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Investigation into Pharmacological Profile and Mechanism of Action of Abscisic acid With Reference to it's Possible Therapeutic Usefulness

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In

PHARMACY

(Faculty of Medicine)

By

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FEBRUARY, 2012

Certificate

This is to certify that the thesis entitled **"Investigation into Pharmacological Profile and Mechanism of Action of Abscisic acid With Reference to it's Possible Therapeutic Usefulness"** represents bonafide work of **MR. DEVANG BHIKHUBHAI SHETH**, carried out under my guidance and supervision. The work mentioned in this thesis was carried out at R. K. College of Pharmacy, Rajkot; S. K. Patel College of Pharmaceutical Education and Research, Mehsan and Maliba Pharmacy College, Bardoli during the period of years 2007-2012. The work is up to my satisfaction.

Date: 27/02/2012

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DECLARATION BY THE CANDIDATE

I, Devang B. Sheth, hereby, declare that Saurashtra University, Rajkot shall have the right to preserve, use and disseminate this thesis, in print or electronic format, for academic/research purpose.

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Ph.D. 7 of Saurashtra University

The contents of this thesis are my own work, carried out under supervision of Dr. T. R. Desai. It leads to some contribution in pharmacy, supported by necessary references.

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DECLARATION

I, Devang B. Sheth, hereby, declare that the thesis entitled **"Investigation into Pharmacological Profile and Mechanism of Action of Abscisic acid With Reference to its Possible Therapeutic Usefulness"** is a bonafide research work, carried out by me, under the guidance of Dr. T. R. Desai. This work is original and has not been submitted in part or full for any degree/diploma to other University**.**
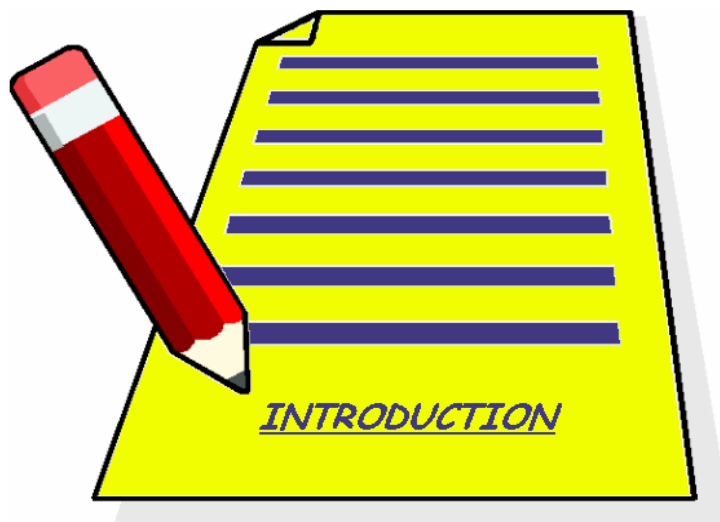
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INDEX

Introduction

Drug research is a unique multi-disciplinary process leading to the development of novel therapeutic agents for disease states that have unmet needs. Traditional health care systems, using medicinal plants, can be recognized and used as a starting point for the development of novelty in drugs (NCE). The plants are rich reservoir of potential leads for drug discovery against various disorders. The desire of searching new potential candidates for various disease have opened new venue in the field of drug discovery.

One such target could be phytohormones – a field which is relatively untouched so far. In plants, each developmental process integrates a network of signaling events that are regulated by different phytohormones, and interactions among hormonal pathways are essential to modulate their effect. As human hormones significantly affect the activity of every cell in the body such as influence on mental activity, physical agility, and body build and stature; plant hormones also do play vital functions in wide varieties of plant kingdom. The hormone concept as developed for animals cannot easily be translated to plants. Still, plants have regulated growth, determined steps of differentiation, different metabolic rates in cells, and communication between cells (Cerana, 2006; Farnsworth, 1976). Phytohormones regulate most of the life cycle events in plants at genetic level including germination, cell division and extension, flowering, fruit ripening, seed dormancy and death.

Plant biologists believe that hormones exert their effects via specific receptor sites in target cells, similar to the mechanisms found in animals (Farnsworth, 1976). The number of different plant hormones is rather small when compared to animals. Many animal hormones, especially the macromolecular ones have a very limited action spectrum that has its root in the selectivity and the cell-specific or tissue-specific distribution of the respective receptors. In contrast the receptors for plant hormones have wide-spread distribution.

Many of plant hormones have been tested for their usefulness in disease status. Plant stress hormones activate cellular responses, including cell death, to diverse stress situations in plants. Researchers have found that some plant stress hormones share the ability to adversely affect human cancer cells. For example, sodium salicylate has been found to suppress proliferation of lymphoblastic leukemia, prostate, breast, and melanoma human cancer cells (Flescher and Eliezer, 2005). Jasmonates induced death

Devang B. Sheth

in leukemic cells isolated from the blood of chronic lymphocytic leukemia (CLL) patients and increased significantly the survival of lymphoma-bearing mice (Fingrut and Flescher, 2002; Flescher and Eliezer, 2005). Methyl jasmonate has been found to induce cell death in a number of cancer cell lines. Steroidal plant hormone, 24-epibrassinolide can be used for the treatment of infections caused by virus responsible for human immune deficiency (HIV). Plant hormones have beneficial effects on the human immune system, particularly the gibberellins and abscisic acid. Natural hormone replacement therapy (NHRT) is used to treat hormone imbalances and deficiencies in some cases.

Recent studies on some phytohormones, both in-vivo and in-vitro, have opened a new avenue to discover new chemical entities in the field of phyto-pharmacology.

One such promising molecule is abscisic acid. Since its discovery in 1965, abscisic acid (ABA) has received considerable attention as an important phytohormone, and more recently, as a candidate medicinal in humans. In plants it has been shown to regulate important physiological processes such as response to drought stress, and dormancy. The discovery of ABA synthesis in animal cells has generated interest in the possible parallels between its role in plant and animal systems. ABA recognition in plants has been shown to occur at both the intra- and extracellularly but little is known about the perception of ABA by animal cells. A few ABA molecular targets have been identified *in vitro* (e.g., calcium signalling, G protein-coupled receptors) in both plant and animal systems.

The isoprenoid phytohormone abscisic acid (ABA), commonly known as one of the five major classes of plant hormones, plays important roles during many phases of the plant life cycle including seed development and dormancy, in plant responses to various environmental stresses and host response. ABA is unique from other phytohormones such as auxins, giberellins, and cytokinins in that it is a single compound, rather than a class of phytohormones, and is now known to be ubiquitous in *Viridiplantae* (green plants). ABA activity has also been reported in fungi (Tsavkelova et al., 2006), marine sponges (Zocchi et al., 2001; Zocchi et al., 2003) and most recently in human cells (Bruzzone et al., 2007; Magnone et al., 2009). ABA research has taken a new turn outside the world of plant physiology with the publication of a series of papers showing biological activity in mammalian cells and

pre-clinical studies suggesting a possible role for ABA as potential therapeutic agent. ***Hence, in the present project we resolved to explore pharmacological profile of abscisic acid which, on further investigations, could lead to discovery of its therapeutic potential.***

Over the past 30 years, as obesity rates have surged towards epidemic proportions, health practitioners have reported an attendant rise in the number of patients diagnosed with insulin resistance (Finkelstein et al., 2003), a condition characterized by the diminished ability of muscle or other peripheral tissues to uptake glucose from the bloodstream. According to the most recent World Health Organization estimates, 1.6 billion adults are overweight and 300 million are obese. One of the consequences of insulin resistance is chronic hyperglycemia, which induces the micro and macrovascular disorders associated with Type 2 Diabetes (T2D) (Vasudevan, 2006). In addition to T2D, however, researchers are finding that the costs of insulin resistance extend to many chronic diseases, including coronary vascular disease (CVD), atherosclerosis, and hypertension (Center for Disease Control and Prevention, 2005).

Because even moderate insulin resistance puts one at an elevated risk for developing CVD and other insulin resistance-related chronic diseases, a number of questions have arisen concerning when patients should begin taking an anti-diabetic medication and also what type of medication they should be prescribed. The more traditional anti-diabetic treatments include the sulfonylureas and α-glucosidase inhibitors, which reduce the consequences of chronic hyperglycemia by increasing pancreatic insulin secretion and inhibiting intestinal glucose absorption, respectively (Center for Disease Control and Prevention, 2005). These drugs, however, are designed to manage hyperglycemia rather than treat it, and they do not have any significant effect on the progression of insulin resistance. Because of this, they are often used in conjunction with the newer class of anti-diabetic treatments known as the "insulin sensitizers."

As their name implies, "insulin sensitizers" increase the sensitivity of peripheral tissues to the actions of endogenous insulin, thereby directly counteracting the effect of obesity. The two main classes of insulin-sensitizing drugs include the biguanides (i.e. metformin) and thiazolidinediones (TZDs, i.e. rosiglitazone and pioglitazone). While both are classified as "insulin sensitizers," biguanides and TZDs act through

Devang B. Sheth

distinct molecular mechanisms. Biguanides lower hepatic glucose production and increase intramyocellular fatty acid oxidation by increasing the activity of the signal transduction protein AMP kinase (AMPK) (Zhou et al., 2001). Conversely, TZDs serve as synthetic ligands for a key metabolic regulator and transcription factor known as peroxisome proliferator-activated receptor γ (PPAR γ) (Lehmann et al., 1995). PPAR γ is a member of the nuclear receptor superfamily, which consists of 48 ligand-induced transcription factors that respond to nutrients, xenobiotics, and various hormones and endogenously produced compounds, and is found in high concentrations in white adipose tissue (WAT), immune cells, and the colonic epithelium (Braissant et al., 1996; Desvergne and Wahli, 1996). PPARs, which in addition to PPAR γ also include PPARs α and δ, are endogenously activated by fatty acids and products of lipid metabolism (Desvergne and Wahli, 1996).

One notable difference between the biguanides and TZDs is that the latter, perhaps due to activating a central metabolic regulator, have the added benefits of being anti-hypertensive and anti-atherogenic (Wang et al., 2005; Dobrian et al., 2004; Scheen and Lefebvre, 2005). Comparisons between biguanides and TZDs have indicated that TZDs are more effective in improving glycemic control and in enhancing whole body insulin sensitivity (Seufert et al., 2004; Knowler et al., 2005). Therefore, with regard to the insulin sensitizing medications, PPAR γ agonists in particular appear to represent a more promising avenue for future drug design and disease treatment/prevention.

While TZDs have shown effectiveness in improving insulin sensitivity in patients with both T2D and prediabetes (Dumasia et al., 2005), these compounds, as are the case with many pharmaceutical agents, are associated with a number of unwanted side effects which limit their desirability and availability to millions of potential users (Nesto et al., 2003). For instance, in some recent clinical trials pioglitazone has been shown to increase the incidences of both edema and congestive heart failure (Dormandy et al., 2005; Scheen and Lefebvre, 2005). TZDs are also associated with weight gain and, in the past, hepatotoxicity (Nesto et al., 2003). Recently, a study in the New England Journal of Medicine showed that use of rosiglitazone was associated with a 43% increase in myocardial infarction and 64% increase in risk of cardiovascular mortality (Nissen SE and Wolski, 2007). With regard to TZDs, there

Devang B. Sheth

are still many questions concerning how PPAR γ ligands improve systemic insulin sensitivity.

Abscisic acid is structurally similar to thiazolidinediones. Moreover, PPAR γ -responsive genes were found to be induced or activated by abscisic acid in 3T3-L1 preadipocytes *in vitro* (Guri et al., 2008; Lehmann et al., 1995). It is also found that ABA could be used as a nutritional supplement to combat type II diabetes and obesity-related inflammation (Guri et al., 2007). ***In the light of these facts, in present project we resolved to investigate pharmacological effects of abscisic acid on insulin resistance.***

Cancer is a class of diseases in which, a group of cells display the traits of uncontrolled growth (growth and division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). (Fodde R and Smits R, 2002; Merlo *et al.*, 2006). Today it is believed that cancer is a leading cause of death where it accounts for 7.6 million deaths which are about 13% of all deaths in 2008 (WHO, 2011). The main types of cancer leading to overall cancer mortality each year are lung (1.3 million deaths/year), stomach (803000 deaths), colorectal (639000 deaths), liver (610000 deaths) and breast (519000 deaths) (Garcia et al., 2007). More than 70% of all cancer deaths occurred in low- and middle-income countries (Boyle and Levin, 2008; WHO, 2011). Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO, 2011). Breast cancer, for instance, is the most common cancer disease in woman globally. The American Cancer Society estimated that in year 2008, about 182,460 women in the USA will be diagnosed with it and 40,480 women will die of breast cancer (Kelly, 2008). Breast cancer is currently the top cancer in women worldwide, both in the developed and the developing world (WHO, 2011).

Most of anti-cancer drugs lead to serious adverse effects like immune suppression, hair loss, nausea, vomiting, etc. They are also associated with occurrence of irreversible degenerative cardiomyopathy, hepatotoxicity, nephrotoxicity, ototoxicity, and encephalopathy which limits their clinical usefulness (Rang et al., 2005). There are many kinds of drugs that have been discovered and used as an alternative treatment to stop the progressive growing and spreading of the breast cancer such as

Devang B. Sheth

selective estrogen receptor modulators (e.g. Tamoxifen) and aromatase inhibitors (e.g. anastrozole, exemestane) (Newman and Cragg, 2006). Possible side effects of SERMs include fatigue, hot flashes, increased chances of endometrial cancer and vaginal dryness. Side effects of aromatase inhibitors include joint and muscle pain, as well as an increased risk of bone thinning (osteoporosis) (Rang et al., 2005). Clearly, these serious side effects limit the overall clinical utility of these compounds. Even targeted drug treatments, which attack specific abnormalities within cancer cells, like trastuzumab and bevacizumab also are associated with serious adverse drug effects like cardiotoxicity, high blood pressure, sore mouth, skin rashes etc. Thus, search for novel and safe drug in breast cancer is desirable. The discovery of a new alternative medicine which is more to natural basis is highly welcome.

Recently, there is increasing interest in the search for plant based hormones for the development of new pharmaceuticals, along with the increase of deadly illness such as cancer (Newman et al., 2003). Many phytohormones such as jasmonates and salicylates have been shown to suppress growth of various types of cancer cells (Flescher and Eliezer, 2005).

Limited information is available in the peer-reviewed literature regarding the possible role of ABA in the prevention and treatment of cancer. However, a patent was issued to Livingston-Wheeler in 1976 for its use as an anti-cancer compound (Livingston, 1976). The patent describes the use of a solid myeloid leukemia C1498 transplantation model in C57BL/6 mice to investigate the anti-cancer efficacy of ABA. The inventor reported increased survival rates at 14 days post-transplantation in mice treated with ABA in comparison to control mice. The inventor also proposed that ABA "neutralized" the human chorionic gonadotropin (hCG) that reportedly coated cancer cells, thereby facilitating anti-cancer immune responses ABA may represent a potential cancer treatment due to its ability to modulate calcium signalling (Livingston, 1976). More specifically, modulates pathways in plants and animals involving cADPR which control the increase in $[Ca^{+2}]_{int}$ (Wu et al., 1997). In plants, ABA depolarizes plasma membranes, which activates potassium ion channels and thereby extrudes K^+ outside the cells (Schroeder et al., 1987). Ion channel depolarization is dependent on calcium, which is an ABA second messenger (Schroeder et al., 2001). The increase in calcium concentrations also leads to increased nitric oxide (NO) production (Bodrato et al., 2009). As calcium signaling is

Devang B. Sheth

a key regulator of apoptosis, changes in calcium distribution in the cell activate cellular cascades which lead to cell death (Hajnoczky et al., 2003).

Mechanistically, some of the pathways activated by ABA and those modulated by chemotherapeutic agents used for treating cancer are strikingly similar. For instance, several chemotherapeutic agents modulate pathways leading to increased intracellular calcium concentrations. Medications such as staurosporine, doxorubicin, tamoxifen, and etoposide act as anti-cancer agents which lead to the death of cancer cells by increasing $[Ca^{+2}]_{int}$ resulting in oxidative stress followed by apoptosis (Kruman et al., 1998; Panaretakis et al., 2005; Parihar et al., 2008).

Moreover, epidemiologic studies demonstrated that the consumption of diet rich in abscisic acid is associated with a decreased risk of cancer (Riboli and Norat, 2003; Wigmore, 1985). Dr. Ann Wigmore mentioned that wheatgrass contains a variety of elements that is believed to be anticancer and abscisic acid is one of them. She reported that wheatgrass and controlled diet cured breast cancer of few patients. Even in experimental animal showed that even a tiny amount of abscisic acid has tremendous effect on cancer (Wigmore, 1985). Tan et al. (2006) reported in a China patent that ABA effectively inhibits the proliferation of tumour cells; stagnates the cells in S phase, stops cell division, and induces differentiation of tumour cells or reverts cancerous cells to normal cells. ABA was reported to inhibit proliferation and induced differentiation in human SMMC- 7221 cells (Ma et al., 2006) and in nude mice transplanted with human hepatocarcinoma (Lu et al., 2007). Thus, exploring the role of ABA in cancer prevention and treatment in the context of well controlled, mechanistically oriented projects may result in the development of novel ABA-based chemotherapeutic approaches against cancer. Additionally, abscisic acid is a close relative of Vitamin A. All these findings and observations suggest strong anti-cancer potential of abscisic acid.

Low cytotoxicity to healthy cells and high cytotoxicity to cancerous cells is the ultimate goal of many chemotherapy drugs. In-vitro cell based cytotoxicity assay is an easy and cost effective tool for early stage of drug discovery. Most of the commonly used cytotoxic anticancer drugs were discovered through random high-throughput screening of synthetic compounds and natural products in cell based cytotoxicity assays. *In vitro* cytotoxicity assays has been used to rapidly evaluate the potential

Devang B. Sheth

toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound. ***Hence, in the present project, we decided to screen abscisic acid for potential in-vitro anticancer activity in breast cancer cell-lines.***

Calcium is the most important regulator of cell function. Many physiological mechanisms and drugs operate, directly or indirectly, by influencing the free intra cellular concentration of calcium. Different types of Ca^{+2} channels are involved in important physiological functions such as contraction of muscles, activation of various enzymes, release of neurotransmitters and hormones, etc. Intracellular calcium is usually bound to a protein called calmodulin. The calcium-calmodulin complex acts together with a further component (RE, response element, also a protein) as a protein kinase catalyzing the phosphorylation of numerous proteins that themselves control independent but partially parallel developmental processes, differentiation, and movements within the cell (Rang et al., 2005). Abnormal Ca^{+2} signalling is involved in many pathophysiological conditions, such as, cardiac dysrhythmias, hypertension, epileptogensis, ischaemic cell death, and endocrine disorders. Many clinically important drugs like anti-hypertensive agents (e.g. nifedine, verapamil, diltiazem); anti-epileptic drugs (e.g. ethosuximide, sodium valproate, gabapentin, pregabalin); antidysrhythmic agents (e.g. class-IV antidysrhythmic agents), spasmolytic agents, etc. act directly or indirectly by blocking calcium release (Rang et al., 2005).

Over the past few years, evidence from a variety of sources has suggested that calcium ions are involved in the molecular events underlying the action of growth substance in plants (De Silva et al., 1985). Ca^{+2} is a second messenger and intracellular regulator which regulates of growth and development in plants (Hepler, 2005).

Recent studies of the control of stomatal aperture by abscisic acid have suggested that its cellular action depends on the availability of calcium ions, which appear to function as secondary messengers. In plants, it has been demonstrated that ABA stimulates the release of intracellular calcium in conjunction with the up-regulation of cyclic ADP-ribose (Wu et al., 1997). In plants, ABA depolarizes plasma membranes, which activates potassium ion channels and thereby extrudes K^+ outside the cells

Devang B. Sheth

(Schroeder et al., 1987). Ion channel depolarization is dependent on calcium, which is an ABA second messenger (Schroeder et al., 2001). The increase in calcium concentrations also leads to increased nitric oxide (NO) production (Bodrato et al., 2009). Huddart et al. (1986) found that ABA may serve as a universal Ca^{+2} agonist across taxonomic kingdoms based on experiments with various smooth muscle preparations and a cyanobacterium.

In contrast, Lynch et al (1991) reported that abscisic acid (ABA) analogue SD217595 inhibits K^+-induced phasic and tonic contractions of rat bladder detrusor smooth muscle strips. This dual inhibition was hypothesized due to blockade of two subtypes of voltage-operated calcium channels with T- and L-type characteristics. This was further supported by finding that ABA is without significant Ca^{+2} modulatory activity in rat prostatic and epididymal vas deferens smooth muscle preparation but the ABA analogue SD217595 possesses strong Ca^{+2} entry blocking ability (Masters et al., 1994). Thus, role of abscisic acid in relevance to its Ca^{+2} channel modulatory action is controversial. ***Hence, in the present study, we attempted to assess effects of Ca^{+2} channel modulatory action of abscisic acid, using isolated rat ileum and in-vivo effect on various cardiac parameters in rat.***

Devang B. Sheth

Review of Literature

3.1 Plant hormones

Hormones in plants are called phytohormones. They are small molecules distributed within tissues from cell to cell, as in the case of auxins, *via* vascular bundles (as in the case of cytokinin), or via the intercellular space (ethylene). A number of results indicate that phytohormones enter cells and regulate intracellular processes, though hardly anything about their intracellular distribution or about their transport from one compartment into another is known. It remains open, too, whether they are stored in one or the other compartment, and whether they become biologically active by being set free from such compartments.

They regulate most of the life cycle events in plants, such as germination, cell division and extension, flowering, fruit ripening, seed dormancy and death. Five plant hormones have long been identified: auxin, cytokinin, gibberellin, abscisic acid, and ethylene. Recent discoveries of other plant hormones include brassinosteroids, salicylates, and jasmonates (Kletter et al., 1997).

Auxins are primarily responsible for protein synthesis and promote the growth of the plant's length. The most common auxin, indoleacetic acid (IAA), is usually formed near the growing top shoots and flows downward, causing newly formed leaves to grow longer. Auxins stimulate growth toward light and root growth (Kokate et al., 2007).

Gibberellins, which form in the seeds, young leaves, and roots, are also responsible for protein synthesis, especially in the main stem of the plant. Unlike auxins, gibberellins move upward from the roots (Kokate et al., 2007). Cytokinins form in the roots and move up to the leaves and fruit to maintain growth, cell differentiation, and cell division. Among the growth inhibitors are abscisic acid, which promotes abscission, or leaf fall; dormancy in buds; and the formation of bulbs or tubers, possibly by preventing the synthesis of protein.

Ethylene, another inhibitor, also causes abscission, perhaps by its destructive effect on auxins, and it also stimulates the ripening of fruit.

Brassinosteroids act with auxins to encourage leaf elongation and inhibit root growth. Brassinosteroids also protect plants from some insects because they work against

Devang B. Sheth

some of the hormones that regulate insect molting. Salicylates stimulate flowering and cause disease resistance in some plants. Jasmonates regulate growth, germination, and flower bud formation. They also stimulate the formation of proteins that protect the plant against environmental stresses, such as temperature changes or droughts.

3.2 Abscisic acid

3.2.1 Introduction

The isoprenoid phytohormone abscisic acid (ABA), commonly known as one of the five major classes of plant hormones, plays important roles during many phases of the plant life cycle including seed development and dormancy, in plant responses to various environmental stresses and host response. ABA is unique from other phytohormones such as auxins, giberellins, and cytokinins in that it is a single compound, rather than a class of phytohormones, and is now known to be ubiquitous in *Viridiplantae* (green plants) (Bassaganya-Riera et al., 2010). It was called "abscisin II" originally because it was thought to play a major role in abscission of fruits. At about the same time another group was calling it "dormin" because they thought it had a major role in bud dormancy. Eventually, the roles of ABA in bud dormancy and abscission are now found to be minor. Though ABA generally is thought to play mostly inhibitory roles, it has many promoting functions as well (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992). Abscisic acid has previously been extracted from leaves of Lupin (*Lupinus cosentinii*), Apricot (*Prunus armeniaca*), Avocado (*Persea Americana*), Sunflower (*Helianthus annuus*), Grapevine (*Vitis vinifera*), Tomato (*Lycopersicon esculentum*), Spinach (*Spinada oleracea*), Orange (*Citrus sinensis*) and Mango (*Mangifera indica*) (Loveys BR and van Dijk HM, 1988). ABA and its metabolites have also been isolated from *Brassica napus* and *Brassica* rapa seed (Zhou et al., 2004).

3.2.2 History of abscisic acid

In 1963, abscisic acid was first identified and characterized by Frederick Addicott and his associates. It was first isolated in studies relating plant compounds with bud dormancy and fruit abscission (cotton), and hence was initially known as dormin or abscissin. Ironically, the roles of ABA in bud dormancy and abscission are now considered to be minor. Two compounds were isolated and called abscisin I and

[19]

abscisin II. Abscisin II is presently called abscisic acid (ABA) (Addicot, 1963). Two other groups at about the same time discovered the same compound. One group headed by Philip Wareing was studying bud dormancy in woody plants. The other group led by Van Steveninck was studying abscission of flowers and fruits from lupine. Plant physiologists agreed to call the compound abscisic acid (Salisbury and Ross, 1992).

3.2.3 Biosynthesis and Metabolism

ABA is a naturally occurring compound in plants. It is a sesquiterpenoid (15-carbon) which is partially produced via the mevalonic pathway in chloroplasts and other plastids. Because it is sythesized partially in the chloroplasts, it makes sense that biosynthesis primarily occurs in the leaves. The production of ABA is accentuated by stresses such as water loss and freezing temperatures. It is believed that biosynthesis occurs indirectly through the production of carotenoids. Carotenoids are pigments produced by the chloroplast which have 40 carbons. Breakdown of these carotenoids occurs by the following mechanism:

Violaxanthin is a carotenoid which has forty carbons. It is isomerized and then split via an isomerase reaction followed by an oxidation reaction. One molecule of xanthonin is produced from one molecule of violaxanthonin and it is uncertain what happens to the remaining biproduct. The one molecule of xanthonin produced is unstable and spontaneously changed to ABA aldehyde. Further oxidation results in ABA.

Activation of the molecule can occur by two methods. In the first method, an ABA-glucose ester can form by attachment of glucose to ABA. In the second method, oxidation of ABA can occur to form phaseic acid and dihyhdrophaseic acid.

The transport of ABA can occur in both xylem and phloem tissues. It can also be translocated through paranchyma cells. The movement of abscisic acid in plants does not exhibit polarity like auxins. ABA is capable of moving both up and down the stem (Walton and Li, 1995; Salisbury and Ross, 1992).

Devang B. Sheth

3.2.4 Functions of Abscisic Acid in plants

The following are some of the physiological responses known to be associated with abscisic acid (Davies, 1995; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).

- Stimulates stomatal closure, in order to reduce transpiration and prevent water loss. The action of ABA can specifically target guard cells for induction of stomatal closure but may also produce a systemic response during periods of drought stress. The regulation of stomatal opening and closure is critical to a plants ability to control water loss. Thus, ABA plays a major role as an endogenous messenger in the regulation of plant's water status (McAinsh, 1990).

- Inhibits fruit-ripening

- Encourages seed dormancy by inhibiting cell growth – inhibits seed germination

- Inhibits the uptake of Kinetin

- Activates the pathogen resistance response defense

- Induces senescence in already-damaged cells and their proximate neighbors

- ABA is generated to control seed germination and further developmental processes and as a signaling molecule to induce plant response to abiotic stresses such as salt, cold, drought, and wounding. It quickly puts a plant, organ, tissue or individual cell in a defensive posture (whatever this entails) in response to rapidly-developing nutrient or environmental stress that threatens their survival.

- Decreases metabolism in response to a newly-developing deficiency of nutrient or adverse environmental condition, such that the condition becomes survivable at the new lower level of metabolism.

- Possibly induces cell dormancy or senescence by a climactic increase or sustained level, stimulating the synthesis of gibberalic acid and/or ethylene.

- A climatic rise or sustained level of ABA may be a prerequisite for the synthesis of any gibberallic acid and/or ethylene in that its presence indicates unusable or unsurvivable levels of Water, Sugar, Minerals and/or essential gases.

3.2.5 Abscisic Acid – Biological significance and pharmacological role

Recent studies of some phytohormones both in-vivo and in-vitro has opened a new door to work-out new leading chemical entities in the field of pharmacology. The most promising among these plant hormones is abscisic acid. Following are some reported information which claims the possible role of abscisic acid as a pharmacological molecule and its therapeutic usefulness:

1. ABA was reported to stimulate several functional activities (phagocytosis, reactive oxygen species and nitric oxide (NO) production, and chemotaxis) of human granulocytes through a signaling pathway sequentially involving a pertussis toxin (PTX)-sensitive G protein/receptor complex, protein kinase A activation, ADP-ribosyl cyclase phosphorylation, and consequent cyclic-ADP-ribose overproduction, leading to an increase of the intracellular Ca^{+2} concentration. The increase of free intracellular ABA and its release by activated human granulocytes indicated that ABA should be considered as a new pro-inflammatory cytokine in humans. ABA behaves as a pro-inflammatory endogenous cytokine capable of stimulating granulocyte functions (N. LeBrasseur, 2007; Bruzzone, 2007).

2. ABA (free and conjugated) has been detected in the brain of mammals (Le Page-Degivry, 1986).

3. ABA could be used as a nutritional intervention against type II diabetes and obesity-related inflammation (Guri et al., 2006). PPAR γ -responsive genes were found to be induced or activated by abscisic acid in 3T3-L1 preadipocytes *in vitro* (Guri et al., 2008; Lehmann et al., 1995). ABA is produced by pancreatic islets and acts as an endogenous insulin secretor at nanomolar concentrations with cyclic ADP ribose as second messenger (Bruzzone et al., 2008).

4. ABA intake exerted an anti-atherosclerotic and anti-hypertensive effect in ApoE-/- mice by suppressing macrophage and CD4+ T cell infiltration into the aortic walls (Guri et al., 2009).

5. A patent was issued to Livingston-Wheeler in 1976 for its use as an anti-cancer compound (Livingston, 1976). The patent describes the use of a solid myeloid leukemia C1498 transplantation model in C57BL/6 mice to investigate the anti-cancer efficacy of ABA. The inventor reported increased

survival rates at 14 days posttransplantation in mice treated with ABA in comparison to control mice. The inventor also proposed that ABA "neutralized" the human chorionic gonadotropin (hCG) that report. Role of abscisic acid as an anti-cancer drug has also been claimed by Ann Wigmore (Wigmore, 1985).

6. ABA was reported to inhibit proliferation and induced differentiation in human SMMC-7221 cells (Ma et al., 2006) and in nude mice transplanted with human hepatocarcinoma (Lu et al., 2007). Tan et al. (2006) reported in a China patent that ABA effectively inhibits the proliferation of tumour cells; stagnates the cells in S phase, stops cell division, and induces differentiation of tumour cells or reverts cancerous cells to normal cells.

7. ABA has a profound effect on some mammalian tissues and on a cyanobacterium. Studies on smooth muscles from the vas deferens and bladder of rat showed that 10^{-6}M ABA enhanced field stimulation responses by about 25%. This effect was inhibited by the calcium-channel blocker, nifedipine. In K^+-depolarized bladder smooth muscle in which the fast calcium channels were voltage-inactivated, 10^{-6}M ABA augmented contracture tension, and enhanced the slow tonic phase of the response which is known to be dependent on the activity of slow calcium channels (Huddart et al., 1986).

8. Masters et al. (1994) reported that ABA is without significant Ca^{+2} modulatory activity in rat prostatic and epididymal vas deferens smooth muscle preparation but the ABA analogue SD217595 possesses strong Ca^{+2} entry blocking ability. Lynch et al. (1991) also reported that abscisic acid (ABA) analogue SD217595 inhibits K^+-induced phasic and tonic contractions of rat bladder detrusor smooth muscle strips.

9. Magnone et al. (2009) reported ABA increases NF-κβ activation and MCP-1 secretion in cultured human monocytes.

10. ABA induced a significant increase in prostaglandin E_2 (PGE_2) production, induced chemokinesis or cell migration and stimulated the release of several cytokines known to mediate the trophic and immunomodulatory properties of mesenchymal stem cells (MSC). In MSC, ABA production and release were stimulated by specific growth factors (e.g., bone morphogenetic protein-7), by inflammatory cytokines, and by lymphocyte conditioned medium (Scarfi et al., 2008).

11. United States Patent-3958025: "Tablets of abscisic acid and a carrier are used to treat a vitamin deficiency of abscisic acid in man, animal and the avian species" is also been filed (www.freepatentsonline.com).

12. It was observed that abscisic acid apparently is non-toxic in the mouse even when administered I.P. in amounts of up to 10% by weight of the mouse (www.freepatentsonline.com).

13. Guri et al. (2010) reported that ABA prevents experimental inflammatory bowel disease (IBD). ABA significantly ameliorated disease activity, colitis and reduced colonic leukocyte infiltration and inflammation. These improvements were associated with the decreased expression of VCAM-1, E-selectin, and mucosal addressin adhesion marker-1. ABA also increased the numbers of CD4 and CD8 T-lymphocytes in the blood as well as MLN and regulatory T cells in the blood.

Devang B. Sheth

3.3 Insulin resistance

3.3.1 Diabetes mellitus

Diabetes mellitus (DM) comprises a group of common metabolic disorders that share the phenotype of hyperglycemia. Several distinct types of DM exist and are caused by a complex interaction of genetics, environmental factors, and life-style choices. Depending on the etiology of the DM, factors contributing to hyperglycemia may include reduced insulin secretion, decreased glucose utilization, and increased glucose production. The defect in metabolic regulation associated with DM causes secondary pathophysiological changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system. In the United States, DM is the leading cause of end-stage renal disease (ESRD), non-traumatic lower extremity amputations, and adult blindness. With an increasing incidence worldwide, DM will be a leading cause of morbidity and mortality for the foreseeable future.

3.3.1.1 Classification

DM is classified on the basis of the pathogenic process that leads to hyperglycemia, as opposed to earlier criteria such as age of onset or type of therapy. The two broad categories of DM are designated type 1 and type 2. Type 1A DM results from autoimmune beta cell destruction, which leads to insulin deficiency. Individuals with type 1B DM lack immunologic markers indicative of an autoimmune destructive process of the beta cells (Atkinson and Maclaren, 1994). However, they develop insulin deficiency by unknown mechanisms and are ketosis prone (Maclaren et al., 1988). Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Previously the terms *insulin-dependent diabetes mellitus* (IDDM) and *non insulin-dependent diabetes mellitus* (NIDDM) were used for type-1 and type-2 DM, which are now obsolete. Since many individuals with type 2 DM eventually require insulin treatment for control of glycemia, the use of the term NIDDM generated considerable confusion.

Devang B. Sheth

Etiological Classification: (Kasper et al., 2005)

1. **Type 1 diabetes mellitus (formerly IDDM)**

   Autoimmune type 1 diabetes mellitus (type 1 A)

   Non-autoimmune or idiopathic type 1 diabetes mellitus (type 1 B)

2. **Type 2 diabetes mellitus (formerly NIDDM)**

3. **Other specific types**

   Specific defined gene mutations

   Maturity onset diabetes of the youth (MODY)

   MODY 1 hepatic nuclear factor 4α gene mutations

   MODY 2 glucokinase gene mutations

   MODY 3 hepatic nuclear factor 1α gene mutations

   MODY 4 pancreatic determining factor X gene mutations

   MODY X unidentified gene mutations

   Maternally inherited diabetes and deafness (MIDD)

   Mitochondrially leucine t RNA gene mutations

   Insulin gene mutations

   Insulin receptor gene mutations

   Secondary to pancreatic diseases

   Chronic pancreatitis

   Tropical diabetes

   Neoplasia

   Pancreatectomy

   Secondary to endocrinopathies

   Acromegaly

   Cushing's syndrome

   Glucagonoma

   Pheochromocytoma

   Hyperthyroidism

   Secondary to immune suppression

   Due to infections

   Congenital rubella

   Cytomegalo virus

Devang B. Sheth

Drug or chemical induced diabetes

> Glucocorticoids
>
> Diuretics
>
> Diazoxide
>
> Ca^{+2}-channel blockers
>
> β_2-adrenergic receptors agonists
>
> Phenytoin
>
> α-interferons
>
> Clonidine
>
> Thyroid hormones, etc.

3.3.1.2 Pathogenesis

3.3.1.2.1 Type 1 DM

Type 1A DM develops as a result of the synergistic effects of genetic, environmental, and immunologic factors that ultimately destroy the pancreatic beta cells. Individuals with a genetic susceptibility have normal beta cell mass at birth but begin to lose beta cells secondary to autoimmune destruction that occurs over months to years. This autoimmune process is thought to be triggered by an infectious or environmental stimulus and to be sustained by a beta cell–specific molecule. In the majority of individuals, immunologic markers appear after the triggering event but before diabetes become clinically overt. B-cell mass then begins to decline, and insulin secretion becomes progressively impaired, although normal glucose tolerance is maintained. Features of diabetes do not become evident until a majority of beta cells are destroyed (>80%) (Maclaren et al., 1988). At this point, residual functional beta cells still exist but are insufficient in number to maintain glucose tolerance. The events that trigger the transition from glucose intolerance to frank diabetes are often associated with increased insulin requirements, as might occur during infections or puberty. After the initial clinical presentation of type 1A DM, a "honeymoon" phase may ensue during which time glycemic control is achieved with modest doses of insulin or, rarely, insulin is not needed. However, this fleeting phase of endogenous insulin production from residual beta cells disappears as the autoimmune process destroys the remaining beta cells, and the individual becomes completely insulin deficient.

Devang B. Sheth

3.3.1.2.2 Type 2 DM

Insulin resistance and abnormal insulin secretion are central to the development of type 2 DM. Although controversy remains regarding the primary defect, most studies support the view that insulin resistance precedes insulin secretory defects and that diabetes develops only if insulin secretion becomes inadequate.

3.3.1.2.2.1 Metabolic Abnormalities- Insulin Resistance

The decreased ability of insulin to act effectively on peripheral target tissues (especially muscle and liver) is a prominent feature of type 2 DM and results from a combination of genetic susceptibility and obesity (Umpierrez et al.,1995; Dagogo-Jack et al.,1997). Insulin resistance is relative, however, since supernormal levels of circulating insulin will normalize the plasma glucose. Insulin dose-response curves exhibit a rightward shift, indicating reduced sensitivity, and a reduced maximal response, indicating an overall decrease in maximum glucose utilization (30 to 60% lower than normal individuals). Insulin resistance is believed to play a major role in its pathogenesis, causing progressive β-cell dysfunction (Polonsky et al .,1996 ; Haffner et al.,1990; Warram et al.,1990; Lillioja et al.,1993).

Insulin resistance impairs glucose utilization by insulin-sensitive tissues and increases hepatic glucose output; both effects contribute to the hyperglycemia. Increased hepatic glucose output predominantly accounts for increased FPG levels, whereas decreased peripheral glucose usage results in postprandial hyperglycemia. In skeletal muscle, there is a greater impairment in nonoxidative glucose usage (glycogen formation) than in oxidative glucose metabolism through glycolysis. Glucose metabolism in insulin-independent tissues is not altered in type 2 DM. The precise molecular mechanism of insulin resistance in type 2 DM has not been elucidated. Insulin receptor levels and tyrosine kinase activity in skeletal muscle are reduced, but these alterations are most likely secondary to hyperinsulinemia and are not a primary defect. Therefore, postreceptor defects are believed to play the predominant role in insulin resistance. Polymorphisms in IRS-1 may be associated with glucose intolerance, raising the possibility that polymorphisms in various postreceptor molecules may combine to create an insulin-resistant state. The pathogenesis of insulin resistance is currently focused on a PI-3-kinase signaling defect, which reduces translocation of GLUT4 to the plasma membrane, among other abnormalities.

Devang B. Sheth

Of note, not all insulin signal transduction pathways are resistant to the effects of insulin [e.g., those controlling cell growth and differentiation and using the mitogen-activated protein (MAP) kinase pathway]. Consequently, hyperinsulinemia may increase the insulin action through these pathways, potentially accelerating diabetes-related conditions such as atherosclerosis. Another emerging theory proposes that elevated levels of free fatty acids, a common feature of obesity, may contribute to the pathogenesis of type 2 DM (Prentki et al., 1996; Shimabukuro et al., 1998). Free fatty acids can impair glucose utilization in skeletal muscle, promote glucose production by the liver, and impair beta cell function.

➢ Insulin Resistance Syndrome

The insulin resistance condition comprises a spectrum of disorders, with hyperglycemia representing one of the most readily diagnosed features. The *metabolic syndrome*, the *insulin resistance syndrome*, or *syndrome X* are terms used to describe a constellation of metabolic derangements that includes insulin resistance, hypertension, dyslipidemia [low high-density lipoprotein (HDL) and elevated triglycerides], central or visceral obesity, type 2 diabetes or IGT/IFG, and accelerated cardiovascular disease (Saltiel and Kahn, 2001). Insulin resistance is a serious medical problem that leads to type 2 diabetes when pancreatic β-cells fail to compensate by increasing the amount of secreted insulin (DeFronzo, 1997).

At the physiological level, obesity, inactivity, and aging are common causes of insulin resistance. Although moderate compensatory hyperinsulinemia might be well tolerated in the short term, chronic hyperinsulinemia exacerbates insulin resistance and contributes directly to β-cell failure and diabetes (Pessin et al., 2000; Shulman, 2000). Importantly, the β-cell failure probably does not arise from overwork but rather from dysregulated growth and survival signals that accompany insulin resistant states.

➢ Symptoms of metabolic syndrome

Many people are unaware that they have metabolic syndrome (Syndrome X), even though the American Heart Association estimates that 20-25% of the adult population of the U.S. suffers from this disorder – between 58 and 73 million men and women. Metabolic Syndrome is characterized by having at least three of the following symptoms:

Devang B. Sheth

- Insulin Resistance (when the body can't absorb blood sugar or insulin properly)

- Abdominal fat – in men this means a 40 inch waist or larger, in women 35 inches or larger

- High blood sugar levels – at least 110 milligrams per deciliter (mg/dL) after fasting

- High triglycerides – at least 150 mg/dL in the blood stream

- Low HDL (the "good" cholesterol) – less than 40 mg/dL

- Pro-thrombotic state (e.g. high fibrinogen or plasminogen activator inhibitor in the blood)

- Blood pressure of 130/85 mmHg or higher

The American Heart Association states that the "underlying causes of Metabolic Syndrome are being overweight, physical inactivity and genetic factors." Researchers have found a connection between Metabolic Syndrome and other conditions such as obesity, high blood pressure and high levels of LDL "bad" cholesterol, all of which are risk factors for Cardiovascular Disease. Studies have shown, for example, an increased link between Metabolic Syndrome and atherosclerosis, which occurs when fatty deposits called plaque cling to the interior walls of the arteries, leading to blockages that can cause heart attacks or stroke. People with Metabolic Syndrome are also more prone to developing Type 2 Diabetes, as well as PCOS (Polycystic Ovarian Syndrome) in women and prostate cancer in men. All these findings substantially raise the bar on the seriousness of Metabolic Syndrome, making it even more important that doctors correctly diagnose the condition and instruct their patients about one of its underlying causes – Insulin Resistance. At present, there is no single pharmaceutical drug that can reverse the symptoms of Metabolic Syndrome. A complete system of elements is needed to treat the major factor in causing this condition, namely Insulin Resistance.

Devang B. Sheth

3.3.1.3 Treatment

Table – 3.1: Oral Glucose-Lowering Therapies in Type 2 DM
(UKPDS, 1998; American diabetes association, 2002)

	Mechanism of Action	Examples	Agent-Specific Advantage	Agent-Specific Disadvantages	Contraindications
Insulin secretagogues	↑ Insulin secretion				
(a)Sulfonylureas		Tolbutamide Glimepiride Glipizide Glyburide	Lower fasting blood glucose	Hypoglycemia weight gain, hyperinsulinemia	Renal/liver disease
(b) Meglitinide		Repaglinide	Short onset of action, lower postprandial glucose	Hypoglycemia	Liver disease
Biguanides (Kirpichnikov, 2002; Knowler, 2002)	↓Hepatic glucose production, weight loss, ↑glucose utilization	Metformin	Weight loss, improved lipid profile, no hypoglycemia	Lactic acidosis, diarrhea, nausea, possible increased cardiovascular mortality	Serum creatinine >1.5 mg/dL (men), >1.4 mg/dL (women), radiographic contrast studies, seriously ill patients, acidosis
α-Glucosidase inhibitors	↓Glucose absorption	Acarbose, miglitol	No risk of hypoglycemia	GI flatulence, ↑liver function tests	Liver/renal disease
Thiazolidinediones	↓Insulin resistance, ↑glucose utilization	Rosiglitazone, pioglitazone	↓Insulin and sulfonylurea requirements, ↓triglycerides	Frequent hepatic monitoring for idiosyncratic hepatocellular injury (see text)	Liver disease, congestive heart failure
Medical nutrition therapy and physical activity (Clement, 2004)	↓Insulin resistance	Low-calorie, low-fat diet, exercise	Other health benefits	Compliance difficult, long-term success low	

Type 1 DM is treated with Insulin whereas in type 2 DM oral hypoglycemic agents are used. The table-3.1 shows the whole treatment regimen for insulin resistance (Type 2 DM).

Devang B. Sheth

3.3.2 Abscisic acid and insulin resistance

PPAR-γ agonists, such as thiazolidinediones (TZDs), have been shown to be very effective in improving systemic insulin sensitivity. Abscisic acid is structurally similar to TZDs. It is found that ABA could be used as a nutritional supplement to combat type II diabetes and obesity-related inflammation (Guri et al., 2007). Dietary ABA-increased mRNA expression was found to increase the expression of PPAR-γ and its responsive genes (i.e., adiponectin, aP2, and CD36) in WAT. No side effects were observed in ABA-fed mice, such as excess weight gain and fluid retention, which are commonly observed with TZDs (Guri et al., 2007). Moreover, PPAR γ - responsive genes were found to be induced or activated by abscisic acid in 3T3-L1 pre-adipocytes *in vitro* (Lehmann et al., 1995; Guri et al., 2008). ABA is also produced by pancreatic islets and acts as an endogenous insulin secretor at nanomolar concentrations with cyclic ADP ribose as second messenger (Bruzzone et al., 2008; Bassaganya-Riera et al., 2010).

Devang B. Sheth

3.4 Cancer

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display the traits of uncontrolled growth (growth and division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are selflimited, do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. (Fodde and Smits, 2002; Merlo *et al*., 2006)

3.4.1 Pathways to cancer

There are many distinct types of cancer, there are believed to be six essential alterations to normal cell physiology, which together define the progression of most human malignancies (Hanahan *et* al., 2000).

(1) Self-sufficiency in growth signals

Normal cell proliferation depends upon the presence of growth factors produced outside of the cell. However, one of the key characteristics of the tumor cell is its capacity for proliferation without dependence on external growth factors. Tumor cells may proliferate by either internal production of growth factors or by responding to levels of external growth factors not usually sufficient to produce proliferation in normal cells (Jin *et* al., 2005).

(2) Insensitivity to antigrowth signals

In normal tissue, the stability of the cell population is maintained by a host of signals and factors inhibiting cell proliferation and differentiation. For cancer cells to survive and replicate, these antigrowth signals must be avoided (Hanahan et al., 2000, Jin et al., 2005).

(3) Tissue invasion and metastasis

Up to 90% of cancer deaths are due to metastatic disease. Once cancer cells leave the primary tumor and travel through the body, the ability to invade and colonize distant sites to form metastases is dependent on acquiring the ability to overcome the normal suppressors of invasion (Hanahan et al., 2000).

Devang B. Sheth

(4) Limitless potential for replication

Many, if not all, normal human cells are programmed to limit their own replication. However, for cells to form a potentially life-threatening tumor, the mechanisms that normally limit replication must be disrupted. For a tumor cell population to expand, it must develop unlimited replicative potential, effectively gaining "immortality''.

(5) Sustained angiogenesis

In normal tissue, continued cell function is dependent on the availability of oxygen and nutrients and the removal of metabolic waste through the capillary beds. Angiogenesis, the process by which new blood vessels are formed, is not an inherent property of most cells in small, localized neoplasms. To develop into larger, potentially metastatic tumors, angiogenic ability must be acquired (Bergers et al., 2003).

(6) Evading apoptosis

In normal tissue, the stability of the cell population is maintained through a process of programmed cell death, or apoptosis, which is latent in virtually all cell types throughout the body. Acquiring resistance to apoptosis is one of the key mechanisms by which cancer cells maintain proliferation and is thought to be a critical survival factor for the majority of tumors (Ghobrial et al., 2005).

3.4.2 Treatment

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient (performance status). A number of experimental cancer treatments are also under development. (Dolmans et al., 2003; Kumar et al., 2005)

Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Radiation can also cause damage to normal tissue. (Kleinman and Liau, 2001)

Table – 3.2: Classification of currently available anticancer drugs

Alkylting agents	*Nitrogen mustards:* Chlorambucil, Chlormethine, Cyclophosphamide, Ifosfamide, Melphalan, Bendamustine *Nitrosoureas :* Carmustine, Fotemustine, Lomustine, Streptozocin *Platinum:* Carboplatin, Cisplatin, Oxaliplatin, Triplatin tetranitrate *Alkyl sulfonates:* Busulfan, Treosulfan *Hydrazines:* Procarbazine, Dacarbazine, Temozolomide *Aziridines :* ThioTEPA
Anti-metabolites	*Folic acid:* Aminopterin, Methotrexate, Pemetrexed, Raltitrexed *Purine:* Cladribine, Clofarabine, Fludarabine, Mercaptopurine, Pentostatin, Thioguanine *Pyrimidine:* Capecitabine, Cytarabine, Decitabine, Fluorouracil,
Mitotic inhibitor	*Taxane:* Docetaxel, Larotaxel, Paclitaxel *Vinca:* Vinblastine, Vincristine, Vindesine, Vinorelbine
Cytotoxic antibiotics	*Anthracycline family:* Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitoxantrone, Pixantrone, Valrubicin *Streptomyces:* Actinomycin, Bleomycin, Mitomycin
Topoisomerase inhibitors	*Camptotheca:* Camptothecin, Irinotecan, Rubitecan *Podophyllum:* Etoposide, Teniposide
Monoclonal antibodies	*Receptor tyrosine kinase:* Cetuximab, Panitumumab, Trastuzumab *CD20:* Rituximab, Tositumomab *Other:* Alemtuzumab, Bevacizumab, Gemtuzumab
Photosensitizers	Aminolevulinic acid, Methyl aminolevulinate, Porfimer sodium, Verteporfin
Tyrosine kinase inhibitors	Axitinib, Bosutinib, Cediranib, Dasatinib, Erlotinib, Gefitinib, Imatinib, Lapatinib, Lestaurtinib, Nilotinib, Semaxanib, Sorafenib, Sunitinib, Vandetanib
Retinoids	Alitretinoin, Tretinoin
Others	Fusion protein (Aflibercept) - Altretamine, Amsacrine, Anagrelide, Arsenic trioxide, Asparaginase (Pegaspargase), Bexarotene, Bortezomib, Celecoxib, Denileukin diftitox, Elesclomol, Estramustine, Irofulven, Ixabepilone, Masoprocol, Mitotane, Oblimersen, Testolactone, Tipifarnib, Trabectedin

3.4.3 Breast cancer

Breast cancer is a cancer that starts in the cells of the breast. Worldwide, breast cancer is the second most common type of cancer after lung cancer (10.4% of all cancer incidence, both sexes counted) and the fifth most common cause of cancer death. The most common pathologic types of breast cancer are invasive ductal carcinoma, malignant cancer in the breast's ducts, and invasive lobular carcinoma, malignant cancer in the breast's lobules. Breast is composed of identical tissues in males and females, breast cancer also occur in males. Incidences of breast cancer in men are approximately 100 times less common than in women, but men with breast cancer are considered to have the same statistical survival rates as women. No etiology is known for 95% of breast cancer cases, while approximately 5% of new breast cancers are attributable to hereditary syndromes. In particular, carriers of the breast cancer susceptibility genes, BRCA1 and BRCA2, are at a 30-40% increased risk for breast and ovarian cancer, depending on in which portion of the protein the mutation occurs. Breast cancer screening is an attempt to find unsuspected cancers. The most common screening methods are self and clinical breast exams, X-ray mammography, and breast Magnetic resonance imaging (MRI). (Paull et al., 2001)

BRCA1 (**breast cancer 1, early onset**) is a human gene that belongs to a class of genes known as tumor suppressors, which maintains genomic integrity to prevent uncontrolled proliferation. The multifactorial BRCA1 protein product is involved in DNA damage repair, ubiquitination, transcriptional regulation as well as other functions. Variations in the gene have been implicated in a number of hereditary cancers, namely breast, ovarian and prostate. The *BRCA1* gene is located on the long (q) arm of chromosome 17 at band 21, from base pair 38,449,843 to base pair 38,530,933 (map). (Starita and Parvin, 2003)

➢ **DNA Damage Repair**

The BRCA1 protein is directly involved in the repair of damaged DNA. In the nucleus of many types of normal cells, the BRCA1 protein is thought to interact with RAD51 during repair of DNA double-strand breaks, though the details and significance of this interaction is the subject of debate. These breaks can be caused by natural radiation or other exposures, but also occur when chromosomes exchange genetic material during a special type of cell division that creates sperm and eggs

(meiosis). The BRCA2 protein, which has a function similar to that of BRCA1, also interacts with the RAD51 protein. By influencing DNA damage repair, these three proteins play a role in maintaining the stability of the human genome.

BRCA1 directly binds to DNA, with higher affinity for branched DNA structures. This ability to bind to DNA contributes to its ability to inhibit the nuclease activity of the MRN complex as well as the nuclease activity of Mre11 alone. This may explain a role for BRCA1 to promote higher fidelity DNA repair by NHEJ. BRCA1 also colocalizes with γ-H2AX (histone H2AX phosphorylated on serine-139) in DNA double-strand break repair foci, indicating it may play a role in recruiting repair factors. (Paull et al., 2001; Durant and Nickoloff, 2005)

➢ **Transcription**

BRCA1 was shown to co-purify with the human RNA Polymerase II holoenzyme in HeLa extracts, implying it is a component of the holoenzyme. Later research, however, contradicted this assumption, instead showing that the predominant complex including BRCA1 in HeLa cells is a 2 megadalton complex containing SWI/SNF. SWI/SNF is a chromatin remodeling complex. Artificial tethering of BRCA1 to chromatin was shown to decondense heterochromatin, though the SWI/SNF interacting domain was not necessary for this role. BRCA1 interacts with the NELF-B (COBRA1) subunit of the NELF complex. (Ye et al., 2001)

➢ **Other roles**

Research suggests that both the BRCA1 and BRCA2 proteins regulate the activity of other genes and play a critical role in embryo development. The BRCA1 protein probably interacts with many other proteins, including tumor suppressors and regulators of the cell division cycle.

➢ **Mutations and Cancer Risk**

Certain variations of the *BRCA1* gene lead to an increased risk for breast cancer. Researchers have identified more than 600 mutations in the *BRCA1* gene, many of which are associated with an increased risk of cancer. These mutations can be changes in one or a small number of DNA base pairs (the building blocks of DNA). Those mutations can be identified with PCR and DNA sequencing.

In some cases, large segments of DNA are rearranged. Those large segments, also called large rearrangements, can be a deletion or a duplication of one or several axons in the gene. Classical methods for mutations detection (sequencing) are unable to reveal those mutations. Other methods are proposed: Q-PCR. Multiplex Ligation-dependent Probe Amplification (MLPA), and Quantitative Multiplex PCR of Shorts Fluorescents Fragments (QMPSF). New methods have been recently proposed: heteroduplex analysis (HDA) by multi-capillary electrophoresis or also dedicated oligonucleotides array based on comparative genomic hybridization (array-CGH). (Tapia et al., 2008)

A mutated *BRCA1* gene usually makes a protein that does not function properly because it is abnormally short. Researchers believe that the defective BRCA1 protein is unable to help fix mutations that occur in other genes. These defects accumulate and may allow cells to grow and divide uncontrollably to form a tumor. In addition to breast cancer, mutations in the *BRCA1* gene also increase the risk of ovarian, fallopian tube and prostate cancers. Moreover, precancerous lesions (dysplasia) within the fallopian tube have been linked to *BRCA1* gene mutations. (Hogervorst et al., 2003)

BRCA2 (Breast Cancer Type 2 susceptibility protein) is a human gene that is involved in the repair of chromosomal damage and belongs to a class of genes known as tumor suppressor genes. Tumor suppressor genes regulate the cycle of cell division by keeping cells from growing and dividing too rapidly or in an uncontrolled way. Although the structures of the BRCA1 and BRCA2 genes are very different, their functions appear to be similar. The proteins made by both genes are essential for repairing damaged DNA. The BRCA2 protein binds to and regulates the protein produced by the RAD51 gene to fix breaks in DNA. These breaks can be caused by natural and medical radiation or other environmental exposures, but also occur when chromosomes exchange genetic material during a special type of cell division that creates sperm and eggs (meiosis). The BRCA1 protein also interacts with the RAD51 protein. By repairing DNA, these three proteins play a role in maintaining the stability of the human genome. Like BRCA1, BRCA2 probably regulates the activity of other genes and plays a critical role in embryo development. The BRCA2 gene is located on the long (q) arm of chromosome 13 at position 12.3 (13q12.3), from base pair 31,787,616 to base pair 31,871,804. (Orelli and Bishop, 2001)

➢ Related conditions

Certain variations of the BRCA2 gene cause an increased risk for breast cancer. Researchers have identified about 450 mutations in the BRCA2 gene, many of which cause an increased risk of cancer. BRCA2 mutations are usually insertions or deletions of a small number of DNA base pairs (the building material of chromosomes) in the gene. As a result of these mutations, the protein product of the BRCA2 gene is abnormally short and does not function properly. Researchers believe that the defective BRCA2 protein is unable to help fix mutations that occur in other genes. As a result, mutations build up and can cause cells to divide in an uncontrolled way and form a tumor. (Zou et al., 1999)

People who have two mutated copies of the BRCA2 gene have one type of Fanconi anemia. This condition is caused by extremely reduced levels of the BRCA2 protein in cells, which allows the accumulation of damaged DNA. Patients with Fanconi anemia are prone to several types of leukemia (a type of blood cell cancer); solid tumors, particularly of the head, neck, skin, and reproductive organs; and bone marrow suppression (reduced blood cell production that leads to anemia). In addition to breast cancer in men and women, mutations in BRCA2 also lead to an increased risk of ovarian, Fallopian tube, prostate, and pancreatic cancers, as well as malignant melanoma. In some studies, mutations in the central part of the gene have been associated with a higher risk of ovarian cancer and a lower risk of prostate cancer than mutations in other parts of the gene. Several other types of cancer have also been seen in certain families with BRCA2 mutations. (Yoshida et al., 2004)

➢ Discovery of BRCA2

The BRCA2 gene was discovered in 1995 by Professor Michael Stratton and Dr. Richard Wooster (Institute of Cancer Research, UK). The Wellcome Trust Sanger Institute (Hinxton, Cambs, UK) collaborated with Stratton and Wooster to isolate the gene. In honour of this discovery and collaboration, the Wellcome Trust has participated in the construction of a cycle path between Addenbrooke's Hospital site in Cambridge and the nearby village of Great Shelford. It is decorated with over 10,000 lines of 4 colours representing the nucleotide sequence of BRCA2. It makes-up part of the National Cycle Network route 11, and can be seen from the Cambridge-London Liverpool Street train. (Duncan et al., 1998)
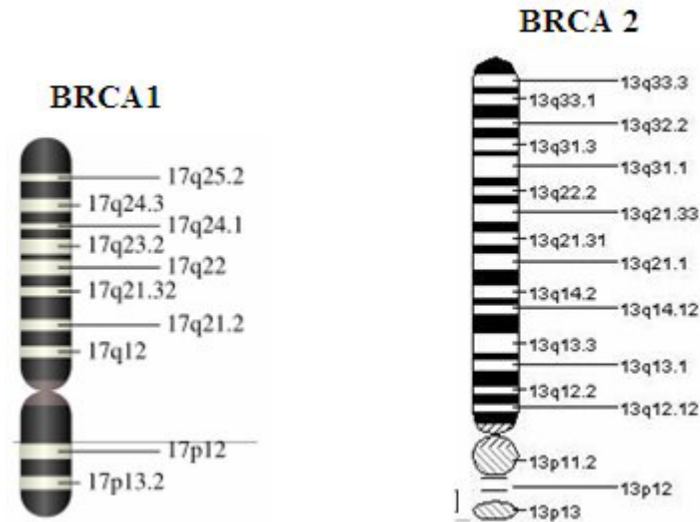
Devang B. Sheth

Figure 3.1: Location of gene BRCA1 on chromosome 17 and BRCA2 on chromosome 13 (Zou et al., 1999)

Statistics show that the incidence of breast cancer is rapidly increasing in urban India and has overtaken cancer of the cervix as the number one cancer in our major metros according to a study conducted on women and cancer in India.

The number of breast cancer cases is estimated to have reached 1.2 million worldwide. Snacking, fatty food, late marriage and delayed motherhood are some factors that can lead to a high risk of breast cancer. It is now clear that in postmenopausal women an enzyme called aromatase present in peripheral tissues such as fat, muscle and liver produces oestrogen that triggers breast cancer.

3.4.4 Cell line and its role in in-vitro screening method

3.4.4.1 Cell line

Specific cells that can grow indefinitely given the appropriate medium and conditions i.e. living cells that are maintained *in vitro* in artificial media of serum and nutrients for the study and growth of certain strains, experiments in controlling diseases, or study of the reaction to certain drugs or agents (Walton et al., 1975). Human tumor cell line panels combined with rapid high-throughput cytotoxicity testing have proven to be valuable tools for drug screening and early drug evaluation and investigation of drug resistance mechanisms. The National Cancer Institute (NCI) pioneered the utilization of large human tumor cell line panels for drug screens, after phasing out

their previously used animal models. The disease-oriented cell line panel used by NCI consists of 60 different cell lines, which consists of seven sub panels representing common solid tumors, leukemia and lymphomas (Shoemaker et al., 1988; Suggitt and Bibby, 2005). To date, more than 100 000 compounds and a large number of natural product extracts have been tested in their short-term growth inhibition assay (Shoemaker et al., 1988; Bussey et al., 2006). Typically, compounds are applied to the cell lines in a wide concentration range, and concentrations that inhibit/kill e.g. 50 % of the cells (IC_{50}) are determined. The IC_{50} concentrations for a drug in many cell lines provide a drug specific profile, which can be compared to profiles from other drugs. This approach has successfully been used for drug mechanism classification of standard drugs, and assignment of drug action to investigational drugs and discovery of new classes of chemotherapeutic compounds (Dhar et al., 1996; Paull et al., 1989; Weinstein et al., 1997).

3.4.4.2 Concepts in mammalian cell culture

➢ **Isolation of cells**

Cells can be isolated from tissues for *ex vivo* culture in several ways. Cells can be easily purified from blood; however only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by enzymatic digestion with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as explant culture.

Cells that are cultured directly from a subject are known as *primary cells*. With the exception of some derived from tumours, most primary cell cultures have limited lifespan. After a certain number of population doublings cells undergo the process of senescence and stop dividing, while generally retaining viability (Freshney, 2005).

An established or immortalized cell line acquire the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. There are numerous well established cell lines representative of particular cell types (Walton et al., 1975).

➢ **Maintaining cells in culture**

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2) in a cell incubator. Culture conditions vary widely for each cell type and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrient components. The growth factors used to supplement media are often derived from animal blood, such as calf serum. These blood-derived ingredients pose the potential for contamination of derived pharmaceutical products with viruses or prions. Current practice is to minimize or eliminate the use of these ingredients where possible. Some cells naturally live without attaching to a surface, such as cells that exist in the bloodstream. Others require a surface, such as most cells derived from solid tissues. Cells grown unattached to a surface are referred to as *suspension cultures* for example, U-937, HL60 etc. Other *adherent cultures* cells can be grown on tissue culture plastic, which may be coated with extracellular matrix components (e.g. collagen or fibronectin) to increase its adhesion properties and provide other signals needed for growth. Example of adherent cell lines are HEp-2, HEK-293, MCF-7 etc. (Suggitt and Bibby, 2005).

➢ **Manipulation of cultured cells**

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues (Roberts and Spoon, 1990):

- Nutrient depletion in the growth media
- Accumulation of apoptotic/necrotic (dead) cells.
- Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as contact inhibition.
- Cell-to-cell contact can stimulate promiscuous and unwanted cellular differentiation.

These issues can be dealt with using tissue culture methods that rely on sterile technique. These methods aim to avoid contamination with bacteria or yeast that will

compete with mammalian cells for nutrients and/or cause cell infection and cell death. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics can also be added to the growth media (Cabrera et al., 2006). Amongst the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells.

Table – 3.3: List of commonly used cell lines (Freshney, 2005)

Cell line	Meaning	Organism	Origin tissue	Morphology
HEK-293	*Human embryonic kidney*	Human	Kidney (embryonic)	Epithelium
HeLa	*Henrietta Lacks*	Human	Cervical cancer	Epithelium
CHO	*Chinese hamster ovary*	Hamster	Ovary	Epithelium
MCF-10A	*Michigan Cancer Foundation*	Human	Mammary gland	Epithelium
Peer	---	Human	T cell leukemia	---
HL-60	*Human leukemia*	Human	Myeloblast	Bloodcells
A-549	---	Human	Lung carcinoma	Epithelium
Jurkat	---	Human	T-Cell Leukemia	Blood cells
HEp-2	*Larynx Cells*	Human	Larynx epithelial	Epithelial and Adherent
U-937	*Histolytic lymphoma*	Human	Monocytic Lymphoma	Suspension

(1) Media changes

The purpose of media changes is to replenish nutrients and avoid the build up of potentially harmful metabolic byproducts and dead cells. In the case of suspension cultures, cells can be separated from the media by centrifugation and resuspended in fresh media. In the case of adherent cultures, the media can be removed directly by aspiration and replaced (MacLeod et al., 1999).

(2) Passaging cells

Passaging or sub culturing cell culture involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells

diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this was historically done with a mixture of trypsin-EDTA; however other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture (Chatterjee, 2007).

3.4.4.3 MCF-7 cell line:

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation – 7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers (Soule et al., 1973). The MCF-7 cell-lines retain several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Growth of MCF-7 cells is inhibited by tumor necrosis factor alpha (TNF-α). Treatment with anti-estrogens can modulate the secretion of insulin-like growth factor binding proteins. MCF-7 and two other breast cancer cell lines, named T-47D, MDA-MB231, account for more than two-thirds of all abstracts reporting studies on mentioned BCC lines, as concluded from a Medline-based survey (Dickson et al., 1986; Osborne et al., 1987). PIK3CA helical mutations were identified in MCF-7, but with low AKT activation.

The stem line chromosome numbers ranged from hypertriploidy to hypotetraploidy, with the 2S component occurring at 1%. There were 29 to 34 marker chromosomes per S metaphase; 24 to 28 markers occurred in at least 30% of cells, and generally one large submetacentric (M1) and 3 large subtelocentric (M2, M3, and M4) markers were recognizable in over 80% of metaphases. No DM was detected. Chromosome 20 was nullisomic and X was disomic (Dickson et al., 1986; Osborne et al., 1987).

Characteristics

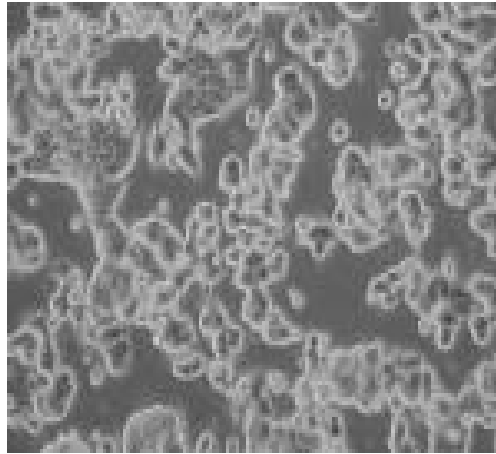|		
Primary tumor	:	Invasive breast ductal carcinoma
Origin of cells	:	Pleural effusion
Presence of estrogen receptors	:	Yes
Proliferative response to estrogens	:	Yes
Presence of progesterone receptor	:	Yes
Phenotype	:	Luminal epithelial

[44]

Figure 3.2: MCF-7 cells under light microscope (10 X)

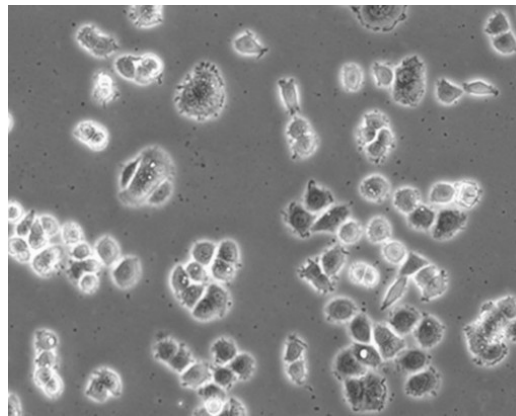**3.4.4.4 MDA-MB-468 cell line:**

Figure 3.3: MDA-MB-468 cells under light microscope (10 X)

Characteristics

MDA-MB-468 cell line was isolated in 1977 by R. Cailleau et al. from a pleural effusion of a 51-year-old Black female patient with metastatic adenocarcinoma of the breast. Although the tissue donor was heterozygous for the G6PD alleles, the cell line consistently showed only the G6PD A phenotype. There is G -> A mutation in codon 273 of the p53 gene resulting in an Arg -> His substitution (Avila et al., 1994). MDA-MB-468 expresses receptors for epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) (Bates et al., 1990). In vitro, the MDA-MB-468 cell line is commonly used in EGF signalling study. The cell line has abundant activity in both the chemo-invasion and chemotaxis assay. MDA-MB-468 cell line is an example of ER negative cell line.

Devang B. Sheth

3.4.5 *In-vitro* cytotoxicity assays

To study cytotoxic activity of a compound, cytotoxic assays are carried out. It is now well-documented that apoptosis or programmed cell death is the key mechanism by which chemotherapeutic agents exert their cytotoxicity (Larson R., 2003).

These assays are principally of two types.

1. **Radioactive and non-radioactive assays** that measure increases in plasma membrane permeability, since dying cells become leaky.
2. **Colorimetric assays** that measure reduction in the metabolic activity of mitochondria; mitochondria in dead cells cannot metabolize dyes, while mitochondria in live cells can metabolize it and so are distinguished.

Depending on the knowledge of physiological events occurring in cell cycle and death, assay type is chosen and used. A number of methods have now been developed to study apoptosis in cell populations (Boyd, 1985).

Cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they also allow direct extrapolation of quantitative data to similar *in vivo* situations (Freshney, 2001; Bacon et al., 1990) This refers to the *in vitro* assessment of material to determine whether or not it releases toxic chemicals in sufficient quantities to kill cells either directly or indirectly through the inhibition of cell metabolic pathways.

➤ **Common Basic Steps of in-vitro Assays**

Although the techniques for testing drug sensitivities of tumor cells differ, each employ four common basic steps:

  i.   Isolation of cells,

  ii.   Incubation of cells with drugs,

  iii.   Assessment of cell survival, and

  iv.   Interpretation of the result (Brown and Markman, 1996)

- ➢ **Ideal characteristics of in vitro methods** (Gupta, 2003)

  - An ideal in vitro screening method should be simple economical, reproducible, rapid and sensitive.

  - The assay should be applicable to large number of tumor types and test compounds.

  - The choice of cell lines should be representative of clinical situation as close as possible.

  - The range of drug concentration used in vitro should be comparable to that expected for in vivo treatments.

  - The assay should be able to process a large number of samples quickly and in automated fashion.

  - Data acquisition should be simple, easily interpreted and applied.

- ➢ **Advantages**

The development of in-vitro cytotoxicity assays has been driven by the need

  - To rapidly evaluate the potential toxicity of large numbers of compounds,

  - To limit animal experimentation whenever possible, and

  - To carry out tests with small quantities of compound.

  - Most cost effective and easier to manage.

The most promising advantage of in-vitro methods over in-vivo method is, here culture can be cultivated under a controlled environment (pH, temperature, humidity, oxygen carbon-dioxide balance etc.) resulting in homogeneous batches of cells and thus minimizing experimental errors. (Freshney, 2001; Gupta, 2003)

- ➢ **Limitation of in vitro methods:**

  - They often furnish false positive results (compounds show no activity *in vivo*) and false negative results (compounds show no activity *in vitro* but show activity *in vivo* as they need to be biotransformed *in vivo* to pharmacologically active compounds).

  - A second pitfall is that role of pharmacokinetic in determining drug effects cannot be evaluated *in vitro*.

  - Geometry of solid tumors *in vivo* is very different from that of cells growing *in vitro* in suspension or mono layer culture.

Devang B. Sheth

3.4.5.1 Methodological Aspects

The cell culture is a model of a target tissue in the human body and mimics the response of human cells to exposure to chemicals. Provided that time and degree of exposure (dosage for animals and concentration/ exposure time in cell tests) in the experiments correspond to human exposure, both models can potentially predict any type of chemical interference with corresponding aspects of the human body (Ekwall et al., 1990). *In vitro* Cell toxicity of chemicals can be predicted by observing few of changes as follows:

(1) Morphological alteration: The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture. Therefore, it is not surprising that morphological alterations are used as an index of toxicity. Different types of toxic effects may require investigative tools of different levels of sensitivity. (Walton and Buckley, 1975)

(2) Cell growth alteration: Another indicator of toxicity is altered cell growth. The effect of chemicals on the capability of cells to replicate is used as an index of toxicity; the concentration of the substances at which 50 per cent of the cells do not multiply is called the median inhibitory dose (ID_{50}). A more specific measure of replication is plating efficiency-the ability of cells (100-200 per dish, 60 mm diameter) to form colonies after 10-15 days of culture in the presence of a toxic agent gives more complete information, indicating both cell survival and ability to reproduce (Nardone, 1977).

(3) Cell reproduction: Cell reproduction can be measured by several parameters including cell count, DNA content, protein content, or enzyme activity (e.g. ornithinedecarboxylase) (Costa, 1979). Each of these parameters can be measured by more or less sophisticated means. Examples are the assay of DNA content by biochemical methods and incorporation of radio labeled precursors.
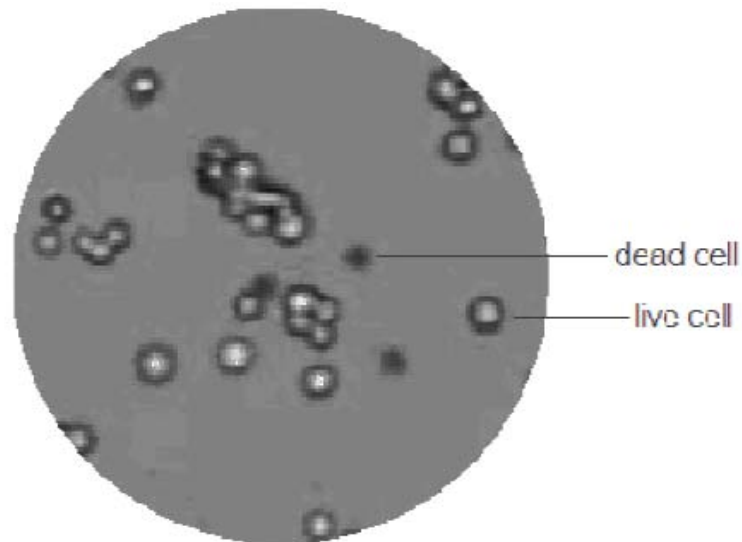
Devang B. Sheth

Figure 3.4(a): Observations of viable and non viable cell under microscope, where viable cells appear as small, round and refractive while Non-viable cells appear as swollen, larger, and dark.
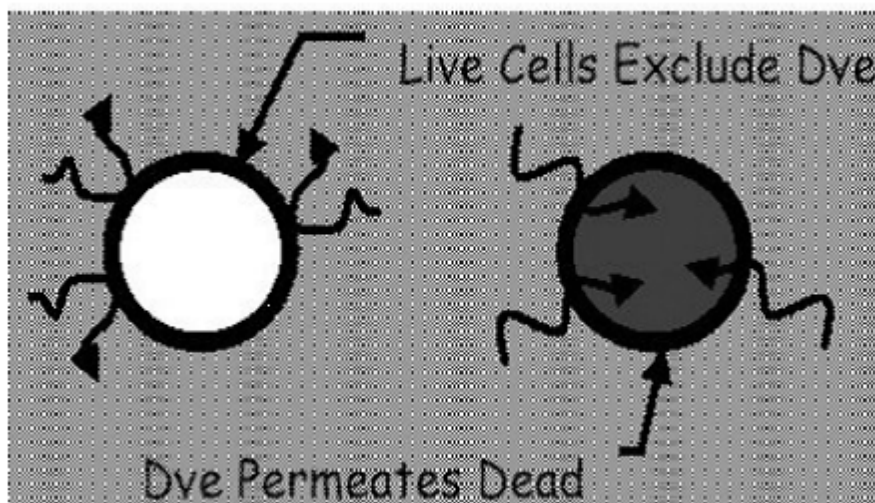

Figure 3.4(b): Mechanism of dye exclusion, in which white colour live cell exclude dye and dark blue coloured dead cell allow dye to permeate

(4) **Cell viability:** Another crude index of toxicity is cell viability measured by using vital dyes such as trypan blue which enters dead cells only or neutral red that is actively taken up by living cells; the latter is commonly used in biomaterial testing by the agar overlay method (Guess et at., 1965).A count of dead and vital cells in comparison with the control provides an index of

Devang B. Sheth

lethality of the test compound. The release of SlCr is another index of lethality measuring membrane functions (Holden et al., 1973).

(5) Measurement of Biochemical or Metabolic cell alterations: Other indices of toxicity to basal cell functions involve measurement of biochemical or metabolic cell alterations. The pathways of energy transmission and their alterations, 2° consumption or ATP levels are usually measured by the Clark electrode (Harmon and Sanborn, 1982) and by the luciferase assay respectively.

One parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH activity is determined in an enzymatic test. The first step is the reduction of NAD^+ to $NADH/H^+$ by the LDH catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl) - 3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan (Elferink, 1979; Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988; Lappalainen et al., 1994).

Another parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. Tetrazolium salts are reduced only by metabolically active cells. Thus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) can be reduced to a blue colored formazan (Mosmann T, 1983). Table-3.4 represents various categories of viability assay and their principles.

Drug exposure:

Selection of drug concentrations for *in vitro* cytotoxic drug resistance assays is an important issue. There are at least three ways to approach the issue of appropriate drug exposure under assay conditions. One alternative is to find drug concentrations that mimic the *in vivo* situation, by means of comparison of intracellular uptake *in vivo* and *in vitro* (Sundman-Engberg B *et al*., 1990). A full concentration-effect curve can be aimed at, and the estimated IC50 value, can be used as the measure of activity (Fruehauf J and Bosanquet A, 1993). This will result in a better estimation of the

difference between resistant and sensitive samples, but for appropriate testing, an increased number of cells will be required.

Table – 3.4: *In Vitro* cytotoxicity assays and theirs principle

Sr. No.	Category of viability assay	Type of assay	Principles	Reference
1.	Membrane integrity assay	1. Trypan blue dye exclusion assay 2. Fluorescent dyes assay 3. LDH leakage assay	The determination of Membrane integrity via dye exclusion from live cells	Riss *et al.,* 2004
2.	Functional Assay	1. MTT, XTT assay 2. Crystal violet/ Acid phosphatise (AP) assay 3. Alamar Blue oxidationreduction assay 4. Neutral red assay 5. [3H]-thymidin/ BrdU Incorporation	Examining metabolic components that are necessary for cell growth	Marshall *et al.,* 1995
3.	Protein assay	SRB assay	Based on measurement of total protein content.	Coppeta and Rogers, 1998
4.	DNA labeling Assay	Fluorescent conjugates	Simultaneous cell selection and viability assay	Negoescu *et al.,* 1998
5.	Morphological Assay	Microscopic observation	Determination of morphological change	Kerr *et al.,* 1972
6.	Reproductive assay	Colony formation assay	Determination of growth rate	Franken *et al.,* 2006

3.4.5.2 Overview on *in-vitro* cytotoxicity assay used in experiment

Colorimetric assay are mainly useful in determination of cellular proliferation, viability and activation. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Such an example is based on the capability of the cells to incorporate a radioactively labeled substance ([3H]-thymidine), or to release a radioisotope such as [51Cr] after cell lysis. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts MTT and XTT are especially useful for assaying the quantification

Devang B. Sheth

of viable cells. Both, MTT and XTT work by being to a formazan dye only by metabolic active cells (Weyermann, 2005).

MTT assay

It is a laboratory test and a standard colorimetric assay for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials. This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble substrate 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in range of cell lines. (Cory et al., 1991).
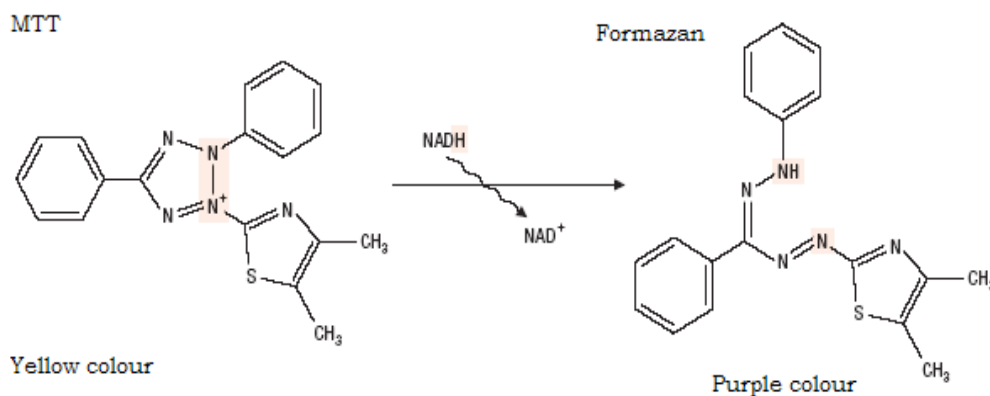

Figure 3.5: Principle of MTT assay

➢ **Application**

MTT used for the nonradioactive, spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well-plate format. It can be used for:

- Measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients.
- Analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds.

- Assessment of growth-inhibitory antibodies and physiological mediators. (Wilson, 2000)

➢ **Advantages of MTT assay**

- Rapid, versatile, quantitative and highly reproducible
- Adaptable to large-scale screening; relevant for most cells
- MTT reduction correlates to indices of cellular protein and earlier cell number
- More sensitive and earlier predictor of toxicity than classical LDH or neutral red measurements

➢ **Disadvantage of MTT assay**

- Production of the MTT product is dependent on the MTT concentration in the medium. The kinetics and degree of saturation are dependent on cell type.
- Assay is less effective in the absence of cell proliferation.
- MTT cannot distinguish between cytostatic and cytocidal effect.
- Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance.
- Test is less effective if cells have been cultured in the same media that has supported growth for a few days, which leads to underestimation of control and untreated samples (Bernas et al., 2002).

3.4.6 Abscisic acid and cancer

Many phytohormones such as jasmonates and salicylates have been shown to suppress growth of various types of cancer cells (Flescher and Eliezer, 2005). Limited information is available in the peer-reviewed literature regarding the possible role of ABA in the prevention and treatment of cancer. A patent was issued to Livingston-Wheeler in 1976 for its use as an anti-cancer compound (Livingston, 1976). The patent describes the use of a solid myeloid leukemia C1498 transplantation model in C57BL/6 mice to investigate the anti-cancer efficacy of ABA. The inventor reported increased survival rates at 14 days post-transplantation in mice treated with ABA in comparison to control mice. The inventor also proposed that ABA "neutralized" the human chorionic gonadotropin (hCG) that reportedly coated cancer cells, thereby facilitating anti-cancer immune responses ABA may represent a potential cancer treatment due to its ability to modulate calcium signalling (Livingston, 1976).

Several chemotherapeutic agents modulate pathways leading to increased intracellular calcium concentrations. Medications such as staurosporine, doxorubicin, tamoxifen, and etoposide act as anti-cancer agents which lead to the death of cancer cells by increasing $[Ca^{+2}]_{int}$ resulting in oxidative stress followed by apoptosis (Kruman et al., 1998; Panaretakis et al., 2005; Parihar et al., 2008). Thus, these pathways activated by ABA and those modulated by chemotherapeutic agents used for treating cancer are noticeably similar.

Moreover, epidemiologic studies demonstrated that the consumption of diet rich in abscisic acid is associated with a decreased risk of cancer (Riboli and Norat, 2003; Wigmore, 1985). Dr. Ann Wigmore mentioned that wheatgrass contains a variety of elements that is believed to be anticancer and abscisic acid is one of them. She reported that wheatgrass and controlled diet cured breast cancer of few patients. Even in experimental animal showed that even a tiny amount of abscisic acid has tremendous effect on cancer (Wigmore, 1985). Tan et al. (2006) reported in a China patent that ABA effectively inhibits the proliferation of tumour cells; stagnates the cells in S phase, stops cell division, and induces differentiation of tumour cells or reverts cancerous cells to normal cells. ABA was reported to inhibit proliferation and induced differentiation in human SMMC- 7221 cells (Ma et al., 2006) and in nude mice transplanted with human hepatocarcinoma (Lu et al., 2007). Additionally, abscisic acid is a close relative of Vitamin A. Retinoids (derivatives of Vitamin A) are currently used for treatment for breast cancer. Thus, exploring the role of ABA in cancer prevention and treatment in the context of well controlled, mechanistically oriented projects may result in the development of novel ABA-based chemotherapeutic approaches against cancer.

Devang B. Sheth

3.5 Cacium channel and abscisic acid

3.5.1 Types: (Rang et al., 2005)

Table – 3.5: Types and functions of calcium channel

Gated by	Types	Characteristics	Location / Function	Drug effects
Voltage	L	High activation threshold, slow inactivation	Plasma membrane of many cells; main Ca^+ source for contraction in smooth and cardiac muscle.	Blocked by dihydropyridines, verapamil, diltiazem Activated by Bay-K 8644
	N	Low activation threshold, slow inactivation	Main Ca^+ source for transmitter release by nerve terminals	Blocked by ω-conotoxin
	T	Low activation threshold, fast inactivation	Widely distributed; important in cardiac pacemaker & atria (role in dysrhythmias)	Blocked by mibefradil
	P/Q	Low activation threshold, slow inactivation	Nerve terminals; transmitter release	Blocked by ω-agatoxin
	R	Low activation threshold, fast inactivation	-	-
Inositol tri-Phosphate (IP_3)	IP_3 receptor		Located in ER/SR; mediates Ca^{2+} release in response to IP_3 produced by GPCR activation	Not directly targeted by drugs; some experimental blockers known; responds to GPCR agonist/antagonists in many cells
Ca^{+2}, Sensitized by cADPR	Ryanodine receptor	Directly activated in striated muscle via dihydropyridine receptor of T-tubules	Located in ER/SR; mediates Ca^{2+}-evoked Ca^{2+} release in muscle; also activated by the second messenger cADPR	Activated by caffeine (high concentrations), blocked by ryanodine
Store depletion	Store operated channels	Indirectly coupled to ER/SR Ca^{+2} stores	Located in plasma membrane	Activated indirectly by agents that deplete intracellular stores (e.g. GPCR agonists); not directly targeted by drugs
NAADP	-	Activated by NAADP	Located in lysosomes No clear function	

3.5.2 Physiological role

Calcium is the most important regulator of cell function. Many physiological mechanisms and drugs operate, directly or indirectly, by influencing the free intra cellular concentration of calcium. Different types of Ca^{+2} channels are involved in important physiological functions such as contraction of muscles, activation of various enzymes, release of neurotransmitters and hormones, gene transcription, etc. Intracellular calcium is usually bound to a protein called calmodulin. The calcium-calmodulin complex acts together with a further component (RE, response element, also a protein) as a protein kinase catalyzing the phosphorylation of numerous proteins that themselves control independent but partially parallel developmental processes, differentiation, and movements within the cell (Rang et al., 2005). Abnormal Ca^{+2} signalling is involved in many pathophysiological conditions, such as, cardiac dysrhythmias, hypertension, epileptogensis, ischaemic cell death, and endocrine disorders.

3.5.3 Drugs acting through Ca^{+2} channels

3.5.3.1 Ca^{+2} channel blockers

Many clinically important drugs like anti-hypertensive agents (e.g. nifedine, verapamil, diltiazem); anti-epileptic drugs (e.g. ethosuximide, sodium valproate, gabapentin, pregabalin); antidysrhythmic agents (e.g. class-IV antidysrhythmic agents), spasmolytic agents (e.g. dantrolene), etc. act by blocking calcium release (Rang et al., 2005). Many other drugs (like G-protein coupled receptor antagonists) act indirectly by blocking Ca^{+2} channels.

3.5.3.2 Ca^{+2} channel openers

Direct Ca^{+2} channel openers (e.g. Bay-K 8644) are more useful as experimental tool rather than as therapeutic agent. Variety of Ca^{+2} channel openers are useful in experimental pharmacology to produce hypertension, arrhythmia, epilepsy, spasm, etc. There are many drugs acting indirectly by opening Ca^{+2} channel like GPCR (G-protein coupled receptor) agonists, positive inotropic agents (e.g. digoxin), hormones, etc.

3.5.4 Abscisic acid and Calcium channels

Over the past few years, evidence from a variety of sources has suggested that calcium ions are involved in the molecular events underlying the action of growth substance in plants (De Silva et al., 1985). Ca^{+2} is a second messenger and intracellular regulator which regulates of growth and development in plants (Hepler, 2005).

Recent studies of the control of stomatal aperture by abscisic acid have suggested that its cellular action depends on the availability of calcium ions, which appear to function as secondary messengers. In plants, it has been demonstrated that ABA stimulates the release of intracellular calcium in conjunction with the up-regulation of cyclic ADP-ribose (Wu et al., 1997). In plants, ABA depolarizes plasma membranes, which activates potassium ion channels and thereby extrudes K^+ outside the cells (Schroeder et al., 1987). Ion channel depolarization is dependent on calcium, which is an ABA second messenger (Schroeder et al., 2001). The increase in calcium concentrations also leads to increased nitric oxide (NO) production (Bodrato et al., 2009). Huddart et al. (1986) found that ABA may serve as a universal Ca^{+2} agonist across taxonomic kingdoms based on experiments with various smooth muscle preparations and a cyanobacterium.

In contrast, Lynch et al (1991) reported that abscisic acid (ABA) analogue SD217595 inhibits K^+-induced phasic and tonic contractions of rat bladder detrusor smooth muscle strips. This dual inhibition was hypothesized due to blockade of two subtypes of voltage-operated calcium channels with T- and L-type characteristics. This was further supported by finding that ABA is without significant Ca^{+2} modulatory activity in rat prostatic and epididymal vas deferens smooth muscle preparation but the ABA analogue SD217595 possesses strong Ca^{+2} entry blocking ability (Masters et al., 1994). Thus, role of abscisic acid in relevance to its Ca^{+2} channel modulatory action is controversial.

Devang B. Sheth

In nutshell the objectives of the present project were:

- ➢ To investigate pharmacological effects of abscisic acid on fructose induced insulin resistance

- ➢ To screen abscisic acid for *in-vitro* anti-cancer activity in breast cancer cell-lines

- ➢ To assess Ca^{+2} channel modulatory action of abscisic acid, using isolated rat ileum and in-vivo effect on various cardiac parameters in rat

- ➢ To carry out preliminary toxicological study of abscisic acid in rats

Devang B. Sheth
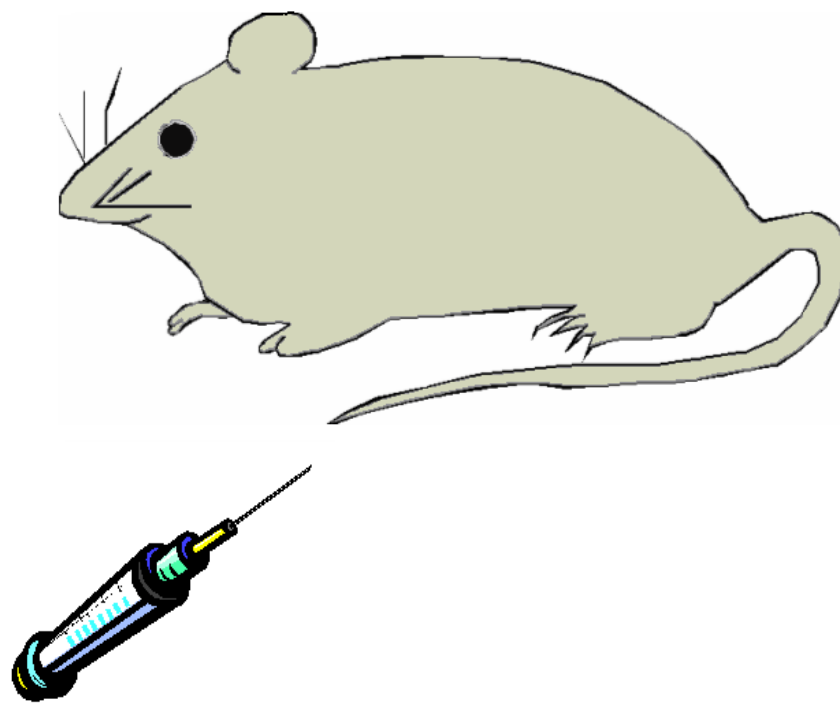
Materials & Methods

5.1 Sources of chemicals

2 – cis, 4 – trans abscisic acid (98%, synthetic) was purchased from Sigma Aldrich, USA.

All the routine reagents, routine chemicals were obtained from Ranbaxy chemicals Ltd, Delhi; Qualigens chemicals Ltd, Bombay; S.D. Fine chemicals, Boisar.

5.2 Experimental models

5.2.1 Anti-diabetic activity (Fructose-induced insulin resistance in rats)

5.2.1.1 Indirect type molecular modeling

3D structural similarity between abscisic acid and thiazolidinedione was checked by indirect type of molecular modeling study using PC based Discovery Studio (Version 2.1, Accelrys Inc. USA).

5.2.1.2 Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of R. K. College of Pharmacy, Rajkot and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol No. RKCP/COL/RP/09/02).

Ten week old male Sprague-Dawley rats (200 ± 25 gm) were housed in-group of 3 animal in cages and maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and provided free access to palleted diet and purified drinking water ad libitium, unless specified.

Parameters assessed:

> **Serum:** Fasting glucose, fasting insulin, fasting insulin resistance index (FIRI), oral glucose tolerance test (OGTT), total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, VLDL cholesterol.
>
> **Liver:** Glycogen
>
> **Muscle:** Glycogen

Devang B. Sheth

5.2.1.3 Experimental design

Rats (n=24) were randomized into following groups:

1) Normal control (CON): They were administered with vehicle (water) for 30 days. They were fed with standard laboratory diet and water *ad libitum.*

2) Disease control (FRU):- They were administered with vehicle (water) for 30 days. They were fed with standard laboratory diet and 10% fructose in water for 30 days.

3) Standard (PIO + FRU):- Pioglitazone (10 mg/kg/day, p.o.) was administered for 30 days along with 10% fructose in water.

4) Test (ABA + FRU):- Abscisic acid (1 mg/kg/day, p.o.) was administered for 30 days along with 10% fructose in water.

Rats were weighed and their food/water intake was recorded weekly. On 29th day Oral glucose tolerance test was performed. On 30th day animals were used for serum collection followed by tissue collection. Serum and tissue were subjected to different biochemical analysis.

5.2.1.4 Oral glucose tolerance test (OGTT)

Method of Shrwaikar et al. was used for the OGTT. The oral glucose tolerance test was performed in overnight fasted rats. Rats divided into four groups (n=6) were administered drinking water (normal control and disease control), pioglitazone and abscisic acid respectively. Glucose (2g/kg) was fed 30 min after the administration of drug. Blood was withdrawn from the retro orbital plexus under light ether anesthesia at 30, 60,120 min of glucose administration.

5.2.1.5 Collection of serum and detection of serum parameters

The blood samples were withdrawn from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept at 4ºC until used.
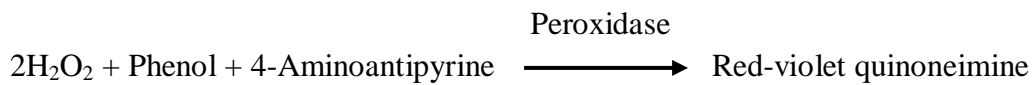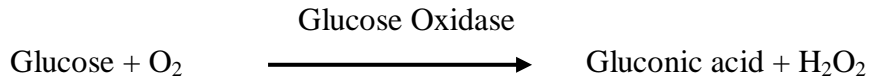
The serum was estimated for fasting glucose and fasting insulin as described following:

Devang B. Sheth

5.2.1.5.1 Fasting glucose (Teitz NW, 1970)

In vitro quantitative determination of glucose in serum was done using enzymatic kit (ACCUCARE™, Lab – Care Diagnostics India Pvt. Ltd.).

Principle:

Enzymatic colorimetric determination of glucose according to the following reactions:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red-violet quinoneimine}$$

Procedure:

Pippette in the tubes labeled as follows:

	Blank	Standard	Sample
Sample	-	-	10 µl
Standard	-	10 µl	-
Reagent	1000 µl	1000 µl	1000 µl

Mix and incubate for 15 min. at 37º C for 30 min. at room temperature. Measure absorbance of sample (AT) and standard (AS) against reagent blank at 505 nm. The colour is stable for at least 30 min. at room temperature.

Calculations:

$$\text{Fasting Glucose (mg/dl)} = \frac{(AT)}{(AS)} \text{ x Concentration of standard}$$

5.2.1.5.2 Fasting insulin

Serum fasting insulin was estimated at Saurashtra Pathology Laboratory – Rajkot using radio immune assay (RIA) kit.

Principle:

The radioimmunoassay method is based upon the competition of unlabelled insulin in the standardized samples and radio iodinated (I-125) insulin for the limited binding sites on a specific antibody.

Devang B. Sheth

At the end of incubation, the antibody bound and free insulin are separated by second antibody-polyethylene glycol (PEG) aided separation method. Insulin concentration of samples is quantified by measuring the radioactivity associated with the bound fraction of sample and standard.

5.2.1.5.3 Fasting insulin resistance index (FIRI) (Duncan et al., 1995)

Fasting insulin resistance index (FIRI) was calculated by following formula

FIRI= fasting blood glucose in mg/dl x fasting insulin in uU/ml) / 25

5.2.1.5.4 Estimation of total cholesterol

(CHOD/POD-Phosphotungstate method)

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit (Monozyme India Limited).

Principle:

Cholesterol esterase (CHE) hydrolyses cholesterol ester. Free cholesterol is oxidized by the cholesterol oxidize (CHO) to choloest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce pink colored quinoneimine dye. The intensity of color produced is proportional to cholesterol concentration.

Procedure:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Total Cholesterol (Tc) as shown below;

	Blank	Standard	Total Cholesterol
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Specimen	-	-	20 µl
A) Mix and incubate at 37ºC for 5 minutes (or) RT for 10 minutes.			
Distilled water	2.0 ml	2.0 ml	2.0 ml

Devang B. Sheth

Read the absorbance of standard, total cholesterol against Blank at 505 nm.

Calculations:

Total Cholesterol (mg/dl) = Abs. of Tc / Abs. of S X 200

5.2.1.5.5 Estimation of triglycerides (GPO Method)

In vitro quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit (Reckon diagnostics (India) Pvt. Ltd.).

Principle:

Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in reaction catalyzed by glycerol kinase (GK). G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a quinoneimine purple colored dye.

The absorbance of this dye in solution is proportional to the concentration of triglycerides in sample.

Procedure:

	Blank	Standard	Test
Working reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	20 µl	-
Specimen	-	-	20 µl
A) Mix and incubate at 37ºC for 15 minutes.			
Distilled water	1.5 ml	1.5 ml	1.5 ml

Mix and read the absorbance of test and standard against reagent blank at 546 nm.

Devang B. Sheth

Calculations:

Triglycerides (mg/dl) = Abs. of Test/ Abs. of Std. X   50

5.2.1.5.6 Estimation of HDL-cholesterol (CHOD/POD-Phosphotungstate method)

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit (Monozyme India Limited.)

Principle:

The VLDL and LDL fractions of serum sample are precipitated using PTA and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester cholesterol. Then cholesterol is oxidized by cholesterol oxidase (CHO) to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) reacts with 4-aminoantipyrine and phenol to produce a red colored complex, whose absorbance is proportional to HDL-cholesterol concentration.

Procedure:

Pipette into a centrifuge tube:

Serum	0.2 ml
Precipitating Reagent	0.3 ml

Mix well and allow standing at RT for 5 minutes. Centrifuge at 3000 rpm for 10 minutes to get a clear supernatant. If the supernatant is not clear (high TGL level) dilute the sample 1:1 with normal saline. Pipette into 3 test tubes labeled Blank (B), Standard (S) and HDL Cholesterol (H) as shown below;

	Blank	Standard	HDL Cholesterol
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Supernatant	-	-	20 µl
A) Mix and incubate at 37ºC for 5 minutes (or) RT for 10 minutes.			
Distilled water	2.0 ml	2.0 ml	2.0 ml

Read the absorbance of standard, total cholesterol against Blank at 505 nm.

Devang B. Sheth

Calculations:

HDL Cholesterol (mg/dl) = Abs. of H / Abs. of S X 50

5.2.1.5.7 Estimation of LDL-cholesterol (Tiwari and Gode, 1990)

Estimation of LDL-cholesterol was done using the Friedewald formula.

LDL cholesterol = total cholesterol – (HDL cholesterol + VLDL cholesterol)

5.2.1.5.8 Estimation of VLDL-cholesterol (Tiwari and Gode, 1990)

Estimation of VLDL-cholesterol was done using the Friedewald formula.

VLDL cholesterol = triglycerides / 5

5.2.1.6 Liver and Muscle Glycogen (Stafford et al., 1955)

The liver and thigh muscle were removed and blotted on filter paper to remove blood, weighed, dropped into flasks containing 10 ml hot 30% potassium hydroxide and digested on a hot plate. The digest was diluted to 100 ml and a 50-fold dilution of an aliquot was used for analysis. Ten ml 0.2% anthrone in 95% sulfuric acid was slowly added to 5 ml of liver digest dilution with cooling. The mixture was heated in a boiling water bath for 10 min and then placed into cold water. Optical density was measured in a spectrophotometer at 620 μm using the anthrone-reagent as blank. Calibration curves were established using glucose as standard.

Devang B. Sheth

5.2.2 Anti-cancer activity

5.2.2.1 Indirect type molecular modelling

3D structural similarity between abscisic acid and vitamin A was checked by indirect type of molecular modeling study using PC based Discovery Studio (Version 2.1, Accelrys Inc. USA).

5.2.2.2 Material

5.2.2.2.1 Reagents

1. Trypan blue Dye (Hyclone, Lot No: 029K2358, 100 ml)
2. Triton X100 (MP Biomedicals, Lot No: 8009H, 100 ml)
3. DMSO cell culture grade (Bioworld, Lot No: 1388B230, 500 ml)
4. Sodium bicarbonate (Bioworld, Lot No: 1775B29)
5. Amphotericin B (Himedia, Lot No: 1397893, 100 ml)
6. Penicillin and Streptomycin solution stabilized (Sigma, Lot No: 1208029, 100 ml)
7. EDTA (MP Biomedicals, Lot No: YY02022B207Y)
8. DPBS modified 1X (Dulbecoo's phosphate buffer saline without Ca+ and Mg+) (Himedia, Lot No: LW537, 100 ml)
9. Trypsin 1X Gamma irradiated (SAFC Bioscience, Lot No: 8NO535, 500 ml)
10. Methotrexate (MP Biomedicals, Cat no. 102299, Lot no. R27204)
11. Triton – X 100 (Bioworld, Cat no. 730208, Lot no. 18278075)
12. Iso Propanol (Finar Chemicals, Cat no. 11390, Lot no. 19075330)

5.2.2.2.2 Media

1. DMEM (Dulbecoos Modified Eagels medium, low glucose with glutamine) (MP Biomedical, Lot No: C1478)
2. FBS (Fetal Bovine Serum, South American origin, 500 ml) (Quaditive, Lot No: 103128, 500 ml)
3. SFM HEKTM (Hyclone, Lot no: ARF26635, 500ml)
4. Fluid thioglycolate media (TGM) (Himedia, Lot No: YHI25)
5. Tryptone Soya broth (TSB) (Himedia, Lot No: YH031)

Devang B. Sheth

5.2.2.2.3 Cell lines

1. MCF-7 celline (Breast cancer cell line)
2. MDA-MB-468 (Breast cancer cell line)
3. HEK293T (Human Embryonic Kidney cell line - Normal cell line)

5.2.2.2.4 Glass wares and plastic wares

1. 96-well microtiter plate (Flat Bottom, U Bottom, V Bottom)
2. Tissue culture flasks (75 cm^2 T Flask vented and 150 cm^2 T Flask vented)
3. Falcon tubes (15 ml, 50 ml), Cryotubes (2 ml), Cell scrapper
4. Micro tips (Blue 1000 μl, Yellow 200 μl, White 10 μl) (Volex)
5. Reagent bottles (100 ml, 250 ml, 500 ml, 1000 ml)
6. Haemocytometer cell counting chamber

5.2.2.2.5 Equipments

1. Fluorescence inverted microscope (Leica DM IL, Germany)
2. Biosafety cabinet classII (Esco, Singapore)
3. Cytotoxic safety cabinet (Esco, Singapore)
4. CO2 incubator (RS Biotech, mini galaxy A, Scotland)
5. Deep freezer (Dairei, Denmark)
6. ELISA plate reader (Thermo, USA)
7. Micropipettes (Eppendorff, Germany)
8. RO water system (Millipore, USA)
9. Electronic water bath (Genei, India)

5.2.2.2.6 Cell proliferation kit

1. MTT Dye Powder (Serva Electrophoresis, Cat no. 20395, Lot no. 080364)

5.2.2.3 Methods

5.2.2.3.1 Characterization of Cell Lines and Culture Media

Characterization is essential not only when deriving new lines, but also when a cell line is obtained from a cell bank or other laboratory. Cultures were examined under an inverted phase microscope before start of experiments and frequent assessments are made of the viability of the cell population throughout the experimental periods.

Devang B. Sheth

5.2.2.3.1.1 Testing for Microbial Contamination

The two methods generally used by us in our laboratory to check for bacterial and fungal contamination. Detection carried out using special media like Fluid thiogycolate media (TGM) and Tryptone Soya broth (TSB) and direct observation using Grams stain.

Contamination by bacteria, yeast or fungi was detected by an increase in turbidity of the medium and/or a decrease in pH (yellow in media containing phenol red as a pH indicator). Cells were inspected daily for presence or absence of microbial growth.

5.2.2.3.1.2 Protocol (Freshney, 2001)

- Cell lines were cultured in the absence of antibiotics prior to testing using 25 cm^2 non-vented T-flask.
- In case of adherent cell line, attached cells were bringing in to into suspension using a cell scraper. Suspension cell lines were tested directly.
- 1.5 ml test sample (cells) were Inoculated in to two separate test tubes of each containing Thioglycollate Medium (TGM) and Tryptone Soya broth (TSB).
- 0.1 ml *E. Coli,* 0.1 ml *B. Subtilis and* 0.1 ml *C. Sporogenes* inoculated in to separate test tubes (duplicate) containing (TGM) and (TSB). These were act as positive controls where as two separate test tubes of each containing (TGM) and (TSB) un-inoculated as negative controls.

Broths were incubated as follows:

- For TSB, one broth of each pair were incubated at 32 ℃ the other at 22 ℃ for 4 days.
- For TGM, one broth of each pair were incubated at 32 ℃ the other at 22 ℃ for 4 days.
- For the TGM inoculated with *C. Sporogenes* incubate at 32 ℃ for 4 days.

Note: Test and Control broths were examined for turbidity after 4 days.

Criteria for a Validity of results: If control broths show evidence of bacteria and fungi within 4 days of incubation in all positive control broths and the negative control broths show no evidence of bacteria and fungi.

Devang B. Sheth

Criteria for a Positive Result: Test broths containing bacteria or fungi show turbidity.

Criteria for a Negative Result: Test broths should be clear and show no evidence of turbidity.

5.2.2.3.2 Preparation of media

5.2.2.3.2.1 Preparation of DMEM

13.37 gm of DMEM powder was added in 1litre of distilled water and then it was stirred continuously until clear solution formed. To this, $NaHCO_3$ was added to maintain pH 7.0 – 7.2 and then solution was filtered using membrane filtration assembly. It was sterile in autoclave and stored in reservoir bottle in refrigerator at 4^oC.

5.2.2.3.2.2 Preparation of the Trypsin dilution

5 ml of Trypsin solution was pipette out in to 50 ml falcon centrifuge tube containing 45 ml of PBS using 10 ml pipette.

5.2.2.3.3 Determination of cell viability, density and population doubling time

The quantification of cellular growth, including proliferation and viability, has become an essential tool for working on cell-based studies.

5.2.2.3.3.1 Cell viability by Trypan Blue Dye Exclusion Method

The viability of cells was determined by the Trypan Blue dye exclusion method. It takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue. (Freshney, 2001)

5.2.2.3.3.2 Haemocytometer Cell Counts

1. Hemocytometer and cover slip were cleaned and wiped with 70% alcohol. Then cover slip was placed on haemocytometer.
2. In separate 2 ml centrifuge tube, cell suspension (cells in culture media) was added. Then two fold dilution of reaction mixture was prepared by mixing aliquot of 0.1 ml cell suspensions with 0.1 ml trypan blue.

3. Afterwards 0.1 ml of cell suspension was then placed in to chamber of haemocytometer.

4. By using a Lieca inverted microscope, numbers of cells were counted in 1 mm^2 area with use of 10X objective lens.

5. Viable and non-viable cells were counted in both halves of the chamber.

5.2.2.3.3.3 Calculations

(1) Total number of viable cells = $A \times B \times C \times 10^4$

(2) Total dead cell count = $A \times B \times D \times 10^4$

Where,

A = Vol. Of cell solution (ml)

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead/stained cells

10^4 = Conversion of 0.1 mm^3 to ml

(3) Total cell count = Viable cell count + dead cell count

➢ **% viability** = (Viable cell count / Total cell count) $\times$ 100

Note: Cell quantification, module 4B:1, Hemocytometer cell counts and viability studies, 1.1 – 1.5 used as reference in counting of cells.

➢ **Cell density: cells/cm^2**

It was particularly important in case of adherent cell line like MCF – 7 and HEK 293T. It was calculated by following equation:

[No. of cells / well or flask] / [surface area of well or flask]

➢ **Population doubling time (PDT)**

It is the time expressed in hours, taken for cell number to double and is reciprocal of the multiplication rate (1/r).

N^H = No. of cells harvested at the end of growth period that is t_2

N^I = No. of cells inoculated at time t_1 = 0

$$n = 3.32 \ (\log N^H - \log N^I)$$

PDT = total time elapsed / no. of generations = 1/r

➢ **Multiplication rate (r)**: No. of generation that occurs per unit time and is usually expressed as population doubling in 24 hours.

$$r = 3.32 \ (\log N^H - \log N^I) / t_2 - t_1$$

5.2.2.4 Preparation of compound dilution

5.2.2.4.1 Preparation of test Sample

Test sample was dissolved in DMSO (2%) solution and appropriately diluted using same solvent to prepare different concentration of test compound in micro molar concentration.

Note: The final concentration of DMSO (2%) used does not interfere cell viability (Li-Jun Yang et al; 2009)

5.2.2.4.2 Dilution of Test Compound

100 μl of 1mM concentration of test compound was added in to 900 μl of complete media and as a result 100 μM concentration of test sample was obtained.

Then 1:3 dilution of test sample was done as shown in Table. It was done by mixing 50 μl of test compound with 100 μl of complete media. For this, initially 100 μl of complete media was added in to well no. 1 – 9. Well 10 contained 150 μl test substance only, from that 50 μl was pipetted out and added into well no. 9 which already contain 100 μl of complete media, which lead to 1:3 dilution of test sample. Same procedure was repeated 9 times in order to get final conc. of test Sample up to 0.005 μm (Table 5.1).

Devang B. Sheth

Table - 5.1: Dilution of test compound used in the assay

Well no. 1-9 contain complete media 100µl										
Well No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Compound dilution	50 µl mixture from well 2	50 µl mixture from well 3	50 µl mixture from well 4	50 µl mixture from well 5	50 µl mixture from well 6	50 µl mixture from well 7	50 µl mixture from well 8	50 µl mixture from well 9	50 µl T.C. from well 10	150 µl T.C.
Final con. (µM)	0.005	0.015	0.045	0.13	0.41	1.23	3.7	11.1	33.3	100

Where, T.C. = Test compound

5.2.2.4.3 Reference substance

Doxorubicin, a cytotoxic substance used in anti-neoplasic therapy, was evaluated in the same cellular system to obtain cytotoxicity parameters, allowing us to classify the thiazole derivatives according to their relative toxicity. M.W. of Doxorubicin is 545.3 gm/mol. Stock solution of it was prepared with 1 ml of DMSO, which produces stock solution of Doxorubicin of 10 mM concentration. Stock solution was further diluted for 10 times using DMSO to obtain 100 µM solution.

Table - 5.2: Plate assignment

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.005 µM/ml	0.01 µM/ml	0.04 µM/ml	0.13 µM/ml	0.41 µM/ml	1.23 µM/ml	3.7 µM/ml	11.11 µM/ml	33.33 µM/ml	100 µM/ml	NC	PC
B	0.005 µM/ml	0.01 µM/ml	0.04 µM/ml	0.13 µM/ml	0.41 µM/ml	1.23 µM/ml	3.7 µM/ml	11.11 µM/ml	33.33 µM/ml	100 µM/ml	NC	PC

Where, PC = Positive control (cells + media; no drug),

NC = Negative control (only complete media; no cells; no drug)

5.2.2.5.1 Experimental setup

5.2.2.5.1.1 Cell lines and culture medium

MCF-7, MDA – MB468 and HEK293T cell cultures derived from National Centre for Cell Science (NCCS) - Pune, were used in these experiments. Stock cells of these cell lines were cultured in DMEM, supplemented with 10% FBS (fetal bovine serum). Along with media cells were also supplemented with 5 % HBSS, penicillin, streptomycin and Amphotericin – B, in a humidified atmosphere of 5 % CO_2 at 37 °C until confluence reached. The cells were dissociated with 0.2 % trypsin, 0.02 % EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 cm^2 tissue culture flasks, than in 75 cm^2 and finally in 150 cm^2 tissue culture flask and all cytotoxicity experiments were carried out in 96 microtitre well-plates. 2×10^4 cells/well was added in to each well of 96 well-plates. It was calculated as followed:

5.2.2.5.1.2 Calculation for number of cells in 96 well plates

For this we need to calculate for no. of cells required for 100 wells≈ 96 well,

No. of cells / well $\times$ 100

$$= 2 \times 10^4 \times 100$$
$$= 2 \times 10^6 \text{ cells / plate}$$

Total volume of media for 100 wells

$$= \text{volume of media / well} \times 100$$
$$= 100 \text{ μl} \times 100$$
$$= 10 \text{ ml}$$

Therefore, we need a total of 2×10^6 cells in 10 ml of medium, then aliquot the required volume of cell suspension in to each wells.

5.2.2.5.1.3 Design of experiment

Cell lines in exponential growth phase were washed, trypsinized and re-suspended in complete culture media. Cells were seeded at 2×10^4 cells / well in 96 well microtitre plate and incubated for 24 hrs during which a partial monolayer forms. The cells were then exposed to various concentrations of abscisic acid (as indicated in plate assignment) and standard doxorubicin. Control wells were received only maintenance

Devang B. Sheth

medium. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2, 75% relative humidity for a period of 24 hrs. Morphological changes of drug treated cells were examined using an inverted microscope at different time intervals and compared with the cells serving as control. At the end of 24 hrs, cellular viability was determined using MTT assay.

5.2.2.5.2 Screening of Test Compound by MTT Assay

5.2.2.5.2.1 Protocol

- Cells were pre incubated at a concentration of 1×10^6 cells / ml in culture medium for 3 hrs at 37°C and 6.5% CO_2, 75% relative humidity.
- Cells were seeded at a concentration of 5×10^4 cells / well in 100 μl culture medium and various amounts of compound (final concentration e.g. 100 μM – 0.005 μM) were added into microplates (tissue culture grade, 96 wells, flat bottom).
- Cell cultures were incubated for 24 hrs at 37 °C and 6.5% CO_2.
- 10 μl MTT labeling mixture was added and incubate for 4 hrs at 37 °C and 6.5% CO_2, 75% relative humidity.
- 100 μl of solubilization solution was added to each well and incubate for overnight.
- Absorbance of the samples was measured using a microplate (ELISA) reader.
- The wavelength to measure absorbance of the formazan product is between 540 and 600 nm according to the filters available for the ELISA reader, used (The reference wavelength should be more than 650 nm).

5.2.2.5.2.2 Data interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes. After 24 hrs, the cytotoxicity data was evaluated by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95 % confidence limit and R^2 were used to define dose-response curves and to compute the

concentration of chemical agents needed to reduce absorbance of the formazan by 50 % (IC_{50}).

Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

$$\% \text{ Viability} = (A_T - A_B) / (A_C - A_B) \times 100 \ldots \ldots \ldots (1)$$

Where,

A_T = Absorbance of treated cells (drug)

A_B = Absorbance of blank (only media)

A_C = Absorbance of control (untreated)

There by,

$$\% \text{ Cytotoxicity} = 100 - \% \text{ cell survival} \ldots \ldots \ldots (2)$$

➢ **Determination of IC_{50} Value**

According to the FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In our study, IC_{50} is a concentration of drug at which 50 % of cell population die.

For primary screening, we use a threshold of 50 % cell growth inhibition as a cut off for compound toxicity against cell lines. IC_{50} is determined from plot of Dose Response curve between log of compound concentration and percentage growth inhibition. IC_{50} value has been derived using curve fitting methods with *Graph Pad Prism* as stastical software (Ver. 5.02) (Vanicha vichai *et al.*; 2006). IC_{50} values were calculated using the nonlinear regression program Origin The average of two (duplicates manner) were taken in determination.

Graph was plotted by keeping log concentration of drug on X axis and % cell growth inhibition or % cytotoxicity Y axis. IC_{50} was estimated as a concentration of drug at 50 % position on Y axis. The relationship should be sigmoidal, log drug concentration on the X axis and 'response / measurement' of the Y axis.

Devang B. Sheth

5.2.3 Ca^{+2} channel modulatory action

5.2.3.1 Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of R. K. College of Pharmacy, Rajkot and Maliba Pharmacy College, Bardoli with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol No. RKCP/COL/RP/09/02; MPC 1206).

5.2.3.2 Effects on isolated rat ileum

5.2.3.2.1 Material
5.2.3.2.1.1 Apparatus

1. Mammalian organ bath
2. Isotonic frontal writing lever
3. Recording drum
4. Aeration tube cum tissue holder
5. Dissection box

5.2.3.2.1.2 Chemicals

1. Tyrode solution
2. Barium chloride
3. Papaverine

5.2.3.2.2 Method (Goyal et al., 2010)

Healthy Wistar male rats weighing 230-250 gm (n=3) were kept for overnight fasting. They were sacrificed as per CPCSEA guidelines. The abdomen was quickly opened and a piece of ileum is isolated. It was placed in a petridish containing Tyrode solution maintained at 37º C. The mesentry of ileum was removed and the lumen of ileum was cleaned by passing warm Tyrode through it from a pipette held at an angle of about 20-30 degrees. The tissue was mounted in mammalian organ bath and connected to isotonic frontal writing lever. The tissue was allowed to stabilize for 30 min. The responses for abscisic acid at concentration of 1,10 and 100 µg/ml were taken alone and in presence of BaCl$_2$ / papaverine.

5.2.3.3 Effect on cardiovascular system parameters

5.2.3.3.1 Material

5.2.3.3.1.1 Apparatus

1. Power Lab
2. Blood pressure transducer
3. Pair of scissors
4. Burette

5.2.3.3.1.2 Chemicals

1. Saline
2. Heparin
3. Urethane
4. Verapamil

5.2.3.3.2 Method (Ordodi et al., 2005)

Healthy Wistar male rats (230-250 gm) were anesthetized with urethane (1200 mg/kg). Femoral vein was cannulated with fine polyethylene catheter for administration of the drug. Tracheostomy was performed and blood pressure (BP) was recorded from left common carotid artery using pressure transducer by direct method on Chart data system (Power Lab/4SP, AD Instrument, Australia). Heparinized saline (100 IU/ ml) was filled in the transducer and in the fine polyethylene catheter cannulated to the carotid artery to prevent clotting. After 30 min of stabilization, mean change in blood pressure, heart rate and ECG were recorded for before and after administration of abscisic acid (0.1 mg/kg). Effect of abscisic acid was checked in presence of verapamil (0.1 mg/kg)/ Calcium gluconate (0.1 mg/kg).

5.3 Preliminary toxicity testing

The rats (n=6) were treated with abscisic acid (1 mg/kg/day, p.o.) for 30 consecutive days. These rats were observed during the treatment period for over clinical signs of toxicity and stress as compared to control (vehicle treated) rats. On 30th day, blood was collected by retro-orbital method under light ether anaesthesia. Collected uncoagulated blood was used for measurement for total RBC count, white blood cell count (total and differential), platelet count and haemoglobin content (Ghai, 2000).

The animals (n=4) were then sacrificed. Part of liver, heart, lung (right) and kidney (right) tissues were dissected out and kept immediately in 10% formalin for storage. Histopathology studies were performed.

5.4 Statistical analysis

All the values are expressed as mean S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using computer based fitting program (Prism, Graphpad). Differences were considered to be statistically significant when $p < 0.05$.

Devang B. Sheth

Results

6.1 Anti-diabetic activity (Fructose-induced insulin resistance in rats)

6.1.1 Indirect type molecular modelling

3D structural similarity between abscisic acid and thiazolidinedione was confirmed by indirect type of molecular modeling study. Both the structures were generated, energy minimized and superimposed (Figure-6.1) using PC based Discovery Studio (Version 2.1, Accelrys Inc. USA). All geometries were fully optimized by minimizing the energy with respect to geometrical variables without symmetry constraints, using a 0.01 kcal/mol gradient. The r.m.s.d. observed was 0.325. The low root mean square distance (r.m.s.d.) value suggests good 3D similarity between abscisic acid and thiazolidinedione.
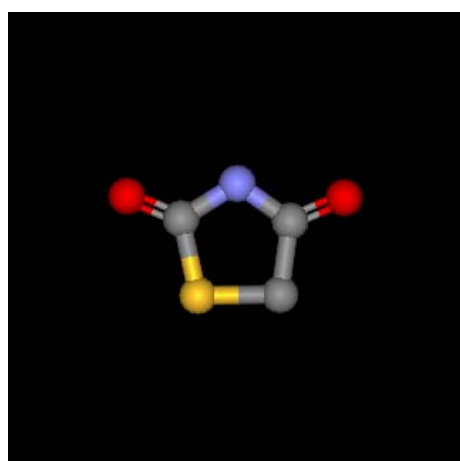
3D structure of thiazolidinedione   **3D structure of abscisic acid core**
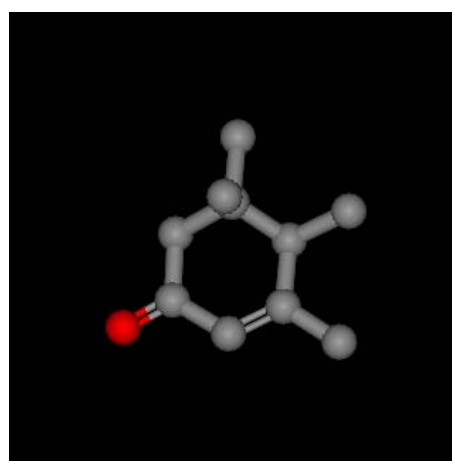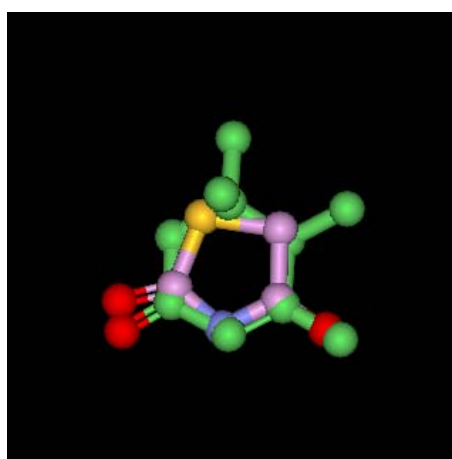

Figure-6.1: 3D Overlay of abscisic acid (green) and thiazolidinedione (pink)

6.1.2 Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) showed that at all time intervals fructose treatment significantly (p<0.01) increased the serum glucose levels as compared to normal group. Abscisic acid as well as pioglitazone treatment significantly (p<0.01) decreased the fasting serum glucose levels as compared to fructose treated animals (Table-6.1, Fig-6.2).

Table-6.1: Effect of Abscisic acid (1 mg/kg/day, 30 days) on oral glucose tolerance test (OGTT) in fructose-induced insulin resistance in rats

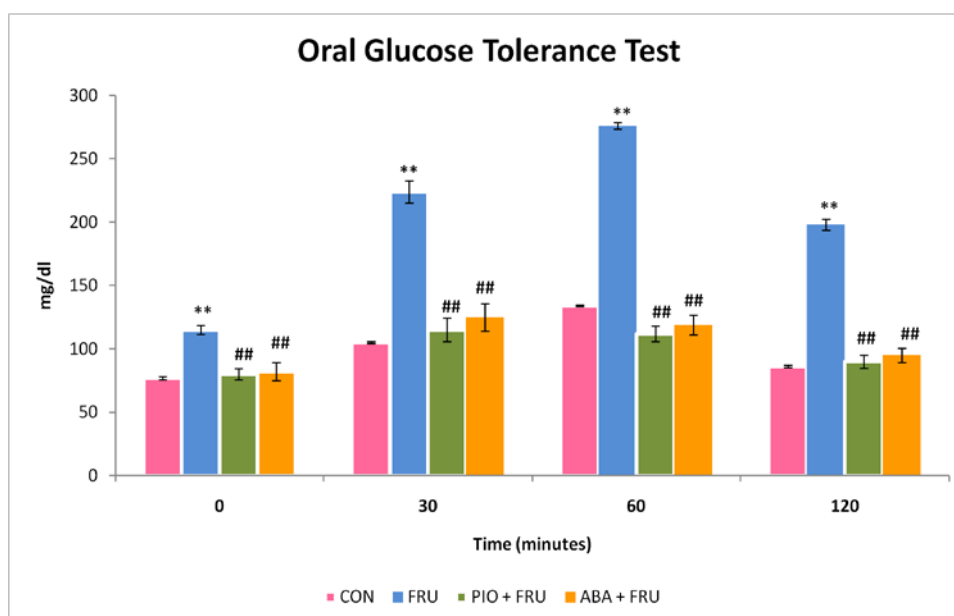| Treatment (n=6)	Time (minutes)			
	0	30	60	120
Normal control (CON)	77 ± 1.23	105 ± 3.45	134 ± 4.33	86 ± 7.21
Disease control (FRU)	115 ± 4.55[**]	224 ± 8.64[**]	276 ± 9.23[**]	198 ±10.76[**]
Standard (PIO + FRU)	80 ± 0.54[##]	115 ± 2.53[##]	112 ±6.27[##]	90 ± 7.63[##]
Test (ABA + FRU)	82 ± 0.98[##]	125 ± 4.33[##]	119 ±5.22[##]	95 ± 5.74[##]

Figure-6.2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on oral glucose tolerance test (OGTT) in fructose-induced insulin resistance in rats

All values represents Mean ± SEM; n=6

** Significantly different from normal control (p < 0.01)
Significantly different from disease control (p < 0.01)

Devang B. Sheth

6.1.3 Serum parameters

Serum parameters were found to be increasing significantly with administration of 10% fructose feeding for 30 days. Fructose significantly increased serum fasting glucose (p<0.01), serum insulin (p<0.05) and FIRI (p<0.01) as compared to control group (n=6).

Pioglitazone and abscisic acid both exhibited significantly reduction in serum fasting glucose as compared to disease control. Treatment with abscisic acid (1 mg/kg, p.o.) and pioglitazone (10 mg/kg, p.o.) for 30 days in fructose fed rats resulted in serum fasting glucose, 82 ± 0.98 and 80 ± 0.54 mg/dl, respectively as compared to 115 ± 4.55 mg/dl in disease control (Table-6.2, Fig-6.3). Abscisic acid showed significant anti-hyperglycaemic activity by bringing back serum fasting glucose near to normal as identical to pioglitazone.

Both abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment resulted in significant decrease (p<0.05) in fasting insulin, 24 ± 0.26 and 23 ± 0.42 µU/ml, respectively as compared to 29 ± 0.15 µU/ml in disease control (Table-6.2, Fig-6.4). Fasting insulin resistance index (p<0.01) was also found to be reduced as compared to disease control group (Table-6.1, Fig-6.5), indicating improvement in insulin resistance produced by fructose feeding.

Table-6.2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting glucose, serum fasting insulin and fasting insulin resistance index in fructose-induced insulin resistance in rats

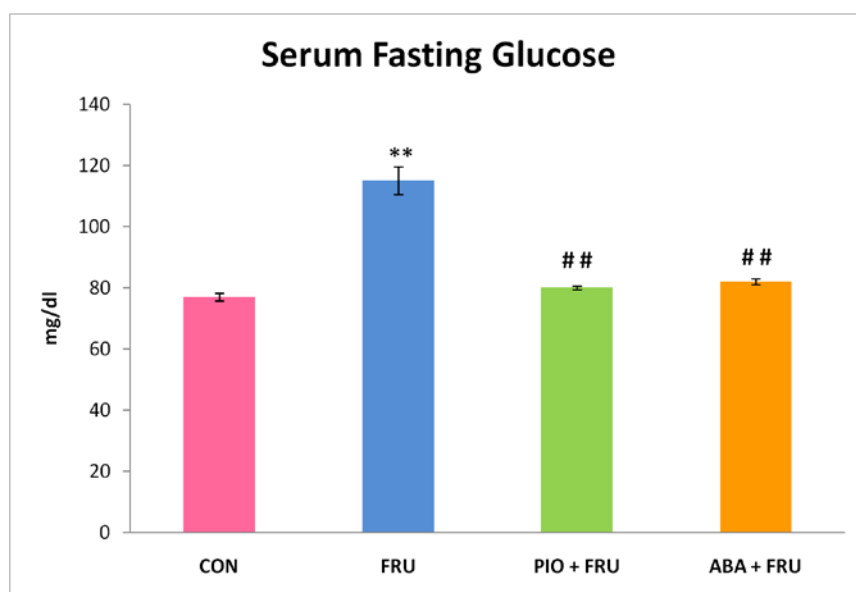| Treatment (n=6)	Serum fasting glucose (mg/dl)	Serum fasting insulin (µU/ml)	Fasting insulin resistance index (FIRI)
Normal control (CON)	77 ± 1.23	22 ± 0.38	67.76
Disease control (FRU)	115 ± 4.55 [**]	29 ± 0.15 [*]	133.4 [**]
Standard (PIO + FRU)	80 ± 0.54 [##]	23 ± 0.42 [#]	73.6 [##]
Test (ABA + FRU)	82 ± 0.98 [##]	24 ± 0.26 [#]	78.72 [##]

Figure-6.3: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting glucose in fructose-induced insulin resistance in rats

All values represents Mean ± SEM; n=6

**   Significantly different from normal control (p < 0.01)
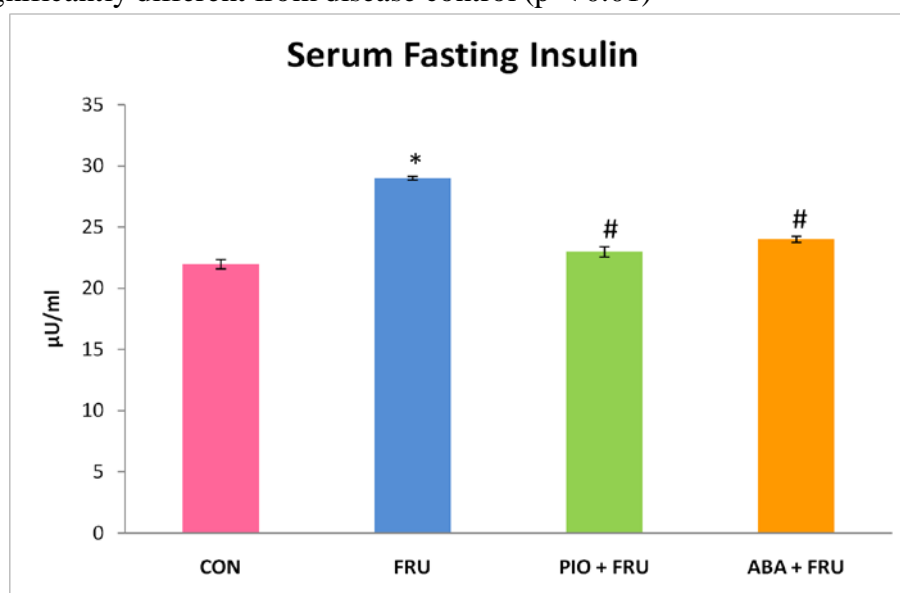\#\#   Significantly different from disease control (p < 0.01)

Figure-6.4: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting insulin in fructose-induced insulin resistance in rats

All values represents Mean ± SEM; n=6

*   Significantly different from normal control (p < 0.05)
\#   Significantly different from disease control (p < 0.05)

Devang B. Sheth

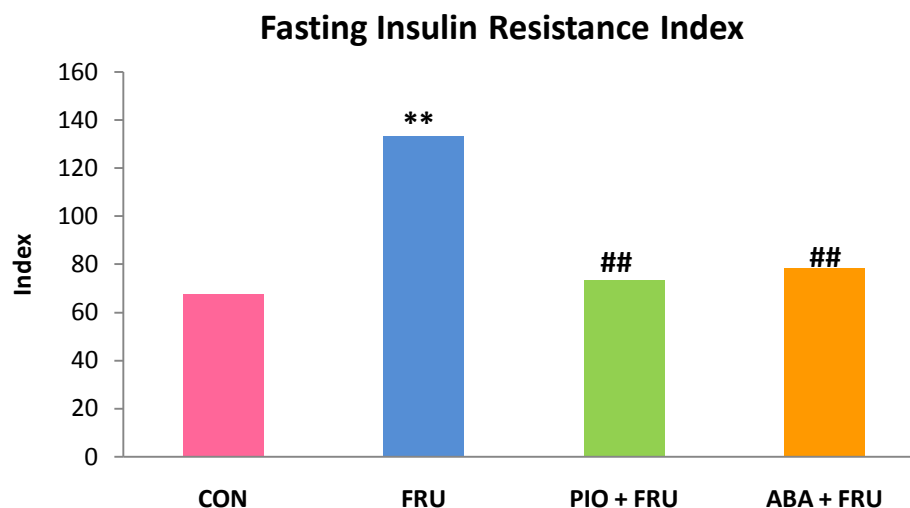## Fasting Insulin Resistance Index

Figure-6.5: Effect of Abscisic acid (1 mg/kg/day, 30 days) on fasting insulin resistance index in fructose-induced insulin resistance in rats
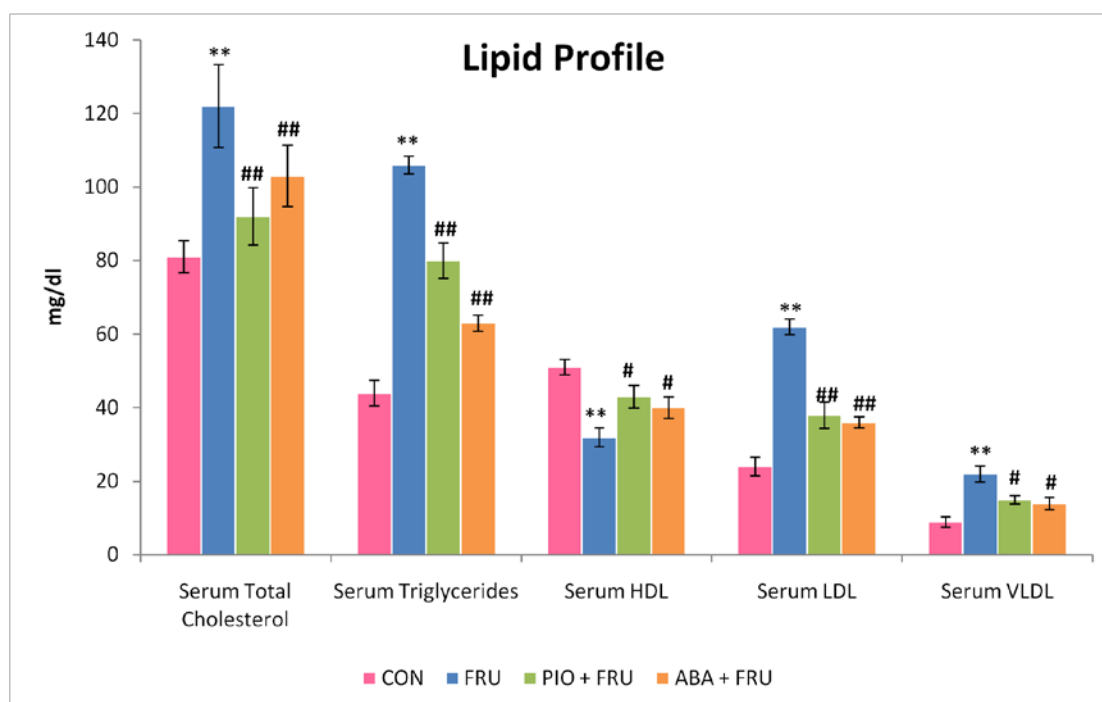

Figure-6.6: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum lipid profile parameters in fructose-induced insulin resistance in rats

All values represents Mean ± SEM; n=6

*   Significantly different from normal control ($p < 0.05$)
#   Significantly different from disease control ($p < 0.05$)
**   Significantly different from normal control ($p < 0.01$)
Significantly different from disease control ($p < 0.01$)

Devang B. Sheth

Fructose feeding significantly decreased serum HDL (p<0.01) and increased serum total cholesterol (p<0.01), serum triglycerides (p<0.01), serum LDL (p<0.01) and serum VLDL (p<0.01) as compared to control group (n=6). Both abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment resulted in significant increase (p<0.05) in serum HDL as compared to disease control (Table-6.3, Fig-6.6). Both abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment decreased the rise in total serum cholesterol (p<0.01), serum triglycerides (p<0.01), serum LDL (p<0.01) and serum VLDL (p<0.05) observed with fructose feeding significantly. Although abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment both decreased lipid abnormalities significantly, the effect of abscisic acid was apparently more on serum triglycerides as compared to pioglitazone treatment (Table-6.3, Fig-6.6).

Table-6.3: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum lipid profile parameters in fructose-induced insulin resistance in rats
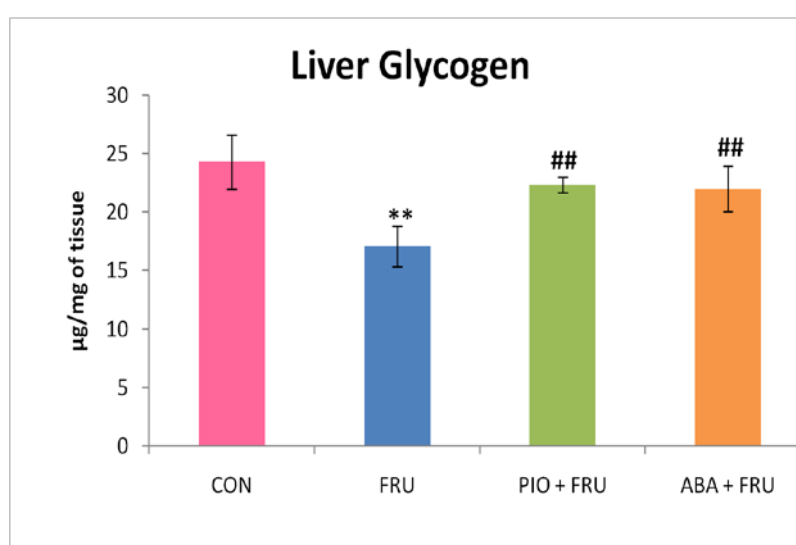
Lipid profile parameters (mg/dl)	Treatment (n=6)			
	Normal control (CON)	Disease control (FRU)	Standard (PIO+FRU)	Test (ABA+FRU)
Serum Total Cholesterol	81 ± 4.36	$122 \pm 11.23^{**}$	$92 \pm 7.76^{\#\#}$	$103 \pm 8.32^{\#\#}$
Serum Triglycerides	44 ± 3.44	$106 \pm 2.43^{**}$	$80 \pm 4.76^{\#\#}$	$63 \pm 2.14^{\#\#}$
Serum HDL	51 ± 2.11	$32 \pm 2.54^{**}$	$43 \pm 3.12^{\#}$	$40 \pm 2.87^{\#}$
Serum LDL	24 ± 2.54	$62 \pm 2.14^{**}$	$38 \pm 3.56^{\#\#}$	$36 \pm 1.53^{\#\#}$
Serum VLDL	9 ± 1.43	$22 \pm 2.13^{**}$	$15 \pm 1.13^{\#}$	$14 \pm 1.66^{\#}$

6.1.4 Liver and muscle glycogen

Liver and muscle glycogen stores were found to be decreasing (p<0.01 and p<0.05 respectively) with fructose supplement, as compared to control group, indicating development of insulin resistance. Both abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment increased liver and muscle glycogen stores significantly (p<0.01 and p<0.05 respectively), as compared to disease control, showing considerable increase in insulin sensitivity (Table-6.4, Fig-6.7 and 6.8).

Devang B. Sheth

Table-6.4: Effect of Abscisic acid (1 mg/kg/day, 30 days) on liver and muscle glycogen in fructose-induced insulin resistance in rats

Treatment (n=6)	Liver glycogen (µg/mg of tissue)	Muscle glycogen (µg/mg of tissue)
Normal control (CON)	24.3 ± 2.3	6.2 ± 0.35
Disease control (FRU)	17.1 ± 1.74 **	5.15 ± 0.78 *
Standard (PIO + FRU)	22.35 ± 0.65 ##	5.95 ± 0.4 #
Test (ABA + FRU)	22 ± 1.95 ##	6.12 ± 0.89 #

Figure-6.7: Effect of Abscisic acid (1 mg/kg/day, 30 days) on liver glycogen in fructose-induced insulin resistance in rats
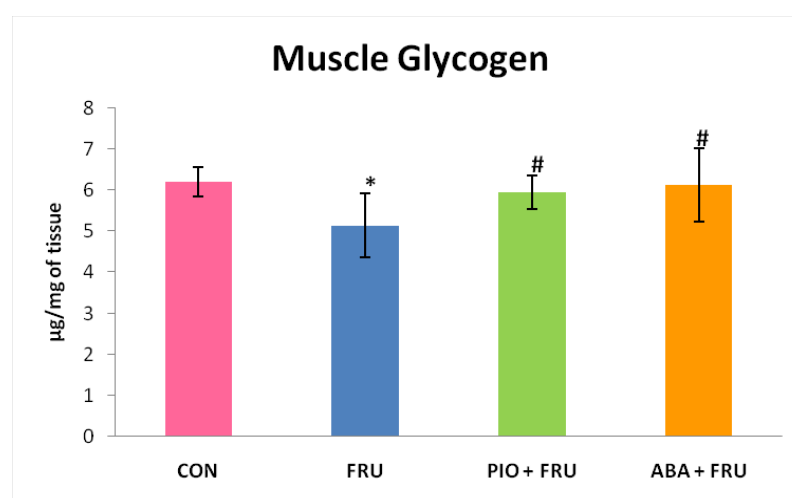
Figure-6.8: Effect of Abscisic acid (1 mg/kg/day, 30 days) on muscle glycogen in fructose-induced insulin resistance in rats

Devang B. Sheth

6.2 Anti-cancer activity

6.2.1 Indirect type molecular modelling

3D structural similarity between Vitamin A and Abscisic acid was confirmed by indirect type of molecular modeling study. Both the structures were generated, energy minimized and superimposed (Figure-6.9) using PC based Discovery Studio (Version 2.1, Accelrys Inc. USA). All geometries were fully optimized by minimizing the energy with respect to geometrical variables without symmetry constraints, using a 0.01 kcal/mol gradient. The r.m.s.d. observed was 0.219. The low r.m.s.d. value suggests good 3D similarity between Vitamin A and Abscisic acid.
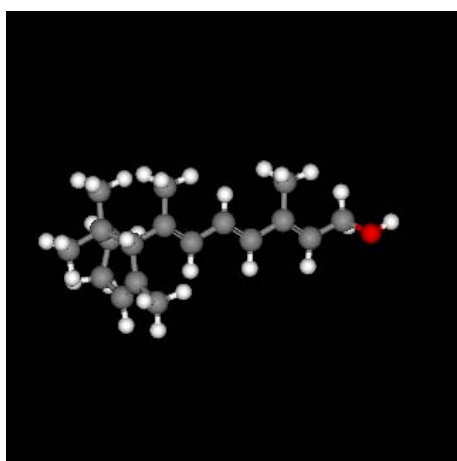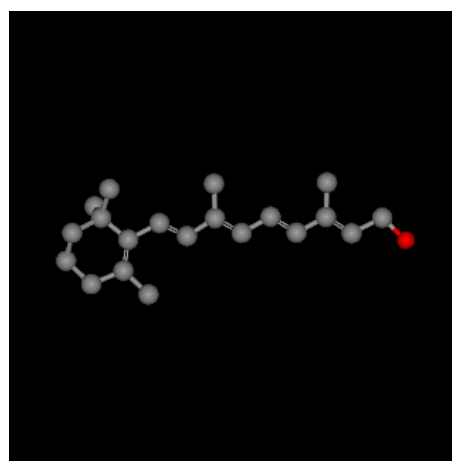
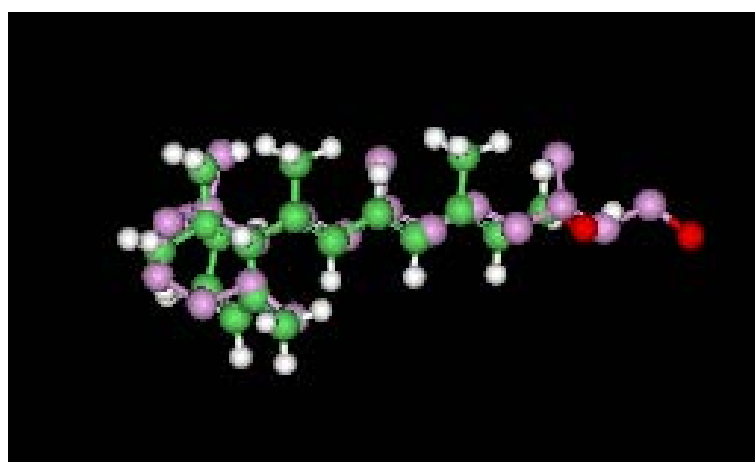**3D structure of abscisic acid**   **3D structure of vitamin A**

Figure-6.9: 3D Overlay of abscisic acid (green) and vitamin A (pink)

6.2.2 Characterization of cell lines and culture media

Characterization of cell lines was performed for detection of microbial and cross contamination. Cell lines used in our experiments were free from any kind of microbial or fungal contamination (Table-6.5), which in essential in order to continue our screening experiments.

Table-6.5: Results for characterization of cell lines

Cell line	% Viability		PDT (hrs)	Microbial contamination	Cross contamination	pH
	Stock	After				
MCF – 7	64.46	87.20	23.4	No contamination	No contamination	7.0
MDA-MB-468	78.34	89.89	28.3	No contamination	No contamination	7.0
HEK 293T	60.46	87.20	19.3	No contamination	No contamination	7.5

From viability studies and Population doubling time (PDT), we have concluded that the cell lines derived from NCCS, Pune were initially free from cross contamination.

6.2.3 Cell viability, density and population doubling time

The quantification of cellular growth, including proliferation and viability, has become an essential tool for working on cell-based studies.

HEK 293T

At the time of subculture, density of HEK 293T cell line derived from NCCS, Pune was around 1.8×10^7 cells / flask and viability was 61.81 %, which was not suitable for cytotoxicity study, considering requirement of cell viability greater than 90 %. In order to increase the viability and cell density of HEK 293T cell line, sub-culturing was done by using complete media and additional 5 % FBS. As a result, on the fourth day morning cell density was increased up to 6.4×10^7 and viability was around 87.98 % which was suitable for cytotoxicity screening. PDT (Population Doubling Time) for HEK 293T was 19.3 hrs. Table-6.6 represents results for subculture of HEK 293T cell.

Table-6.6: Results for sub-culturing of HEK 293T cell line

Day	1st	2nd	3rd	4th
Viable cell count	34	43	70	77
Non-viable cell count	21	18	17	11
% Viability	61.81	70.79	80.16	87.98
Cells/ml	3.6×10^5	5.4×10^5	9×10^5	12.8×10^5
Total cells in flask (50 ml)	1.8×10^7	2.7×10^7	4.5×10^7	6.4×10^7
Viable cells in flask (50 ml)	9.81×10^6	16.95×10^6	30.2×10^6	46×10^6
pH	7.5	5.0	4.5	5.0
PDT	19.3 hrs			

Average PDT for HEK 293T was found to be 19.3 hrs. As the population of cells in the flask increase, more amounts of media were consumed by cells for growth purpose and this lead to acidic pH of the media, which requires continuous addition of media for maintenance of pH and nutritional requirements. Subculturing was performed every 3rd or 4th day i.e. twice in week.

MCF-7 cell line

Table-6.7: Results for sub-culturing of MCF-7 cell line

Day	1st	2nd	3rd
Viable cell count	27	68	77
Non-viable cell count	18	27	11
% Viability	60.46	71.40	87.20
Cells/ml	3.4×10^5	10.2×10^5	30.2×10^5
Total cells in flask (50 ml)	1.7×10^7	5.1×10^7	15.1×10^7
Viable cells in flask (50 ml)	10.7×10^6	38×10^6	139×10^6
pH	7.5	7.5	7
PDT	23.4 hrs		

MCF-7 is adherent cell line, viability of which was around 60.46 % and seeded density was around 1.7×10^7, which was equivalent to HEK 293T viability but still requires passaging for cytotoxicity screening.

On subculture, after 48 hrs that on 3rd day morning, viability was increased up to 87.2 % and cell density was 15.1×10^7 which was found to be suitable to carry out experiments. PDT was around 23.4 hrs, so sub culturing was carried out three times in week for MCF-7 cell line. Table-6.7 represents result for sub-culturing of MCF-7 cell line.

MDA-MB-468

In case of MDA-MB-468 cell line, seeded density was around 1.7×10^7 and viability was 62.96% which was higher than HEK 293T viability but still requires passaging for cytotoxicity screening.

Table-6.8: Results for sub-culturing of MDA-MB-468 cell line

Day	1st	2nd	3rd
Viable cell count	27	68	164
Non-viable cell count	17	51	151
% Viability	62.96	72	92.07
Cells/ml	3.4×10^5	10.2×10^5	30.2×10^5
Total cells in flask (50 ml)	1.7×10^7	5.1×10^7	15.1×10^7
Viable cells in flask (50 ml)	10.7×10^6	38×10^6	139×10^6
pH	7.5	7.5	7
PDT	28.3 hrs		

On subculture, after 48 hrs that on 3rd day morning, viability was increased up to 92.07% and cell density was 15.1×10^7 which were quite good to carried out experiments. PDT was around 28.3 hr. So sub-culturing was carried out three times in week for MDA-MB-468 cell line. Table-6.8 represents result for sub-culturing of MDA-MB-468 cell line.

6.2.4 Screening by MTT assay

All the compounds were evaluated *in vitro* against panel of three cell lines consisting of HEK 293T (Human Embryonic Kidney cell line - Normal cell line), MCF – 7 (Human breast cancer cell line) and MDA-MB-468 (Human breast cancer cell line) cell lines by MTT assay. Results for each test samples were reported as the percent of growth inhibition (IC_{50})**.** For both abscisic acid and doxorubicin, Dose Response

Curve (DRC) against both cell line was plotted with 10 analysis point i.e. with 10 different drug concentrations (Figure 6.10 and 6.11). The concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using GraphPad Prism software (V-5.02).

Table-6.9: IC_{50} value (µM) of abscisic acid and doxorubicin against breast cancer cell lines by MTT assay

Drug	MCF-7	MDA-MB468	HEK 293T (Normal cell line)
Abscisic acid	48.39	38.52	>100
Doxorubicin	20.23	23.56	>100

Results as mentioned in Table 6.9 indicate abscisic acid show IC_{50} value below 100µM against both MCF-7 cell line and MDA-MB-468. Doxorubicin shown comparable IC_{50} values against both cancer cell lines where as in case of HEK 293T (as normal cell line), doxorubicin was found to be inactive, which confirm the cytotoxicity of doxorubicin against cancer cell line and inactiveness against HEK293T cell line. IC_{50} value of abscisic acid for MDA-MB-468 was found to be 38.42 µM, which is comparable with standard IC_{50} value i.e.23.56 µM. Similarly for MCF-7 cell line IC_{50} value was found to be 48.39 µM, which is also comparable with standard doxorubicin IC_{50} value 20.23 µM. Abscisic acid does not produce cytotoxic effect significantly with HEK 293T (i.e. normal cell line-Human Embryonic Kidney cell line) with IC_{50} value >100 µM suggesting inactiveness against HEK293T cell line.
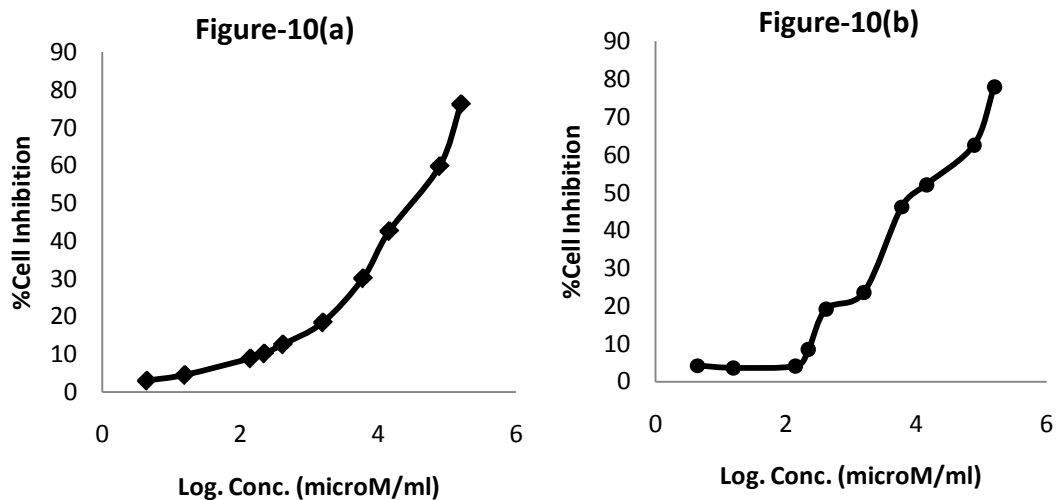
Figure-6.10 (a): DRC of doxorubicin by MTT assay (MCF-7)

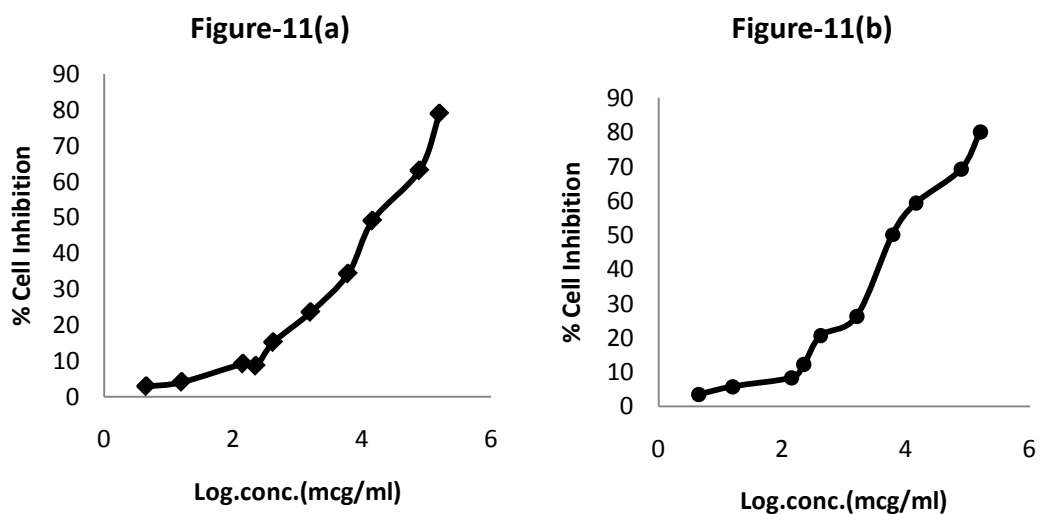**Figure-6.10 (b)**: DRC of doxorubicin by MTT assay (MDA-MB468)

Figure-6.11 (a): DRC of abscisic acid by MTT assay (MCF-7)

Figure-6.11 (b): DRC of abscisic acid by MTT assay (MDA-MB468)

Devang B. Sheth

6.4 Ca^{+2} channel modulatory action

6.4.1 Effects on isolated rat ileum

Abscisic acid did not produce any effect in isolated rat ileum at concentration of 1, 10 and 100 µg/ml. ABA also had no effect on contractile response produced by 1% BaCl$_2$, indicating that ABA is without significant Ca^{+2} modulatory activity in this smooth muscle preparation (Table-6.10).

Table-6.10: Effect of abscisic acid (alone and in presence of 1% BaCl$_2$) on isolated rat ileum preparation

Drug	Dose	Concentration	Height (in mm)
Abscisic acid (ABA)	0.1ml	1 µg/ml	-
	0.5 ml	1 µg/ml	-
	0.1ml	10 µg/ml	-
	0.5 ml	10 µg/ml	-
	0.1ml	100 µg/ml	-
	0.5 ml	100 µg/ml	-
BaCl$_2$	0.1 ml	1%	22
ABA + BaCl$_2$(0.1 ml, 1%)	0.1 ml	1 µg/ml	20
	0.5 ml	1 µg/ml	21
	0.1ml	10 µg/ml	20
	0.5 ml	10 µg/ml	19
	0.1ml	100 µg/ml	19
	0.5 ml	100 µg/ml	20

6.4.2 Effect on cardiovascular system parameters

Abscisic acid produced negligible increase in heart rate in anaesthetized rat (n=6). Normal values before administration of ABA (0.1 ml, 0.1 mg/kg) showed 318 ± 3.54 beats/min and after administration of ABA resulted in 326.67 ± 3.99 beats/min. ABA administration resulted in slight increase in systolic (135.83 ± 3.57) and diastolic (89.67 ± 1.68) blood pressure as compare to normal systolic (128.67 ± 2.89) and

Devang B. Sheth

diastolic (88.33 ± 1.86) blood pressure, which was found to be statistically insignificant. These effects of abscisic acid were blocked by, Ca^{+2} channel blocker, verapamil (Table-6.11). ECG data were found to be normal after administration of ABA.

Table-6.11: Effect of abscisic acid (0.1 mg/kg, i.v.) and verapamil (0.1 mg/kg, i.v.) on heart rate and blood pressure

Parameter	Heart rate (beats/min)	Blood pressure (mmHg)	
		Systolic	Diastolic
Normal	318 ± 3.54	128.67 ± 2.89	88.33 ± 1.86
Abscisic acid	326.67 ± 3.99	133.83 ± 3.57	89.67 ± 1.68
Verapamil + Abscisic acid	319.25 ± 4.63	127.98 ± 0.29	87.2 ± 1.26

All values represents Mean ± SEM; n=6

Devang B. Sheth

6.5 Preliminary toxicity testing

Treatment with abscisic acid (1 mg/kg/day, p.o.) for 30 consecutive days resulted in no changes in weight in rat. Further there were no signs of toxicity indicated as abnormal changes in histopathology of heart, lung (right) and kidney (right) tissues of rat (n=4). Hematological data (n=6) revealed increase in total RBC count, Hb content and total WBC count which was found to be statistically insignificant (Table-6.12).

Table-6.12: Effect of abscisic acid (1 mg/kg/day, p.o., 30 days) on weight and hematological parameters in rats (n=6)

Parameter	Control	ABA treated
Weight (gm)	249.75 ± 2.5	251.25 ± 4.15
RBC (x 10^6/cmm)	7.4 ± 0.23	7.6 ± 0.88
Hb (g/dl)	13 ± 0.29	13.2 ± 0.26
WBC (x 10^3/cmm)	8.3 ± 0.15	8.67 ± 0.43
Neutrophils (%)	15 ± 0.42	14 ± 0.32
Lymphocytes (%)	76 ± 0.65	77 ± 0.24
Eiosinophils (%)	5 ± 0.22	4 ± 0.17
Monocytes (%)	3 ± 0.46	4 ± 0.41
Basophils (%)	1 ± 0.25	1 ± 0.25
Platelets (x 10^5/cmm)	6.5 ± 0.76	6.47 ± 0.82

All values represents Mean ± SEM; n=6

Devang B. Sheth

Discussion

Recent studies on some phytohormones, both in-vivo and in-vitro, have opened new avenues to discovery of new chemical entities, in the field of phyto-pharmacology. Abscisic acid (ABA) is an important phytohormone that regulates plant growth, development, dormancy and stress responses. Recently, it has been discovered that ABA is produced by a wide range of animals including sponges (*Axinella polypoides*), hydroids (*Eudendrium racemosum*), human parasites (*Toxoplasma gondii*) and various mammalian cells (*leukocytes, pancreatic cells, and mesenchymal stem cells*). Since its discovery in early 1960's, abscisic acid (ABA) has received considerable attention as an important phytohormone, and more recently, as a therapeutic candidate. Hence, in the present project we attempted to explore pharmacological profile of abscisic acid which, on further investigations, could lead to discovery of its therapeutic potential.

In recent decades the prevalence of insulin resistance and type II diabetes has seen a precipitous growth (Center for Disease Control and Prevention, 2005). In our study abscisic acid was found to be structurally similar to thiazolidinedione as indicated by low root mean square distance (r.m.s.d.) in indirect molecular modeling. After confirmation of structural similarity, abscisic acid was screened for anti-diabetic activity, induced by fructose feeding in rat.

Fructose feeding for 30 days resulted in hyperglycaemia, hyperinsulinaemia, dyslipidemia (decreased levels of HDL and increased levels of LDL, VLDL, total cholesterol and triglycerides) and decreased peripheral uptake of glucose. The resultant increase in fasting insulin resistance index (FIRI) is also indicative of aggravation of insulin resistance. Earlier fructose was considered as a glucose alternative in diabetic patients. But later on it was revealed that chronic use of fructose culminates in development of metabolic syndrome, including induction of insulin resistance. Research in metabolism of fructose has unmasked difference between short-term positive effects, and the negative effects of chronic use of fructose. Long-term derogatory effects include changes in digestion, absorption, plasma hormone levels, appetite, and hepatic metabolism, leading to precipitation of insulin resistance, diabetes, obesity, and inevitably cardiovascular disease (Moyer and Rodin, 1993).

Fructose is a potent regulator of glycogen synthesis and liver glucose uptake. Therefore any catalytic improvements are due to hepatic glucokinase and glucose uptake facilitation. However, as mentioned, the beneficial effects do not continue with chronic fructose utilization. Because of its lipogenic properties, excess fructose in the diet can cause glucose and fructose malabsorption, and greater elevations in triglycerides and cholesterol compared to other carbohydrates (Hallfrisch J, 1990). Of the key importance is the ability of fructose to bypass the main regulatory step of glycolysis, the conversion of gluctose-6-phosphate to fructose 1,6-bisphosphate, controlled by phosphofructokinase. Thus, while glucose metabolism is negatively regulated by phosphofructokinase, fructose can continuously enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate, and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates, and resultant excess energy flux due to unregulated fructose metabolism, will promote the overproduction of TG (Mayes, 1993). It has been further reported that fructose causes metabolic syndrome mediated through leptin, adiponectin, and free fatty acids (Heather et al., 2005).

Pioglitazone as previously reported has been shown to prevent the rise in serum biochemical parameters leading to normal levels of glucose, insulin and lipid profile. TZDs serve as synthetic ligands for a key metabolic regulator and transcription factor known as peroxisome proliferator-activated receptor γ (PPAR γ) (Lehmann et al., 1995). Following ligand binding, PPAR-γ heterodimerizes with retinoid X receptor (RXR), another member of the nuclear receptor superfamily, and subsequently binds to a PPAR response element (PPRE) on DNA that initiates the transcription of responsive genes. With regard to insulin sensitizers, TZDs appear to be more effective in improving glycemic control, controlling metabolic dyslipidaemia and in enhancing insulin sensitivity (Seufert et al., 2004; Knowler et al., 2005).

In our study, abscisic acid treatment for 30 days largely prevented the abnormalities produced by fructose feeding. Decrease in insulin resistance was observed to mimic pioglitazone treatment. Abscisic acid (ABA) was found to be decrease insulin resistance induced by fructose feeding. ABA decreased serum fasting glucose, significantly. This is in agreement with previously reported study involving use of dietary abscisic acid ameliorating glucose tolerance and obesity-related inflammation in db/db mice fed with high-fat diets (Guri et al., 2007). Abscisic acid not only

Devang B. Sheth

decreased rise in serum fasting glucose and insulin (observed with fructose feeding) but also promoted levels of liver and muscle glycogen contents. Thus, abscisic acid decreases blood sugar level, reduces over-burdening of pancreatic β-cells (by decreasing supernormal serum insulin) and increases uptake of glucose in peripheral tissues like liver and muscle. This is clearly indicative of insulin sensitizing action, probably via PPAR-γ receptor action. The similarities in structure of ABA and TZDs by indirect molecular modeling software studies are also suggestive of PPAR-γ receptor agonistic action. Hence, our result confirms previous report indicating activation of PPAR-γ responsive genes by abscisic acid in 3T3-L1 pre-adipocytes *in vitro* (Guri et al., 2007).

Generally, type 2 diabetes mellitus (T2DM) patients suffer from both hyperglycemia and dyslipidemia. The insulin resistant state is commonly associated with lipoprotein abnormalities that are risk factors for atherosclerosis, including hypertriglyceridemia, high levels of very low density lipoprotein (VLDL), low levels of high-density lipoprotein cholesterol (Ruotolo G and Howard, 2002), and small, dense LDL (Friedlandre et al., 2000). Clinical studies implicate hyperglycemia in the onset of microangiopathic complications of T2DM, including kidney failure, retinopathy and peripheral neuropathy. However, the major cause of mortality in T2DM patients is atherosclerotic macrovascular disease, which culminates in myocardial infarction. Such cardiovascular disease appears to result, in large part, from diabetic dyslipidemia (Koyama, 2004).

Many of the genes induced by PPAR-γ, such as aP2 and CD36, are important in fatty acid transport, adipogenesis and lipid uptake. Studies have shown that PPAR-γ is an essential participant in the differentiation of pre-adipocytes into adipocytes, particularly in the subcutaneous adipose tissue depots. This function has been proposed to enhance systemic insulin sensitivity by inhibiting lipid efflux from adipose tissue into tissues such as the liver, pancreas, skeletal muscle, which are important for the proper regulation of glucose homeostasis (Guri et al., 2006; de Ferranti et al., 2008). Synthetic PPAR-γ agonists, such as thiazolidinediones (TZDs), have been shown to be very effective in improving insulin sensitivity as well as lipid dyslipidaemia (Braissant et al., 1996). Present study confirms effect of pioglitazone on reducing dyslipidaemia through PPAR-γ activation. Similar pattern of results have been observed with ABA treatment in our study. Although both pioglitazone and

Devang B. Sheth

ABA decreased TG, LDL, and total cholesterol significantly; effect of ABA was remarkably better on levels of TG as compared to pioglitazone. Increased delivery of triglycerides or non-esterified fatty acids to the muscle interferes with the utilization of glucose, through the principles of Randle cycle (Randle, 1998), impairing the insulin action. However, in present study we were not been able to co-relate hypetriglyceridaemia with hyperinsulinaemia as suggested by FIRI, which was found to be same with ABA and pioglitazone treatment. Present study also supports earlier finding that ABA-supplementation was associated with significant improvements in hepatic steatosis and plasma triglyceride levels (Guri et al., 2008). Further, as reported earlier (Guri et al., 2007) we found that ABA treatment is not associated with hepatotoxicity and weight gain suggesting substantial safety as compared to TZDs. Our results indicate that administration of abscisic acid may be beneficial for preservation of functional efficiency of pancreatic β-cells, probably by improving insulin action and thereby preventing induction of insulin resistance. The putative action of ABA on PPAR-γ receptors is most probable mechanism for its therapeutic effect on insulin resistance. Further studies are warranted to establish safety and efficacy of abscisic acid in management of diabetes mellitus.

Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO, 2011). Breast cancer is the most common cancer disease in woman globally. In our study abscisic acid was found to be structurally similar to vitamin A as indicated by low r.m.s.d. in indirect molecular modeling. After confirmation of structural similarity, abscisic acid was screened for *in-vitro* anti-cancer activity in breast cancer cell-lines.

In the present study, doxorubicin exhibited comparable IC_{50} values against both cancer cell-lines, where as in HEK 293T (i.e. Normal cell line - Human Embryonic Kidney cell line), doxorubicin was inactive, confirming cytotoxicity of doxorubicin against cancer cell-line and inactivity against HEK293T cell-line. Abscisic acid has significant cytotoxicity activity against cell line studied. IC_{50} value of abscisic acid for MDA-MB 468 and MCF-7 cell-line is comparable with std. IC_{50} value of doxorubicin. Further, abscisic acid did not produce cytotoxic effect with HEK293T cell-line.

Devang B. Sheth

Over 60% of breast cancer cases are estrogen receptor (ER) positive, which is highly dependent on estrogen for growth. The usage of MCF-7 breast cancer cells lines is widely used nowadays in numerous researches for the anti-cancer properties. MCF-7 cells are the most commonly used model of estrogen positive breast cancer (Soule et al., 1973). On other hand, MDA-MB 468 cell-line is ER negative breast cancer cell-line. Therefore, in this study we assessed effect of abscisic acid on both ER positive (i.e. MCF-7) as well as ER negative (i.e. MDA-MB 468) cell-lines.

Retinoids (derivatives of vitamin A) are signalling molecules that play important roles in cell growth, differentiation and death. Retinoids inhibit growth of breast cancer cell-lines in culture and inhibit breast tumor growth in animal models as well as humans. Therefore we hypothesized to have same observation with abscisic acid. Retinoid signals are mediated through the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), with each family represented by three distinct receptor genes designated α, β, and γ – which themselves act as ligand-dependent transcription factors (Pfahl, 1994; Mangelsdorf and Evans, 1995; Chambon, 1996). They have been used successfully to cure acute promyelocytic leukaemia (APL) and can suppress carcinogenesis in a variety of tissue types (e.g. breast, skin, lung and oral cancers) (Mangelsdorf et al., 1996). Following stimulation by retinoids, RAR-RXR heterodimers and RXR-RXR homodimers can form. The receptor dimers bind to retinoic acid response elements or retinoid X response elements in the promoter sequences of target genes, and they modulate gene transcription. Inhibition of breast tumor cell growth by retinoids is greater for ER-positive cells than ER-negative cells (Simeone and Tari, 2004). We observed that abscisic acid exerts cytotoxic action for both ER positive (MCF-7) as well as ER negative (MDA-MB 468) breast cancer cell-line indicating that it is not acting through RAR and/or RXR signalling mechanisms. Thus, anti-cancer activity of abscisic acid may not be connected with estrogen receptor expression. Further, earlier reports have proposed that ABA "neutralizes" the human chorionic gonadotropin (hCG), which is a negatively charged glycoprotein that reportedly coats cancer cells and prevents immune cells (the outer membranes are normally negatively charged) from getting close and attack the cancer cells (Livingston, 1976). Thus, abscisic acid may be facilitating anticancer immune responses.

Devang B. Sheth

Cytotoxic effects of ABA could also be due to its ability to modulate calcium signalling. ABA modulates pathways in plants and animals involving cADPR which control the increase in $[Ca^{+2}]_{int}$ (Wu et al., 1997). In plants, ABA depolarizes plasma membranes, which activates potassium ion channels and thereby extrudes K^+ outside the cells (Schroeder et al., 1987). Ion channel depolarization is dependent on calcium, which is an ABA second messenger (Schroeder et al., 2001). The increase in calcium concentrations also leads to increased nitric oxide (NO) production (Bodrato et al., 2009). As calcium signalling is a key regulator of apoptosis, changes in calcium distribution in the cell activate cellular cascades which lead to cell death (Hajnoczky et al., 2003). Coincidently, some of the pathways activated by ABA and those modulated by chemotherapeutic agents used for treating cancer are noticeably similar. For instance, several chemotherapeutic agents modulate pathways leading to increased intracellular calcium concentrations. Medications such as staurosporine, doxorubicin, tamoxifen, and etoposide act as anti-cancer agents which lead to the death of cancer cells by increasing $[Ca^{+2}]_{int}$ resulting in oxidative stress followed by apoptosis (Kruman et al., 1998; Panaretakis et al., 2005; Parihar et al., 2008). Unlike some chemotherapeutic agents which act as cell killers, ABA may regulate cancerous cells by stimulating the immune system or inducing cancer cells to undergo apoptosis without significant toxic effects on normal cells. Further *in-vivo* studies are warranted for finding efficacy and molecular mechanisms for anti-cancer effect.

In plants, it has been demonstrated that ABA stimulates release of intracellular calcium in conjunction with the upregulation of cADPR (Wu et al, 1997). ABA functions through a markedly similar signalling pathway in both plants and animals, which is thought to involve cADPR and Ca^{+2} functioning as intermediates. As calcium is an important regulator of cell function, we tried to check effect of abscisic in smooth muscle and CVS parameters.

In our study we found that abscisic acid neither produced any contractile response nor reduced contractile response of $BaCl_2$ on isolated rat ileum preparation. Current findings suggest that ABA is not producing any significant effect on smooth muscle preparation. Our finding supports previous report claiming that ABA is devoid of any substantial Ca^{+2} channel modulatory activity in rat smooth muscle preparations (Masters et al., 1994). Administration of ABA in *in-vivo* anaesthetized rat showed insignificant increase in heart rate and blood pressure.

[100]

In our study, abscisic acid did not show any observable toxicity in our study, suggesting a safe profile for its potential therapeutic use.

Thus, the findings of our study suggest that administration of abscisic acid may be beneficial for protecting functional efficiency of pancreatic β cells, probably by facilitating action of insulin and potent anti-cancer activity due to its putative immune-stimulant activity or through its ability to modulate calcium signalling process leading to apoptosis.

Devang B. Sheth

Conclusions

Results of our project suggest that administration of abscisic acid may be beneficial for protecting functional efficiency of pancreatic β cells, probably by facilitating action of insulin and thereby preventing emergence of insulin resistance. The putative action of ABA on PPAR-γ receptors is most probable mechanism for its therapeutic effect in insulin resistance. Further studies are warranted to establish safety and efficacy of abscisic acid in management of diabetes mellitus.

In the present project, abscisic acid exhibited potent anti-cancer activity in *in-vitro* cell line study. We observed that abscisic acid exerts cytotoxic action in both ER positive (MCF-7) as well as ER negative (MDA-MB 468) breast cancer cell line studies indicating that its action is not mediated through RAR and/or RXR signalling mechanisms. Thus, anti-cancer activity of abscisic acid is not related to estrogen receptor expression. Cytotoxic effects of ABA may be due to its immune-stimulant activity or its ability to modulate calcium signalling process that may lead to apoptosis.

Abscisic acid did not show any observable toxicity in our study, suggesting a safe profile for its potential therapeutic utility.

We conclude that pharmacologically, abscisic acid is a therapeutically promising phytohormone with beneficial effects in ailments like diabetes mellitus and cancer along with a high safety profile.

Devang B. Sheth

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Annexures

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CERTIFICATE

This is certify that the research project no. **RKCP/COL/RP/09/02** entitled *"Investigation into pharmacological profile and mechanism of action of Abscisic acid with reference to its possible therapeutic usefulness"* has been approved by IAEC committee during meeting on 7th March 2009.

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Ref No: MPC/૬/૬ /2011-12

Date: 21/02/2012

CERTIFICATE

This is certify that the research project no. **MPC 1206** entitled *"Investigation into pharmacological profile and mechanism of action of Abscisic acid with reference to its possible therapeutic usefulness"* has been approved by IAEC committee.

[124]

Devang B. Sheth

Effect of Abscisic Acid in Fructose-Induced Insulin Resistant Rats

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ABSTRACT:

Insulin resistance is a metabolic disorder that prevails worldwide and plays a role in pathophysiology of most common human diseases including type 2 diabetes mellitus, hypertension, obesity, dyslipidemia and coronary heart disease. Insulin resistance can be induced by fructose-rich diet in rats. One of most effective medications in treatment of insulin resistance has been thiazolidinediones (TZDs). TZDs are reported to have some troublesome adverse effects. Abscisic acid is a phytohormone with structural similarity to TZDs. We investigated effects of 2-cis, 4-trans abscisic acid on glucose, lipid profile, and serum insulin levels in wistar rats fed with high fructose. The animals were divided into 4 groups: normal control, disease control (10% fructose in drinking water, 30 days), standard treated (Pioglitazone 10mg/kg p.o. with 10% fructose in drinking water, 30 days) and Abscisic acid (ABA) treated (1mg/kg p.o. with 10% fructose in drinking water, 30 days) group (n=6). Fructose significantly increased serum fasting glucose (p<0.01), serum insulin (p<0.05), FIRI (p<0.01), liver and muscle glycogen (p<0.01) as compared to control group. Standard as well as ABA treated group significantly reduced serum glucose (p<0.01), serum insulin (p<0.05), FIRI (p<0.01), liver and muscle glycogen (p<0.01) as compared to disease control group. Our results show that administration of abscisic acid may be advantageous for preservation of the functional efficiency of pancreatic β cells, probably by improving insulin action via PPAR γ agonistic action and thereby preventing induction of insulin resistance.

Keywords: Abscisic acid, Thiazolidinediones, Insulin resistance.

1. INTRODUCTION

Type II diabetes is an insulin resistance disease[1,2]. TZDs are a class of the most effective insulin-sensitizing drugs that increase the sensitivity of peripheral tissues to endogenous insulin. TZDs serve as synthetic ligands, and activate the key metabolic regulators as well as the transcription factor PPAR-γ[3]. Therefore, PPAR-γ agonists are considered to be a promising target for future drug design and treatment of diseases related to insulin resistance and the related conditions of hyperlipidemia, hyperglycemia, and hyperinsulinemia.

Since its discovery in the early 1960's, abscisic acid (ABA) has received considerable attention as an important phytohormone, and more recently, as a candidate medicinal in humans. Abscisic acid has been reported to be a partial peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist[4,5]. PPAR-γ responsive genes were found to be induced or activated by abscisic acid in 3T3-L1 preadipocytes *in vitro*[3,4]. ABA could also be used as a nutritional intervention against type II diabetes and obesity-related inflammation[4]. More over current TZDs are associated with weight gain and, in the past, hepatotoxicity[6]. Recently, a study in the New England Journal of Medicine showed that use of rosiglitazone was associated with a 43% increase in myocardial infarction and 64% increase in risk of cardiovascular mortality[7]. Thus there is surge for need of novel insulin sensitizer without considerable adverse effects. Therefore, we studied effect of abscisic acid on in a fructose-induced insulin resistance in rat.

2. MATERIAL AND METHODS

2 – cis, 4 – trans abscisic acid (98%, synthetic) was purchased from Sigma Aldrich, USA. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of R. K. College of Pharmacy, Rajkot and with permission from CPCSEA, Ministry of Social Justice and Empowerment, Government of India.

Ten week old male Sprague-Dawley rats (200 ± 25 gm) were housed in-group of 3 animal in cages and maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and provided free access to palleted diet and purified drinking water ad libitium, unless specified. Rats (n=24) were randomized into 4 groups as shown in table 1.

Table 1: Group specification and dose for individual treatment

Group No.	Group Specification	Treatment (n=6, 30 days)
I	Normal control (CON)	Vehicle (water)
II	Disease control (FRU)	10% fructose in water *ad libitium*
III	Standard (PIO + FRU)	Pioglitazone (10 mg/kg/day, p.o.) + 10% fructose in water *ad libitium*
IV	Test (ABA + FRU)	Abscisic acid (1 mg/kg/day, p.o.) + 10% fructose in water *ad libitium*

Rats were weighed and their food/water intake was recorded weekly. On 30th day animals were used for serum collection followed by tissue collection.

2.1 Serum fasting glucose, serum fasting insulin and FIRI:

Serum glucose was measured by using enzymatic kit (ACCUCARE™, Lab – Care Diagnostics India Pvt. Ltd.). Serum fasting insulin was estimated at Saurashtra Pathology Laboratory – Rajkot using radio immune assay (RIA) kit. Fasting insulin resistance index (FIRI) was calculated by following formula[8]:

FIRI = fasting blood glucose in mg/dl x fasting insulin in uU/ml) / 25

2.2 Liver and Muscle Glycogen[9]:

The liver and thigh muscle were removed and blotted on filter paper to remove blood, weighed, dropped into flasks containing 10 ml hot 30% potassium hydroxide and digested on a hot plate. The digest was diluted to 100 ml and a 50-fold dilution of an aliquot was used for analysis. Ten ml 0.2% anthrone in 95% sulfuric acid was slowly added to 5 ml of liver digest dilution with cooling. The mixture was heated in a boiling water bath for 10 min and then placed into cold water. Optical density was measured in a spectrophotometer at 620 μm using the anthrone-reagent as blank. Calibration curves were established using glucose as standard.

2.3 Statistical Analysis:

Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using computer based fitting program (Prism, Graphpad). Differences were considered to be statistically significant when $p < 0.05$.

3. RESULTS

Serum parameters were found to be increasing significantly with administration of 10% fructose feeding for 30 days. Fructose significantly increased serum fasting glucose ($p<0.01$), serum insulin ($p<0.05$) and FIRI ($p<0.01$) as compared to control group (n=6). Pioglitazone (10mg/kg, p.o.) and abscisic acid (1mg/kg, p.o.) both exhibited significantly reduction in serum fasting glucose as compared to disease control. Abscisic acid showed significant anti-hyperglycaemic activity by bringing back serum fasting glucose near to normal as identical to pioglitazone treatment.

Both abscisic acid and pioglitazone treatment resulted in significant decrease ($p<0.05$) in fasting insulin respectively as compared to in disease control (Table-2, Fig-2). Fasting insulin resistance index ($p<0.01$) was also found to be reduced as compared to fructose treated group (Table-2, Fig-3) indicating improvement in insulin resistance produced by fructose feeding.

Table 2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting glucose, serum fasting insulin and FIRI in fructose induced insulin resistance in rats

Treatment (n=6)	Serum fasting glucose (mg/dl)	Serum fasting insulin (μU/ml)	Fasting insulin resistance index (FIRI)
Normal control (CON)	77 ± 1.23	22 ± 0.38	67.76
Disease control (FRU)	115 ± 4.55 **	29 ± 0.15 *	133.4 **
Standard (PIO + FRU)	80 ± 0.54 ##	23 ± 0.42 #	73.6 ##
Test (ABA + FRU)	82 ± 0.98 ##	24 ± 0.26 #	78.72 ##

All values represents Mean ± SEM; n=6

*   Significantly different from normal control (p < 0.05)

\#   Significantly different from disease control (p < 0.05)

** Significantly different from normal control (p < 0.01)

\#\# Significantly different from disease control (p < 0.01)

Figure 1: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting glucose in fructose induced insulin resistance in rats
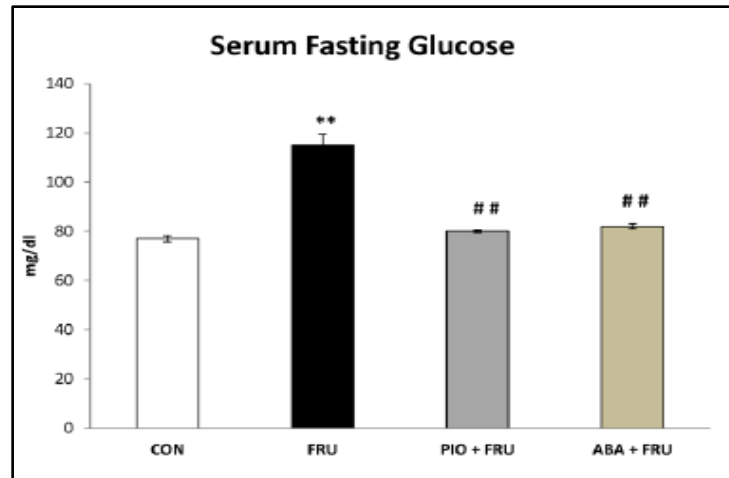

Figure 2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum fasting insulin in fructose induced insulin resistance in rats
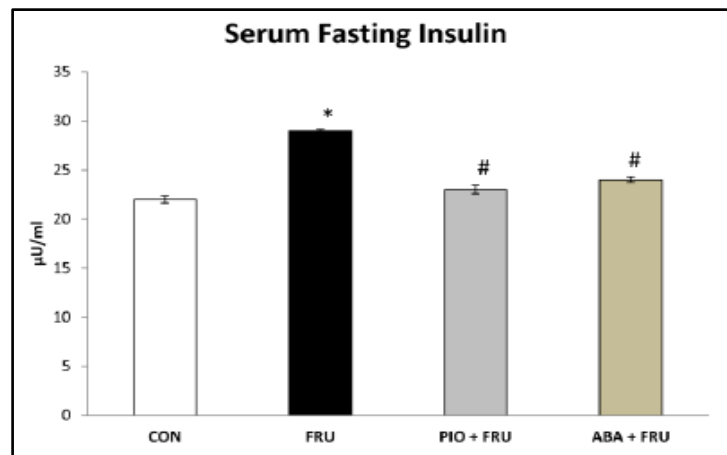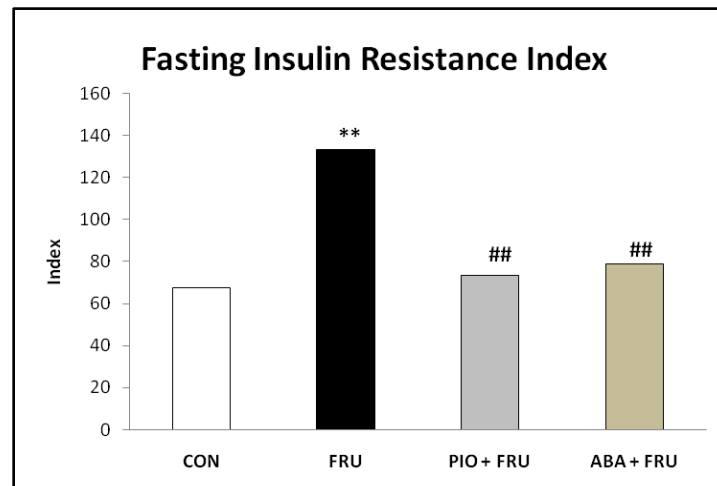

Figure-3: Effect of Abscisic acid (1 mg/kg/day, 30 days) on fasting insulin resistance index in fructose induced insulin resistance in rats

Liver and muscle glycogen stores were found to be decreasing (p<0.01 and p<0.05 respectively) with fructose supplement, as compared to control group, indicating development of insulin resistance. Both abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment increased liver and muscle glycogen stores significantly (p<0.01 and p<0.05 respectively), as compared to disease control, showing considerable increase in insulin sensitivity (Table-3, Fig-4 and 5).

Table 3: Effect of Abscisic acid on liver and muscle glycogen in fructose induced insulin resistance in rats

Treatment (n=6)	Liver glycogen (µg/mg of tissue)	Muscle glycogen (µg/mg of tissue)
Normal control (CON)	24.3 ± 2.3	6.2 ± 0.35
Disease control (FRU)	17.1 ± 1.74 [**]	5.15 ± 0.78 [*]
Standard (PIO + FRU)	22.35 ± 0.65 [##]	5.95 ± 0.4 [#]
Test (ABA + FRU)	22 ± 1.95 [##]	6.12 ± 0.89 [#]

Figure 4: Effect of Abscisic acid on liver glycogen in fructose induced insulin resistance in rats
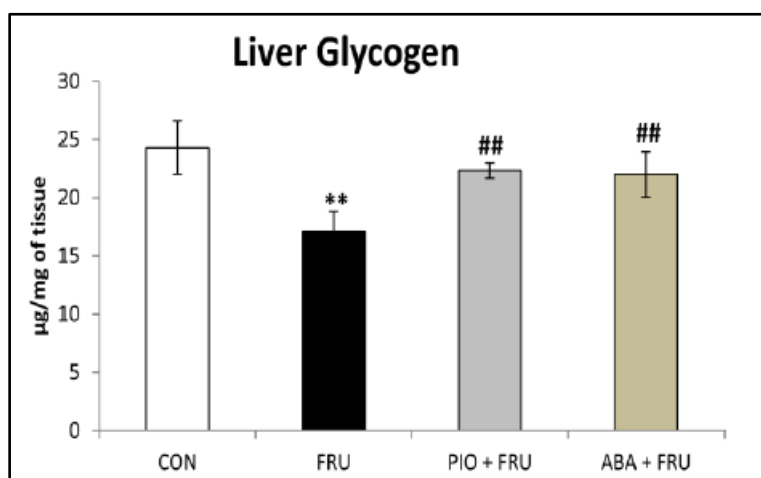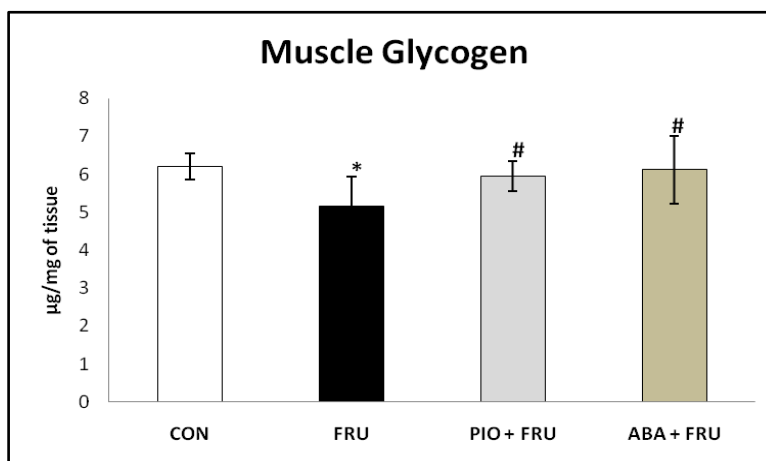

Figure 5: Effect of Abscisic acid (1 mg/kg/day, 30 days) on muscle glycogen in fructose induced insulin resistance in rats

4. DISCUSSION

In the recent decades the prevalence of insulin resistance and type II diabetes has seen a precipitous growth[10]. Abscisic acid can ameliorate the symptoms of type II diabetes, targeting PPAR-γ in a similar manner as the thiazolidinediones class of anti-diabetic drugs.

Earlier fructose was considered as one of the glucose alternative in diabetic patients. But afterwards it was found that upon chronic usage fructose causes metabolic syndrome including insulin resistance. The long-term negative effects can include changes in digestion, absorption, plasma hormone levels, appetite, and hepatic metabolism, leading to development of insulin resistance, diabetes, obesity, and inevitably cardiovascular disease[11]. Same results were obtained in our study indicated by hyperglycaemia, hyperinsulinaemia and decreased glycogen content in peripheral tissues.

Abscisic acid was found to be decreasing the insulin resistance induced by the fructose feeding. Abscisic acid not only decreased the rise in serum fasting glucose and insulin (observed with fructose feeding) but also increased the level of liver and muscle glycogen content. Thus, abscisic acid decreases blood sugar level, reduces over-burden on pancreatic β cells (by decreasing supernormal serum insulin) and increases uptake of glucose in peripheral tissues like liver and muscle. This clearly is indicative of insulin sensitizing action probably via PPAR-γ receptor action. Our result confirms previous report showing activation of PPAR-γ responsive genes by abscisic acid in 3T3-L1 preadipocytes *in vitro*[4].

Our results show that administration of abscisic acid may be advantageous for preservation of the functional efficiency of pancreatic β cells, probably by improving insulin action and thereby preventing induction of insulin resistance. Further studies are needed to establish safety and efficacy of abscisic acid in management of diabetes mellitus.

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EFFECT OF ABSCISIC ACID ON DYSLIPIDEMIA IN FRUCTOSE-INDUCED INSULIN RESISTANT RATS

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ABSTRACT

Insulin resistance is a metabolic disorder that prevails worldwide and plays a role in pathophysiology of most common human diseases including type 2 diabetes mellitus, hypertension, obesity, dyslipidemia and coronary heart disease. One of most effective medications in treatment of insulin resistance has been thiazolidinediones (TZDs) which act by acting through activation of PPAR-γ. TZDs have been shown to be very effective in improving insulin sensitivity as well as lipid dyslipidaemia through induction of genes such as aP2 and CD36, which are important in fatty acid transport, adipogenesis and lipid uptake. Abscisic acid (ABA), a phytohormone, is recently shown to possess insulin sensitizer activity through PPAR-γ activation. As most of the PPAR-γ agonists have the added benefits of being anti-atherogenic, we also tried to prove action of ABA in dyslipidaemia induced by high fructose feeding. We investigated effects of 2-cis, 4-trans abscisic acid on glucose (OGTT) and lipid profile. The animals were divided into 4 groups: normal control, disease control (10% fructose in drinking water, 30 days), standard treated (Pioglitazone 10mg/kg p.o.) and abscisic acid treated (1mg/kg p.o.) (both along with 10% fructose in drinking water, 30 days, n=6). Fructose significantly impaired glucose tolerance (p<0.01) and increased levels of serum total cholesterol, TG, VLDL (p<0.01) while decreased serum HDL (p<0.01) as compared to control group. Standard as well as ABA treated group significantly improved glucose tolerance (p<0.01), increased HDL (p<0.01) and decreased serum LDL, VLDL, and TG as compared to disease control group. Our results show that administration of ABA not only improve glucose tolerance but also aids in improving lipid profile leading to beneficial effects in insulin resistance or metabolic syndrome.

INTRODUCTION

Generally, type 2 diabetes mellitius (T2DM) patients suffer from both hyperglycemia and dyslipidemia. The insulin resistant state is commonly associated with lipoprotein abnormalities that are risk factors for atherosclerosis, including hypertriglyceridemia, high levels of very low density lipoprotein (VLDL), low levels of highdensity lipoprotein cholesterol[1], and small, dense LDL[2]. Clinical studies implicate hyperglycemia in the onset of microangiopathic complications of T2DM, including kidney failure, retinopathy and peripheral neuropathy. However, the major cause of mortality in T2DM patients is atherosclerotic macrovascular disease, which culminates in myocardial infarction. Such cardiovascular disease appears to result, in large part, from diabetic dyslipidemia[3].

TZDs are a class of the most effective insulin-sensitizing drugs that increase the sensitivity of peripheral tissues to endogenous insulin by activation of PPAR-γ[4,5]. Activation of PPAR-γ receptor not only treats hyperglycaemia, but also leads to decrease in atherogenic potential. They are considered to be a promising target for future drug design and treatment of diseases related to insulin resistance and the related conditions of hyperlipidemia, hyperglycemia, and hyperinsulinemia.

Since its discovery in the early 1960's, abscisic acid (ABA) has received considerable attention as an important phytohormone, and more recently, as a candidate medicinal in humans. Abscisic acid has been reported to be a partial peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist[5,6,7]. Abscisic acid is previously reported to ameliorate glucose tolerance in insulin resistant rat[6]. Improvement in lipid profile can be beneficial in insulin resistance and/or metabolic syndrome. Therefore, we studied effect of abscisic acid on dyslipidaemia in fructose-induced insulin resistance in rat.

MATERIAL AND METHODS

2 – cis, 4 – trans abscisic acid (98%, synthetic) was purchased from Sigma Aldrich, USA. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of R. K. College of Pharmacy, Rajkot and with permission from CPCSEA, Ministry of Social Justice and Empowerment, Government of India.

Ten week old male Sprague-Dawley rats (200 ± 25 gm) were housed in-group of 3 animal in cages and maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and provided free access to palleted diet and purified drinking water ad libitium,

unless specified. Rats (n=24) were randomized into 4 groups as shown in table 1. Rats were weighed and their food/water intake was recorded weekly. On 30th day animals were used for serum collection.

Group No.	Group Specification	Treatment (n=6, 30 days)
I	Normal control (CON)	Vehicle (water)
II	Disease control (FRU)	10% fructose in water *ad libitium*
III	Standard (PIO + FRU)	Pioglitazone (10 mg/kg/day, p.o.) + 10% fructose in water *ad libitium*
IV	Test (ABA + FRU)	Abscisic acid (1 mg/kg/day, p.o.) + 10% fructose in water *ad libitium*

Table 1: Group specification and dose for individual treatment

Oral glucose tolerance test (OGTT)[8]

The oral glucose tolerance test was performed in overnight fasted rats. Rats divided into four groups (n=6) were administered drinking water (normal control and disease control), pioglitazone and abscisic acid respectively. Glucose (2g/kg) was fed 30 min after the administration of drug. Blood was withdrawn from the retro orbital plexus under light ether anesthesia at 30, 60,120 min of glucose administration. Serum glucose was measured by using enzymatic kit (ACCUCARE™, Lab – Care Diagnostics India Pvt. Ltd.).

Serum lipid profile

In vitro quantitative determination of the activity of total cholesterol and HDL-cholesterol in serum was done using enzymatic kit (Monozyme India Limited - CHOD/POD-Phosphotungstate method). *In vitro* quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit (Reckon India diagnostics Pvt. Ltd. - GPO Method). Estimation of LDL-cholesterol was done using the Friedewald formula[9].

LDL cholesterol = total cholesterol – (HDL cholesterol + VLDL cholesterol)

Estimation of VLDL-cholesterol was done using the Friedewald formula[9]

VLDL cholesterol = triglycerides / 5

Statistical analysis

Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using computer based fitting program (Prism, Graphpad). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Serum parameters were found to be increasing significantly with administration of 10% fructose feeding for 30 days. Oral glucose tolerance test (OGTT) showed that at all time intervals fructose treatment significantly ($p < 0.01$) increased the serum glucose levels as compared to normal group. Abscisic acid as well as pioglitazone treatment significantly ($p < 0.01$) decreased the fasting serum glucose levels as compared to fructose treated animals (Table-2, Fig-1). Abscisic acid showed significant improvement in glucose tolerance produced by fructose feeding.

Treatment	Time (minutes)			
(n=6)	0	30	60	120
Normal control (CON)	77 ± 1.23	105 ± 3.45	134 ± 4.33	86 ± 7.21
Disease control (FRU)	$115 \pm 4.55^{**}$	$224 \pm 8.64^{**}$	$276 \pm 9.23^{**}$	$198 \pm 10.76^{**}$
Standard (PIO + FRU)	$80 \pm 0.54^{\#\#}$	$115 \pm 2.53^{\#\#}$	$112 \pm 6.27^{\#\#}$	$90 \pm 7.63^{\#\#}$
Test (ABA + FRU)	$82 \pm 0.98^{\#\#}$	$125 \pm 4.33^{\#\#}$	$119 \pm 5.22^{\#\#}$	$95 \pm 5.74^{\#\#}$

Table-2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on oral glucose tolerance test (OGTT) in fructose induced insulin resistance in rats
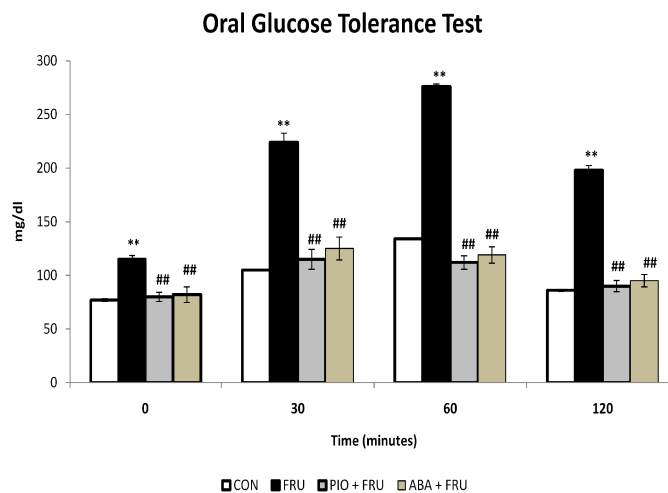

Figure-1: Effect of Abscisic acid (1 mg/kg/day, 30 days) on oral glucose tolerance test in fructose induced insulin resistance in rats

Fructose feeding significantly decreased serum HDL ($p < 0.01$) and increased serum total cholesterol ($p < 0.01$), serum triglycerides ($p < 0.01$), serum LDL ($p < 0.01$) and serum VLDL

(p<0.01) as compared to control. Both abscisic acid and pioglitazone treatment resulted in significant increase (p<0.05) in serum HDL as compared to disease control. Abscisic acid and pioglitazone treatment both decreased the rise in total serum cholesterol (p<0.01), serum triglycerides (p<0.01), serum LDL (p<0.01) and serum VLDL (p<0.05) observed with fructose feeding significantly. Although abscisic acid (1mg/kg) and pioglitazone (10mg/kg) treatment both decreased lipid abnormalities significantly effect of abscisic acid was apparently more on serum TG as compared to pioglitazone treatment (Table-3, Fig-2).

Lipid profile parameters (mg/dl)	Treatment (n=6)			
	Normal control (CON)	Disease control (FRU)	Standard (PIO+FRU)	Test (ABA+FRU)
Serum Total Cholesterol	81 ± 4.36	$122 \pm 11.23^{**}$	$92 \pm 7.76^{\#\#}$	$103 \pm 8.32^{\#\#}$
Serum Triglycerides	44 ± 3.44	$106 \pm 2.43^{**}$	$80 \pm 4.76^{\#\#}$	$63 \pm 2.14^{\#\#}$
Serum HDL	51 ± 2.11	$32 \pm 2.54^{**}$	$43 \pm 3.12^{\#}$	$40 \pm 2.87^{\#}$
Serum LDL	24 ± 2.54	$62 \pm 2.14^{**}$	$38 \pm 3.56^{\#\#}$	$36 \pm 1.53^{\#\#}$
Serum VLDL	9 ± 1.43	$22 \pm 2.13^{**}$	$15 \pm 1.13^{\#}$	$14 \pm 1.66^{\#}$

Table-3: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum lipid profile parameters in fructose induced insulin resistance in rats

All values represents Mean ± SEM; n=6
*Significantly different from normal control (p < 0.05), #Significantly different from disease control (p < 0.05), ** Significantly different from normal control (p < 0.01), ## Significantly different from disease control (p < 0.01)
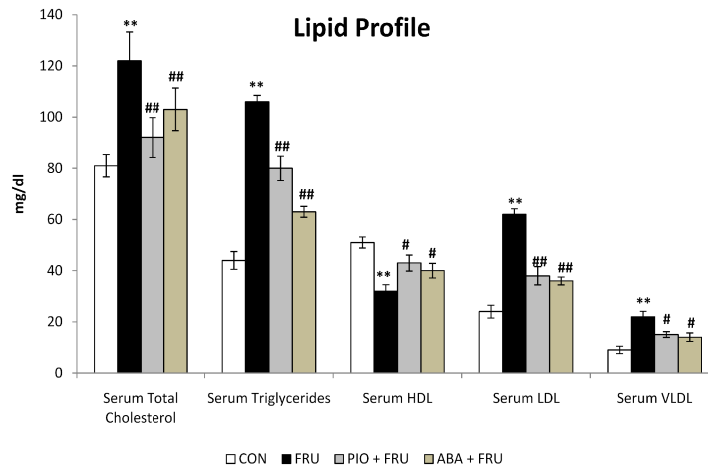

Figure-2: Effect of Abscisic acid (1 mg/kg/day, 30 days) on serum total cholesterol, triglycerides, HDL, LDL and VLDL in fructose induced insulin resistance in rats

DISCUSSION

Insulin resistance is a metabolic disorder that prevails worldwide and plays a role in pathophysiology of most common human diseases including type 2 diabetes mellitus, hypertension, obesity, dyslipidemia and coronary heart disease. Research has proved that mortality due to cardiovascular disease associated with diabetic dyslipidaemia is increased[3]. Chronic fructose feeding is associated with insulin resistance and metabolic abnormalities. ABA was shown to decrease serum fasting glucose and glucose intolerance significantly. This is in compliance with previously reported study showing use of dietary abscisic acid in ameliorating glucose tolerance in db/db mice fed high-fat diets[6]. Our study confirms anti-hyperglycaemic activity of ABA in insulin resistance. It also been reported that fructose causes the metabolic syndrome through involvement of leptin, adiponectin, and free fatty acids[10,11]. Same results were obtained in our study indicated by hyperglycaemia and dyslipidaemia in disease control group. Many of the genes induced by PPAR-γ, such as aP2 and CD36, are important in fatty acid transport, adipogenesis and lipid uptake. Studies have shown that PPAR-γ is an essential participant in the differentiation of pre-adipocytes into adipocytes, particularly in the subcutaneous adipose tissue depots. This function has been proposed to enhance systemic insulin sensitivity by inhibiting lipid efflux from adipose tissue into tissues such as the liver, pancreas, skeletal muscle, which are important for the proper regulation of glucose homeostasis[12,13].

PPAR-γ agonists, such as thiazolidinediones (TZDs), have been shown to be very effective in improving insulin sensitivity as well as lipid dyslipidaemia[14]. Present study confirms effect of pioglitazone on reducing dyslipidaemia through PPAR-γ activation. Similar pattern of results have been observed with ABA treatment in our study. Although both pioglitazone and ABA decreased TG, LDL, and total cholesterol significantly; effect of ABA was remarkably better on levels of TG as compared to pioglitazone. Increased delivery of triglycerides or non-esterified fatty acids to the muscle interferes with the utilization of glucose, through the principles of Randle cycle[15], impairing the insulin action. Thus, reduction in dyslipidaemia especially hypertriglyceridaemia may be correlated with improved insulin sensitivity.

Our results show that administration of ABA not only improve glucose tolerance but also aids in improving lipid profile leading to beneficial effects in insulin resistance or metabolic syndrome. Further studies are needed to establish safety and efficacy of abscisic acid in management of insulin resistance and associated metabolic complications.

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