change	DAF-16 binding site motifs	
				Canonical	DAE
				GTAAAC/TA	CTTATCA
T16G1.6		hypothetical protein	80.61	5	1
E02C1		hypothetical protein	76.63	2	0
T16G1.4		hypothetical protein	23.97	1	2
T16G1.7		hypothetical protein	22.50	1	1
R05D8.8	*dhs-14*	DeHydrogenases	14.07	1	1
F09G2.6	*ugt-36*	UDP-GlucuronosylTransferase	13.84	2	3
C47A10.1	*pgp-9*	P-GlycoProtein related	11.07	1	4
B0213.14	*cyp-34A8*	CYtochrome P450 family	8.79	2	2
Y38E10A.14		hypothetical protein	7.97	3	3
C45H4.2	*cyp-33*	CYtochrome P450 family	6.82	2	1
ZK596.1		hypothetical protein	5.72	3	2
F41B5.2	*cyp-33C7*	CYtochrome P450 family	5.60	1	2
R08F11.3	*cyp-33C8*	CYtochrome P450 family	5.51	0	1
B0213.15	*cyp-34A9*	CYtochrome P450 family	4.60	1	2
T28A11.16		hypothetical protein	4.44	4	0
F13D11.4		dihydroflavonol-4-reductase	4.37	6	2
F47B8.10		hypothetical protein	4.17	2	1
B0285.9	*ckb-2*	Choline Kinase B	3.90	1	0
M88.1	*ugt-62*	UDP-GlucuronosylTransferase	3.86	2	3
T28A11.19		hypothetical protein	3.74	0	1
T10B9.10	*cyp-13A7*	CYtochrome P450 family	3.74	3	2
T23F4.2		hypothetical protein	3.56	0	3
R09B5.11		sugar transporter	3.56	0	3
C01B4.7		hypothetical protein	3.43	1	1
F59C12.4		hypothetical protein	3.37	1	0
C25F9.6		hypothetical protein	3.29	1	1
C33A12.6	*ugt-21*	UDP-GlucuronosylTransferase	3.21	3	3
T20D4.1		hypothetical protein	3.17	1	1
Y19D10A.4		hypothetical protein	3.14	1	1
ZK930.2		hypothetical protein	3.14	0	0
F16F9		lipase	3.11	2	0
B0331.2		phosphate permease like	3.03	4	0
F52E1.8	*pho-6*	hypothetical protein	3.02	3	0
F20G2.1		dehydrogenase	3.00	1	0
F12E12.11		hypothetical protein	2.95	1	0
F59F5.2	*glo-3*	hypothetical protein	2.88	3	5
C04H5.2	*clec-147*	C-type LECtin	2.88	1	3
C08B6.1	*ugt-17*	UDP-GlucuronosylTransferase	2.88	2	1
ZK669		hypothetical protein	2.87	3	1
D1054.8		dehydrogenase	2.86	2	3

C06E4.3		dehydrogenase	2.82	2	0
F55H2.1	*sod-4*	SOD (superoxide dismutase)	2.82	2	1
Y55B1BL.1		hypothetical protein	2.77	2	1
R05D7.5		hypothetical protein	2.74	0	0
R12H7.3	*skr-19*	SKp1 Related (ubiquitin ligase)	2.63	2	2
C37H5.6		hypothetical protein	2.62	1	0
F59B10.4		hypothetical protein	2.55	2	0
ZK813.2		hypothetical protein	2.52	1	0
F32G8.6	*cat-4*	abnormal CATecholamine	2.48	2	2
T22C8.8	*vab-9*	Variable ABnormal morphology	2.44	0	0
T24H10.7	*jun-1*	JUN transcription factor	2.41	3	1
Y41C4A.13		hypothetical protein	2.41	0	1
Y54G2A.O	*ubc-13*	UBiquitin Conjugating enzyme	2.37	4	0
T04C9.4A	*mlp-1*	MLP/CRP family (Muscle LIM Protein/Cysteine-rich Protein)	2.34	4	0
T19D2.2	*prl-1*	hypothetical protein	2.33	1	4
F16B4.2	*F16B4*	hypothetical protein	2.33	1	2
F55A12.7	*apm-1*	AdaPtin, Mu/medium chain	2.29	1	2
C53D5.2		hypothetical protein	2.27	1	0
C47D12.5		hypothetical protein	2.27	4	2
W10G6.2	*sgk-1*	Serum- and Glucocorticoid-inducible kinase homolog	2.26	4	2
K09A9.2	*rab-14*	RAB family	2.24	3	0
F13H6.1		hypothetical protein	2.23	1	1
F36A2.14		hypothetical protein	2.21	2	0
F35C11.4		hypothetical protein	2.21	4	3
K02E2.6		hypothetical protein	2.20	2	0
T07A9.9		hypothetical protein	2.20	1	0
C01G10.5		hypothetical protein	2.18	3	0
K10B4.6	*cwn-1*	WNT family	2.18	1	0
F26A3.1		hypothetical protein	2.17	1	0
C08C3.3	*mab-5*	Homeodomain transcription factor	2.17	2	3
Y37D8A.16		hypothetical protein	2.16	3	0
R74.3	*xbp-1*	X-box Binding Protein homolog	2.16	3	1
W06E11.4	*sbds-1*	Shwachman-Bodian-Diamond Syndrome protein homolog	2.16	1	0
R07H5.4	*sdz-27*	SKN-1 Dependent Zygotic transcript	2.15	4	1
K10D6.4		hypothetical protein	2.14	1	0
H06H21.10	*tat-2*	Transbilayer Amphipath Transporters (subfamily IV P-type ATPase)	2.13	3	3
F54E7.7	*rcn-1*	Regulator of CalciNeurin	2.13	4	1
F58B6.2	*inft-1*	INverted Formin/formin Three-related /// locus:inft-1	2.11	2	1

T28D6.2	*tba-7*	TuBulin, Alpha /// locus:tba-7	2.11	1	1
F44G3.8	*fbxa-143*	F-box A protein	2.10	2	4
F14D2.7		hypothetical protein	2.10	0	0
C45G9		hypothetical protein	2.10	2	0
T19C3.4		Polyposis locus protein 1	2.10	4	1
F58G6.1	*amph-1*	AMPHiphysin homolog	2.09	3	1
K03E6.1	*lim-6*	LIM domain family	2.08	2	2
K03E6.3	*ncs-3*	Neuronal Calcium Sensor family	2.08	1	0
T27A1.5		hypothetical protein	2.07	2	4
C04C3.4		hypothetical protein	2.07	4	0
Y47D9A.2	*scpl-3*	SCP(Small C-terminal domain Phosphatase)-Like phosphatase	2.07	0	0
AV177366	*nhr-97*	Nuclear Hormone Receptor family	2.07	3	2
C14F11.3	*lite-1*	high-energy LIghT unrEsponsive	2.06	2	0
K09C8.1	*pbo-4*	PBOc defective (defecation)	2.05	1	1
H09F14.1		7 transmembrane receptor (rhodopsin family)	2.05	0	0
F53F10.4	*unc-108*	UNCoordinated	2.03	3	2
Y23H5A.6	*ctn-1*	alpha-CaTuliN (catenin/vinculin related)	2.03	0	1
Y77E11A.2		hypothetical protein	2.03	4	3
W02D9.7		hypothetical protein	2.03	1	2
F54C9.1	*iff-2*	Initiation Factor Five (eIF-5A) homologs	2.01	3	0
K10B4.3		hypothetical protein	2.01	2	1
F16B4.9	*nhr-178*	Nuclear Hormone Receptor family	2.01	0	0
Y37H2C.1		hypothetical protein	2.01	0	0
C05D2.8		hypothetical protein	2.00	2	3

Table 7.2: List of genes down regulated on RIF treatment determined by microarray. The analysis along with the number of DAF-16 binding sites in their 5 kb upstream region

Sequence Name	Gene Name	Functional annotation	Fold Change	DAF-16 binding site motifs	
				Canonical	DAE
				GTAAAC/TA	CTTATCA
C57430	*ilys-5*	Invertebrate LYSozyme	-53.71	3	1
C17H12.8		hypothetical protein	-49.38	0	3
C32H11.12	*dod-24*	Downstream Of DAF-16	-47.47	1	2
F55G11.5	*dod-22*	Downstream Of DAF-16	-12.31	3	4
C14C6.5		hypothetical protein	-12.05	0	0
F55G11.4		hypothetical protein	-10.47	3	3
F15E11.14		hypothetical protein	-10.22	1	1
C12C8.1	*hsp-70*	Heat Shock Protein	-9.56	2	0
F56D6.2	*clec-67*	C-type LECtin	-8.85	1	2
Y105C5B.15		hypothetical protein	-8.69	3	2
F01D5.5		hypothetical protein	-7.89	2	2
F35E8.13		hypothetical protein	-7.64	3	1
F49F1.1		hypothetical protein	-7.39	1	2
F01D5.1		hypothetical protein	-6.82	1	5
C45B2.1		hypothetical protein	-6.76	1	1
AU113368	*clec-85*	C-type LECtin	-6.46	1	0
C32H11.4		hypothetical protein	-6.41	5	2
F42A10.6		hypothetical protein	-6.29	3	4
F53E10.4	*irg-3*	hypothetical protein	-6.18	0	1
ZK896.7	*clec-186*	C-type LECtin	-6.09	1	2
C02A12.4	*lys-7*	LYSozyme	-5.81	2	1
AU112775		hypothetical protein	-5.78	3	1
F55G11.8		hypothetical protein	-5.64	1	3
F40G12.5		hypothetical protein	-5.25	3	0
C05C10.4	*pho-11*	hypothetical protein	-5.11	1	1
F57F4.4		hypothetical protein	-5.09	1	3
Y49E10.18		hypothetical protein	-5.08	0	3
C50B6.7		amylase	-5.07	1	1
F01D5.2		hypothetical protein	-5.04	2	4
C03E10.6	*clec-222*	C-type LECtin	-4.65	2	2
ZK1320.2		hypothetical protein	-4.55	4	2
Y19D10B.A		hypothetical protein	-4.43	2	0
K10C2.3		aspartyl protease	-4.42	1	0
Y53F4B.30	*gst-27*	Glutathione S-Transferase	-4.40	1	2
T08A9.7	*spp-3*	SaPosin-like Protein family	-4.22	1	5
B0365.6	*clec-41*	C-type LECtin	-4.19	1	0
Y102A11A.D		hypothetical protein	-3.97	4	0
W10G11.2		hypothetical protein	-3.78	2	1
F28H7.3		lipase	-3.73	1	3
Y34B4A.C		hypothetical protein	-3.72	0	4
C51901	*unc-15*	UNCoordinated	-3.57	1	1
ZK1320.3		hypothetical protein	-3.53	3	1
W10G11.3		hypothetical protein	-3.30	1	1

CEC7564	*vit-4*	VITellogenin structural gene	-3.27	1	3
F59D8.A	*vit-3*	VITellogenin structural gene	-3.24	1	1
C04F6.1	*vit-5*	VITellogenin structural gene	-3.22	1	1
F35C5.8	*clec-65*	C-type LECtin	-3.19	0	2
Y71G12A.A		hypothetical protein	-3.19	2	2
C58535		hypothetical protein	-3.18	4	1
W04E12.8	*clec-50*	C-type LECtin	-3.11	1	1
F57F5.1		cysteine protease	-3.11	2	1
CEC3896	*nspc-2*	Nematode Specific Peptide family	-3.08	1	0
C17G10.5	*lys-8*	LYSozyme	-3.07	4	0
F56A4.2		C-type LECtin	-3.03	2	1
F56F10.1		hypothetical protein	-2.99	2	0
F55F3.2		hypothetical protein	-2.89	3	3
ZK1320.1	*gstk-1*	Glutathione S-Transferase Kappa protein	-2.78	0	2
ZK896.5		hypothetical protein	-2.76	2	1
F52E1.5		hypothetical protein	-2.70	2	2
F49C12.14		hypothetical protein	-2.67	0	2
F27E5.1		hypothetical protein	-2.60	2	0
C30F12.7		hypothetical protein	-2.59	1	2
K02F3.9		hypothetical protein	-2.52	4	0
M28.4		hypothetical protein	-2.49	0	3
R09A8.4	*col-182*	COLlagen	-2.47	1	1
Y38F1A.9	*oig-2*	One IG domain	-2.46	0	0
Y39B6B.J		hypothetical protein	-2.44	0	1
F21F8.4	*F21F8.4*	protease	-2.43	2	6
AU113026	*ttr-24*	domain	-2.40	5	1
F54D5.3		hypothetical protein	-2.38	0	2
AV181428		ATP transferase	-2.34	1	0
AU113659	*dod-19*	Downstream Of DAF-16	-2.32	1	3
C06B3.3	*cyp-35C1*	CYtochrome P450 family	-2.31	1	1
Y66A7A.6	*gly-8*	GLYcosylation related	-2.30	0	0
CEK120C8R	*mlc-1*	Myosin Light Chain	-2.29	3	1
C14C6.2		hypothetical protein	-2.28	3	1
F55A12.1		hypothetical protein	-2.27	1	1
VZK822L.1	*fat-6*	FATty acid desaturase	-2.26	1	2
C50E3.13		hypothetical protein	-2.25	3	7
CEK111C2F	*ttr-41*	TransThyretin-Related family	-2.25	4	0
F01G10.7	*lipl-7*	hypothetical protein	-2.25	2	1
T20D3.2		hypothetical protein	-2.24	1	0
F42F12.6	*nspc-13*	Nematode Specific Peptide family	-2.23	1	1
Y4C6A.3		zinc finger protein	-2.21	0	0
F02H6.2		hypothetical protein	-2.21	1	0
H12C20.2A	*pms-2*	PMS (Post Meiotic Segregation)	-2.21	5	0
ZC308.4		hypothetical protein	-2.20	3	5
C16H3.2	*lec-9*	gaLECtin	-2.19	2	2

T14B4.4	*tsp-10*	TetraSPanin family	-2.19	2	1
F54F11.2		hypothetical protein	-2.19	1	3
C28C12.6	*spp-10*	SaPosin-like Protein family	-2.18	2	1
C01G5.8	*fan-1*	hypothetical protein	-2.17	2	0
T16H12.4		hypothetical protein	-2.10	1	1
F41D9.2		hypothetical protein	-2.09	4	1
T08A9.10	*spp-6*	SaPosin-like Protein family	-2.07	2	2
F19C7.1		hypothetical protein	-2.06	0	3
Y32B12A.3		hypothetical protein	-2.06	2	2
F32A7.7	*str-245*	Seven TM Receptor	-2.05	1	1
K08E3.3B	*toca-2*	TOCA (Transducer Of Cdc42-dependent Actin assembly)	-2.05	0	0
AU116367	*twk-18*	TWiK family of potassium channels	-2.01	4	0
F42F12.1	*nspc-9*	Nematode Specific Peptide family	-2.01	3	1

Table 7.3: List of primers for real time PCR

Gene Name	Forward Primer	Reverse Primer
T16G1.6	GTT GTT GGA GAC GGA AAT GGA	TGC TCT GGT TAG ACT CTT TCA
DHS-14	TTG TGA CAG GCT CCT CAA ACG	TCA GTC GCC AAA TCA GTT GCA
UGT-36	CGC AGG GCA TAA TGT GAC AT	GAT GAG TCA CGG TAC CAA AAC
PGP-9	TAC AGG CTT CAT GCT TCA TGG	ACT GAG CCA TCA TCT GG
CYP-33C1	GGA TTG GTA ACA CTC CTC	GCA AAC CGT CTG TGC TCT
F13D11.4	CTT GTG CTC ACT AGC AGT TGT	TTG TCT TCT GGA AGT CTC TCG
B0331.2	CGC TGC GAC AAT GTA CTA TA	AGA CCG AGA TAC TTT GAT CC
JUN-1	ACC GGC ATC TGC AAG CGA	GTT GTG GAA GAG TCT GGC GAA
UBC-13	TGC TGG TGG TGT CTT CAA GTT	CTG AAT CGA CAG AAG AAC CGT
SGK-1	TGA GAG GAA AAC AGC CAC	CAT GAC GTG TTT CAC CTC
RAB-14	AAG AAG TTC ATG GCC GAC TGC	TAG ACC ATC AAG GCT CCG GCT
ILYS-5	TCT GCT CCT TTC TGT CGC TAT	TCC AGC CTT CTT TGT TGG TTG
C32H11.12	TTG AAT TGC TCC AGA ACG ATG	ACT GGA GGG CTT CCC AAA TC
F55G11.5a	GGC TAC CAT TTC CAA ACA TAG	CGG GAA ACT ACC TCC TTC AA
HSP-70	ATG GAA AGG TTG AAA TCC TCG	CTT CAT CGA ATC GTC TTC C
F35E8.13	GGA ACT GCT TTT GTC TGG AA	CTG AGC TGC ATT CTT CAT GTC
CLEC-85	TGG CTT CAA TCG TCC GCA CTC	TGC CAT TTT CCA TTC AGC GTG
GST-27	TGG GAA AAT CTC AAA GCC AAA	TCG ACG ATA GCA TCA GTC CAG
VIT-4	GAT CAA GAA GGT CTC TGG A	GTT CTG CTC GTG CTC TTG AAT
CLEC-65	CTA GCA GAA TGG ATA CAG GTC	TTC ACA GTC ACC ACC GGG ATC
LYS-8	TGT CCG TGC ATA CAA CCC AGC	CCT CTT GCA GTC AAT CCT TGG
Hsp-12.6	TGG AGT TGT CAA TGT CCT CG	GAC TTC AAT CTC TTT TGG GAG G
Lys-7	GCC GTC AAA CTT GGC ATC TT	GGG TTG TAT GCA CGA ACG AA
Mtl-1	AGT GTG ACT GCA AAA ACA AGC AA	TCC ACT GCA TTC ACA TTT GTC TC
Sip-1	AAG AGA TCG TTC ACT CGC CAG	AGC CAA GTC GAC GTC CTT TG
Sod-1	AGG TCT CCA ACG CGA TTT TT	CCT GGT CAT TTT CGG ACT TC
Sod-2	CAA CCG ATC ACA GGA GTC G	TTA CAG GCT CCA AAT CAG CA
Sod-3	GGA GTT CTC GCC GTC CG	GTC GAA TGG GAG ATC TGG GAG
Sod-4	ACG CGG TAC TTC AGA CCA AT	GAA GGG ATG CTG TCG TTG TT
Sod-5	CCA CAG GAC GTT GTT TCC AA	ACC TTC GGC TTT CTG GGT AA
Fat-7	ATA GTG TGG CGT AAC GTC GC	TAG AGA GCA AAT GAG AAG ACG
Zk742.4	ACT GGA GGT TGG CAA ACT GC	TAC GAG GCA AAT CTG GCT CA
Scl-1	CAA TCA AGC ATT GTG GAT GC	GGA ATC CAC GAC CAT TTT CC
Actin	CTC TTG CCC CAT CAA CCA TG	CTT GCT TGG AGA TCC ACA TC

List of Publications and Conferences

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- **Golegaonkar Sandeep. B.,** Bhonsle H. S., Boppana R., Kulkarni M. J. Discovery of rifampicin as a new anti-glycating compound by matrix-assisted laser desorption/ionization mass spectrometry-based insulin glycation assay. *Eur J Mass Spectrom* 2010, 16, 221-6

- **Sandeep B. Golegaonkar**, Syed Shamsh Tabrez, Awadhesh Pandit, Shalini S, Jagadeeshaprasad MG, Mahesh J Kulkarni, Arnab Mukhopadhyay. Rifampicin increases C. elegans life span by two distinct mechanisms. **Manuscript under Review in *AGING CELL***

- Suresh K. Kesavan, Shweta Bhat, **Sandeep B. Golegaonkar**, Mashanipalya G. Jagadeeshaprasad, Arati B. Deshmukh, Harshal S. Patil, Santosh D. Bhosale, Mahemud L. Shaikh, Hirekodathakallu V. Thulasiram, Ramanamurthy Boppana & Mahesh J. Kulkarni. Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation. *Scientific Report.* 2013, 3, 2941

- Bhonsle, H. S., Korwar, A. M., Kote, S. S., **Golegaonkar, Sandeep. B.**, Chougale, A. D., Shaik, M. L., Dhande, N. L., Giri, A. P., Shelgikar, K. M., Boppana, R., Kulkarni, M. J. Low Plasma Albumin Levels Are Associated with Increased Plasma Protein Glycation and HbA1c in Diabetes. *J Proteome Res*. 2011, 11(2), 1161-1169

Conferences attended

- Presented poster on **MALDI-TOF-MS based assay for screening of glycation inhibitors** at International Symposium on Mass Spectrometry in Life Sciences on 27- 29 September 2009 held at the National Centre for Biological Sciences (NCBS), Bangalore, India.

- Presented poster on **MALDI-TOF-MS based assay for screening of glycation inhibitors** at 5th Asia Oceania Human Proteome Organization (AOHUPO), 14th Association for the Promotion of DNA Finger Printing and Associated DNA Technologies (ADNAT) and 1st Proteomics Society India (PSI) on 21-25 February 2010 held at CCMB, Hyderabad, India.

- Presented poster on **AGEs in aging – influence of glycation Inhibitors** at Indo-US Bilateral symposium on Aging and Age- related disorders on 3 – 4 March 2011 held at National Institute of Immunology, New Delhi, India.

Curriculum Vitae

Sandeep B. Golegaonkar
Research Scholar,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Dr. Homi Bhabha Road,
Pune – 411008, INDIA
Phone no.: +91-20-25902204
: +91-9226935140
Email: golegaonkar.sandeep@gmail.com
sb.golegaonkar@ncl.res.in

Educational Qualification:

Ph.D in Biotechnology at Savitribai Phule Pune University, Pune, India (Working Place: Biochemical Sciences Division, CSIR-NCL, Pune and National Institute of Immunology, New Delhi) May 2008 to Current

Master of Pharmacy in Pharmaceutical Biotechnology at Bharati Vidyapeeth Deemed University; Poona College of Pharmacy, Pune, India, June 2003 to August 2005

Bachelor of Pharmacy at Dr. B. A. M University, Aurangabad, India**;** Y. B. Chavan College of Pharmacy, Aurangabad, India, June 1998 – June 2002.

Work Experience:

Project Assistant II at CSIR-National Chemical Laboratory, Pune

March 2006 – February 2008

Successfully established aqueous two phase extraction using natural gums
Guar gum derivative was prepared for separation of enzyme and proteins model protein was Bovine serum albumin, lactate oxidase and phytase

Trainee Biotechnologist at USV Ltd, Mumbai, India

September 2005 – February 2006

Purification of recombinant protein (Darbopoeitin)
Hands on experience on SDS-PAGE, Western blotting, AKTA Explorer

M. Pharm Dissertation at Serum Institute of India, Hadpser, Pune, India

July 2004 – June 2005

The project entitled "Analytical methods for development of meningococcal a conjugate vaccine from *Neisseria meningitides*"

Developed free and conjugated protein purification methodology and developed In-house ELISA

Awards and Achievements

Awarded CSIR-SRF fellowship from April 2008.

Technical Skills

Proteomics, Drug metabolomics, pseudo-MRM analysis, Protein characterization, MALDI-TOF-MS, and LC-MS/MS analysis of proteins, 2D electrophoresis, SDS-PAGE, Western blotting, Protein purification, Quantitative RT-PCR, ELISA, and PCR

Software - MS Office, Endnote, Sigma Plot, Unicorn software for AKTA purifier, PLGS (protein lynx global server), protein pilot and Xcaliber

Publications

- **Golegaonkar Sandeep. B.,** Bhonsle H. S., Boppana R., Kulkarni M. J. Discovery of rifampicin as a new anti-glycating compound by matrix-assisted laser desorption/ionization mass spectrometry-based insulin glycation assay. *Eur J Mass Spectrom* 2010, 16, 221-6

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induced diabetic mice by hydralazine mediated transglycation. *Scientific Report.* 2013, 3, 2941

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- Presented poster on **AGEs in aging – influence of glycation Inhibitors** at Indo-US Bilateral symposium on Aging and Age- related disorders on 3 – 4 March 2011 held at National Institute of Immunology, New Delhi, India.

References

Dr. Mahesh J. Kulkarni
Scientist,
Biochemical Sciences Division,
CSIR-National Chemical Laboratory,
Dr. Homi Bhabha Road,
Pune- 411008, India
Phone: +91-20-25902541
Mail: mj.kulkarni@ncl.res.in

Dr. Arnab Mukhopadhyay,
Scientist,
National Institute of Immunology,
Aruna Asaf Ali Marg,
New Delhi- 110068, India
Phone: +91-11-26717121
Mail: arnab@nii.ac.in