10.1 Introduction

Cells are constantly generating ROS during aerobic metabolism. Each cell is equipped with an extensive antioxidant defense system to combat excessive production of ROS. Oxidative stress arises in cells when generation of ROS overcomes cell’s natural antioxidant defenses (Nogueira and Hay, 2013; Sosa et al., 2013). Excessive ROS may be generated as a result of elevated metabolic processes or from toxic insult. High level of ROS induces oxidative damage in lipids, proteins and nucleic acids. There is a growing consensus that oxidative stress contributes to initiation of cellular malignancy and progression of cancer owing to genomic instability, especially due to mutations in proto-oncogenes and tumor suppressor genes and subsequent activation of signal transduction pathways (Sosa et al., 2013; Valko et al., 2006; Ivanova et al., 2013; Weinberg and Chandel, 2009; Naka et al., 2008; Trachootham et al., 2009; Coleman and Tsongalis, 2006). ROS is considered as modulator of signaling pathways mediated through ERK, MAPK, HIF, PI3K, PKC etc (Ivanova et al., 2013; Naka et al., 2008; Wu, 2006; Gopalakrishna and Gundimeda, 2002; Trachootham et al., 2008). Cancer cells are dependent on maintaining high level of ROS that allows pro-tumorigenic cell signaling. Thus, dependency of cancer cells on excessive ROS homeostasis may be potentially exploited to target them therapeutically.

PKC is a housekeeping enzyme; however under oxidative stress it promotes tumorigenesis and malignancy (Glasauer et al., 2014; Hu et al., 2011). PKC contains multiple cysteine residues that are oxidatively activated by ROS (Wu, 2006; Giorgi et al., 2010). Activation of PKC is prerequisite for NADPH oxidase dependent ROS generation (Paulsen and Carroll, 2010; Frey et al., 2006). Regulatory as well as catalytic domains of PKC are differentially activated which regulate different downstream pathways. Oxidation at NH$_2$ terminal regulatory domain activates PKC, whereas oxidation at COOH terminal inactivates PKC (Wu, 2006). Isoenzymes of PKC are divided into three categories namely classical (α, β and γ), novel (δ, ε, η and θ) and atypical (ζ, ι, and λ). Different isoenzymes of PKC may exert similar or opposite cellular effects by differential coupling to signaling pathways (Talior et al., 2005). PKCα and PKCδ are ubiquitously expressed. Upregulation of PKCα is correlated with an increased cell proliferation in various cancers including lymphoma (Caino et al., 2009; Mishra and Vinayak, 2011; Lee et al., 2012; Konopatskaya and Poole, 2010). However, PKCδ is a multifunctional kinase implicated in regulation of cell death (Wu et al., 2008; Zhao et al., 2012). Different apoptotic stimuli have been shown to induce caspase 3 dependant cleavage of PKCδ, resulting in generation of a
constitutively active catalytic fragment which has been implicated in its pro-apoptotic function (Reyland et al., 2007; Ghayur et al., 1996; Kanthasamy et al., 2008).

Elevated ROS level may be modulated by antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, etc. and non-enzymic antioxidants like ascorbate, tocopherols, tocotrienols, carotenoids, natural flavonoids, melatonin, etc. (Nogueira and Hay, 2013; Zhou et al., 2013; Saeidnia et al., 2013; Samoylenko et al., 2013). The objective of present work is to investigate role of QUE in modulation of ROS as well as its effect on PKC isoenzymes especially PKCα and PKCδ, and their downstream signaling pathways in DL mice.

10.2 Results

10.2.1 QUE decreases total ROS level

Elevated level of ROS has been observed in different types of cancer including Dalton’s lymphoma, which results in oxidative stress. Therefore, effect of QUE on ROS level was analyzed using green fluorescence dye H$_2$DCFDA. Results obtained from fluorescence images show gradual downregulation of green fluorescence intensity after QUE treatment suggesting regression of ROS level in ascite cells (Fig 10.1).

![Figure 10.1 Effect of QUE on total ROS level in ascite cells of lymphoma bearing mice](image)

Fluorescence image of ascite cells by H$_2$DCFDA staining. Ascite cells of all six animals of each group were pooled separately and used for total ROS measurement. DL, DLT25, DLT50 and DLT75 represents Dalton’s lymphoma bearing group, Dalton’s lymphoma bearing group treated with 25, 50 and 75mg QUE/Kg BW respectively

10.2.2 QUE inhibits PKC activity

Total PKC activity was found to be decreased in QUE treated DL mice as compared to untreated DL mice. PKC activity was suppressed by QUE in dose dependent manner. All three doses of QUE suppressed PKC activity significantly, approximately by 17.7%, 28.6% and 51.1% with dose of 25, 50 and 75mg QUE/Kg BW respectively (Fig 10.2).
Modulation of PKC signaling by QUE

10.2.3 Protein level of PKCα
Level of PKCα was examined in total protein lysate of ascite cells of DL and QUE treated DL mice by Western blot analysis. All doses of QUE significantly downregulated protein level of PKCα, approximately by 15%, 16% and 36% with dose of 25, 50 and 75mg QUE/Kg BW respectively (Fig 10.3a).

10.2.3.1 QUE declines level of membrane associated PKCα
Inactive PKC resides in cytosol and it is recruited to membrane on activation. This recruitment triggers signaling pathways leading to cell proliferation. Therefore, effect of QUE was analyzed on level of membrane associated PKCα. Densitometric scanning shows decreased level of membrane associated PKCα with QUE treatment. QUE significantly downregulated PKCα.
approximately by 19%, 36% and 43% in membrane fraction of ascite cells with dose of 25, 50 and 75mg QUE/Kg BW respectively (Fig 10.3b).

10.2.4 QUE upregulates PKCδ

PKCδ is known to be pro-apoptotic protein and its decreased level has been reported in many cancers. Cleavage fragment (CF) of PKCδ promotes caspase 3 dependent apoptosis. Therefore, effect of QUE on protein level of PKCδ was analyzed by Western blotting. QUE treatment upregulated CF of PKCδ approximately by 2.02 and 2.34 fold, with dose of 50 and 75mg QUE/Kg BW respectively, which supports pro-apoptotic activity of QUE in DL mice (Fig 10.4). However, 25mg QUE/Kg BW did not show any up regulation of PKCδ.

![Western analysis of PKCδ and β-actin](image)

**Figure 10.4 Effect of QUE on protein level of PKCδ**
(a) Western analysis of PKCδ and β-actin (b) Densitometric scanning of PKCδ after normalization with β-actin; data represent as mean ± S.E.M. of three independent experiments. *** denotes significant differences at the level of p < 0.001 between DL and QUE treated DL groups

10.2.5 Effect of QUE on transcriptional expression of PKC isoenzymes

Effect of QUE was analyzed on transcriptional expression of classical, novel and atypical PKC isoenzymes. Treatment of QUE to DL mice differentially modulates the expression of PKC isoenzymes. It was found to regulate classical PKC signaling by downregulating expression of PKCα, PKCβ and upregulation of PKCγ expression. QUE significantly downregulated mRNA level of PKCα approximately by 17%, 9% and 25% and PKCβ by 44%, 29% and 36%, whereas it upregulated expression of PKCγ approximately by 17%, 18% and 23% with dose of 25, 50 and 75mg QUE/Kg BW respectively. Novel isoenzymes PKCδ were upregulated by QUE approximately by 74%, 62% and 55% with dose of 25, 50 and 75mg QUE/Kg BW respectively. However, PKCη and PKCε expression was decreased by QUE treatment with 75 and 25mg QUE/Kg BW respectively. QUE modulates atypical PKC through downregulation of PKCζ and upregulation of PKCι in ascite cells (Fig 10.5).
Modulation of PKC signaling by QUE

Figure 10.5 Effects of QUE on mRNA expression of PKC isoenzymes in ascite cells of lymphoma bearing mice
(a) Expression of classical PKC (PKCα, β and γ) (b) Expression of novel PKC (PKCζ, η and ε), and (c) Expression of atypical PKC (PKCξ and ι); 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 DL and QUE treated DL groups.

10.3 Discussion

Deregulated signaling of PKC promotes cancer growth by loss of apoptosis and increased cell proliferation. Oxidative stress modulates PKC activity. QUE in this context may play a key role to overcome oxidative stress in cancer cell. QUE has been shown to suppress DL growth via downregulating cell proliferation and energy metabolism (Maurya and Vinayak, 2015). QUE is found to downregulate total ROS level in ascite cells progressively with increase in QUE dose. The result indicates that reduced level of ROS promotes suppression of tumor growth. Further, reduced ROS level is correlated with downregulation of total PKC activity. PKC activation is differentially regulated by cellular oxidants (Giorgi et al., 2010; Gopalakrishna and Gundimeda, 2002). Antioxidant gets oxidized during their course of action, and oxidized form of antioxidants bind to catalytic domain causing inactivation of PKC.

PKC family consists of multiple isoenzymes with different distribution and functions (Rosen et al., 2012; Konopatskaya and Poole, 2010; Gutcher et al., 2003). PKCα has emerged as an important isoenzyme to promote cell survival. Cellular depletion of PKCα is reported to induce apoptosis (Li et al., 2013; Kang 2014; Mauro 2002; Martiny and Fabbro, 2007; Lahn et al., 2004;
Zhang et al., 2005). Although, mechanism behind PKCα preventing apoptosis is still a matter of debate, Bcl-2 and Raf-1 has been shown to mediate the anti-apoptotic function of AKT in regulation of PKCα. In present study, decreased level of PKCα in total lysate of ascite cells of DL mice after QUE treatment is in accordance with reduced total PKC activity. The result supports earlier findings showing flavonoids to downregulate PKC (Gamet et al., 1999; Granado et al., 2008; Wu et al., 2009). Further, decrease in membrane associated PKCα by QUE suggests its anti-proliferative activity.

Different apoptotic stimuli such as TNF-α, UV radiation induce cleavage of PKCδ, resulting in generation of a constitutively active catalytic fragment which has been implicated in its apoptotic function (Zhao et al., 2012; Basu and Pal, 2010; Zhu et al., 2010). PKCδ is reported to cause DNA damage induced apoptosis in HeLa cells (Basu et al., 2001). Upregulated level of CF of PKCδ in QUE treated DL mice correlates with activation of caspase 3. The result indicates that QUE promotes apoptosis in DL mice via PKCδ mediated caspase 3 activation. PKCδ null mice develop normally, suggesting that PKCδ is not required for normal cell proliferation (Jackson and Foster, 2004). PKCδ deficient mice were found resistant to cell death (Zhao et al., 2012). Our results indicate that QUE suppresses uncontrolled cell growth as well as cell survival via improving apoptotic potential.

Transcriptional gene expression of PKC isoenzymes suggest that QUE suppresses DL ascites cell growth via modulating conventional, novel and atypical PKC pathways. Oxidative stress plays an important role to maintain high proliferation rate of cancerous cells. Downregulated expression of PKCα by QUE treated DL mice as compared to untreated shows its antioxidant property. PKCα suppresses cell death during oxidative stress by phosphorylating Bcl-2, whereas PKCβ supports progression of lymphoma by inducing cell proliferation and angiogenesis (Murray et al., 2009; Ruvolo et al., 1998; Popla et al., 2001). PKCβ signals via phosphorylating AKT protein which in-turn phosphorylates GSK3, thus inducing cell proliferation (Kawakami et al., 2004). Decreased expression of PKCβ in QUE treated DL mice may be related to low phosphorylation of AKT and GSK3. It has been reported that PKCι mediates activation of NF-E2-related factor 2 (Nrf2) which acts as transcription factor and regulates synthesis of antioxidative proteins (Numazawa et al., 2003). Upregulated PKCι expression by QUE suggests that QUE reduces oxidative stress. Under oxidative stress, cell stimulates production of stress activated proteins that help cell to survive in cancer microenvironment. A170 is an oxidative stress inducible protein which has a structural domain to interact with PKCζ (Kim et al., 2005). PKCζ is observed to be
reduced with QUE treatment to DL mice. Reduced oxidative stress by QUE may inhibit cell survival via PKCζ pathway.

10.4 Summary

Overall results show that QUE suppresses cell proliferation, survival and induces apoptosis by modulating ROS mediated PKC signaling. The findings may provide a base for using QUE as a chemotherapeutic drug in prevention of cancer.