11.1 Introduction

Overexpression of multiple signal mediator molecules including ROS has been implicated in pathogenesis of hepatocellular carcinoma (HCC). ROS participate in carcinogenesis by mutation of proto-oncogenes and tumor suppressor genes and subsequent activation of signal transduction pathways (Wu et al., 2006; Sosa et al., 2013). Tumor suppressor proteins repressively regulate cell cycle and/or promote apoptosis (Sherr, 2004). Tumor suppressor gene p53 and proto-oncogene BAX are most extensively studied genes known to modulate apoptosis (Trachootham et al., 2008; Trachootham et al., 2009). p53 prevents genetic mutation and genomic instability. Moreover, p53 is a transcription factor that regulates expression of many pro-oxidant and antioxidant genes. Downregulation and loss of p53 are associated with redox imbalance, increased production of ROS, mutagenesis and aggressive tumor growth (Trachootham et al., 2008; Trachootham et al., 2009; Suzuki and Matsubara, 2011). Activation of tumor suppressors could be a significant strategy for targeting cancer. Overexpression of pro-inflammatory enzyme COX-2 has been reported in a number of cancers including prostate, colorectal, breast, pancreatic and lung cancers (Ristimaki et al., 2002; Secchiero et al., 2005; Sobolewski et al., 2010). COX-2 has been suggested to play a role at different steps of cancer progression.

PKC is one of the signaling enzymes which is positively regulated by ROS and plays a crucial role in a variety of pathophysiological states including tumor progression. PKC may act as downstream effector of PI3K. PI3K mediated signaling cascade regulates cell proliferation, cell survival, differentiation and apoptosis (Maurya and Vinayak, 2015; Vanhaesebroeck et al., 2010).

HCC is one of major health threats and third leading causes among cancer related death worldwide (Raza and Sood, 2005; Villanueva et al., 2010). HCC is characterized by genetic alterations affecting multiple signaling cascades resulting in uncontrolled growth of hepatocytes. HepG2 cells display a large part of cellular functions similar to those of normal hepatocytes, such as expression of hepatocyte specific cell surface receptors and synthesis and secretion of plasma proteins (Zhao et al., 2013; Dehn et al., 2004; Roe et al., 1993). Because of high degree of morphological and functional differentiation, HepG2 cell line is a suitable model to study hepatocarcinogenesis and drug targeting (Goya et al., 2007).

Cancer cells survive through evading apoptosis or promoting proliferation, invasion and metastasis. Therefore, present study was aimed to analyze anticarcinogenic action of QUE in
Evaluation of anti-carcinogenic action of QUE by in vitro analysis in HepG2 cells

HepG2 cell line via regulation of ROS mediated proto-oncogenes PI3K and PKC as well as tumor suppressor p53. *In vitro* study of effect of QUE on ROS mediated proto-oncogenes PI3K and PKC as well as tumor suppressor p53 on HepG2 cells will be useful to dissect out the molecular mechanism of regulating signaling pathways by use of specific inhibitors. The result obtained will be useful to study regulation of lymphoma by QUE in DL ascite cell culture.

11.2 Results

11.2.1 QUE induces cytotoxicity and morphological alterations

Cytotoxicity of QUE in HepG2 cells was assessed by MTT assay, which measures mitochondrial dehydrogenase activity as an index of cell viability. Results revealed a dose dependent reduction of cell viability of HepG2 cells following QUE treatment. Cell viability significantly decreased by approximately 12.9, 22.5, 36.7, 49.2 and 60% with 20, 40, 60, 80 and 100µM QUE respectively (Fig 11.1). IC<sub>50</sub> of QUE was found to be approximately 80µM. Therefore, three doses of 40, 60 and 80µM QUE were selected for subsequent study.

![Figure 11.1 Effect of QUE on cell viability/cytotoxicity in HepG2 cells](image)

MTT assay after 24h of QUE treatment on HepG2 cells; data represent as mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups.

11.2.2 QUE induces morphological alterations

High degree of morphological differentiation in HepG2 cell line was employed as suitable parameter for drug targeting. Untreated HepG2 cells displayed normal and hexagonal shape. QUE treated HepG2 cells were observed to be round shaped, condensed and beaded as compared to untreated cells at 12 and 24h (Fig 11.2a, b). Dose dependant morphological alteration was observed with QUE treated HepG2 cells at both time points.
Evaluation of anti-carcinogenic action of QUE by in vitro analysis in HepG2 cells

Figure 11.2 Effect of QUE on morphological alterations in HepG2 cells
(a) Morphological changes after 12 h and (b) after 24 h of QUE treatment on HepG2 cells observed by Inverted Microscope

11.2.3 QUE attenuates ROS formation
Elevated level of ROS formation has been observed in different types of cancers including DL, which results in oxidative stress (Qian et al., 2005; Das and Vinayak, 2014). Therefore, effect of QUE on ROS level was analyzed as arbitrary fluorescence units/mL using green fluorescent dye, H$_2$DCFDA. ROS level was decreased approximately by 8.4, 29.5 and 44.5% with 40, 60 and 80µM QUE respectively (Fig 11.3).

Figure 11.3 Effect of QUE on ROS formation in HepG2 cells
Homogenates of HepG2 cells from 3 set of each group were pooled separately and used for total ROS measurement. Data represent as mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups

11.2.4 QUE downregulates phosphorylation of p85α
PI3K signaling cascade has been linked to cell proliferation, survival and differentiation. Effect of QUE was investigated on activation of regulatory subunit as phospho-p85α. Level of phospho-p85α was significantly decreased approximately by 21.4 and 41.9% with 60 and 80µM QUE respectively (Fig 11.4).
Evaluation of anti-carcinogenic action of QUE by in vitro analysis in HepG2 cells

Figure 11.4 Effect of QUE on phosphorylation of p85α in HepG2 cells
(a) Western analysis of phospho-p85α and β-actin (b) Densitometric scanning of phospho-p85α after normalization with β-actin; data represent mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups.

11.2.5 QUE attenuates total PKC activity
Total PKC activity was compared between control and QUE treated HepG2 cells in terms of phosphorylated PKC. Level of phosphorylated PKC was decreased, whereas that of non-phosphorylated PKC was increased after QUE treatment (Fig 11.5a, b). Decrease in activity of PKC was found to be approximately 29.7, 39.5 and 47.8% in HepG2 cells with 40, 60, 80μM QUE respectively (Fig 11.5c).

Figure 11.5 Effect of QUE on total PKC activity in HepG2 cells
(a) PepTag assay of PKC (b) Densitometric scanning of phosphorylated and non-phosphorylated band and (c) Total PKC activity; data represent as mean ± S.E.M. of three independent experiments. *, ** and *** denotes significant differences at the level of p < 0.05, p < 0.01 and p < 0.001 respectively, between QUE treated and control groups.

64
11.2.6 QUE downregulates PKCα protein expression

PKCα has been implicated with increased cell proliferation in different cell lines (Lee et al., 2012; Konopatskaya et al., 2010; Wu et al., 2008). Therefore, PKCα protein expression was examined to analyze role of QUE in cell proliferation of HepG2 cells. Level of PKCα was decreased upon QUE treatment in a dose dependent manner, which was approximately by 18, 26 and 41% with 40, 60 and 80µM QUE respectively (Fig 11.6).

![Figure 11.6](image)

**Figure 11.6 Effect of QUE on PKCα protein expression in HepG2 cells**

(a) Western analysis of PKCα and β-actin (b) Densitometric scanning of PKCα after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups

11.2.7 QUE induces p53 protein expression

Tumor suppressor p53 is most frequently inactivated in cancer (Suzuki and Matsubara, 2011). QUE was found to significantly induce level of p53 in HepG2 cells by approximately 18, 29, and 44% with 40, 60 and 80µM QUE respectively (Fig 11.7). Induced level of p53 suggests cancer preventive potential of QUE.

![Figure 11.7](image)

**Figure 11.7 Effect of QUE on p53 protein expression in HepG2 cells**

(a) Western analysis of p53 and β-actin (b) Densitometric scanning of p53 after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups
Evaluation of anti-carcinogenic action of QUE by in vitro analysis in HepG2 cells

11.2.8 QUE suppresses COX-2 protein expression

Inflammatory enzyme COX-2 is involved in cancer progression by regulating proliferation, apoptosis and angiogenesis (Ristimaki et al., 2002; Secchiero et al., 2005; Sobolewski et al., 2010). QUE was found to significantly downregulated protein expression of COX-2 by approximately 14.7 and 53.2% with 60 and 80µM QUE respectively (Fig 11.8).

**Figure 11.8 Effect of QUE on COX-2 protein expression in HepG2 cells**
(a) Western analysis of COX-2 and β-actin (b) Densitometric scanning of COX-2 after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. * and *** denotes significant differences at the level of p < 0.05 and p < 0.001 respectively, between QUE treated and control groups.

11.2.9 QUE induces BAX expression

BAX is one of the best known pro-apoptotic genes, downstream to p53. Expression of BAX was upregulated by approximately 1.37, 1.22, 1.44, 2.39 and 1.59 fold with 10, 20, 40, 60 and 80µM QUE respectively (Fig 11.9).

**Figure 11.9 Effect of QUE on BAX mRNA expression in HepG2 cells**
(a) RT-PCR of BAX and GAPDH (b) Densitometric scanning of BAX after normalization with GAPDH; M denotes 100 bp marker. Data represent as mean ± S.E.M. of three independent experiments. *** denote significant differences at the level of p < 0.001 between QUE treated and control groups.
11.3 Discussion

Reduced cell viability and morphological changes in HepG2 showing decreased cell volume, cell shrinkage and formation of cytoplasmic blebs indicated onset of apoptosis on QUE treatment. Untreated cells showed a high confluence of monolayer cells as compared to QUE treated cells. We have earlier reported reduced cell viability and improved longevity of DL mice by QUE treatment (Maurya and Vinayak, 2015). Excess ROS production provides a sustained oxidative microenvironment to cancerous cells and thus promotes cancer growth. Declined ROS level by QUE suggests its anticarcinogenic action.

Several stress activated proto-oncogenes are responsible for increased cell proliferation/cell viability and suppressed apoptosis (Sosa et al., 2013). Hyperactivated signaling of PI3K and PKC promotes cancer growth by loss of apoptosis and increased cell proliferation. In present study, ROS mediated PI3K-PKC signaling pathway was analyzed and its modulation by antioxidant QUE was validated in HepG2 cells.

PI3K controls a variety of biological responses such as induced cell proliferation, growth and cell survival. PI3K family have been divided into three classes (class I, class II and class III) based on structural features and lipid substrate preferences. Class IA PI3K is widely expressed and found to be hyperactivated in various cancers (Zhang et al., 2015; Faes and Dormond, 2015; Vanhaesebroeck et al., 2010). PI3K is a heterodimer, comprised of a p110α catalytic subunit and p85α regulatory subunit. The p85α subunit regulates catalytic subunit p110α. p85α stabilizes and inactivates kinase activity of PI3K at basal level whereas on activation it recruits PI3K to phospho-tyrosine residues of activated receptors (Vanhaesebroeck et al., 2010; Geering et al., 2007). Downregulation of phospho-p85α is correlated with inactivation of PI3K by decreasing tyrosine kinase activity (Cohen et al., 1990). Inhibition of PI3K activity is suggested through competitive inhibition of ATP binding site of p85α (Walker et al., 2000). Inactivation of PI3K activity by QUE suggests its key role in lowering survival of HepG2 cells. Our previous report of decreased phosphorylation of p85α with constant protein and mRNA expression in DL mice supports the findings (Maurya and Vinayak, 2015). Increased ROS production was presumably associated with PKC activation. Decreased PKC activity by QUE treatment as indicated by total phosphorylated PKC in comparison to non-phosphorylated PKC in HepG2 cells is correlated with downregulation of ROS level as well as PKC activity suggesting anticarcinogenic action of QUE.
PKC family comprises multiple isoenzymes with different distribution and functions (Rosen et al., 2012; Giorgi et al., 2010). PKCα is important isoenzyme to promote cell survival. Cellular depletion of PKCα is reported to inhibit cell growth and to induce apoptosis (Mishra and Vinayak, 2013; Mishra and Vinayak, 2014; Sharma and Vinayak, 2012). Decreased level of PKCα as well as total PKC in HepG2 cells after QUE treatment supported earlier findings (Gamet et al., 1999; Wu et al., 2009; Maurya and Vinayak, 2015). We have previously reported QUE to suppress DL via modulating conventional, novel and atypical PKC isoenzymes (Maurya and Vinayak, 2015). It is suggested that PKCα may function as an inhibitor of p53 in melanoma; however, no direct evidence of regulation of p53 by PKCα is available (Smith et al., 2012). p53 contributes to tumor suppression by arresting cell cycle at G1/S phase, and promoting apoptosis (Trachootham et al., 2008; Trachootham et al., 2009; Porebska et al., 2006). QUE elicited p53 level significantly in HepG2 cells in current study. This result is supported by our previous finding that QUE induces p53 level in DL cells (Maurya and Vinayak, 2015). Our results suggest that PKCα might regulate cell proliferation by inhibition of cell cycle at G1/S phase through upregulation of p21 involving p53. Further, p53 knockout mice have been associated with decreased BAX expression in several tissues (Miyashita and Reed, 1994). BAX is one of the established downstream targets of p53 that induces apoptosis. Enhanced BAX gene expression by QUE treatment reflects the potential of QUE to induce apoptosis in HepG2 cell. Promoter of BAX gene has been reported to contain several consensus sequences for p53 binding and is strongly activated by p53 (Miyashita and Reed, 1995). Hence, it may be suggested that QUE mediated modulation of p53 leads to apoptosis through BAX. Activated BAX causes release of cytochrome c and activation of caspases. Our previous report shows modulation of caspase 3 dependant activity of PKCδ by QUE leading to apoptosis (Maurya and Vinayak, 2015). Cytotoxicity exerted by QUE is mainly mediated through induction of apoptosis; however possibility of a small percentage of necrotic death could not be ignored (Sun et al., 2010; Haghiac and Walle, 2005).

Furthermore, p53 is also reported to cause a marked decrease in expression of pro-inflammatory enzyme COX-2 (Subbaramaiah et al., 1999). COX-2 has been suggested to play role at different steps of cancer progression. Over expression of COX-2 has been reported in cancer (Sobolewski et al., 2010; Chiu et al., 2010; Ristimaki et al., 2002; Secchiero et al., 2005; Sobolewski et al.,
Evaluation of anti-carcinogenic action of QUE by in vitro analysis in HepG2 cells

2010). Decreased level of COX-2 by QUE treatment indicates that QUE inhibits HepG2 cell growth via PKC mediated p53 activity in cooperation with BAX and COX-2.

All biochemical, molecular and cellular signaling indices analyzed exhibit dose dependent response of QUE treatment. However, BAX/GAPDH expression was lower with 80µM as compared to 60µM dose. Decreased response to 80µM dose of QUE might be due to possible interference(s) with other signaling molecules.

11.4 Summary

Overall result suggests that oxidative stress induces PI3K and PKC, which in turn attenuate p53 level in HepG2 cells. Reduced level of p53 promotes cell proliferation and attenuates apoptosis through deregulated expression of COX-2 and BAX, respectively. QUE attenuates generation of ROS and in turn, downregulates expression of PI3K, PKC and COX-2. Additionally, it enhances the expression of p53 and BAX. Overall, QUE attenuates cell proliferation and survival of HepG2 cells and elicits apoptosis by enhancing the expression of p53 and BAX through downregulation of ROS, PI3K, PKC and COX-2.

Schematic representation of anticarcinogenic mechanism of QUE in HepG2 cells