CHAPTER 1

INTRODUCTION:

Cancer is the second largest killer after cardiovascular diseases in the developed world and the incidence of cancer is rising very sharply in the developing countries. The latest projections based on the GLOBOCAN 2012, estimate a substantive increase to 19.3 million new cancer cases per year by 2025, and 21 million by 2030 (Global status report on non-communicable diseases 2010). It has been shown in cancer registries in India that the most frequently reported cancer sites in males are; lung, esophagus, stomach and larynx; whereas in females, cancer of cervix, breast, ovary and esophagus are common. Tobacco-related cancers account predominantly for almost one-third of all cancers in India (Misra et al., 2008). Cancer development in humans involves a complex succession of events. In this multistep process, the genomes of incipient cancer cells function inappropriately either by acquiring mutant alleles or dysregulated expression of proto-onco genes, tumor-suppressor genes, microRNA and other genes that control, directly or indirectly, cell proliferation (Hahn and Weinberg, 2002, Evan and Vousden, 2001, Esquela-Kerscher and Slack, 2006). The ability that cancer cells acquire in comparison to the healthy cells are: resistance to growth inhibition, proliferation without dependence on growth factors, replication without limit, supporting angiogenesis, evading apoptosis and invading to metastasize. The mechanism by which these capabilities are acquired by cancer cells vary considerably in various types of tumors, however, most of the physiological changes associated with these mechanisms involve alteration of signal transduction pathways (Gupta et al., 2010). Of many types of cancers found all around the globe, one of the cancer type which is characterized by late clinical presentation, early and aggressive local invasion, metastatic potential, strong resistance to chemo- and radiation- therapy; and most importantly, a very poor overall prognosis, is pancreatic cancer (Li et al.,
2004). No specific molecular mechanism that underlies pancreatic tumorigenesis and non-induction of apoptosis under chemotherapeutically resistant situations is understood.

Attempts have been made to improve upon the efficacy of clinically validated drugs in combination regimen to enhance their response towards refractory tumors, including pancreatic cancer (Tempero et al., 2011). Beside 5-Flourouracil, Gemcitabine (GCB), an inhibitor of ribonucleotidereductase and DNA synthesis is a widely used chemotherapeutic drug for pancreatic cancer, which has shown failures after multiple cycles of therapy because of the emergence of drug resistance (Kang and Saif, 2008, el-Kamar et al., 2003). Treating pancreatic tumors that are refractory to gemcitabine therapy is a challenge for oncologists. Hence, it is essential to expedite novel approaches of induction of apoptosis as a step towards an effective intervention in this deadly disease. Efforts, as in other cancers to target the key processes, such as cell division, carcinogen metabolism, apoptosis, differentiation, has generated and promoted an interest in dietary phytochemicals for potential cancer chemoprevention, especially in pancreatic cancer development.

Numerous lines of evidence have revealed that cancer is a lifestyle disease and 80% of all cancers are preventable by changing the lifestyle (Ralhan et al., 2009). The lifestyle adopted by people today with changed food habits puts them at risk. A new health paradigm is evolving, placing more emphasis on the positive aspects of diet. Consumption of junk food has increased manifold, leading to a number of diseases; and one of them is obesity that is now recognized as a global issue. A mechanistic link between diet and pancreatic cancer comes from its long-recognized interrelationship (Rajendran et al., 2011). “Let food be thy medicine and medicine be thy food”, quoted by Hippocrates about 2,500 years ago, is certainly the tenet of today. Nutraceuticals are the emerging class of natural products, fading the line between food and drugs. The concept of “nutraceutical” arose first in the survey
from U.K., Germany and France, where exercise or hereditary factors were rated less by the consumers than the diet to achieve good health. The term “nutraceutical” was coined from “nutrition” and “pharmaceutical” by Stephen De Felice, founder and chairman of the Foundation for Innovation in Medicine (FIM), Cranford, NJ in 1989. According to De Felice, nutraceuticals are defined as, “a food (or a part of food) that provide health or medical benefits, including the treatment and or prevention of a disease” (Das et al., 2012).

Among various dietary agents one of the dietary agent, which could be used in combination with GCB for treatment of pancreatic cancer, is Betulinic acid (BA), a naturally occurring penta-cyclic-triterpene with a variety of biological activities including potent anti-tumor properties (Fulda, 2009). BA is contained in the outer bark of various plants throughout the plant kingdom, including white-barked birch trees (Fulda, 2009), with anti-inflammatory, anti-viral, and anti-neoplastic activities (Zuco et al., 2002). Anticancer activity of BA has been linked to its ability to directly trigger mitochondrial membrane permeabilization, which is a central event in the apoptotic process, raising the hope to bypass the resistance to conventional chemotherapeutics (Fulda and Kroemer, 2009). Thymoquinone (TQ), another potential anticancer-nutraceutical agent, is a bioactive compound derived from black seed (Nigella sativa) oil. In folklore medicine, consumption of TQ seed has been associated with diverse therapeutic benefits in bronchial asthma, headache, dysentery, gastrointestinal problems, eczema, hypertension and obesity (Padhye et al., 2008). In the context of cancer, TQ is reported to exhibit anti-proliferative effects on cell lines, derived from breast, colon, ovary, larynx, lung, myeloblastic leukemia and osteosarcoma (Norwood et al., 2006, Gali-Muhtasib et al., 2008, Shoib et al., 2003, Wilson-Simpson et al., 2007, El-Mahdy et al., 2005, Roepke et al., 2007, Rooney and Ryan, 2005); and anti-metastasis effect in human pancreatic cancer. It has been shown to suppress migration and invasion of PANC-1 cells in a dose-dependent manner (Wu et al.,
2011) and down-regulate NF-kappa B and MMP-9 expression. Biological activity of thymoquinone (TQ) has also been shown in pancreatic cancer cells *in-vitro*. This has revealed its chemo-sensitizing effect after the pre-exposure of cells to TQ (25μmol/L) for 48 hrs., followed by gemcitabine or oxaliplatin, resulting in 60% to 80% growth inhibition as compared to 15% to 25% of inhibition with gemcitabine or oxaliplatin alone (Banerjee et al., 2009).

Here, this work investigates the apoptotic effect of BA or TQ with GCB, independently and in combination, and screens the synergistic effect of dietary molecules BA and TQ in combination with drug GCB, on human adenocarcinoma cells, MIA PaCa-2, (GCB sensitive) and PANC-1 (GCB resistant). In addition, the role of non-coding small RNAs, the microRNAs (miRNAs), emerging to provide a new insight in cancer treatment in combination with dietary agents and drugs, is expedited for their efficacy in cancer treatment. Addition of microRNA in the treatment regimen could accelerate the synergistic potential of dietary molecules at specific low doses of drug both in drug sensitive and resistant cells. microRNA was used with certain low doses of BA, TQ, GCB individually and in combination (BA+GCB; TQ+GCB) to accelerate the synergistic potential of dietary molecules in inducing apoptosis in both sensitive and resistant pancreatic cancer cell lines, providing an alternative approach to the treatment of pancreatic cancers.

Cancer cells consume more glucose and produce a large amount of lactate even in a well-oxygenized environment; the process termed as aerobic glycolysis or “Warburg effect, and hence predominantly depend on the reprogramming of their metabolic needs (Warburg et al., 1927, Warburg, 1956). While normal differentiated cells under normoxic conditions maximize ATP production by mitochondrial oxidative phosphorylation of glucose, cancer cells generate much less ATP from glucose by aerobic glycolysis. In spite of being less efficient in ATP production, glycolysis is a much more rapid process in cancer cells (Curi
et al., 1988, Pfeiffer et al., 2001). Here, pyruvate kinase (PK) catalyzes the last reaction with transfer of a high-energy phosphate group from phosphoenolpyruvate (PEP) to ADP, producing ATP and pyruvate which is reduced to lactate by lactate dehydrogenase (LDH) in the cytosol. Pyruvate kinase (PK) consists of four isoforms, of which PKM2 expresses predominantly in cancer cells (Wong et al., 2013); as reported in renal (Brinck et al., 1994), colon (Christofk et al., 2008), lung (Schneider et al., 2002) and other cancer cells. PKM2 has been shown to act as a marker for: renal cell carcinoma (RCC) (Wechsel et al., 1999, Oremek et al., 1999), breast cancer (Luftner et al., 2000), testicular cancer (Pottek et al., 2000), lung carcinoma, cervical cancer, and gastrointestinal tumors (Mazurek et al., 2005), urological tumors (Roigas et al., 2001); and with a possible detection in the feces of patients with gastric and colorectal cancers (Hardt et al., 2003). In recent past, mass spectrometry has further demonstrated a predominant presence of PKM2 in: hepatocellular carcinoma, RCC, bladder carcinoma, lung carcinoma, colorectal cancer and follicular thyroid adenoma (Bluemlein et al.).

In this study, a synergistic approach was adopted for induction of apoptosis in cancer cells in culture. The potential of dietary molecules, Betulinic acid (BA) and Thymoquinone (TQ), was used to sensitize human adenocarcinoma cells, Mia PaCa-2 and PANC1, to Gemcitabine induced cell death with different combination schedules. Underlying mechanism of their action, especially with respect to PKM2 expression and activity was assessed. Also, two microRNAs (miR-101 and miR-24-2) were used with low doses of dietary molecules in combination with the low doses of the drug to enhance the synergistic effect of the drug at minimal doses.
AIMS & OBJECTIVES:

The aim of studying a synergistic effect of dietary molecules/nutraceuticals, such as Betulinic acid (BA) and Thymoquinone (TQ), in combination with clinically validated cancer chemotherapeutic agents, such as Gemcitabine (GCB), the sensitive and resistant pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, were used with the following objectives:

1. Calculate IC$_{50}$ of clinically validated anticancer drug(s) when used alone and in combination with dietary molecules.

2. Establish the best possible combination of anticancer drugs and dietary molecules using isobolograms and CI index.

3. Establish the molecular mechanism of enhanced efficacy with anticancer drugs when used in combination with dietary molecules.