Effect of Benzoylsalicylic Acid on IKK-Beta Kinase and NF-κB Pathway in Murine Macrophage raw 264.7 Cells

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Abstract

The transcription factor NF-κB regulates a large array of genes of immune and inflammatory responses. Deregulated NF-κB signalling is implicated in the pathogenesis and broad spectrum of human inflammatory disorders and malignancies. The mechanism for NF-κB activation is the inducible degradation of IkB, triggered through its site-specific phosphorylation by a multi-subunit IkB kinase (IKK) complex. Aspirin (acetylsalicylic acid) a well-known anti-inflammatory agent that binds to ATP binding pocket of IKKβ and inhibits its kinase activity. However, several side effects of aspirin due to the inactivation of COX-1 limits the therapeutic usage of ASA. Here we have demonstrate the effect of a plant phenolic compound benzylosalicylic acid (BzSA) isolated first time in plants a potent anti-viral compound inhibits Tobacco mosaic virus (TMV) and enhance the plant defense response (Samuel et all 2016&2017) inhibits the IKKβ mediated NF-κB pathway higher than aspirin. Our in-vitro COX enzymatic assays with BzSA have shown less COX-1 and high COX-2 inhibition as compared to ASA. Western blotting analysis of Raw 264.7 cells that were pre-treated with BzSA down-regulated pIKK-β, pIkB, NF-κBp65, TNF-α, COX-1, COX-2, 5-LOX, IL-1β, and IL-6 higher than ASA. Therefore, our observations suggested that the potencial therapeutic value of BzSA an upcoming new inhibitor of NF-KB pathway and the dual inhibitor of COX2/5-LOX without effecting the useful COX-1. Hence useful as an anti-inflammatory agent like ASA.

1.0 Introduction

Inflammation is a part of complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants and also a critical component of tumour progression (Coussens and Werb, 2002; Grivennikov et al., 2010). Several researchers has been reported since several decades that the malignancies are initiated by infections, chronic irritation and inflammation (Coussens and Werb, 2002; Grivennikov et al., 2010; Kuper et al., 2000; Schoppmann et al., 2002). NF-κB is a well known protein complex that controls transcription of DNA, cytokine production and cell survival (Lawrence, 2009; Oeckinghaus and Ghosh, 2009). NF-κB is found in almost all animal cell types and involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL and bacterial or viral antigens (Brazier, 2006; Gilmore, 1999, 2006; Perkins, 2007). In most cell types, NF-κB -IkB inactive complex resides in the cytoplasm and its activation requires IkB kinase mediated phosphorylation of IkB kinase and its ubiquitin mediated degradation. NF-κB plays a key role in regulating the immune responses to infection and acts as a critical components of immunoglobulins. Incorrect regulation of NF-κB leading to various cancers, inflammatory, cardiovascular, autoimmune diseases, septic shock, viral infection and improper immune development (Viatour et al., 2005). Bacterial Lipopolysaccharide (LPS) can induce NF-κB pathway (Zhang and Ghosh, 2000). The IkB kinase (IKK) is an enzyme complex and a part of the the upstream NF-κB signal transduction cascade such as IKK-α and IKK-β involved in the cellular response to inflammation. The catalytic activities of IKK-α and IKK-β make essential contributions to IkB-α phosphorylation, and dissociation of cytosolic IkB-NF-κB inactive complex and translocation of active phospho-NF-κB P65 to the nucleous (Israel, 2010; Zandi et al., 1997)
and thus acts as a transcription factor for the induction of TNFα, COX-2, iNOS as well as cytokines like IL-1, IL-2 and IL-6 (Aggarwal, 2006; Esposito and Cuzzocrea, 2009; Liu et al., 2013; Pahl, 1999). Thus, identification of new NF-κB inhibitors are the promising therapeutics to prevent inflammation and cancer diseases (Andres et al., 2013; Xu et al., 2011). Previously, it was reported that ASA and sodium salicylate inhibit the IKK-β and therefore blocks the activation of NF-κB pathway (Yin et al., 1998). NF-κB is a hallmark of inflammatory responses and plays a fundamental role in inflammation and associated cancers (Pikarsky et al., 2004). Literature survey suggested that a number of chemical activators targets the genes of NF-κB pathway (Pahl, 1999). Chebulagic acid (CA) a natural plant compound shown the anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages through NF-κB inhibition and also MAP kinase phosphorylation (Reddy and Reddanna, 2009). NF-κB controls many physiological processes including inflammation, immunity, apoptosis and angiogenesis (Aggarwal, 2006; Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). Baicalein a natural compound suppressed TNF-α- induced NF-κB activation and its target gene products (Li et al., 2016).

Cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-LOX) are the key enzymes of arachidonic acid (AA) metabolism (Dannhardt and Kiefer, 2001; Greene et al., 2011). COX enzymes catalyses the conversion of AA to prostaglandins (PGs) and thromboxanes (Seibert et al., 1994). COX-1 constitutively expressed in mast cells and also different organs whereas, COX-2 localized primarily in inflammatory cells and tissues and the selective COX-2 inhibitors are exceedingly beneficial anti-inflammatory drugs (Seibert and Masferrer, 1994; Seibert et al., 1994). Literature suggested that offensive up-regulation of COX-2 and iNOS has been associated with pathophysiology of certain types of human cancers as well as inflammatory disorders (Surh et al., 2001). ASA is a widely used non-steroidal anti-inflammatory (NSAID) drug world wide (Alfonso et al., 2014; Wentz et al., 1976). The anti-thrombic and anti-ulcerogenic side effects of ASA largely due to the acetylation of Ser 530 in COX-1 and Ser 516 in COX-2 (Kalgutkar et al., 1998). Inhibition of COX-1 by ASA and other NSAIDs causes side effects (Brune and Patrignani, 2015). Recent studies have suggested that ASA works as a chemo preventive agent in several cancers (Goodman and Grossman, 2014). Besides COXs, ASA also acetylates several other bio-molecules and affects their function (Alfonso et al., 2014; Rainsford et al., 1983). Previous reports suggested that chemically synthesized ASA like molecules selectively inactivate COX-2 more effectively than ASA (Beauchamp et al., 2005; Kalgutkar et al., 1998; Kalgutkar et al., 2000). Rofecoxib and celecoxib are selective inhibitors of COX-2 whereas meloxicam inhibit COX-2 by different mechanism (Hawkey, 2001). Several reports suggested that the phytochemicals like curcumin, epigallocatechin gallate (EGCG), resveratrol inhibit COX-2 and are proven to be effective anti-inflammatory and anti-cancer agents (Surh et al., 2001).

To the best of my knowledge we isolated first time BzSA from the seed coats of *Givotiarottleriformis* and shown its role in plant systemic defense against TMV virus (S. Kamatham et al., 2016). The plant *G.rottleriformis* tree species belongs to Euphorbiaceae family and are known have anti-rheumatism, anti-psoriasis and anti-dandruff medicinal properties. Phytochemical analysis of *G.rottleriformis* seed coats provides the evidences to the medicinal value of this plant as we identified important
pharmaceutical molecules such as salicylic acid (SA), benzoic acid (BA), gallic acid (GA) and methylgallate (MG) (Kamatham et al., 2016; Samuel Kamatham, 2015). The purified GA and MG from seed coats of this plant exhibit anti-cancer potential against the proliferation of A431 skin cancer cell line without effecting the normal HaCaT cell line (Samuel Kamatham, 2015).

The present study have shown the anti-inflammatory and anti-cancer potential of BzSA in RAW 264.7 cells. Structurally BzSA is a natural and ASA is a synthetic analogue of SA (Fig. 1). The effect of BzSA on COX-1 and COX-2 inhibition was studied and compared with ASA. This study shown the evidences of BzSA down-regulated IKK-β and the down-stream NF-κB pathway higher as compare to ASA in a dose-dependent manner.

2.0 Materials And Methods

2.1 Cell lines and reagents

RAW 264.7 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. DMEM, Fetalbovine serum (FBS), Phosphate-buffered saline (PBS), Penicillin, Gentamycin and Streptomycin was purchased from Gibco, Ltd, (BRL Life Technologies, Inc., Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] and Dimethylsulphoxide (DMSO), Lipopolysaccharide (LPS), Poly-L-lysine, Glutaraldehyde, Proteinase inhibitor K, Propidium iodide (PI), Phenylmethylsulfonylfluoride (PMSF), Leupeptin, Aprotinin, Pepstatin A, Trypsin, Tween-20, Triton X-100, TMPD (N,N,N,N'-tetramethyl p-phenylenediamine) purchased from Sigma (St. Louis, USA). All the antibodies, chemicals and reagents used in this study were purchased from Cell signalling (USA), Santacruz (USA) and Abcam (USA), Merck and Sigma (USA).

2.2 Cell culture

RAW 264.7 murine macrophase cells were cultured as a monolayer in petridish and supplemented with DMEM medium containing 10% heat inactivated FBS, 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and maintained in a humidified atmosphere with 5% CO2 at 37 ºC. The cells were subcultured alternative days and the exponentially growing cells were used for the treatments.

2.3 MTT assay

RAW 264.7 cells (5X10³ cells per well) were seeded in 96 well plates and incubated in the presence or absence of BzSA or ASA (0.001, 0.01, 0.1, 1.0, and 10 mM) for 24h and 48h. In a final volume of 100µl, 20µl of MTT (5mg/ml in PBS) was added to each well and incubated for an additional 3h at 37 ºC. Then the culture medium was removed from the wells and added 200µl of DMSO followed by dissolved the purple blue formazan crystals. The optical color density was quantified at 570nm on ELISA multi-mode plate reader (SYNERGYMX, Biotech). All the experiments are repeated three times under the same conditions.
2.4 Preparation of whole cell extract and Western blotting analysis

RAW 264.7 cells were treated either with BzSA or ASA (200 and 400µM; dissolved DMEM medium containing 0.01% DMSO) for 24h followed by stimulated with 25ng/mL LPS for 2h. DMSO and LPS treated cells were maintained as positive and negative controls. After the pre-treatment, the cells were washed with 1X PBS and re-suspended in a RIPA lysis and extraction buffer containing 1X protease inhibitor cocktail followed by incubation for 30min at 4°C with a frequent vortexing. Then the lysate was centrifuged at 12,000 rpm for 20min and collected the supernatant and the total protein concentration was estimated by Bradford protein assay (Bradford, 1976) and stored at -20°C for further use. The protein samples (20µg) was resolved on 12% SDS-PAGE and then transferred onto nitrocellulose (NC) membrane. Then the NC membrane were incubatedin 5% (w/v) non-fat dry milk powder at RT for 1h to block nonspecific sites and incubated with primary antibody of interest ( both phospho and total IKK-α, IKK-β, IkBα, IkBα, NF-κBp65 and TNF-α, COX-1, COX-2, iNOS and IL1β) overnight at 4°C under shaking followed by washing with TBST for 3 times 10 min each. The NC membrane were then incubated with respective secondary antibody conjugated with HRP for 1h at RT followed by washing with TBST for 3 times10 min each. Finally, the blot were developed by adding HRP substrate followed by recorded using gel documentation system (Bio-rad).

2.5 Isolation of COX-1 enzyme

COX-1 enzyme was isolated from Ram seminal vesicles according to Hemler et al; 1976 (Hemler and Lands, 1976), with a slight modifications. In brief, Ram seminal vesicles were homogenized with a grinder in Tris-HCl (pH 8.0) buffer for 1min and then the homogenate was filtered through cheese cloth and centrifuged at 13,000g at 4°C for 30 min. Finally, 0.01% sodium azide was added and stored in small aliquots at -80°C and used as a COX-1 enzyme.

2.6 Isolation of COX-2 enzyme

The enzyme COX-2 was isolated according to Reddy et al; 2000 (Reddy et al., 2000) with slight modifications. In brief, the human recombinant COX-2 enzyme was expressed in Sf-9 cells, harvested the cells and sonicated for 3min in 50mM Tris-HCl buffer (pH 7.2) followed by centrifuged at 100,000 g at 4°C for 80 min using ultracentrifuge (Hitachi, Himac CP-100α). Then the cell pellet was resuspended in 2.5 mM Tris-HCl buffer (pH 7.2), 0.8% Tween-20, 1mM phenol, and 0.5% glycerol, and stored in small aliquots at -80°C and used as a COX-2 enzyme.

2.7. COX-1 and COX-2 enzyme activity

The enzymatic activities of both COX-1 and COX-2 were measured according to Copeland et al; 1994 (Copeland et al., 1994) with slight modifications based on the a chromogenic assay and oxidation of N,N,N”,N”-tetramethyl-p-phenylene diamine (TMPD) during the reduction of PGG₂ to PGH₂. In brief, the assay mixture contained Tris-HCl buffer (100mM, pH 8.0), hematin (15µM), EDTA (3µM), enzyme (COX-1 or COX-2) and the test compounds (BzSA or ASA). Then the assay mixture was pre-incubated for 15min
at 25°C and the reaction was initiated by addition of arachidonic acid (AA) and TMPD in a final volume of 1ml. The enzyme activity was measured after 1min by estimated initial TMPD oxidation by monitoring absorbance at 603nm. A low rate of non-enzymatic TMPD oxidation was observed in the absence of COX-1 or COX-2 enzymes and are treated as control reaction and were normalised from the test experimental values while calculating the percentage of inhibition. Each experiment were repeated three times under the same conditions.

### 2.8 5-LOX assay

Similarly, we purified the 5-LOX enzyme from potato tubers and assayed according to Reddanna et al., 1990 (Reddanna et al., 1990). Enzyme activity was measured using polarographic method with a Clark’s oxygen electrode on Strathkelvin Instruments, model 782, RC-300. The typical reaction mixture contained 50–100µl of enzyme and 10µl of the substrate (133µM of AA) in a total volume of 3ml with 100 mM phosphate buffer pH 6.3. The rate of decrease in oxygen concentration was taken as a measure of enzyme activity. Stock solutions of BzSA and ASA were prepared freshly in DMSO before use. Various concentrations of BzSA and ASA were prepared and the LOX reaction was initiated by the addition of substrate. The reaction was allowed to proceed at 25ºC and the maximum slope generated was taken for calculating the enzyme activity. The percentage of inhibition was calculated by comparison of LOX activity in the presence or absence of inhibitor. The concentration of the test compound causing 50% inhibition (IC$_{50}$) was calculated from the concentration-inhibition response curve. The experiment were repeated for three times under the same conditions.

### 3.0 Results

#### 3.1.1 Cytotoxicity assay

The cytotoxic effect of BzSA was determined and compared with ASA in Raw 264.7 cells (Fig. 2a-f). Raw 264.7 cell that were pre-treated with BzSA have shown its cytotoxic effect with an IC$_{50}$ value of 3.0 mM at 24h and 48h (Fig. 2a-c). In contrast, ASA shown its cytotoxic affect with an IC$_{50}$ value of 5.0 mM at 24h and 48h respectively (Fig. 2d-f).

#### 3.1.2 Effect of BzSA on COX-1/COX-2 and 5-LOX enzyme activity

In order to determine the effect of BzSA on COX-1, COX-2 and 5-LOX enzyme activity we perform in vitro enzymatic assays. Interestingly, 4.2 mM BzSA showed 10% COX-1 and 35% COX-2 inhibition (Table 1). Whereas, 4.2 mM ASA shown 95% COX-1 and 11% COX-2 enzyme (Table 1). However, BzSA and ASA have no inhibition effect on 5-LOX enzyme activity even at increasing concentrations (Table 1).
### Table 1
COX-1, COX-2 and 5-LOX enzyme assays with BzSA and ASA.

| Treatment | %COX-1 Inhibition | %COX-2 Inhibition | %5-LOX inhibition |
|-----------|-------------------|-------------------|-------------------|
| BzSA 4.2mM | 10%               | 35%               | 0.0               |
| ASA 4.2mM  | 95%               | 11%               | 0.0               |

### 3.1.3 Effect of BzSA and ASA on COX-1 expression

To examine the effect of BzSA on COX-1 expression we performed western blotting in RAW 264.7 cells that were stimulated with LPS. Previous reports has been suggested that COX-1 required to maintain the body physiology and constitutively expressed in the gastrointestinal tract and many other tissues in the body including lung, kidney, stomach, platelets and monocytes etc. Interestingly, in our results pretreatment of BzSA and ASA does not effect the COX-1 expression upon LPS stimulation (Fig. 3).

### 3.1.4 COX-2

COX-2/5-LOX dual inhibitors are the promising pharmaceutical value for the development of potent drugs to cancer and various inflammatory diseases. RAW 264.7 cells that were pretreated with BzSA down-regulated LPS stimulated COX-2 completely at 200µM (Fig. 3). Whereas cells that were pretreated with ASA down-regulated LPS stimulated COX-2 less than BSA in a dose dependent manner (Fig. 3). These results highlight BzSA is a potent COX-2 inhibitor than ASA.

### 3.1.5 5-LOX

5-lipoxygenase (5-LOX) pathway is the major source of potent proinflammatory leukotrienes (LTs) issued from the metabolism of arachidonic acid (AA), and the best known for their roles in the pathogenesis of asthma. Dual COX-2/5-LOX inhibitors are promising drugs to treat inflammatory diseases. In our results, RAW 264.7 cells that were pretreated with 200µM BzSA down-regulated the expression of 5-LOX enzyme (Fig. 3). Whereas, RAW 264.7 cells that were pretreated with ASA reduced 5-LOX expression low as compared to BzSA (Fig. 3). These results suggest that BzSA is a potent 5-LOX inhibitor.

### 3.2.1 Inhibition of IKK complex by BzSA

The activation of catalytic kinase subunits of IKK kinase complex (IKKα and IKKβ) is a regulatory step in two signalling pathways known as classical (canonical) pathway and the alternative (non-canonical) pathway, leading to the activation of NF-κB. The IKK mediated phosphorylation of IκB and proteosomal degradation are leading to the activation of NF-κB dimers, nuclear translocation and induction of target gene expression. Here we have shown the effect of BzSA on catalytic kinase subunits of IKK kinase complex (IKKα and IKKβ) in RAW 264.7 cells in a dose responsive manner.

### 3.2.2. IKK-α/β inhibition by BzSA

In order to determine the effect of BzSA on NF-κB pathway and its regulatory IKK-α/β complex, we examine the effect of BzSA and ASA on LPS stimulated IKK-α/β in RAW 264.7 cells. Interestingly, BzSA
down-regulated LPS stimulated phospho IKK-α/β higher than ASA in a dose responsive manner (Fig. 4). However, the total IKKα and IKK-β levels were remains same in BzSA, ASA and the controls (Fig. 4).

### 3.2.3 IκB-α

The activation of NF-κB required phosphorylation and ubiquitination of phospho-IκB-α as an inhibitor of NF-κB by IKK-α/IKK-β kinases. Western blotting analysis of IκB-α revealed that BzSA pre-treatment reduced LPS stimulated IκB-α completely as compared to ASA treatment (Fig. 4) and the total IκB-α levels were increased in BzSA pre-treatment over ASA (Fig. 4).

### 3.2.4. NF-κB

In order to determine the effect of BzSA on NF-κB activation, RAW 264.7 cells were pre-treated with BzSA and assess the inhibition of LPS stimulated p-NF-κBp65. Interestingly, down-regulation of p-NF-κB-p65 levels in BzSA pre-treated cells were reduced in a dose dependent manner upon LPS stimulation (Fig. 4). Whereas, in ASA pre-treated cells there was no much reduction of LPS stimulated p- NF-κB-p65 levels were noticed as compared to BzSA (Fig. 4).

### 3.3.1. BzSA inhibits NF-κB- and its responsive gene expression in RAW 264.7 cells

Nuclear translocation of p-NF-κBp65 triggers the expression of inflammatory mediators such as COX-1, COX-2, 5-LOX, TNF-α, iNOS and cytokines such as IL-1β and IL-6, whose expression play an important roles in immune, stress, apoptosis, cell proliferation, cell differentiation and development. Here pre-treatment of BzSA down-regulated the expression of LPS stimulated inflammation mediators and cytokines in RAW 264.7 cells.

#### 3.3.1. TNF-α

TNF-α is an inflammation responsive marker protein and TNF-α antagonists may be effective in treating various inflammatory disorders. Inhibition of TNF-α proved to be an effective therapy for patients with rheumatoid arthritis and other forms of inflammatory disease including psoriasis, psoriatic arthritis, ankylosing spondylitis and inflammatory bowel disease. A moderate reduction of reduction of TNF-α in BzSA and ASA pretreated RAW 264.7 cells were noticed (Fig. 5).

#### 3.3.2 iNOS

Inducible nitric oxide synthase (iNOS) is one of the three key enzymes that generate nitric oxide (NO) from the amino acid arginine. iNOS-derived NO is a free radical, whose predominant function is that of a messenger through cGMP. iNOS-derived NO plays an important role in numerous physiological (e.g. blood pressure regulation, wound repair and host defence mechanisms) and pathophysiological (inflammation, infection, neoplastic diseases, liver cirrhosis, diabetes) conditions and associated with malignant disease. In particular, pivotal effects such as malignant transformation, angiogenesis, and metastasis are modulated by iNOS. Interestingly, BzSA pre-treatment down-regulated LPS stimulated iNOS in RAW
264.7 cells in a dose wise (Fig. 5). Similarly, ASA pre-treatment were also down-regulated iNOS completely at 4mM (Fig. 5).

### 3.3.3 BzSA inhibits IL-1β and IL-6

Interleukin-1β (IL-1β) and interleukin-6 (IL-6) are major inducers of hepatic inflammation and the acute phase response. Over expression of IL-6 has been implicated in the pathology of a number of diseases including multiple myeloma, rheumatoid arthritis, castleman's disease, psoriasis, and post-menopausal osteoporosis. Hence selective antagonists of IL-6 may offer therapeutic benefits. Interestingly, BzSA pretreatment completely down-regulated LPS stimulated IL-6 in RAW 264.7 cells even at 200µM concentration (Fig. 5). Whereas ASA inhibit the expression of LPS stimulated IL-6 in a dose dependent manner, however less effective compared to BzSA (Fig. 5). Similarly, our results shown that BzSA pre-treated cells down-regulated IL-1β and IL-6 in a dose dependent manner and found to be better response compared to ASA (Fig. 5).

### 4.0 Discussion

The present study have evaluated the anti-inflammatory properties of BzSA in RAW 264.7 cells as compared to a well known anti-inflammatory agent ASA. BzSA (Benzoylsalicylic acid) is first time purified from *G. rottlerifirmis* seed coats (Kamatham et al., 2016) where as ASA (Acetylsalicylic acid) a synthetic analogue of SA (Salicylic acid; Fig. 1). BzSA develops plants immunity and induced disease resistance in tobacco and Arabidopsis against TMV virus plants (Kamatham et al., 2016). Further more various chemically synthesized analogues of BzSA have shown a powerfull defence resistance against TMV virus even at low concentrations (Samuel et al, 2017). In order to determine the stability of BzSA We treated SA decient Arabidopsis NahG plants with BzSA and check the defense response and surprisingly we were still able to see the defense response even in the absence of SA which indicates that BzSA is a stable molecule and does not undergo deacylation in the plants after treatment (Samuel et al 2016).

The cytotoxicity MTT assay results of RAW264.7 cells were shown that the IC$_{50}$ value of BzSA slight higher than aspirin (Fig. 2). Aspirin indued a decrease in cell viability in a time and dose dependant manner (Bellosillo et al., 1998). Interestingly, BzSA have shown more COX-2 enzyme inhibition activity over ASA (Table 1). NSAID$s$ including aspirin, indomethacin and ibuprofen are non-selective inhibitors of COX-1 and COX-2, whereas, celecoxib and rofecoxib selectively inhibit COX-2 enzyme activity (Rao and Knaus, 2008). NSAID$s$ are potent anti-inflammatory and anti-cancer agents that acts through the inactivation of the COX enzymes, mostly COX-2 and thus directed the prostaglandins (PGs) synthesis at the site of inflammation (Rao and Knaus, 2008; Willoughby et al., 2000). BzSA have no effect on 5-LOX enzyme inhibition (Table 1). In view of the importance of dual COX-2/5-LOX, identification of specific inhibitors of COX-2/5-LOX proteins have therapeutic advantage (Fiorucci et al., 2001; Ranjbar et al., 2016). Interestingly, BzSA inhibits COX-2 and 5-LOX expression higher than aspirin (Fig. 3). COX-2 expression is highly restricted and selectively induced by pro-inflammatory cytokines at the site of inflammation and
thus involved in the production of pro-inflammatory PGs (Crofford, 1997; Rao and Knaus, 2008; Seibert and Masferrer, 1994).

NF-κB induce 5-LOX enzyme expression during the inflammation process (Pahl, 1999). 5-LOX enzyme catalyzes AA metabolism in the leucocytes (Steinhilber, 1999). Leukotrienes (LTs) are the metabolic products of AA metabolism, which possess a potent pro-inflammatory activity and thus might be involved in cardiovascular diseases (de Gaetano et al., 2003). Decreased expression of 5-LOX gene enhanced cell death in breast cancer cells and therefore plays an essential role in reducing the tumor proliferation (Kumar et al., 2016). The 5-LOX enzyme inhibitor zileuton reduces inflammatory reaction in the ischemic brain damage through the activation of P13K/Akt signaling pathway (Tu et al., 2016). A report suggest that 7-subsituted coumarin derivatives are potential 5-LOX inhibitors (Srivastava et al., 2016).

Interestingly, the inhibition effect of BzSA on COX-1 enzymatic activity very low as compared to ASA (Table 1). However, BzSA and ASA have no effect at COX-1 expression even increasing concentrations (Fig. 3). One of the major disadvantage of ASA is inhibition of COX-1 and associated anti-thrombic and ulcerogenic side effects (Bianchi Porro and Pace, 1988; Undas et al., 2007). COX-1 expressed constitutively in almost all cell types in the human body and thus produced anti-inflammatory PGs which are important to maintain the homeostatic functions such as integrity of the gastric mucosa, platelet function, and renal blood flow (Allison et al., 1992). These results encouraging that the dentification of selective inhibitors of COX-2 with out effecting COX-1 are useful therapeutics.

In order to determine that BzSA is a potent inhibitor of NF-KB pathway we are pre-treated Raw 264.7 cells with BzSA and analysed the IKK-α/IKK-β an essential up-stream signaling of NF-NF-KB. Surprisingly, BzSA down-regulated IKK-α/β expression higher than ASA (Fig. 4). NF-κB pathway is under the control of IKK-kinase complex. IKKα/β phosphorylates IκB-α an inhibitor of NF-κB (Adli et al., 2010; Aggarwal, 2006; Karin and Delhase, 2000) and reduced phosphorylation of IκB and NF-κB pathway. ASA binds to IKK-β and inhibit ATP binding and phosphorylation of IκB (Aggarwal, 2006; Yin et al., 1998). In our results we also observed the similar action of BzSA on IKKα/β (Fig. 4). Phosphorylation of IκB-α by IKKα/β brings IκB-α-NF-κB inactive complex to active form and leading to the activation and localization of NF-κB in to nucleus (Aggarwal, 2006; Huang and Hung, 2013; Yamamoto and Gaynor, 2001; Yin et al., 1998). A dose wise IκBα inhibtion and reduction in p-NF-κB p65 was observed in BzSA higher than ASA treatment and supported by previous observations (Fig. 4) (E.Britta-Mareen Traenckner et al., 1995; Vancurova and Vancura, 2012). NF-κB-DNA binding subunits are released and translocated into the nucleus thereby activates a wide range of inflammation responsive genes (Hayden and Ghosh, 2008; Rao and Knaus, 2008; Vallabhapurapu and Karin, 2009). Benzo[b]thiophen-2-yl-3-bromo-5-hydroxy-5H-furan-2-one (BTH) inhibited NF-κB and STAT3 phosphorylation (Andres et al., 2013). Isoorientin is a natural flavone reduced NF-κB in RAW 264.7 cells (Sumalatha et al., 2015). NF-κB recognized as a key player involved in many steps of cancer initiation and progression (Hoesel and Schmid, 2013; Karin, 2009).

In order to validate the effect of BzSA on NF-κB pathway we tested a series of NF-κB targeted inflammatory mediaters. BzSA suppressed COX-2 (Fig. 3). COX- 2 expression depends on NF-κB
activation (Lim et al., 2001). Previously it was reported that pro and anti-inflammatory nature of COX-2 (Poligone and Baldwin, 2001). The expression of inflammatory mediators such as TNF-α, COX-2, 5-LOX, iNOS and cytokines such as IL-1β and IL-6 require translocation of NF-κB from cytosol to nucleus (Rao and Knaus, 2008). TNF-α is a pleiotropic cytokine, which is a therapeutic target for inflammatory diseases therefore TNF-α inhibitors are therapeutic advantages in inflammatory disorders and malignant diseases (Esposito and Cuzzocrea, 2009; Parameswaran and Patial, 2010). Previously, it was reported that ASA inhibits TNF-α gene expression in RAW 264.7 cells through the suppression of active NF-κB binding to the TNF-α promotor (Shackelford et al., 1997). No significant inhibition of TNF-α by BzSA and ASA noticed (Fig. 5). Literature suggested that induced expression of TNF-α activated by NF-κB in different cell lines (Ke et al., 2007; Parameswaran and Patial, 2010; Wajant et al., 2003). Similarly, the expression of iNOS depends on the nuclear localization of activated NF-κB. BzSA suppressed iNOS higher than ASA (Fig. 5). Previously, it was suggested that inducible nitric oxide synthase (iNOS) is activated by several immunological stimuli and the resultant accumulated nitric oxide cytotoxic to the cells. Furthermore, iNOS mutants exhibited reduced immune response to carrageenin and showed resistance to LPS induced mortality (Vig et al., 2004; Wei et al., 1995). Increased iNOS associated with malignant diseases, particularly malignant transformation, angiogenesis and metastasis (Lechner et al., 2005).

Interestingly, in our study we observed more suppression of IL-1β in BzSA treated cells as compared to ASA (Fig. 5). Interleukins are the cytokines important in the regulation of immune responses, inflammatory reactions and hematopoiesis. Over activation of immune system causes several inflammatory disorders. Anti-inflammatory agents including ASA, anti-cytokine therapies, small molecules and many drugs under clinical trials. IL-1β and IL-6 are the the pro-inflammatory cytokines responds to inflammation. IL-1β induces IL-6 in peripheral blood monocytes (Tosato and Jones, 1990). Pro-inflammatory cytokines such as TNF-α, IL-1β and vascular growth factor (VEGF) plays a central role in inflammation (Dinarello, 2010). In our results we also noticed remarkable reduction in IL-6 by BzSA in a dose dependent manner over ASA (Fig. 5). Previously reports demonstrated that IL-1β and TNF-α induce IL-6 and most of the biological activities associated with IL-1 and IL-6 (Tosato and Jones, 1990).

**Conclusions**

Our findings highlighte the anti-inflammatory and anti-cancer therapeutic proterties of BzSA in RAW 264.7 cells. Interestingly, BzSA exhibited more COX-2 inhibition activity and low COX-1 inhibition activity over ASA. BzSA suppressed NF-κB pathway and the mechanism involves the inhibition of up-stream IKK-complex is similar to ASA (Fig. 6). Hence we suggested that the BzSA is a potencial anti-inflammatory and anti-cancer agent like aspirin.

**Abbreviations**

BzSA, benzyolsalicylic acid; SA, salicylic acid; ASA, acetylsalicylic acid; COX, cyclo-oxygenase; 5-LOX, 5-lipooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin, NF-κB, nuclear factor
κB; IκB, inhibitor of NF-κB; IKKα, IkB kinase α; IKKβ, IkB kinase β; IL-1β, interleukin-1β; interleukin-6 iNOS, inducible nitric oxide synthase.

**Declarations**

**Conflict of interest Statement:**

We wish to confirm that there is no conflicts of interest associated with this publication and no significant financial support for this work that could have influenced its outcome.

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Representative chemical composition of (a) Salicylic acid (SA), (b) Acetylsalicylic acid (ASA) and (c) Benzoyl salicylic acid (BzSA).
Figure 2

Determination of cytotoxic effect of BzSA and ASA in RAW 264.7 cells by MTT assay. RAW 264.7 cells were cultured and pre-treated with increasing concentrations of BzSA and ASA (0.001, 0.01, 0.1, 1, 10, 100mM) and the cytotoxicity of BzSA and ASA were measured as cell death after 24h and 48h. (a) showing the cytotoxic effect of BzSA with an IC50 value of 3.0 mM after 24h of BzSA pre-treatment. (b) Showing the cytotoxic effect of ASA with an IC50 value of 5.0 mM after 24h of ASA pre-treatment. (c) Showing the percentage of (%) cell viability in BzSA and ASA pre-treated RAW 264.7 cells after 24h pre-treatment. (d&e) Showing the IC50 value of BzSA and ASA pre-treated RAW 264.7 cells after 48h. (f) Showing the percentage of cell viability in BzSA and ASA pre-treated cells after 48h pre-treatment. Values representing here are average of 3 independent experiments.
Figure 3

Effect of BzSA on COX-1, COX-2 and 5-LOX expression in RAW 264.7 cells. Raw 264.7 cells were pre-treated with BzSA in a dose-dependent manner for 24h followed by stimulated with LPS. The whole cell protein lysate was prepared and performed western blotting analysis. Blots showing the effect of BzSA and ASA on LPS stimulated COX-1, COX-2 and 5-LOX expression in BzSA pre-treated cells as compared to ASA. DMSO 0.01% /ml and 0.25µg/ml LPS treated RAW 264.7 cells was used as controls. Beta-actin served as a loading control.
Figure 4

Effect of BzSA on IKK- complex (IKK-α / IKK-β), IκB-α and NF-κB in RAW 264.7 cells. Raw 264.7 cells were cultured and pre-treated with BzSA and ASA for 24h and then the cells were stimulated with 0.25μg/mL LPS. Then the whole cell protein lysate of treated and untreated cells were prepared and performed western blotting analysis. The p-IKKβ/β and IKKβ down-regulated in BzSA treated cells as compared to ASA. pIκB-α and total-IκB down-regulated in BzSA treated cells as compared to ASA. NF-κBp65 down-regulated in BzSA pre-treated cells upon LPS stimulation as compared to ASA. DMSO, LPS stimulated RAW 264.7 cells was used as control. Beta-actin served as a loading control.
Figure 5

Effect of BzSA on TNF-α, iNOS, IL-1β and IL-6 expression in RAW 264.7 cells. Raw 264.7 cells pre-treated with BzSA and ASA for 24h followed by stimulated with LPS. The whole cell protein lysate was prepared and performed western blotting analysis. Down-regulation of TNF-α, iNOS, IL-1β and IL-6 in BzSA pre-treated cells upon stimulation with LPS as compared to ASA. DMSO 0.01% /ml and 0.25µg/ml LPS treated RAW264.7 cells was used as controls. Beta-actin was served as a loading control.
Figure 6

Proposed mechanism of NF-κB pathway inhibition in BzSA pre-treated RAW 264.7 cells upon LPS stimulation.