Eicosanoids serve as intermediates in growth factor signaling pathways. Most tumor cells produce AA metabolites and these compounds have been found to modulate a wide range of biological factors that induce growth and invasiveness of tumors (Hong et al, 1999). It is well-established that human malignancies frequently overexpress COX-2 and produce high levels of the COX-2 metabolite, PGE$_2$ (Arunasree et al, 2008; Krysan et al, 2005; Subbaramaiah et al, 2002; Kirschenbaum et al, 2001; Fujita et al, 1998; Soslow et al, 2000; Huang et al, 1998; Wolff et al, 1998; Kutchera et al, 1996). COX-2 overexpression has since been found in many human cancers including breast (Half et al, 2002; Soslow et al, 2000), esophageal (Li et al, 2000; Zimmermann et al, 1999), lung (Hosomi et al, 2000; Hida et al, 1998), prostate (Uotila et al, 2001; Yoshimura et al, 2000), bladder (Ristimaki et al, 2001; Mohammed et al, 1999), skin (Tang et al, 2001; Neufang et al, 2001) and pancreas (Basu et al, 2004; Tucker et al, 1999; Molina et al, 1999). LOX activation may be involved in both pro- and antitumorigenic effects (Muller et al, 2002; Shureiqui et al, 2001). Several line of evidences indicate 12-lipoxygenase (12-LOX) as a key regulator of human cancer development. Overexpression of 12-LOX has been detected in a variety of tumors including breast, renal, pancreatic, and prostate cancers (Nie et al, 2006; Yoshimura et al, 2004; Natarajan et al, 1998; Gao et al, 1995).

Many of the AA metabolites are clastogenic and act as tumor promoters in murine models of skin carcinogenesis and are induced following UVB irradiation (Bickers et al, 2006). Elevated levels of eicosanoids (prostaglandins and leukotrienes) have been shown to be associated with a wide array of dermatological diseases, such as psoriasis, UV-induced erythema, and contact sensitivity (Wang et al, 2001). Besides their role as indicators of neoplastic
development, eicosanoids also act as reporters of skin irritation (Marks et al, 2000). A number of studies indicate overexpression of 12-R-LOX in skin cancers. However, a comprehensive study on the metabolism of AA via the LOX and COX pathways on epidermoid carcinoma are lacking. In the present study the expression of various genes involved in LOX and COX pathways were analysed in human epidermoid carcinoma cell line, A431.

5.1 12-R-LOX and COX-2 overexpressed in A431 cells compared to NIH3T3 cells

Recent investigations have shown that tumors of different histogenesis considerably differ in the metabolism of AA (Kudryavtsev et al, 2005). Thus, the expression and role of AA metabolizing enzymes would be different in different cancers and thus it is very important to know the expression profile of AA metabolizing enzymes first. Therefore, our first question was to investigate the expression of various AA metabolizing enzymes in A431 cells. This study clearly showed the overexpression of 12-R-LOX and COX-2 in A431 cells with no expression in the normal skin fibroblasts suggesting their significance in the pathology of skin cancer. The expression of 12-S-LOX on the other hand, was slightly higher in A431 cells when compared to the normal 3T3 cells. This forms the first report on the overexpression of 12-R-LOX in epidermoid carcinoma cell line, A431.

In recent years, it has become clear that multiple forms of lipoxygenases are expressed in skin of mice and humans (Virmania et al, 2002; Krieg et al, 1998; Boeglin et al, 1998; Sun et al, 1998; Jisaka et al, 1997; Krieg et al, 1995; Chen et al, 1994). In mouse skin, there is a TPA-inducible 8-lipoxygenase and at least three isoforms of 12-lipoxygenase with various designations; a `platelet type’ 12-lipoxygenase (P-12LO), epidermal-type 12- lipoxygenase (e-12LOX-1), and a 12-(R)-lipoxygenase (e-12LOX-2). P-12 LOX expression has been demonstrated in epidermis \textit{in vivo} and in cultured keratinocytes (Krieg et al, 1995; Chen et al,
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In neoplastic epidermal preparations, the predominant expression of P-12 LOX was reported (Krieg et al., 1995) and in human skin immunohistochemical evidence was provided for increased expression in germinal layer keratinocytes in psoriatic scales (Hussain et al., 1994). A constitutive expression of p12-S-LOX was previously demonstrated both in mouse and human epidermis (Heidt et al., 2000; Krieg et al., 1995; Chen et al., 1994). In addition, p12-S-LOX-deficient mice have been shown to be less sensitive for tumor induction according to the initiation-promotion protocol (Muller et al., 2002; Virmani et al., 2001). The discrete expression of 12-R-LOX on day 16.5 of fetal life of mouse embryos indicates a critical function during embryonic development of skin (Sun et al., 1998). A preferential expression of 12-R-LOX and e-LOX-3, in stratifying epithelia was observed, which exhibited only weak signals in all other tissues (Heidt et al., 2000).

8-LOX is also constitutively up regulated in skin tumors (Muga et al., 2000; Furstenberger et al., 1991). It is a murine homolog of human 15S-LOX-2 and metabolizes AA and LA to 8-HETE and 9-HODE, respectively (Burger et al., 1999; Jisaka et al., 1997; Furstenberger et al., 1991; Gschwendt et al., 1986). 8S-LOX plays a role as a pro-differentiating, anti-tumorigenic, and tumor suppressing gene in mouse skin carcinogenesis (Kim et al., 2005).

While COX-2 expression in normal skin was usually very low and restricted to regions of differentiated epidermis (Muller-Decker et al., 1999; Buckman et al., 1998), studies on mouse and human skin carcinogenesis revealed that overexpression of COX-2 contributes to the development of skin cancers (Higashi et al., 2000; Muller Decker et al., 1999, 1998; Buckman et al., 1998). COX-2 overexpression occurs not only in the tumor cells but also in the tumor vasculature (Edelman et al., 2008; Masferrer et al., 2000). COX-1 is constitutively expressed in most tissues including mouse and human skin (Neumann et al., 2007; Muller-Decker et al., 1999, 1998). COX-1 and COX-2 can play very different roles in skin
tumor development, depending on the nature of the agents used to induce tumors. While COX-1 is important in phorbol ester promotion (Tiano et al., 2002), it does not contribute to UV carcinogenesis (Pentland et al., 1999). COX-2, on the other hand, is a prerequisite for skin tumor development with both protocols. COX-2 thus remains a viable target for topical skin cancer prevention (Fischer et al., 2007). There is also evidence of the aberrant expression of COX-2 in tumors of non-epithelial origin, such as malignant melanomas (Muller Decker et al., 2007; Denkert et al., 2001). The targeted disruptions of the genes encoding either COX-1 or COX-2 (Langenbach et al., 1995; Morham et al., 1995) lead to altered epidermal differentiation and reduced skin tumorigenesis (Tiano et al., 2002). The constitutive expression of COX-2 in murine epidermis confers both alopecia and a skin tumor resistance phenotype. These unexpected findings suggest that the role of PGs in normal skin physiology and skin tumor development is complex and not well understood (Bol et al., 2000).

In the present study also predominant expression of COX-2 was observed in human epidermoid carcinoma cell line, A431 but no expression in the normal 3T3 cells, suggesting a possible role for COX-2 in regulating the growth. In the light of dominant expression of 12-R-LOX and COX-2 in skin cancer cells, (A431), but not in normal 3T3 cells, further studies were undertaken to analyse the effect of metabolites of 12-LOX (12-(R)-HETE and 12-(S)-HETE) and COX-2 (PGE\textsubscript{2}) as well as inhibitors of 12-LOX (Baicalein) and COX-2 (Celecoxib).

5.2 12-LOX and COX-2 regulate the growth of A431 cells

The specific 12-LOX inhibitor baicalein and COX-2 inhibitor, celecoxib, alone or in combination, effectively suppressed the growth of A431 cells as indicated by the lowered incorporation of thymidine into DNA in comparison to that in untreated cells. 12-(R)-HETE and PGE\textsubscript{2} increased the incorporation of thymidine in a dose dependent manner. The addition of exogenous 12-(S)-HETE,
on the other hand, increased the incorporation of thymidine into DNA in a dose dependent manner which was in accordance with the earlier report (Kudryavtsev et al, 2005). Similar increase in the proliferation of prostate (Pidgeon et al, 2002) and gastric cancer cells (Hong et al, 2001) was reported in response to 12-(S)-HETE. Very limited data exist regarding 12-(R)-HETE and tumorigenesis. 12-(R)-HETE was shown to induce the proliferation of colon cancer cells in vitro (Bortuzzo et al, 1996). PGE\(_2\) is known to promote tumor-cell proliferation (Ye et al, 2005). 12(S)-HETE has been found as a determinant of metastastic potential of B16a melanoma cells and other tumor cells such as Clone A and Walker 256 (Chen et al, 1994; Liu et al, 1994). The above studies clearly demonstrate the role of 12-LOX and COX-2 in the regulation of A431 cell proliferation. However, the molecular mechanisms underlying the regulation of A431 cell proliferation and the mode of cell death by 12-LOX and COX-2 pathways are not clear. So our next step was to elucidate the molecular mechanisms of apoptosis induced by baicalein and celecoxib.

Apoptosis or programmed cell death is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues. Deregulation of this pathway occurs in cancer, autoimmune diseases, and neurodegenerative disorders. Two major apoptotic pathways have been identified thus far, the death receptor-mediated (extrinsic) and mitochondria-mediated (intrinsic) pathways (Green et al, 2000; Strasser et al, 2000). The former is initiated by triggering of cell surface death receptors of the tumor necrosis factor receptor superfamily by their ligands and the activation of caspase-8. The latter is initiated by specific chemicals, growth factor deprivation, or irradiation. It involves cytochrome c release from mitochondria and activation of capase-9. The Bcl-2 protein family plays an important role in regulating apoptosis. This family includes bad and bax proteins, which are presumed to form pores in the outer mitochondrial
membrane through which cytochrome c can be released into the cytosol (Kluck et al, 1997). Anti apoptotic Bcl-2 family members such as Bcl-2 and Mcl-1 appear to provide negative regulation of apoptosis by impeding bad/bax induced pore formation and cytochrome c release in response to death-inducing stimuli, thereby preventing apoptosis. In contrast, the proapoptotic Bcl-2 family members such as bax, bad, bik and bak promote cytochrome c release and enhance apoptosis (Yin et al, 2000; Marzo et al, 1998). Once caspase-8 and caspase-9 are activated, they can further activate the downstream effector caspases, including caspase-3, caspase-6, and caspase-7, which can then cleave their respective substrates and induce characteristic apoptotic changes. Induction of apoptosis is the characteristic of chemotherapy and irradiation therapy of human cancers. The ability to induce apoptosis in tumor cells is a very attractive feature of antitumor agents (Houghton et al, 1999; Rioux et al, 1998). Caspases exist in cells in an inactive zymogen form called procaspases and can be activated by proteolytic cleavage to produce active caspases. Activated caspases can cleave their substrates after specific aspartic acid residues. Caspase-3 is the most important among these proteases. The substrates for caspase-3 include PARP, retinoblastoma protein, actin, and laminin (Tong et al, 2002; Rosen et al, 1997).

5.3 12-LOX and COX-2 inhibition induce apoptosis in A431 cells via the intrinsic pathway

The mode of cell death induced by the baicalein (12-LOX inhibitor) and celecoxib (COX-2 inhibitor) was evaluated by analyzing cell and nuclear morphology, tunnel assay and Propidium iodide staining. These findings demonstrate that baicalein and celecoxib induce apoptosis by activating the intrinsic death pathway involving alterations in the mitochondrial membrane potential, decrease in the Bcl-2/Bax ratio, release of cytochrome c, activation of caspase 3 and PARP cleavage. Baicalein (BE) has been reported to induce apoptosis and inhibit proliferation in several cell types including gastric, pancreatic,
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and prostate cancer cells via the inhibition of 12-LOX (Tong et al., 2002; Pidgeon et al., 2002; Wong et al., 2001). In MCF-7 cells, BE suppressed 17β-estradiol-induced transactivation, and induced apoptosis (Po et al., 2002. It has been reported that baicalein could make an S-phase arrest in cell cycle of lung squamous carcinoma CH27 cells (Lee et al., 2005) and induce apoptosis in many human cancer cell lines, e.g. hepatoma cells Hep3B and HepG2 (Chang et al., 2002), pancreatic cancer cells MiaPaCa-2 and AsPC-1 (Tong et al., 2002), breast cancer cells MCF-7 (Tong et al., 2002) and prostate cancer cell lines (Chen et al., 2001). Although several biological activities of BE have been reported, intracellular molecules involved in modulation of apoptosis induced by baicalein are still undefined (Chow et al., 2006). A recent report demonstrated that baicalein induced a mitochondria-dependent caspase-3 and caspase-9 activation, and consequently led to apoptotic cell death in human myeloma cells (Ma et al., 2005). Treatment with highly selective COX-2 inhibitors results in a reduction in the size and number of colonic lesions in several animal models (Kawamori et al., 1998) and suppression of carcinogenesis of human colon, prostate and esophageal cancers (Zimmermann et al., 1999). The mechanism by which COX-2 inhibitors suppress carcinogenesis is attributed to its modulation of prostanoid production which affects cell proliferation, tumor growth and immune responsiveness. PGE$_2$, a major product of COX-2, induces bcl-2 expression and inhibits apoptosis and, conversely, that COX-2 inhibitors induce apoptosis (Zimmermann et al., 1999; Sheng et al., 1998a). While the involvement of prostanoids in carcinogenesis is clear, another study showed that selective inhibition of COX-2 activity suppresses the production of PGE$_2$ but does not alter the progress of the morphological transformation of rat fibroblasts (Sheng et al., 1998b), suggesting the existence of distinct signal pathways, PGE$_2$-dependent and PGE$_2$- independent, in the regulation of carcinogenesis (Higashi et al., 2000). Diclofenac induced apoptosis in three of four
cutaneous SCC cell lines as shown by DNA fragmentation and caspase activation (Fecker et al., 2007). Similarly inhibition of COX-2 was shown to inhibit growth of a human cutaneous squamous cell carcinoma cell line (Thompson et al., 2001) and induce apoptosis in K562 cell line (Subhasini et al., 2005). In addition to the induction of apoptosis, celecoxib has the potential to inhibit tumor angiogenesis and metastasis and hence might serve as an ideal agent for long-term maintenance therapy. COX/LOX dual inhibition has been evaluated in several experimental models and found to be potentially beneficial (Edelman et al., 2008; Cianchi et al., 2006; Ye et al., 2005; Wenger et al., 2002). In the present study we show that both 12-LOX and COX-2 inhibition in the combination treatment is more effective in inducing apoptosis when compared to either baicalein or celecoxib alone. Interestingly celecoxib could inhibit the RNA expression of 12-R-LOX which might be one of the reasons for the additive effect of baicalein and celecoxib observed in the present study. This forms the first report for the combination effect of LOX and COX inhibitors on epidermoid carcinoma.

The proposed mechanism of action of baicalein and celecoxib in inducing apoptosis in A431 cells is outlined below (Figure 31).
5.4 12-LOX and COX-2 inhibition induce apoptosis via the inactivation of ERK/AP-1 and Akt/NF-κB pathway

The foregoing studies clearly demonstrate the induction of apoptosis by the inhibition of either 12-LOX by baicalein or COX-2 by celecoxib or by their combined inhibition in A431 cells by intrinsic death pathway involving alterations in mitochondrial membrane potential. However, it is not clear how these effects are mediated by baicalein and celecoxib. The signaling pathways that govern cell proliferation, survival and oncogenesis are of prime interest in cancer biology (Rayet et al, 1999). Most of the signals for survival trigger growth factor receptors that activate the ERK and PI3K/Akt pathways and promote cell growth (Jin et al, 2007; Kennedy et al, 1997; Xia et al, 1995). The mitogen-activated protein kinase (MAPK) family consists of extra cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38MAPK, which are involved in mediating the processes associated with cell growth, survival and death (McCawley et al, 1999; Wada et al, 2004). JNK and p38 MAPK pathways are activated in response to
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chemicals and environmental stress (Davis et al, 2000; Nagata et al, 1999; Roulston et al, 1998; Minden et al, 1997), while the ERK cascade is activated by mitogenic stimuli, such as growth factors, cytokines, and phorbol esters, and is critical for proliferation and survival (Johnson et al, 2002; Chang et al, 2001). However, ERK signaling has been suggested to be proapoptotic in cells undergoing apoptosis (Choi et al, 2003; Xiao et al, 2002; Bacus et al, 2001; Wang et al, 2000). Recent evidence indicates that the MAPK family protein kinases are important mediators of apoptosis induced by stressful stimuli (Johnson et al, 2002; Chang et al, 2001). Among MAPK subfamilies, ERK is controversial in its role in cell death. While some studies showed that the ERK activation mediates survival response that counteracts cell death, other studies reported that the ERK activation is associated with apoptotic signaling pathways (Yang et al, 2007; Choi et al, 2003; Xiao et al, 2002; Bacus et al, 2001; Wang et al, 2000).

In highly specialized cells, which participate in the constant renewal of the skin epithelium, a basal level of ERK activity is absolutely indispensable for normal homeostasis (Bourcier et al, 2006). Given the role of ERK in the development of hyperproliferative skin lesions (Hasse et al, 2002), we asked whether alteration in ERK activity could play a role in apoptosis mediated by baicalein and celecoxib. The data presented here show that baicalein and celecoxib induce apoptosis in parallel with the inactivation of ERK in A431 cells. Similar down regulation of ERK activity associated with the inhibition of proliferation and induction of apoptosis was reported in gastric cancer (Chen et al, 2008) and primary AML blasts (Lunghi et al, 2003). The metabolites 12-(R)-HETE, 12-(S)-HETE and PGE$_2$ on the other hand increased the p-ERK levels and prevented apoptosis. Also, PGE$_2$ was shown to stimulate ERK kinase pathway in NSCLC cells (Krysan et al, 2005) and 12-(S)-HETE to promote phosphorylation of ERK in A431 cells (Szekeres et al, 2000). There are no reports on 12-(R)-HETE and ERK activation. Since it has been

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reported that down regulation of phosphorylated ERK protein expression level is consistent with inhibition of ERK activation (Vial et al, 2005), blockage of the function of ERK via the inhibition of 12-LOX and COX-2 pathways might be associated with baicalein and celecoxib induced apoptosis.

The phosphoinositide 3-kinase (PI3K)/Akt pathway is activated in a wide variety of cancers and results in enhanced resistance to apoptosis through multiple mechanisms. Inhibition of PI3K decreases cell survival and enhances the effects of chemotherapeutic drugs in many types of cancer cells (Jin et al, 2007; Wang et al, 2002; Hu et al, 2002; Asselin et al, 2001). Akt, also known as PKB (protein kinase B) (Bellacosa et al, 1991; Coffer et al, 1991) is a serine/threonine protein kinase that has been shown to regulate cell survival signals in response to growth factors, cytokines, and oncogenic Ras (Downward et al, 1998; Franke et al, 1997; Marte et al, 1997). Akt becomes activated via the phosphoinositide-3-OH kinase (PI3K) pathway (Downward et al, 1998; Fruman et al, 1998; Hawkins et al, 1997) and by other upstream kinases, including the Ca2+ and calmodulin-dependent protein kinase (Yano et al, 1998). Akt inhibits cell death pathways by directly phosphorylating and inactivating proteins involved in apoptosis, including Bad, pro caspase 9, and members of the Forkhead transcription factor family (Madrid et al, 2000; Brunet et al, 1999; Tang et al, 1999; Kops et al, 1999; Cardone et al, 1998; Datta et al, 1997; del Peso et al, 1997). The Akt signaling pathway, however, has been increasingly documented as a prime determinant of tumor promotion and progression in several cell types, including skin (Segrelles et al, 2002). Consequently, Akt-dependent deregulation of the apoptotic response to UVB could be a key event in the multistep process of skin carcinogenesis (Decraene et al, 2004).

In the present study the level of phosphorylated Akt was decreased in A431 cells after treatment with celecoxib. These findings are similar to earlier
reports where celecoxib treatments showed antiproliferative, antiangiogenic, and proapoptotic effects by decreasing PI3K/Akt phosphorylation in metastatic breast cancer cells (Basu et al, 2004). The regulation of Akt with baikalein treatment is not well understood. The results were further validated by the use of ERK specific inhibitor (U0126) and PI3 Kinase inhibitor (Wortmanin) and the metabolites of 12-LOX and COX-2. U0126 and Wortmanin pretreatment sensitized the cells to baikalein and celecoxib resulting in increase in caspase-3 activity and massive apoptosis suggesting the role of these two survival pathways in A431 cells. The metabolites 12-(S)-HETE, 12-(R)-HETE and PGE₂, on the other hand, increased the phospho ERK and phospho Akt levels suggesting that they can stimulate cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways in A431 cells. There are earlier reports where 12-(S)-HETE stimulates cancer growth through activation of p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways in human pancreatic cancer cells (Ding et al, 1999a; Ding et al, 1999b; Tong et al, 2002). PI 3-kinase pathway is an important signaling pathway for 12-LOX to increase VEGF expression in human prostate cancer cells. Increased 12-LOX expression or activity leads to the increased formation of 12(S)-HETE, which subsequently activates PI 3-kinase (Szekeres et al, 2000; Nie et al, 2006).

Akt is frequently activated in a wide variety of human cancers and has been shown to confer resistance to conventional cancer therapies. Hence, there is considerable rationale for targeting the Akt pathway to develop anti-cancer drugs. Interest in the role of Akt in cancer has increased enormously over the past decade, and it is now evident that activation of the Akt pathway is one of the most common molecular alterations in human malignancy. Importantly, many consequences of hyperactive Akt signaling are considered as hallmarks of cancer (Altomare et al, 2005). 12-(S)-HETE, 12-(R)-HETE and PGE₂ activate the ERK
and Akt levels whereas baicalein and celecoxib treatment results in their decrease. Baicalein or celecoxib or their combination with U0126 may become powerful anticancer agents for the treatment of human squamous carcinoma.

Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) are key transcription factors that orchestrate expression of many genes involved in inflammation, embryonic development, lymphoid differentiation, oncogenesis, and apoptosis (Fujioka et al., 2004; Li et al., 2002; Shaulin et al., 2002). Nuclear factor kappa B (NF-κB) is a pleiotropic transcriptional factor involved in the inducible expression of a wide variety of genes, particularly those that promote cell growth and survival and that protect cells from apoptotic death stimuli (Woods et al., 2002; Chen et al., 1999). NFκB might be involved in keratinocyte transformation and skin carcinogenesis (Budunova et al., 1999; Pandolfi et al., 1992). It has been demonstrated that epidermal inflammation and hyperplasia play a critical role in skin tumor promotion and NF-κB is one of the well-known mediators of these effects (Ouyang et al., 2006; Budunova et al., 1999). Continuous activation of NF-κB factors is also emerging as a hallmark of various types of solid tumors, including breast, ovarian, colon, pancreatic, thyroid, bladder and prostate carcinomas as well as melanomas (Chinenov et al., 2001). The present study clearly demonstrates a regulatory role for COX-2 and 12-LOX in the activation of NF-κB as evidenced by its down-regulation with baicalein and celecoxib and up regulation with 12-(R)-HETE, 12-(S)-HETE and PGE₂ treatments. Baicalein inhibited the survival of primary myeloma cells, especially MPC-1 immature myeloma cells in vitro, and induced apoptosis in myeloma cell lines through the down regulation of IkB- α phosphorylation (Ma et al., 2005). NF-κB activation has been also identified in squamous cell carcinomas of the head and neck. Inhibition of NF-κB activity in these tumors inhibits cell survival and tumor growth. In solid tumors, high levels of c-Rel have been found in non-small cell lung carcinoma.
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(Mukhopadhyay et al., 1995) and breast cancer (Dolcet et al., 2005; Sovak et al., 1997). PI3K/Akt signaling has been proposed to induce NF-κB-mediated upregulation of COX-2 following IκB phosphorylation and degradation (Wu et al., 2005; Sheu et al., 2005; St-Germain et al., 2004; Chang et al., 2002).

The activator protein 1 (AP-1) and MAPK signaling pathways are believed to play an important role in cancer chemoprevention and chemotherapy due to their involvement in tumor cell growth, proliferation, apoptosis, and survival. The transcriptional factor activator protein 1 (AP-1) has been proposed to play important roles in carcinogenesis and cancer development (Amit et al., 2003; Oya et al., 2003; Bremner et al., 2002; Shaulian et al., 2002; Heiss et al., 2001; Hsu et al., 2000). AP-1 activity can be regulated by several mechanisms including the activation of the mitogen-activated protein kinase (MAPK) pathways (Xu et al., 2005). ERK activation usually leads to elevated AP-1 activity via c-fos induction. This results in increased synthesis of c-fos, which upon translocation to the nucleus dimerizes with the pre-existing Jun proteins to form AP-1 dimers (Gopalakrishnan et al., 2006; Karin et al., 1996; Karin et al., 1992). AP-1 is one of the major eukaryotic transcription factors involved in regulating COX-2 expression (Chun et al., 2004; Subbaramaiah et al., 2002, 2001; Guo et al., 2001). It was recently reported that the stimulation of young human keratinocytes with EGF increases the AP-1 DNA-binding activity mediated through ERK activation, whereas blocking ERK activation by PD098059 inhibited the AP-1 DNA binding activity (Kim et al., 2006; Shi et al., 2005).

We observed a decrease in AP-1 levels in the nuclear fractions of the baicalein and celecoxib treated cells and up regulation in cells treated with 12-(R)-HETE, 12-(S)-HETE and PGE$_2$. The present study, thus clearly demonstrates a regulatory role for COX-2 and 12-LOX in the activation of AP-1. The proposed
signaling pathways involved in the activation of AP-1 by baikalein and celecoxib are presented in the Fig. 32.

Figure 32: Schematic overview of the proposed signaling pathways involved in the survival and apoptosis in A431 cells mediated by 12-LOX and COX-2 pathways.

The molecular mechanisms underlying anticancer activities of inhibitors of COX and LOX are very complex, and manifest differentially in different cell types. The antitumor effects of baikalein and celecoxib may not only be observed in cells which express 12-LOX and COX-2 but also in cells which do not express these enzymes suggesting that their effects may be independent of inhibitors of COX-LOX pathways. Indeed, many in vitro cell culture and animal experiments have shown that the anticancer activity exerted by certain NSAIDs is independent of their COX-2 inhibitory properties (Kim et al, 2004; Tegeder et al, 2001). In the present study the observed effects of baikalein and celecoxib appear to be mediated through the respective metabolites 12-(S)-HETE, 12-(R)-HETE and PGE₂.
5.5 Baicalein and Celecoxib reduce the tumor weight of A431 xenografts in Swiss mice

*In vitro* studies on A431 cells have revealed the overexpression of 12-LOX and COX-2 suggesting the possible role in the regulation of cell growth. Further studies have clearly demonstrated the induction of apoptosis in A431 cells by baicalein (12-LOX inhibitor) and/or celecoxib (COX-2 inhibitor). These encouraging results under *in vitro* conditions prompted us to evaluate the efficacy of baicalein and celecoxib in reducing/arresting the growth of A431 xenografts in Swiss mice. The *in vivo* studies confirm the inhibitory effect of LOX/COX inhibitors on human epidermoid cancer xenografts in Swiss mice. The decrease in the tumor weight in animals treated with both baicalein and celecoxib was maximum suggesting that the treatment of skin cancer by dual inhibition of 12-LOX and COX-2 is highly effective. Baicalein and celecoxib induced apoptosis is A431 xenografts as seen in the TUNEL assay. It was demonstrated that skin tumor promotion caused by ultraviolet B radiation can be decreased by up to 89% by inhibiting blocking cyclooxygenase-2 (COX-2) with celecoxib (Thompson et al, 2001). A similar study showed that Celecoxib can decrease new tumor formation by 44% in mice that already have tumors (Thompson et al, 2001). Baicalein may be acting through the inhibition of 12-LOX activity and expression, as 12-LOX was reported in xenografts of melanoma (Fischer et al, 2002) and in skin tumors developed by an initiation/promotion protocol (Akunda et al, 2007; Fischer et al, 1999). COX-2 inhibitors were also effective in retarding tumor progression and metastasis in mouse models of injected breast cancer cell lines and in xenograft models of human breast cancer cells in nude mice (Basu et al, 2004; Kundu et al, 2002; Rozic et al, 2001; Blumenthal et al, 2001). It was shown that dietary celecoxib had a significant chemopreventive activity against UV-induced skin carcinogenesis in SKH-HR-1 hairless mice (Fischer et al, 1999) and blocked additional tumor formation after the onset of photocarcinogenesis in hairless mice (Chun et al,
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2004; Pentland et al, 1999). The inhibitory effect of LOX inhibitors on human pancreatic cancer xenografts in athymic mice was reported (Tong et al, 2002). The present study also confirms the anti-tumor effect of 12-LOX and COX-2 inhibition on A431 xenografts in Swiss mice.

In summary, the present study demonstrates the predominant expression of 12-R-LOX and COX-2 in human epidermoid carcinoma cell line, A431. Further studies reveal that baicalein (a 12-LOX inhibitor) and celecoxib (a COX-2 inhibitor) significantly reduce the cell proliferation and induce apoptosis in A431 cells via the intrinsic death pathway involving reduction in the Bcl-2/Bax ratio, release of cytochrome c, activation of caspase 3 and PARP cleavage. The apoptosis induced by baicalein and celecoxib was mediated by ERK/AP-1 and Akt/NF-κB pathway. The in vivo studies confirm the inhibitory effect of 12-LOX /COX-2 inhibitors on human epidermoid cancer xenografts in Swiss mice. Our findings suggest that 12-LOX and COX-2 have a critical role in the regulation of growth of epidermoid carcinoma and their inhibitors may be of potential therapeutic importance.