The approach to the discovery of new anticancer drugs has recently evolved from a dependence on empiric cell-based screening for antiproliferative effects to a more mechanistically based approach that targets the specific molecular lesions thought to be responsible for the development and maintenance of the malignant phenotype in various forms of cancer. The challenges in developing better and effective cancer drugs are reduction of toxicity and improving efficacy and selectivity (cancer-specific targets) by exploiting the differences between cancer cells and normal cells. Past few years several thousands of new chemical entities undergo screening in various cell cultures but eventually do not achieve drug status due to severe side-effects. Ideally, one of the criteria for a drug being good is that it should not exhibit any undesirable side-effects on normal cells (Albrecht *et al.*, 2008). Our results indicated that the multi-parameter assay platform established in this study could be used to provide broader description of the drug property in addition to specific activities. Synthetic compounds are often used as highly potent drugs to treat acute leukemia. (Suh *et al.*, 2003; Hallak *et al.,* 2009; Kang *et al.*, 2008).

Apoptosis is the result of an active, gene-directed process, and this phenomenon should be manipulated to develop drugs that interact with cell death proteins. The understanding of apoptosis process has provided the basis for novel, targeted therapies that specifically induce cell death in carcinoma cell lines or enhance the

cytotoxic effects of established chemotherapeutic agents in these cells (Pettersson *et al.*, 2000). Ability to manipulate the machinery of cell death is an obvious goal of medical research and effect on regulation of apoptosis might lead to new possibilities for cancer treatment (Tamatani *et al.*, 2007). Research has shown enough evidences that synthetic products are a potential source of powerful antileukemic compounds.

Similarly, soft corals are also capable to produce various chemical substances, which are toxic to both predators and some neighbouring hard corals. Some of these chemicals have properties which are beneficial to humans, and can be exploited for various medicinal properties including drugs useful for cancer chemotherapy. In the present search for synthetic quinazolinyl derivative, a biologically active DMQA was powerful cytotoxic compound, which induced apoptosis on human monocytoid leukemia cells (THP-1) and can be further developed as drug for cancer chemotherapy.

The results obtained by applying MTT and Trypan blue exclusion assays demonstrated that DMQA exhibits marked concentration dependent cytotoxic activity on various tumor cell lines which are found to be much less compared to the IC_{50} obtained on normal proliferating cell line PBMC (207.93 μM). The IC_{50} value was found to be least on Thp-1 cell line (2.21 μM) and was selected to study the apoptotic pathway. DMQA is a novel synthetic quinazolinyl derivative that exerts a broad spectrum of activity against U937 (human

monocyte lymphoma), THP-1 (human acute monocytoid), HL60 (human myeloid leukemia cells), HeLa (human cervical cancer), Mia-Pa-Ca-2 (human Caucasian pancreatic carcinoma) A-375 (human melanoma), B16-F10 (Mouse Melanoma) and A-431 (epidermal carcinoma) cell lines.

Apoptosis, often limited to a single cell or a small group of cells, is characterized by chromatin condensation and internucleosomal DNA degradation. Furthermore, cell shrinkage is accompanied by an extensive blebbing of the cell membrane which results in the separation of the cell into a number of membrane-bound vesicles (apoptotic bodies). Structural alterations of the cell surface (e.g. translocation of phosphatidylserine to the outer cell membrane) ensure that apoptotic cells are recognized and phagocytised by either macrophages or adjacent epithelial cells [Duvall *et al.*, 1985]. The numbers of early apoptotic cells were increased in a concentration-dependent manner on THP-1 cells treated with DMQA (Fig). The apoptotic features induced by DMQA include inhibition of proliferation, structural and biochemical features that characterize chromatin condensation, fragmentation and cell shrinkage as demonstrated by Trypan blue exclusion assay and fluorescence microscopy (Fig).

Cell death process (apoptosis) is a characterized by morphological and biochemical features occurring at different stages and therefore, more high-throughput methods are clearly needed when analyzing

numerous samples. Annexin V can be used in a simple assay for early detection of apoptosis. In normal cells, Phosphatidylserine (PS) is located on the inner surface of the plasma membrane. Apoptotic cells bind this phospholipid- binding protein with high affinity due to the externalization of phosphatidylserine on the external leaflet of the plasma membrane during apoptosis (Martin *et al.*, 1995; Fadok *et al*; 1992). The presence of phosphatidylserine on the outer leaflet of apoptotic cells membrane was assessed using Annexin V-cy3/6CFDA staining to quantify the amount of cells in the early stage of apoptosis. The numbers of early apoptotic cells were increased in a concentration-dependent manner on THP-1 cells treated with DMQA (Fig. 3.12).

Imbalance between cell growth and cell death has been proposed to be involved in tumor formation (Smith and Fornace 1996). Accordingly, in response to various types of DNA damage, the cell cycle checkpoints and cell death signals are activated to stop cell growth and to eliminate multiplication of the genetically altered cells. Two checkpoints in the cell cycle, G1 and G2, play a very important role in the regulation of cells proceeding to S and M phases, respectively (Fritsche *et al.*, 1993). Damaged cells stop DNA replication at G1 or G2 phase, presumably allowing the repair systems to function before the next round of cell cycle. Activation of the apoptotic cell death pathway is a safeguard to remove unrepairably damaged cells. Several cellular effector molecules, including p53, are

involved in arresting damaged cells at these checkpoints and inducing apoptosis. Up-regulation of the p53 protein is a common cellular response in many cell types exposed to various DNA damaging agents (Thompson CB, 1995; O'Connor, PM, 1997). Cell cycle analysis showed a sub-G1 population on DMQA treated THP-1 cells, which indicated that the cell death was caused by apoptosis. The maximum number of cells arrested in sub G1 phase were observed at 24h (36.36%) compared to 6h (6.11%) indicating a time dependent effect of DMQA (Fig 3.13).

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. Elegant biochemical work identified the DNA fragmentation factor (DFF) as a major apoptotic endonuclease for DNA fragmentation in vitro. DNA cleavage during apoptosis occurs at sites between nucleosomes, protein-containing structures that occur in chromatin at ~200-BP intervals. DNA fragmentation is often analyzed using agarose gel electrophoresis to demonstrate a "ladder" pattern at ~200-BP intervals. Apoptosis induced by DMQA clearly demonstrated a dose dependent increase in DNA ladder formation (Fig. 3.15).

In addition to the cell membrane changes and death receptors, mitochondria play a central role in the transduction of apoptotic signals triggered by many stimuli. Mitochondrial participation in apoptosis was connected with the collapse of membrane potential that was considered a point of no return in the death cascade (Renz, 2001)

and mitochondrion has thus been becoming the target for chemotherapeutic agents (Costantini *et al.*, 2000). Etoposide, doxorubicin, camptothecin and cisplatin (Sancho *et al.*, 2003) are wonderful examples of mitochondrial-mediated apoptosis. More recently, many novel secondary metabolites with potential chemotherapeutic development include halichondrin B (Towle *et al.*, 2001), Peloruside A (Hood *et al.*, 2002) and Spongistatin 1 (Schyschka *et al.*, 2008) were of marine origin.

The reduction of Δψmt is an early event in the apoptotic cascade and may be a sign of mitochondrial swelling and disruption of the outer mitochondrial membrane (Ly *et al.*, 2003). To address if the title compounds, affect upon irradiation the Δψmt, the fluorescence of the dye JC-1 a reliable mitochondrial potential probe was monitored (Salvioli *et al.*, 1997). Translocation of cytochrome c from the mitochondrial intermembrane space to the cytosol occurs in consequence of outer mitochondrial membrane permeabilisation (OMP). Inner mitochondrial membrane permeabilisation (IMP) is manifested as a dissipation of the transmembrane potential (Δψm). In most cases mitochondrial dysfunction implicates both, OMP and IMP (Ella *et al.*, 1998). Determination of a reduction in Δψm by using the fluorochrome JC-1 indirectly evidenced reduction in mitochondrial membrane potential in a concentration dependent manner (1-3μg/ml for 12h) in Thp-1 cells treated with DMQA (3.17). The decrease of Δψm preceded the release of cytochrome c.

Bcl-2 family members are major regulators of the mitochondria-initiated caspase activation pathway [Forte and Bernardi, 2006] and are classified as two groups in this family, anti-apoptotic members (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic members (e.g. Bid and Bcl-xS) (Chan and Yu *et al.*, 2004). Inadequate Bcl-2 protein expressions are also likely to enhance tumor cell survival. The present study was designed to gain insights into the role of these Bcl-2 family members in DMQA-mediated apoptosis. It is a known phenomenon that Bcl-2 family of proteins regulates apoptosis and that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death [Jacobson and Raff, 1995]. DMQA mediated apoptotic cell death in Thp-1 cells revealed an increase in the expression of proapoptotic protein Bax and a decrease in the level of antiapoptotic protein Bcl-2 (Fig. 3.18). Together these data clearly indicate that DMQA induces mitochondrial dysfunction in Thp-1 cells.

Caspases, intracellular cystein proteases, play an essential role in the initiation and execution phases of chemical-induced apoptotic cell death. However, the number of studies, proposing an additional caspase-independent cell death is growing (Mathiasen *et al.*, 1999, Roberts *et al.*, 1999, Nylandsted *et al.*, 2000). As it is confirmed in the present work, A decrease in level of executioner procaspases-3 and initiator procaspase-9 was observed in THP-1 cells exposed to DMQA, indicating that apoptosis was dependent upon successive activation of

caspases. In contrast, involvement of mitochondria has been found in caspase-9 activation [Green and Reed, 1998]. (Fig.3.18).

PARP is a protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death. This inactivation of PARP has been proposed to prevent depletion of NAD (a PARP substrate) and ATP, which are thought to be required for later events in apoptosis. PARP is a 113 kDa nuclear protein which can exist as a homo- or hetero-dimer, and is strongly activated by DNA strand breaks. This protein acts as a molecular “nick sensor” and functions in base excision repair, poly(ADPribosyl)ation of acceptor proteins involved in chromatin architecture and DNA metabolism, and participates in protein modification to enhance or repress transcription. PARP was subsequently shown to be cleaved into 89- and 24-KDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis in a variety of cells (Kaufmann *et al.*, 1993; Nicholson *et al.*, 1995). Such cleavage essentially inactivates the enzyme by destroying its ability to respond to DNA strand breaks. Western blotting analysis revealed that DMQA (24h) induced cleavage of PARP into 85KD fragment in concentration dependent manner (1, 2 and 3 μg/ml) in Thp-1 cells (Fig. 3.18). It is evident that induction of caspase-3 led to the cleavage of PARP (115 kDa), a substrate of caspase-3 to produce a fragment (85-kDa) with a concomitant decrease of 115-kDa protein, which occurs at apoptosis onset (Lazebnik *et al.*, 1994).

Active caspase recognizes several molecules as substrates during apoptosis. For example, ICAD (inhibitor of caspase-activated deoxyribonuclease) is inactivated while CAD (caspase-activated deoxyribonuclease) is indirectly activated by caspase-3. Caspases recognize specific peptide sequences containing an aspartic acid, and cleave these substrate proteins immediately following this aspartic residue. The tri- peptide sequence "VAD" is broadly recognized by all group 1 caspases. Z-VAD-FMK is a powerful, irreversible and cell permeable inhibitor for caspases. Z-DEVD-FMK is synthetic peptide inhibitor that irreversibly inhibits caspase-3 and related protease/caspase activity and blocks apoptosis. DEVD is the specific recognition sequence found in poly (ADP-ribose) polymerase (PARP). Z-LEHD-FMK is a powerful, irreversible and cell permeable inhibitor for caspase-9. The tetra-peptide sequence "LEHD" is preferentially recognized by caspase-9.

Caspase inhibitors are important tools in the investigation of many biologic processes utilizing whole cells, cell lysates, and *in vivo* systems. Caspase inhibitors act by binding to the active site of caspases [45]. Flowcytometric analysis of Thp-1 cells pretreated with Z-DEVD-FMK and Z-LEHD-FMK exhibited a sub-G1 peak Inhibition in DMQA induced cell death (Fig. 3.19)