1. Introduction

Breast cancer originates in the cells of breast, as a group of cancer cells that can then invade surrounding tissues or spread (metastasize) to other areas of the body; often it can be divided into non-invasive and invasive types. It is estimated that, worldwide over 5,08,000 women died due to breast cancer in 2011. Although breast cancer is thought to be a disease of the developed countries, almost 50% of breast cancer cases and 58% of death occurs in less developed countries. In India, the average age of developing a breast cancer has undergone a significant shift (age 30-40) over last few decades. Twenty five years back, the maximum incidence (30%) of breast cancer in India was in the age among 50-60, but the current statistics furnishes an alarming condition that the highest incidence (44%) is between the ages of 30-50 (WHO Press:2013). Further, the incidence of breast cancer in the year 2012, it was reported that 2,32,000 and 1,45,000 cases were diagnosed in United States (USA) and India respectively. Evidently, the breast cancer burden in India has almost reached about 2/3rd of that of the United States (USA) and is steadily rising (WHO Press: 2013).

Cancer metabolism encompass an extensively rewired metabolic pathways to which do not only permit cancer cells to survive under adverse conditions such as hypoxia, but enable their proliferation, progression, invasiveness and subsequent distant metastasis. Hence, addressing the functional nodes of metabolic alterations would provide effective target and has been explored for diagnostic, prognostic and therapeutic targeting in cancer management. Among the metabolic alterations, “de novo lipogenesis” is an imperative hallmark which provides cancer cells with membrane building blocks, signaling lipid molecules, post-translational modifications of proteins as well as energy supply to support rapid cell proliferation. Additionally, increased lipogenesis involves modulation of several lipogenic enzymes at both transcriptional and post-transcriptional level and is linked to other cancer-associated metabolic changes such as metastatic process and in epithelial to mesenchymal transition (Swinnen et al., 2006; Ounier et al., 2014).
The human genome contains the genes for two different ACCs (Acetyl-CoA carboxylase) namely ACC-α (ACC1) and ACC-β (ACC2), which differ in both tissue distribution and functions. The differences in tissue distribution designates that ACC1 (Acetyl-CoA carboxylase1) involves in fatty acid synthesis whereas ACC2 regulates oxidation of fatty acids. In the cytosol, ACC1 employed as rate-limiting enzyme, which catalyzes ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA which is a critical bi-functional molecule. To be precise, employs substrate for FASN (fatty acid synthase), which is catalyzes the terminal steps in the ‘de novo’ biogenesis of fatty acids and an inhibition of CPT-I (carnitine palmitoyltransferaseI) for fatty acid beta-oxidation (Swinnen et al., 2006).

Many tumors express high levels of ACC1, including breast, colorectal and endometrial cancers (Daniel et al., 2010; Javier et al., 2007; Eiji et al., 2010). BRCA1 (breast cancer associated1), a tumor suppressor protein concerned in many cellular functions, for instance of DNA repair, cell cycle checkpoint, transcription and ubiquitination. It consists of four major protein domains namely Znf C3HC4-RING domain, BRCA1 serine domain and two BRCA1 C-terminal (BRCT) domains. The multiple repeats BRCT domain of the BRCA1 protein is an elongated structure about 70 Å long, 30-35 Å wide and known to contain 90-100 amino acid residues (Glover et al., 2004).

Structural studies of BRCT domain have revealed a conserved structure for the repeat, composed of a four-stranded parallel β-sheet flanked by a pair of α-helices on one face (α1 and α3), and a single α-helix (α2) on the opposite face. In this arrangement, the N-terminal half of the one BRCT domain forms a pocket for pSer as the C-terminal half of the same domain generates a hydrophobic pocket for Phe(Williams et al., 2004). Many conformations revealed that BRCT repeats of BRCA1 recognize phosphorylated peptides via a pocket which accommodates the phosphorylated serine (Williams et al., 2004; Clapperton et al., 2004; Shiozaki et al., 2004; Varma et al., 2005). Notably, this interaction affects ACC1 activity through phospho-dependent manner. Consequently, serial reactions hypothesized as starvation of phospholipids and long chain fatty acids, which increases energy demand in cytosolic region and starvation followed by oxidative stress mediated apoptosis of cancer cells. Further, the decision between apoptosis and growth arrest
following ACC1 inhibition is greatly influenced by p53 status. ACC1 inhibitors are more effective at initiating apoptosis in tumor cells with non-functioning p53, whereas cells with intact p53 function tend to exhibit cytostatic responses (Menendez and Lupu, 2007).

Albeit, there is a need to design a delivery system for peptides which are indented to biological system, to not only protect the peptide from enzymatic degradation but also aid in enhancing its absorption into tumor site without altering its nature. Nano-carriers are promising carrier systems for delivery of peptides. They exhibit high permeability properties because of their smaller size and higher surface to volume ratio (Gupta et al., 2013). In order to improve the bioavailability of peptides, new ranges of biodegradable polymeric nanoparticles are being used which can enhance the stability, control the release and pharmacokinetic parameters. The nature of tumor microenvironment basically allows entrapment and accumulation of nanoparticles and they retained in tumor site at high concentrations for prolonged period. The fundamental principle behind this phenomenon is the inflammatory condition of tumor. In tumor tissue level several factors are collectively supports the entrapment and accumulation of nanoparticles in tumor site. Which includes, increased infiltration rate, excess production of superoxide and nitric oxide, defective vascular architecture, whimsical blood flow and slow venous return. Besides, the BRCA1 protein has nuclear-cytoplasm shuttling nature that requires a versatile cargo which can target cytosol and nucleus as well. The nuclear targeting of cancer cells is complex owing to rapid changes in nuclear pore complex size, which is varying according to cell cycle. Nuclear targeting substance requires being stable more time in the cytosol and it needs to escape from hydrolytic enzymes of endocytic pathway. Consequently, the nano-carriers are the paramount delivery system to conquer the problems in nuclear targeting. It is also observed that, among natural delivery systems, such as CPPs (cell-penetrating peptides) not only the type of chemical groups, but their relative arrangement also plays a key role in their interaction with cell membranes. Such peptides, while crossing at the cell-membrane barrier found to be ineffective owes to their random-coil conformation, display an ability to penetrate or fuse with membranes after adopting an amphipathic α-helical structure (Lindgren et al., 2000). Nevertheless, the problem associated with
interaction of CPPs with tumor cell membrane is still uncertain. This issue might be easily answered by loading the CPPs into the polymeric system which is having high number of charged functional groups. Hence, the uptake of positively charged particles will be higher despite the favorable interaction between the negatively charged cell membrane. Moreover, the synthetic nanoparticles have been conjugated to natural cell-membrane-penetrating motifs that can chaperon the cargo inside the cell into the cytosol or nucleus.

In most of the cases, the current clinical cancer therapies after an inconsistent period of time, resistance to therapy is not only common but also anticipated. Current therapy does not kill all cancer cells, but instead, some cells enter a state known as senescence (programmed growth arrest). Though, there is an urgent need of reinforce the current cancer therapy with an effective molecule with suitable formulation the present study is intended.
2. Aim and objectives of the research

The aim of the current research is to formulate and evaluate a BRCT domain targeting nano formulated peptide.

The objectives of the study were as follows:

- To identify peptide for potential phosphorylation of BRCT domain by in silico approach, to assess the toxicity and develop analytical method for quantification of peptide pACC1.
- To formulate and evaluate the peptide pACC1 loaded polymeric nanoparticle and study the embryotoxicity by Danio rerio model.
- To evaluate the apoptotic effect of peptide pACC1 loaded chitosan nanoparticles using MCF7 cancer cells.
- To explicate the anti-tumorigenic effect of peptide pACC1 loaded chitosan nanoparticles on DMBA induced mammary carcinoma rat model.
3. Review of literature

3.1. History of cancer

Cancer is supposed as old as the human race, but paleopathologic findings indicate that tumors existed in the early Miocene times, long before men on Earth. Even though, ultimate reliance in history of cancer requires documents. In medicine, the earliest written description of diseases and cancer (breast cancer), is found in the Edwin Smith Papyrus that was written approximately on 3000 BC (Hajdu, 2011; wozniak et al., 2012). Indian medical scriptures wrote about Arbud (tumor) is a disease in which utsedh (swelling) is a common feature. In 16th and 17th centuries, it became acceptable to dissect dead bodies and wilhelm Fabry (Father of German surgery) believed that breast cancer was caused by a milk clot in the breast duct. The Dutch professor Francois de la Boe Sylvius believed that all disease was the outcome of chemical processes, and that acidic lymph fluid was the cause of cancer. Latter, Dutch surgeon and may or of Amsterdam Nicolaes Tulp, assumed that cancer was a poison that slowly spreads, and concluded that it was contagious (American Cancer Society, 2014). Current contemporary medical science defines “cancer is the uncontrolled growth and spread of cells. It can affect almost any part of the body. The growths often invade surrounding tissue and can metastasize to distant sites” (WHO-2014).

3.2. Breast cancer

Breast cancer is an uncontrolled growth that occurs in breast tissues and the most common type is ductal carcinoma, which begins in the lining of the milk ducts. Another type of breast cancer is lobular carcinoma, which begins in the lobules (milk glands) of the breast. The disease occurs almost entirely in women, but men can get it, too (Abeloff et al., 2008). The less common breast cancer types are inflammatory breast cancer, Triple-negative breast cancer, Paget disease of the nipple, Phyllodes tumor and Angiosarcoma (Santen and Mansel, 2005). It is estimated that in the USA in 2014 approximately 232,670 cases of female breast cancer will be diagnosed, and approximately 40,000 women will die from the disease (Caplan and Delay, 2014).
3.2.1. Current clinical treatments

The current clinical treatments are mainly based on stage, histology, and biomarkers (Howard and Bland, 2012). The main types of treatment for breast cancer are:

3.2.1.1. Surgery

The surgical removal of one or both breasts, partially or completely may be employed. Currently, there are several surgical approaches to mastectomy which includes Simple mastectomy, Modified radical mastectomy, Extended Radical Mastectomy, Radical mastectomy (Halsted mastectomy), Skin-sparing mastectomy, Prophylactic mastectomy and Nipple sparing/subcutaneous mastectomy. However, the side effects of a mastectomy like wound infection, hematoma (buildup of blood in the wound), and the seroma (buildup of clear fluid in the wound) are obvious (Adam et al., 2014).

3.2.1.2. Radiation therapy

Radiation is used often to the people who have undergone conservative surgery such as lumpectomy. Conservative surgery is designed to leave as much of the breast tissue in place as possible. Hence, the additional radiation might be given for five days a week over five to six weeks (Vallis and Tannock, 2004).

3.2.1.3. Chemotherapy

The use of cytotoxic chemotherapy in both advanced and early stage breast cancer have made significant progress in the last two decade with several landmark studies identifying clear survival benefits for newer therapies (Hassan et al., 2010). Chemotherapy may be classified into several types such as Alkylating agents, Anti-metabolites, Anti-microtubule agents, Topoisomerase inhibitors and cytotoxic antibiotics. The drawbacks of cancer chemotherapy is may be associated with nausea, vomiting, hair loss, cognitive dysfunction, fatigue, premature menopause, lowered resistance to infections, changes in sexual functioning and reductions in quality-of-life ratings (Kayl and Meyers, 2006). The fast-dividing cells of the body are also affected by cancer chemotherapy, such as RBC, WBC of blood and the cells of oral mucosa, stomach and intestines.
3.2.1.4. Hormone therapy

Hormone therapy for breast cancer includes drugs that block the effect of estrogen on cancer cells, drugs that lower the production of estrogen in the body (aromatase inhibitors) and ovarian suppression to prevent the ovaries from producing hormones (Santen and Yue, 2014).

3.3. Molecular targets for breast cancer therapy

In recent years, the altered molecular events of cancer have led to the recognition of novel molecular targets and development of targeted therapies.

3.3.1. EGFR (Epidermal Growth Factor Receptor)

EGFR is a family of transmembrane receptor tyrosine kinases, involved in regulation of cell proliferation and survival. EGFR family includes four receptors: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. EGFR and HER2 are over expressed in about 40% and 25% of breast cancers (Munagala et al., 2011). EGFR over expression in breast cancer is associated with large tumor size, poor differentiation and poor clinical outcomes (Sainsbury et al., 1987; Salomol et al., 1995). Hypoxia of tumor environment and EGFR has been shown to promote EMT (epithelial-mesenchymal transition), process in which cells undergo morphological change from polarized epithelial phenotype to a mesenchymalfibroblastoid phenotype (Radisky, 2005). After sustained EGFR inhibition, oncogene signatures such as Ras and MYC were dramatically decreased in triple negative breast cancer (Lee et al., 2012). In addition, subtypes of breast carcinoma showed increased EGFR copy number and/or EGFR protein expression in the heterogeneous category of metaplastic carcinoma (Reis-Filho et al., 2006). The EGFR inhibition are achieved by different types of agents such as Monoclonal antibodies (Cetuximab, Panitumumab), Tyrosine kinase inhibitors (Gefitinib, Erlotinib, Lapatinib, Canertinib), Antibody based immunoconjugates (Trastuzumab-Emtansine, EQ75-ADR), Antisense oligodeoxynucleotides (GEM 231) and other novel agents (FR18, Affibodies, Nanobodies, Peptides) (Yewale et al., 2013). Though, anti-EGFR therapy is temporary showing initial massive reduction in tumor but is later regrows rapidly and turns into refractory to therapy (Ma et al., 2011).
3.3.1.1. HER2 (Receptor tyrosine-protein kinase erbB-2)

In recent years, the HER2 protein has become an important breast cancer biomarker and target of therapy for the patients. It has been shown to have clinical activity and has led to longer survival times in patients with HER-2 over expressing in MBC (Metastatic Breast Cancer) as well as early breast cancer (Slamon et al., 2001). Selective tyrosine kinase inhibitors for HER2 in development were Neratinib (Phase III), Canertinib (Phase II), BIBW 2992 (Phase II), TAK-285(Phase I), BMS 599626 (Phase I) and CP 724714 (Phase I) (Roy and Perez, 2009).

3.3.2. VEGF (Vascular Endothelial Growth factor)

VEGF is a powerful inducer of cell migration, invasion, vascular permeability and vessel formation. Five related glycoproteins namely, VEGFA, VEGFB, VEFGC, VEGFD, and placental growth factor (PGF) act via three receptor tyrosine kinases namely VEGFR-1, VEGFR-2, and VEGFR-3. Angiogenesis, the development of new blood vessels from an existing circulation to tumor tissue, is a prerequisite for tumor growth and metastasis. The levels of VEGF in breast cancer tissue associates with microvessel density and angiogenesis plays a significant role in metastasis of breast cancer (Byrne et al., 2007). Existing VEGF inhibitors target the VEGF pathway in various ways including: (1) direct inhibition of VEGF protein (anti-VEGF monoclonal antibodies; bevacizumab and ranibizumab); (2) prevention of VEGF receptor binding (VEGF receptor antagonists; alfibercept/VEGF-Trap and pegaptanib); and (3) inhibition of VEGF receptor function through inhibition of tyrosine kinase (tyrosine kinase inhibitors [TKIs]; sunitinib, sorafenib, vandetanib, and pazopanib). Many new anti-angiogenic therapies are under investigation that includes: ramucirumab, cediranib, nintedanib (BIBF 1120), pazopanib, brivanib, ABT-869, axitinib, ABT-751, and NPI-2358 (Weidemann et al., 2013).

3.3.3. IGFs (Insulin-like Growth Factors)

The IGFs plays a major role in cancer cell proliferation, survival and confers resistance to cytotoxic, hormonal, and targeted therapies in diverse tumor types, including breast cancer. The IGFs, the primary mediator of the biological actions of IGF-I, have been detected in a majority of primary breast tumor samples with over
expression in 30 to 40% of breast cancers. In addition, several clinical studies also support the role of IGFs in development of breast cancer (Hankinson and Schernhammer, 2003). The compounds targeting IGF-I components using specific examples are: growth hormone releasing hormone antagonists (e.g. JV-1-38), growth hormone receptor antagonists (e.g. pegvisomant), IGF-1R antibodies (e.g. CP-751, 871, AVE1642/EM164, IMC-A12, SCH-717454, BIIB022, AMG 479, MK-0646/h7C10), and IGF-1R tyrosine kinase inhibitors(e.g. BMS-536942, BMS-554417, NVP-AEW541, NVP-ADW742, AG1024, potent quinolinyl-derived imidazo (1,5-a) pyrazine PQIP, picropodophyllin PPP, Nordihydroguaiaretic acid Insm-18/NDGA) (Hewish et al., 2009).

3.3.4. Other molecular targets
3.3.4.1. Farnesyltransferase (EC 2.5.1.58)

The enzyme farnesyltransferase is involved in post-translational modification of Ras, thereby making it active for signal transduction. Although the Ras protein is mutated in only <5% of breast cancers, there are multiple aberrant pathways that lead to activation of wild-type Ras signaling. Moreover, farnesyltransferase inhibitors have consistently demonstrated efficacy in tumors regardless of their Ras mutational status. Thus, the role of other protein targets in mediating the antitumor effect of farnesyltransferase inhibitors is being elucidated (Dy and Adjei, 2002).

3.3.4.2. Cyclin-dependent Kinase (CDK)

The cyclin-dependent kinases are heterodimeric complexes composed of a catalytic kinase subunit and a regulatory cyclin subunit, and comprise a family divided into two groups based on their roles in cell cycle progression and transcriptional regulation. Cyclin A- dependent kinases 2 and 1 and cyclin B-cdk1 complexes are required for orderly S-phase progression and the G2 to M transition respectively (Akin et al., 2014). Cyclin-dependent kinase inhibitors are categorized as selective and non-selective inhibitors of CDK. CDK inhibitors have been tried as both monotherapy and combination therapy as well.
3.3.4.3. MMPs (Matrix Metalloproteinases)

MMPs are promising candidates with vast diagnostic and prognostic value. MMPs also employ as indicators of tumor recurrence, metastatic spread, and response to primary and adjuvant therapy for breast cancer (Bartsch et al., 2003).

3.3.4.4. Histone Deacetylase

Histone deacetylase inhibition leads to differentiation and/or apoptosis of tumor cells by interfering with the function of histone deacetylase and transcriptional down-regulation of ER (Estrogen receptors) and its response genes in ER-positive cancer cells (Galluzzi et al., 2013).

3.4. Metabolic targets

Accumulating evidence suggests that the metabolic alterations of each neoplasm represent a molecular signature that intimately accompanies and allows for dissimilar facets of malignant transformation. During the past decade, targeting cancer metabolism has emerged as a promising strategy for the development of selective anticancer agents. Our progress is towards an improved understanding of the metabolic alteration that occurs in cancer, hence development of therapeutic measures targeting this important aspect of tumour biology, has been hindered by profound misconceptions.

3.4.1. Metabolic alterations of cancer cells

Cell proliferation requires bioenergetic resources and biosynthetic activity needed for the duplication of all macromolecular components (DNA, membranes, and proteins) to ensure successful passage through the cell cycle. To meet this challenge, metabolic activities are reorganized in proliferating cells resulting in an anabolic shift in cellular metabolism. Similar to highly proliferating normal cells, malignant cells also exhibits a profound imbalance towards anabolic metabolism. Compared to normal cells, malignant transformation is associated with an increased rate of intracellular glucose import, and a higher rate of glycolysis associated with reduced pyruvate oxidation and increased lactic acid production. In addition, cancer cells have increased gluconeogenesis, increased glutaminolytic activity, reduced fatty
acid oxidation, increased de novo fatty acid synthesis, increased glycerol turnover, modified amino acid metabolism, and increased pentose phosphate pathway activity (Zhao et al., 2013). Mounting evidence supports the idea that dysregulated cellular metabolism is linked to drug resistance in cancer therapy.

3.5. Lipogenesis and cancer

Malignant transformation alters both biosynthetic and bioenergetic requirements for cells. Continuous de novo lipogenesis provides cancer cells with membrane building blocks, signaling lipid molecules, posttranslational modifications of proteins as well as energy supply to support rapid cell proliferation. The endogenously synthesized fatty acids facilitates the formation of detergent-resistant membrane microdomain for signal transduction, intracellular trafficking, polarization and migration required for cancer cells. Further, the newly synthesized lipids molecules, such as phosphatidic acid (PA), diacylglycerol (DAG), and lysophosphatidic acid (LPA), also mediate signal transduction in cancer cells (Notarnicola et al., 2014). Additionally, the lipogenesis also helps in posttranslational protein modifications which lead to localization and function of various signaling proteins that includes Phosphatidylinositol (PI) and GPI anchored proteins. Lipogenesis encourages some GPI-anchored proteins, such as urokinase-type plasminogen activator (uPA)-receptor (uPAR) and membrane anchored serine protease matriptase (also known as MT-SP1 and epithin) where all these proteins have close association in cancer proliferation. In addition to directly targeting enzymes involved in fatty acid synthesis, their activities might be reduced by controlling transcription levels. The master transcriptional regulators of fatty acid synthesis are sterol regulatory element-binding protein 1 (SREBP-1) transcription factors (Horton et al., 2002). At normal levels; SREBP-1c activates the fatty acid biosynthetic pathway with responsive genes including ACLY, ACC, FAS, SCD-1, and GPAT. Consequently, inhibition of SREBP-1 in cancer cells could decrease fatty acid synthesis gene expression and possibly prevent cancer cell proliferation. Augmented evidences denotes disturbance in lipid metabolism leads generation ROS, which results in oxidative stress and apoptosis in breast cancer. It is likely that differences in the ability to obliterate cells from these beneficial products will determine the effect of these destructive factors
on breast cancer (Barrera, 2012). Prostaglandins (i.e., prostaglandin E) accumulation reinforces the procarcinogenic roles played by proinflammatory signaling during tumorigenesis.

Remarkably, a tight cross-talk exists between lipid metabolism and modulation of the expression of the vital proinflammatory mediator, cyclo-oxygenase 2 (COX-2), which is constitutively overexpressed in cancer (Cerella et al., 2010). During late breast cancer growth, fast multiplying carcinoma cells require higher energy and angiogenic needs. By up-regulating lipogenesis, core malignant cells can maintain the appropriate energy balance necessary to meet the nutritional demands of an expanding normal or malignant epithelial cell population (McCready et al., 2014). Hence, the lipogenesis plays a vital role in all the stages of cancer.

Figure 1. Fatty acid biosynthesis pathways in tumor cells. Initially pyruvate is converted as CoA, which is then entering into citric acid cycle in mitochondria. Depending on the oxygen availability, citrate can be fully oxidized to generate ATP by oxidative phosphorylation, or it can be transported to the cytoplasm where it is converted back to acetyl-CoA (the requisite building block for FA synthesis). A portion of the acetyl-CoA is carboxylated to malonyl-CoA by ACC1, the primary rate-limiting enzyme and site of pathway regulation. FASN, the main biosynthetic enzyme, performs the condensation of acetyl-CoA and malonyl-CoA to produce the 16-carbon saturated fatty acid palmitate (Menendez and Lupu, 2007) and other saturated long-chain fatty acids, which are used for the synthesis of various cellular lipids such as phospholipids, triglycerides and cholesterol esters, or for the acylation of proteins.
3.6. Peptides in cancer treatment

Over the years peptides have been evolved as promising therapeutic agents in the treatment of cancer, diabetes, and cardiovascular diseases. Moreover application of peptides in a variety of other therapeutic areas is growing rapidly. Current research focuses on developing peptides that can (1) serve as tumor targeting moieties, (2) permeabilize membranes with cytotoxic consequences, (3) binding with DNA and induce apoptotic cascades and (4) Inhibiting metabolic enzymes by phosphorylation etc. There are hundreds of peptides in the pipe line and from the year 2000, peptides entering clinical study were most frequently analysed for cancer (18%) and metabolic disorders (17%) (Thundimadathil, 2012). The ability of peptides to bind to different receptors and its potential role playing in biochemical pathways would makes peptide as a potential diagnostic tool and biomarker in cancer progression. In the last decade, the idea of peptide vaccinations against cancer has transformed into clinical studies to induce anticancer immunity. In this case, peptide sequences are derived from the protein sequence of candidate tumor-associated or specific antigens. Principally, tumor cells tend to express antigens which can be recognized by the host’s immune system (T cells). Many of the antigens expressed by breast, prostate and ovary cancer cells have already been identified and molecularly characterized. Hence, these antigens can be injected into cancer patients in an attempt to induce a systemic immune response that may result in the destruction of the cancer cells.

Direct use of peptide as a therapeutic agent to treat cancer is gaining momentum in the recent years. Anti-cancer therapy of peptides perhaps achieved by variety of mechanisms includes inhibition of tumor angiogenesis, beneficial protein-protein interactions with enzymes and proteins of cytosol and blocking signal transduction pathways or gene expression and competitive binding with receptors of surface as well as nucleus. Recently, many peptides gained good clinical attention in cancer treatment such as octapeptides that specifically target the protein-protein interface of thymidylate synthase, Pt (IV)-peptide that conjugates as a good inhibitor of cellular proliferation, bombesin/gastrin-releasing peptides that bind selectively to the G-protein-coupled receptors on the cell surface and cyclized RGD pentapeptide (cyclo-
[Arg-Gly-Asp-DPhe-(NMeVal)] that is selective for αv integrins, which are important in angiogenesis. RGD pentapeptide is currently under phase II trial for the treatment of glioblastoma and refractory brain tumors in children (Thundimadathil, 2012).

3.7. Delivery of peptides

During the past two decades, the revolutionary expansion of methods in biotechnology has facilitated the availability of therapeutic peptides for chronic diseases including cancer. Nevertheless, the development of suitable dosage forms for increased bioavailability is still in hunt. Even now, injections remain the common method for administrating therapeutic peptides because of their poor oral bioavailability and less stability in blood plasma. However, oral route would be preferred to any other route because of high levels of patient acceptance and long term compliance, which increases the therapeutic value of the peptide. Designing a formulation for effective peptide delivery is not only because of interest in delivery of the peptide in oral route, it is to deliver the peptides in effective manner to the target also. Chemical modification of peptide also a choice of peptides for enhanced enzymatic stability and/or membrane penetration. It can also be used to minimize immunogenicity. Peptide modification can be done by direct modification of exposed amino acid groups of peptides (Fix, 1996). Liposomes are taken up by cells effectively in in vitro and in situ experiments, but there are only few studies that prove their delivery to solid tumors and also plasma resistant lipids extend is limited (Adessi and Sotto, 2002). Peptide-mediated targeting liposomes is an another milestone in cancer targeted drug delivery, each peptide-conjugated liposome can efficiently deliver 15,000 anticancer drug molecules directly into endosome compartments with low pH; the liposomes then break down and release the encapsulated drug into the intracellular space of the target tumor cells (Lu et al., 2013). From the clinical prospective, the potential ability of liposome-encapsulated peptides to enter cytoplasm or lysosomes of live cells is of primary importance for the treatment of inherited diseases caused by the abnormal functioning of some intracellular enzymes, especially in liver and CNS cells. Mucoadhesive polymer system is an another widely used system to deliver the peptides. In this system, the
mucoadhesive properties of formulation can provide an intimate contact with the mucosa at the site of peptide uptake is required. Hence, preventing a presystemic metabolism of peptides is easily achieved. TAT (transactivator of transcription) peptide (residues 1-72 or 37-72) approach is that conjugating the peptide of interest to TAT peptide and deliver it into the cytosol. TAT fusion peptides displayed potential to treat disorders pertaining to oxidative stress. TAT fusion proteins could also deliver biologically active exogenous HSP70 (heat shock protein 70), required for the cytoprotection against cellular stressors. TAT is also used to transduce a biologically active neuroprotectant Bcl-xL in cerebral ischemia TAT and Bcl-xL fusion protein resulted in robust protein transduction in cultures and also delivered the protein across the blood brain barrier. Intravenous administration of M2pep fusion peptides with KLAKLAKKLAKLAK (KLA), a proapoptotic peptide, to tumor-bearing mice selectively reduces TAM (Tumor-associated macrophages) populations and prolongs survival (Cieslewicz et al., 2013). Though, there are many peptides delivered into biological system by various method for tumor suppression, still the effective delivery of peptides is uncertain.

3.8. Biocompatible polymers for peptide delivery

Biocompatible polymers may be defined as synthetic or natural polymers which are compactable in the in vivo system or it may degraded by enzymatically or non-enzymatically into non-toxic by-products, further excreted via normal physiological pathways. For example, a suitable molecular weight is required for renal clearance from 30,000 to 40,000, depending on the polymer used. If the administered polymer’s size is larger than this, then the polymer must undergo degradation. Either chemical or enzymatic biodegradation would provide fragments suitable for renal clearance. Chemical degradation is referred to acid catalyzed degradation such as in the stomach. Biocompatible polymers intended to a biological system as a peptide carrier, desires to hold certain compulsive parameters such as permeability, biodegradability, biocompatibility and tensile strength, even though, these properties are interdependent to certain degree. The advantages of selection of biocompatible polymers for peptide delivery are (1) They provide a constant controlled rate peptide release at prescribed period of time intervals, (2) Polymer carrier would degrade into
non-toxic, absorbable subunits which would be subsequently metabolized and excreted form body and (3) The diffusion coefficient influenced by polymer permeability. Polymer-Peptide Conjugate Hydrogels (PPC) is physically-bonded networks capable of imbibing large quantities of water. There are many advantages of using PPC hydrogels for biomaterials including the combination of the best properties of peptides and that of synthetic polymers is noteworthy (Tzokova et al., 2009). The integration of polymers into drug delivery system facilitates the synthesis of reduced size particles. Although a broad range of micro encapsulation techniques have been developed for the preparation of polymeric systems, the selection of suitable technique is also an important step to decide the architectural designs in terms of size and shape of the system. Importantly, the size modification of polymeric system offers various benefits to peptides including stability, selectivity, tissue entrapment and macrophage escaping etc.

3.9. Mechanism of peptide release from polymeric system

The important function of polymeric carriers is to transport the loaded components to the site of action. Protections of peptide from degradation caused by other biological components sequester the active peptide preventing its arrival at the action place. Basically, biodegradable polymers contain labile bonds such as ester-, amide-, and anhydride-bonds that are prone to hydrolysis or enzymatic degradation that results in release of content in desired point. Principally, there are two typical modes of degradation of polymer coat is expected are surface degradation and bulk degradations. In surface-degrading polymer, degradation is confined to the outer surface of the device (Dorati et al., 2007). In a bulk-degrading polymer, the degradation occurs homogeneously throughout the material (Haller, 1980). The degradation of semicrystalline polymers perhaps occur in two stages: (1) The first stage consists of water infusion into the amorphous regions with random hydrolytic scission of labile bonds and (2) The second stage starts when most of the amorphous regions are degraded. Polyester is a class of polymers like PLGA that shows initial burst effect, which may lead to rapid release of surface associated drug molecules. Later, material degradation via chain scission, and the third phase was mainly the result of polymer erosion which leads to the loss of bulk materials (Grizzi et al.,
1995) Some of the biocompatible polymers may be triggered by the environments such as pH, ionic strength, enzyme-substrate, magnetic, thermal, electrical, ultrasound that are categorized as stimuli-triggered biodegradable polymers (Kim et al., 2009). Most of case, the polymer dissolution involves two transport processes, such as solvent diffusion and chain disentanglement. Though, polymer dissolution plays vital role in the scission of polymer chains, it also leads to the loss of bulk material (Fu and John, 2010). There are number of kinetic models, which described the overall release of content from the dosage forms. They basically categorized into three methods such as (1) Statistical methods, (2) Model dependent methods and (3) Model independent methods. In the statistical method, dissolution profile data are represented in both graphical and numerical manner. Statistical methods are also used by considering repeated measure as time and percent dissolved is the dependent variable. Model dependent methods are based on different mathematical functions, which can describe dissolution profile. The model dependent approaches include zero order, first order, higuchi, hixson-crowell, korsmeyer-peppas, baker-lonsdale, weibull, hop-fenberg, gompertz and regression models. Model independent approach uses a difference factor and a similarity factor to compare dissolution profiles (Suvakanta et al., 2010).

3.10. Gelatin as peptide carrier

Gelatin is a natural polymer derived from collagen obtained from various animal by-products. The biocompatible and biodegradable nature of gelatin is highly appreciated in pharmaceutical and medical areas. It contains 18 non-uniformly distributed amino acids with both charges. The inherent cationic property of gelatin is basically due to lysine and arginine residues. Gelatin is basically divided into two main types: Type A, which is derived from collagen by acid pre-treatment with IEP (Isoelectric point) of 7-9 and Type B, which is derived from collagen liming (alkaline process) with IEP of 4.6-5.4. The bonds of gelatin in the form of easily break and make, that will be a unique advantage of gelatin among the other biodegradable polymers. Despite gelatin being one of the polymers recognized for millennia, questions about its structure and functionality are still being discussed today. Gelatin hydrogel has been proven to be an appropriate carrier for the
sustained release of peptides. The W9 peptide inhibits osteoclastogenesis and accelerates bone morphogenetic protein (BMP)-2-induced ectopic bone formation which is efficiently delivered by formulating gelatin hydrogel with W9 peptide (Abdullah et al., 2013). TAT peptide-gelatin nanoparticle-mediated CGRP gene delivery treats cerebral vasospasm after subarachnoid hemorrhage (Tian et al., 2013). The bovine serum albumin was successfully loaded using gelatin, by using two-step desolvation method particles with a mean diameter of 200-300 nm and EE% (Entrapment efficiency) of 87.4% were achieved (Azimi et al., 2014). Microsphere composites were prepared by encapsulating protein-loaded gelatin nanoparticles in PLGA microspheres with the aid of a phase separation method and a solvent extraction method. The protein loading efficiency achieved is 93.2% for the nanoparticle-microsphere composite which was prepared by the phase separation method, while it is 31.31% for the composite prepared by the solvent extraction method (Li et al., 1997).

3.11. Sodium alginate as peptide carrier

Sodium alginate consists of chiefly sodium salt of alginic acid, which is a mixture of polyuronic acids composed of residues of D-mannuronic acid and L-guluronic acid units. These units are interlinked by β-1, 4 and α-1, 4 glycosidic bonds to form a chain. Due to the equatorial-equatorial bonding, mannuropyranosyluronic region looks flat and ribbon-like structure, similar as cellulose conformation. The gulopyranosyluronic acid regions have a wrinkled conformation due to axial-axial glycosidic bonds. Alginates consist of large number of free hydroxyl and carboxyl groups distributed along its backbone which makes it a better candidate for chemical modification (Tripathi and Mishra, 2012). Sodium alginate is widely used in cosmetics, food products, and pharmaceutical formulations, such as tablets and topical products. It is generally regarded as a non-toxic and non-irritant material, although excessive oral consumption may be harmful. Augmented reports displays hydrogel systems used for delivery of proteins and peptides is very effective. Peptide ICD-85 (combination of 3 peptides isolated partially from two different venoms) loaded sodium alginate nanoparticles were prepared and investigated for their ability to inhibit proliferation of cancer cells (Moradhasel et al., 2013). ICD-85 peptide
loaded sodium alginate nanoparticles can reduce its necrotic effect on primary lamb kidney cells (Mirakabadi and Moradhaselis, 2013). Alginate microspheres with narrow size distribution and good sphericity were successfully prepared by shirasu porous glass membrane emulsification technique and a two-step solidification process. Insulin as a model protein was successfully loaded in the microsphere by three methods and reported (Zhang et al., 2011). Subtilsin, BSA, or lysozyme like model proteins was formulated as sodium alginate nanoparticles by using single step operation with spray drying with particle size of few microns in diameter (Erdinc et al., 2007). Caffeine and antioxidant peptides loaded with sodium alginate by spray drying method using calcium chloride as a cross linker, the 4 μm mean sized particles are reported as peptidic nanoparticles that was not released in the simulated gastric medium (Bagheri et al., 2014).

3.12. Chitosan as peptide carrier

Chitosan is a cetylized unit) and N-acetyl-D-glucosamine (acetylated unit). Being obtained by the partial delinear polysaccharide polymer composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylation of chitin). Chitosan is the most abundant basic biopolymer and is structurally similar to cellulose, which is composed of only one monomer of glucose. The solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups in the polymeric chain of chitosan. The biodegradability of chitosan is ensured by the enzymes which are able to hydrolyse glucosamine-glucosamine, glucosamine-N-acetyl-glucosamine and N-acetyl-glucosamine-N-acetyl-glucosamine linkages (Kean and Thanou, 2009). Chitosan is known to be degraded in vertebrates predominantly by lysozyme and by certain bacterial enzymes in the colon (Zoldners et al., 2005).

Chitosan particles have been studied for nasal delivery of proteins (Teijeiro et al., 2009; Wang et al., 2009; Mahkam et al., 2010). It was noticed earlier that insulin-loaded chitosan nanoparticles enhanced nasal absorption of proteins to a greater extent than relevant chitosan solutions. Insulin-loaded nanoparticles were made by spray-drying a mannitol/lactose solution to yield~1-3 μ powders for alveolar deposition (Grenha et al., 2007; Zhang et al., 2008). An inhalable chitosan based
powder formulation of salmon calcitonin containing mannitol was prepared by using spray-drying method. The dissolution rate of the protein decreased when formulated with chitosan, which might be due to irreversible complex formation between the protein and chitosan during the drying process (Yang et al., 2007). The nanocarrier system which can transfer chitosan nanoparticles loaded with either small peptides such as the caspase inhibitor Z-DEVD-FMK or a large peptide like basic fibroblast growth factor across the blood brain barrier was prepared and it is demonstrated that this nanomedicine formulation is rapidly transported across the blood brain barrier (Caban et al., 2012). Human parathyroid hormone 1-34 (PTH 1-34) used to treat osteoporosis was loaded into chitosan nanoparticles. Further, PEGylated (PEG-CS-PTH NPs) aggregates of 200-250 nm which in turn comprised of 20 nm individual nanoparticles were obtained and evaluated for in vitro and in vivo (Narayanan et al., 2013). The release of silk peptide occurred as an initial burst followed by prolonged release up to 10 days. These nanospheres were suggested for pH dependent. Chitosan based nanocomplexes were prepared by ionic cross-linking with TPP in different acidic media under mild conditions. The self-assembly and ionic interactions of chitosan and TPP were affected by reaction media; chitosan-based nanofibers could be obtained in adipic acid medium while nanoparticles were formed in acetic acid (Hu et al., 2002). Chitosan hydrogel nanoparticles loaded with VEGF peptides (81-91 fragments) was demonstrated for individualized treatment of myocardial ischemia (Hwang et al., 2014).

3.13. Nanoparticles for peptide delivery in cancer tissue

Peptide therapeutics are well admired candidates for anticancer target because of advantages like small molecular size, synthesis and modification process simplicity, tumor penetrating potential, and biocompatibility. Peptides also demonstrate some critical drawbacks, such as unfavorable short plasma half-life, poor oral bioavailability and different administration routes, which could affect the peptides pharmacokinetics and biological activities (Borghouts et al., 2005). Nanoparticles possess higher ability in delivery of proteins and nucleic acids principally into tumor cells and moreover it can preferentially accrue in the tumor tissues greater than they do in normal tissues, this phenomenon is an significant driving force for cancer targeted drug delivery (Grant and Leone, 2012). Under pathological conditions,
leakiness of tumor vasculature is a positive prospect for facilitating tissue penetration of nanoparticles; furthermore peptide based nanoparticles serves to the peptides for their endosomal escape (Matsumura and Maeda, 1986). Hence, nano carriers provide greater improvement for the peptides to conquer above mentioned drawbacks. The selection of biocompatible polymer is an imperative step in delivery of peptides in effective manner. Principally they yield a constant release still with a simple monolithic device; the slow matrix degradation can retain the level of peptides in plasma, thereby increase the permeability of peptides in tissues.

Figure 2. Nanoparticles delivery in cancer tissue. The smaller size of the nanoparticles possesses extreme thermal energy and it can rapidly accumulate into the tumor site. The loosely arranged cells of tumor enable the vascular leaking. Hence, the particles perhaps allowed to move up to tumor core. In the cytosol the loaded peptides prevented from cytosolic enzymes and peptides may enter into the nucleus via nuclear pores.


Currently available animal models for human breast cancer may be categorized into three main groups: (a) Xenograft models; (b) Chemically induced, virally induced or ionizing radiation-induced models; and (c) Genetically engineered animals (GEM) such as transgenics and knockouts. More complex models rely on a combination of particular methodologies used to generate these three main types of mammary cancer model (Laurince and Key, 2005). The mammary tumors in rats arise in the epithelium of the terminal end buds, which are comparable structures to the terminal ductal lobular
units in the human breast (Grubbs et al., 1986). Among the three different mode of induction of carcinogenesis chemical induced animal models are widely accepted for the pharmacological research purpose. Mammary glands of several rat strains, mainly Sprague-Dawley and Wistar-Furth, are susceptible to transformation induced by chemical carcinogens, and the 2 most widely used active chemical inductors of mammary carcinogenesis are 7, 12-dimethylbenz (a) anthracene (DMBA) and N-methylnitrosurea (Russo and Russo, 1996; Dias et al., 2000). Among this DMBA is highly lipophilic compound and requires metabolic activation for its carcinogenicity. Several tissues are capable of activating DMBA, and these include the mammary gland. In the breast, DMBA is converted to epoxides, active metabolites with a capacity for damaging the DNA molecule, the main event in carcinogenesis initiation. With the higher cellular proliferative index of types 1 and 2 lobules, there is higher metabolic activity and more epoxide formation. Thus the reason till now DMBA induced mammary carcinoma models were widely used in the biomedical research (Clarke. 1997; Russo et al., 2003; Balogh et al., 2003).

![Figure 3. Mechanism of DMBA carcinogen. Mammary carcinomas induced in rats by means of chemical carcinogens, mainly DMBA, seem to provide a good model to evaluate the therapy against cancer. Further the tumor resulted by DMBA administration bears a close resemblance to human cancer in their histologic and hormone response pattern. The mechanism of DMBA as a carcinogen is metabolically activated to its ultimate carcinogenic metabolite, dihydrodiol epoxide, which subsequently bind to adenine and guanine residues of DNA and form adducts, contributing to carcinogenesis.](image-url)
4. Materials and methods

4.1. Computational feature

All in silico analysis were performed using HP Workstation Z220 with Next generation 22 nm processors, including Intel Xeon processor E3-1200v2 family with 16GB RAM, 1TB Hard disk, NVIDIA Quadro 2000, Windows 7 Ultimate 64 bit. Accelry’s Discovery Studio 3.5 (AD 3.5), CLC Genomic Workbench 5.1 and Biosolve IT softwares were used for in silico analysis.

4.2. Materials

Peptide pACC1 was purchased from BioConcept Labs (Delhi, India), Gelatin was obtained from Central Drug House (Delhi, India), sodium alginate, chitosan (Medium molecular weight), sodium tripolyphosphate and HPLC grade chemicals used for HPLC analysis were procured from Sigma Aldrich (USA). RPMI-1640 medium and fetal bovine serum (FBS) was purchased from GIBCO (USA). Trypsin, Methylthiazolylldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals, Mumbai. Anti-IKKα, anti-IKKβ, anti-NFkB, anti-ACC1, anti-FASN, anti-Bcl2, anti-Bax, anti-p53, anti-Caspase-3, anti-PRAP and anti-β-Actin were obtained from cell signaling (USA). Secondary antibodies of respective primary antibodies and plasmid DNA (pUC19) were purchased from Genei, Bangalore, India. All other chemicals used were of analytical grade.

4.3. Identification of Target Protein

The crystal structure of the peptide target of BRCT domain was salvaged from RCSB Protein Data Bank [PDB ID-1VKX] (http://www.rcsb.org/pdb).

4.4. Protein-Protein docking

4.4.1. Selection of peptide

Four well known peptides for the target of BRCA1 without bearing in mind the primitive future mechanism were selected as follows.
4.4.1.1. MDC1

The MDC1 peptide holds phosphoserine which smooth the progress of interaction with BRCT domain. The MDC1-BRCT binds pSer-Gln-Glu-Tyr-COO(−) at the C-terminus of the histone variant gamma H2AX via direct recognition of the C-Terminal carboxylate, While BRCA1 recognizes pSer-X-X-Phe motifs either at C-terminal or internal sites within target proteins may assist protein signaling at DNA damage foci through specific interaction with serine-phosphorylated protein partner (Campbell et al., 2010).

4.4.1.2. BATT

The BRCTs of BRCA1 also believed to interact with BAAT1 peptide. As the result, phosphorylation of Ser239 in human BRCA1 is recognized specifically by BRCT domains, whereas a S239A substitution abrogates the BRCA1 binding to BATT1 and leads to G2-M checkpoint defect, indicating that this interaction is essential for the ATR function in checkpoint control (Liu and Ladias, 2013).

4.4.1.3. ATRIP

The BRCA1-Complex is composed of CtIP and the MRN complex, and is formed in a cell cycle-dependent manner during S and G2 phases of the cell cycle. BRCA1-C is involved in DNA end resection to generate ssDNA needed for HR-mediated DNA repair mechanism. In addition to these well-studied complexes, BRCTs also interact with ATRIP (PEACpSPQFG) may possess vital role in cell cycle check point (Liu and Ladias, 2013).

4.4.1.4. pACC1

The earlier reports by Shen and Tong (2008) suggested the crystal structure at 3.2 Å resolutions of human BRCT domains in complex with a phospho-peptide from human, which provide a molecular evidence for direct interactions between BRCA1 and ACC1. The pACC1 peptide is bound in an extended conformation, located in a groove between the tandem BRCT domains.
4.5. Protein docking using ZDOCK in Accelrys Discovery Studio 3.5

ZDOCK is a rigid body protein-protein docking algorithm based on Fast Fourier Transform correlation technique, which is used to explore the rotational and translational space of a protein-protein system (Chen et al., 2003). Here the crystal structure of BRCT domain used as a receptor protein and peptides as a ligand protein was subjected to dock in ZDOCK to calculate the binding energy.

4.6. Toxicity prediction

The toxicity analysis were performed by using ToxinPred, an in silico method using QMS calculator which allows the users to submit query peptide in FASTA format and to optimize the peptide sequence to get maximum/minimum/desired toxicity is principally upon the Quantitative Matrix based position specific scores. It will also help the user to tweak any residue from the predecessor peptide to attain the analog with desired property (highest/lowest toxicity) (Gupta et al., 2013).

4.7. Purity analysis of peptide pACC1

Mass spectrometric analysis of pACC1 peptide was performed (Isotopx, PhonixIMS, UK) and the data were analyzed using Flex Analysis software. Obtained spectra was tested for purity using ‘X! Tandem’ software.

4.8. Genotoxicity testing for pACC1 peptide

Plasmid nicking assay was carried out by the method of Kitts et al., (2000). Briefly, the pUC19 (2.5 ng in 10 mMTris-HCl Buffer, pH 7.5) was added to peptide pACC1 (20, 40, 80 ng) in a microfuge tube. The tube containing 2.5 µl of pUC19 were mixed with 215 µM of H2O2 which served as negative control (Chaudhary et al., 2005) and incubated for 2 hrs. Further, samples were electrophoresed in 0.8% (w/v) agarose gel for 40 min and viewed under UV transilluminator. The images were analyzed with Phoretix 1D and ImageJ software for construal.

4.9. Hemolytic assay for peptide pACC1

Hemolytic activity was evaluated as described by Andra et al., (2008) with slight modifications. Human erythrocytes were washed (until the supernatant become
colorless) with PBS by centrifugation at 8000 ×g for 10 min, then the erythrocytes were re-suspended and diluted to 10 times of the original volume with PBS kept as stock. Then, 150 µl of peptide pACC1 (2-16 µg/mL) in PBS was incubated with 150 µl of stock erythrocyte suspension (4% v/v) for 60 min at 37 °C. After the incubation period, the reaction mixtures were centrifuged at 1,000 ×g for 10 min to remove intact erythrocytes. The supernatant of released hemoglobin was measured at 540 nm using a microplate reader. The experiments were performed in triplicates and the haemolytic activity was expressed as a percentage of haemolysis using the following equation.

\[
\% \text{ of Haemolysis} = \left( \frac{(\text{Abs sample} - \text{Abs buffer})}{(\text{Abs maximum} - \text{Abs buffer})} \right) \times 100
\]

Abs sample: RBC with peptide solution in PBS. Abs buffer’s: Red blood cells in PBS ‘Absmax’: red blood cells with 1% (v/v), TritonX-100 in PBS. No haemolysis (0%) and full haemolysis (100%) were observed in the presence of PBS and Triton X-100 (1% v/v) respectively. Finally the IC₅₀ value was calculated by using PRISM version-5.0 software.

4.10. HPLC method development for peptide pACC1

Mobile phase A: 1% (v/v) of Triflouro acetic acid (TFA) in water and Mobile phase B: 0.1% (v/v) of TFA in 80:20 ratios of Acetonitrile and water were used. The flow rate was set at 1.0 mL/min and the wavelength at 220 nm. The injection volume was kept as 20 µl the sample solution was prepared by accurately weighed 5 mg peptide pACC1 in 5 mL of ultrapure water in a volumetric flask.

The analytical validation parameters were selected according to the “Validation of Analytical Procedures (Q2, R1) of ICH Harmonized Tripartite Guideline (ICH 2005). Analytical validation parameters were accuracy, precision (repeatability and reproducibility) and specificity, sensitivity (limit of detection, LOD; limit of quantification, LOQ) and linearity.

4.10.1. Accuracy

Three different concentrations of peptide pACC1 including high, medium and low concentrations were used for determination of accuracy. Accuracy was expressed as
the percentage of coefficient of variation (% CV). All unknown values was found for the three different concentrations of peptide were lower than 2 % (n=3).

4.10.2. Precision

The precision structure was evaluated in stipulations of repeatability and reproducibility. For injection repeatability, six analyses of one concentration were performed in the same sample. For system repeatability and reproducibility, analysis of six diverse samples of one concentration was performed in the same day and 2 different days.

4.10.3. Sensitivity

Sensitivity was calculated based on the residual standard deviation (RSD) of the retort and the slope. Average values of six calibration curves were used for this purpose. The LOD and LOQ were calculated by using following equations.

\[ \text{LOD} = 4.1 \times (\text{SD/Slope}) \]  
\[ \text{LOQ} = 10 \times (\text{SD/Slope}) \]

4.10.4. Linearity

The retention time (t\text{R}) was 13.99517 min at 220 nm. The Mobile phase A 1% (v/v) of TFA in water and Mobile phase B 0.1% (v/v) of TFA in 80:20 ratio of acetonitrile and water solvent system. The calibration curve was prepared using 5-35 µg/mL concentrations of peptide pACC1 and the average values (n=6) were used to prepare the calibration curve.

4.11. Preparation of nanoparticles

With an objective to evaluate the polymer peptide compatibility, a pilot study was carried out with three peptide compatibles.

4.11.1. pACC1-Chitosan nanoparticles

Chitosan peptide nanoparticles (PCN) were prepared by ionic cross linking of chitosan with sodium tripolyphosphate (TPP) anions (Haliza et al., 2012). Chitosan
(2 mg/mL) was dissolved in acetic acid (0.25% v/v) with constant stirring at 10 °C (12 hrs). Aqueous solution of TPP (0.75% w/v) was added (2:1 ratio) into chitosan solution containing 100 μg/mL of pACC1 peptide (Previously dissolved with HPLC grade water) with continuous stirring for 6 hrs (4 °C). The resultant dispersion was centrifuged at 13000 ×g for 20 min at 4 °C. The supernatant was then removed and Peptide Chitosan Nanoparticles (PCN) were collected and freeze-dried (-55 °C).

4.11.2. pACC1-gelatin nanoparticles

Gelatin peptide nanoparticles (GPN) were prepared by two-step desolvation method with slight modifications (Azimi et al., 2014; Ofokansi et al., 2010). Briefly, gelatin (100 mg) was dissolved in distilled water (5 mL) under constant heating at 37 °C and adjusted to pH 4. Peptide pACC1 (1 mg) was added into the gelatin solution and acetone (12 mL) was added. Further, an aqueous solution of glutaraldehyde (25% v/v) was added as a cross-linking agent and the solution was stirred continuously for 6 hrs at 4 °C. The resultant was sonicated (sonitvibra cell, UC130, USA. amp-20, pulser-5 sec) and centrifuged (13000 ×g) for 30 min at 4 °C. The particles were washed thrice (3000 ×g) with distilled water and freeze-dried.

4.11.3. pACC1-sodium alginate nanoparticles

Sodium alginate peptide nanoparticles (SPN) were prepared by controlled ionic gelation method (Catarina et al., 2006; Saeed et al., 2013). Briefly, 2 mL of calcium chloride (18 mM) was added to 38 mL of sodium alginate solution (0.1% w/v) to promote gelation. Ten milliliters of peptide pACC1 (0.1% w/v) was added to calcium alginate solution to form nanoparticles. The suspension was stirred for 2 hrs and kept overnight for stabilization. The nanoparticles subjected to centrifugation (13000 ×g) for 45 min at 4 °C.

4.12. Peptide polymer compatibility study

4.12.1. FT-IR analysis

The pellets of peptide and potassium bromide were prepared by compressing the powder at 20 psi for 1 min on KBr press and the spectra were recorded in the wave...
carried on peptide pACC1, pure polymers (Chitosan, sodium alginate and gelatin), formulations containing both peptide and polymers were performed to study the peptide pACC1 and polymer interaction.

4.12.2. X-ray diffraction studies

X-ray diffraction studies were performed for chitosan and PCN to confirm the non-existence of crystalline structure. XRD studies were performed on the samples by exposing them to Cuk α1 radiation (40 KV, 30 MA) and the scanning rate was 5°/min over a range of 4-90° and with an interval of 0.1°.

4.13. Characterization of PCN

The Chitosan nanoparticles were formulated by keeping constant peptide pACC1 concentration at different ratios of chitosan. Different concentrations of chitosan (0.1, 0.2, 0.3, 0.4, and 0.5% (w/v)) were obtained by dissolving in acetic acid (0.25% v/v) under constant stirring (250 rpm) for overnight. Twenty milliliter of TPP 0.75% (w/v) was added into 10 mL chitosan solution containing 0.20 mg of peptide pACC1 and stirring was raised to 320 rpm for 6 hrs at 4 °C. The resultant dispersion was subjected to sonication (Sonitvibra cell, UC130, USA. Amplitude-20, Pulser-4 sec) for 10 min at 4 °C. Further, the dispersion was washed thrice (2200 ×g) and re-dispersed in HPLC grade water (LiChrosolv) and again centrifuged at 13000 ×g for 20 min at 4 °C to obtain five different peptide polymer ratio formulations (PCN-J01 (1:0.5), PCN-J02 (1:1), PCN-J03 (1:1.5), PCN-J04 (1:2) and PCN-J05 (1:2.5)) Finally, the nanoparticles were freeze dried (Delvac-lyo1550, INDIA) and stored at -20 °C for further characterization.

4.13.1. Process yield

The freeze dried formulations (PCN-J01 to PCN-J05) were weighed in order to calculate the process yield as previously described by Nesalin and Smith (2012). The results were expressed as mean values of three replicates.
4.13.2. Association and Loading efficiency

Association Efficiency (AE) and Loading Efficiency (LE) were calculated based on previous methods with slight modification (Ma et al., 2002). Briefly, PCN were pelletized at 5800 xg and supernatant was quantified for peptide by HPLC method which was described earlier. The formulae mentioned below were used to calculate AE and LE.

\[
AE = \frac{\text{Total amount of peptide} - \text{Peptide in supernatant}}{\text{Total amount of peptide}} \times 100
\]

\[
LE = \frac{\text{Total amount of peptide} - \text{peptide in supernatant}}{\text{Weight of recovered nanoparticles}} \times 100
\]

4.13.3. In vitro peptide release

In vitro release of formulations (PCN-J01 to PCN-J05) was determined by plasma simulation (Narayanan et al., 2013) and dialysis membrane method (Hu et al., 2002) with slight modifications. Briefly, the formulations was redispersed in 10 mL of NaCl (0.9% w/v) with final peptide concentration of 20 μg/mL. One milliliter of the above mentioned mixture was added to 10 mL of PBS (0.5M, pH 7.4) and 10 mL of suspended blood cells were added. The suspension was kept in orbital shaker at 37 °C. One milliliter of released solution was collected at different time intervals and replaced with fresh solution. The harvested solutions were centrifuged and supernatant was used to analyze peptide content using HPLC method. The release rate was calculated using following equation.

\[
\text{Peptide release}\% = \frac{\text{Amount of Peptide released at time } t}{\text{Amount of Peptide loaded in the nanoformulation}} \times 100
\]

In dialysis membrane method, 2 mg of formulations (PCN-J01 to PCN-J05) was redispersed in 10 mL of PBS (0.5M pH 7.4), placed in dialysis membrane (cut-off 10 kDa) and dialyzed against PBS (0.5M pH 7.4). Two milliliter of released solution was collected at different time intervals, replaced with fresh solution and analyzed using HPLC by calibration curve method.
4.13.4. Size distribution and zeta potentials

The size distribution and zeta potentials of formulations (PCN-J01 to PCN-J05) were measured by Zeta Sizer (Malvern, Mastersizer 2000, UK). Sample was dispersed in water (pH 7.4) and the particles were counted in 4.8 mm calibrated area with the count rate of 210.3 Kcps (kilo counts per second) for 70 sec. The analysis was performed in triplicates and mean values were reported.

4.13.5. Kinetic modeling

In order to determine the suitable drug release kinetic model describing the mechanism dissolution profile, the result of in vitro release obtained from dialysis membrane and plasma simulation methods were fitted with various kinetic equations such as zero order, first order, Higuchi’s model and Peppas plot methods using following equations.

i) Zero order release rate kinetics

To study the zero-order release kinetics the release rate data were fitted to the following equation.

\[ F = Kt \]

Where ‘F’ is the drug release, ‘K’ is the release rate constant and ‘t’ is the release time. The plot of % drug release versus time is linear.

ii) First order release rate kinetics

The release rate data were fitted to the following equation

\[ \log (100-F) = kt \]

(A plot of log % drug release versus time is linear)

iii) Higuchi release model

To study the Higuchi release kinetics, the release rate data were fitted to the following equation.

\[ F = k \sqrt{t} \]

Where ‘k’ is the Higuchi constant

In higuchi model, a plot of % drug release versus square root of time is linear.
iv) Korsmeyer and Peppas release model

The release rate data were fitted to the following equation,

\[ \frac{M_t}{M_\infty} = K t^n \]

Where, \( \frac{M_t}{M_\infty} \) is the fraction of drug released, ‘K’ is the release constant and ‘t’ is the release time.

‘n’ is diffusion exponent, if ‘n’ is equal to 1.0, the release is zero order.

If ‘n’ is equal to 0.5 the release is best explained by Fickian diffusion, and if 0.5 < n < 1.0 then the release is through anomalous diffusion or non-fickian diffusion (Swellable and Cylindrical Matrix). In this model, a plot of \( \log \left( \frac{M_t}{M_\infty} \right) \) versus \( \log \) (time) is linear.

Table: 1 Interpretation of diffusional release mechanisms

<table>
<thead>
<tr>
<th>Diffusion exponent (n)</th>
<th>Drug transport mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Fickian diffusion</td>
</tr>
<tr>
<td>0.5&lt;n&lt;1.0</td>
<td>Anomalous (Non-Fickian) diffusion</td>
</tr>
<tr>
<td>1.0</td>
<td>Case II transport</td>
</tr>
<tr>
<td>n&gt;1.0</td>
<td>Super Case II transport</td>
</tr>
</tbody>
</table>

4.13.6. Surface morphology

Scanning Electron Microscopy (SEM) (TESCAN, VEGA3 SBU, Czech) of the chitosan nanoparticles were performed to observe the particle size and surface morphology. The size and shape of the nanoparticles were further characterized using the Transmission Electron Microscope (TEM) (Jeol, JEM2100, Japan).

4.13.7. Plasma Stability of PCN-J04

Fresh blood (Wistar rat) was collected in heparinized tubes and plasma was separated by centrifugation (1800 xg for 15 min at 4 °C). Separated plasma was incubated at 37 °C for 30 min in 0.9% (w/v) of NaCl solution and PCN-J04 (10 μg) was added. Plasma solutions (1 mL) were collected at regular intervals and stored at
1

TPP Addition

Formulated PCN

Freeze-drying

In vitro Release Analysis by Dialysis Membrane Method
-20 °C until use. The peptide pACC1 content was analyzed by HPLC after centrifugation (17000 xg) at 20 min.

4.13.8. Hemocompatibility of PCN-J04

Hemocompatibility was evaluated as previously reported by Pooja et al., (2014). Briefly, whole blood was collected from a wistar rats and anti-coagulated with sodium citrate (ratio of blood to anticoagulant taken was 9:1). Erythrocytes were isolated by centrifuging whole blood at 1000 xg for 10 min. The erythrocytes were washed thrice with saline before use. PCN-J04 were mixed with RBCs in different concentrations (2-24 μg/mL) and then incubated for 2 hrs at 37 °C and the supernatant was collected by centrifugation at 1500 xg for 5 min. Hemoglobin release was monitored spectrophotometrically (Systronics, 2203, INDIA) at 541 nm. TritonX-100 (1% v/v) and 0.9% (w/v) NaCl were taken as positive and negative controls, respectively.

The hemolysis rate was calculated as follows

\[
\% \text{ hemolysis} = \frac{(\text{OD}_{\text{test}} - \text{OD}_{\text{neg}})}{(\text{OD}_{\text{pos}} - \text{OD}_{\text{neg}})} \times 100
\]

Where \(\text{OD}_{\text{test}}\), \(\text{OD}_{\text{neg}}\), and \(\text{OD}_{\text{pos}}\) are the absorbance of the test sample, negative control, and positive control, respectively. Triplicate measurements were performed for each sample.

4.13.9. Embryo toxicity of PCN-J04

The Danio rerio was maintained as female/male (2:1 ratio) in 23×30×23 cm glass aquarium at an average temperature of 26 ± 1 °C and 14/10 hrs light dark cycle (Zhu et al., 2008). The spawning was goaded in the morning 6-7 (+5.30 GMT). After 30 min, the embryos were collected and maintained in E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl and 0.33 mM MgSO₄). Healthy embryos (n=96) were collected and exposed to PCN-J04, 4 -5 hours post fertilization (hpf). Each well contained 12 embryos with different concentrations of PCN-J04 (1, 3, 10, 30, 100, 300, 1000 mg/L). Throughout the experiment, the developmental status of the Danio rerio embryos and larvae was observed under compound microscope (Olympus, MLXi, Japan) at different time intervals. The toxicity was assessed with multiple
parameters such as embryo/larva survival, hatching rate and malformations including tissue ulceration, pericardial edema and body arcuation.

4.13.10. Genotoxicity of PCN-J04

The genotoxicity studies for PCN-J04 were carried out by using plasmid pUC19 (Kitts et al., 2000). The PCN-J04 (20 and 40 ng) were suspended in double distilled water and added to 3 µl of pUC19 (1 ng) plasmid and the reaction mixture were run on 0.8% agarose gel for 30 min. Instead of PCN-J04, H2O2 (215 µM) served as positive control. The results were analyzed using ImageJ software (Version-1.47, USA).


The human breast cancer cell lines (MCF-7) was purchased from National Center for Cell Sciences (NCCS) Pune, INDIA. Cell line was grown as monolayer cultures maintained in Dulbecco’s modified Eagle’s medium (GIBCO BRL) supplemented with heat inactivated 10% Fetal bovine serum (GIBCO BRL) and 2 mM L-glutamine (Sigma, USA), 100 units/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C in atmosphere of 5% CO2 incubator at 95% air humidified.

4.14.2. Preparation of tests

The test solutions of PCN-J04 and TOFA (5-Tetradecyloxy-2-furonic acid) was prepared in DMSO and stored at -20 °C until use. The concentrations used for the study was freshly prepared for each experiment with a final DMSO concentration of 0.1% (v/v). All the subsequent experiments were performed as minimum of three replicates.

4.14.3. Assessment of cell viability

Cell viability was analyzed using conventional MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphe-nyl tetrazolium bromide) reduction assay. Cells were treated with PCN-J04 and TOFA. The viability was assessed based on the detection of mitochondrial dehydrogenase activity in viable cells (Mosmann, 1983).
Principle

The MTT assay is measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Regents

- MTT (5 mg/mL in PBS) filter and keep dark, prepare freshly
- Acidic isopropanol (0.1N HCl in absolute isopropanol)
- 96-well plate (flat bottom)

Procedure

MCF-7 cells were cultured in 96 well plates approximately (3× 10^3 cells/well) plated in each well and incubated for 24 hrs till attains 90% confluence. The media was replaced with an equal amount of fresh media containing different concentrations of PCN-J04 and TOFA (suspended previously with DMSO). After 24 hrs of incubation, MTT solution was added to the plate at a final concentration of 5 mg/mL and incubated for 4 hrs in dark at 37 °C. After incubation, the medium was removed and the cells were suspended with DMSO (200 μl). The absorbance of formazan reduction product was calculated by measuring optical density at 570 using reference wavelength of 650 nm in ELISA reader (Bio-Rad, Hercules, CA, USA). The obtained IC_{50} concentrations were used for subsequent experiments.

4.14.3. Measurement of intracellular ROS formation

The intensity of intracellular peroxides was quantified by loading the cells with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). The esterified fluorescent probe penetrated into the intracellular matrix of the cells, reacts with ROS and oxidized to form fluorescent dichlorofluorescein (DCF). Intracellular ROS content was measured by following the method of Halliwell and Whiteman (2004).
Reagents

- Phosphate buffered saline (PBS)
- 10 μM 2,7-diacetyl dichlorofluoresceindiacetate (DCFH-DA)

Procedure

Treated and untreated cells (3x 10^3 cells/mL) were incubated with 100 μl of DCFH-DA (10 μM) for 30 min at 37 °C. Cells were rinsed twice with PBS and photographed on fluorescent microscope (450-490 nm; blue filter, Nikon, Eclipse TS100, Japan). Fluorescence estimations were done with excitation and emission filters set at 485 ± 10 and 530 ± 12.5 nm respectively using spectrofluorometer (Shimadzu RF-5301 PC). Initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were calculated using the formula [(Ft30-Ft0)/(Ft0X100)] and the values are expressed in percentage increase in fluorescence intensities at 0(Ft0) and 30(Ft30) min.

4.14.4. Apoptosis analysis using dual staining

Fluorescent probes acridine orange (AO) and ethidium bromide (EB) were used to analyze apoptosis by fluorescence microscope. Acridine orange, a permeable dye stains all the cells and ethidium bromide, a non-permeable dye enters into the cell only when cell membrane disintegrates and it intercalates with DNA forming an orange-red complex. Apoptosis assay was performed by following the method of Kasibhatla et al., (2006).

Reagents

- PBS
- Acridine orange (AO) 3 mg/mL in ethanol
- Ethidium bromide (EB) 5 mg/mL in ethanol

Procedure

After treatments, medium was decanted from the 6 well plates; cells were washed with PBS twice and stained with AO and EB (1:1). These cells were incubated for 20 min at room temperature and washed with warm PBS to remove excess dye.
Cellular morphology was observed using fluorescent microscope (λEx/λEm=490/530 nm) and photographed. Fluorescent intensity was measured at 535 nm using spectrofluorometer.

4.14.5. DNA fragmentation assay

DNA fragmentation assay was carried out for TOFA and PCN-J04 samples to substantiate the apoptosis cascades which results from treatment by confirming oligonucleosomal fragments.

Principle

This protocol provides a qualitative method for assessing cell death by detecting DNA fragments using agarose gel electrophoresis. One of the classic features of apoptosis is the cleavage of the genomic DNA into oligonucleosomal fragments represented by multiples of 180-200 bp. Visualizing these fragments can aid in characterizing an apoptotic event. This method should always be combined with more quantitative methods in order to compare the degree of apoptosis among the experimental samples.

Reagents

- Lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 0.2% Triton X-100, pH 7.5)
- TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5)
- Saturated phenol
- Chloroform
- Isoamyl alcohol

Procedure

MCF-7 cells (1X10^6) were plated in 6 well culture plates. When the cells reached 70% confluency, the IC_{50} concentration of TOFA and PCN-J04 were added and the cells were incubated for 3, 6 and 12 hrs. After inhibition, cells were harvested and pelleted by centrifugation. The harvested cells were washed twice with ice cold PBS. The cell pellet was lysed with ice cold lysis buffer (10 min) and centrifuged (13000 ×g) for 10 min at 4 °C. Then, the supernatant (containing RNA and
fragmented DNA, but not intact chromatin) was extracted first with phenol and then with phenol:chloroform:isoamyl alcohol (24:25:1) as described by Sambrook and Russell (2001). The aqueous phase was brought to 300 mM NaCl and DNA was precipitated with 2 volume of ethanol. The pellet was washed with 70% ethanol, air-dried, and then dissolved in 20 μl of TE buffer. Following digestion of RNA with RNase A (0.6 mg/mL, at 37 °C for 30 min), The DNA samples was be obtained were electrophorized by 2% agarose gel 50V. The DNA ladders were visualized and documented (Alpha image viewer).

4.14.6. Flowcytometric analysis

To investigate the effect of PCN-J04 and TOFA on the cell cycle distribution, MCF-7 cells (1×10^5 cells/mL) were treated with IC_{50} concentrations of PCN-J04 and TOFA. The cells were harvested in 3, 6, and 24 hrs intervals and washed with PBS and fixed in 75% (v/v) ice cold ethanol at kept 4 °C till use. After washing twice with cold PBS, cells were suspended in PBS containing 40 μg/mLpropidium iodide (PI) and 0.1mg/mL RNase A followed by shaking at 37 °C for 30 min Krishan and Rapid (1975). Cells (10000) were analysed by flowcytometry (Becton-Dickinson San Jose, USA) on the FL2-A detectors and the data were consequently calculated using Win MDI 2.9 software (TSRI, La Jolla, USA) to determine the percentage of cells in apoptosis, G0/G1 phase, S phase and G2/M phase.

4.14.7. Preparation of protein extract

After treatment, cells were lysed in 100 mL of ice-cold radio immunoprecipitation assay (RIPA) buffer (1% Triton-X 100, 0.1% SDS, 0.5% deoxycholate, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF) followed by centrifugation at 10000 xg at 4 °C for 10 min to remove insoluble material. The supernatant was used for estimation of protein and western blot experiments.


Protein in the cells/tissue extract was determined by the method of Lowry et al., (1951).
Principle

Blue purple color complex will be produced by phenolic group of tyrosine and tryptophan residues in a protein with absorption at 660 nm in presence of Folin’s reagent which consists of sodium tungstate molybdate and phosphate. Thus, the intensity of the color formed depends upon the amount of protein present.

Reagents

- Alkaline copper reagent:
  - Reagent A: 2% (w/v) sodium carbonate in 0.1 N NaOH
  - Reagent B: 0.5% (w/v) copper sulphate
  - Reagent C: 1% (w/v) sodium potassium tartarate. 50 mL of reagent A was mixed with 0.5 mL of reagent B plus 0.5 mL of reagent C just before use.
- Folin’s Ciocalteu reagent (FCR): The commercial reagent was diluted in the ratio of 1:2 with distilled water.
- Stock standard: 1 mg of bovine serum albumin/1 mL of water.
- TCA 10% (w/v).

Procedure

To 0.5 mL of crude protein extract, equal volume of TCA was added and centrifuged (1000 xg, 4 °C) for 10 min and precipitate was dissolved in 1 mL of 0.1 N NaOH. To this aliquot, 4.5 mL of alkaline copper reagent was added and allowed to stand for 10 min under dark at 37 °C. To this mixture, 0.5 mL of FCR was added and the blue color developed was read at 640 nm after 20 min. A standard curve was obtained with BSA and was used to estimate the tissue protein level values are expressed as mg/dl.

4.14.9. Western blot analysis

Principle

Western blot analysis was used to identify specific proteins from complex tissue homogenate/cells. The proteins were separated based on the size/charge and
transferred to a solid support (PVDF/Nitrocellulose membranes). Following transfer, the protein of interest can be detected by incubation of the membrane with antibodies (primary) specific to the target protein followed by detection with an enzymatically labeled secondary antibody by adding an appropriate substrate.

Regents

- Stacking buffer (Tris-HCl 0.5M, pH 6.8)
- Separating buffer (Tris base 1.5M, pH 8.8)
- Transfer buffer (Tris base 25 mM, glycine 192 mM and methanol 20% v/v)
- PBST (NaCl 137 mM, KCl 2.7 mM, Na2HPO4 10mM, KH2PO4 1.8mM + Tween20 0.1% v/v)
- Developing solution (10 μl of 30% v/v H2O2+10 mL of 0.05% w/v 3,3’-Diamino benzidine (DAB) in PBS)

Procedure

Proteins were electrophoresed in 10% SDS-polyacrylamide gels and electro transferred to PVDF membrane by semi-dry transfer (Medox, Trans-Blot® INDIA, 0.8 mA/cm²) with aid of transfer buffer. After blocking of membrane with 5% (w/v) non-fat dry milk in PBST at 4 °C for overnight, blots were incubated with primary antibody against Bcl-2, Bax, caspase-3, p53, PRAP, IKKa, IKKβ, NF-κB, FASN, ACC1 at a dilution of 1:5000 and β-actin at 1:2000 overnight at 4 °C. After washing thrice with PBST, the membranes were incubated with anti-rabbit HRP conjugated secondary antibody (1:10000) the immunoreactive bands were visualised by the addition of DAB as substrate. Densitometry analysis was done using ‘ImageJ’ software.

4.14.10. Gene expression analysis

Principle

The reverse transcriptase-polymerase chain reaction is one of the most powerful techniques in molecular biology. RT-PCR selectively amplifies the first strand of cDNA that has been synthesized in vitro by RNA polymerase from mRNA templates by reverse transcription.
Materials and methods

Procedure

Total RNA was extracted from MCF-7 cells using Trizol reagent (GeNei, India). Purity and concentration of RNAs were ensured spectrophotometrically. One microgram of total RNA from each sample was reverse transcribed (RT) to cDNA according to the manufacturer’s protocol (AuPreP TM Gold cDNA Synthesis Kit, Life Technologies Pvt. Ltd., INDIA). Polymerase chain reaction (PCR) was performed with PCR ready mix (GeNet Bio, Korea) according to the manufacturer’s instruction. The sequences of the sense and anti-sense primers for ACC1 (Forward 5'-CCGGCAAGTTGGTTCCG-3' Reverse 5'-ACTTCCACAAACCAGCGTCT-3') FASN (Forward 5'-TCTACACCACCATCCTGAACAAA-3' Reverse 5'-AGCCAAAGGATGATGCC-3') and β-Actin (Forward 5'-TGTTACCAACTGGGACGACA-3' Reverse 5'-ACATCTGCTGGAAGGTGGAC-3') were designed by using primer 3 software. The experimental conditions used for RT-PCR. The RT-PCR products were resolved in 2.5 % agarose gels with marker (QuantDNA™ 50-2k), stained with ethidium bromide and visualized in UV transilluminator. The gene expression was shown as the ratio of densitometric value between control and different hours of PCN-J04 and TOFA treated cells.

4.15. In vivo evaluation of PCN-J04

4.15.1. Experimental animals and ethics statement

Forty eight outbred virgin female Sprague-Dawley (SD) rats (21 days old) were purchased from NIN (National Institute of Nutrition), Hyderabad housed in polypropylene cages and maintained in 12-hrs light/12-hrs dark cycle, 50-55% humidity and 25 ± 2 °C. Food and water were available ad libitum. The study was conducted in accordance with the “Guide for the care and use of laboratory animals” and approved by Institutional Animal Ethical Committee of Periyar University (Vide. No. 1085).

4.15.2. Experimental induction of mammary carcinoma with DMBA

The 120 mg of DMBA (Sigma Aldrich, USA) was dissolved in 24 mL of Olive oil mixed well till straw yellow colour solution is obtained. DMBA solution was given to animals orally with the help of oral feeding needle at a dose of 25 mg/kg (Fukamachi et al., 2004). After the induction of DMBA the excess amount of
DMBA, the area was cleaned and chemically inactivated by using a dilute solution of sodium carbonate. Other materials which were used during carcinogen administration were disposed by incineration in compliance with institutions bio safety guidelines.

4.15.3. Experimental design and protocol

Healthy SD rats were divided into six groups. Each group consists of eight animals,

- Group 1: control rats received saline alone (p.o);
- Group 2: negative control rats were treated with DMBA alone 25 mg/kg (p.o);
- Group 3: rats were treated with DMBA and after one month of PIP (Post Induction Period) onwards 10 mg/kg/week of chitosan nanoparticles (i.v route);
- Group 4: rats were treated with DMBA and Tamoxifen 10 mg/kg/day (p.o) after one month of PIP;
- Group 5: rats were treated with DMBA and Orlistat 250 mg/kg/day (p.o) after one month of PIP;
- Group 6: rats were treated with DMBA and after one month of PIP the animals treated with PCN-J04 5mg/kg/week (i.v route) containing of peptide pACC1 (336.78 mg of PCN-J04 contains 12 µg of pACC1)

At the end of experiment (120 days), animals were sacrificed with an excess of diethyl ether anesthesia, the breast tissues were processed for further studies.

4.15.4. Tumor analysis

Body weight and food intake were recorded weekly. All rats were palpated for mammary tumors once a week at the beginning of 20 days after DMBA administration. The number, size and location of tumor were recorded. After histological diagnosis, the time of appearance of the cancers was determined. All the rats were monitored daily for morbidity and a moribund state were promptly killed. In addition, rats with tumor burden exceeding 10% of body weight, tumors >15 ± 20 mm in diameter, tumors that impaired normal movement of the animals and
Mammary Carcinoma Induced by DMBA
ulcerating tumors were immediately killed during the study. At end of the experiment the tumor volume was measured (Tae et al., 2002).

4.15.5. Biochemical analysis

4.15.5.1. Extraction of Lipids

Lipids from the breast tissues were extracted using standard protocol described by Folch et al., (1957). Briefly, The breast tissues were rinsed in ice-cold physiological saline thoroughly and dried by pressing between the folds of filter paper then, tissues were chopped with a razor blade into small pieces, transferred to 50 mL polypropylene tubes containing methanol 99% (5 mL) and homogenized with tissue homogenizer (Cole-Parmer, IKA T 25 ULTRA-TURRAX, India) at 1500 rpm for 5 min. After a vigorous agitation, chloroform was added and the final composition of extracting solvent was maintained 2:1 of chloroform-methanol. The excess of water (for 1 gm tissue 1 mL of water) was added, agitated well and left for 30 min at 40 °C. Then the content was centrifuged at 2500 ×g for 10 min and the clean supernatant was then transferred to an amber glass vial for further analysis. The above extraction was repeated for four times.

4.15.5.2. Estimation of total lipids

Thin layer chromatography (TLC) was performed (Watson, 2006) by spotting of the cellular total lipid extract to a 5 x10 cm silica gel 60 Å aluminum sheet (Merck) and developed with hexane/diethyl ether/acetic acid (80:20:2). Lipids were visualized with iodine vapor and imaged. The densitometry values were calculated using ImageJ software (Version-1.47, USA).

4.15.5.3. Estimation of total cholesterol

The levels of total cholesterol in the lipid extract were estimated by the method of Zlatkis et al., (1953).

Principle

Lipid extract was treated with ferric chloride-acetic acid reagent to precipitate the proteins. The protein free supernatant was treated with concentrated H₂SO₄. A reddish purple colour formed can be read colorimetrically.
Reagents

- Glacial acetic acid
- Ferric chloride 0.05% (w/v) in acetic acid
- Concentrated sulphuric acid

Procedure

Lipid extract (0.5 mL) was evaporated to dryness (37 °C). To the extract, 5.0 mL of ferric chloride-acetic acid reagent was added and vortex well. Three milliliter of concentrated H₂SO₄ was added and the absorbance was read at 560 nm after 20 min. A series of standards containing total cholesterol in the range of 3-15 µg were made to 5.0 mL with the reagent and a blank containing 5.0 mL of the reagent without extract was prepared. The values were expressed as mg/dL.

4.15.5.4. Estimation of free fatty acids

FFAs (Free fatty acids) levels in lipid extract were estimated by the method of Falholt et al. (1973).

Principle

FFA can be extracted with chloroform-heptane-methanol mixture to eliminate interference from phospholipids. The copper soaps of FFA are determined colorimetrically with diphenylcarbazide. The recovery of free fatty acids is 100.4%.

Reagents

- Extraction solvent (chloroform-heptane-methanol solvent, 5:5:1).
- Copper reagent (Aqueous solution copper nitrate 500 mM +10 mL of 1M triethanolamine + 6.0 mL of 1M NaOH+33g of NaCl. pH 8.1)
- Phosphate buffer (0.5 M, pH 7)
- diphenylcarbazide solution (40 mg of diphenylcarbazide +10 mL of ethanol)
Procedure
An aliquot of 0.5 mL of lipid extract was evaporated to dryness (37 °C). To the extract, accurately 1.0 mL of phosphate buffer, 6.0 mL of extraction solvent and 2.5 mL of copper reagent were added and shaked vigorously for 90 sec and kept aside for 15 min. Then, the tubes were centrifuged (1500 xg, 10 min) and 3.0 mL of upper layer was transferred to another tube containing 0.5 mL of diphenylcarbazide solution and mixed carefully. The absorbance was read at 550 nm after 15 min. One milliliter of phosphate buffer was treated as blank. The levels of FFAs were expressed as µmoles/mg protein.

4.15.5.5. Estimation of Phospholipids

Phospholipids (PLs) levels in lipid extract were estimated by the method of Zilversmit and Davis (1950).

Principle
Phospholipids were digested with concentrated sulphuric acid to liberate the lipid bound phosphorous as inorganic phosphorous. It then reacts with ammonium molybdate to form phosphomolybdic acid. This upon treatment with ANSA (1-amino 2-naphthol 4-sulfonic acid) forms a stable blue colour can be read colorimetrically.

Reagents

- Ammonium molybdate 2.5% (w/v) in 5 N sulphuric acid
- Amino napthol sulfonic acid (ANSA) (500 mg of amino napthol sulfonic acid in 195 mL of 15% (w/v) sodium bisulphite and 5.0 mL of 20% (w/v) sodium sulphite solution)
- Standard- organic phosphorus solution

Procedure
An aliquot of 0.5 mL of the lipid extract was pipetted out into a Kjeldahl flask and evaporated to dryness. To the extract 1.0 mL of 5 N sulphuricacid was added and digested till the appearance of light brown colour. Two to three drops of
concentrated nitric acid was added and the digestion continued till it became colourless. The Kjeldahl flask was then cooled and 1.0 mL of distilled water was added and kept in water bath for 5 min. Then, 1.0 mL of 2.5% ammonium molybdate and 0.1 mL of amino naphthol sulphonic acid were added. The volume was then made upto 5.0 mL with distilled water and the absorbance was measured at 660 nm within 10 min. The levels of PLs were expressed as µg/g protein.

4.15.5.6. Estimation of Triglycerides

Triglycerides were estimated by GPO/PAP method “Crest biosystems” kit following manufactures protocol.

Principle
Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3-phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The oxygen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglycerides presents in the sample.

\[
\text{Lipoprotein lipase} \\
\text{Triglycerides} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol + free fatty acids} \\
\text{Glycerol + ATP} \xrightarrow{\text{Glycerol-3-Phosphate Oxidase}} \text{Glycerol 3-phosphate + ADP} \\
\text{Glycerol 3-phosphate + O}_2 \xrightarrow{\text{Peroxidase}} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine + phenol} \xrightarrow{} \text{Red quinoneimine dye + H}_2\text{O}_2
\]
4.15.5.7. Estimation of High-density lipoprotein (HDL) and Low-density lipoprotein (LDL)

High-density lipoprotein (HDL) and Low-density lipoprotein (LDL) were estimated by PEG (polyethylene glycol) precipitation method using “Crest biosystems” kit following manufactures protocol.

Principle

When the tissue extract is reacted with the polyethylene glycol contained in the precipitating reagent, all the very low density lipoproteins (VLDL) and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent. Calculation of VLDL and LDL-cholesterol were using the below formulae.

TVLDL cholesterol = TG/5
LDL cholesterol = Total cholesterol – (HDL cholesterol + VLDL cholesterol)
The values were expressed as mg/dL

4.15.5.8. Preparation of mitoplasts

The mitochondria from breast tissues were isolated as described previously by Ronald et al., (2003) with slight modifications. Briefly, breast tissues (weighed quantity) were homogenized with sucrose/mannitol buffer (2.0 mM HEPES, pH 7.5, 70 mM sucrose, 220 mM D-mannitol, and 0.5 mg/mL of BSA and centrifuged at 5000 ×g for 30 min (4 °C) to remove nuclei and cellular debris. The supernatant was then centrifuged at 12,000 ×g to collect the mitochondria and treated with digitonin (0.1 mg/mg of protein). The isolated mitoplasts were used for analyzing mitochondrial enzymes.

4.15.5.9. Estimation of hexokinase (E.C. 2.7.1.1)

The hexokinase activity was estimated the protocol by Brandstrup et al., (1957).

Principle

The hexokinase can be estimated by quantifying glucose-6-phosphate formed from glucose.
Reagents

- Reaction mixture-1 mL (0.005 M) glucose solution +0.5 mL (0.072 M) ATP solution+0.1 mL (0.05 M) magnesium chloride solution + 0.4 mL (0.0125 M) potassium dihydrogen phosphate solution + 0.4 mL (0.1 M) potassium chloride solution + 2.5 mL of Tris-HCl buffer (0.01M, pH 8.0) + 0.4mL (0.5 M) sodium fluoride solution.
- TCA-10% (w/v)
- GOD/POD (Glucose oxidase/Peroxidase) reagent kit

Procedure

The reaction mixture was pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of 2 mL of mitochondrial suspension into reaction mixture. 1 mL of the reaction mixture was immediately removed to the tubes containing 1 mL of 10% TCA which was considered as zero time. A second aliquot was removed after 30 min incubation at 37 °C and the glucose were estimated by GOD/POD kit (KEE GAD, Biogen) method as per the manufactures protocol. The enzyme activity was expressed as nmoles of glucose phosphorylated/min/mg protein.

4.15.5.10. Estimation of phosphoglucoisomerase (E.C.5.3.1.9)

The phosphoglucoisomerase activity was estimated the protocol described by Horrocks et al., (1963).

Principle

The proportion of ketose and its 6-ester present in the end product justifies the use of an arbitrary D-fructose standard.

Reagents

- Borate buffer (10 M, pH 7.5)
- Buffered substrate (3 mg disodium glucose- 6-phosphate per millilitre of buffer)
- Chromogenic agent (HCl, resorcinol-thiourea reagent and distilled water, 7:1:1)
Procedure

One milliliter of freshly prepared buffered substrate was taken in two tubes. One hundred microliter of mitochondrial suspension was added in a tube as “test” another tube kept as “blank” (Borate buffer). Both the tubes were incubated exactly for 30 min at 37 °C in a water bath. Meanwhile, 1 mL of the standard solution (fructose in 0.25% (w/v) benzoic acid (0.25 M) in ethanol) and 1 mL of distilled water were added to test tubes named as “standard” and “standard blank”. Nine milliliter of chromogenic agent was added in all the tubes and placed in a 75 °C water bath for 15 min and allowed to cool. The absorbance was measured at 410 nm.

The calculation of the activity is given by

\[
\text{(Test - blank)} / \text{(Standardblank)} \times 100 \text{ nmoles of fructose formed/min}
\]

4.15.5.11. Estimation of Aldolase (E.C. 4.1.2.13)

The Aldolase activity was estimated by the method of Sibley and Lehninger (1949).

Principle

Aldolase can be estimated by measuring the coloured reaction of triose phosphates with Dinitrophenyl hydrazine.

Reagents

- Reaction mixture (1 mL Tris buffer (0.1 M, pH 8.6) + 0.25 mL fructose-1,6-diphosphatase solution (0.05 M, pH 8.6) + 0.25 mL hydrazine solution (0.56 M, pH 8.6))
- Dinitrophenyl hydrazine (0.56 M, pH 8.6)
- TCA 10 % (w/v)

Procedure

To 2.5 mL of solution (1.5 mL of reaction mixture + 1 mL water), 0.5 mL of mitochondrial suspension was added and the reaction was stopped after 30 min by the addition of 2 mL of 10 % (w/v) TCA. The blank (except fructose-1, 6-diphosphatase) was prepared in a similar manner and centrifuged (5000 ×g, 10 min). One milliliter of supernatant was transferred into fresh tube and 1μl of NaOH
(0.75 N) was added. After 10 min, dinitrophenyl hydrazine (2 mL) was added and tubes were placed at 38 °C for 10 min. Seven milliliters of NaOH was added to give a total volume of 10 mL. The absorbance was read at 540 nm after 10 min of addition of alkali. The percentage transmission was plotted against mm\(^3\) Fructose-1, 6-diphosphate (FDP) split/hrs. The value obtained was represented in nmoles as 22.4 mm\(^3\) of FDP = 1 μmole of FDP = 2 nmoles of glyceraldehyde formed.

4.15.5.12. Estimation of glucose-6-phosphatase (E.C.3.1.3.9)

The activity of glucose-6-phosphatase was estimated by the protocol as described by Cori and Cori (1952).

Principle

The glucose 6-Phosphate reacts with water molecules in presence of glucose-6-phosphatase to form glucose and inorganic phosphate molecules. Hence, quantification of inorganic phosphate will be proportional to amount of glucose-6-phosphate utilized.

Reagents

- Reaction mixture (3 mL potassium citrate (0.1 M, pH 6.8) + 5 mL glucose-6-phosphate (0.01 M, pH 6.8))

Procedure

To the reaction mixture (5 mL) glucose-6-phosphate (test) and with distilled water (control) were added instead. Both the tubes were incubated at 30 °C for 1 hr and 1 mL of 10% (w/v) of TCA was mixed and filtered. Inorganic phosphate was determined in 1 mL of the filtrate.

4.15.5.13. Estimation of fructose-1,6-biphosphatase (E.C. 3.1.3.11)

The activity of fructose-1,6-biphosphatase was estimated by the protocol by Gansede and Gansedo (1971).
Principle

Fructose-1, 6-biphosphatase was assayed by estimation of inorganic phosphate liberated.

Reagents

- Reaction mixture (1.2 mL Tris-HCl buffer (0.1 M, pH 7.0) + 0.1 mL Substrate: Fructose 1,6- bisphosphate (0.05 M) + 0.25 mL MgCl₂ (0.1 M) + 0.1 mL KCl (0.1 M) + 0.25 mM EDTA (0.001 M)).

Procedure

To the 2 mL of reaction mixture, 0.1 mL of mitochondrial suspension was added, incubated for 5 min at 37 °C, 1 mL 10% TCA was added and centrifuged (2000 xg, 4 °C, 10 min). An aliquot of the supernatant, 0.3 mL of distilled water and 0.5 mL of ammonium molybdate were added. After 10 min incubation, 0.2 mL of ANSA reagent was added. The tubes were shaked well, incubated at 37 °C for 20 min and then blue color developed was read at 620 nm. The values were expressed as nanomoles of Pi liberated/min/mg protein.

4.15.5.14. Estimation of isocitrate dehydrogenase (E.C.1.1.1.42)

The activity of isocitrate dehydrogenase was estimated the protocol described earlier by King (1965).

Principle

Isocitrate dehydrogenase activity is determined using isocitrate as the substrate in an enzyme reaction, which results in a colorimetric product proportional to the enzymatic activity present. One unit of isocitrate dehydrogenase is the amount of enzyme that will generate 1.0 μmole of NADH per min at pH 8.0 at 37 °C.

Reagents

- Tris-HCl (pH 7.5, 0.1 M)
- Trisodiumisocitrate (0.1 M)
- Manganese chloride (0.015 M)
- NADP⁺ (0.001 M)
- DNPH (0.001 M) in 1N HCl
- EDTA (0.005 M)
- NaOH (0.4 N)

Procedure

To 0.1 mL of Tris-HCl, 0.2 mL of trisodium isocitrate, 0.3 mL of manganese chloride, 0.2 mL of mitochondrial suspension and 0.2 mL of NADP⁺ were added. Instead of mitochondrial suspension and 0.2 mL of water served as control. After 60 min of incubation, 1 mL of DNPH followed by 0.5 mL of EDTA was added and kept at 37 °C for 20 min. Ten milliliter of NaOH was added and read at 390 nm. A standard containing α-ketoglutarate was run simultaneously. The isocitrate dehydrogenase activity was expressed as μmoles of α-ketoglutarate liberated/min/mg protein.

4.15.5.15. Estimation of α-Ketoglutarate dehydrogenase (E.C.1.2.4.2)

The activity of α-Ketoglutarate dehydrogenase was estimated the protocol by Reed and Mukherjee (1969).

Principle

The α-Ketoglutarate dehydrogenase complex catalyzes the hydrolysis of S-succinyl-CoA to succinate and CoA-SH. The reaction rate is dependent upon the presence of thiamine pyrophosphate and NADH.

Reagents

- Potassium phosphate buffer (1 M, pH 6.0)
- α-Ketoglutarate (0.5 M)
- Thiamine pyrophosphate (0.002 M)
- Magnesium chloride (0.003 M)
- Potassium ferricyanide (0.25 M)
- TCA 10% (w/v)
- Dupanol 4% (w/v)
Materials and methods

- Ferric ammonium sulphate-dupanol reagent (1.7 g of ferric ammonium sulphate was dissolved in 10 mL of water and this mixture was filtered. To this solution, 27 mL of 85% (v/v) Orthophosphoric acid was added and made up to 140 mL with water).
- Potassium ferrocyanide 0.01% (w/v)

Procedure

To 0.15 mL of phosphate buffer, 0.1 mL of each thiamine pyrophosphate, magnesium sulphate, α-Ketoglutarate and potassium ferricyanide were added. The total volume was made up to 1.2 mL with water followed by 0.2 mL of mitochondrial suspension and incubated at 30 °C for 20 min. Then 1 mL of TCA was added, centrifuged (5000 ×g, 15 min, 4 °C) and 0.2 mL of mitochondrial suspension was added to the control after the addition of TCA. To the supernatant, 0.1 mL of potassium potassiumferricyanide, 1mL of dupanol, then incubated for 20 min at 25 °C and measured at 540 nm. A standard containing potassium ferrocyanide was run simultaneously.

4.15.5.16. Succinate dehydrogenase (E.C.1.3.5.1)

The activity of succinate dehydrogenase was estimated the protocol described earlier Slater and Bonner (1952).

Principle

The rate of reduction of potassium ferricyanide was measured in the presence of enough potassium cyanide to inhibit cytochrome oxidase by following the rate of decrease in the absorbance.

Reagents

- Phosphate buffer (0.3 M, pH 7.6)
- EDTA (0.03 M)
- Sodium succinate (0.4 M)
- BSA 3% (w/v)
- Potassium ferricyanide (0.075 M)
- Potassium cyanide (0.03 M)
Procedure

The reaction mixture containing 1 mL of phosphate buffer, 0.1 mL of EDTA, 0.1 mL of BSA, 0.3 mL of sodium succinate, 0.2 mL of potassium ferricyanide and 0.1 mL of potassium cyanide were added made upto 2.8 mL with water. The reaction was started by the addition of 0.2 mL of mitochondrial suspension. The change in absorbance was recorded at 15 sec interval for 5 min at 420 nm. The succinate dehydrogenase activity was expressed as μmoles of succinate oxidized/min/mg protein.

4.15.5.17. Estimation of Malate dehydrogenase (E.C.1.1.1.37)

The activity of Malate dehydrogenase was estimated by Mehler et al., (1948).

Principle

\[ \text{Malate dehydrogenase} \]

\[ \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-Malate} + \text{NAD} \]

The disappearance of NADH is measured spectrophotometrically at 340 nm.

Reagents

- Tris-HCl buffer (0.25 M, pH 7.4)
- NADH (0.005 M)
- Oxaloacetate (0.0076 M)

Procedure

To 0.3 mL of buffer, 0.1 mL of NADH and 0.1 mL of oxaloacetate were added and the total volume was made upto 2.9 mL with water. The reaction was started by adding 0.1 mL of mitochondrial suspension. The change in optical density was measured at 340 nm in an interval of 15 sec for 5 min in a spectrophotometer. The enzyme activity was expressed as nmoles of NADH oxidized/min/mg protein.

4.15.5.18. Estimation of Lactate

Prewtighed frozen breast tissue samples were homogenized in 20 volumes of ice cold water:acetone (1:1.2). The samples were centrifuged (4000 xg) for 30 min at 28 °C and supernatants were delipidated with finely powdered solid MgO (Sabater et
al., 2014). The lactate estimation was carried out using RandoxmonzaLC2389 kit (Randox, INDIA) followed by manufactures instructions.

4.15.6. Oxidative stress enzymes levels

4.15.6.1. Assay of Superoxide Dismutase (SOD) (E.C.1.15.1.1)

Superoxide dismutase activity was measured by the method of Kakkar et al., (1984).

Principle

The assay is based on the inhibition of nitrobluetetrazolium (NBT) reduction. Illumination of riboflavin in the presence of O₂ and electron donor like methionine generates superoxide anions and this has been used as the basis of assay of SOD. The reduction of NBT by superoxide radicals to blue colored formazan can be followed at 560 nm.

Reagents

- Phenazinemethosulphate (PMS) (186 μM)
- NADH (780 μM)
- Sodium pyrophosphate buffer (0.025M, pH 8.3)
- Nitrobluetetrazolium (NBT) (300 μM)
- n-butanol
- Potassium phosphate buffer (50 mM, pH 6.4)
- TCA 10% (w/v)

Procedure

Reaction mixture contained 0.1 mL of cytosolic fraction, 1.2 mL of sodium pyrophosphate buffer, 0.1 mL PMS, 0.3 mL of 300 μM NBT, 0.2 mL NADH. Reaction was started by addition of NADH. After incubation at 30 °C for 90 seconds, the reaction was then stopped by the addition of 0.1 mL TCA, stirred with 4.0 mL of n-butanol. Then the mixture was allowed to stand for 10 min, centrifuged (3000 xg, 10 min) and butanol layer was separated. Colour intensity of the chromogen in the butanol layer was measured at 560 nm and SOD was expressed as units/mg protein.
4.15.6.2. Estimation of Catalase (E.C.1.11.1.6)

Catalase activity was assayed as described by Luck (1963).

Principle

Catalase catalyzes the decomposition of H₂O₂. In the ultraviolet range H₂O₂ shows a continual increase in absorption with decreasing wavelength. The decomposition of H₂O₂ can be followed directly by the decrease in extinction at 240 nm.

Reagents

- Phosphate buffer (0.05 M, pH 7.0)
- H₂O₂ (0.75 M)

Procedure

In this assay, the reaction mixture consists of 0.05 M phosphate buffer (2.5 mL), 0.019M hydrogen peroxide (50 µl) and 20 µl of cytosolic fraction. Change in absorbance was recorded at 240 nm. Enzyme activity was expressed as Units/mg protein.

4.15.6.3. Estimation of Glutathione peroxidase (E.C.1.11.1.9)

Glutathione peroxidase (GPx) activity was measured according to Lawrence and Burk (1976).

Principle

The activity of GPx was determined by measuring the decrease in GSH (Glutathione) content after incubating the sample in the presence of H₂O₂ and NaN₃.

\[
H₂O₂ + 2 \text{GSH} \rightarrow 2\text{H}_2\text{O} + 2 \text{GSSG (Glutathione disulfide).}
\]

Reagents

- Phosphate buffer (0.05 M, pH 7.0)
- EDTA (1 mM)
- Sodium azide (1 mM)
- Glutathione reductase (1 U/mL)
Materials and methods

- Glutathione (1 mM)
- NADPH (0.2 mM)
- H2O2 (0.25 mM)

Procedure

In this assay, the total reaction mixture consists of 1.44 mL phosphate buffer, 0.1 mL of EDTA, 0.1 mL of sodium azide, 0.05 mL of glutathione reductase (GR), 0.1 mL of glutathione, 0.1 mL of NADPH, 0.01 mL of hydrogen peroxide, 0.1 mL of cytosolic fraction. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as units of NADPH oxidized/mg of protein.

4.16. Histological examination

4.16.1. Hematoxylin and eosin stain (HE stain)

Principle

The principle of HE staining is to determine different parts of the tissue components in contrasting colours, such as pink, blue, deep red and orange red. Haematoxylin can be oxidized to Haematein, it requires a mordant, which is usually a metal, to enable Haematein dye to be well demonstrated on the acidic nucleus to give a blue colour. Eosin has the ability to differentiate between the cytoplasm of diverse tissue types, connective tissue fibers and matrices.

Procedure

Mammary tissues were surgically excised and immediately fixed in 10% (v/v) formalin fixative and embedded in paraffin wax. Five micron thick sections were cut under microtome (Leica, RM2135, Germany), spread on glass slides and cleared with xylene. The sections were rehydrated with series of ethanol, rinsed with water then stained using hematoxylin and washed with running water (20 min). The slides were counterstained with eosin and dehydrated with series of ethanol. Finally slides were then mounted in DPX, viewed under microscope (Olympus, MLXi, Japan) and photographed.
4.16.2. Staining of mast cells (Toluidine blue)

Principle

Mast cells should stain red-purple (metachromatic staining) and the background stain blue (orthochromatic staining). Metachromasia, tissue elements staining a different color from the dye solution, is due to the pH, dye concentration and temperature of the basic dye. Blue or violet dyes will show a red color shift, and red dyes will show a yellow color shift with metachromatic tissue elements.

Procedure

The paraffin sections were cleared with xylene, rehydrated serial of alcohol and stained with toluidine blue (2-3 min), washed with water (3 changes). The sections were dehydrated quickly through 95% and 2 changes of 100% alcohol (10 dips each since stain fades quickly in alcohol). Cleared with xylene (2 changes, 3 min) finally slides were then mounted in DPX, viewed under microscope (Olympus, MLXi, Japan) and photographed.

4.17. Immunohistochemistry

Principle

Immunohistochemistry (IHC), refers to the detection of proteins in cells by exploiting the principle of antibodies binding specifically to antigens in biological tissues. This technique allows visualization of antigen via sequential application of a specific antibody (primary antibody) to the antigen, a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site.

Procedure

Sections were deparaffinized in xylene and rehydrated. The IHC for epidermal growth factor receptors (ErbB-1) were carried out with the aid of ScyTek (PolyTek™) kit protocol. Concurrently, the receptor tyrosine-protein kinase erbB-
2(HER2) IHC was also been performed using Novolink™ polymer detection system (Leica) kit by following the manufacturer’s protocol.

4.18. Ultrastructural analysis using Transmission Electron Microscopy

After fixation with glutaraldehyde (2.5% (v/v)) and paraformaldehyde 2% (w/v), breast sections were cut into approximately 1 mM cubes and post fixed in 1% osmium tetraoxide for 2 hrs at 4 °C. The cubes were then dehydrated in series of ethanol and treated twice with propylene oxide for 10 min (37 °C). The tissues were infiltrated with EPON mixture (Shell, Chemical Co., USA.) and propylene oxide (1:1) for 2 hrs at 37 °C, then embedded in EPON mixture containing Taab/812, followed by polymerization at 60 °C for 24 hrs. Ultra microtome (Leica, EM UC7, Germany) was used to cut tissues at 0.5 μm sections and stained with 0.5% toluidine blue for viewing the cells. Then the 60 nm ultra-thin sections were cut and mounted on copper grids (300 mesh). The sections were double stained with uranyl acetate and lead citrate and then examined by Transmission Electron Microscope (Philips, CM10, Deutschland) and photographed.

4.19. Statistical analysis

All data were expressed as mean ± Standard Error (SE) of number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using Graphpad PRISM (Version-5.01, USA) and the individual comparison were obtained by “Dunnett” or “Bonferroni” (Chapter II) comparisons. A value of P<0.05 was considered to indicate a significant difference between groups. Values not sharing common superscript are significantly different with each other at P< 0.05.
5. Results

5.1. In silico evaluation of phospho-peptides interaction on BRCT binding

The human BRCA1 protein consists of major protein domains such as Znf C3HC4-RING domain, the BRCA1 serine domain and BRCT domains. Although most BRCT domain-containing amino acids that participate in DNA-damage checkpoint (or) DNA-repair pathways (or) controlling ACC1 level in the cytosol (or) all the three (Yu et al., 2003) and is appear to act as a phospho-peptide binding domain. The ability to recognize phospho-peptides has been well characterized, both functionally and structurally, in a number of proteins. BRCT domain interactions may participate in more dynamic signaling processes or may require additional interactions of proteins/peptides within oligomeric complexes and/or from other protein domains, to stabilize these interactions, evidently more evaluation studies will be required to elucidate the mechanism of BRCT domain phospho-peptide interactions (Leung and Glover, 2011). Our understanding, docking studies were still lacking with different phospho-peptides on BRCT domain. Hence, the current study may provide a comprehensive understanding of interactions BRCT domain with phospho-peptides. Moreover, the abilities of peptides to translocate through cell membranes can be accompanied by toxic effects resulting from membrane perturbation at higher peptide concentration. Consequently, in this chapter also evaluated the toxicity of the peptides which can potentially interact with BRCT domain by in silico methods.

5.1.1. Protein docking with BRCT domain

The Z Rank score displayed in table-2 denotes the attraction gain of hydrogen bonds. The result shows equal interactions with MDC1 and pACC1. Whereas, ATRIP defeat the MDC1 with the Z score of 10.76, in contrast BATT1 peptide displayed very poor ‘Z’ score and it defeats rest of three peptides excluding pACC1 in the Z Rank score.
<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Peptide</th>
<th>Sequence of Amino acids</th>
<th>Z Rank Score</th>
<th>‘Z’ Score</th>
<th>Hydrogen Bond Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>BATT1</td>
<td>LQGDpSSLFVA</td>
<td>-60.322</td>
<td>8.08</td>
<td>P: ARG5: N - C: ASN1774: O</td>
</tr>
</tbody>
</table>

Table 2. Z Rank scores of peptides. Z Rank by using Accelrys Discovery Studio Client 3.5 (P: Peptide and C: BRCT domain)

Figure 4. Interaction of phosphopeptides with BRCA1-BRCT domain. (A) ATRIP Peptide with BRCT, (B) BATT1 Peptide with BRCT, (C) MDC1 Peptide with BRCT and (D) pACC1 peptide with BRCT.
The docking studies with Accelrys Discovery Studio 3.5 shows, affinity between different peptides to BRCT domain (Figure-4). The pink and green manifestation in concert denotes the hydrogen bonding. In this peptide pACC1 shows higher degree of pink-green manifestation whereas, peptide MDC1 and pACC1 shows equal number of hydrogen bonding ability with BRCT domain.

5.1.2. In silico toxicity analysis

The QMS score for prediction of toxicity, results exhibits closer variations between minimum and maximum QMS scores for MDC1, BATT1 and ATRIP. Whereas, peptide pACC1 was displayed greater degree of variation between in QMS scores shown in table-3. Basically, the quantitative matrix was generated by ToxinPred considering the probability or frequency of amino acid at particular position. Hence, the arrangement of amino acids is responsible of these scores.

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Peptide</th>
<th>Sum of Origin score</th>
<th>Sum of Maximum score</th>
<th>Sum of Minimum Score</th>
<th>Variations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MDC1</td>
<td>5.00</td>
<td>158.40</td>
<td>-81.00</td>
<td>239.40</td>
</tr>
<tr>
<td>2</td>
<td>BATT1</td>
<td>-2.10</td>
<td>158.40</td>
<td>-81.00</td>
<td>239.40</td>
</tr>
<tr>
<td>3</td>
<td>ATRIP</td>
<td>4.30</td>
<td>150.00</td>
<td>-76.40</td>
<td>226.40</td>
</tr>
<tr>
<td>4</td>
<td>pACC1</td>
<td>4.20</td>
<td>223.30</td>
<td>-100.10</td>
<td>323.40</td>
</tr>
</tbody>
</table>

Table.3. QMS Score by ToxinPred

5.2. Initial toxicity and purity evaluation

Analysis of peptide purity is extremely important before used for any clinical or biological research. Further, investigating toxicity associated with highly interesting peptides becomes progressively significant and thorough initial assessment of cytotoxicity, in vitro is a first step towards advancing the delivery of peptides into the clinics. The present chapter describes results of genotoxic and hemotoxic potentials.
5.2.1. MASS Spectrometry of peptide pACC1

The result of MASS spectrometry (Figure-5) shows the characteristic peaks at 110, 255.1, 355.1, 470, 612.2, 675, 825.9, 957.5 and 1044.4 \times 10^5. It was found that peptide pACC1 was of highest purity with respect of significant peaks (confirmed by x!Tandem) and the net charge of the peptide was found to be negative (-2).

![Figure 5. MASS spectra of peptide pACC1](image)

5.2.2. DNA nicking assay

The results show in the DNA nicking assay describes clearly there is no significant damage in the DNA (2.5 ng) even at higher concentration (80 ng) in lane-5. The super coiled DNA does not get much affected by the peptide shown in figure-6. Further, the results evidenced that DNA strand interruptions by peptide pACC1 was not been significant (lanes 3-4) when compared with lane-5 (Figure-7).

![Figure 6. DNA Nicking Assay of Peptide pACC1.](image)
Note: Lane 1: Control pUC19 plasmid; Lane 2: H₂O₂ + Control pUC19 plasmid; Lane 3: 20 ng peptide + Control pUC19 plasmid; Lane 4: 40 ng peptide + Control pUC19 plasmid; Lane 5: 80 ng peptide + Control pUC19 plasmid.

Figure 7. Densitometry values of supercoiled DNA

5.2.3. Hemolytic Assay

The peptide pACC1 was evaluated for non-toxic effect on hemolysis (RBC) at varying concentrations (2-16 µg/mL). It was shown the peptide does not elicit any toxic effect on hemolysis even at higher concentration (16 µg/mL), which may not be possible in in vivo distribution (4 µg/mL) shown in figure-8.

Figure 8. Peptide pACC1-Heam interaction
5.3. Peptide pACC1 quantification method development

Incorrect quantification of peptides may lead to false conclusions in research and may also adversely affect the dosage that is prescribed. As of our knowledge, the quantification of peptide pACC1 was not available in the literature yet. The results obtained will provide suitable HPLC method for the quantitative analysis of peptide pACC1.

5.3.1. HPLC method development for peptide pACC1 quantification.

The HPLC method was developed for peptide pACC1, the accuracy was expressed as the percentage of coefficient of variation (% CV). All the bias were found for the three different concentrations of peptide were lower than 5% (n=3) (Table-4) system repeatability and reproducibility, analyses of six diverse samples of one concentration were performed in the same day and 2 different days. All CV values were found to be lower than 2% (Table-5).

<table>
<thead>
<tr>
<th>Theoretical Concentration (μg/mL)</th>
<th>Area(mAu)</th>
<th>Practical Concentration (μg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>567.98</td>
<td>24.87</td>
<td>99.48</td>
</tr>
<tr>
<td>25</td>
<td>546.37</td>
<td>24.22</td>
<td>92.88</td>
</tr>
<tr>
<td>25</td>
<td>544.13</td>
<td>24.01</td>
<td>96.04</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>24.15</td>
<td>96.133</td>
</tr>
<tr>
<td>% Bias</td>
<td></td>
<td>3.002</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>983.12</td>
<td>99.81</td>
<td>99.81</td>
</tr>
<tr>
<td>100</td>
<td>993.55</td>
<td>99.23</td>
<td>99.23</td>
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<tr>
<td>100</td>
<td>979.21</td>
<td>99.12</td>
<td>99.12</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>99.38</td>
<td>99.386</td>
</tr>
<tr>
<td>% Bias</td>
<td></td>
<td>0.233</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1581.503</td>
<td>198.65</td>
<td>99.32</td>
</tr>
<tr>
<td>200</td>
<td>1588.627</td>
<td>197.87</td>
<td>98.93</td>
</tr>
<tr>
<td>200</td>
<td>1589.888</td>
<td>198.65</td>
<td>99.32</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>198.39</td>
<td>99.195</td>
</tr>
<tr>
<td>% Bias</td>
<td></td>
<td>0.654</td>
<td></td>
</tr>
</tbody>
</table>

Table.4. Accuracy values for three different concentrations of peptide pACC1 (n=3)
5.3.2. Calibration curve preparation for peptide pACC1

The calibration curve was prepared for peptide pACC1 from five sample concentrations within the range of 5-35 µg/mL (Figure-9) and found linear in this range. There were seven series of samples prepared and average values were used to prepare the calibration curve. The R² value was found to be 0.926 (Figure-10) and the peak descriptions were given in figure-11.

![HPLC Chromatogram](image_url)

Figure.9. HPLC chromatogram of peptide pACC1 in different concentrations (5-35 µg/ml)
5.3.3. Linearity ranges of peptide pACC1

The linearity range was found to be 5-35 µg/ml. LOD-5.65 µg/ml and LOQ-17.1322 µg/ml was found respectively (Table-6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range</td>
<td>5-35µg/mL</td>
</tr>
<tr>
<td>Slope</td>
<td>15.181</td>
</tr>
<tr>
<td>Intercept</td>
<td>-1.506</td>
</tr>
<tr>
<td>LOD</td>
<td>5.65 µg/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>17.1322 µg/ml</td>
</tr>
</tbody>
</table>

Table.6. Linearity Parameters of peptide pACC1 analytical method
6.1. Discussion

The docking and toxicity studies may provide immense thought concerning about the ability of therapeutic peptides intended pharmacology. The ZDOCK procedure performs the initial global, systematic search of the orientations of the two protein partners (Chen et al., 2003). Classically, the larger protein (receptor) is kept fixed while moving the smaller protein (ligand) around the receptor. ZDOCK utilizes a grid-based rigid body docking seek in six dimensions with the aid of Fast Fourier Transform (FFT) technique (Li et al., 2011). The rotational search sampling grid can use a 15 degree grid which samples a total of 3600 docked poses, or 6 degree grid which samples a total of 54,000 poses for more accurate results. Thus, the results of pACC1 furnish good Zscore and Z Rank score. Basically, Z Rank score denotes optimized energy scoring function based on weighted energy terms of van der Waals, electrostatics and desolation chemical properties between two proteins. In the present investigation number of interaction is not much influential but the force involved, bond angle and bond length were considerably important. Evidently, the BATT1 peptide scored higher Zscore when compared with other peptides. Dehydrons promotes the removal of water through proteins or ligand binding and notably exogenous dehydration enhances the electrostatic interactions (Fraser et al., 2010).

The quantitative matrix score was used to find the toxicity of the cell penetrating peptides (Gupta et al., 2013). The present study also demonstrates high variation in the minimum and maximum toxicity score for peptide pACC1 when compared to other peptides. The pACC1 possess higher score in maximum QMS score. The high lethality is perhaps attributable to the amino acid arrangements and it can be further confirmed by wet lab analysis.

To assay the peptides for their genotoxic property, various features were studied like single and double strand DNA break, loss of excision repair, cross-linking, alkali-labile sites, point mutations, structural and numerical chromosomal aberrations. Moreover, genotoxic testing in pharmaceuticals are of increasing concerns (Liu et al., 2010). Assays provide a simple, rapid and extremely sensitive method for detecting toxicity of sequence-specific DNA-binding proteins. Proteins which bind
specifically to a labeled DNA fragment retard the mobility of the fragment during electrophoresis. DNA nicking assay in another hand provides uncooked suggestion about affinity, abundance, association rate constants, dissociation constants and binding specificity of DNA-Binding proteins (Yang et al., 2005). Hence, the results from DNA nicking assay describes clearly that there is no significant damage to the DNA even at higher concentration.

The US FDA (http://www.fda.gov/cder/guidance/index.htm) recommends that, even for the excipients intended to biological administration, an in vitro hemolytic study should be performed at the higher concentration for biological administration to test for hemolytic potential. Hence, the present investigation confirms that the peptide pACC1 does not turn out any changes in the hemolysis even at higher concentration which may not be possible in in vivo distribution. The well-known haemolytic peptide, mellittin, has been reported to cause 50% hemolysis at 7.5 μg/mL (Wan et al., 2007). Whereas, peptide pACC1 showed 50% hemolysis at 25.24 μg/mL. Hence, the application of pACC1 peptide might be possible for safe use treatment.

The main interaction considered in HPLC is the interaction between the mobile phase and the stationary phase, which is considered as a trap to attach proteins, peptides and other analytes (Penwell et al., 2012). HPLC method development would be a better choice for peptide quantification. Especially in early development, method optimization is not adequate due to restricted time and often lack of crude material to be spent on such experiments. Low initial purity of the peptide samples is typical fore early development and gives rise to very challenging separations. In this evaluation, validation parameters selected were as accuracy, precision (repeatability and reproducibility), specificity, sensitivity (LOD, LOQ) and linearity. Accuracy and precision of the method have been established by coefficient of variation and all values were found less than two percent. Using this newly developed method, peptide pACC1 can be successfully determined from any formulations which contains peptide pACC1 as ingredient. In the current investigation system, suitability test values have been found between required ranges. Hence, this developed HPLC method can be employed as sensitive method for quantification of peptide pACC1.