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THE ROLE OF RESVERATROL AND ITS ANALOGS IN INFLAMMATION,
PREADIPOCYTE DIFFERENTIATION & NEUROBLASTOMA DIFFERENTIATION

By

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ABSTRACT OF THE DISSERTATION

**THE ROLE OF RESVERATROL AND ITS ANALOGS IN INFLAMMATION,
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Dissertation Director:
Dr. Kuang-Yu Chen

In this dissertation, I have evaluated the role of heat (physical) stress in cell death. It has been previously reported that heat shock causes cancer cell death and is currently used as an adjuvant therapy in cancer treatment (Wust 2002). However, heat therapy is currently limited due to the requirement of high temperatures (45°C) for physiological effect. Improved efficacy of treatment could be achieved by understanding the mechanism of heat shock induced tumor cell death which is not well understood.

A possible mechanism for heat induced tumor cell death could involve activation of proteases by heat which degrades key survival proteins thus leading to loss of viability. It has been previously reported that heat shock induced tumor cell death is associated with loss of eIF5A, a highly conserved survival protein (Takeuchi 2002, Gossiau 2009).

I have independently reconfirmed the results of these studies and further evaluated the role of two protease inhibitors. The findings suggest the possibility that various proteases

get activated by heat shock and multiple proteins, including eIF5A get degraded eventually leading to loss of viability. The findings of this dissertation strengthen the hypothesis that proteases are involved in heat induced tumor cell death and use of small molecule activators of proteases could possibly improve the efficacy of heat-treatment through synergistic/additive mechanism.

I studied the effect of resveratrol ((3,5,4'-trihydroxy-trans-stilbene) and its analogs, MR-4 (3,4,5,4'-tetramethoxy-trans-stilbene) and MC-4 (3,4,5,4'-tetramethoxy-cis-stilbene) in inflammation, neuroblastoma differentiation and preadipocyte differentiation where resveratrol has been reported to be involved. In this dissertation, I have successfully established the effect of MC4 and MR4 in inflammation, neuroblastoma and adipogenesis disease models.

The mechanism of action for these biomolecules remains to be evaluated but it can be speculated that AMPK is a potential upstream target of resveratrol, MC4 and MR4, given that AMPK is known to play a role inflammation, neuroblastoma differentiation and adipogenesis.

Both MC4 and MR4 offer the advantage of improved bioavailability and lower dosage to achieve the same physiological effect as resveratrol making them an attractive target for therapeutics in inflammation, neuroblastoma and adipogenesis.

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And last but not the least my loving family. My deepest gratitude to my mom (Nayana Tiwari) and dad (Satyanarayan Tiwari) for all their love and support. I would like to thank my brother Ashish and sisters Sushma, Rashmi, Sarita and Varsha for encouraging me in many different ways and providing all the confidence and belief that I needed.

DEDICATION

This dissertation is dedicated to my mom and dad for all their support, encouragement.
Their love and belief in me has made everything possible for me.

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Figure A: Overview of biochemical pathways in this dissertation

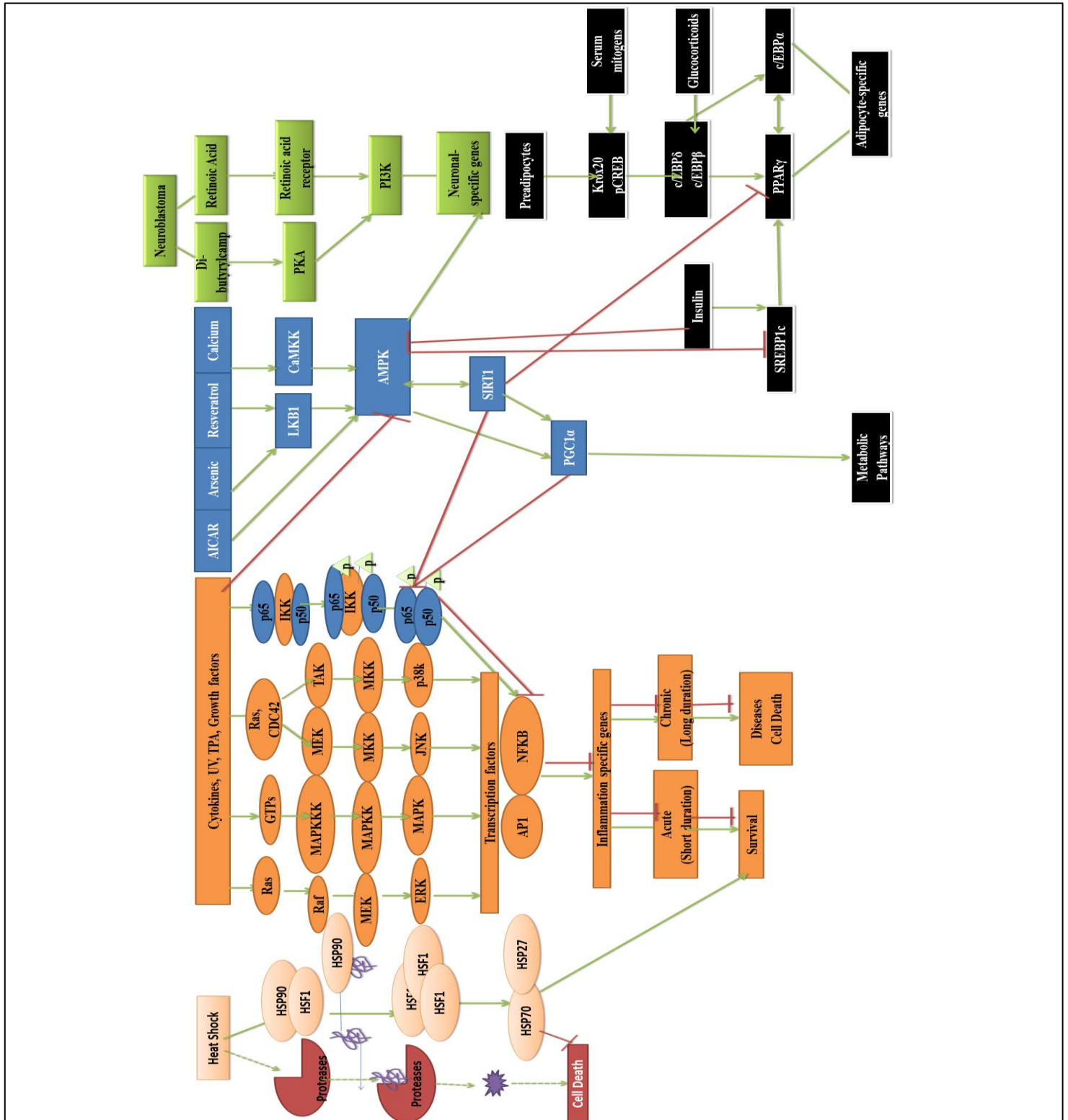
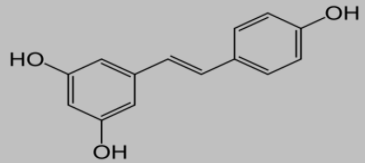
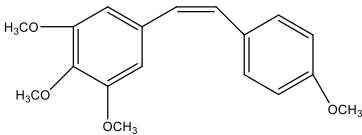
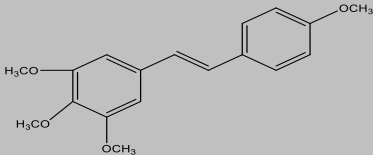
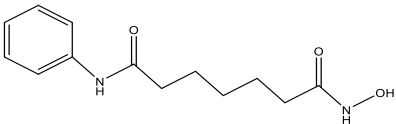
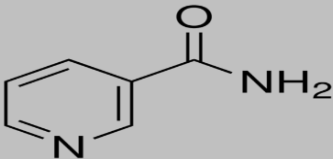
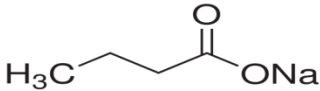
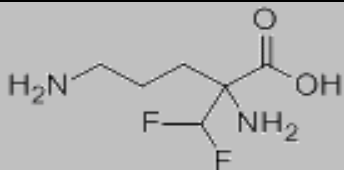
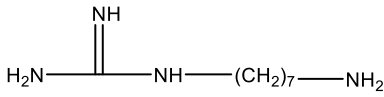
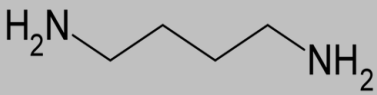
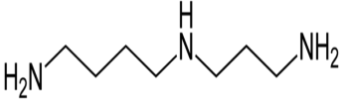
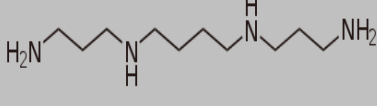
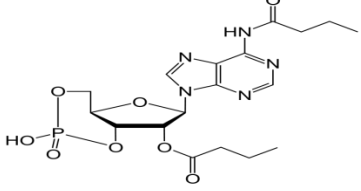
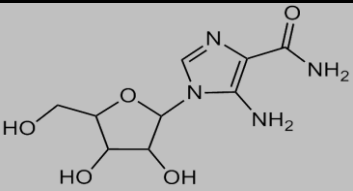
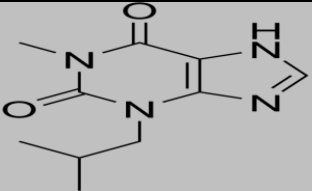
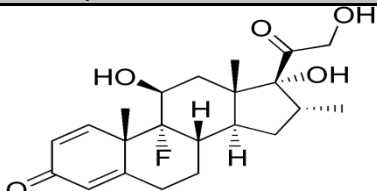
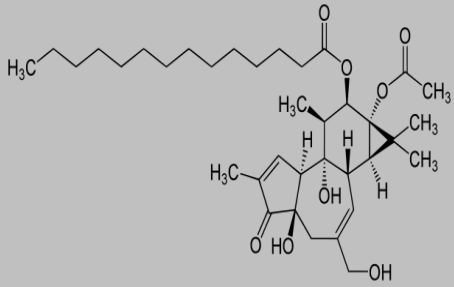
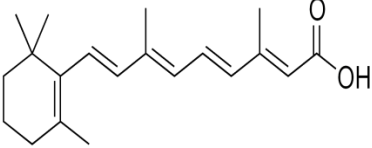


Figure B: Chemical structure of molecules used in this dissertation

Sr. no.	Name	Structure	Function (specifically discussed in this dissertation)
1	Resveratrol (3,5,4'-trihydroxy-trans-stilbene)		Activates: SIRT1 AMPK Downregulates: NF-κB
2	MC4 (3,4,5,4'-tetramethoxy-cis-stilbene)		Not known
3	MR4 (3,4,5,4'-tetramethoxy-trans-stilbene)		Not Known
4	SAHA (Suberoylanilide Hydroxamic Acid)		Downregulates: SIRT1
5	NAM (Nicotinamide)		Downregulates: SIRT1
6	Sodium butyrate		Downregulates: SIRT1
7	DFMO (alpha-difluoromethyl-dl-ornithine)		Inhibits polyamine biosynthesis (via downregulation of ornithine decarboxylase)
8	GC7 (N1-Guanyl-1,7-diaminoheptane)		Inhibits eIF5A modification (via competitive inhibition of DHS) Inhibits preadipocyte differentiation
9	Putrescine		Polyamine

10	Spermidine		Polyamine required for eIF5A modification
11	Spermine		Polyamine
12	Di-butryl-c-AMP		Activates: cAMP Induces neuroblastoma differentiation
13	Calcium	Ca ²⁺	Induces neuroblastoma differentiation
14	Arsenic	As	Activates: AMPK Induces neuroblastoma differentiation
15	AICAR(5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside)		Activates: AMPK Inhibits preadipocyte differentiation Induces neuroblastoma differentiation
16	Insulin	Peptide	Activates: SREBP1c Downregulates: AMPK Induces preadipocyte differentiation
18	IBMX (3-isobutyl-1-methylxanthine)		Activates: cAMP Induces preadipocyte differentiation
19	Dexamethasone		Activates: c/EBPδ Induces preadipocyte differentiation

20	TPA (12-O-Tetradecanoylphorbol-13-acetate)		Activates: NF-κB Induces NF-κB mediated inflammation
21	Retinoic Acid		Induces neuroblastoma differentiation

CHAPTER I: Introduction

Cellular responses to stress

Cellular responses to stimuli in the form of biological, physical, chemical or mechanical stress include cell death, survival, growth and/or differentiation (Fulda 2010). Cell death occurs through one of the three mechanisms; apoptosis (chemical, radiation, oxidative stress), necrosis (Glutamate toxicity, DNA damage) or autophagy (nutrient starvation, infection) dependent on the type of stress. However before the cell activates one of these death pathways, a built-in survival response is activated to restore homeostasis. Stress-dependent survival mechanisms such as heat shock response, inflammation (cytokines, allergens, pathogens) etc. remediate the damaging effects of stress. However, failure of these remediation mechanisms leads to cell death, usually via activation of the apoptotic pathway. The mechanism by which the cell decides to switch from pro-survival to pro-death pathway is poorly understood.

Physical Stimuli induced cell death: Heat Shock

Temperatures higher than physiological conditions result in activation of a pro-survival mechanism called heat shock response. Heat shock response is mediated via heat shock proteins (HSPs). HSPs are conserved proteins that act as molecular chaperones and are activated under stress conditions. The biochemical pathway of heat shock response is represented in Fig. 1.1, briefly inactive heat shock factor-1 (HSF1) exists in the cytoplasm as a monomer bound to heat shock protein 90 (HSP90). Under stress condition

there is accumulation of unfolded protein which compete with HSF1 to bind to HSP90. This causes release of HSF1 resulting in its trimerization and translocation into the nucleus. Activated HSF1 binds to the promoter region of DNA leading to transcription of heat shock proteins, HSP70 and HSP27. Expression of HSP70 and HSP27 leads to blocking of the apoptotic pathways and activation of survival pathways (Fig. 1.1, Fulda 2010).

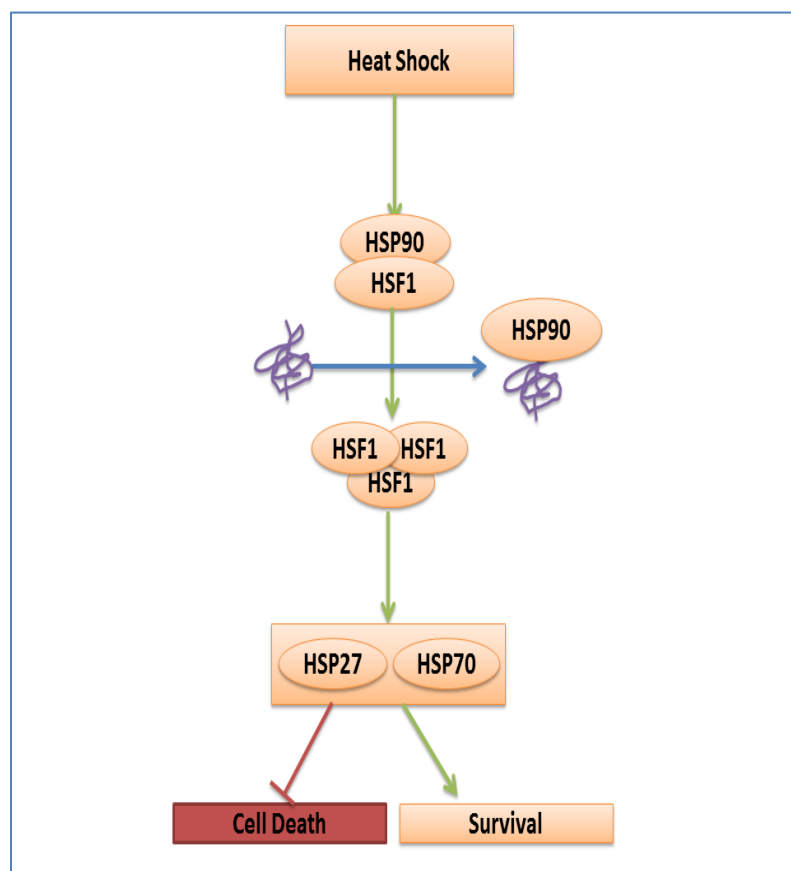


Figure 1.1: Heat Shock Response

Failure of heat shock response to remediate stress, leads to cell death. However, the mechanism by which heat shock results in cell death is poorly understood and is of significance because of its potential application in cancer therapeutics.

Heat therapy (HT) used in cancer treatment involves inducing tissue damage via application of heat to the tumor area. The exact mechanism by which HT induces tissue damage remains to be evaluated, but the current understanding considers DNA damage and blockage of protein synthesis as a plausible mechanism for heat induced tumor cell death (Hilderbrandt 2002). Another plausible mechanism for heat sensitivity of tumor cells could possibly be through activation of a proteolytic system that systematically denatures key survival proteins.

Proteases & Heat Shock:

Hyperthermia is a process that involves heating of tissue, to 41-45°C and is widely used in oncology in combination with chemo or radiation therapy for treating advanced and recurrent cancers (Sardari 2011). Heating of cells or tissues to a high temperature results in irreversible damage making the tissue more sensitive to radiation therapy (RT) and/or Chemotherapy (CT) (ACS 2013) resulting in increased efficacy of treatment (Hilderbrandt 2002, Wust 2002). The mechanism of heat induced tumor cell death remains to be elucidated.

Takeushi (2000) reported that acute heat treatment at 51°C for 30 mins resulted in loss of eIF5A (a conserved cell survival protein). In accordance with this study we observed that heat shock of colon cancer cells (Caco2) was accompanied by loss of eIF5A (Gossiau 2009), while HeLa cells which were resistant to the cytotoxic effect of hyperthermia showed unchanged levels of eIF5A. A possible explanation for the sensitivity of colon

cancer cells to heat shock as evidence by loss of viability and eIF5A, could be that heat shock activates a proteolytic system which degrades eIF5A resulting in loss of viability.

In order to study this hypothesis we evaluated the role of various protease inhibitors in acute heat shock model using colon cancer cells. It was noticed that that use of PMSF (a serine protease inhibitor) partially blocked the loss of eIF5A and restored cell viability in colon cancer cells (Gosslau 2009). The nature of this proteolytic system in heat induced protein degradation remains to be investigated.

Present dissertation aims to discover the potential relationship between proteases and thermo-tolerance in colon cancer cells in hope of finding novel targets for cancer treatment via modulation of protease activity. A secondary aim is to evaluate the role of eIF5A modification on heat induced tumor cell death because as previously reported (Takeushi 2002, Gosslau 2009) loss of eIF5A correlates with loss of viability in heat shocked cancer cells.

Pathological Stimuli induced survival: Inflammation

Inflammation is a patho-physiological cell survival mechanism of the body in response to irritant stimuli such as cytokines, pathogens, allergens etc. and it functions by neutralizing the irritant and initiating tissue repair to restore homeostasis. While acute inflammation is a desirable response because it is a short-term response that aids tissue healing, chronic inflammation is a maladaptive response in disease conditions such as arthritis, cancer etc. which results in tissue damage and manifests as a disease symptom.

The biochemical pathway of inflammation (Bode 2010, Fig. 2) involves a sequential TPA-induced or UV-induced activation of various GTP proteins, MAP kinase kinase kinases (MKKKs), MAP kinase kinases (MKKs), MAP kinases (MAPKs). Other pathways include activation of ERK and/or p38 and/or JNK which finally leads to activation of transcription factors such as NF- κ B or AP1. NF- κ B is a pivotal transcription factor that is involved in inflammation and exists in an inactive state in the cytoplasm. An appropriate stimulus such as TPA or TNF α causes I κ B kinase to bind to NF- κ B and phosphorylate NF- κ B upon which I κ B is released from the complex and degraded. Released NF- κ B protein moves to the nucleus, where it binds to DNA-regulatory regions and promotes transcription of various inflammatory genes including those encoding cytokines, growth factors necessary for tissue degradation and repair.

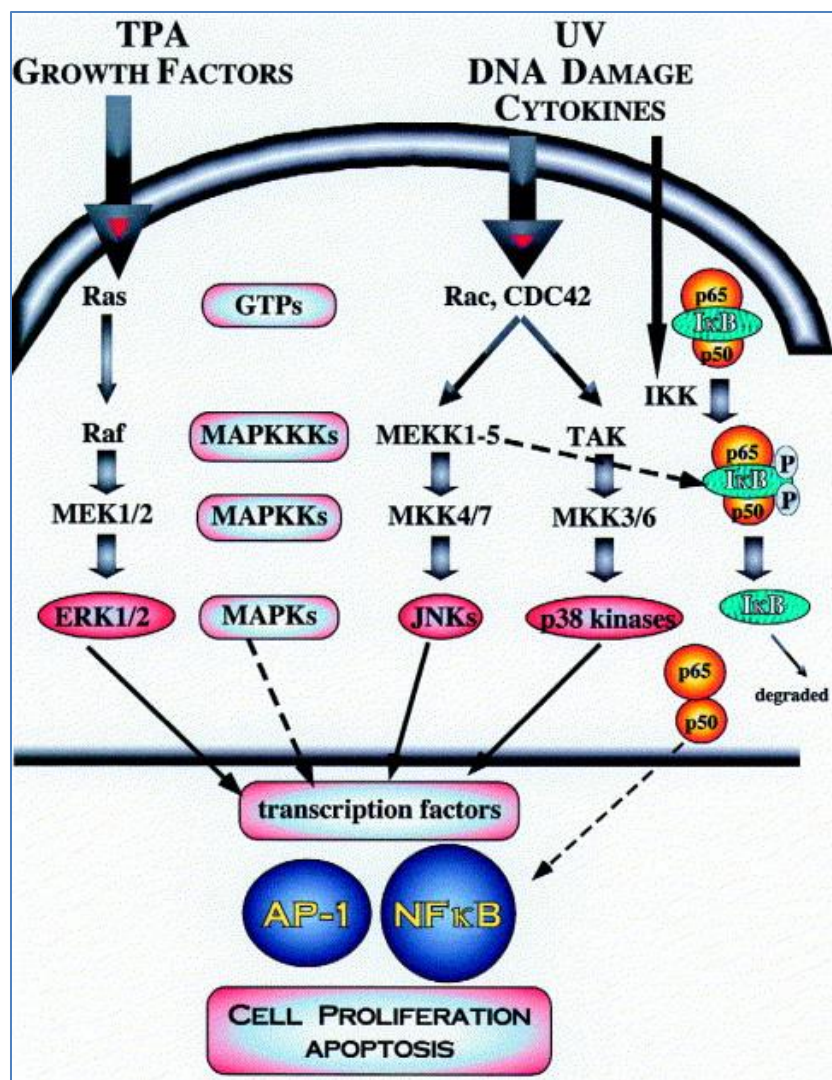


Figure 1.2: Inflammation Pathways

Failure of inflammation, a desirable pro-survival mechanism (acute inflammation) to remediate stress becomes a maladapted, destructive response (chronic inflammation) resulting in tissue carnage, especially in disease conditions. Management of chronic inflammation requires mediation by use of various anti-inflammatory drugs.

Non-selective inhibitors of inflammation include non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen etc. which have been traditionally used because of their easy availability (over-the-counter) and low to none sedation & addiction incidences. However, use of NSAIDs has been reported to have significant adverse events such as higher incidences of gastrointestinal and renal bleeding, ulceration, perforation and atherothrombosis (Ivanenkov 2008).

Selective inhibitors of inflammation target a pro-inflammatory factor cyclooxygenase-2 (COX-2). However currently available FDA approved COX-2 inhibitors, celecoxib and rofecoxib, have been associated with fatal cardiovascular adverse events (Chen 2008).

Various adverse events, lack of responsiveness and resistance to existing anti-inflammatory drugs, necessitate development of new anti-inflammatory drugs.

Resveratrol & Inflammation:

The discovery of resveratrol as a bioactive molecule is attributed to a report that followed the “French paradox” in an attempt to solve the observation that French population had a lower incidence of cardiovascular diseases despite a high fat diet. The cardioprotective benefits were attributed to consumption of red wine rich in polyphenolic chemicals such as resveratrol (de Lange 2007). Resveratrol (trans-3, 4', 5-trihydroxystilbene) is a non-flavonoid, polyphenolic phytochemical with demonstrated ability as anti-cancer (Aluyen 2012), anti-oxidant (Gulcin 2010) and anti-inflammatory agent (Udenigwe 2008).

Resveratrol has been shown to have anti-inflammatory properties, both in-vitro and in-vivo (Udenigwe 2008). The mechanism of resveratrol induced anti-inflammatory effect is possibly via down-regulation of pro-inflammatory factors such as Cyclooxygenase-2 (COX-2), NF- κ B, interleukins etc.

The mechanism by which resveratrol down-regulates pro-inflammatory factors such as NF- κ B is suspected to be mediated via activation of silent mating type information regulation 2 homolog-1(SIRT1). SIRT1 is a histone deacetylase which is known to be activated by resveratrol (Baur 2006). Activation of SIRT1 leads to deacetylation of the RelA/p65 subunit of NF- κ B leading to suppression of NF- κ B and thus repression of inflammation (Chung 2010, Fig. 1.3)

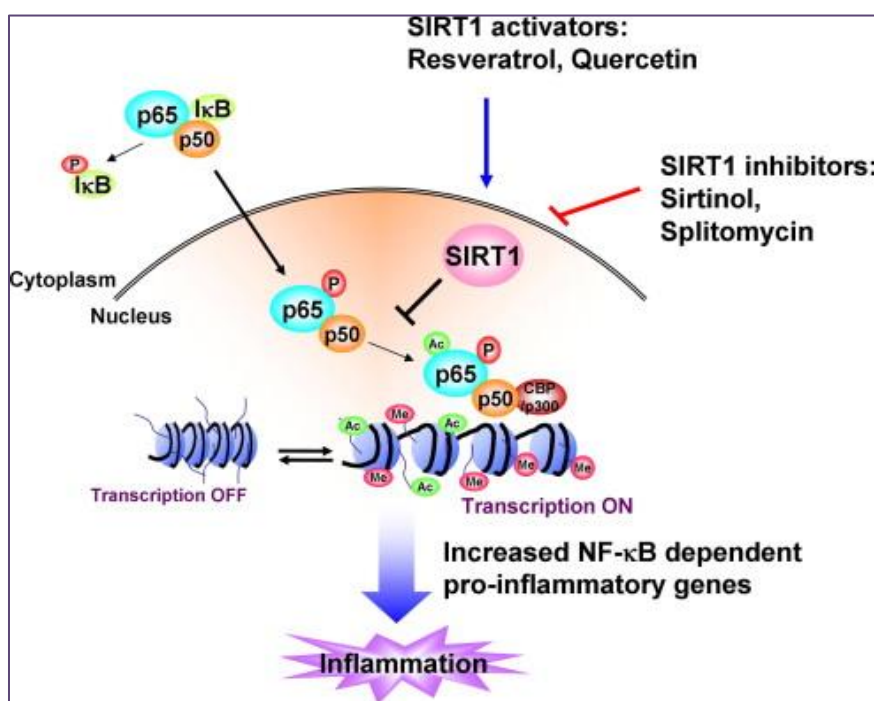


Figure 1.3: Role of SIRT1 in inflammation

Resveratrol in clinical trials:

Resveratrol is currently in phase 1 clinical trial for evaluation of safety and pharmacokinetic/pharmacodynamic profile in humans (Tome-Carneiro 2013). However the results of clinical studies have been limited due to the low bioavailability of resveratrol which is a characteristic of its polyphenolic structure. The trihydroxy functionality of resveratrol is highly susceptible to enzymatic degradation and leads to its rapid metabolism in vivo and hence the low bioavailability (Fig. 1.4). One of the ways to improve bioavailability is to increase dosage but higher dosage correlates with more undesirable side-effects, not to mention potential toxicity issues.

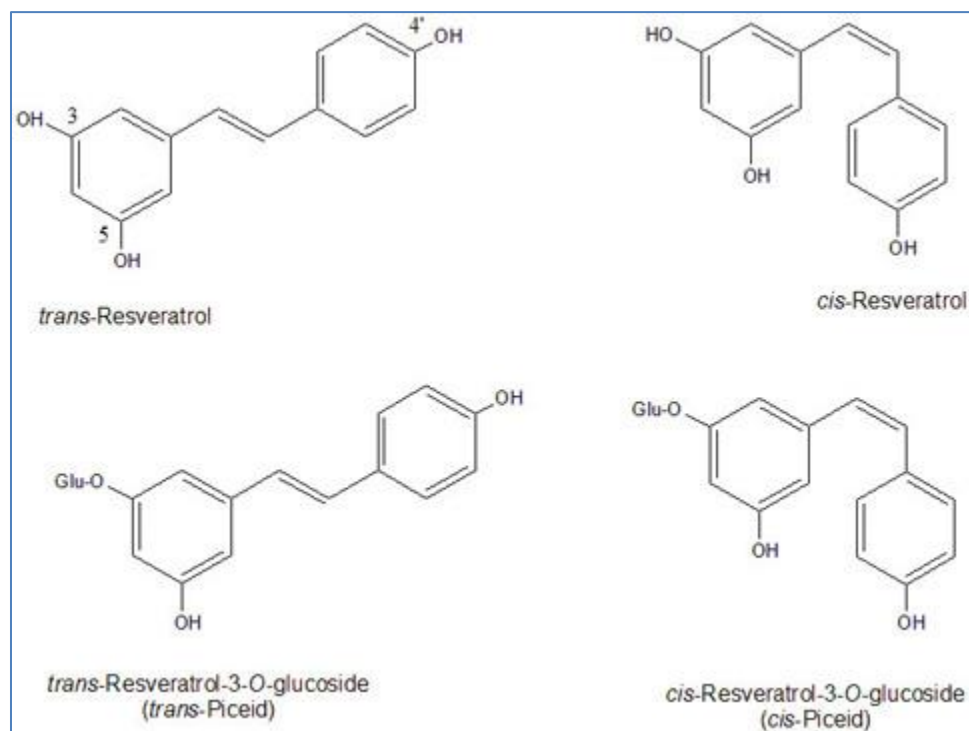


Figure 1.4: Metabolites of resveratrol

We have synthesized various derivatives of resveratrol that offer potential to overcome this low bioavailability issue associated with resveratrol. Two such analogs (Table 1.1)

are MC4 a cis-isomer of methoxylated-resveratrol and MR4 a trans-isomer of methoxylated-resveratrol which have been previously reported (Gossiau 2005) to mimic cytotoxicity of resveratrol in cervical cancer cell line (HeLa) at 20nM and 2μM respectively.

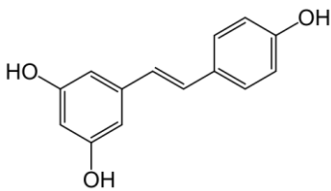
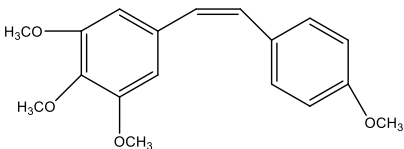
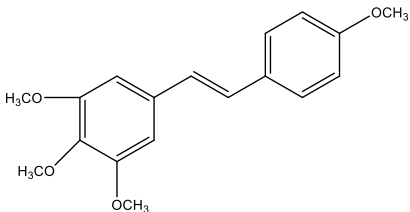
Resveratrol (3,5,4'-trihydroxy- <i>trans</i> -stilbene)	MC4 (3,4,5,4'-tetramethoxy-cis-stilbene)	MR4 (3,4,5,4'-tetramethoxy- <i>trans</i> -stilbene)
		

Table 1.1: Chemical structure of resveratrol, MC4 and MR4

Present study aims to evaluate the role of these two potent analogs of resveratrol as anti-inflammatory agents in order to establish a biosimilar role for these analogs in comparison with resveratrol.

Chemical Stimuli & Differentiation

In addition to causing cell death or activating cell survival mechanisms, various stimuli such as growth factors, chemicals etc. are capable of inducing cellular growth or differentiation. Differentiation is the process by which a cell (precursor) undergoes

differential gene expression to produce a phenotypically different cell capable of specialized function (Boehler 2002).

Regulated cellular differentiation is essential for normal disease-free survival, however unregulated differentiation leads to disease conditions such as tumor (arrested neuroblastoma differentiation) or obesity (hyper differentiation of precursors of fat-cells). Regulation of these differentiation systems via small molecules offers potential for therapeutics in cancer and obesity.

Neuroblastoma differentiation:

Neuroblastoma is a malignant childhood tumor originating in the peripheral nervous system that accounts for 6% of all childhood tumors. A critical event in neuroblastoma pathogenesis is arrest of neuroblast differentiation, which is a process by which neuroblasts convert into mature neural cells. Interestingly, neuroblastoma is characterized by a unique capacity of spontaneous and complete regression, at least partly through differentiation in select patient population and is regarded as a cancer due to block of cellular differentiation (Tee 2012). The histology of neuroblastoma reveals that tumors with more differentiated cells are less aggressive than their immature undifferentiated counterparts (Abemeyor 1989).

The clear relationship between neuroblastoma differentiation and favorable clinical prognosis is suggestive of the treatability of aggressive neuroblastoma by inducing differentiation response as part of the treatment regime.

The biochemical pathway of neuroblastoma differentiation is not well understood. However, in-vitro studies have shown that neuroblastoma differentiation can be achieved by systematically restricting cell growth through depletion of growth factors and introducing small molecules (Fig.1.5). Dibutyryl-c-AMP is known to induce neuronal differentiation via activation of tyrosine hydroxylase leading to activation of pKA and PI3k resulting in neuro-specific gene expression (Prasad 1975). Calcium on the other hand is known to function through activation of the phosphatidylinositol second-messenger system during neuronal differentiation (Reboulleau 1985). Retinoic acid binds to retinoic acid receptor and activates the PI3K/AKT pathway during neuroblastoma differentiation (Sidell 1982). Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) a natural polyphenol has been previously reported to induce neuroblastoma differentiation via AMPK activation (Dasgupta et. al., 2007). Arsenic has been shown to induce neuroblastoma differentiation by inhibiting the L1KB-AMPK pathway (Wang et. al., 2010).

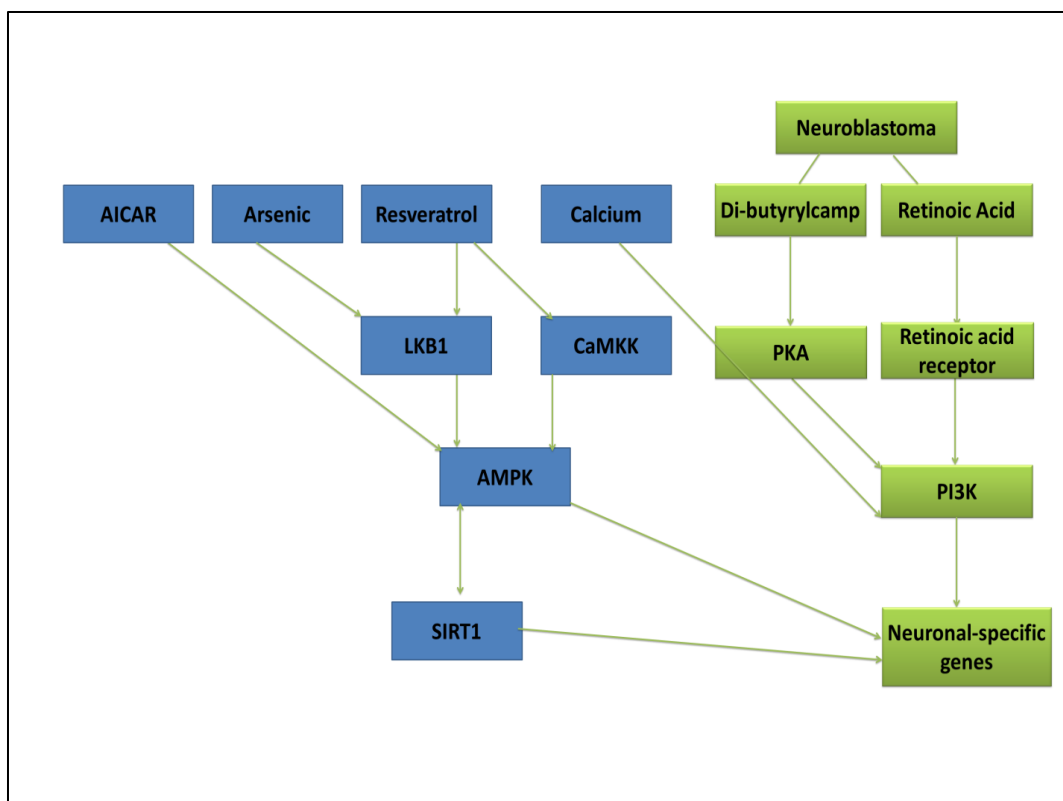


Figure 1.5: Neuroblastoma differentiation overview

Resveratrol & Neuroblastoma differentiation

Resveratrol, a naturally occurring polyphenol is known to induce neuroblastoma differentiation by activation of AMPK (Dasgupta 2007). Present thesis aims to evaluate the role of two methoxy derivatives of resveratrol, MC4 (3, 4, 5, 4'-tetramethoxy-cis-stilbene) and MR4 (3, 4, 5, 4'-tetramethoxy-trans-stilbene) in neuronal differentiation in order to establish a biosimilar role for these analogs. Both MC4 and MR4 offer an improved pharmacokinetic/pharmacodynamic (PK/PD) profile as compared to resveratrol (Lin and Ho 2009), which is currently facing low bioavailability issues in pre-clinical studies limiting the translation of its bioactivity under physiological conditions.

In an effort to improve the efficacy of neuroblastoma differentiation, I evaluated the combinatorial effect of calcium and resveratrol, MC4 and MR4 on neuroblastoma differentiation.

Both calcium and stilbene analogs are known inducers of neuroblastoma differentiation, so assuming that calcium and resveratrol have different biochemical targets in the neuroblastoma differentiation; we would expect a synergistic/additive effect. This approach was evaluated because it offers a multi-targeted efficacious treatment regime for neuroblastoma.

Preadipocyte Differentiation:

Obesity is a condition where excess body fat is associated with health risks such as diabetes, atherosclerosis, hypertension etc. The mechanism by which body accumulates fat is either by expanding existing fat (adipose) tissue called adipose hypertrophy or by creating new fat cells (adipocytes) called adipose hyperplasia (Jo 2009). Hypertrophy (increase adipose volume) precedes hyperplasia (increase in adipose number) to meet the need for excess fat storage as obesity progresses which is why hyperplasia is often associated with severe-extreme obesity.

Hyperplasia is achieved by a cellular process called adipogenesis or preadipocyte differentiation in which precursors of fat cells (preadipocytes) undergo growth arrest and subsequent terminal differentiation into fat cells (adipocytes) capable of accumulating

and storing excess fat. Various factors affect differentiation of preadipocytes including growth factors, hormones, cell-cell/cell-matrix interactions (Gregoire 1998).

Biochemically preadipocyte differentiation is characterized by a cascade activation of eight major transcriptional factors: krox20, pCREB, cEBP δ , cEBP β , KLF5, PPAR γ , SREBP1 and cEBP α (Fig.1.6 adapted from Farmer 2006) leading to adipocyte-specific gene expression. Various small molecule inducers of these transcriptional factors such as cyclic AMP (pCREB), glucocorticoids (cEBP δ), Insulin (SREBP1c) etc. lead to a cascade of biochemical events resulting in adipocyte formation. Understanding of this mechanism of adipogenesis has led to successful adaptation of preadipocyte precursor cells such as 3T3-L1/3T3-F442A and a cocktail of these chemical inducers (IBMX, Dexamethasone and Insulin) to in-vitro culture systems studying obesity.

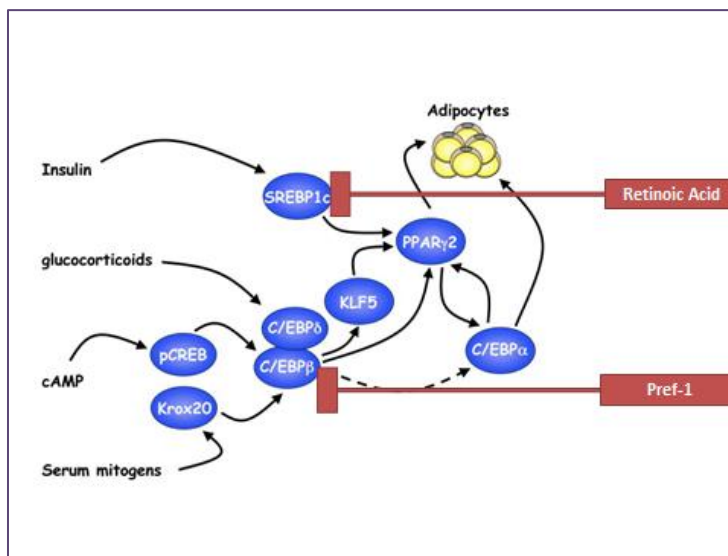


Figure 1.6: Transcriptional regulation of preadipocyte differentiation

Resveratrol & preadipocyte differentiation:

Resveratrol, a natural product found primarily in the grapevines, was discovered as a result of an epidemiological study that found lower incidences of coronary heart diseases in French population despite consumption of a high saturated fat diet. This apparent discrepancy known as the French paradox was explained to be a result of moderate wine consumption, especially red wine which contains a stilbene analog, resveratrol among other flavanols and phenolic acids (Kopp 1998).

Following this discovery, resveratrol has been extensively studied in preventing and/or treating metabolic disorders such as obesity and diabetes. (Fig 1.6) shows a systematic representation of effects induced by resveratrol and leading to reduced blood glucose level and diminished lipid accumulation (Szkudelska 2010). Resveratrol primarily functions through activation of cyclic-Adenosine Monophosphate (cAMP), Adenosine monophosphate activated-protein kinase (AMPK) and Sirtuins (SIRT1 stands for silent mating type information regulation 2 homolog-1) resulting in anti-adipogenic activity.

The confirmation of health benefits of resveratrol in in-vitro and in-vivo studies has led to clinical investigations of resveratrol in humans for multiple indication including metabolic disorders. However, despite the high bioactivity observed during preliminary research studies, the pre-clinical evidence of resveratrol benefits has been limited due to the low bioavailability issues (Tome-Carneiro 2013). The main reason for low bioavailability of resveratrol is characteristic of its polyphenol structural framework

which leaves free-hydroxyl groups vulnerable to enzymatic activity of sulphatase and glucuronidase (Walle 2004).

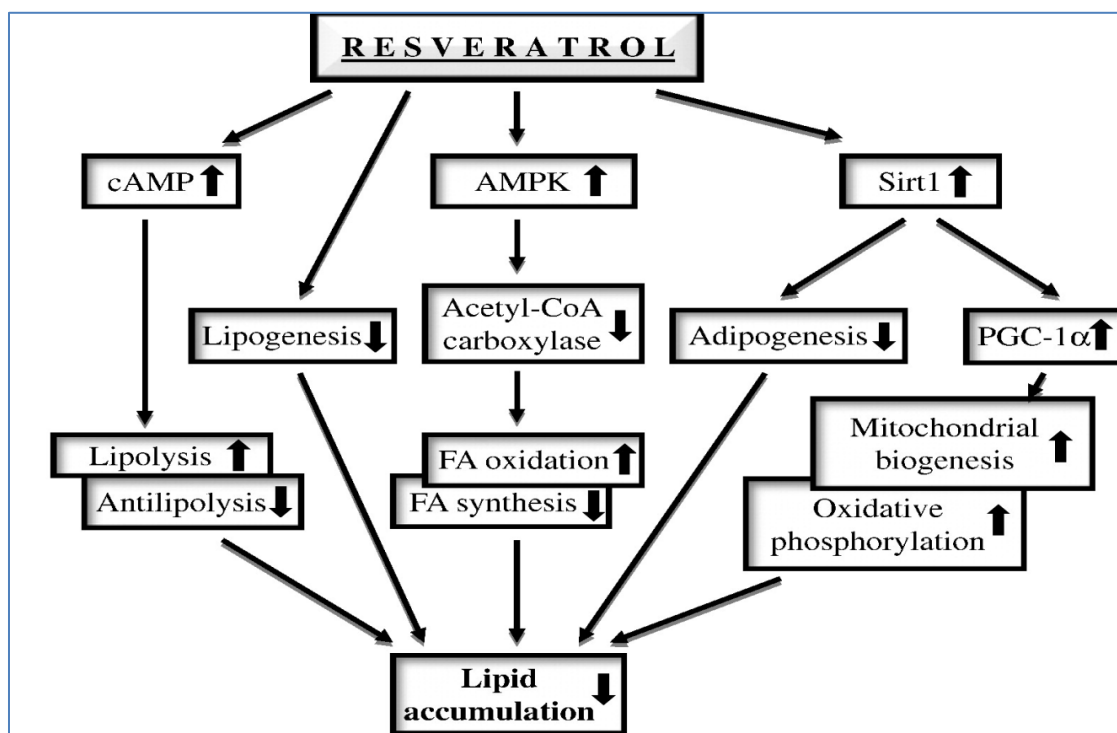


Figure 1.7: Resveratrol in obesity

One of the approaches we devised to improve the low bioavailability of resveratrol was through synthesis of derivatives of resveratrol, including two methoxy derivatives, MC4 (3, 4, 5, 4'-tetramethoxy-cis-stilbene) and MR4 (3, 4, 5, 4'-tetramethoxy-trans-stilbene) which make these molecules resistant to the enzymatic activity responsible for the rapid metabolism and hence enhanced bioavailability (Gossiau 2005).

This dissertation aims to evaluate these two methoxy-derivatives of resveratrol in preadipocyte differentiation with aim of establishing the bioactivity of these analogs in mimicking the biochemical pathway of resveratrol induced anti-adipogenic activity.

eIF5A and preadipocyte differentiation:

It has been previously reported that α -difluoro methyl ornithine (DFMO), a competitive inhibitor of the enzyme Ornithine decarboxylase (ODC) is known to inhibit preadipocyte differentiation (Erwin 1984, Bethell 1981).

Objectives:

The present thesis, primarily explores the role of external stimuli on cellular responses such as death (heat shock), survival (inflammation) and differentiation (neuroblastoma and adipogenesis). Specifically, I want to evaluate the role of proteases in heat-induced tumor cell death with an aim to increase the efficacy of heat therapy by modulation of protease activity. A second objective is to evaluate the role of resveratrol and its analogs, MC4 and MR4 on cell survival (inflammation) and cell differentiation (neuroblastoma and preadipocyte) with an aim to establish biosimilarity of MC4 and MR4 with resveratrol in these disease models for potential anti-cancer, anti-obesity and anti-inflammatory applications. A secondary goal of this dissertation is to evaluate other small molecules for potential anti-cancer (neuroblastoma) and anti-obesity (preadipocyte differentiation) properties. The specific objectives of this thesis are listed below:

Role of Proteases in Heat Induced Tumor Cell Death

Objective 1: To determine the role of proteases in hyperthermia: As previously reported, serine-protease inhibitor PMSF, offered thermal protection in heat induced cell death in colon cancer (Gosslau 2009). The aim of this study is to determine the role of proteases in heat shocked cancer cells by using other small molecule protease inhibitors such as EDTA (metalloproteinase inhibitor) and Leupeptin (a cysteine, serine and threonine protease inhibitor) in order to determine the selectivity/specificity of proteases in heat shock induced cell death. A second aim is to support the findings by Gosslau et. al., that a heat induced proteolytic system was involved in thermal killing of colon cancer cells.

Role of Resveratrol, MC4 and MR4 in Inflammation

Objective 1: To establish biosimilarity of MC4 and MR4 with resveratrol in inflammation: Resveratrol (3, 5, 4-trihydroxy-trans-stilbene), a phytoalexin present in grapes, peanuts, and pines, has antioxidant and anti-inflammatory activities. Present study aims to evaluate the role of resveratrol and its methoxy analogs, MC4 and MR4, to inhibit TPA-induced inflammation in NF- κ B-Jurkat GFP (njg) cells with the purpose of evaluating their role as anti-inflammatory agents. Both MC4 and MR4 offer a superior pharmacokinetic/pharmacodynamics profile as compared to resveratrol (Lin and Ho 2009) and establishing a biosimilarity between these molecules offers potential for clinical use of these analogs instead of resveratrol.

Role of Resveratrol, MC4, MR4 and Calcium in neuroblastoma differentiation

Objective 1: To establish biosimilarity of MC4 and MR4 with resveratrol in neuroblastoma differentiation: It has been reported that resveratrol induces neuroblastoma differentiation (Dasgupta 2007). The aim of our study is to reconfirm the effect of resveratrol on neurite outgrowth in neuro-2a cells and evaluate the effect of two potent resveratrol analogs (MC4 and MR4), on neuronal differentiation with the purpose of establishing the biosimilarity of these analogs in tumor differentiation.

Objective 2: To determine the effect of calcium ions in neuroblastoma differentiation: It has been previously determined that calcium ions induce the differentiation of neuroblastoma cells (Reboulleau 1986). The aim of our study to reconfirm the role of calcium in neuroblastoma differentiation and evaluate the potential biochemical pathway of calcium induced neuronal differentiation.

Objective 3: To determine the combinatorial effect of resveratrol and its analogs and calcium in neuroblastoma differentiation: Both resveratrol and calcium have been reported to induced neuronal differentiation and the aim of present study is to evaluate the combinatorial effect of resveratrol, MC4 and MR4 and calcium on neuronal differentiation with the purpose of maximizing the efficacy of neuronal differentiation by a multi-drug program. Evaluation of synergistic or additive effect of these inducers on neuronal differentiation would further elucidate the biochemical targets of these two classes of inducers and enhance our understanding of the neuronal differentiation pathway.

Role of eIF5A and Resveratrol, MC4 and MR4 in preadipocyte differentiation

Objective 1: To establish the role of polyamines and eIF5A in preadipocyte differentiation. It has been previously established that DFMO, a polyamine biosynthesis inhibitor, reduced lipid accumulation in differentiation preadipocytes (Erwin 1984, Bethell 1981) implicating a role for polyamines in adipogenesis. eIF5A modification is a unique polyamine-dependent pathway and the aim of our study is to investigate the role of eIF5A in preadipocyte differentiation. eIF5A is a highly conserved, survival protein; however its function remains largely unknown. A secondary aim of this study is to gather intelligence on the functions of this crucial protein.

Objective 2: To establish the biosimilarity of resveratrol analogs (MC4 & MR4) with resveratrol in preadipocyte differentiation. It has been previously established that resveratrol plays an inhibitory role in preadipocyte differentiation (Pang 2006). The aim of our study is to reconfirm the inhibitory effect of resveratrol on preadipocyte differentiation and evaluate the effect of two potent resveratrol analogs (MC4 and MR4), on adipogenesis with the purpose of establishing the biosimilarity of these analogs in normal cell differentiation.

Chapter 2: Role of Proteases in Heat Induced Tumor Cell Death

Introduction:

Heat shock response is triggered by an increase in the physiological temperature (37°C) and functions to restore homeostasis by inhibiting apoptotic pathway and promoting pro-survival pathway through a group of conserved proteins called heat shock proteins (HSPs). One of the effects of heat shock is inhibition of protein synthesis (except for HSPs) and accumulation of misfolded proteins in the cell. HSPs bind to the misfolded proteins and allow for proper folding or target them to the lysosome for degradation (Dikomey and Franzke, 1992; Morimoto 1994; Sakaguchi 1995; Xu 1998; Stein 1999).

Failure of this mechanism to remediate the deleterious effects of heat treatment results in cell death. However the mechanism of cell death induced by heat treatment is poorly understood and is of significance due to its potential in cancer therapy.

Heat treatment & cancer cells:

It has been previously reported that cancer cells are sensitive to heat shock treatment (van der Zee 2002) with minimal injury to healthy tissue. Several clinical trials have shown the efficacy of heat treatment (HT) in cancer therapy (Hilderbrandt 2002, Wust 2002). However, HT has limited application in cancer therapy due to the high temperatures (44-51°C) required for cell death and is mainly used as an adjuvant therapy to chemo or radiation.

Due to the variations in cancer-types, low tolerance of heat treatment and practical hindrances in application of heat to tumor area, heat therapy is inefficient as a stand-alone

therapy. An approach to improving the efficacy of heat treatment could possibly be achieved via combinatorial treatment using various small molecules capable of mimicking heat treatment induced apoptotic pathway and thus allowing for synergistic or additive effect on tumor cell death. This kind of combinatorial approach would possibly allow for achieving heat induced tumor cell death at a lower temperature.

In this study, we have evaluated the role of small molecules to mitigate or enhance the effect of heat induced tumor cell death. The molecules selected were modulators of two biochemical targets involved in heat shock mediated survival (SIRT1) and death (eIF5A).

SIRT1 & Heat Shock:

SIRT1, is a histone deacetylase known to bind to heat shock protein factor-1 (HSF1). Activation of SIRT1 results in the binding of SIRT1 to HSF1 and maintaining it in a deacetylated form which negatively controls the DNA-binding activity of HSF1 (Fig. 2.1 Westerheide 2009). Trimerization and binding of HSF1 is required for activation anti-apoptotic and pro-survival heat shock proteins, HSP70 and HSP27, failure of which leads to cell death. Based on this interaction of SIRT1 to promote cell death in heat shock response, we evaluated the role of small molecule inhibitors of SIRT1, Sodium butyrate, SAHA (Suberoylanilide Hydroxamic Acid), NAM (nicotinamide) and an activator of SIRT1, Resveratrol on heat induced tumor cell death in HeLa and HCT116 cells. The hypothesis that underlies use of these molecules is that SIRT1 inhibitors (SAHA, NAM, Sodium butyrate) would allow for activation of HSF1 and protect the loss of viability of heat induced tumor cells through inhibition of the heat shock response pathway. SIRT1

activator (Resveratrol) on the other hand would inactivate HSF1 (through interaction with SIRT1) and enhance the loss of viability in heat-induced tumor cell death.

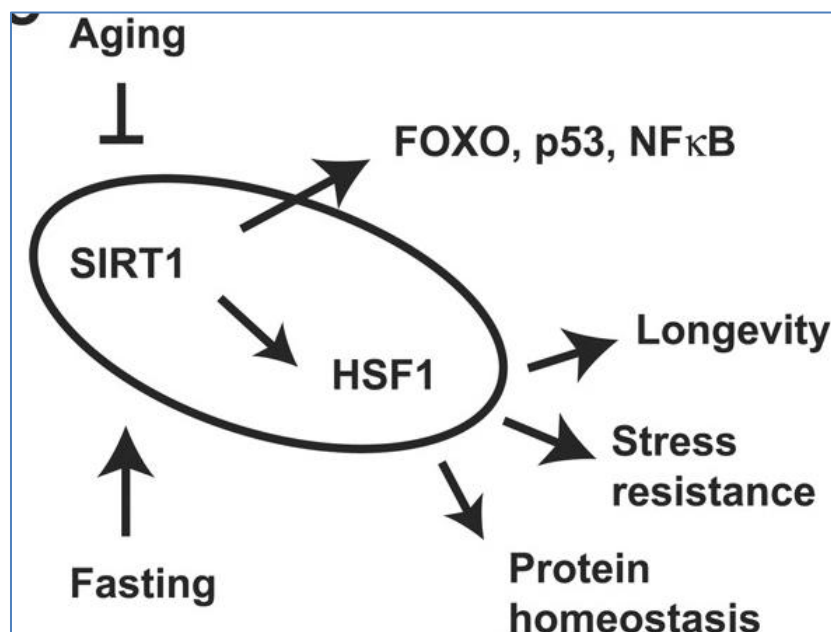


Figure 2.1: Role of SIRT1 in regulation of HSF1

eIF5A & Heat Shock:

Takeuchi *et al* (2002) reported that treatment of pancreatic cancer cells with high temperature (51°C) results in loss of a small protein, discovered to be eIF5A and the loss of eIF5A was correlated to pancreatic tumor cell death. eIF5A is a highly conserved, survival protein which is required for normal cell growth and proliferation (Chen 1997) .

Gossiau *et. al*, (2009) showed that colon cancer cells (Caco-2) were sensitive to heat shock (51°C for 30 mins) and the loss of eIF5A was correlated to loss of viability in this cell line as compared to cervical cancer cells (HeLa) which were resistant to heat shock and remained viable.

The eIF5A gene is highly conserved, from yeast to human. The eIF5A protein contains an unusual amino acid derived from posttranslational modification of a unique lysine residue using spermidine as the substrate. Fig. 2.2 illustrates the biochemistry of this unique posttranslational modification, wherein deoxyhypusine synthase (DHS) catalyzes the transfer of 4-aminobutyl moiety from spermidine to a specific lysine residue, resulting to a deoxyhypusine residue and deoxyhypusine hydroxylase catalyzed the hydroxylation of deoxyhypusine to hypusine (Chen 1997). Deletion of genes encoding eIF5A or DHS in yeast produces lethal phenotype (Schnier 1991; Sasaki 1996), indicating that eIF5A is an essential protein and that hypusine modification is necessary for its essential function.

Inhibition of DHS using specific inhibitors such as GC7 (N1-guanyl-1,7-diaminoheptane) in mammalian cells leads to growth arrest, suggesting that eIF5A plays an important role in growth regulation (Park 1994; Chen 1997). To further test the role of eIF5A modification in heat induced tumor cell death, we evaluated the effect of two inhibitors of eIF5A modification, DFMO (difluoro methyl ornithine) and GC7 on heat induced tumor cell death. The underlying hypothesis for use of DFMO and GC7 is that if indeed eIF5A modification is involved in heat induced tumor cell death, inhibition of eIF5A modification would enhance the efficacy of heat shock treatment.

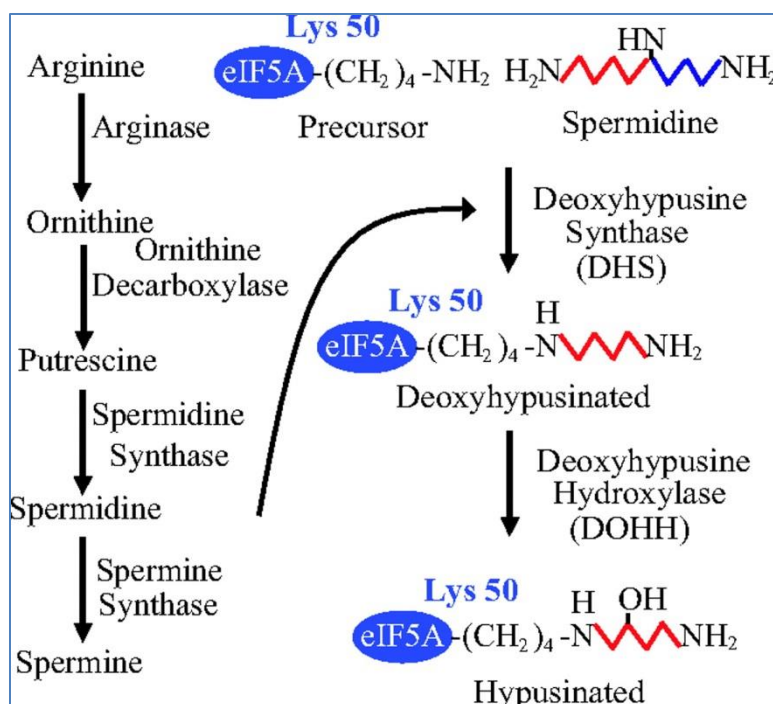


Figure 2.2: Polyamine biosynthesis and eIF5A modification

Proteases & Heat shock:

Gossiau (2009) reported that treatment of Caco-2 cells with para-methyl sulphonic acid (PMSF), a serine protease inhibitor resulted in partial recovery of viability in heat shocked cells. This study implies a role of proteases in heat induced cancer cell death.

Our hypothesis is that heat shock induces the activation of a proteolytic system which degrades proteins crucial for cell survival and loss of these proteins results in cell death. In order to test this hypothesis, we evaluated the role of various protease inhibitors such as EDTA (metalloproteinase) and Leupeptin (serine, threonine, cysteine protease) in heat shock induced tumor cell death.

Materials & methods:

Cell culture:

Cell culture was performed as described previously (Gosslau 2005). HeLa (cervical cancer), Caco-2 (colon cancer), HCT116 (colon cancer), HT29 (colon cancer), HTB-81 (prostate), neuro-2a (neuroblastoma) were obtained from the American Type Culture Collection (Rockville, MD).

Cell treatment:

Before experiments, cells were seeded in 60 or 35 mm culture dishes until confluence and treated with SAHA, Sodium butyrate, NAM, Resveratrol, DFMO, GC7, PMSF, EDTA and Leupeptin for 24 hours, followed by heat shock.

Heat treatment:

For heat treatment, cells were seeded in 60 or 35 mm culture dishes and a heat treatment at either 44°C or 51°C was applied using a CO₂ incubator (for 44°C) or by transferring cell culture dishes into a water bath for 30 mins (for 51°C) followed by different recovery times in a CO₂ incubator at 37°C (Gosslau *et al.*, 2009).

Western blot analysis:

This procedure was carried out as described previously (Gosslau 2005) for detection of eIF5A and actin. For eIF5A and actin analysis, cells were washed and scraped into 1 ml of PBS. After sonication, the homogenate was exposed to centrifugation at 14,000x g for 5 min. The supernatant was saved and protein concentration was determined by the

Bradford method from Bio-Rad (Hercules, CA). Whole cell extracts (5 μ g) were resolved on a 15% SDS-PAGE system and transferred onto a PVDF membrane using a Trans-Blot Electrophoresis Transfer Cell at 370 mA for 1.5 h. Non-specific protein-binding sites on the membrane were blocked by incubation with 5% (w/v) non-fat dry milk in PBSTween20 and probed with primary anti-eIF5A antibody (1:500 dilution) overnight at 4°C. The membranes were washed in PBS-Tween20 (phosphate buffer saline), followed by incubation with secondary antibody at 1:25,000 dilution (Jao 2006). Primary polyclonal antibody against recombinant human eIF5A was raised in chicken and affinity-purified by recombinant eIF5A proteins. The secondary rabbit against chicken antibody conjugated to horseradish peroxidase (HRP-RAC) was from Amersham Pharmacia (Piscataway, NJ).

Results:

Colon cancer cells (Caco2 and HCT116) were more sensitive to heat treatment as compared to cervical cancer cells (HeLa)

Sensitivity to heat treatment is a function of cancer-type as previously reported (Gossiau 2009). HeLa (Cervical cancer) and Caco-2 and HCT116 (colon cancer cells) were heat shocked at 51°C for 30 min and then allowed to recover at 37°C and the recovery was monitored by cell morphology and metabolic activity. As evident in Fig. 2.3 and 2.4, HeLa cells remained attached and viable during the heat shock and recovery period for up to 48 hrs. Interestingly the colorectal cancer cells (Fig. 2.3 HCT116 and Fig. 2.4 Caco2) were 50-60% detached from substrate and appeared to floating. The cells failed to recover from the heat shock and lost their viability as evidenced by the morphology and viability assay (Fig. 2.3 & 2.4) implying colorectal cancer cells were more sensitive to heat shock as compared to cervical cancer cells.

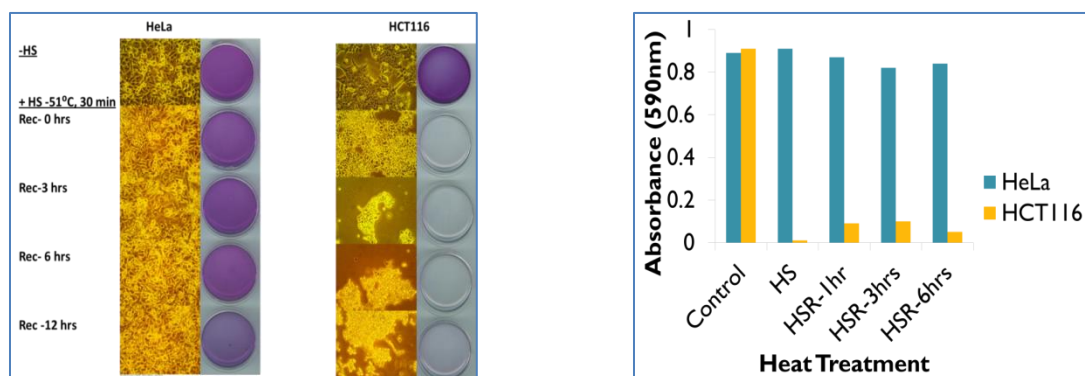


Figure 2.3: Differential effect of acute heat treatment on colon (HCT116) and cervical cancer cells (HeLa)

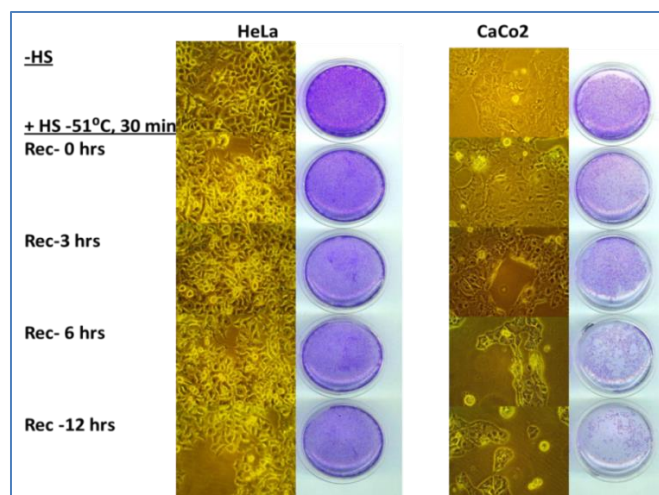


Figure 2.4: Differential effect of acute heat treatment on colon (Caco2) and cervical cancer cells (HeLa)

I investigated the sensitivity of prostate (HTB-81) and neuroblastoma (neuro-2a) cancer cells to heat shock by exposing HTB-81 and neuro-2a cells to 30 mins of heat shock at 51°C followed by recovery at 37°C for a period of 1-6hrs. As evidenced by the morphological and viability data in Fig. 2.5, prostate cancer cells were resistant to heat shock at 51°C and remained viable through the heat shock and recovery period. Neuroblastoma cells were partially resistant to heat shock at 51°C and remained viable through the heat shock and recovery period up to 12 hours (Fig. 2.6).

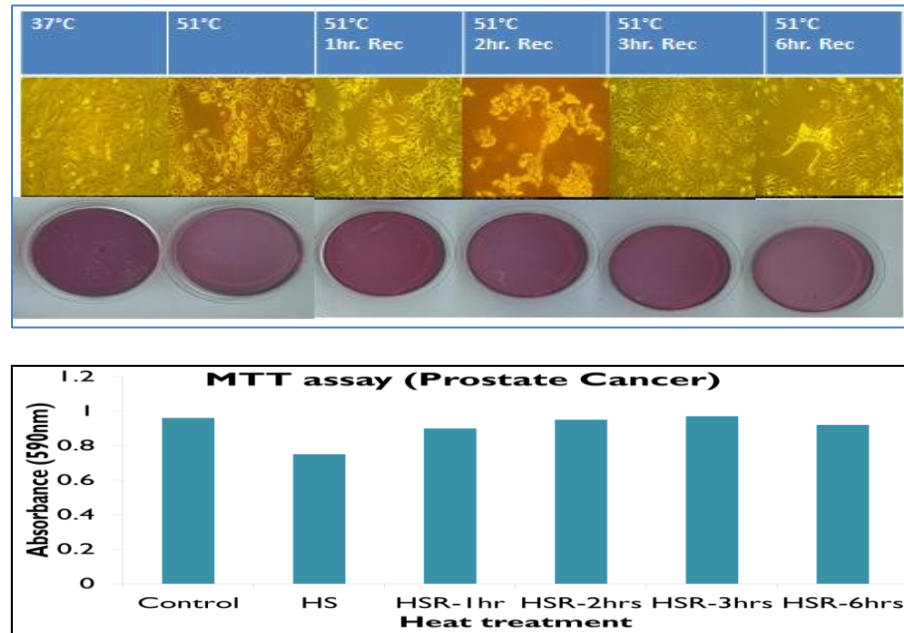


Figure 2.5: Effect of acute heat treatment on Prostate cancer cells (HTB-81)

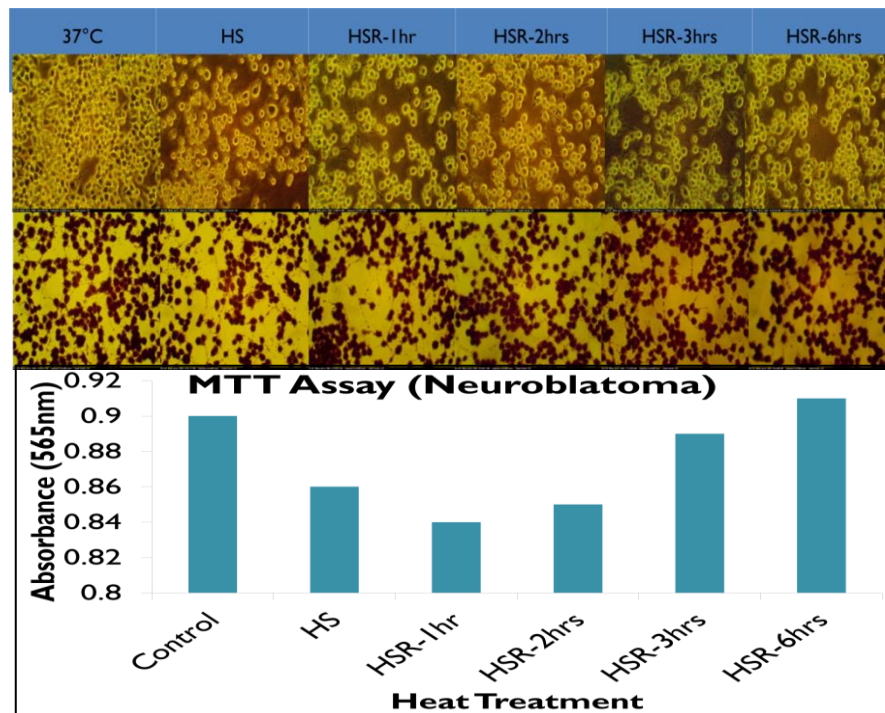


Figure 2.6: Effect of acute heat shock on Neuroblastoma cancer cells (nuero-2a)

Of all the cancer cell lines (prostate, cervical, colorectal and neuroblastoma) tested, colorectal cancer cells were the most sensitive to heat shock at 51°C and to further evaluate the sensitivity of colorectal cancer cells towards heat shock, we varied the temperature at which heat shock was administered followed by a recovery period. Briefly, HeLa and HCT116 cells were heat shocked at 44°C and 51°C for 30 mins followed by a 3 hour recovery at 37°C. As evidenced by morphology and viability assay (MTT) in Fig. 2.7, both cervical and colorectal cancer cells were viable after 30 mins of heat shock at 44°C and remained viable throughout the recovery period. This implies that colorectal cancer cells were sensitive to heat shock at 51°C, but were able to sustain through heat shock at 44°C. Based on this data, we continued to explore this differential behavior in cervical vs. colorectal cancer cells at 51°C where the effect of heat shock was most pronounced.

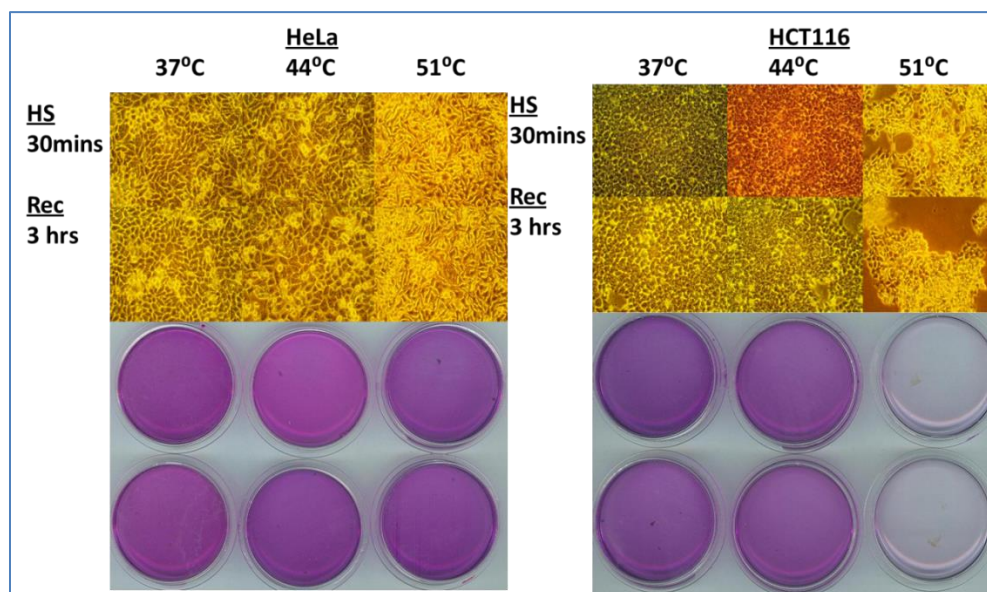


Figure 2.7: Effect of varying temperature (44 vs 51) of heat treatment followed by recovery on cervical (HeLa) and colorectal (HCT116) cancer cells

It was eminent to determine the minimum exposure time of acute heat shock required (51°C) to induce cell death in colorectal cancer cells. To evaluate this, we exposed colorectal cancer cells (HCT116 and HT29) to 10, 20 and 30 min of acute heat shock at 51°C (Fig. 2.8). The morphological evidence suggests that colorectal cancer cells were viable up to 20 mins of heat shock and lost their viability after exposure to 30 mins of heat shock (51°C).

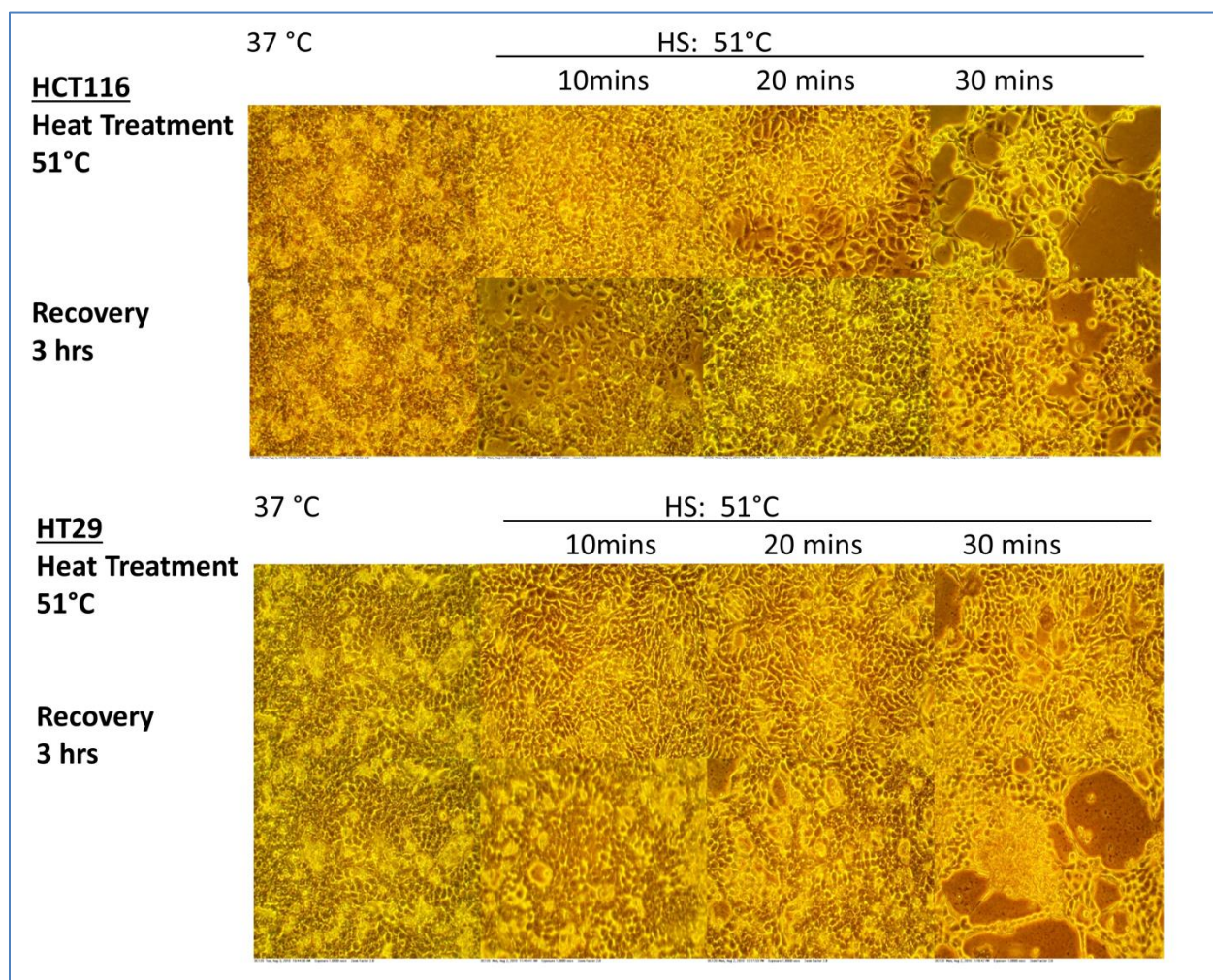


Figure 2.8: Effect of varying time of heat treatment followed by recovery on colorectal (HCT116 and HT29) cancer cells

eIF5A Levels After Heat Shock:

Since there was a correlation between loss of eIF5A after acute heat stress and cell death for Caco-2 and HeLa cells (Gosslau 2009), I determine the eIF5A level in heat treated cervical and colon cancer cells. Western blot analysis of heat shocked samples of colorectal (HCT116 and HT29) cancer cells for loss of eIF5A was carried out and cervical (HeLa) cancer cells were used as control. As evident in Fig 2.9 (51°C) there is a significant loss of eIF5A post-heat shock (51°C) in HCT116 and HT29 cells but not in HeLa cells, implying a role for eIF5A in heat induced cell death of colorectal cancer cells. The findings of these studies are congruent with the previously published data from our lab (Gosslau 2009).

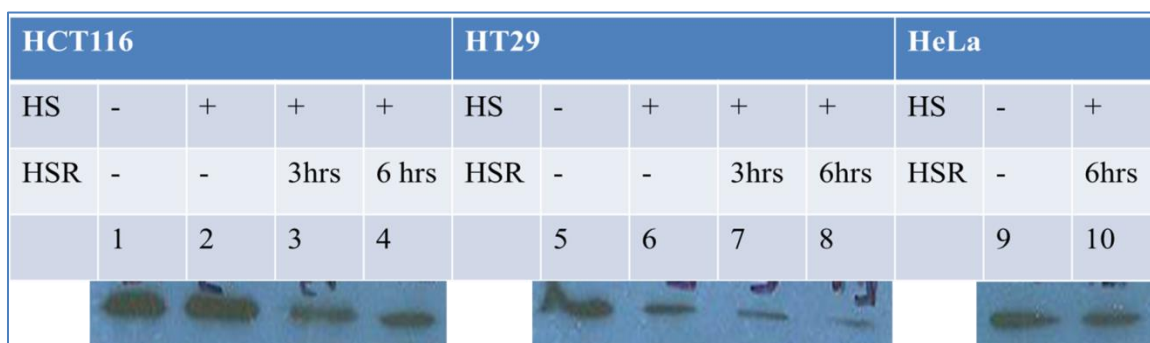


Figure 2.9: Western blot analysis of heat shocked (51°C) samples of colorectal cancer cells (HCT116 & HT29) and cervical cancer (HeLa) cells for loss of eIF5A

Role of small molecules in heat induced cancer cell death

We devised a panel of small molecules to enhance or mitigate the effect of heat induced cancer death. The idea was to determine combinatorial effect of chemical and heat stress for efficient killing of tumor cells, analogous to combining chemotherapy with radiation

therapy, to develop a potent regime for effective cancer therapy. A secondary goal was to determine the role of SIRT1 and eIF5A in heat induced tumor cell death.

As shown in Fig. 2.10, I pre-treated HeLa and HCT116 cells with SIRT1 modulators: 10mM Sodium butyrate, 10 μ M suberoylanilide hydroxamic acid (SAHA) and 10mM nicotinamide (NAM) and eIF5A modulators: 10mM DFMO and 10 μ M GC7 for 24 hours followed by heat shock for 30 mins at 51°C and heat shock recovery for 3 hrs. at 37°C. As evidenced by morphology and viability assay, the small molecules failed to mitigate or enhance the effect of heat shock on both cervical and colorectal cancer cell lines implying that SIRT1 and eIF5A possibly play no significant role in heat induced tumor cell death.

Western blot analysis was carried out to detect the loss of eIF5A in drug-treated and heat shocked cancer cells. As expected, all the heat shocked and heat shock recovered colorectal cancer cells showed loss of eIF5A. However, the drugs by themselves failed to induce loss of eIF5A or cell death as evident from the morphology and viability assay of colorectal cancer cells at 37°C (Fig. 2.10). In addition HeLa cells were resistant to the effects of heat shock, drug treatment and the combinatorial effect of drugs and heat shock.

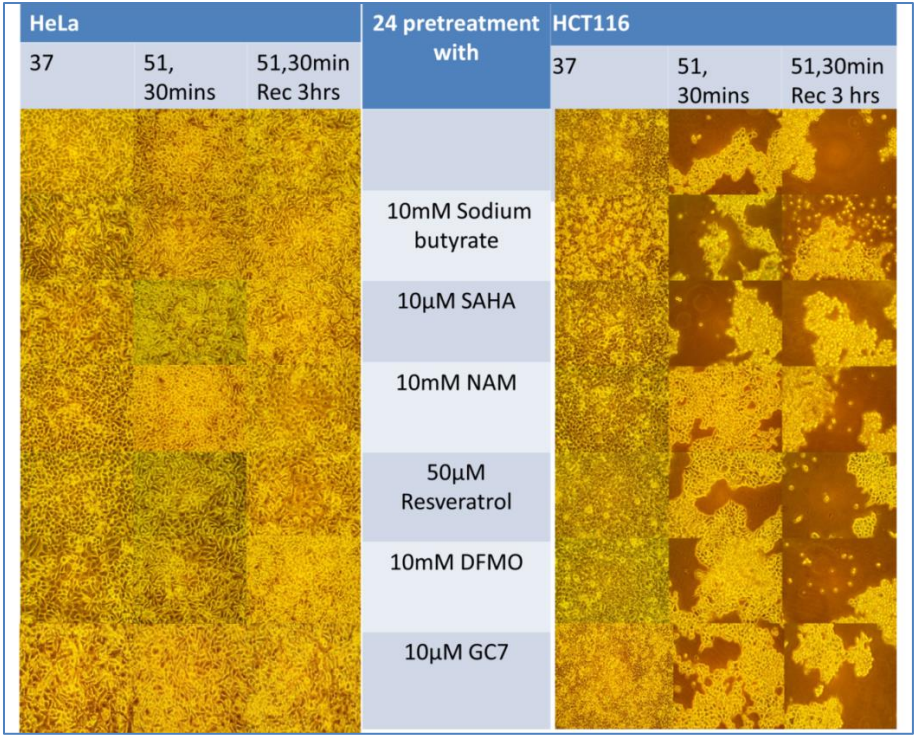


Figure 2.10: Combinatorial effect of small molecules and hyperthermia in cervical (HeLa) and colorectal (HCT116) cancer cells

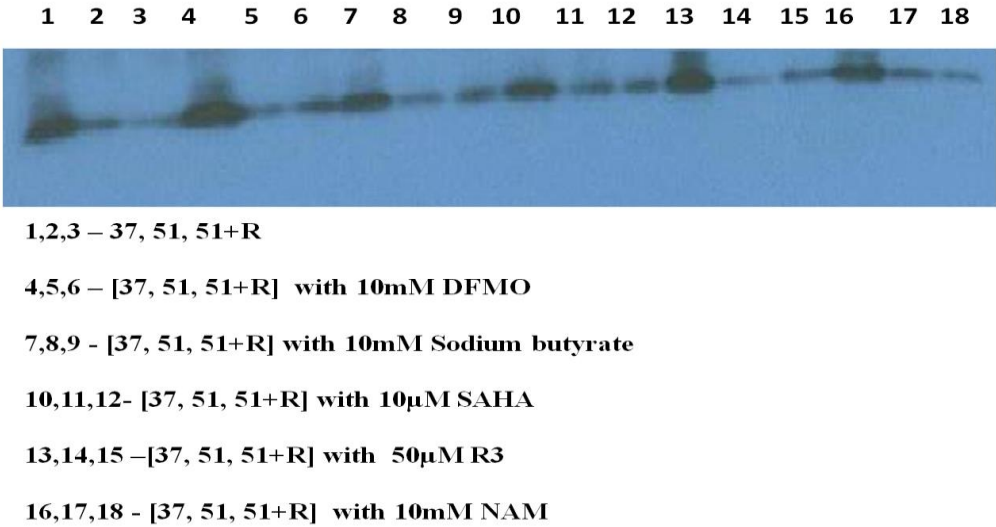


Figure 2.11: Western blot analysis (eIF5A) of drug-treated, heat shocked colorectal (HCT116) cancer cells

Role of SIRT1 in heat induced tumor cell death

As seen in Fig. 2.10, modulation of SIRT1 did not have a significant effect on heat induced tumor cell death indicating the mechanism of cell death induced by heat treatment was independent of SIRT1.

Role of proteases in heat shock induced tumor cell death and loss of eIF5A

All of these experiments suggest that the death of colorectal cancer cells (HCT116) is correlated with loss of eIF5A (Takeuchi 2002, Gossiau 2009, and Fig. 2.9). However blocking of eIF5A modification did not have synergistic or additive effect on heat shock induced tumor cell death (Fig. 2.10 and 2.11). One of the possible mechanisms to explain the loss of eIF5A in heat shocked cancer cells, would involve activation of protease/proteases which would then lead to the degradation of eIF5A resulting in cell death.

Our lab has previously shown to that a serine protease inhibitor, PMSF was partially able to restore cell viability (Gossiau 2009). We further explored the role of proteases in heat induced tumor cell death by pre-treating colorectal (HCT116) cancer cells with other known, non-specific protease inhibitors such as EDTA (metalloprotease inhibitor) and leupeptin (cysteine, serine and threonine peptidase inhibitor). As evidenced in Figs. 2.12 and 2.13, both EDTA and Leupeptin were able to partially rescue the cell viability in heat induced colorectal (Fig. 2.12 HCT116) and (Fig. 2.13 CaCo2) cancer cells.

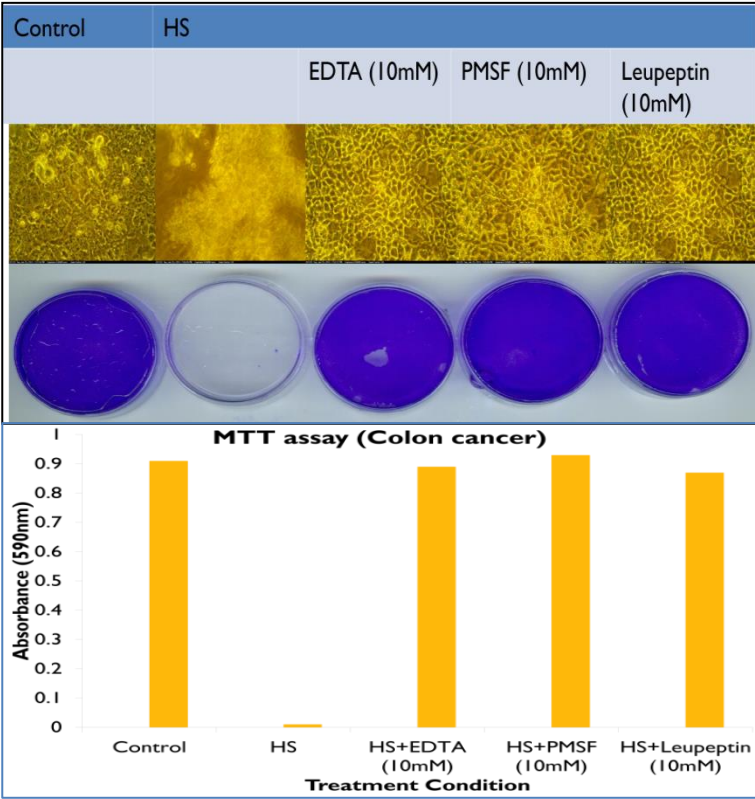


Figure 2.12: Effect of protease inhibitors on heat induced colon cancer cell death

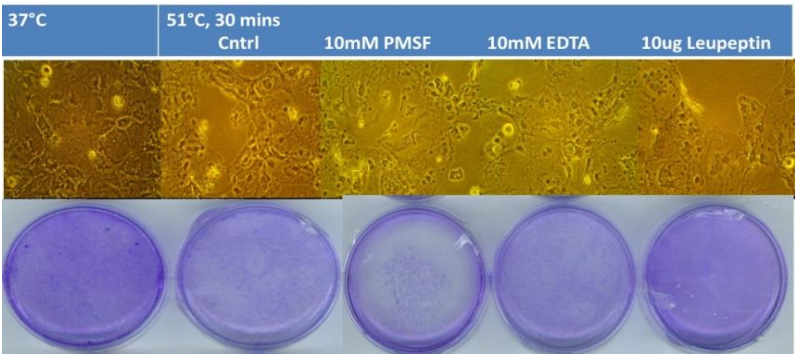


Figure 2.13: Effect of protease inhibitors on heat induced cancer Caco-2) cell death

Discussion:

Colorectal cancer cells were found to be the most sensitive (compared to cervical and prostate) to acute heat shock as demonstrated in previous studies (Gosslau 2009) and independently reconfirmed in this study in comparison with cervical, prostate and neuroblastoma cancer cells. This sensitivity towards acute heat shock was accompanied by loss of viability merely after 30 mins of acute heat shock at 51°C and the cells were incapable of revitalizing post-heat shock known as recovery period at 37°C.

Stress response is a vital mechanism by which cells mediate survival under stress conditions such as temperature changes, starvation, hypoxia (lack of oxygen supply) etc. Under extreme stress condition when this mechanism fails to rescue the cells from the damaging effects of stress, the cells activate the apoptosis pathway leading to cell death.

In the case of colorectal cells, the heat shock response mediated via activation of HSF1 (Butler 2011) was incapable of rescuing the cells from the harmful effects of acute heat shock and the cells lost viability almost immediately at the end of heat shock period and post-recovery period.

It was previously established (Gosslau 2009) that thermal killing of colorectal cancer was associated with a loss of eIF5A, an essential and highly conserved survival protein (Chen 1996) and a serine-protease inhibitor, PMSF was able to partially restore cell viability

during acute heat shock. These findings were independently reconfirmed in this current study.

We argue that the thermal-protective effect of PMSF is a function of its protease inhibition activity possibly on eIF5A, the loss of which has been shown to result in cell death (Chen 1996). To further test the role of protease inhibitors we selected two known protease inhibitors, leupeptin (serine, cysteine and threonine peptidase inhibitor) and EDTA (metalloproteinase inhibitor) and used PMSF (serine protease inhibitor) as control. Interestingly, all three of the protease inhibitors successfully showed partial rescuing of the acute heat shock by restoring cell viability as evidenced by MTT viability assay.

Based on the finding that all of the three different protease inhibitors were capable of thermal protective effect in colorectal cancer cells, it would be logical to derive the implication that more than one protease is activated during acute heat shock and there might multiple protein targets, including eIf5A that are either degraded or modified post-activation of these proteases.

I independently reconfirmed these finding and further proved that other protease inhibitors, EDTA (metalloproteinase) and leupeptin (a cysteine, serine and threonine protease inhibitor) were able to rescue the loss of viability in colon cancer cells. These findings strengthen the hypothesis that proteolytic degradation of crucial survival protein results in heat induced tumor cell death (Fig. 2.14). The exact mechanism, specific proteases and proteins involved in tumor cell death remains to be evaluated.

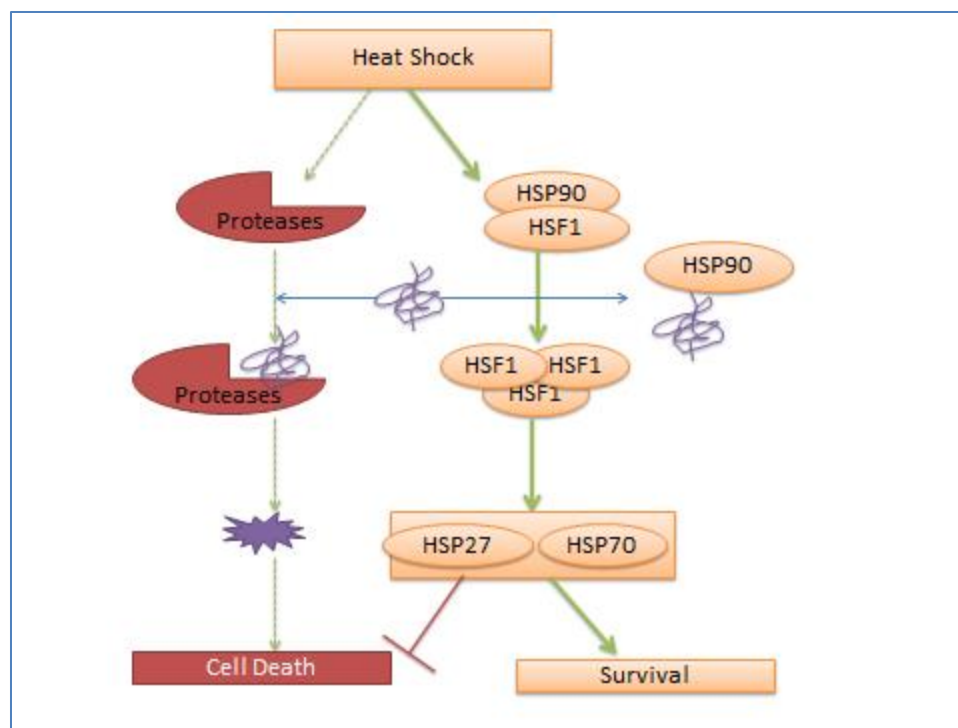


Figure 2.14: Proposed mechanism for role of proteases in heat induced tumor cell death

Conclusions:

- 1) Of all the cancer cell lines (cervical, colorectal, prostate and neuroblastoma) Of all the cancer cells exposed to heat treatment, colon cancer cells were the most sensitive
- 2) On treatment with SIRT1 inhibitors, cervical cancer cells continued to remain viable post-heat treatment and on treatment with SIRT1 activator, Resveratrol, colon cancer cells continued to be sensitive to heat treatment. These results taken together, imply a lack of role for SIRT1 modulation in increasing the efficacy of heat treatment
- 3) Loss of viability in heat treated colon cancer cells is associated with loss of eIF5A expression as measured by western blot analysis indicating a role of eIF5A in heat induced tumor cell death
- 4) On treatment with eIF5A modulation inhibitors, colon cancer cells continued to remain sensitive to heat treatment and cervical cancer cells continued to remain resistant to heat treatment. This finding indicates a lack of role for eIF5A modulation in increasing the efficacy of heat treatment.
- 5) Treatment with protease inhibitors restores viability in heat treated colon cancer cells indicating a role for proteases in heat induced tumor cell and offers potential for modulation of protease activity for improving the efficacy of het treatment

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CHAPTER 3: Role of resveratrol and its analogs in inflammation

Introduction

Inflammation is a patho-physiological cell survival mechanism of the body in response to irritant stimuli such as cytokines, pathogens, allergens etc. which function by neutralizing the irritant and initiating tissue repair to restore homeostasis. While acute inflammation is a desirable response because it is a short-term response that aids tissue healing, chronic inflammation is a maladaptive response in disease conditions such as arthritis, cancer etc. which results in tissue damage and manifests as a disease symptom (Fig. 3.1). Non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to ease chronic inflammation but those are associated with an arsenal of side effects and their safety and tolerability concerns make development of new anti-inflammatory drugs eminent.

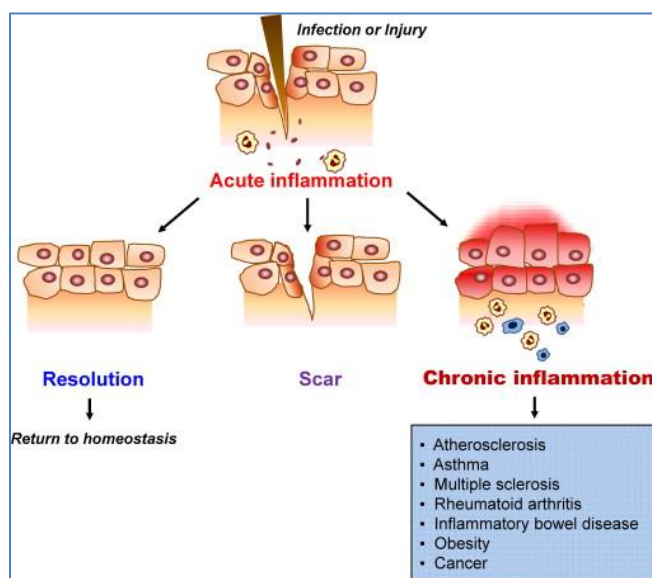


Figure 3.1: Acute vs Chronic Inflammation

The biochemical pathway of inflammation (Fig. 3.2, Bode 2000) involves a sequential TPA-induced or ultraviolet-induced activation of various GTP proteins, MAP kinase kinase kinases (MKKKs), MAP kinase kinases (MKKs), MAP kinases (MAPKs). Other pathways include activation of ERK and/or p38 and/or JNK which finally leads to activation of transcription factors such as NF- κ B or AP1. Activation of these pro-inflammatory transcription factors leads to expression of inflammatory genes.

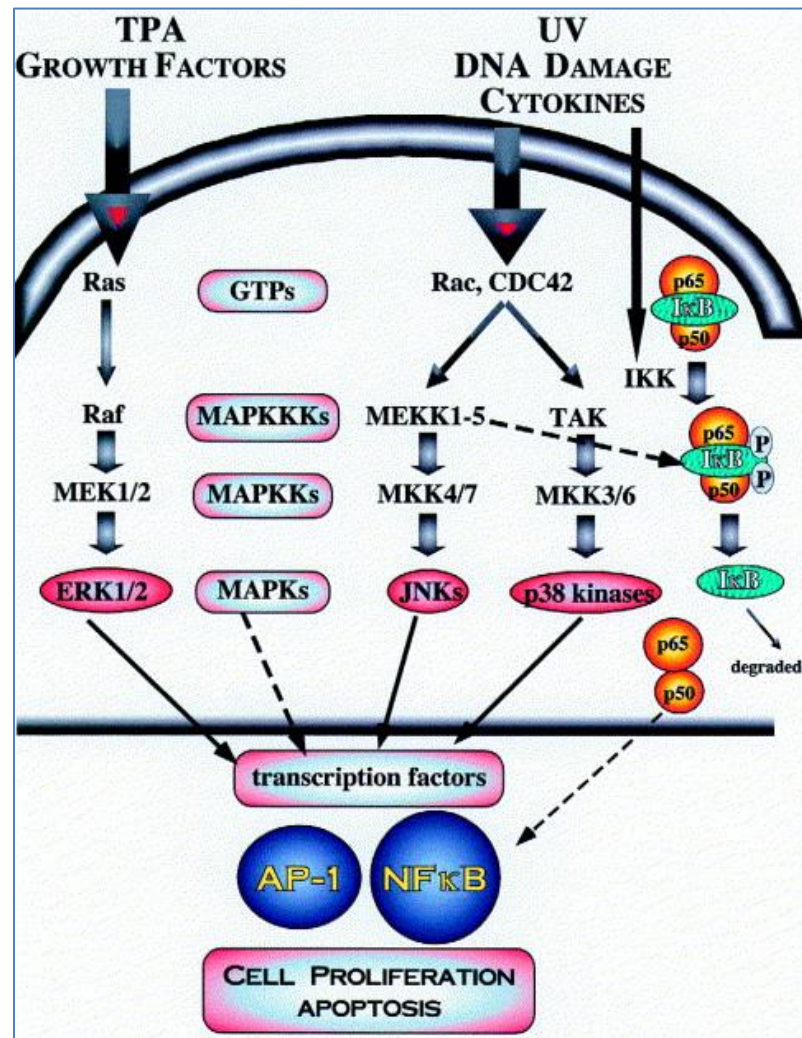


Figure 3.2: Biochemical Pathway of Inflammation

NF- κ B and Inflammation:

NF- κ B is a pivotal transcription factor that is involved in inflammation and can be activated by cytokines such as Tumor necrosis factor- α (TNF), Interleukin-1 (IL-1) etc. which cause activation of I κ K. I κ K causes phosphorylation of I κ B which is then released from the NF- κ B complex and degraded. Release of I κ B from the NF- κ B complex allows for binding of nuclear localization signal (NLS, Importin) which directs the NF- κ B complex to the nucleus. In the nucleus, NF- κ B binds to the promoter region of DNA and causes transcription of pro-inflammatory gene (Tak 2001)

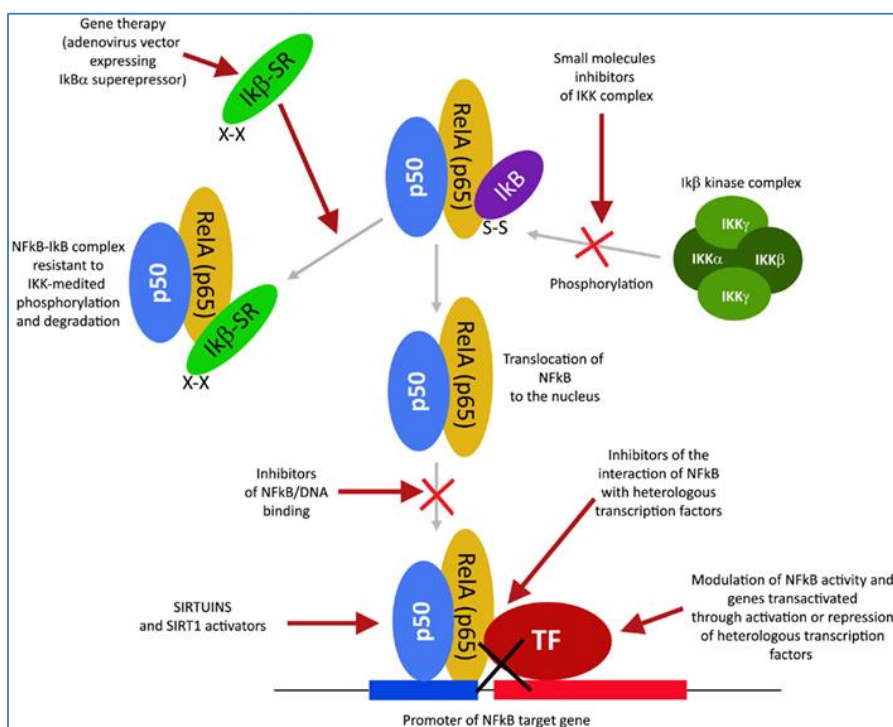


Figure 3.3: NF- κ B activation pathway in inflammation

Resveratrol and Inflammation:

Resveratrol a naturally occurring polyphenol is known to have anti-inflammatory properties (de la Lastra 2005 & Zhu 2011). The mechanism by which resveratrol down-

regulates inflammatory response is possibly through inhibition of NF- κ B via activation of SIRT1, a histone/protein deacetylase which is known to deacetylate the p50/p65 unit of NF- κ B (Yang 2012). Deacetylation of p65 leads to suppression of TNF α -induced NF- κ B activation.

The aim of this study is to determine the effect of MC4 and MR4 on TNF- α induced activation of NF- κ B mediated inflammation. The underlying hypothesis is that since resveratrol is known to inhibit activation of NF- κ B-mediated inflammation, analogs of resveratrol, MC4 and MR4, would potentially have the same inhibitory effect on NF- κ B-mediated inflammation. This would establish a biosimilar role for MC4 and MR4 in inflammation pathways which is of significance given the better drug-like properties of MC4 and MR4.

NF- κ B Reporter Assay:

In order to study the effect of resveratrol, MC4 and MR4 on NF- κ B mediated inflammation response; I used the NF- κ B-Jurkat-GFP-reporter assay (System Biosciences). Briefly stimulation of NF- κ B by TNF- α or TPA (12-O-tetradecanoylphorbol-13-acetate) results in a 30-fold increase in the expression of the reporter gene, GFP (Fig. 3.4)

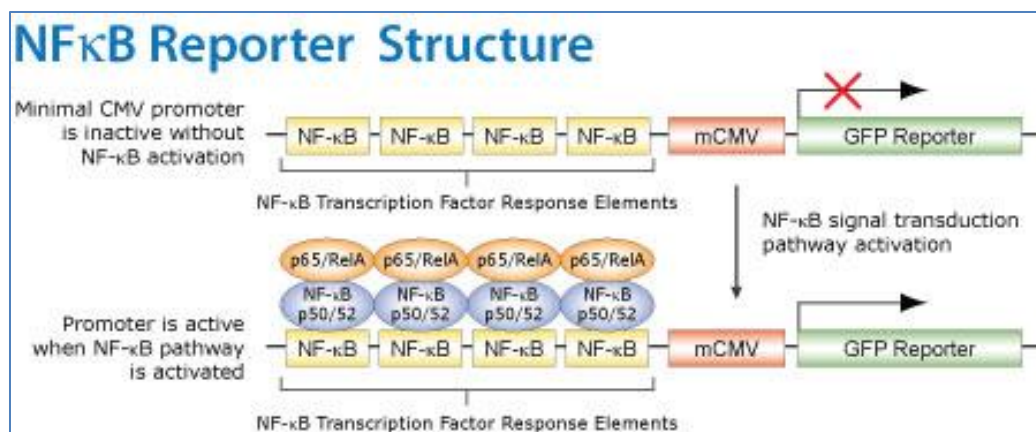


Figure 3.4: NF-κB-Jurkat-GFP Reporter assay (SBI)

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD). Resveratrol was obtained from Sigma-Aldrich and the analogs MC4 and MR4 were synthesized in our lab using the protocol published by Gossiau et. al., (2005).

Tissue Culture

NF-κB/Jurkat/GFP (NJG) cells were cultured in DMEM with 10% FBS at 37°C in a humidified, 10% CO₂ atmosphere. Cells were sub cultured in culture flasks (Falcon, Becton-Dickinson, Franklin Lakes, NJ) and passaged every 3 days. Before experiments, cells were seeded in 60 mm, 35 mm culture dishes or 24- well plates (Falcon, Becton-Dickinson, Franklin Lakes, NJ) as indicated for the different assays. Resveratrol and its analogs MC4 and MR4 dissolved in DMSO were applied to the medium to achieve the indicated final concentrations.

NF- κ B/Jurkat/GFP reporter assay:

NF- κ B/Jurkat/GFPTM Transcriptional Reporter Cell Line was obtained from SBI System Biosciences and the assay was carried out in accordance with the protocol detailed in the user manual. NF- κ B/Jurkat/GFPTM cells were cultured as a suspension in RPMI medium supplemented with 10% FBS and 1% Antibiotic-Antimycotic and maintained at 37°C and 5% CO₂. The cells were treated with varying dose of TPA (1-10ng) or resveratrol (10 μ M-25 μ M), MC4 (10nM) and MR4 (5 μ M) or a combination thereof. The inflammation response was recorded as a function of the intensity of GFP fluorescence measured (Excitation 485 \pm 20, Emission 528 \pm 20) in a Teacan plate reader.

Results

NF- κ B-Jurkat-GFP reporter (njg) cell line purchased were used an inflammation model to evaluate the role of stilbene analogs for anti-inflammatory properties. njg cells were induced to express GFP, a measure of inflammation through NF- κ B activation, using varying concentrations of TPA (1-10ng). As shown in Fig. 3.5, TPA induced inflammation response in njg cells was dose-independent and concentration as low as 1ng TPA effectively reported activation of the inflammatory pathway.

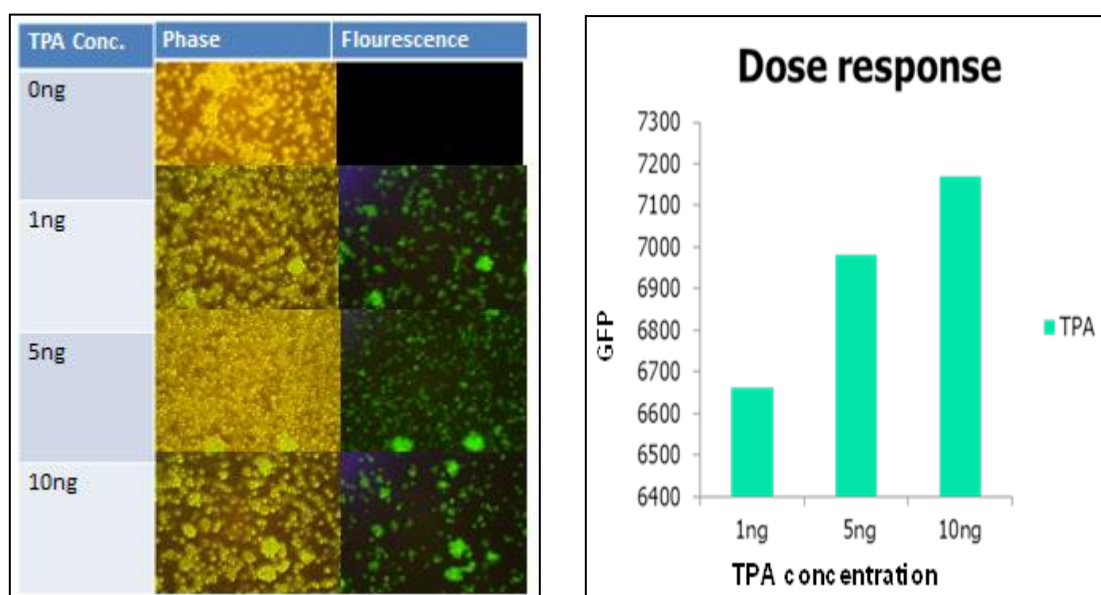


Figure 3.5: Dose response of TPA induced inflammation in NJG cells

In order to test the anti-inflammatory properties of resveratrol, a dose dependent study was carried out using varying concentration of resveratrol (10-50 μ M). As evidenced by Fig. 3.6, resveratrol when used alone was incapable of activating NF- κ B mediated inflammation pathway. The cells were viable as evident from the phase contrast images. This would imply that either resveratrol was incapable of activating the inflammation pathway in njg cells indicating that resveratrol is not an activator of NF- κ B-mediation inflammatory response.

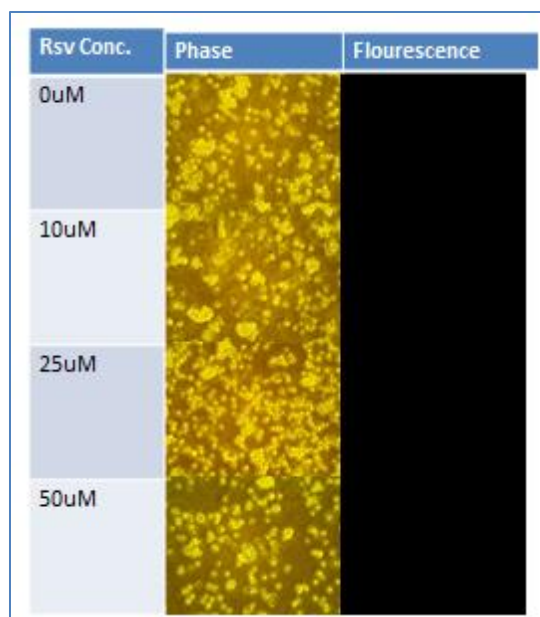


Figure 3.6: Effect of Resveratrol on the inflammation response of NJG cells

In order to evaluate whether resveratrol inhibited or had no effect on njg inflammation response, I pre-treated njg cells with varying concentration of resveratrol (10-50 μ M) and induced inflammation response via addition of TPA (5ng and 10ng). As evidenced by the images obtained through fluorescence microscopy (Fig. 3.7), TPA successfully rescued the inflammation pathway implying a) either resveratrol had no effect on NF- κ B-mediated inflammation response or b) resveratrol inhibited TPA-induced fluorescence but the effect was not visually evident.

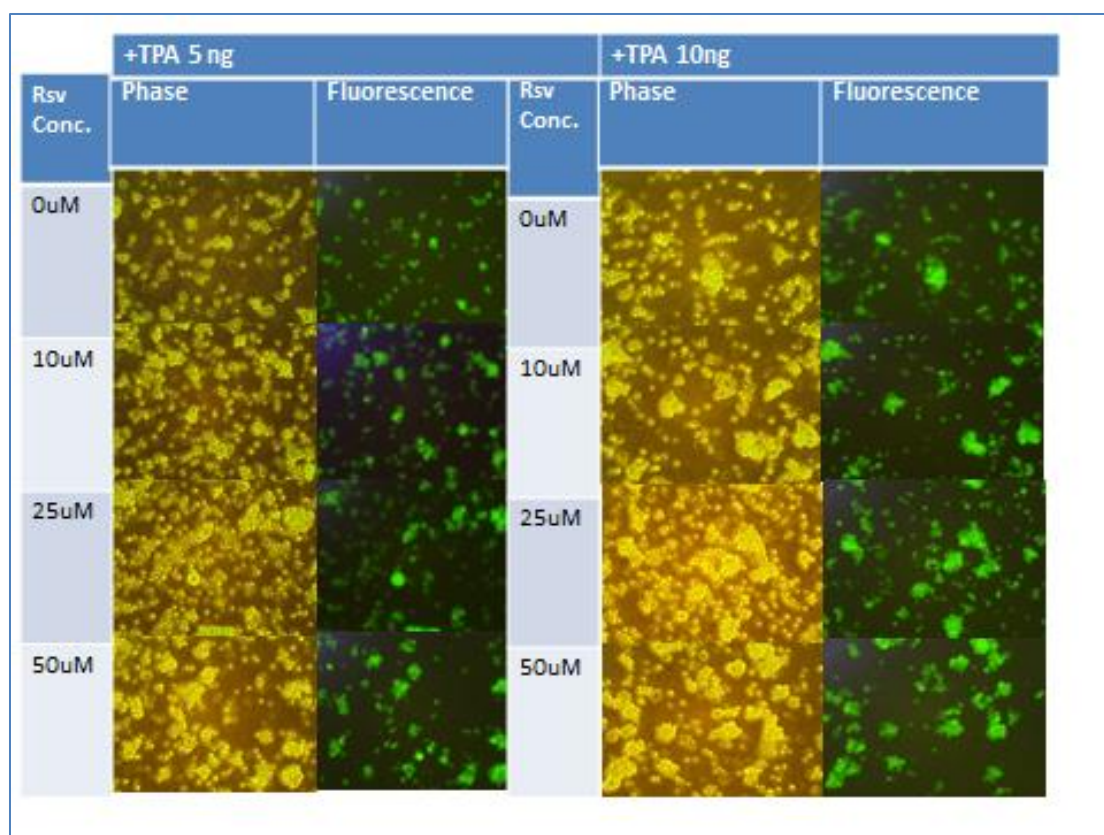


Figure 3.7: The anti-inflammatory effect of resveratrol was rescued by addition of TPA

Quantitative analysis of GFP signal was recorded after adding TPA to njg cells in presence or absence of resveratrol to determine the role of resveratrol in TPA-induced NF- κ B activation.

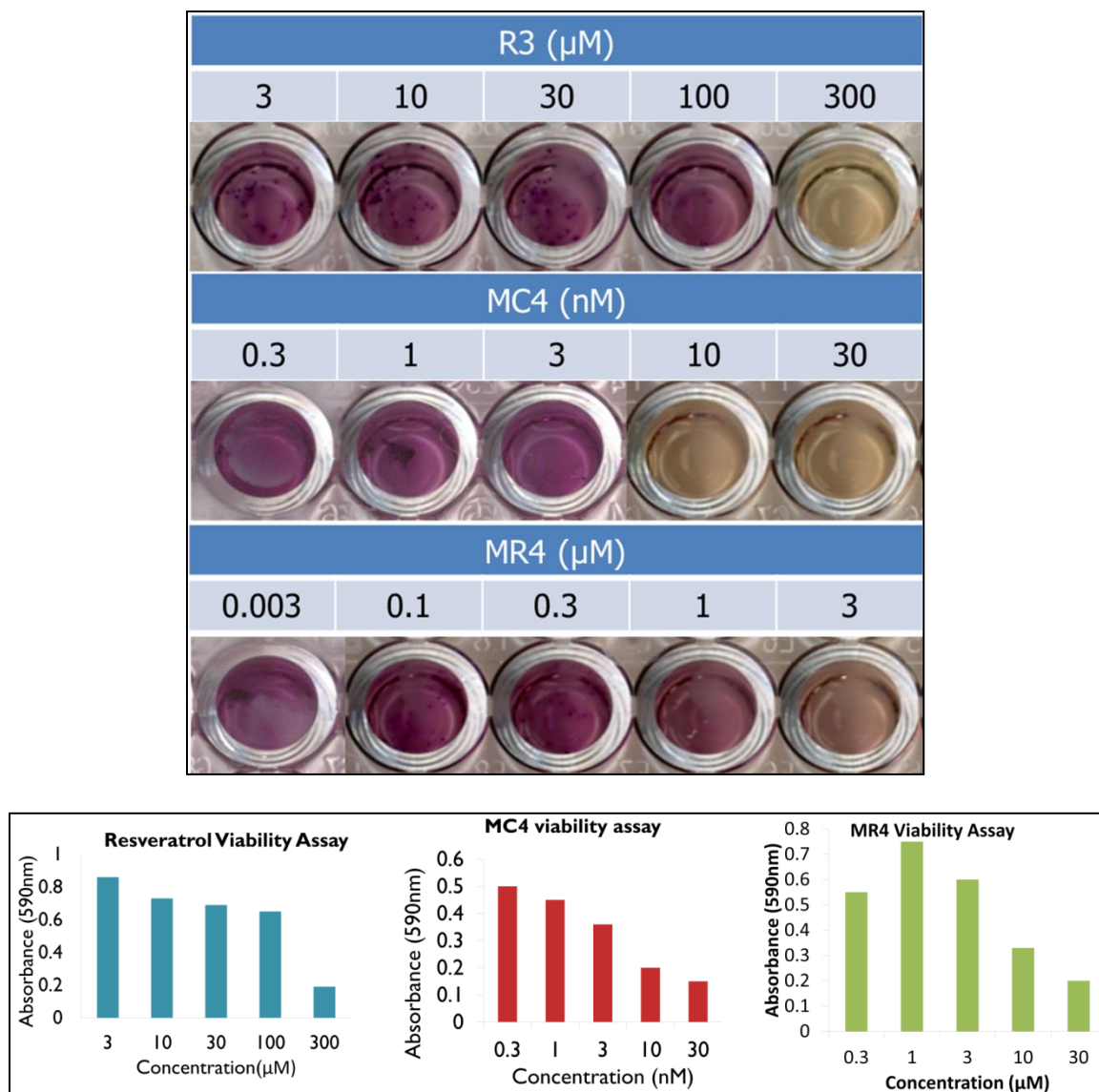


Figure 3.8: Resveratrol, MC4 and MR4 viability assay in njg cells (Dr. Sasaki)

Njg cells were treated with resveratrol, MC4 and MR4 based on the IC_{50} values determined in Fig. 3.8. Fig. 3.9 clearly shows a significant decrease in GFP signal in presence of resveratrol indicating an inhibitory role in NF- κ B-activation. MC4 and MR4 showed similar inhibition in TPA-induced activation of NF- κ B in njg reporter assay (Fig. 3.10 and 3.11). The mechanism of resveratrol, MC4 and MR4 mediated inhibition of inflammation remains to be evaluated.

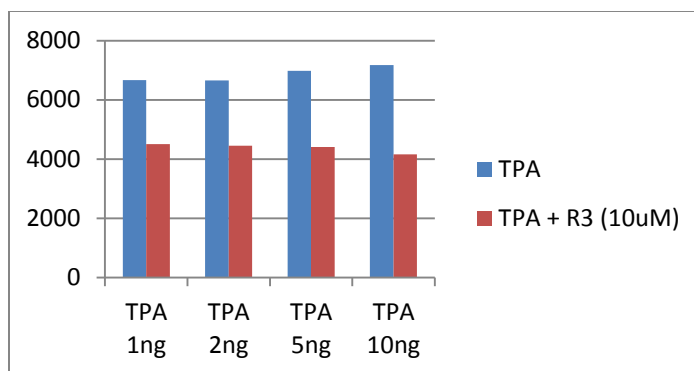


Figure 3.9: Quantification of GFP signal in presence and absence of resveratrol during TPA-induced inflammation in njg reporter cell line

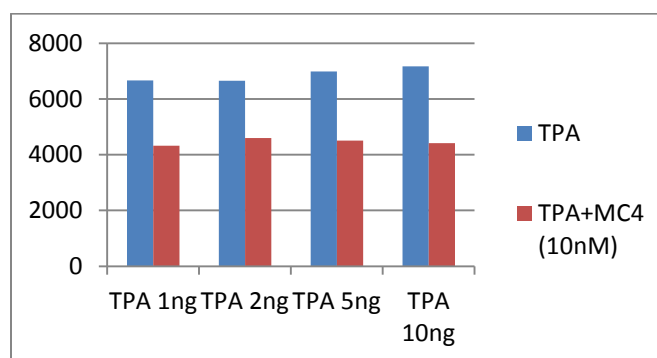


Figure 3.10: Quantification of GFP signal in presence and absence of MC4 (10nM) during TPA- inflammation in njg reporter cell line

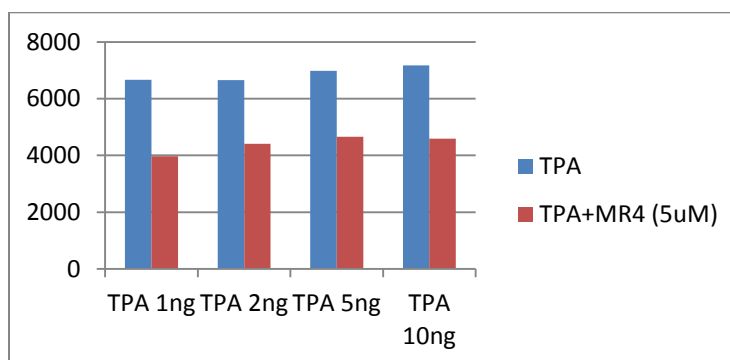


Figure 3.11: Quantification of GFP signal in presence and absence of MR4 (5uM) during TPA- inflammation in njg reporter cell line

Discussion

Inflammation is a patho-physiological response of the body to tissue/cell damage by pathogens, chemicals and physical injury. Acute inflammation is a short-term response mediated by leukocytes that works by removal of the stimuli and repairing the tissue while chronic inflammation associated with disease conditions such as allergies, arthritis, autoimmune responses is a prolonged, maladaptive response that results in tissue destruction (Weiss 2008, Medzhitov 2008).

Resveratrol has been known to inhibit inflammatory response via downregulation of NF- κ B, a pivotal factor in inflammation response (de la Lastra 2005). I reconfirmed the role of resveratrol as an anti-inflammatory agent using NF κ B-Jurkat-GFP reporter assay. This assay allowed for quantitative evaluation of inflammation response mediated by activation of NF- κ B using inducers of inflammatory response such as TPA. Resveratrol and its analogs, MC4 and MR4 mitigated the inflammatory response induced by TPA, possibly through downregulation of NF- κ B. The exact mechanism of resveratrol, MC4 and MR4 induced downregulation of NF- κ B remains to be evaluated.

One possible mechanism of the downregulation of NF- κ B by resveratrol could be through direct or indirect (through AMPK) activation of SIRT1 which is known to deacetylates the p65 unit of NF- κ B (Zhu 2011, Yang 2012). SIRT1 is known to deacetylate the p65 unit of NF- κ B which makes it incapable of binding to the DNA-promote region which is required for the transcription of inflammatory genes. Another possible mechanism could

be through activation of PGC-1 α (via AMPK) which is known to bind with the p65 unit of NF- κ B (Alvarez-Guardia 2010, Eisele 2013) making it incapable of binding to DNA.

Both of these plausible mechanisms possibly involve activation of resveratrol through AMPK (Fig. 3.11) which has been implicated to have an anti-inflammatory role, especially in inflammation related to metabolic disorders (Hoogendijk 2013, Salminen 2011, O'neil 2013).

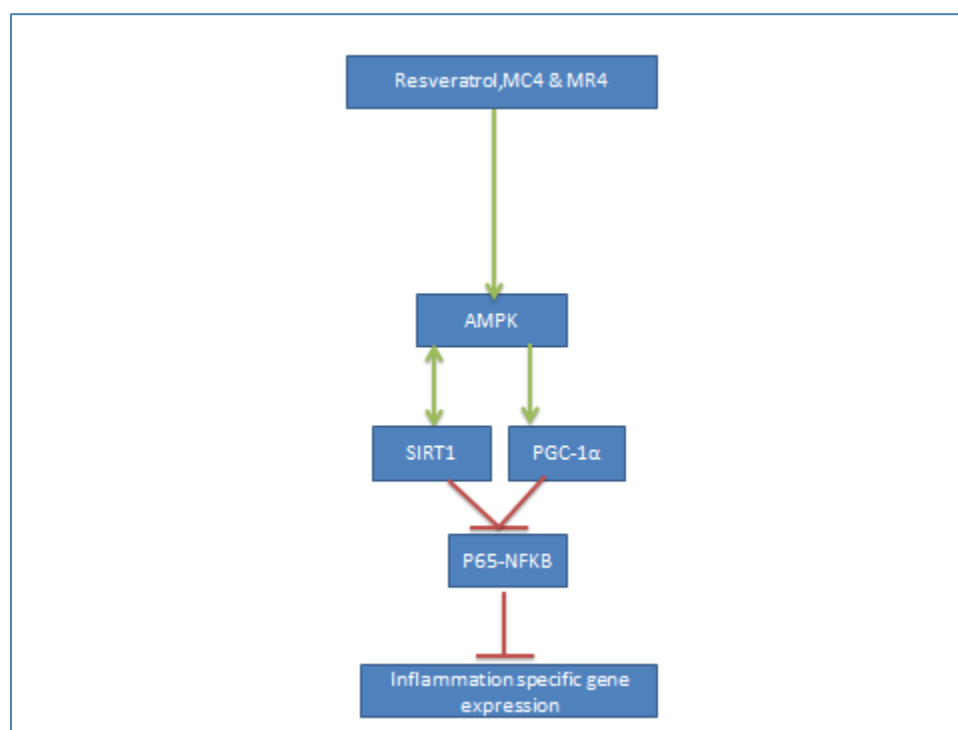


Figure 3.12: Proposed mechanism for anti-inflammatory effect of resveratrol, MC4 & MR4

Resveratrol, Arsenic, AICAR induced inhibition of inflammation is possibly mediated via activation of AMPK which leads to activation of SIRT1 and/or PGC-1 α which in-turn interact with the p65-unit of NF- κ B and down-regulate its expression (Fig. 3.12). In

consensus with this hypothesis, cytokines like TPA are known to down-regulate AMPK expression resulting in activation of the inflammatory pathways (Steinberg 2009).

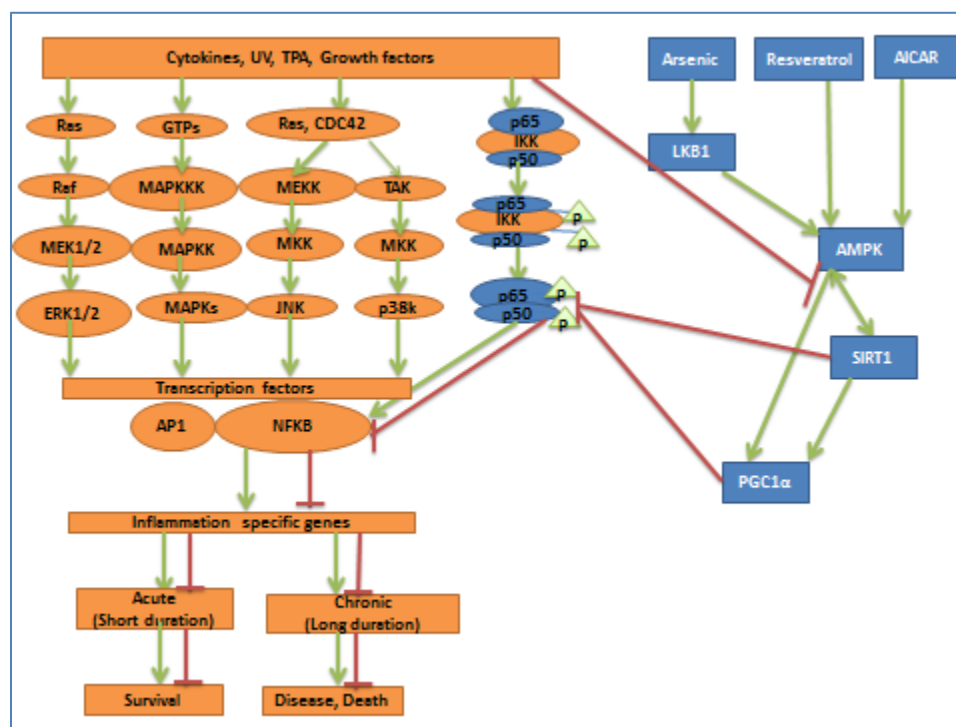


Figure 3.13: Proposed role of AMPK in the inflammation pathway

Overall, we have shown that resveratrol, MC4 and MR4 inhibit NF-κB-mediated inflammation establishing a biosimilar role for resveratrol and its analogs in this disease model. The findings that resveratrol and its analogs MC4 and MR4 show potential anti-inflammatory properties is of significance due to their potential in management of inflammatory response, especially in disease conditions. Further research is required to test the biochemical targets of resveratrol and its analogs in the inflammation pathways, particularly AMPK, followed by in vivo studies to determine bioavailability.

Nevertheless, the data presented here provides a solid foundation for future mechanistic and translation studies in inflammation management.

Conclusions

- 1) MC4 & MR4 inhibit NF- κ B mediated inflammation and offer potential for therapeutics in chronic inflammation
- 2) The possible mechanism of action of resveratrol, MC4 and MR4 on the down regulation of NF κ B is possibly mediated via activation of
 - a) AMPK, a crucial regulator of metabolic homeostasis, known to disrupt the metabolism of inflammation by activation of PGC-1 $^{\alpha}$ and/or SIRT1
 - b) PGC-1 α , a cofactor known to repress the transcriptional activity of NF- κ B by interacting with the p/65 unit of NF- κ B and/or
 - c) SIRT1, a histone and protein deacetylase known to deacetylate p/65 unit of NF- κ B leading to its inactivation

Chapter 4: Effect of chemical stimuli on neuroblastoma differentiation

Introduction:

Neuroblastoma is an early childhood tumor that is most common in infants and accounts for about 7% of all cancers in children. Its prevalence is 1 in 7000 live births and approximately 650 cases diagnosed each year in the United States (ACS). Median age of diagnosis is 18 months, so about 40% are diagnosed in the first year, 75% by age 4 and 98% by age 10 (Brodeur 2003).

Age and perhaps heredity may be considered high-risk factors for neuroblastoma, but there are no known lifestyle-related or environmental causes for this disease. Screening infants for tumor has helped detect a large number of tumors that would normally have gone undetected, however in most cases due to the non-specificity of the diagnostic methods, the outcome does not improve count of early detections or lives saved (ACS).

Existing ultrasound technology is not adequate to detect neuroblastoma pre-birth. All of these hindrances rule out preventative therapy for this disease and diagnosis occurs only when the child shows signs of illness.

Like most cancers, neuroblastoma exhibits extreme heterogeneity and the signs and symptoms vary depending on size, location of the tumor, extent of metastasis, etc. One of the sure signs is formation of a lump or mass most commonly in the abdomen. It is one of the few cancers in children that releases hormones that can cause what is collectively

known as paraneoplastic syndromes, symptoms of which include constant diarrhea, fever, high blood pressure, rapid heartbeat, flushing of the skin, sweating and a more uncommon symptom called *opsoclonus-myoclonus-ataxia*, which causes rapid eye movements, twitching muscle spasms and uncoordinated walking or standing (ACS).

Some other symptoms are of the generic variety including discomfort, pain, swelling, urination etc. which are not necessarily associated with tumor pathology. Because of which by the time neuroblastoma is diagnosed, in 2 out of 3 cases, the cancer has already metastasized.

Neuroblastomas can be diagnosed with a blood or urine test for detecting catecholamines. The nerve cells secrete this hormone which is broken down into metabolites such as homovanillic acid (HVA) and Vanillylmandelic acid (VMA). Presence of these metabolites in the urine is an indicator of neuroblastoma and further diagnosis involves imaging with an X-ray or a CT-Scan and tumor pathology confirmation via biopsy.

Neuroblastoma is essentially a mass of small, round cells called neuroblasts that have little or no neural differentiation. Neuroblastomas develop when normal fetal neuroblasts fail to become mature nerve cells, but instead continue to grow and divide.

Treatment includes stand-alone surgery, chemotherapy, radiation etc. or a combination thereof. Most of these treatment options are non-specific to the tumor type and cause an arsenal of side-effects. Additional treatment option for neuroblastoma patients is retinoid

therapy. Retinoids are chemicals that are related to vitamin A and are known inducers of neuronal differentiation which cause undifferentiated tumor cells to differentiate into normal nerve cells. Currently, this therapy is used to prevent relapse in chemo-treated patients.

Novel neuronal differentiation inducing mechanisms offer a potential for therapy in neuroblastoma. The following chapters evaluate the role of small molecules, particularly resveratrol, MC4, MR4 and Calcium for potential as inducers of neuroblastoma differentiation.

Neuroblastoma differentiation:

Neuroblastomas develop when normal fetal neuroblasts fail to become mature nerve cells, but instead continue to grow and divide. The process by which neuroblasts become nerve cells is known as neuronal differentiation and neuroblastoma is essentially a tumor resulting from the blockage of this differentiation process. There is a clear correlation between the degree of differentiation and clinical prognosis, in that a neuroblastoma with a high degree of differentiation correlates to a less aggressive tumor-type.

Differentiation therapy used for treatment of neuroblastoma involves treatment with small molecule inducers of neuronal differentiation. Various chemical inducers of neuroblastoma differentiation have been reported, however the biochemical pathway of neuroblastoma differentiation is poorly understood (Fig. 4.1). Dibutyl-c-AMP is known to induce neuronal differentiation via activation of tyrosine hydroxylase leading to

activation of pKA and PI3k resulting in neuro-specific gene expression (Prasad 1975). Calcium on the hand is known to function through activation of the phosphatidylinositol second-messenger system during neuronal differentiation (Reboulleau 1986). Retinoic acid binds to retinoic acid receptor and activates the PI3K/AKT pathway during neuroblastoma differentiation (Sidell 1982). Resveratrol (3,5,4'-trihydroxy-trans-stilbene) a natural polyphenol has been previously reported to induce neuroblastoma differentiation via AMPK activation (Dasgupta 2007). Arsenic has been shown to induce neuroblastoma differentiation by inhibiting the L1KB-AMPK pathway (Wang 2010).

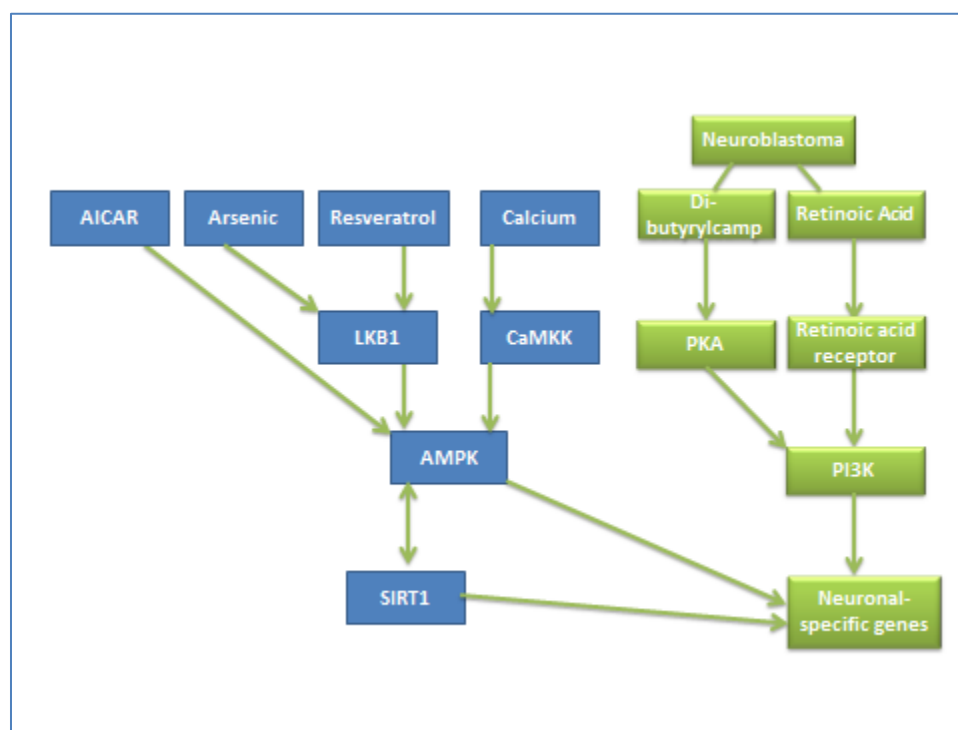


Figure 4.1: Effect of various chemical inducers on neuroblastoma differentiation

In this present thesis, we have evaluated the role of resveratrol and its analogs and calcium in neuroblastoma differentiation.

CHAPTER 4A: Role of Resveratrol and its analogs in neuroblastoma differentiation

Introduction:

Resveratrol is a polyphenol, found in grapes and has been shown to have neuroprotective benefits (Baur 2006). Our research focuses on evaluating the role of two potent analogs of resveratrol, MC4 and MR4 as inducers of neuroblastoma differentiation. MC4 and MR4 have been previously shown to possess biosimilar properties of resveratrol such as toxicity, and growth inhibition in in-vitro tumor models (Gosslau 2005). In this thesis, I evaluated the role of MC4 and MR4 in cellular differentiation systems, particularly neuroblastoma. These analogs of resveratrol offer more potent small molecules for cancer therapeutics by modulating the cell progression from proliferation to differentiation.

As previously published (Gosslau 2008), MC4 is cis-isomer of methoxylated-resveratrol and MR4 is the trans-isomer of methoxylated-resveratrol. The IC_{50} of MC4 and MR4 has been previously reported to be 20nM and 2 μ M respectively using HeLa cells for cytotoxicity assay. The potency of MC4 and MR4 in comparison to resveratrol (50 μ M) makes them an attractive drug targets for cancer therapeutics.

Dasgupta (2007) previously reported that resveratrol induced neuronal differentiation through AMPK activation. The present study aims to reconfirm the results of Dasgupta et. al's and establish the effect of novel and potent analogs of resveratrol MC4 and MR4 on neuroblastoma differentiation and establish the biosimilarity of these analogs in neuroblastoma differentiation system.

Materials and Methods:

Materials:

Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco, Grand Island, NY. Other reagents were purchased from Sigma (St. Louis, MO).

Tissue culture:

Mouse neuro-2a neuroblastoma cells were cultured in Dulbecco's medium containing 10% fetal bovine serum. For proliferation assay, cells were plated at 2×10^5 cells per 35-mm dish in the presence of various chemicals. Cell growth was measured by counting viable cells using the Trypan Blue dye exclusion. For differentiation induction assay, cells were plated in 35-mm dish at 2×10^5 cells/ml with or without added chemicals. After 4 or 5 days, cells were fixed with 5% of trichloroacetic acid and stained with Bacto Gram Crystal Violet solution (Difco, Detroit, MI). Differentiation of neuro-2a cells was monitored by morphological appearance of neurite outgrowth.

Phase contrast Imaging:

Phase contrast images were captured at 10X10 magnification.

Results:

As shown in Fig. 4.2, neuro-2a cells when treated with small molecule inducers such as dibutryl-c-AMP (1mM), exhibit growth arrest as evident by the low cell density and these growth arrested cells produce long neurite-like outgrowths characteristic of differentiation.

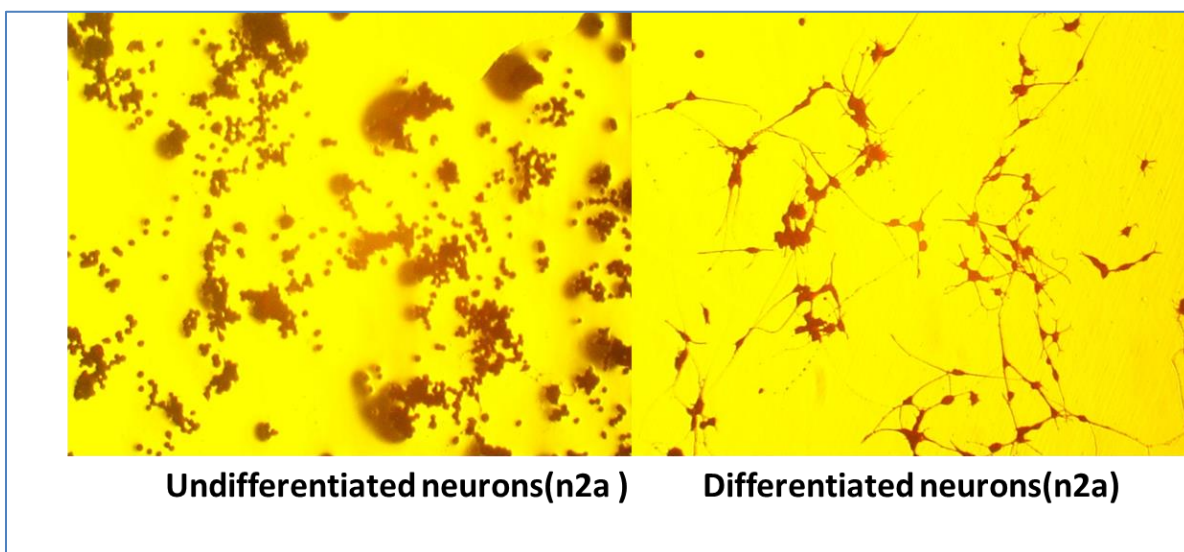


Figure 4.2: Morphology of Differentiated Vs. Undifferentiated Neurons

In order to test the effect of resveratrol and its analogs in neuronal differentiation, a viability assay was carried out to determine the IC_{50} of these chemicals in neuro-2a cells under proliferation and differentiation conditions. A single dose of resveratrol at 25 μ M, MC4 at 2.5nM and MR4 at 2.5 μ M was maintained throughout the period of differentiation (7 days). It was determined that the stilbene analogs at the above mentioned concentrations were toxic to the cells as evident by the Crystal violet assay, neuro-2a cells died under both proliferation and differentiation conditions (Fig. 4.3)

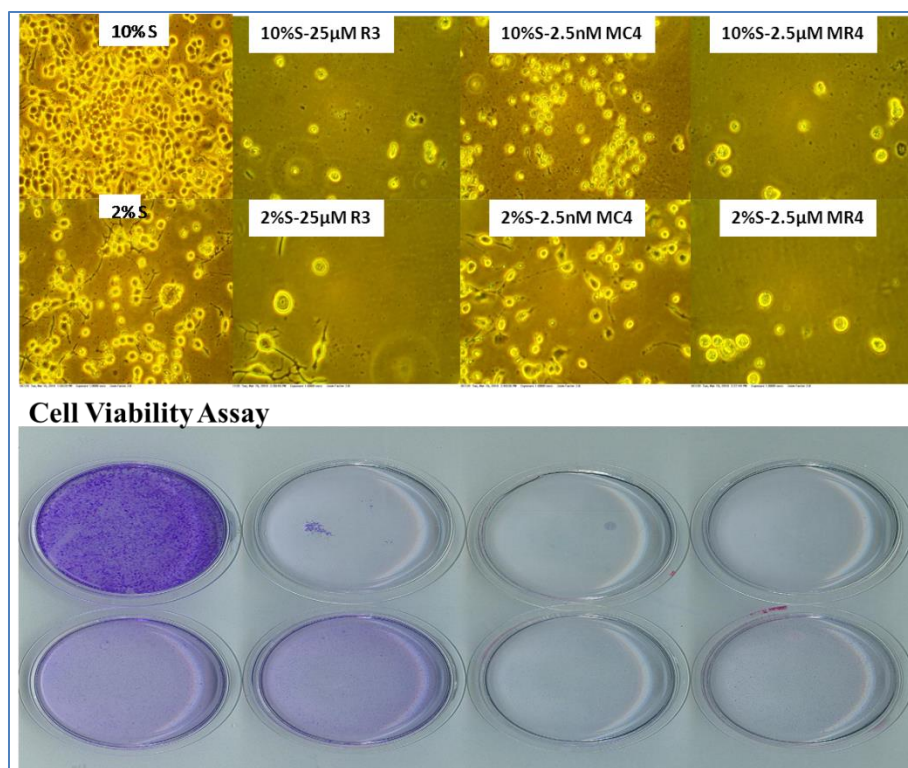


Figure 4.3: Viability assay of resveratrol and its analogs in neuro-2a cells

A more systematic dose-response study was carried out to determine the IC_{50} on resveratrol, MC4 and MR4 in neuro-2a cells under proliferation and differentiation conditions.

Dose-response effect of Resveratrol on neuroblastoma cell differentiation

During the course of the dose-response assay using neuro-2a cells, we noticed that the presence of resveratrol in the culture appeared to promote neurite outgrowth, a hallmark of neuroblastoma cell differentiation. Since Bt_2cAMP is a commonly used reagent for mouse neuroblastoma differentiation, we compared the effects of resveratrol at different concentrations with that of Bt_2cAMP on the cell morphology of neuro-2a cells. As shown in Fig. 4.4, resveratrol alone at 5μM concentration was sufficient to generate an extensive

web of neurite outgrowth. The efficacy of resveratrol in eliciting the morphological change in mouse neuroblastoma cells was comparable to that of Bt_2cAMP .

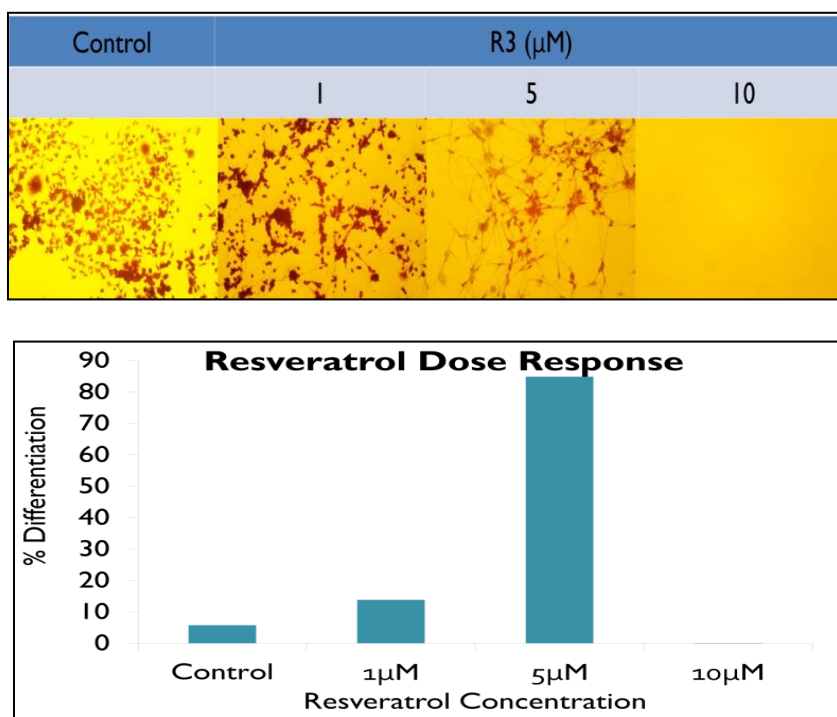


Figure 4.4: Dose response study of Resveratrol in neuronal differentiation

Effect of MC4 and MR4 on the differentiation of neuroblastoma cells

Fig. 4.4, shows that resveratrol at 5 μM caused more than 90% of cells in the culture to generate long neurite outgrowth (>50 μm). The potency of resveratrol in inducing the morphological differentiation of neuro-2a cells prompted us to test the effects of other resveratrol derivatives MC-4 (IC_{50} -20nM) & MR-4 (IC_{50} -2 μM) on neuroblastoma cell differentiation.

Neuro-2a cells were treated with MC4 under differentiation conditions at a dose interval of 1-9nM to evaluate the effect of MC4 on differentiation alone. As shown in Fig. 4.5, MC4 was toxic at a concentration of 7nM and above. However at a lower concentrations, between 3-5nM, the cells exhibited neurite outgrowth.

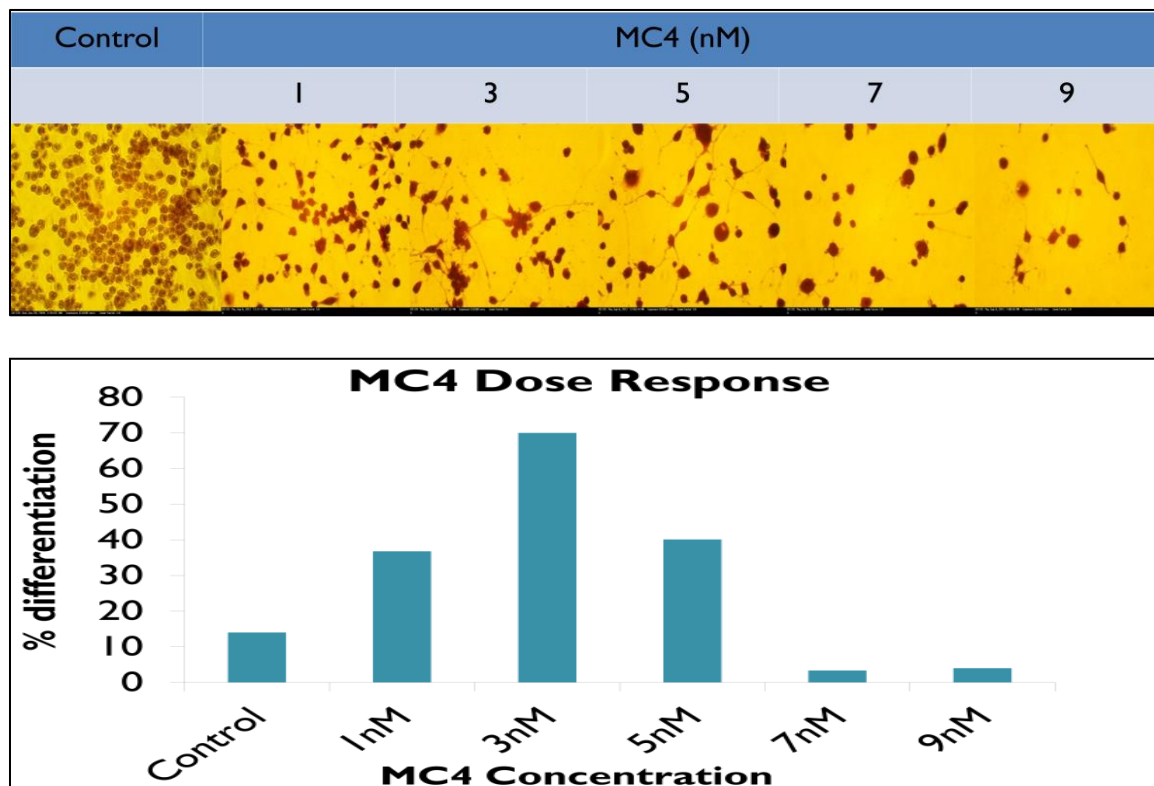


Figure 4.5: Dose response study of MC4 in neuronal differentiation

MR4 induced neuronal differentiation was evaluated by conducting a systematic dose response study (Fig. 4.6). MR4 was toxic at 100nM and above, this concentration was well below the known IC_{50} of MR4 which was previously observed to be at 2 μ M (Gossiau 2005). Neuronal differentiation was observed at a concentration range of 10-50nM as evident by a network of neurite outgrowth.

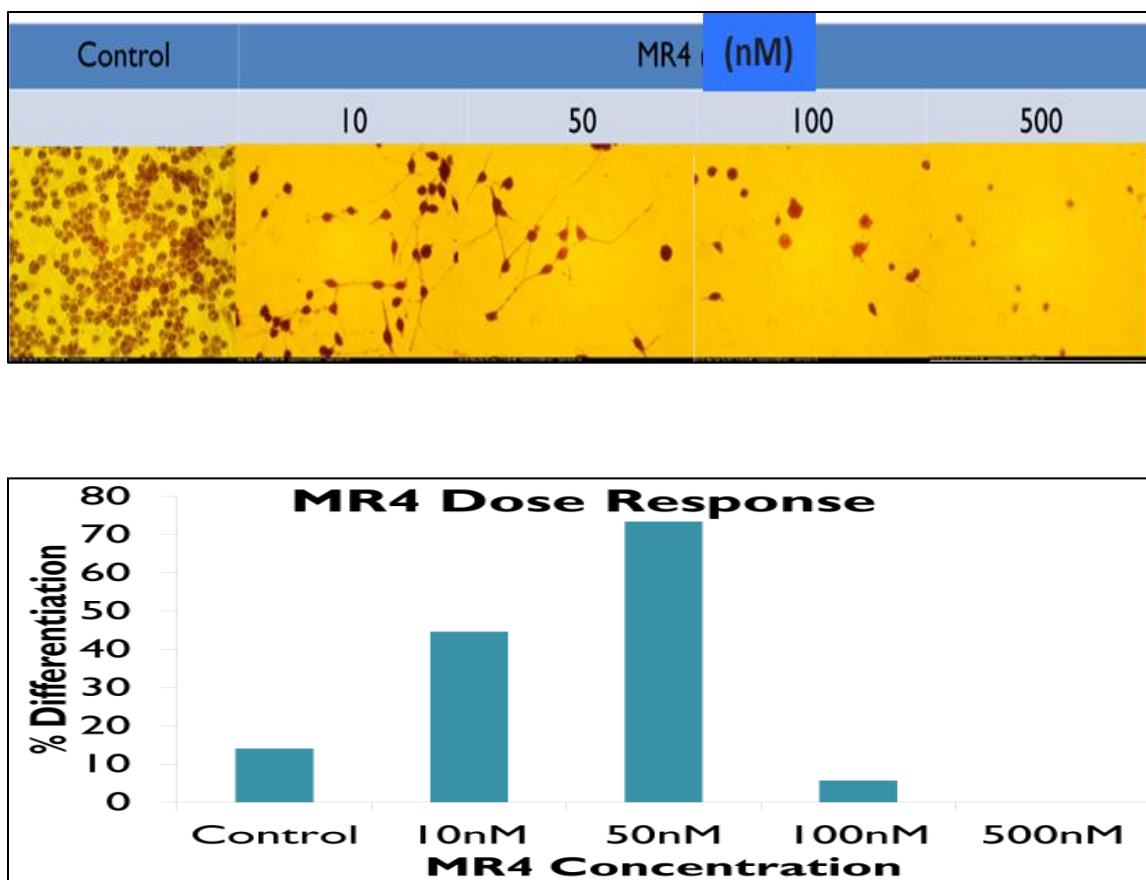


Figure 4.6: Dose response study of MR4 on neuronal differentiation

Effect of Nicotinamide on neuronal differentiation:

Resveratrol is a known activator of SIRT1 (Borra 2005) and in the light of this evidence, we evaluated the role of nicotinamide on neuronal differentiation. Nicotinamide is a small molecule inhibitor of SIRT1 and our hypothesis was that if resveratrol induced neuronal differentiation by activating SIRT1, then the treatment of neuro-2a cells with nicotinamide would inhibit differentiation and promote growth of neuroblastoma. We conducted a systematic dose response study of the effect of nicotinamide in neuro-2a cells (Fig. 4.7). Intriguingly, nicotinamide induced neuronal differentiation at

concentrations between 1-2mM. It inhibited growth at 5-10mM and was toxic above 10mM indicative of a lack of SIRT1 involvement in neuroblastoma differentiation.

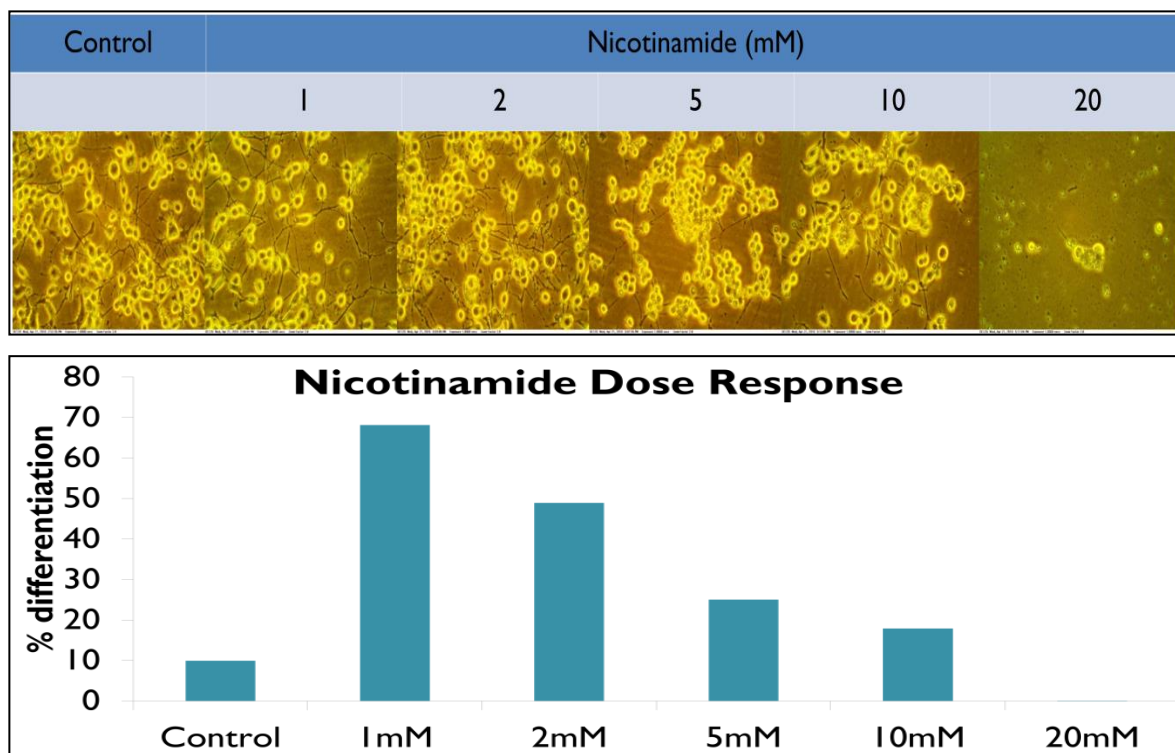


Figure 4.7: Dose response of Nicotinamide on neuronal differentiation

Discussion:

Resveratrol and its potent analogs MC4 and MR4 induced differentiation of neuroblastoma cells as evident from Figs. 4.4, 4.5 and 4.6. Interestingly the concentrations at which the stilbene analogs (R3, MC4 and MR4) were toxic to neuronal cell was well below the established IC_{50} (Gossiau et. al, 2005) refer Table 4.1.

	IC ₅₀ (HeLa, Cervical cancer) (Gossiau et. al. 2005)	IC ₅₀ (neuro-2a, neuroblastoma)	Differentiation inducing conc.
R3	50µM (10-fold)	10µM (2-fold)	5µM
MC4	2µM(1000-fold)	8nM (4-fold)	2nM
MR4	20µM (100-fold)	100nM (2-fold)	50nM

Table 4.1: Comparison of IC₅₀ values for differentiation inducing conc. of resveratrol, MC4 and MR4

Overall there is increased sensitivity to stilbene analogs in neuroblastoma cells as compared to cervical cancer (Table 4.1). This phenomenon could imply one of the two things: either neuroblastoma cells are 10-1000 fold more sensitive to stilbene analogs, which offers a huge therapeutic advantage in using R3, MC4 and MR4 for differentiation therapy in neuroblastoma mainly because it leads to lower dosage, decreased side effects and an overall reduction in cost of treatment.

However, the observed sensitivity in neuroblastoma cells could possibly be a function of the complex differentiation protocol. As described in the methods section, induction of neuronal differentiation involves a combination of growth arrest and external activator or the differentiation pathway. Growth arrest is achieved by lowering the serum concentration (from 10% to 2%) which reduces growth factors available for proliferation and a highly low seeding density (1:50 dilution) which inhibits the crucial cell-cell

contact essential for proliferation. These variations in the cell environment make proliferation difficult and upon receiving external signal to differentiate, they commit to the differentiation pathway. The reduced serum conditions and chemical stress in the form of stilbene analogs makes the cells more sensitive compared to cervical cancer cells.

Comparing the toxicity and functional (differentiation inducing) concentration range, it was evident that there is a very narrow window (between 2-8 fold) that allows the cells to undergo differentiation versus cell death. This observation strengthens the idea that stilbene analogs have multiple downstream targets and activation of these targets is a function of dosage.

Comparing the effect of R3 to its potent analogs, MC4 and MR4 in neuronal differentiation we reach the conclusion that agrees with the previous studies that have established bio-similarity between these molecules (Chapter 2). Resveratrol and its analogs induce neuronal differentiation in much the same manner as MC4 and MR4, although at a much lower concentrations. This suggests that the biochemical target of these molecules is possibly the same given how closely related they are in structure (Table 4.2).

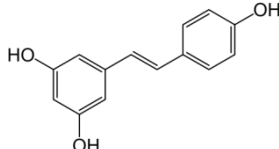
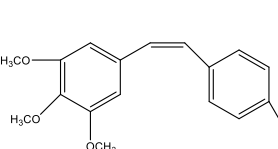
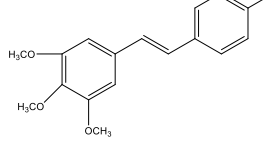
Molecule	R3	MC4	MR4
Chemical Name	3,5,4'-trihydroxy- trans-stilbene	3,4,5,4'-tetramethoxy- cis-stilbene	3,4,5,4'-tetramethoxy- trans-stilbene
Structure			

Table 4.2: Structure of Stilbene analogs

The cis- vs trans-stereochemistry of MC4 and MR4 does not affect the activation of biochemical targets required for neuronal differentiation, disqualifying a receptor-ligand binding mechanism of action.

One of the well-known targets of resveratrol is SIRT1 and we tested the effect of inhibition of SIRT1 on neuronal differentiation. Neuro-2a cells were treated with nicotinamide, a non-specific product inhibitor of SIRT1. The hypothesis being that if SIRT1 is involved in neuroblastoma differentiation, inhibition of SIRT1 by nicotinamide would result in inhibition of neuronal differentiation. As evident from the results of the dose response study of nicotinamide (1-2mM) treated neuro-2a cells continued to undergo neuronal differentiation (Fig. 4.7) suggesting a lack of SIRT1 involvement in neuronal differentiation. This finding is in agreement with the results of Dasgupta et. al,

(2007) that showed that resveratrol mediated neuronal differentiation was independent of SIRT1 activation and possibly mediated via activation of AMPK. It would be interesting to evaluate whether MC4 and MR4 function through activation of AMPK in neuronal differentiation. These potent analogs of resveratrol offer exciting therapeutic potential, especially due to the findings that they are biosimilar to resveratrol and better potency.

Conclusions:

- 1) Resveratrol, MC4 and MR4 effectively inhibit proliferation and induce neuroblastoma differentiation
- 2) Of all the molecules tested, MC4 was the most effective in achieving neuroblastoma differentiation (3nM)

Chapter 4B: Effect of Ca^{2+} ions on neuroblastoma differentiation

Introduction

As established by Dasgupta (2007), resveratrol promotes neuronal differentiation by activating the AMPK pathway; it was eminent to look for other small molecule modulators of AMPK to reconfirm the involvement of AMPK pathway in neuroblastoma differentiation. Calcium is a known modulator of AMPK pathways and moreover, a previous study (Reboulleau 1986) showed that Calcium ions induce neuroblastoma differentiation at a concentration of 20mM.

This study aims to reconfirm the effect of calcium ions on neuroblastoma differentiation and further explore time course of external calcium exposure required to induce non-reversible differentiation of neuroblastoma. Another objective is to evaluate the role of calcium in combination with other small molecules such as resveratrol and its analogs for potential therapy. This will provide insights into the role of calcium in neuroblastoma differentiation and offer potential therapeutic use for calcium in neuroblastoma differentiation.

Materials & Methods

Materials:

Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco, Grand Island, NY. Other reagents were purchased from Sigma (St. Louis, MO).

Tissue culture:

Mouse nuero-2a neuroblastoma cells were cultured in Dulbecco's medium containing 10% fetal bovine serum. For proliferation assay, cells were plated at 2×10^5 cells per 35-mm dish in the presence of various chemicals. Cell growth was measured by counting viable cells using the Trypan Blue dye exclusion. For differentiation induction assay, cells were plated in 60-mm dish at 2×10^5 cells/ml with or without added chemicals. After 4 or 5 days, cells were fixed with 5% of trichloroacetic acid and stained with Bacto Gram Crystal Violet solution (Difco, Detroit, MI). Differentiation of neuro-2a cells was monitored by morphological appearance of neurite outgrowth.

Phase contrast Imaging:

Phase contrast images were captured at 10X10 magnification.

Western blot analysis:

This procedure was carried out as described for detection of SIRT1 and actin. For analysis, cells were washed and scraped into 1 ml of PBS. After sonication, the homogenate was exposed to centrifugation at 14,000x g for 5 min. The supernatant was saved and protein concentration was determined by the Bradford method from Bio-Rad

(Hercules, CA). Whole cell extracts (5 μ g) were resolved on a 15% SDS-PAGE system and transferred onto a PVDF membrane using a Trans-Blot Electrophoresis Transfer Cell at 370 mA for 1.5 h. Non-specific protein-binding sites on the membrane were blocked by incubation with 5% (w/v) non-fat dry milk in PBSTween20 and probed with primary anti-SIRT1 antibody (1:500 dilution) overnight at 4°C. The membranes were washed in PBSTween20, followed by incubation with secondary antibody at 1:25,000 dilution. Primary polyclonal antibody against recombinant human SIRT1 was raised in chicken and affinity-purified by recombinant SIRT1 proteins. The secondary rabbit against chicken antibody conjugated to horseradish peroxidase (HRP-RAC) was from Amersham Pharmacia (Piscataway, NJ).

Semi-quantitative RT-PCR:

Reverse-transcription polymerase chain reaction (RT-PCR) was employed to monitor the expression of SIRT1 gene. The treated cells were harvested 5 days after plating. Total RNA was prepared using RNeasy Kit (Qiagen, Chatsworth, CA), reverse transcribed into cDNA by SuperScript™ RNase H reverse transcriptase (Gibco BRL) using oligo (dT) 12±18 as primer. For PCR amplification, gene specific primers were designed. The sequences of the sense and anti-sense primers for various genes were: Sense: TGGATGATATGAACGCTGTGGCAGA

Antisense: AGAGGTGTTGGTGGCAACTCTGAT. PCR was performed under conditions where the yield of the amplified product was linear with respect to the amount of input RNA. The PCR products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

Results:

Calcium specifically induces neuronal differentiation:

Calcium chloride was added to proliferating (10% FBS) and differentiating (2% FBS) neuro-2a cells at 10mM and 20mM concentration. As shown in figure 4.8, external calcium is not toxic to neuro-2a cells at the maximum concentration tested (20mM) and in the presence of reduced serum medium and 10mM calcium chloride; the neuroblastoma cells exhibit an extensive network of neurite outgrowth.

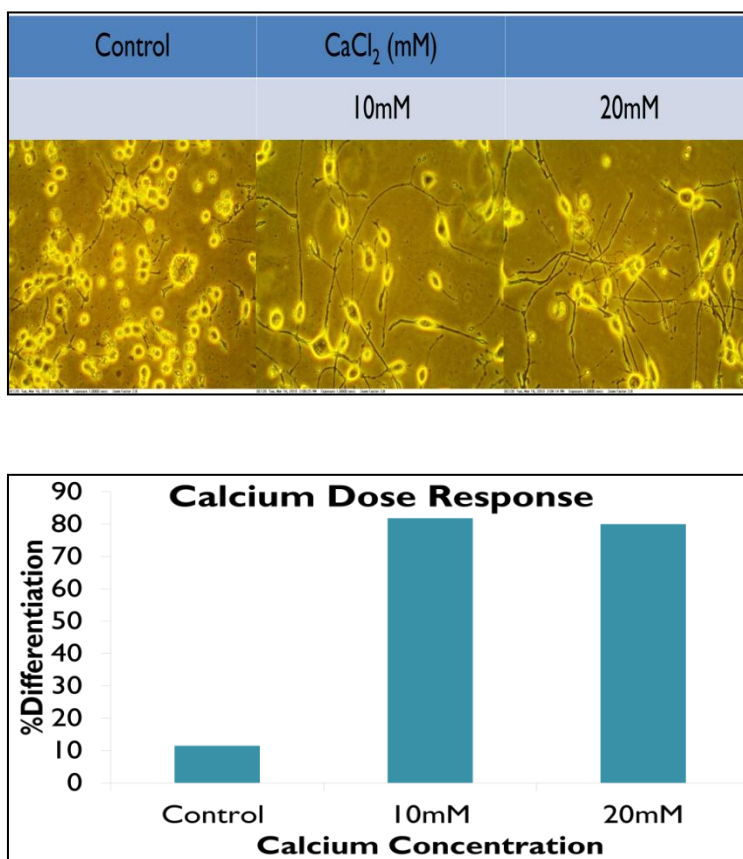


Figure 4.8: Dose response and viability assay of Calcium chloride on neuroblastoma differentiation

In order to establish a robust neuronal differentiation system, I designed a systematic experiment to study the effect of varying serum (1-10%) and calcium chloride (5-20mM) concentration (Fig. 4.9). Dibutyryl-c-AMP was used as a control to compare the effects of calcium chloride on neuronal differentiation. It was observed that 10mM Calcium chloride at 2% serum concentration was optimum for inducing neurite outgrowth in neuro-2a cells. Crystal viability assay was performed to demonstrate cell viability (Fig.4.10).

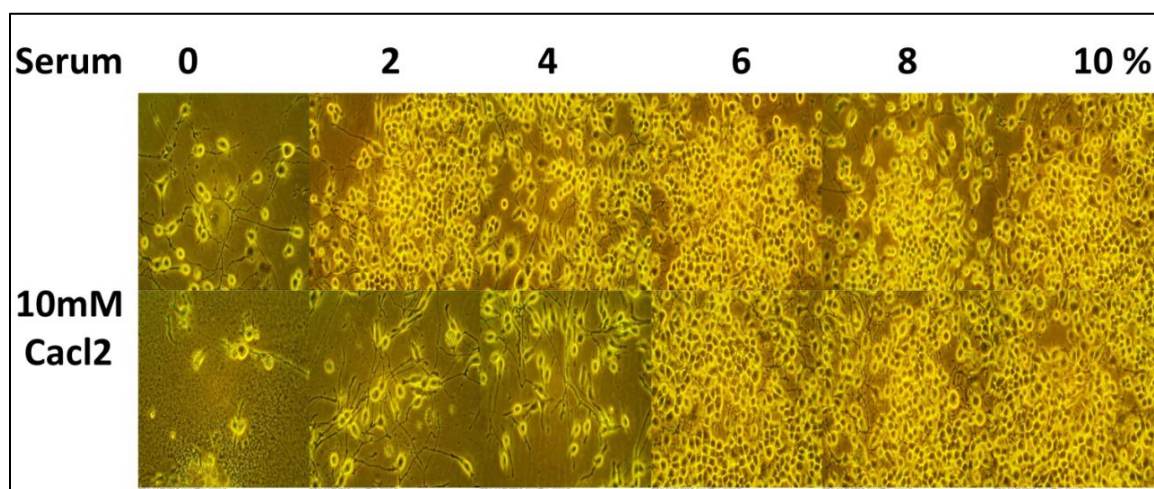


Figure 4.9: Establishing a robust model for calcium induced neuroblastoma differentiation by varying serum concentration

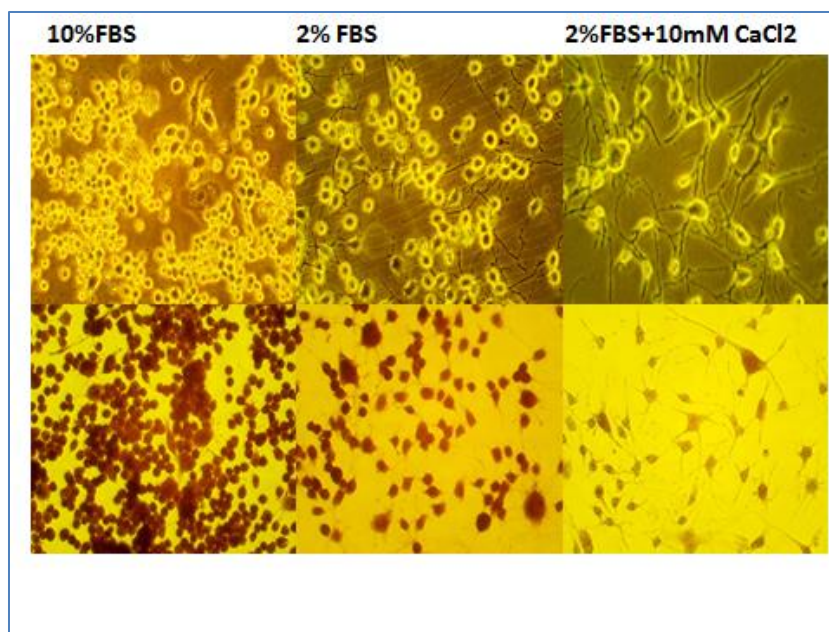


Figure 4.10: Crystal Violet Viability assay for calcium induced neuroblastoma differentiation

Calcium specifically induces neuroblastoma differentiation

It is valid to ask the question as to whether the effect of calcium is specific to neuronal differentiation or is it a function of its divalent metallic ion-like properties. In order to test this hypothesis, we evaluated the effect of Zn and Cu in the form of Zinc chloride and copper chloride to establish 1) specificity of calcium and 2) rule out chloride ion as an inducer of differentiation. As shown in Fig. 4.11, Zn and Cu failed to induce differentiation of neuroblastoma cells confirming our hypothesis that calcium induced differentiation of neuronal cells involves activation of differentiation pathway specifically by calcium.

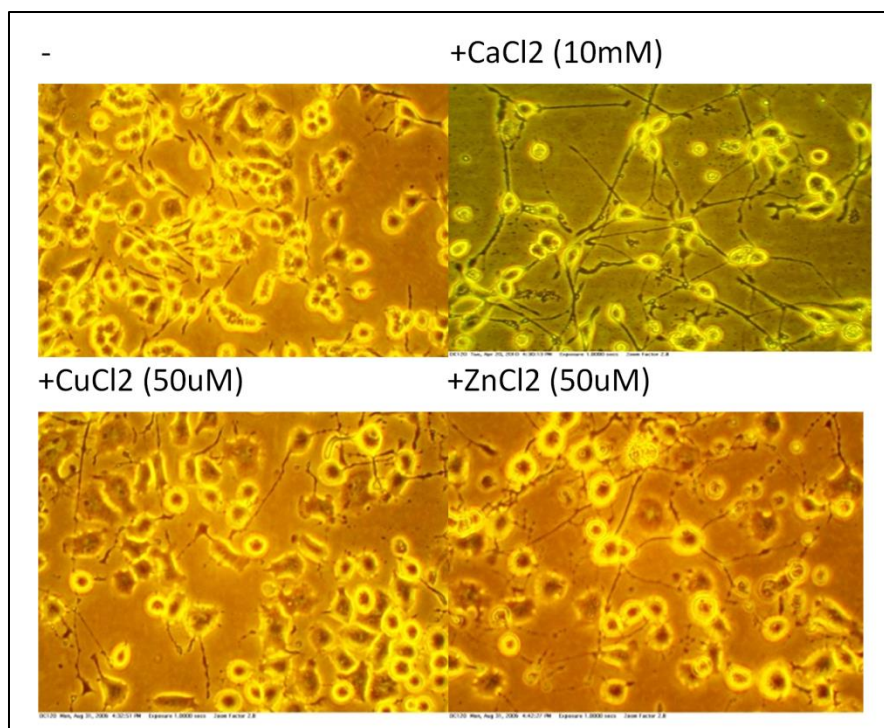


Figure 4.11: Effect of other divalent ions (Cu & Zn) on neuroblastoma differentiation

A constant external source of calcium is required for neuronal differentiation

We further evaluated the minimal exposure time required for calcium induced neuronal differentiation by removing Calcium chloride and replacing with fresh reduced serum medium and allowing the cell to remain in culture. Calcium chloride exposure was varied from 1 Day (24 hrs.) to 7 Days (168 hours) (Figs. 4.12 A & B). A viability assay was carried out at the end of differentiation period (7 days). It was very intriguing to discover that a constant external source of calcium ions was eminent for neurite outgrowth. Neuro-2a cells failed to differentiation if the constant calcium source was depleted and replaced with reduced medium serum.

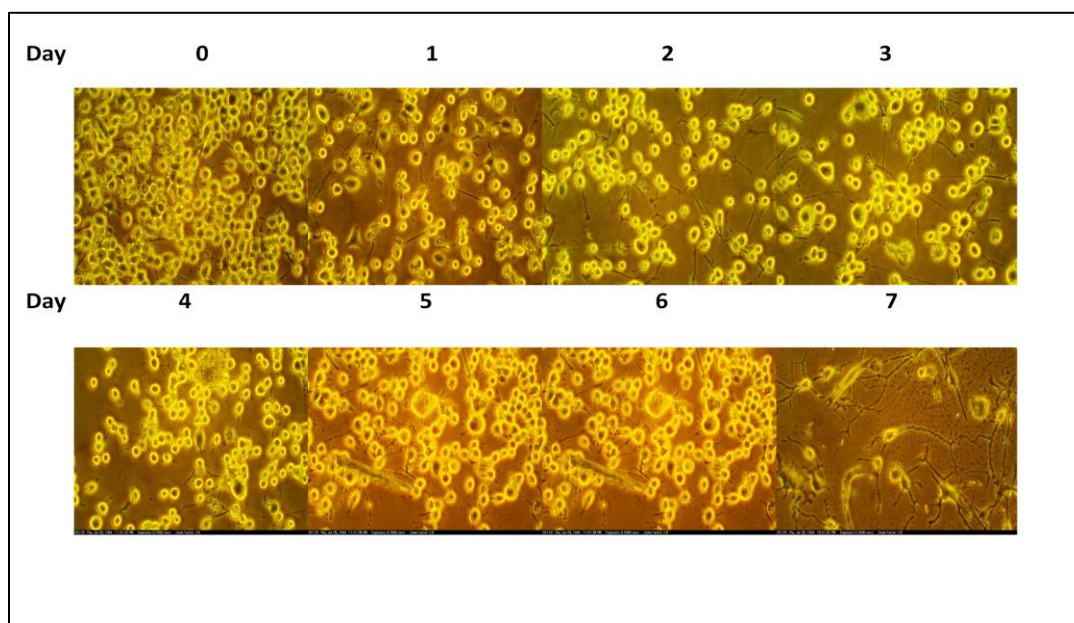
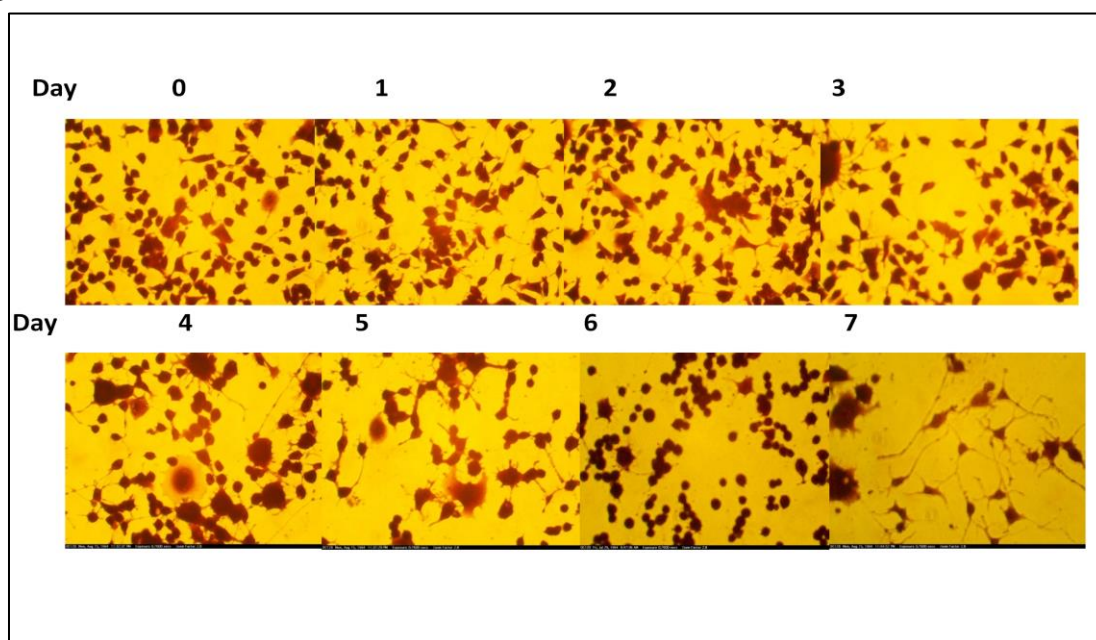
A**B**

Figure 4.12: Time course study to evaluate the effect of calcium chloride on neuronal differentiation, 4.12A: Morphology, 4.12 B: Crystal violet viability assay

Combinatorial effects of stilbene polyphenols and Calcium on neuronal differentiation

Chemotherapy approaches involve combining a complex dose regime for effective and aggressive targeting tumors. In light of the data obtained in the previous chapter where I reported stilbene analogs (Chapter 2A) induced neuronal differentiation and this current study also supporting calcium as an inducer of neuroblastoma differentiation, I designed a study to achieve synergist/additive effect of these small molecules with calcium.

Adding 5 μ M resveratrol in combination with 10mM calcium chloride seemed to increase the number of neurite outgrowths by morphological observation (Fig. 4.13) but the difference was not significant to confirm synergistic/additive effect. MC4 and MR4 showed no synergistic/additive effect with calcium ions on neuronal differentiation (Fig. 4.14 & Fig. 4.15).

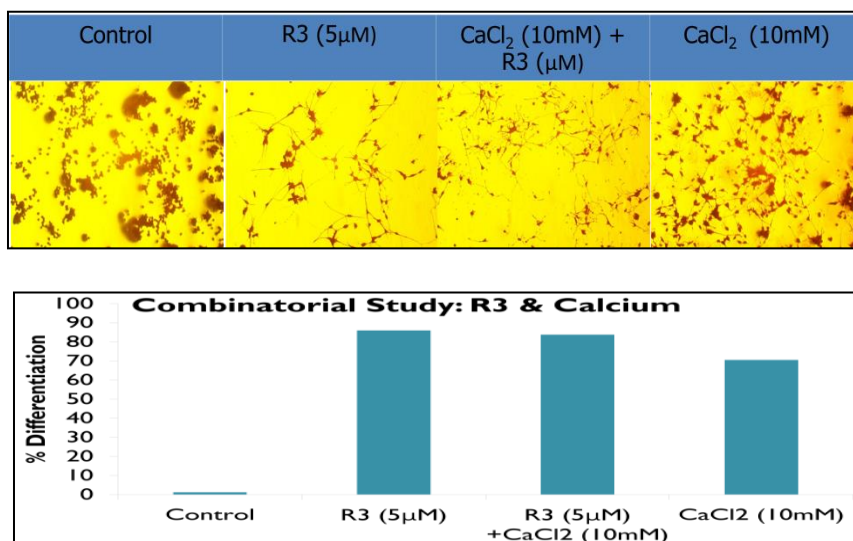


Figure 4.13: Combinatorial effect of resveratrol and Calcium on neuroblastoma differentiation

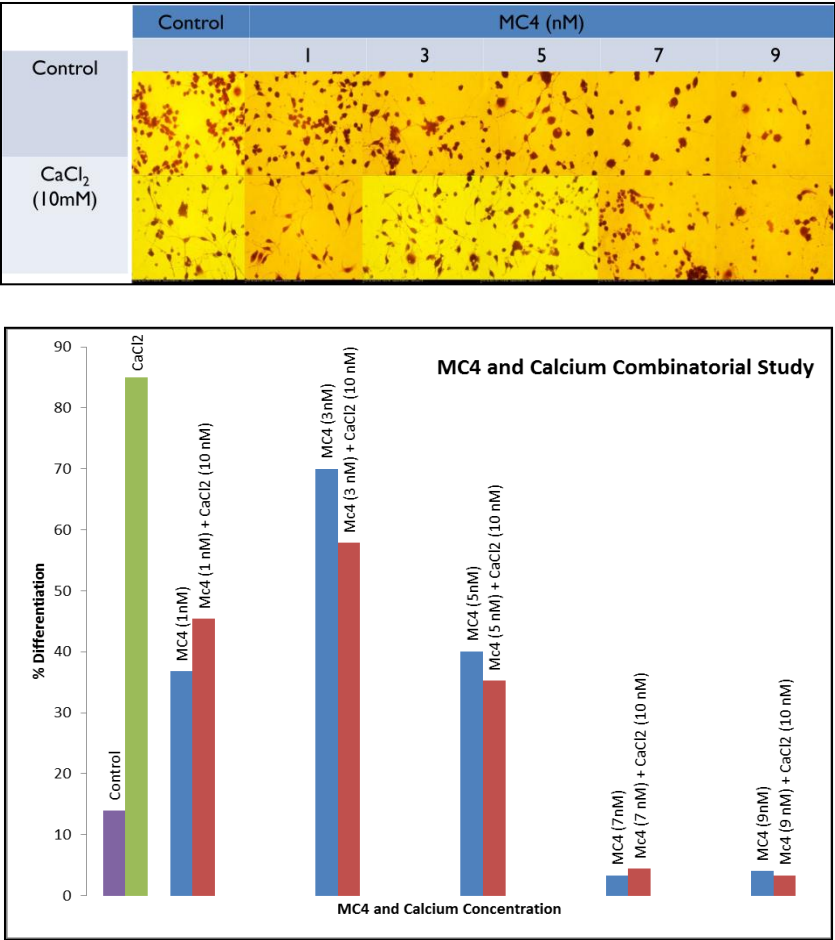


Figure 4.14: Combinatorial dose response study of MC4 and calcium on neuroblastoma differentiation

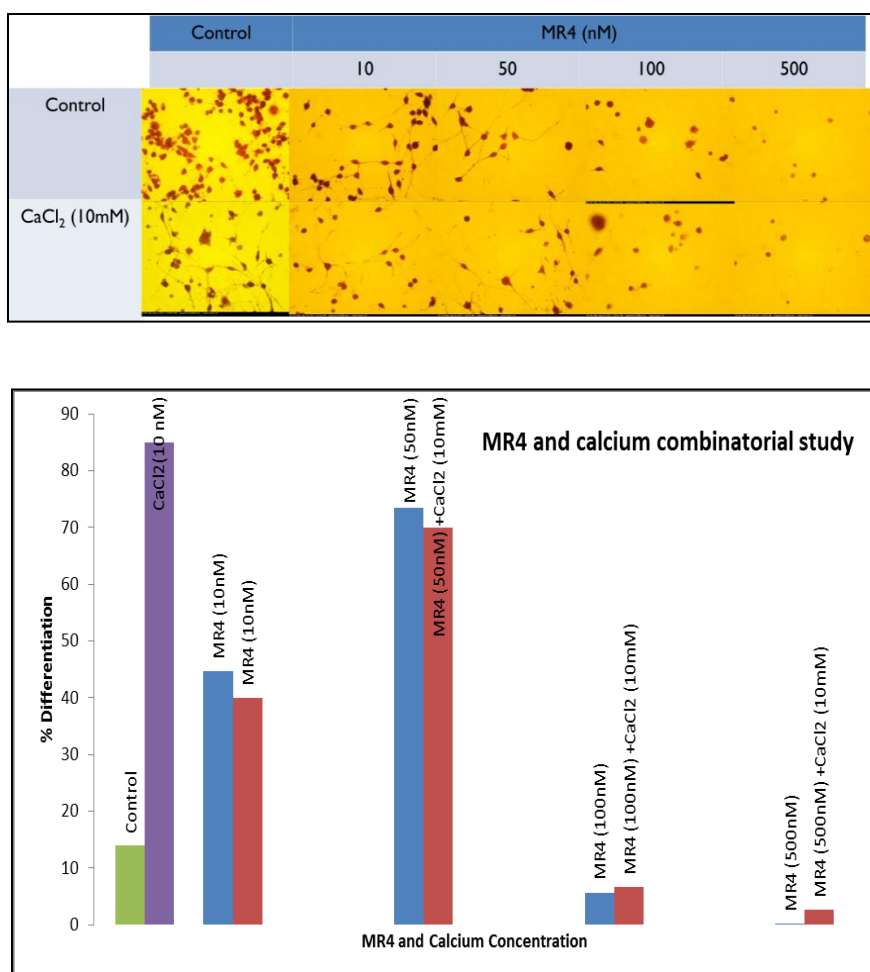


Figure 4.15: Combinatorial dose response study of MR4 and calcium on neuroblastoma differentiation

Biochemical analysis of calcium induced differentiation

Resveratrol has been shown to be an activator of SIRT1 (Baur 2006). Based on the combinatorial studies performed in this thesis (Fig. 4.13, 4.14 & 4.15) we interpreted that resveratrol and calcium possibly activate a similar pathway in neuronal differentiation. I tested this hypothesis by conducting western-blot analysis of samples of undifferentiated and differentiated (calcium induced) neuro-2a cells to detect SIRT1 protein. As shown in Fig. 4.16 and 4.17, samples of neuro-2a cells harvested, lysed and analyzed post 24 hours of treatment as follows-Lane 1 (growth medium), Lane 2 (reduced serum medium) and

Lane (calcium supplemented reduced serum medium). Actin was used as a housekeeping gene. Western blot analysis of SIRT1 (Fig. 4.16), visible as a band at ~150 KDa and we notice an increased expression of SIRT1 in Lane 2 (reduced serum medium), this collaborates well with the existing literature that shows SIRT1 is activated during calorie restriction (Canto & Auwerx 2009). However there was very little to no expression of SIRT1 in lane 3 (calcium supplemented reduced serum medium). RT-PCR analysis of SIRT1 in lane 3 (calcium supplemented reduced serum medium). RT-PCR analysis of SIRT1 (Fig. 4.17) corroborates the results in Fig. 4.16 indicating the absence of a role of SIRT1 in neuronal differentiation. Together this implies that calcium induced differentiation was independent of SIRT1 activation.

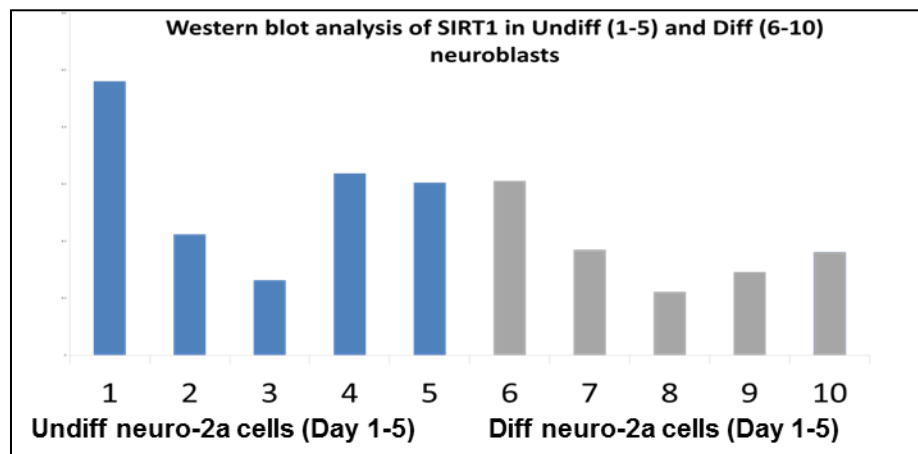
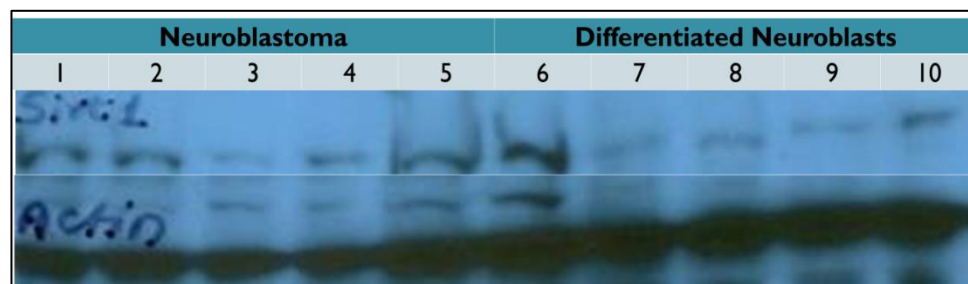
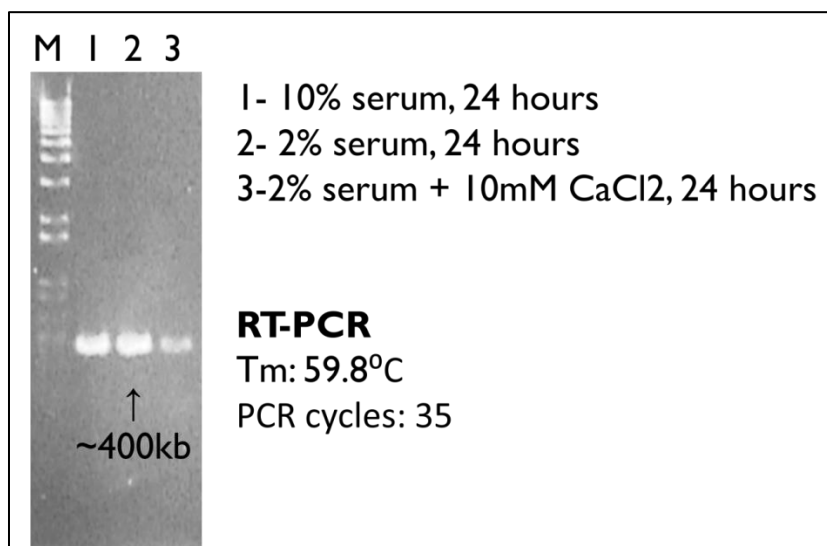


Figure 4.16: Western blot analysis of SIRT1



Samples of neuro-2a cells harvested, lysed and analyzed post 24 hours of treatment as follows-
 Lane 1 (growth medium),
 Lane 2 (reduced serum medium) and
 Lane 3 (calcium supplemented reduced serum medium).
RT-PCR Conditions:
 T_m: 59.8°C
 PCR cycles: 35

Figure 4.17: RT-PCR analysis of SIRT1

Discussion:

Specificity of Calcium induced differentiation of neuro-2a cells

Calcium induced differentiation of neuroblastoma was observed by Reboulleau (1986) using rat CNS neuroblastoma cell line (B50) and this was reassessed by systematic dose-response, viability and time-course studies conducted using a different in-vitro neuroblastoma model, neuro-2a (mouse). The results of this study were congruent to the findings of Reboulleau (1986) indicating that extracellular calcium induced

differentiation of neuroblastoma cells characterized by the presence of extensive neurite outgrowths.

Lacking in Reboulleau's study was evidence for the specificity of calcium as an inducer of neuronal differentiation although he reported previously published citations of calcium dependent differentiation processes.

It was eminent for our study to determine the specificity of calcium ions in inducing neuronal differentiation, by eliminating the effect of chloride ions and using other divalent cations. This was achieved by addition of Zinc chloride and Copper chloride to neuro-2a cells in culture and evaluated for differentiation potential. It was concluded that both Zinc chloride and Copper chloride were incapable of neuronal differentiation as evidence by the lack of neurite processes. This established the specificity of calcium ions in inducing neuro-2a differentiation.

A constant external source of calcium is required for neurite outgrowth in calcium induced differentiation of neuro-2a

I further determined that a constant source of calcium ions in the differentiation medium throughout the differentiation period (7 days) was crucial for neuronal differentiation without which the cells failed to develop neurite extensions and continued to proliferate. This discovery was highly unusual because the current understanding of differentiation models is that differentiation occurs through differential gene expression resulting from extrinsic or intrinsic signals within a cells environment. However once the cell is committed to a differentiation fate and activated the differential gene expression, it

continues to undergo terminal differentiation in the absence of these signals (reference). However it was highly unusual to notice that an external source of calcium ions was required throughout the entire course of differentiation.

Possible rationale and Implications of the stringent requirement for presence of calcium source throughout the differentiation period (7 days)

A possible explanation for this peculiar phenomenon could be that calcium was required for two or more early to late stages of differentiation, disqualifying the notion that neuronal differentiation induced by calcium was an inducer driven cascade of biochemical pathway leading to terminal differentiation. It appears calcium is involved in more than one independent biochemical pathways essential for neuronal differentiation. This hypothesis is supported by similar timing-dependent effect of NGF (neuronal growth factors) on PC12 (neuronal differential model) differentiation where discontinuous stimulation of NGF was required for neurite extension (Chung 2010).

I have established that calcium is required during the later stage of neuronal differentiation. However the question remains whether calcium is required during the early stages of differentiation. This could be easily tested by introducing calcium at a later stage of differentiation (Day 5-7 after addition of reduced serum medium) and monitoring the effect on neurite outgrowths. Results from this proposed experiment, would determine where calcium is required during early stages of differentiation. Another interesting experiment would involve depleting calcium in differentiated neurons and monitoring the effect on existing neurite outgrowths. Considering how differentiation is

a terminal event, it would be highly unusual to notice retraction of neurite extensions and reversion back to progenitor cells which in this case would be neuroblastoma cell type.

Possible mechanism action of calcium induced neuronal differentiation

IP₃

Reboulleau concluded that activation of phosphatidylinositol (IP₃) second messenger system by calcium through the cell membrane is an initial step in a cascade of events involved in neuronal differentiation. However in the light of new findings in this thesis, that implicate a role for calcium during later stages of differentiation, it is plausible that calcium activates more than one biochemical pathway to induce neurite extensions in neuroblastoma.

SIRT1

I evaluated the effect of SIRT1 on calcium induced differentiation through biochemical analysis of SIRT1 (Western-blot, Semi-quantitative PCR). As evidenced by the data (Fig. 4.16 & 4.17) and results of Dasgupta (2007), SIRT1 a known biochemical target of resveratrol, the calcium induced differentiation of neuroblastoma was independent of SIRT1 activation.

AMPK

As detailed in the previous chapter, resveratrol induced neuronal differentiation involves activation of AMPK (Dasgupta 2007). It remains to be seen whether AMPK is a target of Calcium induced differentiation of neuroblastoma.

Combinatorial studies of calcium and resveratrol analogs:

Cancer therapeutics utilize an aggressive, combinatorial approach to maximize efficacy of treatments available such as a regime of chemotherapy in combination with radiation therapy for a multi-targeted attack on tumors. As demonstrated in previous chapter (2A), resveratrol and its analogs were inducers of neuronal differentiation much like calcium induced differentiation of neuro-2a. I conducted combinatorial studies to determine any potential synergistic role for resveratrol and its analogs (MC4 and MR4) with calcium on neuronal differentiation. The purpose of this study is twofold: 1) to determine if the stilbene analogs and calcium have a similar downstream biochemical target which would be evident by the lack of any synergistic effect and 2) to develop a multi-drug regime for effective differentiation of neuroblastoma achieved by targeting multiple biomolecules in the differentiation pathway which would be evident from the synergistic effect of stilbene analogs and calcium.

I have independently reconfirmed the neuronal differentiation inducing effect of resveratrol and calcium using the in-vitro cell model: neuro-2a, which is a mouse neuroblastoma cell line. Given the aggressive nature of cancer, I evaluated the combinatorial effect of calcium and resveratrol and its analogs on neuroblastoma differentiation with the purpose of determining the biochemical target of calcium and developing a potent cocktail of drugs with better differentiation potential than individual drugs. However calcium and resveratrol and its analogs failed to show any significant synergistic effect implying that they had similar biochemical targets, which could

possibly be AMPK (established in previous studies involving resveratrol and neuronal differentiation).

Dose-response studies of stilbene analogs with calcium suggest that there is a lack of synergistic effect of these inducers on neuronal differentiation. Of course, a time-course study of these inducers would provide more conclusive evidence to support the dose-response study. However present data partially supports our hypothesis that stilbene analogs and calcium possibly have similar biochemical targets involved in neuronal differentiation due to which no significant synergistic effect was observed.

It was previously established that calcium possibly activated the phosphatidylinositol second messenger system as an early step during neuronal differentiation (Reboulleau 1986). I reconfirmed the specificity of calcium ions in inducing neuroblastoma differentiation and further proved that a constant source of calcium ions is eminent for neuronal differentiation. This is indicative of a role for calcium ions in both early and late stages of neuronal differentiation. This finding that calcium had more than one biochemical target in neuronal differentiation coupled with the fact that resveratrol and calcium had similar biochemical targets makes us speculative of a role for calcium in activation of AMPK. A possible mechanism for activation of AMPK via calcium could be through CaMKK (Fig. 4.18). It remains to be evaluated if AMPK is a biochemical target in calcium induced neuronal differentiation.

The exact mechanism by which resveratrol activates AMPK is not well understood, but a potential mechanism (Fig. 4.18 and 4.19) involves a cascade of events starting with inhibition of PDE, followed by increase in cAMP levels, leading to activation of PKA, followed by activation of L1KB or CAMKK both of which have been known to activate AMPK (Chung 2012). Interestingly, calcium is known to activate CAMKK potentially leading to activation of AMPK during neuronal differentiation. Additionally, in-vitro neuroblastoma differentiation method involves treatment of neuro-2a cells with inducers under reduced serum conditions. This step is critical for inducing differentiation, which possibly induces AMPK activation which is a crucial energy sensor and responds to low energy levels. My finding that resveratrol and calcium induce neuroblastoma differentiation individually, but fail to show synergistic effect when used in combination, implies a similar biochemical pathway for both resveratrol and calcium in neuronal differentiation. Both resveratrol and calcium possibly activate AMPK via L1KB/CaMKK or CaMKK respectively, mimicking a calorie restriction environment which leads to growth inhibition and activation of differentiation pathway.

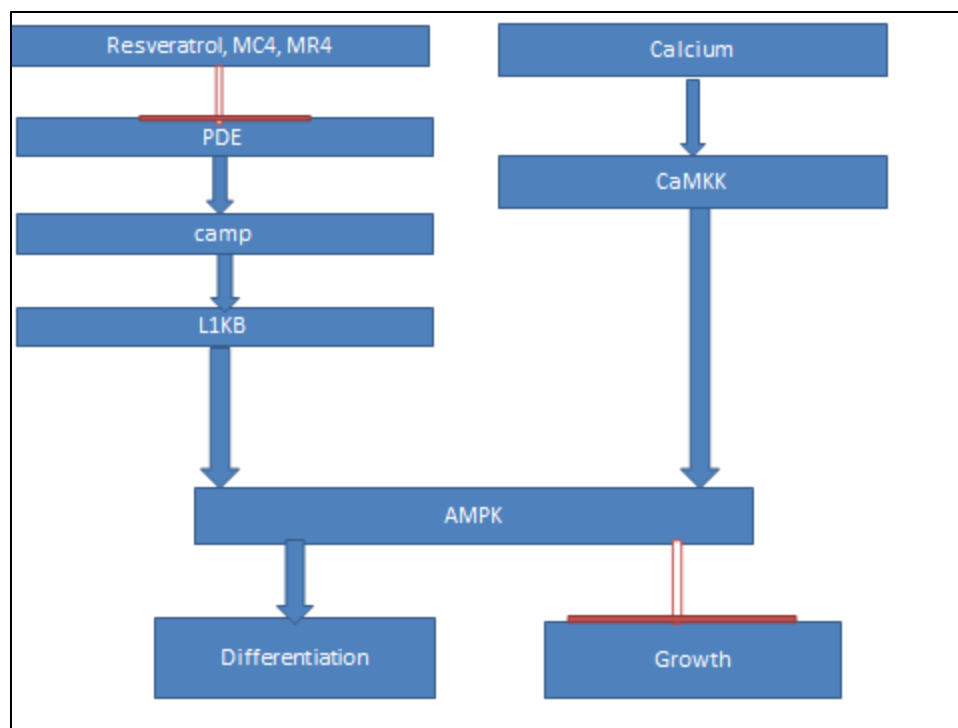


Figure 4.18: Proposed biochemical pathway of resveratrol and calcium induced neuroblastoma differentiation

In addition, we have established a biosimilar effect of two methoxy derivatives of resveratrol, MC4 (3,4,5,4'-tetramethoxy-cis-stilbene) and MR4 (3,4,5,4'-tetramethoxy-trans-stilbene) in neuronal differentiation. It remains to be evaluated if the effect of MC4 and MR4 is mediated via activation of AMPK, similar to resveratrol in the neuronal differentiation pathway. However the biosimilar effects of MC4 and MR4 in neuronal differentiation system offers a pharmacological advantage for clinical studies. Both MC4 and MR offer an improved pharmacokinetic/pharmacodynamic (PK/PD) profile as compared to resveratrol, which is currently facing low bioavailability issues in pre-clinical trials limiting the translation of its bioactivity under physiological conditions.

The low bioavailability of resveratrol is characteristic of its polyphenol structural framework which leaves the free-hydroxyl groups vulnerable to enzymatic activity resulting in rapid metabolism. The methoxy derivatives of resveratrol offer resistance to the enzymatic degradation due to the protective methoxy groups.

All of these findings provide insights into the mechanism of neuroblastoma differentiation which maybe mediated via AMPK activation and offer pharmacologically superior derivatives of resveratrol with potential for treatment in neuroblastoma differentiation.

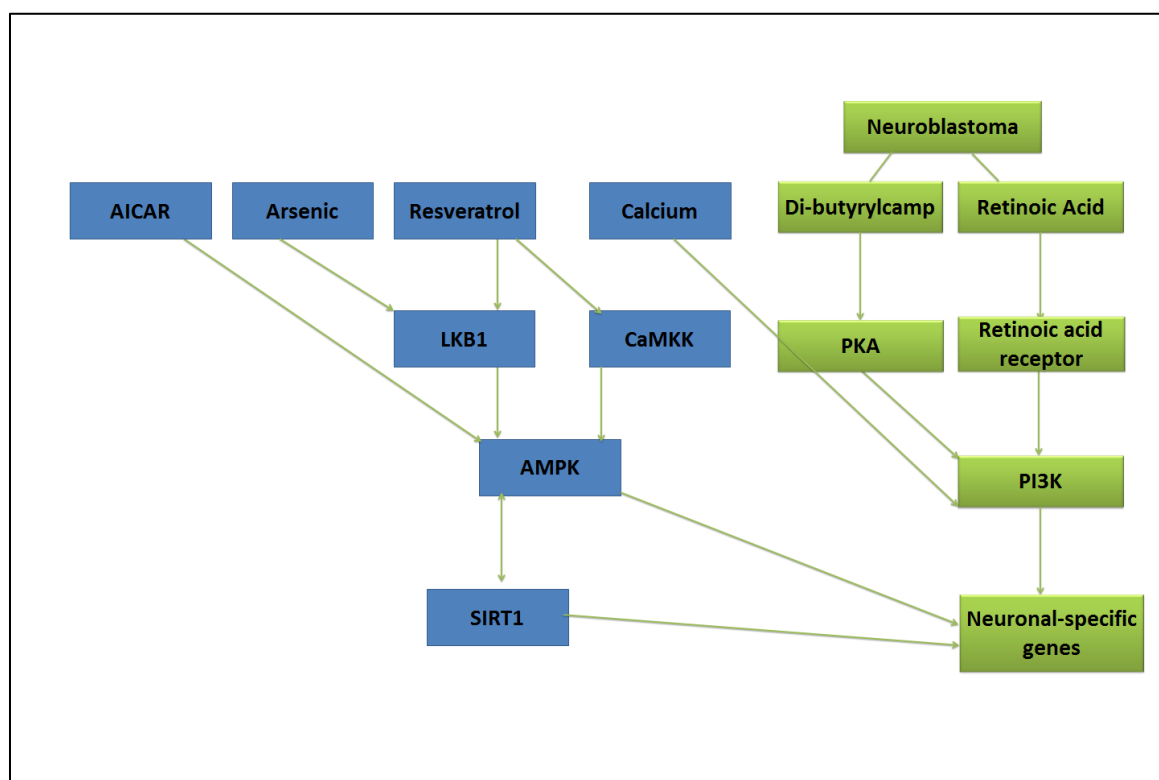


Figure 4.19: Proposed biochemical mechanism of resveratrol, MC4, MR4 and calcium in neuroblastoma differentiation

Conclusions:

- 1) Combinatorial approach of treatment of resveratrol, MC4 and MR4 with calcium did not enhance the efficacy of neuroblastoma differentiation
- 2) Despite lower levels of SIRT1 during neuroblastoma differentiation as evidenced by western blot analysis, RT-PCR, induction of differentiation upon treatment with SIRT1 inhibitor and SIRT1 activator resveratrol, it can be concluded neuroblastoma differentiation is independent of SIRT1 expression

Chapter 5: Effect of chemical stimuli on preadipocyte differentiation

Introduction

Obesity is a condition where storage of excess body fat results in adverse health effects such as increased risk of diabetes or stroke etc. Available treatment options include prescription weight-loss drugs that work on CNS and suppress hunger (Lorcaserin), block absorption and digestion of fat (Orlistat) etc. all of which cause an arsenal of side-effects. Additional treatment options include surgery for removal of excess fat which is highly invasive. All of these limitations of current obesity treatments necessitate the discovery of novel drugs with better bioactivity and lower side effects.

Body accumulates excess fat by two mechanisms, adipose hypertrophy (increase in fat tissue volume) or adipose hyperplasia (increase in fat cell number). Clinical prognosis shows adipose hypertrophy precedes adipose hyperplasia which is associated with severe to extreme obesity. Adipose hyperplasia is achieved by a cellular process called preadipocyte differentiation in which a precursor of fat cell (preadipocyte) is converted into fat cell (adipocyte) capable of accumulating the excess body fat. Inhibition of this process of fat storage in the body offers potential anti-obesity treatments.

The biochemical pathway of preadipocyte differentiation is well understood and involves activation of a cascade of transcription factors, Krox20 (homolog of early growth response 2 (EGR2), pCREB (phosphorylated cyclic-AMP response element binding protein), c/EBP β (cytidine-cytidine-adenosine-adenosine-thymidine-enhancer binding

protein β), c/EBP δ , KLF5, SREBP1c, c/EBP α and PPAR γ , leading to expression of adipocyte-specific genes such as Leptin, Fatty Acid Synthase, Adiponectin etc. The activation of these transcription factors requires various inducers such as serum mitogens (Krox20), cAMP (pCREB), glucocorticoids such as dexamethasone (c/EBP δ) and Insulin (SREBP1c) to commit to a terminal differentiation pathway. Pref-1 blocks expression of pCEBP β and exercises negative control over preadipocyte differentiation. Briefly, activation of Krox20 by serum mitogens or activation of pCREB (Farmer 2006) (Fig. 5.1).

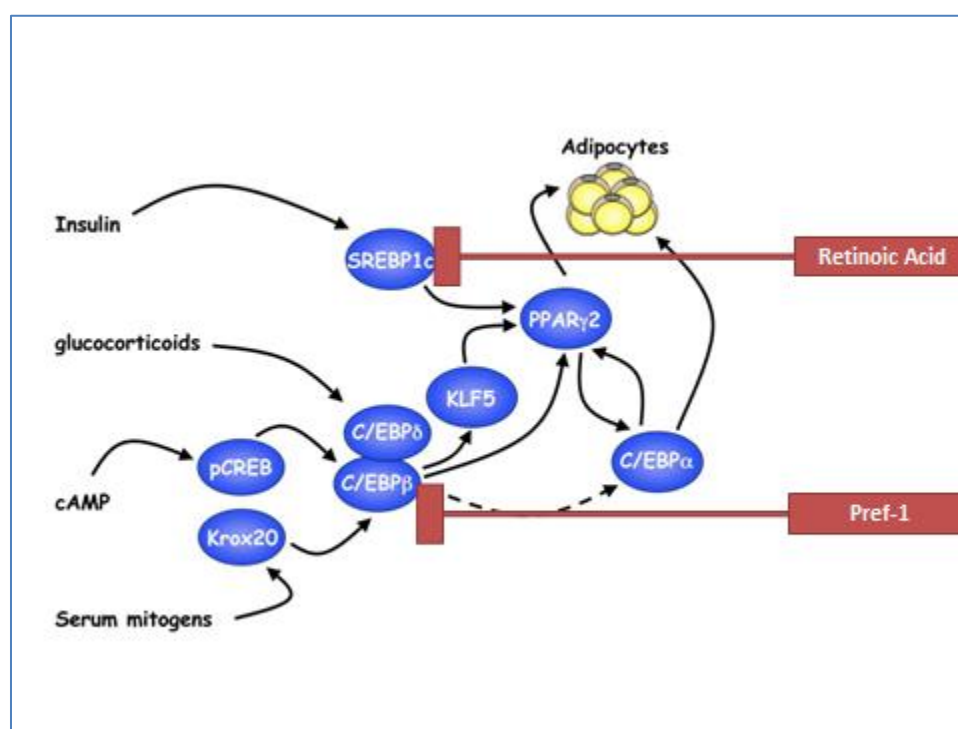


Figure 5.1: Transcriptional Regulation of Preadipocyte differentiation

Given the extensive handle on the preadipocyte differentiation pathway; various inhibitors of preadipocyte differentiation have been reported that target these transcription factors, especially the master regulators, c/EBP α and PPAR γ . There are

several small molecules such as DFMO, resistin, retinoic acid, resveratrol etc. that have been shown to inhibit adipogenesis and are currently in preliminary research.

Polyamines and eIF5A in Preadipocyte differentiation:

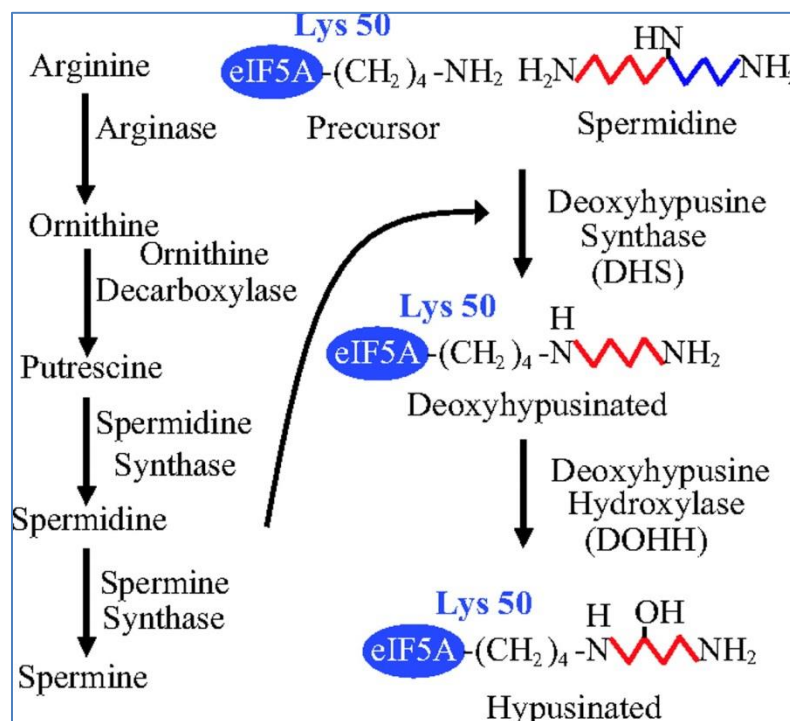


Figure 5.2: Polyamine Biosynthesis & eIF5A

Polyamines are low molecular weight, aliphatic amines present in all eukaryotes and are tightly regulated by a process called polyamine biosynthesis (Fig 5.2). Ornithine is upstream in the biosynthesis pathway and is the source of putrescine, spermidine and spermine. Various enzymes function to synthesize these amines or degrade them and each one provides a regulatory step. For example, synthesis of putrescine from ornithine requires an enzyme called ornithine decarboxylase, but α -Difluoro Methyl Ornithine (DFMO) an inhibitor of this enzyme, attenuates the synthesis of putrescine and other

polyamines downstream. Appropriate levels of polyamines are maintained by synthesis, degradation and uptake of amines. It is known that they are required for cell proliferation and are lucrative targets for cancer and other hyperproliferative diseases (Casero and Marton 2007).

The polyamine- spermidine is required for the modification of eIF5A, another protein crucial for cell viability. Infact, modification of eIF5A is the most specific polyamine dependent pathway (Chen 1997). eIF5A gene is highly conserved, from yeast to human.

The eIF5A protein contains a an unusual amino acid derived from posttranslational modification of a unique lysine residue using spermidine as the substrate Fig. 5.2 illustrates the biochemistry of this unique posttranslational modification, wherein deoxyhypusine synthase (DHS) catalyzes the transfer of 4-aminobutyl moiety from spermidine to a specific lysine reside, resulting to a deoxyhypusine reside and deoxyhypusine hydroxylase catalyzed the hydroxylation of deoxyhypusine to hypusine (Chen 1997). Deletion of genes encoding eIF5A or DHS in yeast produces lethal phenotype (Schnier 1991; Sasaki 1996), indicating that eIF5A is an essential protein and that hypusine modification is necessary for its essential function. Inhibition of DHS using specific inhibitors such as GC7 (N1-guanyl-1,7-diaminoheptane) in mammalian cells leads to growth arrest, suggesting that eIF5A plays an important role in growth regulation (Park 1994; Chen 1996).

Previous studies have shown that modulation of polyamine content may affect differentiation program in a number of cell systems. For example, DFMO was found to induce or promote the differentiation of mouse and human neuroblastoma cells (Chen 1996). On the other hand, DFMO was reported to inhibit the preadipocyte differentiation (Bethell and Pegg 1981, Erwin 1984). In addition, specific inhibition of hypusine formation has been reported to affect the differentiation of neuroblastoma, erythroleukemia cells (Chen 1996), and more recently, PC-12 cells (Huang 2007).

Although it has been shown that DFMO inhibits the differentiation of 3T3-L1 preadipocytes, the published work is preliminary and has not been confirmed in the literature. Present study aims to reconfirm the role of DFMO in inhibition of preadipocyte differentiation and further evaluate whether polyamines or eIF5A are involved in preadipocyte differentiation. This would be significant for determining the biochemical role of eIF5A which is poorly understood and provide a powerful tool for modulation of the preadipocyte differentiation pathway via eIF5A for anti-obesity potential.

Resveratrol & Preadipocyte differentiation:

Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) a natural stilbene derivative, has been shown to inhibit the lipid storage in adipocytes by exerting control over the differentiation and metabolic pathways involved in adipogenesis. Resveratrol is known to activate silent mating type information regulation 2 homolog-1 (SIRT1), a NAD⁺ dependent histone deacetylase which regulates fat mobilization by repressing PPAR γ -2, a key transcription factor in preadipocyte differentiation (Pang 2006, Fischer-Posovszky

2010). However recently it has been reported (Chen 2010 Mitterberger 2013), that resveratrol mediates inhibition of preadipocyte differentiation via activation of 5' adenosine monophosphate-activated protein kinase (AMPK) in a SIRT1 independent manner. AMPK is a key enzyme in cellular energy homeostasis and is known to be activated during calorie-restricted conditions. Activation of AMPK leads to up-regulation of PGC-1 α , a transcription factor that is involved in metabolic pathways. Other small molecule activators of AMPK such as RSVA314 and RSVA405 (Vingtdeux 2011), Ursolic Acid (He 2013) and Genistein, EGCG, and capsaicin (Hwang 2005) have been shown to inhibit adipogenesis. It remains to be evaluated if resveratrol mediates anti-adipogenic effects by activation of AMPK.

Based on the promising results in preliminary in-vitro and in-vivo studies, resveratrol (3,5,4'-tehydroxy-trans-stilbene) has advanced into pre-clinical studies for anti-obesity indications. However, initial pre-clinical studies of resveratrol report a low bioavailability making therapeutic dosage impractical for physiological effects.

Gossiau et. al, (2005) designed and synthesized two-methoxy derivatives of resveratrol, MC4 (3,4,5,4'-tetramethoxy-cis-stilbene) and MR4 (3,4,5,4'-tetramethoxy-trans-stilbene) that circumvent the low bioavailability issue of resveratrol due to protective methoxy groups.

In this study I evaluated the role of resveratrol and its methoxy analogs (MC4 & MR4), in adipogenesis using in-vitro obesity models (3T3-L1 and 3T3-F442A cell lines) in order to establish a biosimilar role for MC4 and MR4 in preadipocyte differentiation.

Materials & Methods:

Materials:

Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, aminoguanidine, putrescine, spermidine and spermine were purchased from (Sigma Aldrich, St Louis, MO, USA). α -Difluoro methyl ornithine (DFMO) was a generous gift from Merrell Dow Research Center (Cincinnati, OH). Resveratrol, MC4 and MR4 were synthesized as previously reported (Gossiau 2005). Oil-O red stain was purchased from (Sigma Aldrich, St Louis, MO, USA) for adipocyte staining.

Cell culture:

3T3-L1 cells were obtained from ATCC. The cells were always worked on in a type II sterile hood to avoid contamination. 70% ethanol was used to further maintain sterility. These cells were cultured in DMEM (GibCo) supplemented by 10% bovine calf serum (Gemini). They were maintained in an incubator at 37°C and 5% CO₂. Cells were sub cultured at 70-80% using Trypsin-EDTA.

Differentiation:

The protocol involved allowing the cells to get confluent and growth arrested (designated as Day 0). Followed by treatment of these cells with differentiation solution A [DMEM (GibCo) supplemented with 10% fetal calf serum (Gemini) + IBMX (Iso-butyl methyl

xanthine, 0.5mM) + DEX (Dexamethasone, 0.25mM)] for 4 days followed by treatment with differentiation solution B [DMEM + 10% FBS + Insulin (10 μ g/mL)] for 5-6 days (Green & Kehinde 1975). Formation of fat cells can be confirmed by observing the cells under a phase contrast microscope seen as round droplets in cells and oil-red staining.

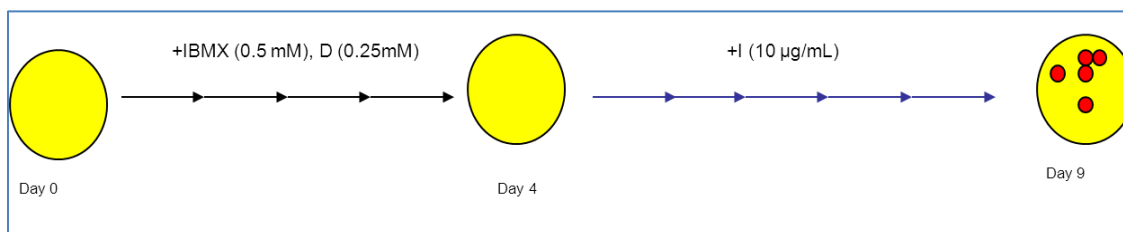


Figure 5.3. Scheme for Preadipocyte Differentiation

Oil-O Red staining assay:

Oil- O red is a lipophilic dye and specifically stains lipids. The petri dishes containing the cells to be stained were washed with PBS (twice). 1ml of Oil-O red (Chemi-Con adipogenesis kit) was added to the dishes and the dishes were returned to the incubator. After about 30 mins of incubation with the dye, the dishes were taken back to the sterile hood. They were washed with a wash solution (Chemi-Con adipogenesis kit) to wash away excess dye and remove background staining. Staining of fat cells by Oil-O Red makes the lipid droplets visible as red spots within the cells. The cells were then imaged with a camera connected to a phase contrast microscope. Also, the stained petri dishes were scanned to view the overall extent of staining (which correlates to fat accumulated).

Results:

Establishing preadipocyte differentiation model:

I used 3T3-L1 and 3T3-F442A cell lines as an in vitro obesity model; these cell lines are committed to differentiating into adipocytes under the influence of chemical inducers such as IBMX, Dexamethasone and insulin. Fig. 5.4 represents morphology of undifferentiated vs. differentiated fat cells. Oil-O Red staining was used to specifically stain the fat droplets.

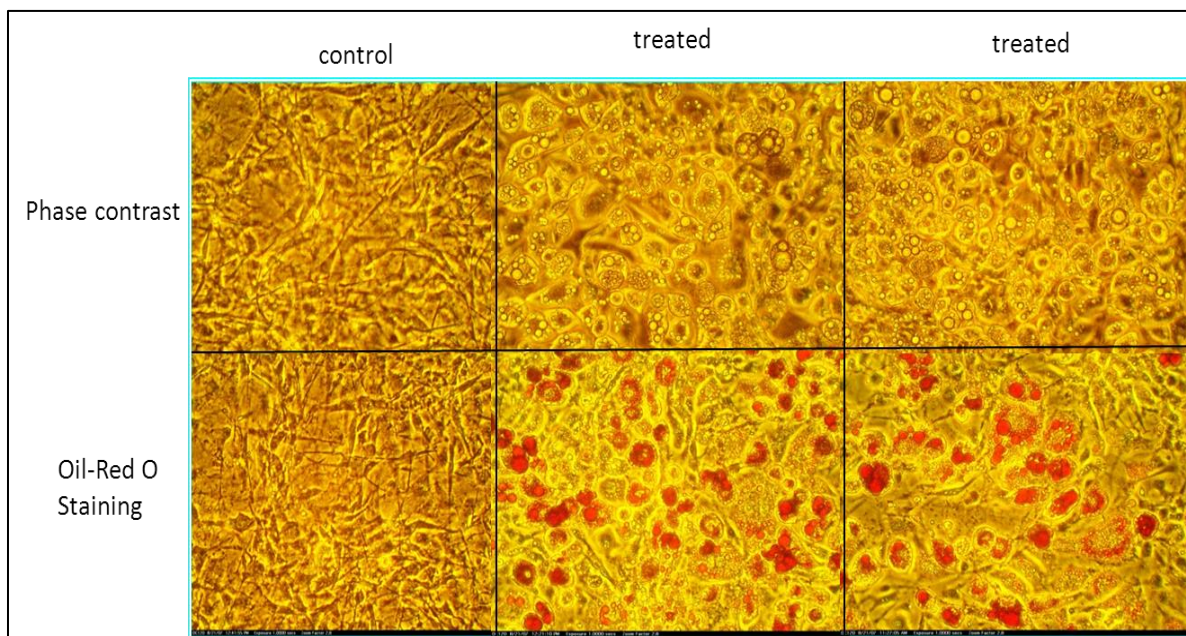


Figure 5. 4: Differentiation of preadipocytes

Effect of DFMO on preadipocyte differentiation:

A dose-response study was carried out to evaluate the effect of DFMO (an irreversible inhibitor of ornithine decarboxylase) on preadipocyte differentiation. 3T3-L1 cells were treated with varying concentration of DFMO (0-10mM) 24-hours prior to the induction of the adipogenic inducers. As shown in Fig. 5.5, DFMO inhibited differentiation of 3T3-L1

cells as previously reported (Erwin et. al., 1981; Bethell and Pegg 1984) at a low, non-cytotoxic concentration of 2mM.

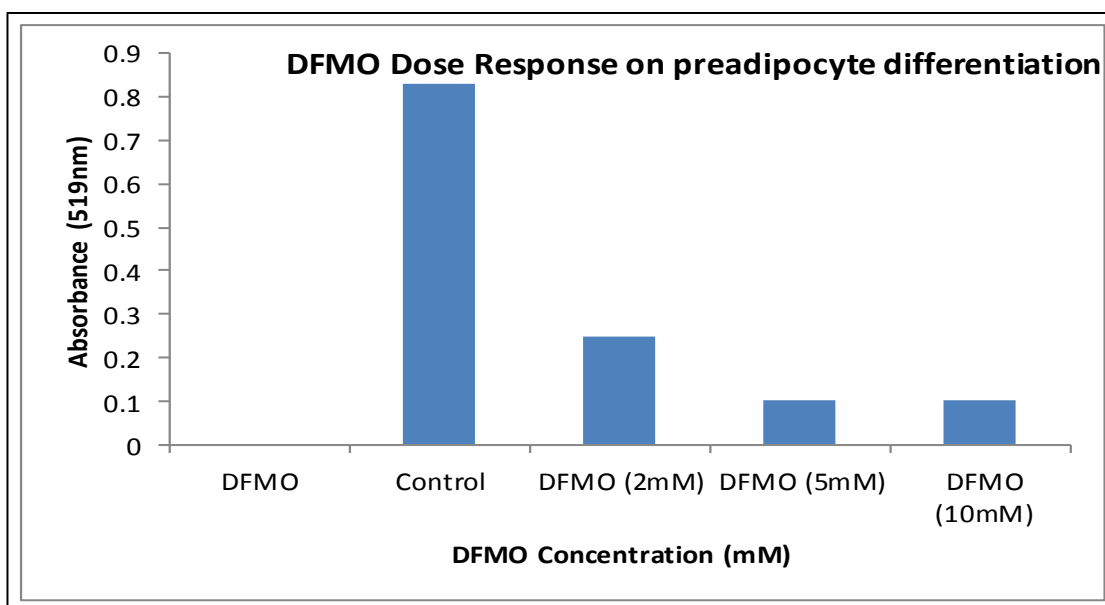
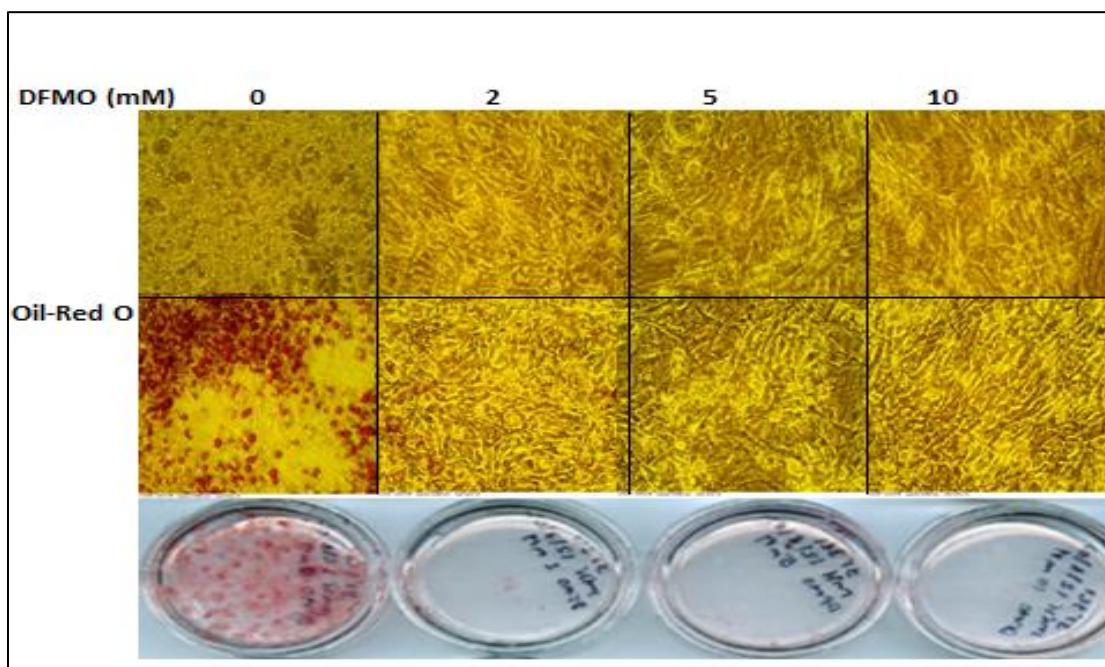


Figure 5. 5: DFMO (2mM) inhibits preadipocyte differentiation

The effect of DFMO on adipocyte formation was evaluated in Mesenchymal stem cells (MSCs) induced to differentiate into preadipocytes using the adipogenic inducers (IBMX, Dexamethasone & Insulin. MSCs isolated from Balb/bc mice were utilized to study the effect of DFMO on adipogenesis. As shown in Fig. 5.6, DFMO inhibited preadipocyte differentiation of MSCs at a concentration of 5mM.

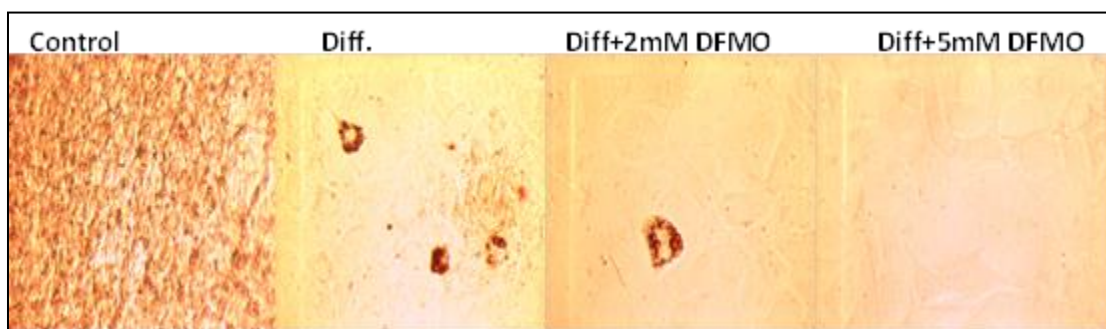


Figure 5.6: DFMO (5mM) inhibits adipogenesis of MSCs

DFMO induced inhibition of preadipocyte differentiation is rescued by addition of external polyamines:

The inhibition of polyamine biosynthesis by DFMO results in depletion of polyamines possibly required for preadipocyte differentiation. If polyamines were essential for preadipocyte differentiation, addition of external polyamines to DFMO treated cells would rescue the polyamine biosynthesis pathway and result in preadipocyte differentiation. In order to test this hypothesis, I evaluated the effect of addition external polyamine in DFMO-inhibited preadipocyte cells. Amino-guanidine (AG) was added to all dishes treated with polyamines to protect the oxidation of polyamines in the medium.

As shown in Fig. 5.7, AG did not have any effect on preadipocyte differentiation.

Addition of external putrescine (20 μ M) or spermidine (20 μ M) rescued the DFMO induced inhibition of preadipocyte differentiation (Fig. 5.7 (3T3-L1) and 5.8 (3T3-F442A) as evidenced by the morphological differentiated cells and Oil-O red staining of accumulated fat.

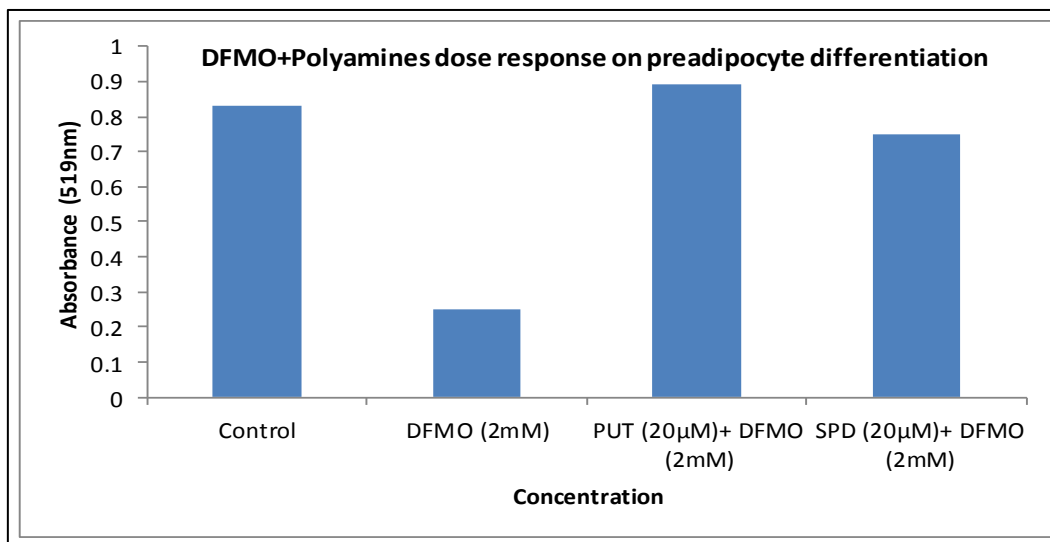
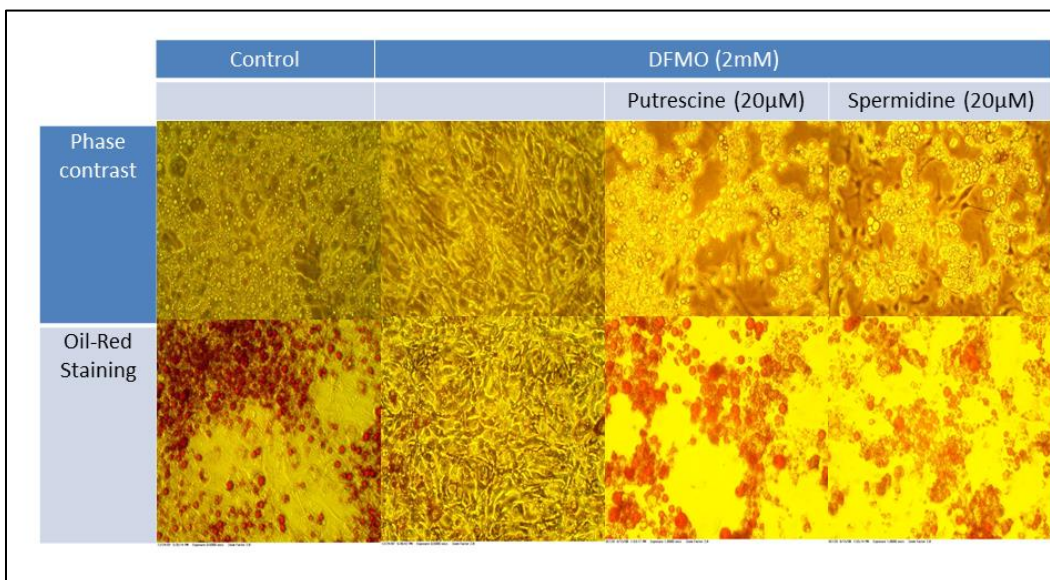


Figure 5.7: Putrescine and Spermidine reverse DFMO induced inhibition of preadipocyte differentiation (3T3-L1)

Polyamines did not significantly enhance preadipocyte differentiation:

As shown in Fig. 5.5, 5.6 and 5.7 polyamines are essential for preadipocyte differentiation. In order to evaluate the role of polyamines in preadipocyte differentiation, I conducted a systematic dose-response study. Polyamines putrescine (10-30 μ M), spermine (10-30 μ M) and spermidine (10-30 μ M) were added 24 hours prior to induction of preadipocyte differentiation. Polyamines by themselves (without adipogenic inducers) failed to induce preadipocyte differentiation (Data not shown). However as shown in Fig. 5.8, 5.9, 5.10, addition of polyamines (with adipogenic inducers) did not significantly enhance preadipocyte differentiation as compared to the control.

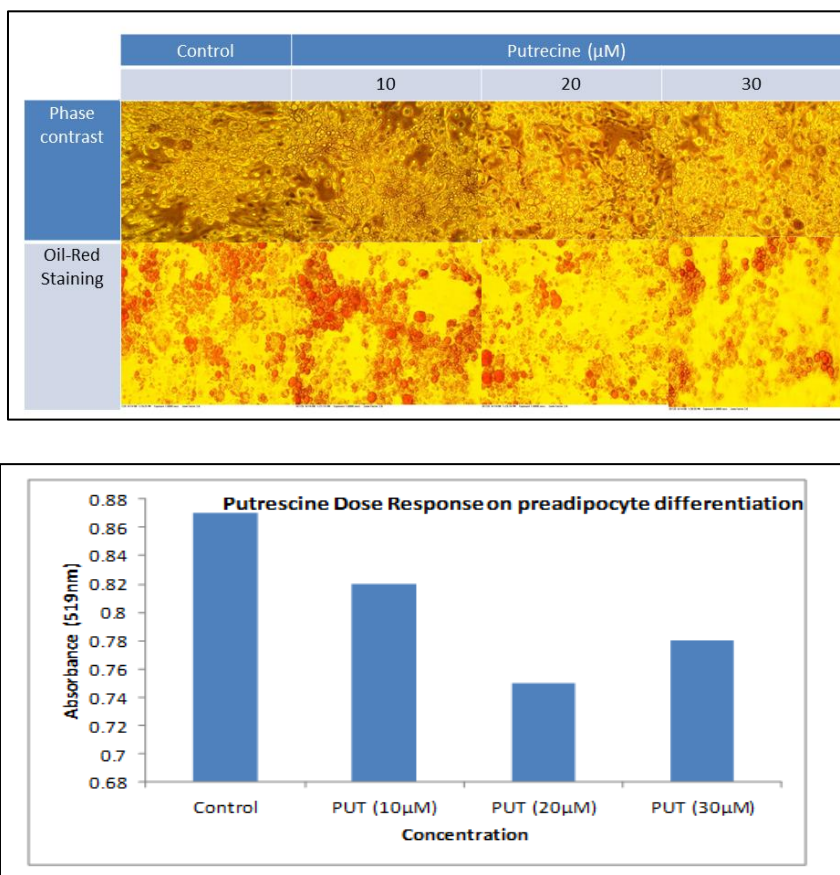


Figure 5.8: Addition of external putrescine did not affect preadipocyte differentiation

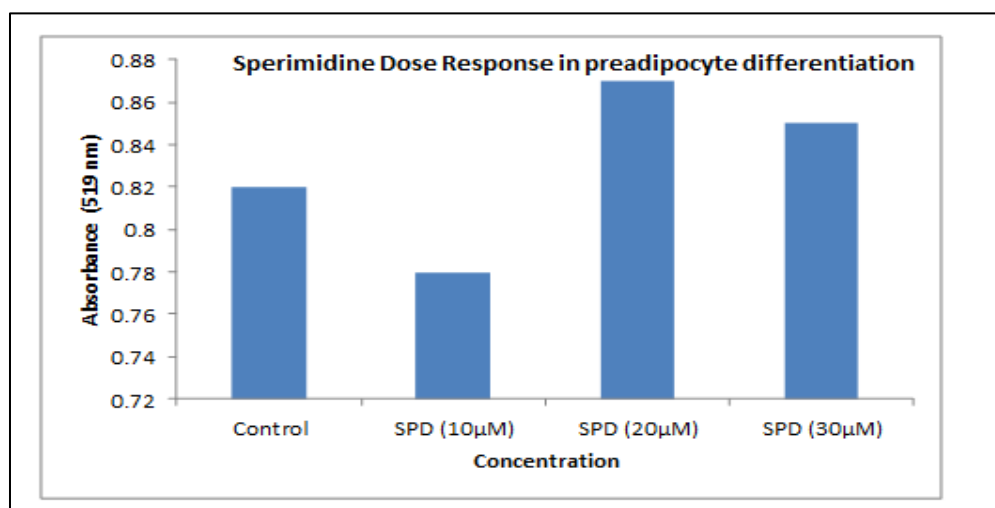
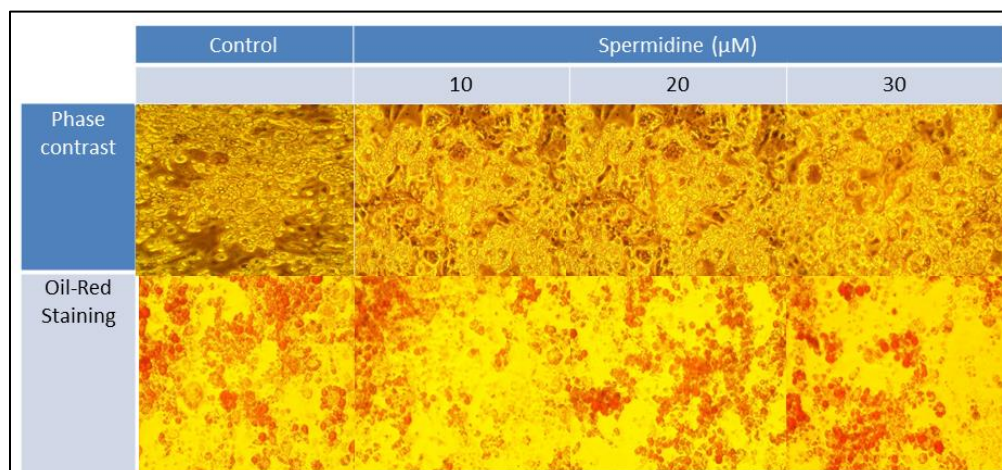


Figure 5. 9: Addition of external spermidine did not affect preadipocyte differentiation

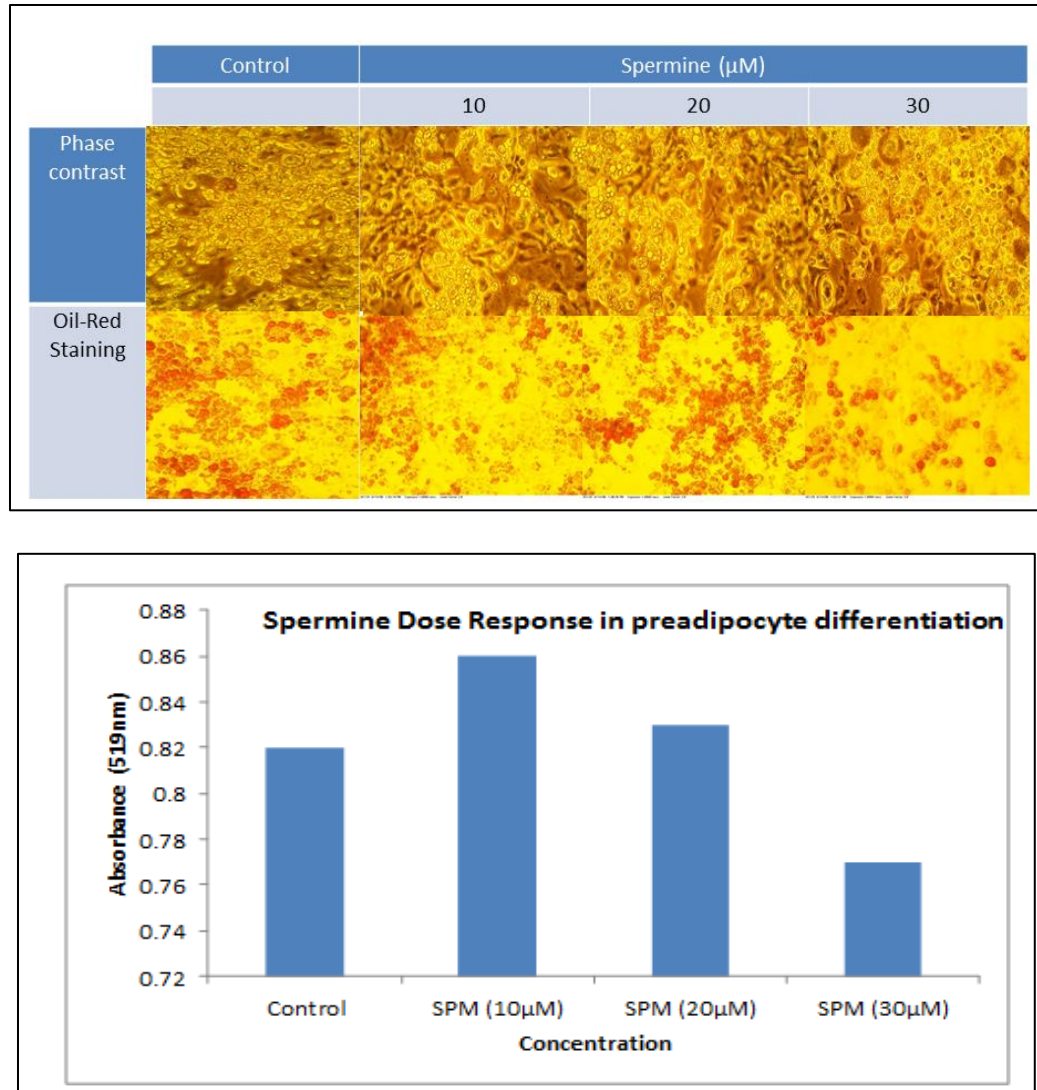


Figure 5.10: Addition of external spermine did not affect preadipocyte differentiation (3T3-F442A)

Given the findings in Fig. 5.4-5.10, it appears that polyamines were required for preadipocyte differentiation, but polyamines did not directly affect the preadipocyte differentiation pathway. Based on this inference I continued to evaluate the role of eIF5A in preadipocyte differentiation because eIF5A modification is a unique, polyamine-dependent pathway and could possibly be involved in preadipocyte differentiation.

GC7 inhibits preadipocyte differentiation:

Since polyamines did not directly affect preadipocyte differentiation, I evaluated the role of eIF5A in preadipocyte differentiation. GC7 is a known inhibitor of eIF5A modification and if eIF5A modification was crucial for preadipocyte differentiation, GC7 would inhibit the process.

To validate this hypothesis, I treated 3T3-L1 cells with varying dose of GC7 (20-100 μ M) for 24 hours prior to induction of differentiation. As shown in Fig. 5.11, GC7 inhibited preadipocyte differentiation at 20 μ M and higher dose of GC7 (50-100 μ M) were toxic to the cells.

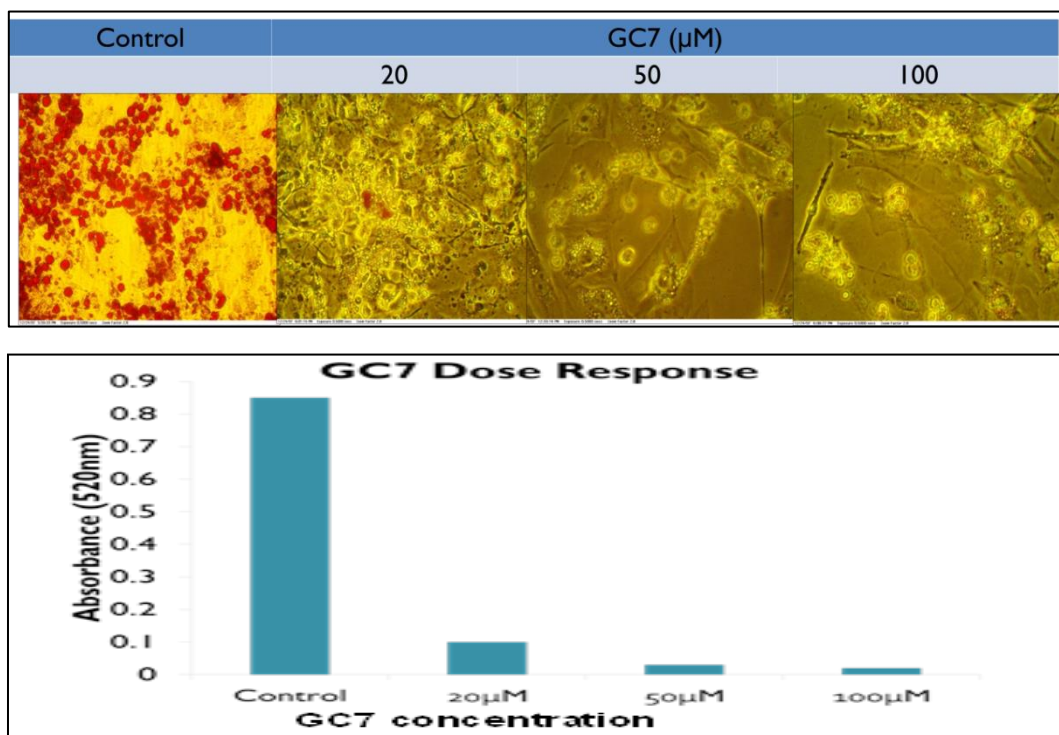


Figure 5.11: GC7 (20 μ M) inhibits preadipocyte differentiation

GC7 induced inhibition of preadipocyte differentiation is rescued by addition of external spermidine: The inhibition of preadipocyte differentiation by GC7 is rescued by addition of spermidine (20 μ M), but not putrescine (20 μ M) as shown in Fig. 5.12. A possible explanation for the specificity of spermidine in reversing GC7 mediated inhibition of preadipocyte differentiation lies in the fact that GC7 is a competitive inhibitor of Deoxyhypusine synthase (DHS) and is very similar in structure to spermidine (Table 5.1). Addition of external spermidine thus allows for eIF5A modification which is possibly crucial for preadipocyte differentiation.

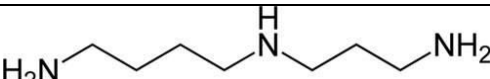
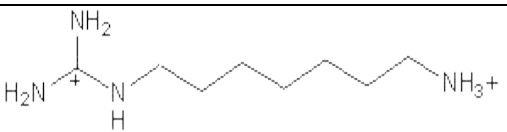
Spermidine	GC7 (N1-Guanyl-1, 7-diaminoheptane)
	

Table 5.1: Chemical Structure of spermidine and GC7

Spermidine and/or eIF5A modification is/are required for preadipocyte differentiation

Based on these finding I conclude that, spermidine or eIF5A modification plays a crucial role in preadipocyte differentiation. The exact mechanism by which spermidine and/or eIF5A modification modulates preadipocyte differentiation remains to be evaluated.

This finding was congruent to the findings of Vuohelainen et . al, (2010) which was unknown at the time this study was conducted.

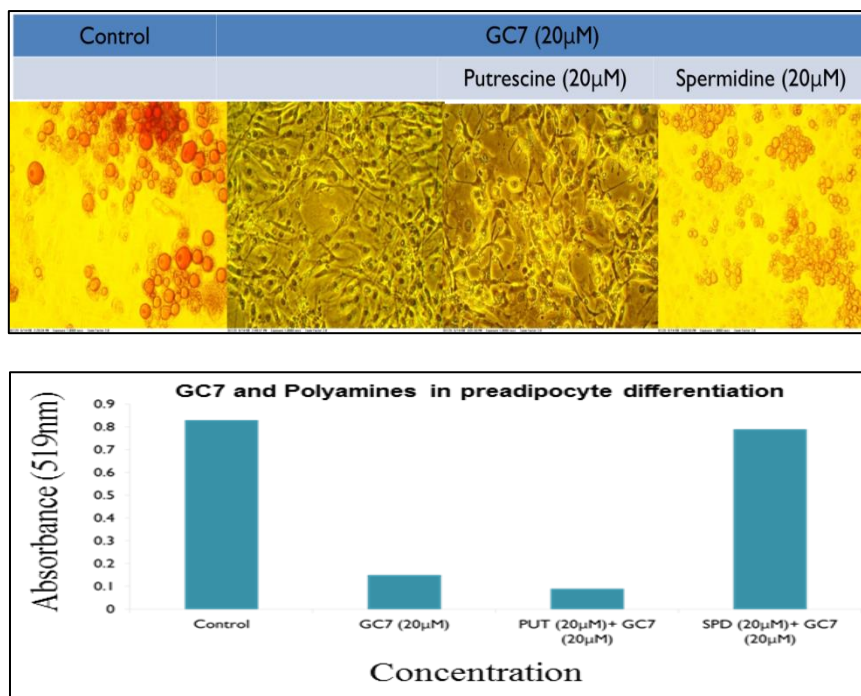


Figure 5.12: Inhibitory effect of GC7 on preadipocyte differentiation is rescued by addition of external spermidine (20 μ M)

Role of resveratrol and its analogs in preadipocyte differentiation:

Rayalam et. al, (2008) reported that resveratrol exhibited a dose-dependent response in differentiation of 3T3L1. A high dose of resveratrol (100 μ M) induced apoptosis while a lower dose (25-50 μ M) inhibited fat accumulation in 3T3-L1. I reconfirmed the inhibitory effect of resveratrol in preadipocyte differentiation and further evaluated the role of two analogs of resveratrol, MC4 and MR4 on preadipocyte differentiation.

In maturing preadipocytes, preliminary experiments with a range of resveratrol concentrations (data not included) showed that concentrations of 100 μ M and above were cytotoxic. Therefore, lower concentrations were used in future experiments. The results

showed that resveratrol at 10-20 μM significantly decreased lipid accumulation (Fig. 5.13). However, resveratrol also decreased cell viability at 50 μM indicating that the effect on lipid accumulation was possibly mediated via inhibition of growth. Oil Red O staining to visualize lipid accumulation in cells after treatment show that resveratrol caused a greater reduction of lipid accumulation when compared to control cells (Fig. 5.13).

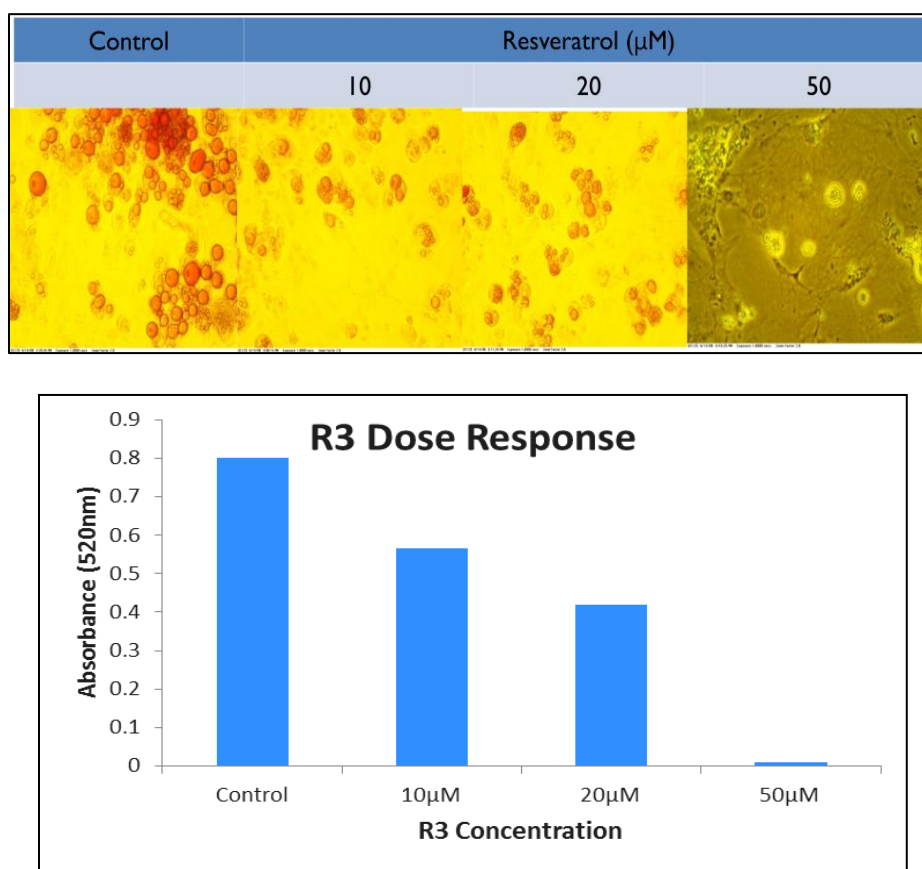


Figure 5.13: Resveratrol inhibits preadipocyte differentiation

Effect of MC4 and MR4 on preadipocyte differentiation

Both MC4 and MR4 inhibit preadipocyte differentiation at relatively low concentration, 0.001 μ M (Fig. 5.14) and 1 μ M (Fig. 5.15) respectively. The exact mechanism by which resveratrol and its analogs inhibit preadipocyte differentiation remains to be evaluated.

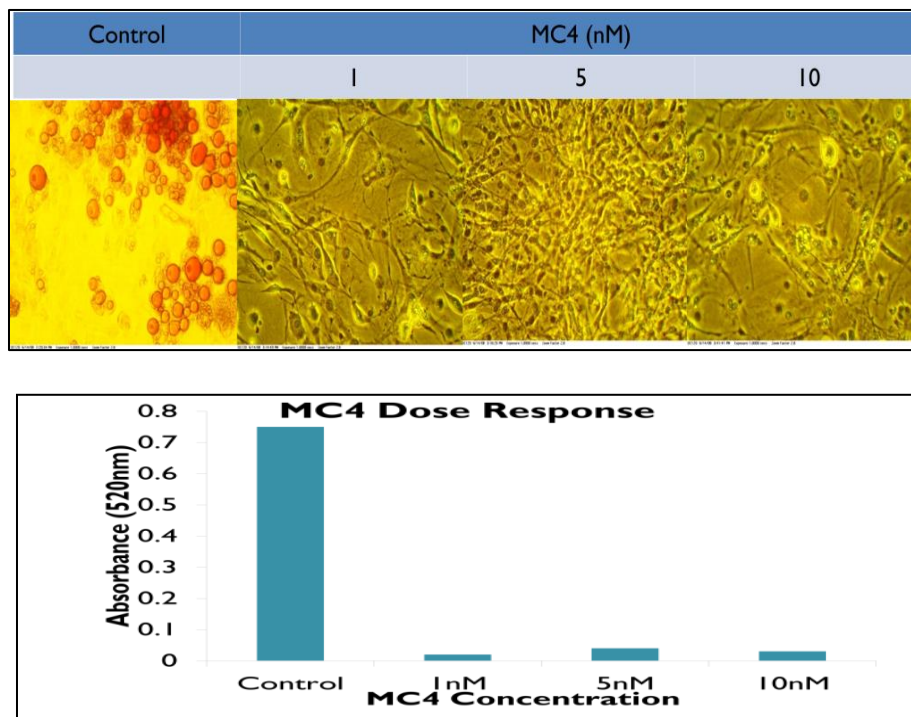


Figure 5.14: MC4 (0.01 μ M) inhibits preadipocyte differentiation

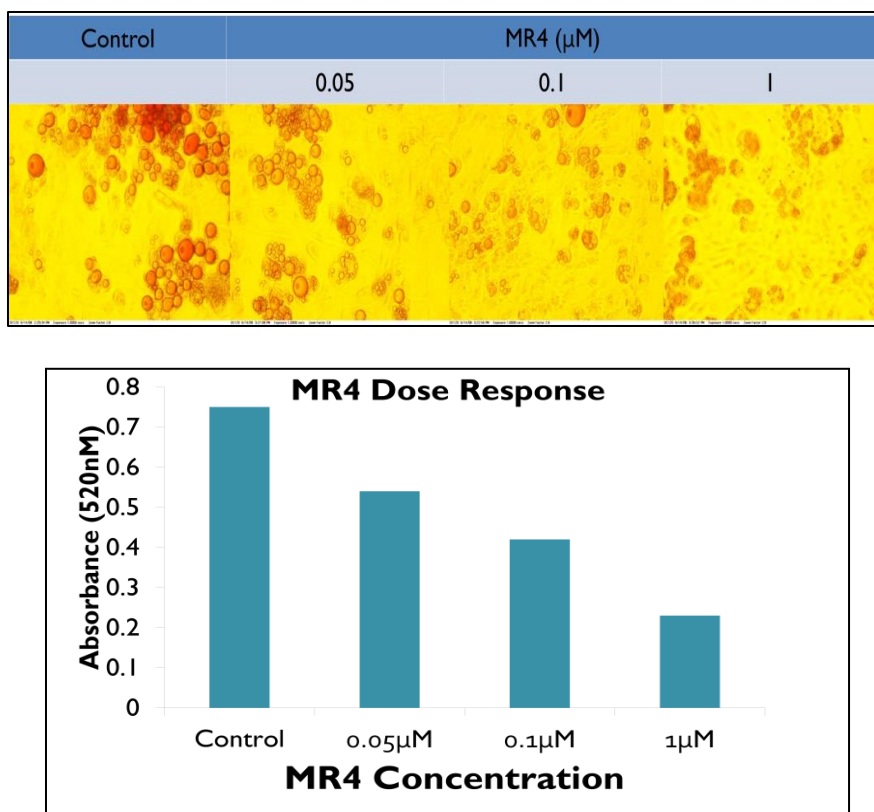


Figure 5.15: MR4 inhibits preadipocyte differentiation

Discussion:

Inhibition of preadipocyte differentiation by using small molecules offers potential for obesity treatment. In this study, I have evaluated the role of two different classes of small molecules targeting different biochemical targets involved in preadipocyte differentiation.

Polyamines and eIF5A in preadipocyte differentiation:

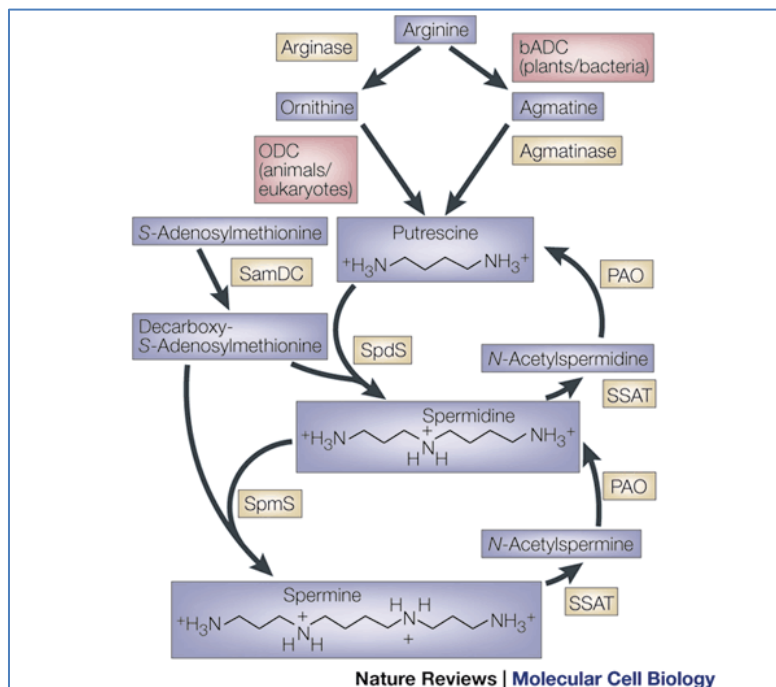


Figure 5.16: Polyamine biosynthesis and degradation pathway

The first class of small molecules includes DFMO and GC7, known to target polyamine biosynthesis and eIF5A modification, respectively. It was previously reported that DFMO inhibits preadipocyte differentiation at a concentration of 5mM (Bethell 1981, Erwin 1984). DFMO is an inhibitor of the enzyme, ornithine decarboxylase (ODC) which converts ornithine to putrescine which is required for the biosynthesis of downstream polyamines, spermidine and spermine (Fig. 5.16). Inhibition of preadipocyte differentiation via DFMO implicates a role for polyamines in preadipocyte differentiation.

In this present study, I have reconfirmed the inhibitory role of DFMO on preadipocyte differentiation using 3T3-L1 and Mesenchymal Stem Cells (MSCs) as in-vitro models of

adipogenesis. DFMO was capable of inhibiting preadipocyte differentiation at 2mM in 3T3-L1 cells (Fig. 5.5) and 5mM in MSCs (Fig. 5.6).

Interestingly, the inhibitory effect of DFMO was completely reversed by addition of external polyamines, putrescine (20 μ M) and spermidine (20 μ M) in both 3T3L-1(Fig. 5.7) and 3T3-F442A (Fig. 5.8) cell lines. This is in congruence with previous studies (Bethell 1981, Erwin 1984).

In order to test the effect of polyamines on preadipocyte differentiation, I conducted a dose-response study in which preadipocytes were treated with putrescine, spermidine and spermine and their effect on preadipocyte differentiation was evaluated. Addition of external polyamine did not have any significant effect on preadipocyte differentiation (Fig. 5.9). This implies that basal level of polyamines was sufficient for the induction of preadipocyte differentiation in presence of adipogenic inducers (IBMX, Insulin and Dexamethasone).

Spermidine has been shown to be essential for preadipocyte differentiation (Vuohelainen 2010), but the specific role of polyamines in preadipocyte differentiation remains to be evaluated. It has been previously reported that stimulation of 3T3-L1 cells with adipogenic inducers (IBMX, Dexamethasone & Insulin) results in a two-fold increase in putrescine and a three-fold increase in spermidine and an increase in ornithine decarboxylase (ODC), however the activities of spermidine synthase (SpdS) and spermine synthase (SpmS) remain unaltered (Erwin 1984). This implies that elevation of

polyamines during differentiation is not only due to changes in the biosynthesis enzymes but also due to degradation and/or excretion of polyamines (Ishii 2012).

A possible mechanism by which spermidine modulates control over preadipocyte differentiation could be through downregulation of c/EBP β . Very recently Hyvonen (2013) et. al. showed that depleting spermidine levels leads to decrease in c/EBP β specifically among other transcription factors involved in preadipocyte differentiation. c/EBP β is required for activation of c/EBP δ , PPAR γ and SREBP1c (Fig. 5.1).

Another possible mechanism by which polyamines modulate preadipocyte differentiation could involve polyamine mediated eIF5A modification. eIF5A is a highly conserved protein that undergoes a unique post-translation modification that involves the polyamine, spermidine (Fig. 5.17). The unhypusinated-eIF5A is converted to Deoxyhypusinated-eIF5A by the enzyme Deoxyhypusine synthase (DHS) by the transfer of spermidine on to the lysine50 residue of eIF5A. Deoxyhypusine is converted to hypusinated-eIF5A by the enzyme Deoxyhypusine hydroxylase (DHH), thus completing the post-translational modification of eIF5A.

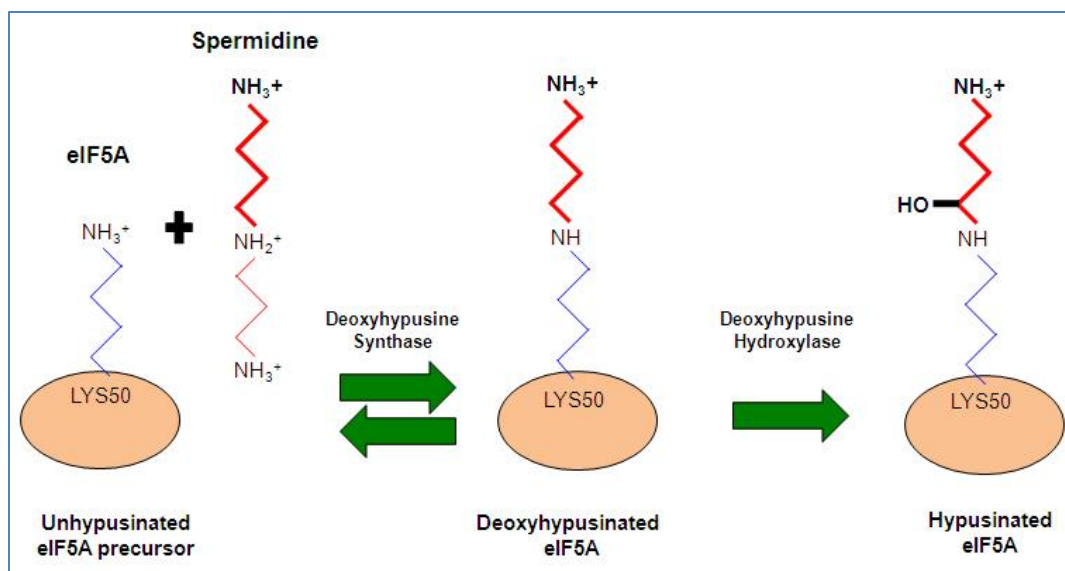


Figure 5.17: Polyamine dependent hypusination of eIF5A

In order to evaluate the role of eIF5A modification on preadipocyte differentiation, I treated 3T3-L1 cells with GC7, an inhibitor of DHS (Fig 5.17). GC7 competes with spermidine and binds to DHS, thus making it unavailable for the first step of eIF5A modification (Table I).

GC7 inhibits preadipocyte differentiation at a concentration of ($20\mu\text{M}$) beyond which it was cytotoxic. The cytotoxicity of GC7 has been previously reported (Jasiulionis 2007). However the role of GC7 in preadipocyte differentiation is a novel finding.

Deactivation of DHS by GC7 could possibly be reversed by addition of external spermidine, since both GC7 and spermidine compete with each other for DHS binding sites. As expected, addition of spermidine specifically rescued the inhibition of

preadipocyte differentiation by GC7. This finding supports the hypothesis that eIF5A modification is involved in preadipocyte differentiation.

A possible mechanism by which eIF5A modification inhibits preadipocyte differentiation is through inhibition of the mitotic clonal division required for preadipocyte differentiation. At a cellular level, preadipocyte differentiation involves growth arrest, post-confluence mitosis, clonal expansion, growth arrest and terminal differentiation (Phrakonkham 2008, Fig. 5.18). Briefly, confluent cells reach growth arrest by cell-cell contact inhibition mechanism and expression of preadipocyte factor-1 (Pref1) during this growth arrested state keeps the cells undifferentiated. Addition of pro-adipogenic factors such as insulin, dexamethasone and IBMX leads to expression of adipogenic transcription factors. c/EBP β is one of the early transcription factors to be expressed and is required for mitotic clonal expression. Downregulation of c/EBP β disrupts the differentiation of preadipocytes by blocking cell proliferation associated with mitotic clonal expansion (Zhang 2004). Expression of c/EBP δ , PPAR γ and c/EBP α leading to terminal differentiation. eIF5A, a key survival protein is required for cell proliferation and inhibition of eIF5A hypusination leads to arrested cell growth in various mammalian cell types (Park 1997). GC7 is known to block growth of normal and cancer cells (Park 1997).

Based on the finding that GC7 inhibits preadipocyte differentiation, it is fair to assume that effect of GC7 on preadipocyte differentiation is through inhibition of post-mitotic clonal expansion which is crucial for preadipocyte differentiation.

In conclusion, DFMO and GC7 inhibit preadipocyte differentiation possibly via interaction with c/EBP β which is required for mitotic clonal division crucial for adipogenesis.

Role of resveratrol and its analogs in preadipocyte differentiation:

Resveratrol (3,5, 4'-trihydroxy-trans-stilbene) a natural stilbene derivative, has been shown inhibit the lipid storage in adipocytes by exerting control over the metabolic pathway involved in preadipocyte differentiation (Pang 2006, Fischer-Posovszky et. al., 2010). I have independently reconfirmed the inhibitory role of resveratrol in preadipocyte differentiation and evaluated the effect of methoxy derivatives of resveratrol, MC4 and MR4 on preadipocyte differentiation using the in-vitro cell model-3T3L1 and 3T3F442A, which are both mouse preadipocyte cell lines.

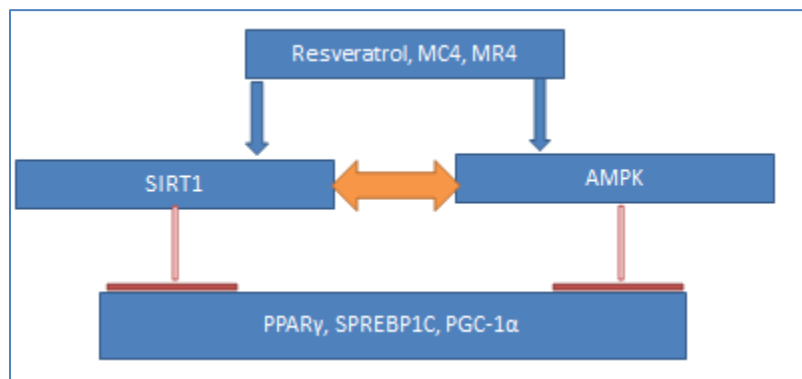


Figure 5.20: Resveratrol mediated inhibition of transcription factors in preadipocyte differentiation

The biochemical pathway of resveratrol and its analogs, mediated inhibition of preadipocyte differentiation is possibly through activation of AMPK. The role of AMPK in preadipocyte differentiation has been previously evaluated using small molecule

activators of AMPK (Hwang et. al., 2005, Vingtdeux 2011, Chen 2011 & He 2013). However the exact mechanism by which these activators of AMPK inhibit preadipocyte differentiation is not well understood. It could be speculated that AMPK decrease expression of PPAR γ (Sozio 2011) and/or SREBP1C (Li 2011, Yap 2011).

These findings suggest that resveratrol and its analogs inhibit preadipocyte differentiation possibly through either direct or indirect (through SIRT1) activation of AMPK and provide a solid framework for pre-clinical studies. In preadipocyte differentiation, insulin acts as an activator of PPAR γ through SREBP1c and this results in expression of adipocyte-specific genes. Insulin negatively regulates AMPK which remains inactive in an energy rich environment. Addition of resveratrol during preadipocyte differentiation activates AMPK which leads to down-regulation of adipocyte-specific genes possibly through PPAR γ and/or c/EBP α and/or SREBP1c.

Understanding of the biochemical mechanism of action of these small molecules and further in vivo evaluation for drug-like properties such as safety, bioavailability etc. offer potential for therapeutics as anti-obesity drugs.

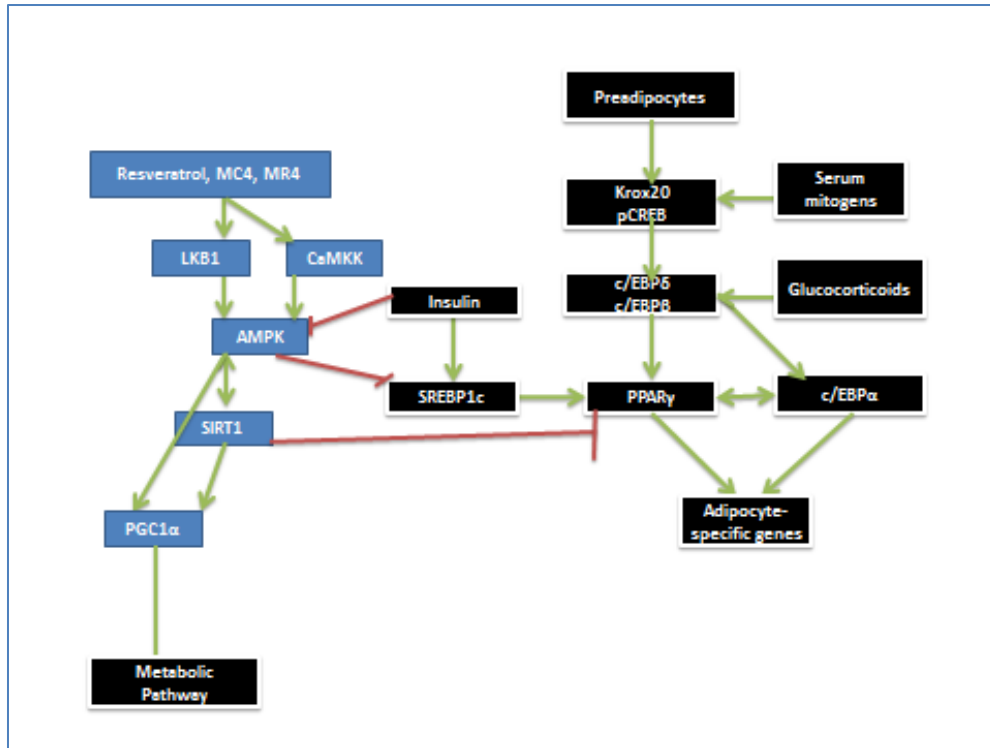


Figure 5.21: Possible mechanism of resveratrol and its analogs in preadipocyte differentiation

Conclusions

- 1) Treatment with Resveratrol, MC4, MR4 and GC7 effectively decreased lipid accumulation which is characteristic of adipocyte formation, a process crucial for regulation of preadipocyte differentiation
- 2) Of all the molecules tested, MC4 showed the highest efficacy (1nM)
- 3) GC7, an inhibitor of an enzyme required for eIF5A modulation inhibits preadipocyte differentiation indicating a mechanistic role for eIF5A, a highly conserved protein with poorly understood functionality, in adipocyte formation and presents a novel target for therapeutics in obesity.
- 4) Spermidine, a polyamine required for eIF5A modulation rescues the inhibitory effect of GC7 on preadipocyte differentiation, indicating a role of polyamines in preadipocyte differentiation and offers a novel target for therapeutics in obesity
- 5) The mechanism of action of resveratrol and its analogs in the obesity pathway is possibly mediated via activation of SIRT1, a histone and protein deacetylase, known to suppress expression of PPAR γ , a transcriptional regulator of preadipocyte differentiation

Chapter 6: Conclusion and Perspective

Major Findings:

Heat shock induced cell death is possibly mediate via activation of a proteolytic system

Heat shock response is a pro-survival mechanism that protects the cells against the damaging effects of heat stress. However the failure of this mechanism to restore homeostasis results in tumor cell death. The exact mechanism by which heat treatment induces cell death, especially in tumor cells is not well understood and is of significance because of its potential application cancer therapy. It has been previously established that cancer cells are sensitive to heat shock and heat shock induced tumor cell death is associated with loss of eIF5A, a highly conserved survival protein (Takeuchi 2002, Gossiau 2009).

The findings from this thesis suggest that possibly various proteases get activated by heat shock and multiple proteins, including eIF5A get degraded eventually leading to loss of viability. It remains to be evaluated which proteases are activated and what other proteins are degraded (besides eIF5A). However the findings of this dissertation strengthen the hypothesis that proteases are involved in heat induced tumor cell death and use of small molecule activators of proteases could possibly improve the efficacy of heat-treatment through synergistic/additive mechanism.

Even though heat therapy is currently used in combination with radiation and chemotherapy, its use is limited due to the high temperatures required for tumor cell

death which is impractical. A potential way to overcome this limitation is to administer heat shock at lower temperatures but magnify the physiological effect by using other mechanisms to mimic the biochemical pathway of heat induced cell death. The finding that proteases activated during heat shock lead to cell death, imply a potential role for protease activators to induce tumor cell death in absence or low heat application (Fig. 6.1). This discovery is significant from a multi-targeted cancer therapeutic approach.

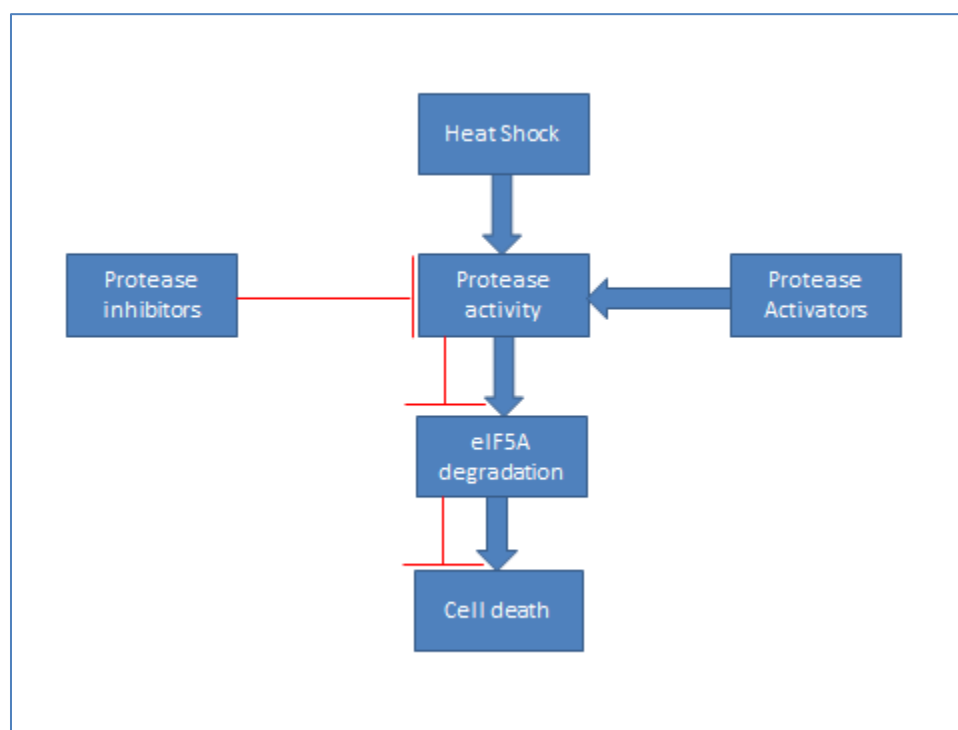


Figure 6.1: Proposed role for modulating protease activity for improved efficacy of heat treatment

Resveratrol-mediated biochemical pathways require activation of AMPK

In this thesis, we evaluated the role of resveratrol and its analogs in various disease models such as obesity (Preadipocyte differentiation), cancer (neuroblastoma differentiation) and inflammation. In all of these disease models, resveratrol and its

analogs showed potential for therapeutics by modulation of key targets in the biochemical pathway of these diseases. Although the mechanism of the bioactivity of resveratrol in these disease models is not well understood, there is strong evidence that it is possibly mediated by activation of AMPK. AMPK acts as an energy sensor and gets activated by factors such as low nutrients, exercise, cAMP etc. and is down regulated by proinflammatory factors (TNF α , TPA), insulin etc. The possible mechanism for resveratrol induced anti-cancer, anti-obesity and anti-inflammatory effect could be as follows,

In neuroblastoma differentiation, the cells with base level AMPK activity continue to proliferate, however upon activation of AMPK through resveratrol, mimics a calorie restricted environment which results in arrested cell growth. Since growth and differentiation are mutually exclusive events, inhibition of growth in neuroblastoma results in the cell committing to a differentiation pathway.

In preadipocyte differentiation, insulin acts as an activator of PPAR γ through SREBP1c and this results in expression of adipocyte-specific genes. Insulin negatively regulates AMPK which remains inactive in an energy rich environment. Addition of resveratrol during preadipocyte differentiation activates AMPK which leads to downregulation of adipocyte-specific genes possibly through PPAR γ and/or c/EBP α and/or SREBP1c.

Induction of inflammation response starts with activation of NF- κ B through pro-inflammatory agents such as TPA, TNF α etc. Activation of NF- κ B leads to expression of inflammation-specific genes responsible for removal of irritant stimuli and tissue healing.

Both TPA and $\text{TNF}\alpha$ are known to down-regulate AMPK leading to activation of a apoptosis particularly in metabolic disorders. Addition of resveratrol during TPA-induced activation of NF- κ B leads to activation of AMPK which is known to down-regulate NF- κ B expression leading to mitigated inflammatory response. The possible mechanism of downregulation of NF- κ B through AMPK involves SIRT1 or PGC1 α , which are known to interact with the p65 unit of NF- κ B.

Thus in conclusion, the biochemical effects of resveratrol in various disease models is possibly mediated via AMPK (Fig. 6.2,). The exact mechanism remains to be evaluated.

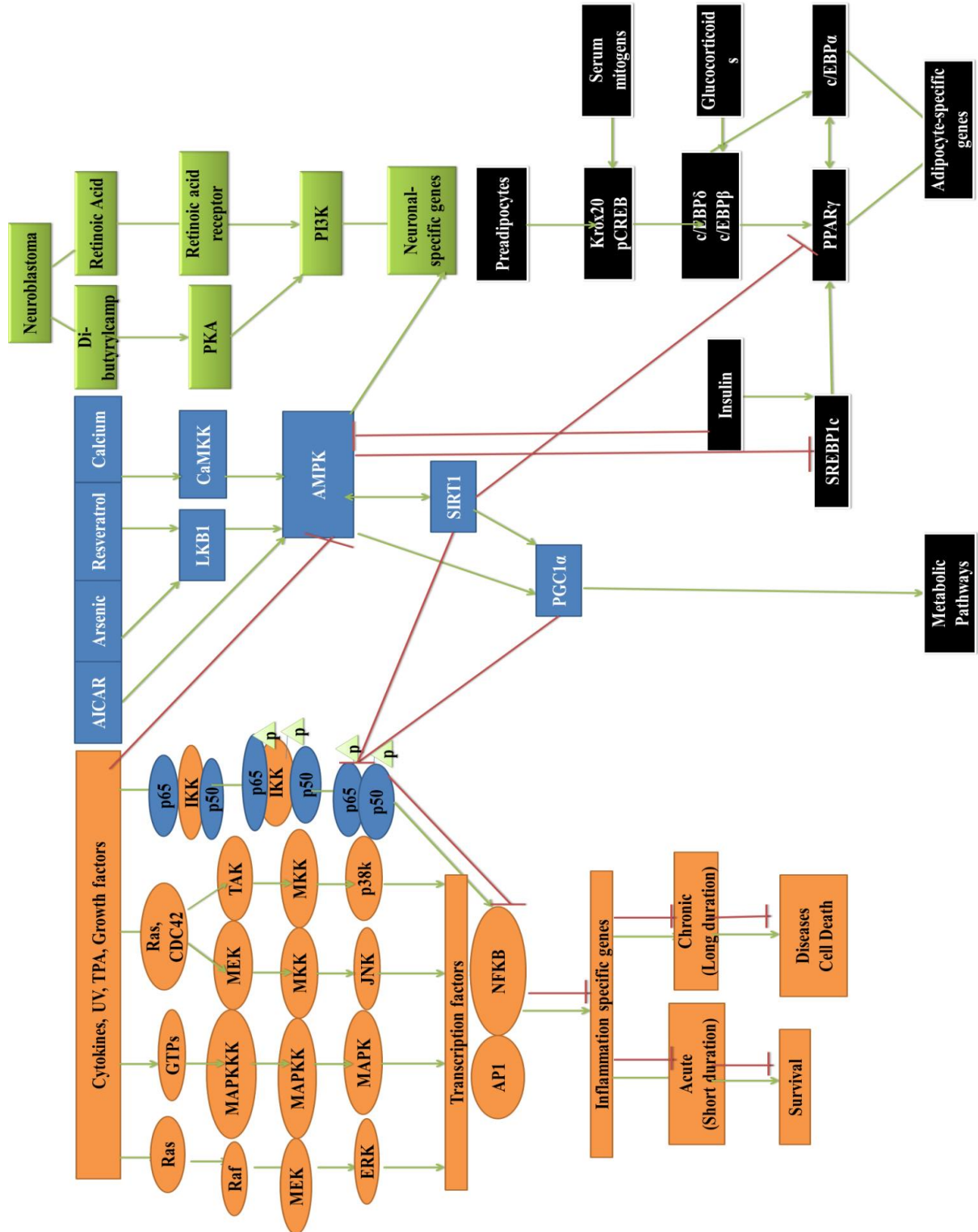


Figure 6.2: Proposed biochemical pathway of Resveratrol, MC4 and MR4 in inflammation, neuroblastoma differentiation and adipogenesis

MC4 & MR4 show biosimilar phenomenological effects as resveratrol in various disease models:

Resveratrol is a versatile molecule implicated to have therapeutic potential in cancer, heart diseases, inflammation, stroke, brain damage and aging to name a few (Baur 2006). I have reconfirmed the role of resveratrol through in-vitro studies in neuroblastoma differentiation, adipogenic differentiation and inflammation models. More importantly, I have established a biosimilar role for novel and potent analogs of resveratrol , MC4 and MR4 in each of these systems. This finding is highly significant owing to the fact that inspite of the highly desirable health benefits offered by resveratrol through diverse mechanism of actions, the pre-clinical/clinical performance of resveratrol has been far from the stellar performance observed in in-vitro studies (Tome-Carneiro 2013).

The current state of clinical data on resveratrol is ridden with issues of low bioavailability of about 2 μ M given a dose of 25mg-oral dose, despite high level of absorption most of the resveratrol was recovered in urine as sulphate and glucuronic acid conjugates (Walle 2004). Approaches to increase bioavailability by dose escalation studies using 5000mg of oral dose have reported an overall increase in the metabolite concentrations but failed to improve the bioavailability (Boocock 2007). These finding imply a rapid metabolism of resveratrol being the limiting factor to poor bioavailability.

Interestingly, the metabolites of resveratrol were mostly dihydroxymetabolites which is typical of most polyphenol, identified as trans- resveratrol- 3- O- glucuronide, and trans-resveratrol- 3-sulfate by Yu (2002) and trans-resveratrol-3-O-sulfate, trans-resveratrol-4'-O-sulfate, and transresveratrol-3-O-4'-O-disulfate by Miksits et. al., (2005) and 3-O-beta-D- and 4'-O-beta-D-glucuronide conjugates of resveratrol.

In the light of these studies, MC4 and MR4 offer the promise of improved bioavailability offered by the protective methoxy- group in 3, 4 and 5 position making them resistant to the action of Beta-glucuronidase and sulfatase. The low IC_{50} of MC4 (20nM) and MR4 (2 μ M) (Gossiau 2005) makes them an attractive alternative to resveratrol (IC_{50} 50 μ M) by offering a low dosage for similar physiological advantages.

Investigations into the potential of methoxy-derivatives of resveratrol has promise in preliminary in-vitro studies by evidenced by high accumulation in intestinal mucosa (Sale 2004) and overall longer half-life and lower clearance in mice studies (Lin 2009)

The biochemical targets of MC4 and MR4 which currently remain unknown. It would of interest to discover if MC4 and MR4 have similar and varied biochemical targets as resveratrol. This would establish the mechanism of action of these exciting molecules beyond the mimicking effect of resveratrol and hopefully advance into clinical trials.

Potent and novel analogs of resveratrol offer a huge pharmacokinetic advantage compared to resveratrol, a property of the protective methoxy groups making them

resistant to enzymatic degradation resulting in improved bioavailability. The findings of this thesis has established a biosimilar role for MC4 and MR4 in neuroblastoma differentiation, adipogenic differentiation and inflammation models offering exciting new small molecules for therapeutic indications in these disease areas without the limiting pharmacokinetic profile of resveratrol an otherwise excellent candidate for clinical testing.

Future work:

- 1) Resveratrol, MC4 and MR4 regulate multiple disease pathways including neuroblastoma proliferation, preadipocyte differentiation and inflammation. Hence a focused and more targeted approach is required for delivering these therapeutic candidates in order to prevent undesirable side-effects
- 2) Determining the molecular targets of Resveratrol, MC4, MR4, particularly AMPK, SIRT1 and PGC-1^α in neuroblastoma proliferation, preadipocyte differentiation and inflammation would allow for developing a targeted approach for therapeutics in these disease areas
- 3) MC4 and MR4 offer potential for improved bioavailability as drug candidates due to presence of methoxy-groups making them resistant to enzymatic degradation. Hence It is imperative to validate MC4 and MR4 in in-vivo disease models as an extension to the findings of significant efficacy in in-vitro disease models for neuroblastoma proliferation, preadipocyte differentiation and inflammation.
- 4) Given the finding that treatment with protease inhibitors protects cancer cells from heat induced death pathway, there is a need to design a combinatorial

approach of treatment with protease activators to improve the efficacy of heat treatment in cancer therapeutics.

This would shed light into the biochemical targets of MC4 and MR4 which currently remain unknown. It would of interest to discover if MC4 and MR4 have similar and varied biochemical targets as resveratrol. This would establish the mechanism of action of these exciting molecules beyond the mimicking effect of resveratrol and hopefully advance into clinical trials.

Potent and novel analogs of resveratrol offer a huge pharmacokinetic advantage compared to resveratrol, a property of the protective methoxy groups making them resistant to enzymatic degradation resulting in improved bioavailability. The findings of this thesis has established a biosimilar role for MC4 and MR4 in neuroblastoma differentiation, adipogenic differentiation and inflammation models offering exciting new small molecules for therapeutic indications in these disease areas without the limiting pharmacokinetic profile of resveratrol an otherwise excellent candidate for clinical testing.

REFERENCES:

- Abemayor, E. and N. Sidell (1989). "Human neuroblastoma cell lines as models for the in vitro study of neoplastic and neuronal cell differentiation." *Environ Health Perspect* 80: 3-15.
- Alarcón de la Lastra, C. and I. Villegas (2005). "Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications." *Molecular Nutrition & Food Research* 49(5): 405-430.
- Aluyen, J. K., Q. N. Ton, T. Tran, A. E. Yang, H. B. Gottlieb and R. A. Bellanger (2012). "Resveratrol: potential as anticancer agent." *J Diet Suppl* 9(1): 45-56.
- Alvarez-Guardia, D., X. Palomer, T. Coll, M. M. Davidson, T. O. Chan, A. M. Feldman, J. C. Laguna and M. Vazquez-Carrera (2010). "The p65 subunit of NF-kappaB binds to PGC-1alpha, linking inflammation and metabolic disturbances in cardiac cells." *Cardiovasc Res* 87(3): 449-458.
- Andrew Tee, P. Y. L., Glenn M. Marshall and Tao Liu (2012). "Neuroblastoma: A Malignancy Due to Cell Differentiation Block." *Neuroblastoma - Present and Future*.
- Bai, L., W.-J. Pang, Y.-J. Yang and G.-S. Yang (2008). "Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes." *Molecular and Cellular Biochemistry* 307(1-2): 129-140.
- Baur, J. A. and D. A. Sinclair (2006). "Therapeutic potential of resveratrol: the in vivo evidence." *Nat Rev Drug Discov* 5(6): 493-506.

- Bethell, D. R. and A. E. Pegg (1981). "Polyamines are needed for the differentiation of 3T3-L1 fibroblasts into adipose cells." *Biochem Biophys Res Commun* 102(1): 272-278.
- Bode, A. M. and Z. Dong (2000). "Signal transduction pathways: targets for chemoprevention of skin cancer." *The Lancet Oncology* 1(3): 181-188.
- Boheler, K. R., J. Czyz, D. Tweedie, H. T. Yang, S. V. Anisimov and A. M. Wobus (2002). "Differentiation of pluripotent embryonic stem cells into cardiomyocytes." *Circ Res* 91(3): 189-201.
- Boocock, D. J., G. E. Faust, K. R. Patel, A. M. Schinas, V. A. Brown, M. P. Ducharme, T. D. Booth, J. A. Crowell, M. Perloff, A. J. Gescher, W. P. Steward and D. E. Brenner (2007). "Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent." *Cancer Epidemiol Biomarkers Prev* 16(6): 1246-1252.
- Borra, M. T., B. C. Smith and J. M. Denu (2005). "Mechanism of human SIRT1 activation by resveratrol." *J Biol Chem* 280(17): 17187-17195.
- Brodeur, G. M. (2003). "Neuroblastoma: biological insights into a clinical enigma." *Nat Rev Cancer* 3(3): 203-216.
- Canto, C. and J. Auwerx (2009). "Caloric restriction, SIRT1 and longevity." *Trends Endocrinol Metab* 20(7): 325-331.
- Casero, R. A. and Marton, L. J (2007). "Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases." *Nature Reviews Drug Discovery* 6: 373-390.

- Chen, K. Y. and A. Y. Liu (1997). "Biochemistry and function of hypusine formation on eukaryotic initiation factor 5A." *Biol Signals* 6(3): 105-109.
- Chen, S., Z. Li, W. Li, Z. Shan and W. Zhu (2011). "Resveratrol inhibits cell differentiation in 3T3-L1 adipocytes via activation of AMPK." *Canadian Journal of Physiology and Pharmacology* 89(11): 793-799.
- Chen, S., Z. Li, W. Li, Z. Shan and W. Zhu (2011). "Resveratrol inhibits cell differentiation in 3T3-L1 adipocytes via activation of AMPK." *Can J Physiol Pharmacol* 89(11): 793-799.
- Chen, Y. F., P. Jobanputra, P. Barton, S. Bryan, A. Fry-Smith, G. Harris and R. S. Taylor (2008). "Cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs (etodolac, meloxicam, celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib) for osteoarthritis and rheumatoid arthritis: a systematic review and economic evaluation." *Health Technol Assess* 12(11): 1-278, iii.
- Chen, Z. P., Y. P. Yan, Q. J. Ding, S. Knapp, J. A. Potenza, H. J. Schugar and K. Y. Chen (1996). "Effects of inhibitors of deoxyhypusine synthase on the differentiation of mouse neuroblastoma and erythroleukemia cells." *Cancer Lett* 105(2): 233-239.
- Chung, J., H. Kubota, Y.-i. Ozaki, S. Uda and S. Kuroda (2010). "Timing-Dependent Actions of NGF Required for Cell Differentiation." *PLoS ONE* 5(2): e9011.
- Chung, J. H., V. Manganiello and J. R. Dyck (2012). "Resveratrol as a calorie restriction mimetic: therapeutic implications." *Trends Cell Biol* 22(10): 546-554.

- Chung, S., H. Yao, S. Caito, J.-w. Hwang, G. Arunachalam and I. Rahman (2010). "Regulation of SIRT1 in cellular functions: Role of polyphenols." *Archives of Biochemistry and Biophysics* 501(1): 79-90.
- Dasgupta, B. and J. Milbrandt (2007). "Resveratrol stimulates AMP kinase activity in neurons." *Proceedings of the National Academy of Sciences* 104(17): 7217-7222.
- de Lange, D. W., S. Verhoef, G. Gorter, R. J. Kraaijenhagen, A. van de Wiel and J. W. Akkerman (2007). "Polyphenolic grape extract inhibits platelet activation through PECAM-1: an explanation for the French paradox." *Alcohol Clin Exp Res* 31(8): 1308-1314.
- Dikomey, E. and J. Franzke (1992). "Effect of heat on induction and repair of DNA strand breaks in X-irradiated CHO cells." *Int J Radiat Biol* 61(2): 221-233.
- Eisele, P. S., S. Salatino, J. Sobek, M. O. Hottiger and C. Handschin (2013). "The peroxisome proliferator-activated receptor gamma coactivator 1alpha/beta (PGC-1) coactivators repress the transcriptional activity of NF-kappaB in skeletal muscle cells." *J Biol Chem* 288(4): 2246-2260.
- Erwin, B. G., D. R. Bethell and A. E. Pegg (1984). "Role of polyamines in differentiation of 3T3-L1 fibroblasts into adipocytes." *Am J Physiol* 246(3 Pt 1): C293-300.
- Farmer, S. R. (2006). "Transcriptional control of adipocyte formation." *Cell Metab* 4(4): 263-273.

- Fischer-Posovszky, P., V. Kukulus, D. Tews, T. Unterkircher, K. M. Debatin, S. Fulda and M. Wabitsch (2010). "Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner." *Am J Clin Nutr* 92(1): 5-15.
- Fischer-Posovszky, P., V. Kukulus, D. Tews, T. Unterkircher, K.-M. Debatin, S. Fulda and M. Wabitsch (2010). "Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner." *The American Journal of Clinical Nutrition* 92(1): 5-15.
- Fulda, S., A. M. Gorman, O. Hori and A. Samali (2010). "Cellular Stress Responses: Cell Survival and Cell Death." *International Journal of Cell Biology* 2010.
- Gossiau, A., M. Chen, C. T. Ho and K. Y. Chen (2005). "A methoxy derivative of resveratrol analogue selectively induced activation of the mitochondrial apoptotic pathway in transformed fibroblasts." *Br J Cancer* 92(3): 513-521.
- Gossiau, A., D. L.-e. Jao, R. Butler, A. Y. C. Liu and K. Y. Chen (2009). "Thermal killing of human colon cancer cells is associated with the loss of eukaryotic initiation factor 5A." *Journal of Cellular Physiology* 219(2): 485-493.
- Gossiau, A., S. Pabbaraja, S. Knapp and K. Y. Chen (2008). "Trans- and cis-stilbene polyphenols induced rapid perinuclear mitochondrial clustering and p53-independent apoptosis in cancer cells but not normal cells." *Eur J Pharmacol* 587(1-3): 25-34.
- GREGOIRE, F. M., C. M. SMAS and H. S. SUL (1998). "Understanding Adipocyte Differentiation." *Physiological Reviews* 78(3): 783-809.

- Gülçin, İ. (2010). "Antioxidant properties of resveratrol: A structure–activity insight." *Innovative Food Science & Emerging Technologies* 11(1): 210-218.
- He, Y., Y. Li, T. Zhao, Y. Wang and C. Sun (2013). "Ursolic Acid Inhibits Adipogenesis in 3T3-L1 Adipocytes through LKB1/AMPK Pathway." *PLoS ONE* 8(7): e70135.
- He, Y., Y. Li, T. Zhao, Y. Wang and C. Sun (2013). "Ursolic Acid Inhibits Adipogenesis in 3T3-L1 Adipocytes through LKB1/AMPK Pathway." *PLoS ONE* 8(7): e70135.
- Hildebrandt, B., P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix and H. Riess (2002). "The cellular and molecular basis of hyperthermia." *Critical Reviews in Oncology/Hematology* 43(1): 33-56.
- Hoogendijk, A. J., S. S. Pinhancos, T. van der Poll and C. W. Wieland (2013). "AMP-activated protein kinase activation by 5-aminoimidazole-4-carbox-amide-1-beta-D-ribofuranoside (AICAR) reduces lipoteichoic acid-induced lung inflammation." *J Biol Chem* 288(10): 7047-7052.
- Hoogendijk, A. J., S. S. Pinhanços, T. van der Poll and C. W. Wieland (2013). "AMP-activated Protein Kinase Activation by 5-Aminoimidazole-4-carbox-amide-1-β-d-ribofuranoside (AICAR) Reduces Lipoteichoic Acid-induced Lung Inflammation." *Journal of Biological Chemistry* 288(10): 7047-7052.
- Huang, Y., D. S. Higginson, L. Hester, M. H. Park and S. H. Snyder (2007). "Neuronal growth and survival mediated by eIF5A, a polyamine-modified translation initiation factor." *Proc Natl Acad Sci U S A* 104(10): 4194-4199.

- Hwang, J. T., I. J. Park, J. I. Shin, Y. K. Lee, S. K. Lee, H. W. Baik, J. Ha and O. J. Park (2005). "Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase." *Biochem Biophys Res Commun* 338(2): 694-699.
- Hyvönen, M, T. K., Janne Weisell, Marko Pietilä, Alex R. Khomutov, Jouko Vepsäläinen, Leena Alhonen and Tuomo A. Keinänen (2013). "Spermidine promotes adipogenesis of 3T3-L1 cells by preventing interaction of ANP32 with HuR and PP2A." *Biochem. J.* 453(3): 467-474.
- Ishii, I., Y. Ikeguchi, H. Mano, M. Wada, A. E. Pegg and A. Shirahata (2012). "Polyamine metabolism is involved in adipogenesis of 3T3-L1 cells." *Amino Acids* 42(2-3): 619-626.
- Ivanenkov, Y. A., K. V. Balakin and Y. Lavrovsky (2011). "Small molecule inhibitors of NF-kB and JAK/STAT signal transduction pathways as promising anti-inflammatory therapeutics." *Mini Rev Med Chem* 11(1): 55-78.
- Jao, D. L. and K. Y. Chen (2006). "Tandem affinity purification revealed the hypusine-dependent binding of eukaryotic initiation factor 5A to the translating 80S ribosomal complex." *J Cell Biochem* 97(3): 583-598.
- Jasiulionis, M. G., A. D. Luchessi, A. G. Moreira, P. P. Souza, A. P. Suenaga, M. Correa, C. A. Costa, R. Curi and C. M. Costa-Neto (2007). "Inhibition of eukaryotic translation initiation factor 5A (eIF5A) hypusination impairs melanoma growth." *Cell Biochem Funct* 25(1): 109-114.

- Jo, J., O. Gavrilova, S. Pack, W. Jou, S. Mullen, A. E. Sumner, S. W. Cushman and V. Periwai (2009). "Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth." *PLoS Comput Biol* 5(3): e1000324.
- Kopp, P. (1998). "Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'?" *Eur J Endocrinol* 138(6): 619-620.
- Li, Y., S. Xu, M. M. Mihaylova, B. Zheng, X. Hou, B. Jiang, O. Park, Z. Luo, E. Lefai, J. Y. Shyy, B. Gao, M. Wierzbicki, T. J. Verbeuren, R. J. Shaw, R. A. Cohen and M. Zang (2011). "AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice." *Cell Metab* 13(4): 376-388.
- Lin, H.-S. and P. C. Ho (2009). "A rapid HPLC method for the quantification of 3,5,4'-trimethoxy-trans-stilbene (TMS) in rat plasma and its application in pharmacokinetic study." *Journal of Pharmaceutical and Biomedical Analysis* 49(2): 387-392.
- Lin, K. H., G. Hsiao, C. M. Shih, D. S. Chou and J. R. Sheu (2009). "Mechanisms of resveratrol-induced platelet apoptosis." *Cardiovasc Res* 83(3): 575-585.
- Luke, A. J. O. N. and D. G. Hardie (2013). "Metabolism of inflammation limited by AMPK and pseudo-starvation." *Nature* 493(7432): 346-355.
- Medzhitov, R. (2008). "Origin and physiological roles of inflammation." *Nature* 454(7203): 428-435.

- Miksits, M., A. Maier-Salamon, S. Aust, T. Thalhammer, G. Reznicek, O. Kunert, E. Haslinger, T. Szekeres and W. Jaeger (2005). "Sulfation of resveratrol in human liver: evidence of a major role for the sulfotransferases SULT1A1 and SULT1E1." *Xenobiotica* 35(12): 1101-1119.
- Mitterberger, M. C. and W. Zwerschke (2013). "Mechanisms of Resveratrol-Induced Inhibition of Clonal Expansion and Terminal Adipogenic Differentiation in 3T3-L1 Preadipocytes." *J Gerontol A Biol Sci Med Sci*.
- O'Neill, L. A. and D. G. Hardie (2013). "Metabolism of inflammation limited by AMPK and pseudo-starvation." *Nature* 493(7432): 346-355.
- Pang, W. J., S. D. Sun, L. Bai, Y. J. Yang and G. S. Yang (2006). "[Effects of resveratrol on pig primary preadipocytes proliferation, differentiation and transcription expression of Sirt1 gene]." *Sheng Wu Gong Cheng Xue Bao* 22(5): 850-855.
- Park, M. H., Y. B. Lee and Y. A. Joe (1997). "Hypusine is essential for eukaryotic cell proliferation." *Biol Signals* 6(3): 115-123.
- Park, M. H., E. C. Wolff, Y. B. Lee and J. E. Folk (1994). "Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines." *J Biol Chem* 269(45): 27827-27832.
- Phrakonkham, P., S. Viengchareun, C. Belloir, M. Lombes, Y. Artur and M. C. Canivenc-Lavier (2008). "Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis." *J Steroid Biochem Mol Biol* 110(1-2): 95-103.

- Prasad, K. N. (1975). "DIFFERENTIATION OF NEUROBLASTOMA CELLS IN CULTURE." *Biological Reviews* 50(2): 129-165.
- Rayalam, S., J. Y. Yang, S. Ambati, M. A. Della-Fera and C. A. Baile (2008). "Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes." *Phytother Res* 22(10): 1367-1371.
- Reboulleau, C. P. (1986). "Extracellular calcium-induced neuroblastoma cell differentiation: involvement of phosphatidylinositol turnover." *J Neurochem* 46(3): 920-930.
- Richard I. Morimoto, A. T., Costa Georgopoulos (1994). "Progress and Perspectives on the Biology of Heat Shock Proteins and Molecular Chaperones."
- Sakaguchi, Y., L. C. Stephens, M. Makino, T. Kaneko, F. R. Strebel, L. L. Danhauser, G. N. Jenkins and J. M. Bull (1995). "Apoptosis in tumors and normal tissues induced by whole body hyperthermia in rats." *Cancer Res* 55(22): 5459-5464.
- Sale, S., R. G. Tunstall, K. C. Ruparelia, G. A. Potter, W. P. Steward and A. J. Gescher (2005). "Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3,4,5,4'-tetramethoxystilbene (DMU-212) on adenoma development in the Apc(Min+) mouse and cyclooxygenase-2 in human-derived colon cancer cells." *Int J Cancer* 115(2): 194-201.
- Salminen, A., J. T. Hyttinen and K. Kaarniranta (2011). "AMP-activated protein kinase inhibits NF- κ B signaling and inflammation: impact on healthspan and lifespan." *Journal of Molecular Medicine* 89(7): 667-676.

- Sasaki, K., M. R. Abid and M. Miyazaki (1996). "Deoxyhypusine synthase gene is essential for cell viability in the yeast *Saccharomyces cerevisiae*." *FEBS Lett* 384(2): 151-154.
- Schnier, J., H. G. Schwelberger, Z. Smit-McBride, H. A. Kang and J. W. Hershey (1991). "Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast *Saccharomyces cerevisiae*." *Mol Cell Biol* 11(6): 3105-3114.
- Sidell, N. (1982). "Retinoic Acid-Induced Growth Inhibition and Morphologic Differentiation of Human Neuroblastoma Cells In Vitro." *Journal of the National Cancer Institute* 68(4): 589-596.
- Society, A. C. (2013). "Hyperthermia to treat cancers."
- Sozio, M. S., C. Lu, Y. Zeng, S. Liangpunsakul and D. W. Crabb (2011). "Activated AMPK inhibits PPAR- α and PPAR- γ transcriptional activity in hepatoma cells." *Am J Physiol Gastrointest Liver Physiol* 301(4): G739-747.
- Stein, U., B. Rau, P. Wust, W. Walther and P. M. Schlag (1999). "Hyperthermia for treatment of rectal cancer: evaluation for induction of multidrug resistance gene (mdr1) expression." *Int J Cancer* 80(1): 5-12.
- Steinberg, G. R., M. J. Watt and M. A. Febbraio (2009). "Cytokine Regulation of AMPK signalling." *Front Biosci (Landmark Ed)* 14: 1902-1916.
- Szkudelska, K. and T. Szkudelski (2010). "Resveratrol, obesity and diabetes." *Eur J Pharmacol* 635(1-3): 1-8.

- Tak, P. P. and G. S. Firestein (2001). "NF-kappaB: a key role in inflammatory diseases." *J Clin Invest* 107(1): 7-11.
- Takeuchi, K., K. Nakamura, M. Fujimoto, S. Kaino, S. Kondoh and K. Okita (2002). "Heat stress-induced loss of eukaryotic initiation factor 5A (eIF-5A) in a human pancreatic cancer cell line, MIA PaCa-2, analyzed by two-dimensional gel electrophoresis." *ELECTROPHORESIS* 23(4): 662-669.
- Tome-Carneiro, J., M. Larrosa, A. Gonzalez-Sarrias, F. A. Tomas-Barberan, M. T. Garcia-Conesa and J. C. Espin (2013). "Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence." *Curr Pharm Des* 19(34): 6064-6093.
- Udenigwe, C. C., V. R. Ramprasath, R. E. Aluko and P. J. H. Jones (2008). "Potential of resveratrol in anticancer and anti-inflammatory therapy." *Nutrition Reviews* 66(8): 445-454.
- van der Zee, J. (2002). "Heating the patient: a promising approach?" *Annals of Oncology* 13(8): 1173-1184.
- Verga, D. S. a. N. (2011). "Cancer Treatment with Hyperthermia " *Current Cancer Treatment-Novel Beyond Conventional Approaches*.
- Vingtdeux, V., P. Chandakkar, H. Zhao, P. Davies and P. Marambaud (2011). "Small-molecule activators of AMP-activated protein kinase (AMPK), RSVA314 and RSVA405, inhibit adipogenesis." *Mol Med* 17(9-10): 1022-1030.
- Vuohelainen, S., E. Pirinen, M. Cerrada-Gimenez, T. A. Keinanen, A. Uimari, M. Pietila, A. R. Khomutov, J. Janne and L. Alhonen (2010). "Spermidine is

indispensable in differentiation of 3T3-L1 fibroblasts to adipocytes." *J Cell Mol Med* 14(6b): 1683-1692.

- Walle, T., F. Hsieh, M. H. DeLegge, J. E. Oatis and U. K. Walle (2004). "HIGH ABSORPTION BUT VERY LOW BIOAVAILABILITY OF ORAL RESVERATROL IN HUMANS." *Drug Metabolism and Disposition* 32(12): 1377-1382.
- Wang, X., D. Meng, Q. Chang, J. Pan, Z. Zhang, G. Chen, Z. Ke, J. Luo and X. Shi (2010). "Arsenic inhibits neurite outgrowth by inhibiting the LKB1-AMPK signaling pathway." *Environ Health Perspect* 118(5): 627-634.
- Weiss, U. (2008). "Inflammation." *Nature* 454(7203): 427.
- Westerheide, S. D., J. Anckar, S. M. Stevens, Jr., L. Sistonen and R. I. Morimoto (2009). "Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1." *Science* 323(5917): 1063-1066.
- Wust, P., B. Hildebrandt, G. Sreenivasa, B. Rau, J. Gellermann, H. Riess, R. Felix and P. M. Schlag (2002). "Hyperthermia in combined treatment of cancer." *The Lancet Oncology* 3(8): 487-497.
- Xin, W., M. Dan, C. Qingshan, P. Jingju, Z. Zhuo, C. Gang, K. Zunji, L. Jia and S. Xianglin (2009). "Arsenic Inhibits Neurite Outgrowth by Inhibiting the LKB1–AMPK Signaling Pathway." *Environmental Health Perspectives* 118(5).
- Xu, M., W. D. Wright, R. Higashikubo and J. R. Roti (1998). "Intracellular distribution of hsp70 during long duration moderate hyperthermia." *Int J Hyperthermia* 14(2): 211-225.

- Yang, H., W. Zhang, H. Pan, H. G. Feldser, E. Lainez, C. Miller, S. Leung, Z. Zhong, H. Zhao, S. Sweitzer, T. Considine, T. Riera, V. Suri, B. White, J. L. Ellis, G. P. Vlasuk and C. Loh (2012). "SIRT1 Activators Suppress Inflammatory Responses through Promotion of p65 Deacetylation and Inhibition of NF- κ B Activity." PLoS ONE 7(9): e46364.
- Yap, F., L. Craddock and J. Yang (2011). "Mechanism of AMPK suppression of LXR-dependent Srebp-1c transcription." Int J Biol Sci 7(5): 645-650.
- Yu, C., Y. G. Shin, A. Chow, Y. Li, J. W. Kosmeder, Y. S. Lee, W. H. Hirschelman, J. M. Pezzuto, R. G. Mehta and R. B. van Breemen (2002). "Human, rat, and mouse metabolism of resveratrol." Pharm Res 19(12): 1907-1914.
- Zhang, J. W., Q. Q. Tang, C. Vinson and M. D. Lane (2004). "Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes." Proc Natl Acad Sci U S A 101(1): 43-47.
- Zhu, X., Q. Liu, M. Wang, M. Liang, X. Yang, X. Xu, H. Zou and J. Qiu (2011). "Activation of Sirt1 by Resveratrol Inhibits TNF- α Induced Inflammation in Fibroblasts." PLoS ONE 6(11): e27081.